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

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23 ABSTRACT

1
2 24 Red mud (RM) is a byproduct of aluminum production, worldwide between 70 and 120
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4 25 million tons are produced annually. We analyzed RM which was released in the course of the
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7 26 Kolonatar disaster in Hungary into the environment in acute and genotoxicity experiments
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9 27 with plants which are widely used for environmental monitoring. We detected induction of
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11 28 micronuclei which reflect chromosomal damage in tetrads of Tradescantia and in root cells of
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14 29 Allium as well as retardation of root growth with contaminated soils and leachates. Chemical
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17 30 analyses showed that RM contains metals, in particular high concentrations of vanadium.
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19 31 Follow up experiments indicated that vanadate causes the effects in the plants. This
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21 32 compound causes also in humans DNA damage and positive results were obtained in
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24 33 carcinogenicity studies. Since it was found also in RM from other production sites our
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26 34 findings indicate that its release in the environment is a global problem which should be
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29 35 studied in more detail.

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42 40 **Capsule abstract:** Our findings indicate that the red mud causes genotoxic effect in plants
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44 41 probably due to the presence of vanadate which is contained at high concentrations in the
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46 42 residue.

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56 46 Key words: red mud, Tradescantia, micronuclei, Allium, vanadium

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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 m³ 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

73 the release of this material into the environment induces chromosomal damage. This question
1
2 74 has been addressed in a human study (Gundy et al., 2013) and in bacterial tests (Gelencser et
3
4 75 al., 2011) but no firm conclusions can be drawn of these studies (for details see discussion).
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7 76 The primary aim of the present study was the investigation of the genotoxic properties
8
9 77 of red mud in two plant bioassays, namely in the micronucleus (MN) test with tetrads of
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11 78 *Tradescantia* (Trad-MN assay) and with root tip cells of *Allium cepa* (A-MN assay). The
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13 79 experiments were conducted with tetrads as they reflect damage in meiotic germ cells. Root
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15 80 tip cells were used as they enable the detection of effects in mitotic cells. It was postulated
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17 81 that differences exist in regard to sensitivity of these cell types towards DNA reactive
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19 82 compounds (Rodrigues et al., 1997). MN are formed as a consequence of chromosomal
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21 83 breakage or aneuploidy and can be monitored in a variety of organisms (Heddle et al., 2011).
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23 84 In addition acute toxic effects were studied in root cells of *A. cepa* by measuring the impact of
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25 85 the material on the root growth and by calculating the division rates of the cells. These
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27 86 bioassays are at present the most widely used genotoxicity tests with higher plants and have
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29 87 been employed in more than 300 investigations for the detection of DNA damaging properties
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31 88 of chemicals and complex mixtures (for reviews see Leme and Marin-Morales, 2009; Misik et
32
33 89 al., 2011). We used these test systems since they provide information on environmental
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35 90 effects and it is known that they are, in contrast to other genotoxicity assays with bacterial
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37 91 indicators and mammalian cells (which are also used for environmental monitoring), highly
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39 92 sensitive towards radiation (Ma and Davies, 2009; Misik et al., 2011) and heavy metals
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41 93 (Knasmuller et al., 1998; Majer et al., 2002; Steinkellner et al., 1998).
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51 94 We investigated the acute cytotoxic and genotoxic activities of red mud and of soils
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53 95 which were contaminated with the material collected from fields and gardens which were
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55 96 used for cultivation of crops and vegetables. Furthermore, we studied also the effects of
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57 97 waters leached from red mud and affected soils. In additional experiments, attempts were
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98 made to identify the compound(s) which cause genotoxic effects. We determined the
1
2 99 concentrations of trace elements in solid samples and in leachates and conducted additional
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4 100 Trad-MN assays with sodium metavanadate (NaVO_3) since vanadium was found to be the
5
6
7 101 most abundant heavy metal in the samples.
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9

102 103 **2. Materials and methods**

104 105 2.1 Sampling

106 Soil samples were collected from the Torna catchment on December 15th 2010. Table
107 1 contains a description of the sampling sites and specifications of their GPS locations. From
108 each site, samples were collected from the 0-5; 5-15 and 15-25 cm horizons to assess if
109 potentially toxic constituents in red mud contaminated the soils.

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

112
113 Table 1

114 Figure 1

115 2.2 Preparation of the leachates.

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

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123 2.3 Measurements of the trace elements in the soil samples and leachates.

124 The detection of the trace elements is described in detail in a recent paper of Renforth
125 et al. (2012). Prior to analysis by XRF the samples was prepared as follows. For major
126 element analysis, samples were prepared as fused beads (after loss on ignition at 1050 °C)
127 with lithium metaborate/tetraborate flux (Johnson - Matthey Spectroflux JM100B) (0.6g
128 sample; 3 g Flux). For minor / trace element analysis approximately 10 g dried sample was
129 prepared as a pressed pellet using ~10-20 drops of 6.6% w/v polyvinyl alcohol in a 1:6 mix of
130 methanol and distilled deionised water as a binder (Moviol 88 solution). Elemental analysis of
131 the soil composition was achieved using a PANalytical Axios Advanced X-ray Fluorescence
132 (XRF) spectrometer (data corrected for loss on ignition; % weight loss after furnace treatment
133 at 1050°C). The aqueous phase produced during water extractions was separated from solids
134 by centrifugation (10 min; 2000 g) followed by membrane filtration (0.45 µm); the filtered
135 samples were then acidified by addition of 2% v/v HNO₃. Aqueous elemental concentrations
136 were then determined using a Perkin Elmer Optima 5300 DV ion-coupled plasma, optical
137 emission spectrometer (ICP-OES).

138

139 2.4 Tradescantia micronucleus assays

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

145 fixed in acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol.

146 Maleic hydrazide (20 mg L⁻¹; MH, Sigma-Aldrich, SL, US) was used as a positive control.

147 The protocol for root absorption assays is described in an article of Steinkellner et al.
148 (1998). The soil which was used in the control cultures was biological, pesticide free (from
149 Composana, Wien, Austria). Intact plants were removed from hydroponic culture,
150 subsequently the roots were rinsed and individual plants with at least 15 inflorescences placed
151 into plastic pots (250 ml, diameter 12 cm) which were filled with the different soil samples.
152 The plants were exposed under standard conditions for 48 h (Ma et al., 1994). Subsequently,
153 15 inflorescences were collected from each soil sample in all experiments and fixed in a
154 solution of acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70%
155 ethanol.

156 To find out if vanadium accounts for the effects which were found in the soils and in
157 the leachates, additional experiments were carried out with sodium metavanadate. The salt
158 was dissolved in 0.1M NaOH (pH 13) at 1000 ppm (colorless solution) since earlier findings
159 indicate that vanadium is present as V⁵⁺ in the soil (Burke et al., 2012). The stock solution
160 was diluted with tap water and different concentrations (0.5-10.0 ppm) were used in
161 experiments with *Tradescantia*.

