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The efficiency of combined CaO/electrochemical treatment in removal of acid mine drainage induced toxicity and genotoxicity

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HIGHLIGHTS

- Acute geno(toxic) impacts of treated and untreated acid mine drainage were assessed.
- Chemical analysis and bioassays on plants and Daphnia were conducted.
- Genotoxic and toxic compounds induced oxidative stress.
- Acute toxicity and genotoxicity were removed following CaO/electrochemical treatment.

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ABSTRACT

Acid mine drainage (AMD) is a by-product of the mining industry that has a detrimental effect on aquatic plant and animal life due to high load of heavy metals and sulfates. In the present study, the toxic and genotoxic potential of AMD prior to and following combination of neutralization/electrocoagulation processes was evaluated using several bioassays and selected parameters. Regardless of pH correction of AMD prior to Daphnia bioassay, high acute toxicity was observed in *Daphnia magna*. The mine leachate also induced strong phyto-, cyto- and genotoxicity to *Allium cepa* roots. Short term exposure to AMD inhibited duckweed growth and chlorophyll *a* content and simultaneously promoted lipid peroxidation and DNA damage despite duckweed capability to upregulate antioxidative defense mechanisms. The results show that observed (geno) toxicity could be related to oxidative stress most probably induced by toxic metal action. However, influence of low pH as a contributing factor in the phytotoxicity of AMD cannot be excluded. The application of combined treatment eliminated genotoxicity and was highly efficient in reducing toxicity of AMD. Thus, the method seems to be suitable for treatment of AMD waters enabling their safe discharge to an aquatic environment.

1. Introduction

Acidic sulfur-rich wastewaters are the by-products of a variety of industrial operations but the major producer of such effluents is the mining industry. Acid mine drainage (AMD) originates in the reaction of sulfide minerals from an open pit or the mine tailings with water and oxygen creating sulfuric acid. Under the acidic conditions trace metals are liberated from the ore/tailings into solution causing acidification/heavy metals pollution of the surrounding soil, groundwater as well as rivers and streams receiving such effluent. Consequently, this activity leads to the to the serious pollution of drinking water sources in the area of the influence as well as loss of the agricultural land which is not suitable for agricultural production due to the pollution with metals and high acidity (Johnson and Hallberg, 2005; Oreščanin et al., 2012). Johnson and Hallberg (2005) summarized the treatment methods of the acid mine drainage which include aeration and lime addition, anoxic limestone drains, off-line sulfidogenic bioreactors, aerobic wetlands, compost reactors/wetlands, permeable reactive barriers and packed bed iron-oxidation bioreactors. The novel treatment procedure developed by Oreščanin et al. (2012) was successfully applied to AMD originating from the sulfide ore mining to reduce pollutant load. However, potency of the method in reduction of AMD toxicity and genotoxicity and thus its suitability and safety of the methods prior to their wide range applications has not yet been evaluated. In several studies, the genotoxic and toxic potential of mine drainage water or mine soils has been determined by use of various test systems (Pereira et al., 2009; Geremias et al., 2012). In the present study, Daphnia, Lemna, comet and Allium were used in assessment of toxic and genotoxic potential of the untreated and treated AMD. *Daphnia magna* immobilization test (ISO 6341, 2012), an acute toxicity test with freshwater crustacean *D. magna*, is used

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internationally for toxicity screening of chemical compounds and the monitoring of industrial effluents. The advantage of the test species is their broad distribution in a wide range of habitats, their relatively short life cycle and the fact that they are relatively easy to culture and maintain in the laboratory though it has to be taken into account that pH values greater than 11.5 or less than 4 cause their total immobilization (Seco et al., 2003). Lemna test (growth inhibition test) is suitable for assessing contaminated waters as duckweeds (*Lemna minor* L.) possess certain physiological properties desirable in ideal test systems such as small size, growth over a wide range of pH (pH 3–10) and vegetative propagation (Landolt, 1986). According to ISO protocol, the test is conducted over a period of 7 days when the growth rate is calculated (ISO 20079, 2005) while here the effects of AMD on duckweed were assessed by estimating growth rate and certain biochemical parameters after 24-h period. Indicators of oxidative stress were used as additional toxicity parameters in duckweed as heavy metals are found to be potent inducers of reactive oxygen species (ROS) production (Fodor, 2002). Both Allium and Comet assays are simple, rapid, inexpensive and sensitive methods for measuring genotoxic effects of either single or mixture of pollutants (Yıldız et al., 2009; Radić et al., 2010a, 2010b) with a difference that latter assay does not require actively dividing cells. However, Allium test also provides useful estimate of the total toxic and cytotoxic effect employing parameters of root growth and cell division. The system operates over a wide pH range (3.5–11.0) without any obvious effects upon the growth of the root systems (Fiskesjö, 1995).