162 Slides were prepared and evaluated as described in the protocol of Ma et al. (1994)
163 The tetrads were stained with a 2% aceto-carmin solution. Early tetrads were analyzed under
164 400-fold magnification. For each experimental point a minimum of five buds with early
165 tetrads was evaluated (300 tetrads were evaluated per slide, 1500 per sample).

166 2.5 Micronucleus assay with *Allium cepa*

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

169 weis, Austroaat, Wien, Austria) were placed in 13 ml glass tubes filled with tap water for
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2 170 24 h in the dark. Subsequently, the roots (lengths ca. 0.5-1 cm) were exposed directly in the
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5 171 soils in the dark for 24 h and then transferred to fresh tap water for another 24 h. At the end of
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7 172 the recovery phase, the maximal root length of each onion was measured and the material was
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9 173 fixed in a mix of acetic acid and ethanol (1/3, v/v) for 24 h and stored in 70% ethanol. MH
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12 174 (10 mg L⁻¹; Sigma-Aldrich, SL, US) was used in all experiments as a positive control.
13

14 175 The root tips were hydrolyzed in a 1:1 mix of HCl (5.0 N) and ethanol (99%) for
15
16 176 3 min and washed in tap water before they were stained with 2% acetocarmine. MN were
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19 177 scored according to the criteria described by Ma et al. (1995). For each experimental point,
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21 178 the MN frequencies were determined in five plants. From each bulb, two slides were made
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24 179 and 500 cells were evaluated per slide (5000 cells per dose). Furthermore, also the mitotic
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26 180 indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The
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29 181 microscopic evaluation was carried out under a light microscope (Nikon YS200, Japan) with
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31 182 400-fold magnification.
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35 36 37 184 2.6 Statistical evaluation

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40 185 The results of the MN experiments were analyzed with one-way ANOVA followed by
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42 186 Dunnett's multiple comparison test. The results of the experiments concerning the mitotic
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45 187 indices (MI) were analyzed with the Kruskal-Wallis test followed by Dunn's comparison test.
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47 188 P-values ≤ 0.05 were considered as significant.
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193 **3. Results**

194
195 3.1 TOC and pH values of the samples.

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

202
203 3.2 Micronucleus assays with Tradescantia (experiments with soils and leachates)

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

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

216
217 Figures 2A and B

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219 3.3 Micronucleus experiments with mitotic root tip cells of *Allium cepa*

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

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

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

233
234 Figures 3A – C

235

236 3.4 Chemical analyses of the soil samples and of the leachates

237 The chemical composition of samples which were collected from S3 and S1 (Table
238 2A-B) and of the corresponding leachates (Table 2C) are shown in Table 2.

239
240 Table 2

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242 The chemical composition of the topmost soil sample (S3 0-5 cm) is similar to that of
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2 243 other samples of red mud which were collected after the Ajka spill (Burke et al., 2012;
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4 244 Ruyters et al., 2011). Several potentially toxic metals including As, Cr, Ni, Pb and V were
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7 245 present at elevated concentrations.
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9 246 The chemical composition of the control sample (S1) is consistent with unaffected
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12 247 soils found in the region (see Table 2c) (Mayes et al., 2011b).
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14 248 The corresponding leachates from S3, contained elevated concentrations of potentially
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17 249 toxic elements, such as V, As, Cu, Al and Cr, notably, V concentrations were an order of
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19 250 magnitude higher than the levels of the other metals (except Al). Also chromium levels were
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27 253 3.5 Tradescantia micronucleus assays with vanadate

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30 254 We found in earlier experiments with Tradescantia that the concentrations of Ni, Cr,
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33 255 Cd, Pb and As, which cause MN induction in tetrads are in the range of hundred to thousand
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35 256 ppm (Knasmuller et al., 1998; Steinkellner et al., 1998). We hypothesized that V, which was
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38 257 detected in higher levels than other metals, may account for the induction of MN, which was
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40 258 seen with the soils and the leachates. Therefore, we tested different concentrations of an
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43 259 aqueous vanadate solution in subsequent Trad MN assays. The results are summarized in
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45 260 Figure 4. It can be seen that exposure of the cuttings to concentrations ≥ 1.0 mg/L caused
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47 261 significant induction of the MN frequencies i.e. an approximately 6-fold increase over the
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50 262 background.
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54 264 Figure 4

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266 3.6 Impact of pH on MN formation in Tradescantia

267 Since the pH values of the soil extracts and of the vanadate solutions were quite high,
268 i.e. in the range between 8.2 and 9.3 (for details see Table 1) we conducted an additional
269 experiment in which plant cuttings were exposed in aqueous solutions (without metals) to
270 different pH values. No indication of pH depended induction of MN by the pH itself was
271 found in this experiment. The numbers of MN per 100 tetrads were 1.7 ± 0.6 ; 2.1 ± 0.6 and
272 1.6 ± 0.6 for pH 8, 8.5 and 9.3 respectively (numbers are means \pm SD of results obtained from
273 1500 tetrads).

274 **4. Discussion**

275
276 The results of this study show that red mud, which was released in large amounts into
277 the environment, causes damage of the genetic material and also acute toxic effects in higher
278 plants.

279
280 4.1 Acute toxic and genotoxic effects of red mud

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

290 gypsum which may limit the nutrient supply and increase the availability of contaminants
1
2 291 (Klebercz et al., 2012). The genotoxic effect of red mud has not been investigated in the
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4 292 invertebrates so far according to our knowledge. However, it is possible that several species
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7 293 may be affected due to the high metal concentration of the material. For example, it is know
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9 294 that earthworms are highly sensitive towards specific contaminants and specific metals.
10
11
12 295 Genotoxic effects were detected with chromium, nickel and cadmium (Bigorgne et al., 2010;
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14 296 Bonnard et al., 2010; Manerikar et al., 2008).

16 297 The results of the present study show clearly that red mud inhibits the division and
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19 298 growth of root tip cells in *A. cepa* (Figures 3B and 3C) and causes induction of MN in this
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22 299 species. Furthermore, we showed that also aqueous leachates of red mud contaminated soils
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24 300 cause induction of MN in both plant bioassays. As shown in Figure 3 a stronger effect was
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26 301 obtained with sample S2 (5-15 cm) as with the sample from upper layer from the same site.
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29 302 This effect can be explained by reduction of MN formation due to inhibition of cell division.
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31 303 Furthermore, it is notable that the effects which were detected with soil exposure are more
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34 304 pronounced as those which were found with the leachates. This observation is in agreement
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36 305 with the results obtained with heavy metals contaminated soils and can be explained by more
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38 306 efficient uptake via the roots by active transport mechanism (for detail see Steinkellner et al.,
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41 307 1998).