The aim of the present study was to evaluate toxic and genotoxic effects of AMD prior to and following CaO/electrochemical treatment using several bioassays as well as selected biochemical indicators of oxidative stress.

2. Materials and methods

2.1. Sampling site and sample treatment

The AMD was collected from the Robule Lake located within Bor copper mining and smelting complex, Serbia. Robule Lake originated by the accumulation of wastewaters leaching from the waste heap of the "Bor" open pit. The volume of 10 L of water was pumped into two acid cleaned polyethylene containers. The samples were kept at +4 °C until the analysis and the purification experiments. Prior to the purification experiments, the water samples were homogenized for 10 min. For each experiment 2.0 L of the effluent was taken and subjected to the pH adjustment by calcium oxide (4 g L^{-1}). Calcium oxide (Lika lime factory, Ličko Lešće, Croatia) used in the treatment was of technical grade with less than 5% impurities and particle size less than 90 μm. The addition of CaO stopped when pH of the solution increased over 7. The suspension was mixed with air bubbles for 10 min and subjected to electrocoagulation process using iron electrode plates (I = 50 A, U = 10 V) for 5 minute aluminum electrode set (I = 50 A, U = 10 V) for additional 10 min. Each electrode set contained of 10 quadratic, 1 mm thick plates (75 × 250 mm) in the parallel arrangement, separated by an electro insulator. The distance between plates was 10 mm. Each even plate was connected to + pole of DC power supply and represented sacrificial anode, while odd plates connected to the − pole of DC served as the cathodes. Either Fe or Al electrode set was placed at the bottom of the reaction vessel perpendicular to the vessel bottom. In the end of electrochemical treatment the suspension was recirculated in strong electromagnetic field for 10 min and subjected to slow mixing for another 15 min while the settlement time was 30 min (Oreščanin et al., 2012).

2.2. Physicochemical parameter analysis

Color was determined according to 8025 APHA platinum–cobalt standard method (adopted from Standard Methods for the Examination of Water and Wastewater) and for accuracy and performance check a 500 platinum–cobalt unit's color standard solution (Cole-Parmer, USA) was used. Turbidity was determined by 8237 absorptometric method and for accuracy and performance check 4000 NTU Formazin stock solution (Cole-Parmer, USA) was used. Suspended solids (SS) were determined by 8006 photometric method, sulfates by SulfaVer 4 method (an equivalent to USEPA method 3754 for wastewater) and manganese by 8149 PAN method. All aforementioned parameters were measured by use of HACH DR890 colorimeter (Hach Company, Loveland, Colorado, USA). pH value and electrical conductivity (EC) were determined by PHT-027 — water quality multiparameter monitor (Kelilong Electron, Fuan Fujian, China). For calibration purpose Cole-Parmer standard solutions were used.

2.3. Metal analysis in water samples

Following digestion procedure described by Oreščanin et al. (2008), untreated and treated samples of the landfill leachate were prepared for the analysis as follows: 100 mL of the leachate was adjusted to pH 3 by the addition of hydrochloric acid (Kemika, Zagreb, Croatia) and ammonium hydroxide (Kemika, Zagreb, Croatia). Following the pH adjustment, 2 mL of 1% (w/v) ammonium-pyrrolidinedithiocarbamate (APDC) (Merck, Schuchardt, Germany) was added into each flask. After the complexation lasted for 20 min, the suspension was filtered through a Millipore HAWP filter (pore size-0.45 μm; diameter-25 mm) using a Millipore micro filtration system. The prepared thin targets were air-dried, protected with a thin mylar foil (2 μm) and analyzed by X-ray spectrometer. All samples were irradiated by X-rays generated from X-ray tube (Oxford instruments) with Mo anode (high voltage: 40 kV; current: 900 μA; cooling medium: Air). The detection of characteristic X-ray radiation from the sample was conducted with Si drift detector-model SXD15C-150-500 (Canberra, Meriden, USA). The calibration model for the qualitative and quantitative analysis of thin targets was created on the basis of the measurements of the standard solutions (Merck) having the concentration range from 10 to 200 μgL^{-1}, prepared and measured in the same way as unknown samples (Oreščanin et al., 2008).