43 308 The only genotoxicity assay conducted so far with red mud is the SOS chromotest
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46 309 which is based on the detection of SOS responses in bacterial indicator cells (Gelencser et al.,
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49 310 2011). Consistently negative results were obtained with samples, which were prepared from
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51 311 fugitive dust from the area where the accident had happened. However, it is notable that it is
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53 312 known from earlier investigations that bacterial assays are insensitive towards heavy metals
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56 313 (Majer et al., 2002).

58 314

315 4.2 Identification of vanadate as the active principle of red mud

1
2 316 The results of the chemical analyses show that red mud contaminated soils and their
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4
5 317 aqueous leachates contain elevated concentrations of V (Table 2C). This observation is in
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7 318 agreement with the results of earlier investigations with material from Ajka (Mayes et al.,
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10 319 2011a; Mayes et al., 2011b; Renforth et al., 2012). In this context, it is notable that vanadium
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12 320 was also detected in red mud from several other aluminium production sites (Fontanier et al.,
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15 321 2012; Gawu et al., 2012; Rajeev et al., 1999; Samal et al., 2012).

16
17 322 Spectroscopic evidence showed that vanadium is present primarily as V^{5+} phases
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19 323 (Burke et al., 2012). Under alkaline conditions (high pH is induced in red mud leachate by
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22 324 dissolution of the NaOH present), V^{5+} is predicted to become readily solubilized in water as
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24 325 vanadate (Peacock and Sherman, 2004).

25
26
27 326 Vanadium is more abundant in red mud than other alkali-soluble trace metals (e.g.
28
29 327 As), therefore its environmental mobility is enhanced relative to those of other carcinogenic
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32 328 heavy metals which are present in the material. Therefore, we conducted Trad-MN assays
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34 329 with sodium metavanadate solutions and compared the concentrations which cause positive
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36 330 results with those contained in the aqueous leachates. The extracts prepared from the upper
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39 331 layer of S3 contained 1.1 mg/L V (Table 2) and the results which were obtained with aqueous
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41 332 vanadate solutions (Figure 4) show, that levels ≥ 1.0 mg/L cause significant induction of the
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44 333 MN frequencies in Tradescantia indicating that vanadate accounts for the genotoxic effects
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46 334 which we detected in the leachate.

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49 335 The solubility of vanadate in the environment is largely controlled by sorption
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51 336 reactions with mineral surfaces, with low solution concentrations expected at circumneutral
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54 337 pH (Peacock and Sherman, 2004). If the pH of red mud affected soils can be controlled to pH
55
56 338 < 9 , high aqueous V concentrations and their accompanying genotoxic effects may be avoided
57
58
59 339 (Lehoux et al., 2013). In this context, it is notable that, several technologies have been

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

344

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

346 As shown in Table 2A-C, a number of other genotoxic and carcinogenic heavy metals
347 such as Ni, Cd, Cr and As were detected in the aqueous extracts. Results of earlier Trad-MN
348 assays show that the concentrations of these metals, which are required to cause significant
349 effects, are 10^2 - to 10^3 -fold higher as the levels which were detected in the leachates. The
350 strongest activity was seen in these experiments with $\text{Cr}^{(\text{VI})}\text{O}_3$ (129) followed by As_2O_3 (98.5),
351 $\text{Ni}^{(\text{II})}\text{Cl}_2$ (645), CdCl_2 (1006) and $\text{Pb}(\text{NO}_3)_2$ (1820); numbers in parenthesis indicate the
352 LOELs in mg/L (Knasmueller et al., 2003; Knasmuller et al., 1998; Steinkellner et al., 1998).
353 Furthermore, the results of the chemical analyses demonstrate that, these effects can be
354 attributed to the presence of soluble vanadium. As mentioned above, we found in the present
355 study significant induction of MN by vanadate in *Tradescantia* already at levels ≥ 1 mg/L.
356 Comparisons with the effects seen with other metals indicate that vanadate is more potent in
357 regard to induction of chromosomal damage. According to our knowledge, it has never been
358 tested before in genotoxicity experiments with higher plants, but earlier findings with *Allium*
359 roots showed that 25 mg/L cause stickiness of the chromosomes and a decrease of the MI
360 (Marcano et al., 2006).

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

364 (2012). The available data show that positive results were obtained in most in vitro
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2 365 experiments with mammalian cells and also in a number of in vivo studies with rodents.
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8 367 4.4 Possible consequences of the release of DNA damaging toxins in red mud to the
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10 368 environment

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13 369 It has been postulated that genotoxins may have an impact on the stability of ecosystems.

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15 370 In this context it is notable, that we found in an earlier study concerning urban air pollution,
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17 that the induction of MN in Tradescantia is paralleled by a decrease of the fertility of wildlife
18 371 plants (Misik et al., 2007). It was also shown by other groups that DNA damaging toxins in
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20 372 the environment cause mutations which lead to transgenerational genomic instability in the
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22 offspring (Glen and Dubrova, 2012). The fact that we detected pronounced induction with
23 373 leachates of red mud indicates that the compounds which induce genetic damage (presumably
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25 374 V^{5+}) contaminate surface and/or ground waters. Therefore, it is likely that they cause
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27 375 environmental damage.
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35 378 In regard to potential effects in humans it is known that DNA damage is causally
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37 379 related to diseases such as cancer, infertility and heritable diseases (Shaugnessy and
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39 DeMarini, 2009) and leads to accelerated ageing. We found in an earlier occupational study
40 380 that exposure of workers to V^{5+} causes induction of MN in peripheral lymphocytes (Ehrlich et
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42 381 al., 2008) which is a reliable biomarker for increased cancer risks (Bonassi et al., 2011).
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44 382 Furthermore, it was shown in a long term carcinogenicity study that inhalative exposure to
45
46 383 this compound causes an increase of the tumor rates in the lungs of rats (Assem and Levy,
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48 384 2009), as a consequence of those results V_2O_5 was classified as a possible (group 2B) human
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50 385 carcinogen by the IARC in 2006 (IARC, 2006).
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387 It can be not excluded that humans living in the area where the accident happened are
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2 388 exposed to the genotoxic components which are contained in red mud. Gundy et al. (2013)
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4 389 analyzed chromosomal aberrations (CA) in lymphocytes of individuals who were either
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7 390 injured by the accident or exposed during cleanup activities. The authors conclude that the
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9 391 exposure does not pose an immediate short term genotoxic hazard but recommend further
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11 392 studies. In fact, no firm conclusions can be drawn from their investigation. The CA
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13 393 frequencies of the exposed group were almost 30% higher as those in an urban control group
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15 394 while no differences were seen with controls from a rural village. Furthermore, it is notable
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17 395 that the exposure periods of the individuals in the study group were quite heterogeneous (i.e.
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19 396 between 9 and 336 hrs) which enhardens the detection of effects.
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24 397 Taken together, the results of the present investigation show for the first time that
25
26 398 DNA damaging toxins were released in the course of the Kolontar disaster and that red mud
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28 399 contains vanadate which causes damage of the genetic material at low concentrations. As
29
30 400 mentioned above, the metal was detected also in samples from other production sites and
31
32 401 environmental pollution has been reported from other countries, for example from Brazil and
33
34 402 India (2011; Gawu et al., 2012; Lima et al., 2009). The release of genotoxins into the
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36 403 environment may affect the stability of ecosystems and have a negative impact on human and
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38 404 environmental health; due to the worldwide production of the residue, the contamination of
39
40 405 the material with DNA reactive and potentially carcinogenic vanadium compounds is a global
41
42 406 problem which should be studied in more detail in the future.
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49 50 51 408 **Acknowledgement**

52
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56
57 410 the collection of the samples.
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3 1 Red mud a byproduct of Aluminum production contains soluble vanadium that causes
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5 2 genotoxic and cytotoxic effects in higher plants
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23 ABSTRACT

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

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

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

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4 48 **1. Introduction**

5 49 Red mud is a by-product of aluminum production with the Bayer process. Its global
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7 50 production is in the range between of 70 to 120 million tons per year (Mayes et al., 2011b).
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9 51 The material consists mainly of iron-, aluminum- and titanium- oxides and hydroxides (Burke
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11 52 et al., 2012; Mayes et al., 2011b). Chemical analyses showed that its contains also
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13 53 radionuclides (e.g. ^{226}Ra , ^{230}Th and ^{40}K), as well as heavy metals including As, Cr, Co, Cd,
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15 54 Ni and V (Mayes et al., 2011a; Ruyters et al., 2011).

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17 55 On Oct 4th 2010, approximately one million m³ of the residue was released into the
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19 56 environment from the aluminum plant Ajkai Timfoldgyar Zrt in Western Hungary. According
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21 57 to the Hungarian Ministry of Interior the “Kolontar disaster” is the biggest environmental
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23 58 catastrophe which ever happened in this country (Ádám et al., 2011). Hundreds of houses
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25 59 were destroyed, 265 individuals were injured and ten died (Gundy et al., 2013).

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27 60 After the accident, attempts were made to investigate the impact of the release of the
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29 61 material into the environment and to assess the health consequences in humans.
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31 62 Ecotoxicological studies were conducted with different plant species and bacteria concerning
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33 63 acute toxic effects (Klebercz et al., 2012; Ruyters et al., 2011); the motility and the
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35 64 concentrations of toxic trace elements were studied in physico-chemical measurements
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37 65 (Burke et al., 2012). Furthermore, studies were conducted to assess the consequences of
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39 66 inhalation of dust particles in humans and rodents (Czovek et al., 2012; Gelencser et al.,
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41 67 2011).

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43 68 Radionuclides as well as certain heavy metals (found in red mud) cause damage of the
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45 69 genetic material (Knasmuller et al., 1998; Minouflet et al., 2005) which may lead to
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47 70 destabilization of ecosystems (Sarkar et al., 2006; Zvereva et al., 2008) and also causes
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49 71 adverse effects in humans such as cancer, ageing, infertility and birth defects in the offspring
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51 72 (Aitken and De Iuliis, 2007; Assem and Levy, 2009). Therefore it is of particular interest if

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3 73 the release of this material into the environment induces chromosomal damage. This question
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5 74 has been addressed in a human study (Gundy et al., 2013) and in bacterial tests (Gelencser et
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7 75 al., 2011) but no firm conclusions can be drawn of these studies (for details see discussion).

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9 76 The primary aim of the present study was the investigation of the genotoxic properties
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11 77 of red mud in two plant bioassays, namely in the micronucleus (MN) test with tetrads of
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13 78 *Tradescantia* (Trad-MN assay) and with root tip cells of *Allium cepa* (A-MN assay). The
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15 79 experiments were conducted with tetrads as they reflect damage in meiotic germ cells. Root
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17 80 tip cells were used as they enable the detection of effects in mitotic cells. It was postulated
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19 81 that differences exist in regard to sensitivity of these cell types towards DNA reactive
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21 82 compounds (Rodrigues et al., 1997). MN are formed as a consequence of chromosomal
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23 83 breakage or aneuploidy and can be monitored in a variety of organisms (Heddle et al., 2011).
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25 84 In addition acute toxic effects were studied in root cells of *A. cepa* by measuring the impact of
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27 85 the material on the root growth and by calculating the division rates of the cells. These
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29 86 bioassays are at present the most widely used genotoxicity tests with higher plants and have
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31 87 been employed in more ~~the~~than 300 investigations for the detection of DNA damaging
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33 88 properties of chemicals and complex mixtures (for reviews see Leme and Marin-Morales,
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35 89 2009; Misik et al., 2011). We used these test systems since they provide information on
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37 90 environmental effects and it is known that they are, in contrast to other genotoxicity assays
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39 91 with bacterial indicators and mammalian cells (which are also used for environmental
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41 92 monitoring), highly sensitive towards radiation (Ma and Davies, 2009; Misik et al., 2011) and
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43 93 heavy metals (Knasmuller et al., 1998; Majer et al., 2002; Steinkellner et al., 1998).

44
45 94 We investigated the acute cytotoxic and genotoxic activities of red mud and of soils
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47 95 which were contaminated with the material collected from fields and gardens which were
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49 96 used for cultivation of crops and vegetables. Furthermore, we studied also the effects of
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51 97 waters leached from red mud and affected soils. In additional experiments, attempts were

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3 98 made to identify the compound(s) which cause genotoxic effects. We determined the
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5 99 concentrations of trace elements in solid samples and in leachates and conducted additional
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7 100 Trad-MN assays with sodium metavanadate (NaVO_3) since vanadium was found to be the
8
9 101 most abundant heavy metal in the samples.

14 103 **2. Materials and methods**

17 105 2.1 Sampling

19 106 Soil samples were collected from the Torna catchment on December 15th 2010. Table
20
21 107 1 contains a description of the sampling sites and specifications of their GPS locations. From
22
23 108 each site, samples were collected from the 0-5; 5-15 and 15-25 cm horizons to assess if
24
25 109 potentially toxic constituents in red mud contaminated the soils.

27 110 Total organic carbon (TOC) was determined in soil samples using a LECO SC-144DR
28
29 111 elemental analyzer after removal of the inorganic carbon fraction using 20% HCl.

33 113 Table 1

35 114 Figure 1

38 115 2.2 Preparation of the leachates.

40 116 Soils were used as sampled (field moist). 50 g of each sample were suspended in 100
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42 117 mL of deionised water in a 250 mL glass beaker for 2 hours using a magnetic stirrer. The
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44 118 extracts were filtered with filter paper before they were tested in stem absorption experiments.
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46 119 The pH values of the leachates were measured with a pH Meter 526 (WTW, Weilheim,
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48 120 Germany) and are listed in Table 1.