2.4. D. magna immobilization test

D. magna Straus. used in the study was obtained from the Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium. Tests were carried out with hatched neonates (<24 h) according to ISO 6341 (2012) protocol. A concentration range of untreated (0.01–5%) and treated AMD (1–100%) was made using dilution water for preparation. Five individuals (less than 24 h old) were exposed to each dilution sample in 4 replicates. After 24 h and 48 h incubation at 20 ± 1 °C, the number of immobile daphnids was recorded. Results were expressed as the concentration of the sample that produced a 50% immobilization (EC50). Toxicity values (EC50) were converted in Toxic Units (TU), i.e. inverse of EC50 expressed in %. The toxicity classification of effluents is reported as follows (Persoone et al., 2003): no acute toxicity TU < 0.4; slight acute toxicity 0.4 < TU < 1; acute toxicity 1 ≤ TU < 10; high acute toxicity 10 ≤ TU < 100; very high acute toxicity ≥ TU 100.

2.5. Allium test

Prior to test start, the outer scales of the bulbs (1.5–2.0 cm diam.) of onion (*Allium cepa*, 2n = 16) and the dry bottom plate were removed without destroying the root primordia. For each test water sample, a series of twelve bulbs were set up and placed in distilled water for 48 h. The bulbs were exposed to untreated AMD prepared in a range of serial dilutions — AMD 5% (pH 4.03), AMD 25% (pH 3.52), AMD 50% (pH 3.0) and AMD 100% (pH 2.70), to treated AMD (pH 6.71) and to distilled water (pH 6.4) which served as a negative control (C). Ethyl methanesulfonate (EMS, 5 mM) (Sigma-Aldrich)

was used as a positive control mutagen. After 24 h of exposure, some root tips were removed from the bulbs, fixed 3:1 in ethanol:glacial acetic acid and kept overnight at 4 °C before being rinsed in tap water and stained in aceto-carmine. Microscope slides were prepared by squashing the stained root tips in 45% (v/v) glacial acetic acid. One slide was prepared per bulb all slides were examined using bright-field microscopy (Zeiss Standard 20) at a total magnification of 1000×. Microscopical investigations were performed on three slides (replicates) with each having 1–2 root tips for each water sample. To obtain mitotic indices (MI), approximately 3000 cells (1000 cells in each of the three slides) were observed for each water sample. The number of chromosomal aberrations was recorded in approximately 300 dividing cells (preferably 100 per slide). Various types of aberrations scored were c-mitosis, laggards, chromosome breaks, anaphase bridges and stickiness. After 48 h of exposure to tested water samples, the root lengths ($n = 6$) were measured and used as an index of general toxicity.