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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 eHNO₃. 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

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3 145 fixed in acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70% ethanol.

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5 146 Maleic hydrazide (20 mg L⁻¹; MH, Sigma-Aldrich, SL, US) was used as a positive control.

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7 147 The protocol for root absorption assays is described in an article of Steinkellner et al.
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9 148 (1998). The soil which was used in the control cultures was biological, pesticide free (from
10
11 149 Composana, Wien, Austria). Intact plants were removed from hydroponic culture,
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13 150 subsequently the roots were rinsed and individual plants with at least 15 inflorescences placed
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15 151 in-to plastic pots (250 ml, diameter 12 cm) which were filled with the different soil samples.
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17 152 The plants were exposed under standard conditions for 48 h (Ma et al., 1994). Subsequently,
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19 153 15 inflorescences were collected from each soil sample in all experiments and fixed in a
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21 154 solution of acetic acid and ethanol (1/3, v/v). After 24 h, they were transferred to 70%
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23 155 ethanol.

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25 156 To find out if vanadium accounts for the effects which were found in the soils and in
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27 157 the leachetes, additional experiments were carried out with sodium metavanadate. The salt
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29 158 was dissolved in 0.1M NaOH (pH 13) at 1000 ppm (colorless solution) since earlier findings
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31 159 indicate that vanadium is present as V⁵⁺ in the soil (Burke et al., 2012). The stock solution
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33 160 was diluted with tap water and different concentrations (0.5-10.0 ppm) were used in
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35 161 experiments with Tradescantia.

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37 162 Slides were prepared and evaluated as described in the protocol of Ma et al. (1994)
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39 163 The tetrads were stained with a 2% aceto-carmin solution. Early tetrads were analyzed under
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41 164 400-fold magnification. For each experimental point a minimum of five buds with early
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43 165 tetrads was evaluated (300 tetrads were evaluated per slide, 1500 per sample).

44 45 46 166 2.5 Micronucleus assay with *Allium cepa*

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48 167 The experiments were carried out according to the standard protocol published by Ma
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50 168 et al. (1995) with slight modifications. Young onion bulbs (diameter 12-21 mm, Schneeball

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3 169 weis, Austrosaat, Wien, Austria) were placed in 13 ml glass tubes filled with tap water for
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5 170 24 h in the dark. Subsequently, the roots (lengths ca. 0.5-1 cm) were exposed directly in the
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7 171 soils in the dark for 24 h and then transferred to fresh tap water for another 24 h. At the end of
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9 172 the recovery phase, the maximal root length of each onion was measured and the material was
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11 173 fixed in a mix of acetic acid and ethanol (1/3, v/v) for 24 h and stored in 70% ethanol. MH
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13 174 (10 mg L⁻¹; Sigma-Aldrich, SL, US) was used in all experiments as a positive control.
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15 175 The root tips were hydrolyzed in a 1:1 mix of HCl (5.0 N) and ethanol (99%) for
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17 176 3 min and washed in tap water before they were stained with 2% acetocarmine. MN were
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19 177 scored according to the criteria described by Ma et al. (1995). For each experimental point,
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21 178 the MN frequencies were determined in five plants. From each bulb, two slides were made
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23 179 and 500 cells were evaluated per slide (5000 cells per dose). Furthermore, also the mitotic
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25 180 indices (MIs) were determined in 1000 cells (100 cells/root) per experimental point. The
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27 181 microscopic evaluation was carried out under a light microscope (Nikon YS200, Japan) with
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29 182 400-fold magnification.
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34 184 2.6 Statistical evaluation

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36 185 The results of the MN experiments were analyzed with one-way ANOVA followed by
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38 186 Dunnett's multiple comparison test. The results of the experiments concerning the mitotic
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40 187 indices (MI) were analyzed with the Kruskal-Wallis test followed by Dunn's comparison test.
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42 188 P-values ≤ 0.05 were considered as significant.
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3 193 **3. Results**
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6 195 3.1 TOC and pH values of the samples.
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8
9 196 It can be seen in Table 1 that values between 8.0 and 9.3 were found in the soil
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11 197 leachates which were contaminated with red mud. Most of the samples had a total organic
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13 198 carbon content between 1.0-1.5%, which is typical for agricultural soils in the Ajka area
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15 199 (Lehoux et al., 2013). The sample from the upper horizon of the site 3 had a substantially
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17 200 lower TOC content (0.22%) and a higher pH (9.33) which is typical for red mud (Lehoux et
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19 201 al., 2013).
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23 203 3.2 Micronucleus assays with Tradescantia (experiments with soils and leachates)
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26 204 The results which were obtained in Trad-MN assays with red mud contaminated soil
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28 205 and with aqueous leachates from these samples are summarized in Fig. 2A and 2B. It can be
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30 206 seen that direct exposure of intact plants in material from the contaminated sites 2 (S2) and 3
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32 207 (S3) caused a significant increase of the MN frequencies. In the case of S3, the strongest
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34 208 effect was seen with material from the upper layer, samples from deeper horizons caused only
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36 209 a statistically not significant increase. With the two samples from S4, clear cut negative
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38 210 results were obtained. As described above (Table 1), this material was collected after
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40 211 remediation (i.e. after removal of the upper layer).
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42 212 Also with some leachates, positive results were obtained (Fig. 2B). Again the strongest
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44 213 effects were seen with extracts prepared with material from the upper layers (S2 and S3); soils
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46 214 from deeper horizons did not cause significant effects, also the sample which was collected
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48 215 from the cleanup site was devoid of activity.
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50
51 216
52 217 Figures 2A and B
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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

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3 242 The chemical composition of the topmost soil sample (S3 0-5 cm) is similar to that of
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5 243 other samples of red mud which were collected after the Ajka spill (Burke et al., 2012;
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7 244 Ruyters et al., 2011). Several potentially toxic metals including As, Cr, Ni, Pb and V were
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9 245 present at elevated concentrations.

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11 246 The chemical composition of the control sample (S1) is consistent with unaffected
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13 247 soils found in the region (see Table 2c) (Mayes et al., 2011b).

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15 248 The corresponding leachates from S3, contained elevated concentrations of potentially
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17 249 toxic elements, such as V, As, Cu, Al and Cr, notably, V concentrations were an order of
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19 250 magnitude higher than the levels of the other metals (except Al). Also chromium levels were
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21 251 relatively high.

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26 253 3.5 Tradescantia micronucleus assays with vanadate

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28 254 We found in earlier experiments with Tradescantia that the concentrations of Ni, Cr,
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30 255 Cd, Pb and As, which cause MN induction in tetrads are in the range of hundred to thousand
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32 256 ppm (Knasmuller et al., 1998; Steinkellner et al., 1998). We hypothesized that V, which was
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34 257 detected in higher levels than other metals, may account for the induction of MN, which was
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36 258 seen with the soils and the leachates. Therefore, we tested different concentrations of an
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38 259 aqueous vanadate solution in subsequent Trad MN assays. The results are summarized in
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40 260 Figure 4. It can be seen that exposure of the cuttings to concentrations ≥ 1.0 ppm-mg/L caused
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42 261 significant induction of the MN frequencies i.e. an approximately 6-fold increase over the
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44 262 background.

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46 263
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3 266 3.6 Impact of pH on MN formation in Tradescantia
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5 267 Since the pH values of the soil extracts and of the vanadate solutions were quite high,
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7 268 i.e. in the range between 8.2 and 9.3 (for details see Table 1) we conducted an additional
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9 269 experiment in which plant cuttings were exposed in aqueous solutions (without metals) to
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11 270 different pH values. No indication of pH depended induction of MN by the pH itself was
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13 271 found in this experiment. The numbers of MN per 100 tetrads were 1.7 ± 0.6 ; 2.1 ± 0.6 and
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15 272 1.6 ± 0.6 for pH 8, 8.5 and 9.3 respectively (numbers are means \pm SD of results obtained from
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17 273 1500 tetrads).
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20 274 **4. Discussion**
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23 276 The results of this study show that red mud, which was released in large amounts into
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25 277 the environment, causes damage of the genetic material and also acute toxic effects in higher
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27 278 plants.
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32 280 4.1 Acute toxic and genotoxic effects of red mud
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34 281 Several ecotoxicological studies have been conducted with red mud from Ajka
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36 282 (Klebercz et al., 2012; Ruyters et al., 2011) and adverse effects were seen in experiments with
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38 283 higher plants; i.e. the material was found to inhibit the root of *Sinapis alba* and affect the
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40 284 shoot yield in barley (Klebercz et al., 2012; Ruyters et al., 2011). In the latter study, the
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42 285 authors monitored also the levels of trace elements which are contained in red mud such as
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44 286 Cu, Cr, Fe and Ni in exposed plants. They detected these metals in the shoots but stress that
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46 287 their levels did not exceed the toxic limits and hypothesize that Na is the prime cause which
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48 288 affected the growth of the indicator plants. Another factor which was made responsible for the
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50 289 toxic effects in *Sinapis* and in experiments with the Ostracod *Heterocypris incongruens* is
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3 290 gypsum which may limit the nutrient supply and increase the availability of contaminants
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5 291 (Klebercz et al., 2012). The genotoxic effect of red mud has not been investigated in the
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7 292 invertebrates so far according to our knowledge. However, it is possible that several species
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9 293 may be affected due to the high metal concentration of the material. For example, it is know
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11 294 that earthworms are highly sensitive towards specific contaminants and specific metals.
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13 295 Genotoxic effects were detected with chromium, nickel and cadmium (Bigorgne et al., 2010;
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15 296 Bonnard et al., 2010; Manerikar et al., 2008).

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17 297 The results of the present study show clearly that red mud inhibits the division and
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19 298 growth of root tip cells in *A. cepa* (Figures 3B and 3C) and causes induction of MN in this
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21 299 species. Furthermore, we showed that also aqueous leachates of red mud contaminated soils
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23 300 cause induction of MN in both plant bioassays. As shown in Figure 3 a stronger effect was
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25 301 obtained with sample S2 (5-15 cm) as with the sample from upper layer from the same site.
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27 302 This effect can be explained by reduction of MN formation due to inhibition of cell division.
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29 303 Furthermore, it is notable that the effects which were detected with soil exposure are more
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31 304 pronounced as those which were found with the leachetes. This observation is in agreement
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33 305 with the results obtained with heavy metals contaminated soils and can be explained by more
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35 306 efficient uptake via the roots by active transport mechanism (for detail see Steinkellner et al.,
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37 307 1998).

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39 308 The only genotoxicity assay conducted so far with red mud is the SOS chromotest
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41 309 which is based on the detection of SOS responses in bacterial indicator cells (Gelencser et al.,
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43 310 2011). Consistently negative results were obtained with samples, which were prepared from
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45 311 fugitive dust from the area where the accident had happened. However, it is notable that it is
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47 312 known from earlier investigations that bacterial assays are insensitive towards heavy metals
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49 313 (Majer et al., 2002).

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3 315 4.2 Identification of vanadate as the active principle of red mud
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5 316 The results of the chemical analyses show that red mud contaminated soils and their
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7 317 aqueous leachates contain elevated concentrations of V (Table 2C). This observation is in
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9 318 agreement with the results of earlier investigations with material from Ajka (Mayes et al.,
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11 319 2011a; Mayes et al., 2011b; Renforth et al., 2012). In this context, it is notable that vanadium
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13 320 was also detected in red mud from several other aluminium production sites (Fontanier et al.,
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15 321 2012; Gawu et al., 2012; Rajeev et al., 1999; Samal et al., 2012).
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17 322 Spectroscopic evidence showed that vanadium is present primarily as V^{5+} phases
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19 323 (Burke et al., 2012). Under alkaline conditions (high pH is induced in red mud leachate by
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21 324 dissolution of the NaOH present), V^{5+} is predicted to become readily solubilized in water as
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23 325 vanadate (Peacock and Sherman, 2004).
24

25 326 Vanadium is more abundant in red mud than other alkali-soluble trace metals (e.g.
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27 327 As), therefore its environmental mobility is enhanced relative to those of other carcinogenic
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29 328 heavy metals which are present in the material. Therefore, we conducted Trad-MN assays
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31 329 with sodium metavanadate solutions and compared the concentrations which cause positive
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33 330 results with those contained in the aqueous leachates. The extracts prepared from the upper
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35 331 layer of S3 contained 1.1 mg/L V (Table 2) and the results which were obtained with aqueous
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37 332 vanadate solutions (Figure 4) show, that levels ≥ 1.0 mg/L cause significant induction of the
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39 333 MN frequencies in Tradescantia indicating that vanadate accounts for the genotoxic effects
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41 334 which we detected in the leachate.
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43 335 The solubility of vanadate in the environment is largely controlled by sorption
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45 336 reactions with mineral surfaces, with low solution concentrations expected at circumneutral
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47 337 pH (Peacock and Sherman, 2004). ~~Therefore, if~~ the pH of red mud affected soils can be
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49 338 controlled to $pH < 9$, high aqueous V concentrations and their accompanying genotoxic
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51 339 effects may be avoided (Lehoux et al., 2013). In this context, it is notable that, several
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3 340 technologies have been developed to recover vanadium from sludge (for review see Rajeev et
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5 341 al., 1999). As mentioned above, we found no impact of the pH on MN frequencies in
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7 342 experiments with waters to which NaOH was added in absence of metal ions. However, the
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9 343 situation may be different in soils which are contaminated with vanadium compounds. In this
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11 344 context, it is notable that, several technologies have been developed to recover vanadium from
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13 345 sludge (for review see Rajeev et al., 1999).
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15 346 16 17 18 347 4.3 Vanadate is an extremely potent mutagen in the Trad MN assay 19