2.6. Lemna test and biochemical parameters in duckweed

L. minor L. was originally collected from Botanical Garden, Faculty of Science, University of Zagreb. For long-term cultivation, *L. minor* plants were sterilized and maintained as stock cultures on Pirson-Seidel nutrient medium (Pirson and Seidel, 1950). The axenic cultures were grown under a 16:8 h light:dark period of cool fluorescent light (90 $\mu Em^{-2}\ s^{-1}$) at 24 ± 2 °C. Prior to experiment for toxicity testing, *L. minor* plants were allowed to adapt to the Steinberg (Steinberg, 1946) medium (pH 5.5) modified according to Altenburger: KNO_3 3.46 mM, KH_2PO_4 0.66 mM, K_2HPO_4 0.072 mM, $MgSO_4$ 0.41 mM, $Ca(NO_3)_2$ 1.25 mM, H_3BO_3 1.94 mM, $ZnSO_4$ 0.63 mM, Na_2MoO_4 0.18 mM, $MnCl_2$ 0.91 mM, $FeCl_3$ 2.81 mM and EDTA-Na_2 4.03 mM for a 2-week period (pre-cultivation). Following adaptation period, several healthy colonies with 2–3 fronds (from stock cultures) were transferred to Erlenmeyer flasks containing modified Steinberg medium prepared with untreated and treated (T) AMD samples (pH 6.65). Untreated AMD was prepared in a range of serial concentrations: 5 (pH 3.3), 25 (pH 3.0), 50 (pH 2.81) and 100% (pH 2.66). Duckweed plants grown on modified Steinberg medium with dH_2O served as a control (C; control media). Prior to cultivation, all leachate samples were filtered using cellulose nitrate membranes (Whatman, pore size 0.45 μm). During pre-cultivation and cultivation, the cultures were grown under a continuous cool fluorescent light of 90 $\mu mol\ m^{-2}\ s^{-1}$ (TLD 36 W/54-765; Philips, Poland) at 24 ± 2 °C. To measure N fronds/N colony ratio (N fr/N col) and growth rate, the frond (FN) and colony numbers were scored at the start (t0) of exposure and 24 h after (t1). Relative growth rate (RGR) was calculated from the following equation: $RGR = (\ln FN_{t1} - \ln FN_{t0})/t1\text{-}t0$. The chlorophyll *a* (Chl a) and *b* (Chl b) levels were determined in 80% (v/v) acetone extracts and calculated by the method of Lichtenthaler (1987). Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content using the thiobarbituric acid method described by Heath and Packer (1968). Antioxidant enzyme activities were analyzed by first homogenizing plant tissue in 50 mM KPO_4 buffer (pH 7) including 1 mM EDTA and polyvinylpolypyrrolidone (Sigma-Aldrich). The homogenates were centrifuged (Sigma Aldrich, 3 K18 centrifuge) at 25,000 *g* for 30 min at 4 °C and supernatants were used for enzyme activity and protein content assays. Total soluble protein contents of the enzyme extracts were estimated according to Bradford (1976) using bovine serum albumin as standard. The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (Sigma-Aldrich) following the method of Beauchamp and Fridovich (1971). Ascorbate peroxidase (APX) activity was done following ascorbate oxidation at 290 nm according to Nakano and Asada (1981). Catalase (CAT) activity was determined by the decomposition of H_2O_2 and according to Aebi (1984). The specific activities of all enzymes were expressed as units per milligram of protein.

Comet assay on *L. minor* was performed according to Gichner et al. (2004) with slight modification (10 min denaturation, 20 min electrophoresis at 1 V/cm, 300 mA). Three slides were evaluated per water sample. For each slide, 50 randomly chosen nuclei were analyzed using a fluorescence microscope with an excitation filter of BP 520/09 nm and a barrier filter of 610 nm. A computerized image-analysis system (Komet version 5, Kinetic Imaging Ltd., Liverpool, UK) was employed. The median tail extent moment (TM) was used as the measure of DNA damage.

2.7. Statistical analysis

Data were compared by analysis of variance (ANOVA) to confirm the variability of data and validity of results, and Duncan's multiple range test was performed to determine the significant differences between treatments ($p < 0.05$). Differences between corresponding controls and exposure treatments were considered statistically significant at $p < 0.05$. Coefficients of determination between different plant parameters and their respective significance level were calculated. Statistical analyses were performed using the STATISTICA 8.0 (StatSoft, Inc., USA) software package.

3. Results and discussion

In the present study, toxic effects of untreated and treated AMD were evaluated by Daphnia, Allium, Lemna and comet tests. As presented in Table 1, the AMD from the Robule Lake (Bor mining complex and smelting/refining of copper) was characterized by low pH value and high concentrations of sulfates and Fe, Mn, Cu, Zn, Cr and Ni which is consistent with the literature (Johnson and Hallberg, 2005; Wang et al., 2012). Treatment with CaO and electrocoagulation by electrochemically generated iron and aluminum flocs resulted in increase in pH and showed over 96% removal efficiency of color, turbidity, suspended solids (SS) and selected heavy metals, over 86% removal efficiency of sulfates and over 76% removal efficiency of EC. The removal of heavy metals and sulfates at the stage of pretreatment with CaO was probably due to formation of the poorly soluble hydroxides and gypsum (Oreščanin et al., 2012).