20 348 As shown in Table 2A-C, a number of other genotoxic and carcinogenic heavy metals
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22 349 such as Ni, Cd, Cr and As were detected in the aqueous extracts. Results of earlier Trad-MN
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24 350 assays show that the concentrations of these metals, which are required to cause significant
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26 351 effects, are 10^2 - to 10^3 -fold higher as the levels which were detected in the leachates. The
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28 352 strongest activity was seen in these experiments with $\text{Cr}^{(VI)}\text{O}_3$ (129) followed by As_2O_3 (98.5),
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30 353 $\text{Ni}^{(II)}\text{Cl}_2$ (645), CdCl_2 (1006) and $\text{Pb}(\text{NO}_3)_2$ (1820); numbers in parenthesis indicate the
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32 354 LOELs in mg/L (Knasmueller et al., 2003; Knasmuller et al., 1998; Steinkellner et al., 1998).
33
34 355 Furthermore, the results of the chemical analyses demonstrate that, these effects can be
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36 356 attributed to the presence of soluble vanadium. As mentioned above, we found in the present
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38 357 study significant induction of MN by vanadate in Tradescantia already at levels ≥ 1 mg/L.
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40 358 Comparisons with the effects seen with other metals indicate that vanadate is more potent in
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42 359 regard to induction of chromosomal damage. According to our knowledge, it has never been
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44 360 tested before in genotoxicity experiments with higher plants, but earlier findings with Allium
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46 361 roots showed that 25 mg/L cause stickiness of the chromosomes and a decrease of the MI
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48 362 (Marcano et al., 2006).
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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 V⁵⁺) 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 V⁵⁺ 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,

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3 387 2009), as a consequence of those results V₂O₅ was classified as a possible (group 2B) human
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5 388 carcinogen by the IARC in 2006 (IARC, 2006).

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7 389 It can be not excluded that humans living in the area where the accident happened are
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9 390 exposed to the genotoxic components which are contained in red mud. Gundy et al. (2013)
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11 391 analyzed chromosomal aberrations (CA) in lymphocytes of individuals who were either
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13 392 injured by the accident or exposed during cleanup activities. The authors conclude that the
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15 393 exposure does not pose an immediate short term genotoxic hazard but recommend further
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17 394 studies. In fact, no firm conclusions can be drawn from their investigation. The CA
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19 395 frequencies of the exposed group were almost 30% higher as those in an urban control group
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21 396 while no differences were seen with controls from a rural village. Furthermore, it is notable
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23 397 that the exposure periods of the individuals in the study group were quite heterogeneous (i.e.
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25 398 between 9 and 336 hrs) which enhardens the detection of effects.

26
27 399 Taken together, the results of the present investigation show for the first time that
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29 400 DNA damaging toxins were released in the course of the Kolontar disaster and that red mud
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31 401 contains vanadate which causes damage of the genetic material at low concentrations. As
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33 402 mentioned above, the metal was detected also in samples from other production sites and
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35 403 environmental pollution has been reported from other countries, for example from Brazil and
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37 404 India (2011; Gawu et al., 2012; Lima et al., 2009). The release of genotoxins into the
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39 405 environment may affect the stability of ecosystems and have a negative impact on human and
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41 406 environmental health; due to the worldwide production of the residue, the contamination of
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43 407 the material with DNA reactive and potentially carcinogenic vanadium compounds is a global
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45 408 problem which should be studied in more detail in the future.

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49 410 **Acknowledgement**
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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' | 7.18 (0-5cm) | 1.36 (0-5cm) |
| | | EO17°28.159' | 7.18 (5-15 cm) | 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' | 9.21 (0-5 cm) | 0.99 (0-5 cm) |
| | | EO 17°27.947' | 9.19 (5-15 cm) | 1.07 (5-15 cm) |
| | | | 8.26 (15-25 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' | 9.33 (0-5 cm) | 0.22 (0-5 cm) |
| | | EO 17° 28.067' | 8.32 (5-15 cm) | 1.05 (5-15 cm) |
| | | | 8.24 (15-25 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' | 8.28 (0-5 cm) | |
| | | EO17°28.253' | 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 ₂ O ₃ | Fe ₂ O ₃ | MnO | MgO | CaO | Na ₂ O | K ₂ O | P ₂ O 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 |