Untreated 1% AMD showed very high (100% at 24 h and 48 h) lethal toxicity to *Daphnia magna*. Calculated 24 h EC50 value for *D. magna* was equaled to 0.21% of untreated AMD in solution. Results indicate that untreated AMD has very high acute toxicity to tested aquatic invertebrates (TU = 476). Immobilization of *D. magna* dramatically increased with time and 48 h EC50 value for *D. magna* was equaled to 0.06% of untreated AMD in solution, i.e. TU = 1666 (very

Table 1
Physicochemical parameters in the acid mine drainage (AMD) before and after the combined CaO/electrochemical treatment.

Measured parameter	Detection limit	AMD	Treated AMD
Color (PtCo)	1	743	–
Turbidity (NTU)	1	30	1
pH	0.01	2.7	6.71
SS ($mg\ L^{-1}$)	1	10	–
EC ($mS\ cm^{-1}$)	0.01	8.92	2.13
Sulfates ($mg\ L^{-1}$)	4.9	10830	1470
Hg ($mg\ L^{-1}$)	0.0001	0.082	0.001
Pb ($mg\ L^{-1}$)	0.0001	0.043	0.001
V ($mg\ L^{-1}$)	0.0001	0.087	0.003
Cr ($mg\ L^{-1}$)	0.0001	12.341	0.004
Mn ($mg\ L^{-1}$)	0.0001	107.304	0.074
Fe ($mg\ L^{-1}$)	0.0001	921.13	0.047
Zn ($mg\ L^{-1}$)	0.0001	58.637	0.029
Co ($mg\ L^{-1}$)	0.0001	0.119	0.001
Ni ($mg\ L^{-1}$)	0.0001	1.97	0.002
Cu ($mg\ L^{-1}$)	0.0001	91.671	0.003

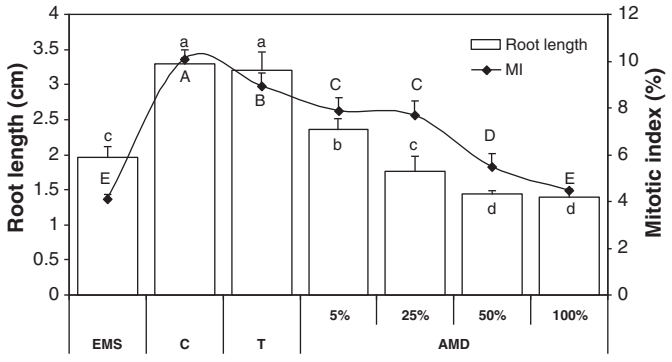

Fig. 1. Root length (cm) and mitotic index (%) of *Allium cepa* root-tip cells following exposure to serial dilutions of untreated AMD (5, 25, 50 and 100% L) or treated (T) AMD. As a negative control dH_2O was used and as a positive one 5% EMS was employed. Standard deviations were presented by error bars. Different letters indicate significantly different values at $p < 0.05$.

high acute toxicity). Similar results were obtained by Kang et al. (2011) who showed that AMD is highly toxic to crustaceans. Such strong acute toxicity of AMD to daphnids could be the result of Cu and Zn (Arambašić et al., 1995) which were detected in AMD in higher quantities. Treatment of AMD had sharply reduced their toxicity. The immobilization of *D. magna* was 15% and 20% after 24 h and 48 h, respectively, in 100% concentration of treated AMD. EC50 was not calculated for treated AMD, as treated AMD had below 50% effect level. Toxic units in case of treated AMD were <0.4, i.e. no acute toxicity.

Untreated AMD from the copper mine was highly toxic and cytotoxic to *A.cepa* (Fig. 1) as significant reduction of root length and cell division, compared to negative control (C), was observed even at the highest AMD dilution (AMD 5%). The general toxicity of undiluted AMD to *A. cepa* was even stronger than with positive control (5 mM EMS). The inhibition of mitotic index could be attributed to the metal induced disturbances in the cell cycle or due to chromatin dysfunction caused by metal-DNA interactions (Glińska et al., 2007). Heavy metals such as Cu, Fe, Mn and SO_4^{2-} individually or in complex mixtures such as AMD, may exert a strong inhibitory effect on root length and cell division (Arambašić et al., 1995; Geremias et al., 2010, 2012). When evaluating the acute toxicity of single metallic (Cu, Pb, Zn) solutions to *A. cepa* roots, Arambašić et al. (1995) showed that Cu was the most toxic. However, the influence of low pH as a contributing factor in the toxicity and cytotoxicity of AMD cannot be disregarded, especially in the case of higher AMD concentrations (50 and 100% AMD) with pH level of 3 or even lower. Regarding genotoxicity of AMD, the number of chromosomal and mitotic aberrations increased in a concentration dependent manner (Table 2). The most frequent abnormalities induced by AMD were stickiness and c-metaphases followed by laggards and chromosome breaks. Sticky chromosomes, which are considered to be a common sign of toxic effects on chromosomes leading to cell death, have been reported in *Allium* roots after treatment with heavy metals such as Cu, Ni and especially Hg (Monte Egito et al., 2007). The other effect frequently observed, c-metaphase, suggests that compounds present in AMD disturb the mitotic spindle, most likely the kinetochore function (Fiskesjö, 1995). Similar distribution of aberrations along with root growth and cell division reduction was noticed in onion roots following exposure to Cu and Co (Yıldız et al., 2009). On the other hand, applied treatment procedure was efficient in removing toxic and genotoxic effects of AMD (Table 2, Fig. 1) while cytotoxicity caused by treated AMD was not completely reduced. The low cytotoxicity noted following the applied treatment might be the result of residual sulfates which were not completely removed by the method. The number of chromosomal abnormalities scored in treated AMD was similar to that in negative control. Also, none of the individually observed aberrations showed statistical significance compared to positive or negative control.

Undiluted AMD and 50% AMD caused strong acute toxicity in *L. minor* plants (Fig. 2) which was manifested in rapid (in less than 24 h) bleaching of plant tissue. Thus, only effects of higher dilutions of AMD were presented in the study (Fig. 2). Following 24-h exposure to 5 and 25% AMD, duckweed growth rate declined for 55 and 62%, respectively, compared to control (Fig. 2A). AMD also induced rapid colony breakup as N fr/N col ratio (Fig. 2B) dropped for 50% with regard to control values. Although the interactions in multimetallic samples considerably limit possibility to explain the results for single metals, the growth reduction and increased colony breakup observed in our study could be due to the suppression of the elongation growth rate of cells exerted by Cu, Zn, Ni, Fe and Cr (Li and Xiong, 2004; Srivastava et al., 2006). However, low pH and substantially high EC might have contributed to inhibition of *L. minor* growth as EC values over 4 mS cm^{-1} were shown to negatively affect duckweed growth (Oron and Willers, 1989; Cayuela et al., 2007). Pigment content has shown to be a very consistent biomarker in relation to growth rates. Both serial dilutions (5 and 25% AMD) affected Chl a in a dose-dependent manner while Chl b significantly declined following duckweed exposure to 25% AMD (Fig. 2C). On contrary, TM and formation of MDA increased in duckweed exposed to AMD in a dose-dependent manner (Fig. 2D). MDA is the decomposition product of polyunsaturated fatty acids of biomembranes and its increase shows the extent of membrane lipid peroxidation. Both Chl a and b strongly correlated (Table 3) with oxidative stress parameters as well as comet assay parameter (TM) suggesting oxidative damage to chloroplast membrane lipids and DNA. Moreover, a strong correlation was observed between TM, MDA and antioxidative enzyme activities (Table 3) indicating DNA damage through oxidative stress reactions. When micronutrients like Cu, Fe and Mn exceed their optimal levels, they become toxic due to increased production of highly toxic hydroxyl radicals via the Fenton reaction (Fodor, 2002). In contrast to redox active, non-redox metals such as Zn, Ni, Cr and Pb do not produce ROS

Table 2
Mitotic and chromosomal abnormalities of *Allium cepa* meristem root cells following 24 h-period exposure to acid mine drainage (AMD) and to AMD purified by a combined CaO/electrochemical treatment (T). As a negative control dH_2O was used and as a positive one 5% ethyl methanesulphonate (EMS).