^aall 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 |

^aall 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 |

^aall values are indicated as mg/L

ppm

Figure 1

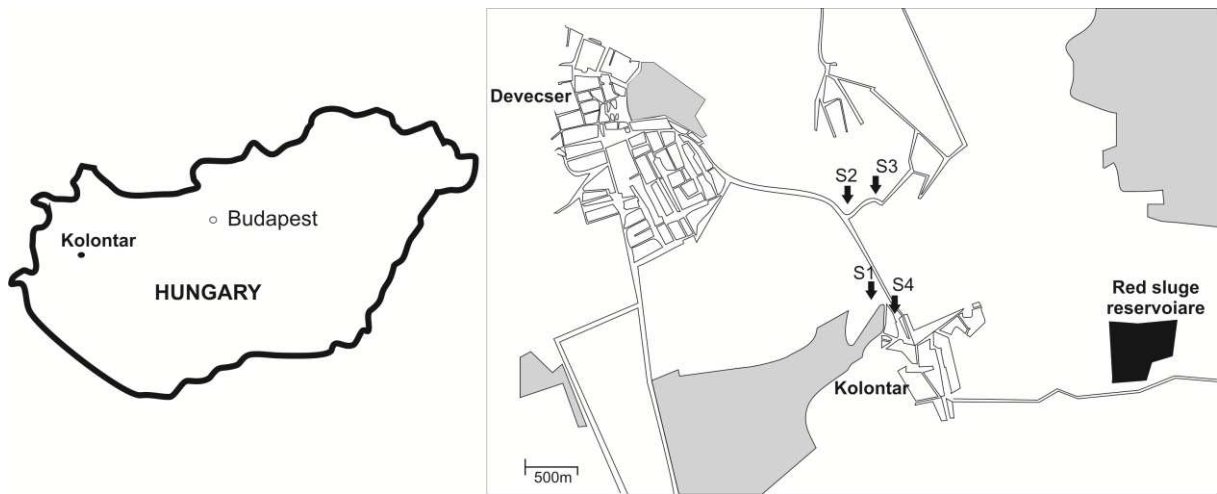


Figure 2A and B

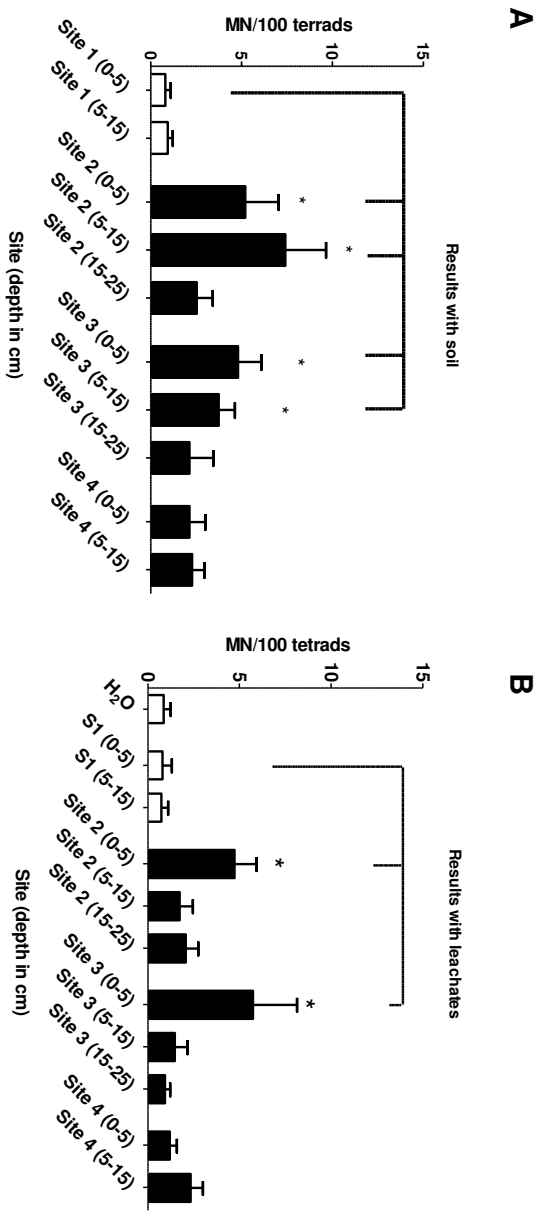


Figure 3A–C

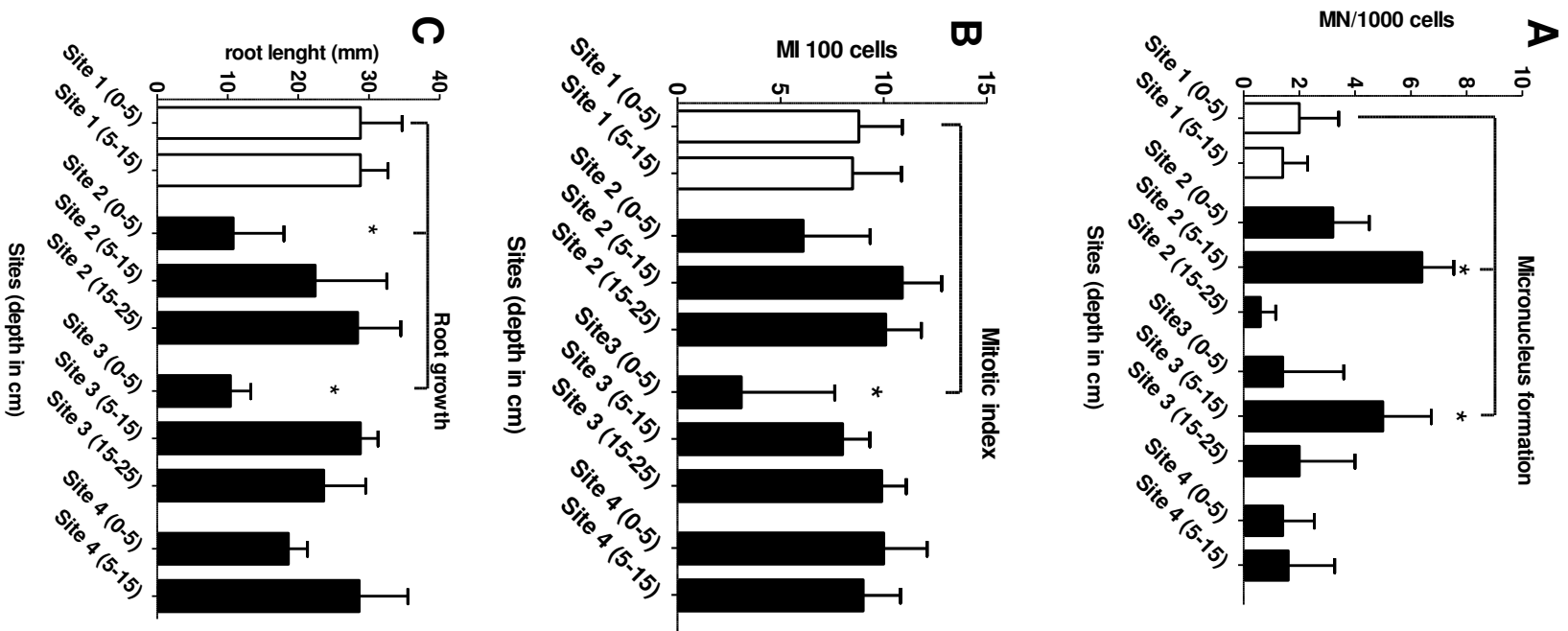
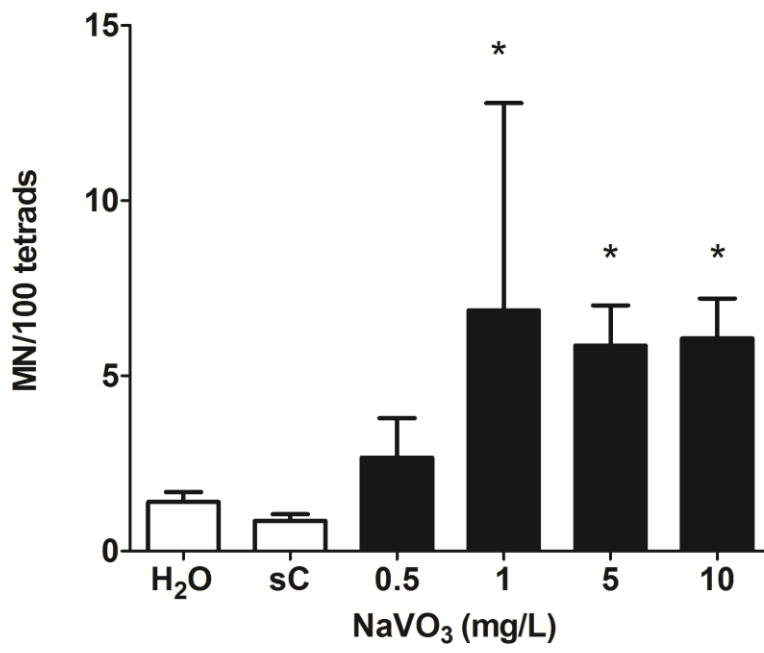
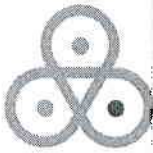


Figure 4





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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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