		Spindle failure			Chromosome abberations	Chromatin abnormalities		
Sample	No. dividing cells	C-mitosis	Laggards	Multipolar spindle	Breaks	Anaphase bridges	Sticky chromosomes	Abberant cells %
C	304	4	1	0	0	0	1	1.9
EMS	126	14*	8*	3	10*	8*	16**	45.2
T	269	2	1	0	0	0	5	2.9
AMD 5%	237	11*	5	1	2	2	17**	16.0
AMD 25%	235	8	4	1	3	3	22**	17.5
AMD 50%	166	11*	8*	1	4	3	19**	27.7
AMD 100%	136	7	5	0	8*	3	19**	30.9

* $p < 0.05$.
** $p < 0.001$.

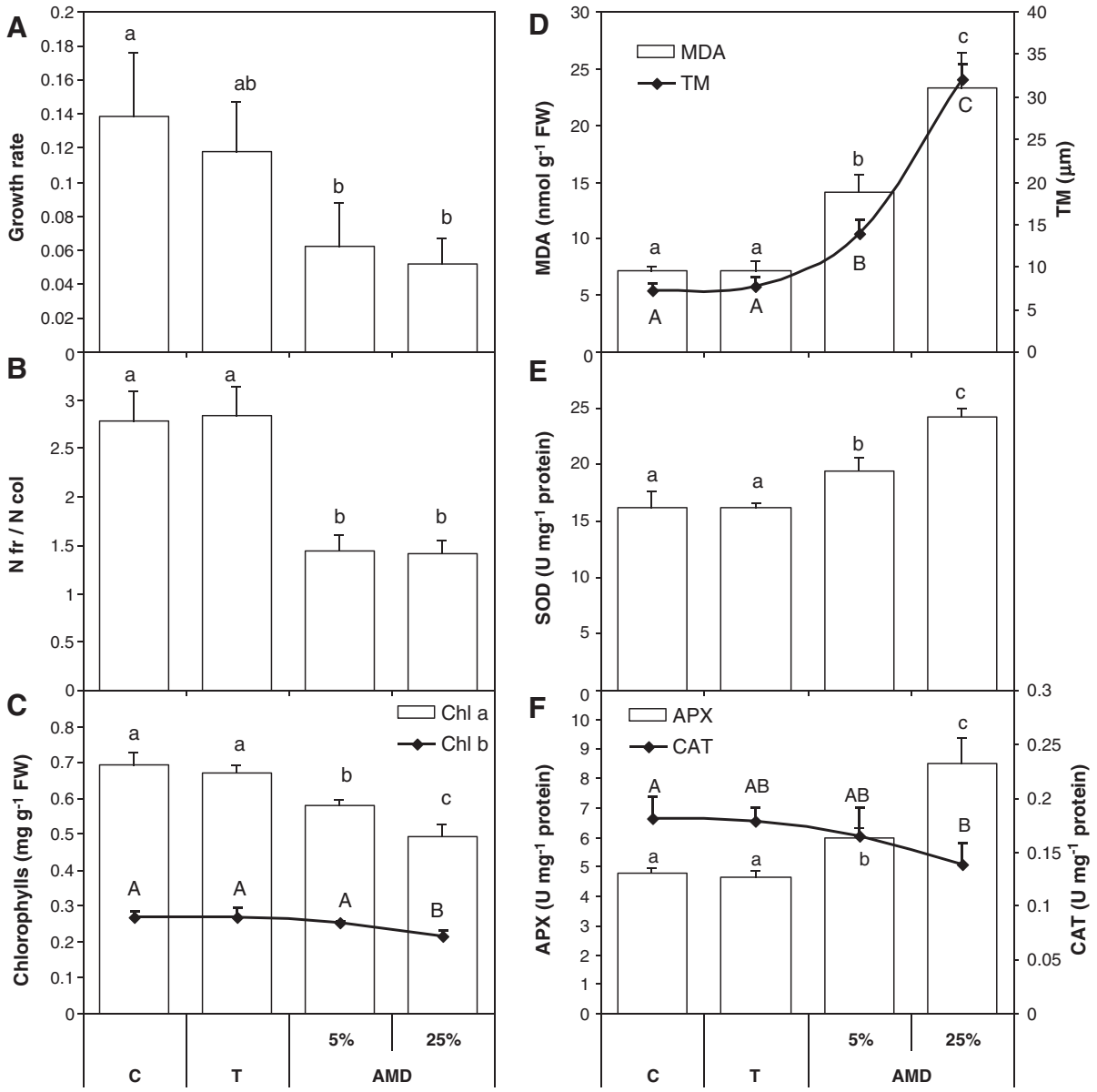

Fig. 2. (A) Relative growth rate and N fr/N colony ratio, (B) contents of chlorophyll *a* (Chl a) and chlorophyll *b* (Chl b), (C) NP thyols, (D) malondialdehyde content (MDA) and DNA damage — tail moment (TM), (E) superoxide dismutase (SOD), (F) ascorbate peroxidase (APX) and catalase (CAT) in duckweed exposed for 24-h period to control media (C), media containing untreated AMD (5 and 25%) or treated (T) AMD. Standard deviations were presented by error bars. Different letters indicate significantly different values at $p < 0.05$.

directly, but generate oxidative stress by interfering with the plant's antioxidant defense system (Radić et al., 2010a). In the present work, antioxidant enzymes SOD and APX exhibited significant increase in their activity following exposure to AMD (Fig. 2E, F). High increase in SOD activity suggests that there was a quick breakdown of superoxide radicals which was followed up by action of APX, a H_2O_2 destroying enzyme. On the other hand, the activity of CAT markedly declined in duckweed exposed to 25% AMD (Fig. 2F) which might be due to metal-induced inhibition of enzyme synthesis or due to a change in the assembly of enzyme subunits.

Table 3
Coefficients of determination (R^2) and their respective significance level (p) between different parameters measured in Lemna, Allium and comet assay.

Parameter	Growth rate		Root length		Nfr/Nkol		Chl a		Chl b		Tail moment	
	R^2	p	R^2	p	R^2	p	R^2	p	R^2	p	R^2	p
MDA	0.83	0.09	0.97	0.015*	0.77	0.123	0.98	0.008*	0.99	0.006*	0.98	0.012*
Tail moment	0.68	0.177	0.86	0.049*	0.61	0.222	0.92	0.040*	0.99	0.003*	–	–
SOD	0.81	0.101	0.96	0.020*	0.75	0.125	0.98	0.012*	0.99	0.003*	0.97	0.016*
APX	0.74	0.14	0.92	0.041*	0.67	0.179	0.95	0.028*	1	<0.001*	0.99	0.003*
CAT	0.88	0.063	0.99	0.006*	0.82	0.092	0.99	0.004*	0.97	0.015*	0.94	0.030*
Abberant cells (%)	1	<0.001*	0.93	0.037*	0.99	0.006*	0.89	0.048*	0.73	0.144	0.67	0.183

* Significantly different at $p < 0.05$.

Similar to results obtained by Allium test, the applied treatment significantly reduced phytotoxicity and genotoxicity of AMD on duckweed as all parameters showed values similar to control.

In conclusion, our results indicate that AMD has the toxic and genotoxic potential that led to adverse effects in microcrustacean *Daphnia* and plants — onion and duckweed. The results obtained also demonstrate that, beside daphnids and *A.cepa*, *L.minor* can also be successfully employed as a test system suitable in assessment of acute (geno)toxic effects of burdened waters such as acid mine drainage. *L. minor* was shown to be even more sensitive than *A.cepa* which is not unexpected as duckweeds take up water and different substances directly through the leafy fronds. Certain indicators displayed a consistency with each other as root length and chlorophylls showed strong correlation with genotoxicity and oxidative stress parameters (Table 3). A simplified comet procedure modified for plant tissue and antioxidative enzymes confirmed their applicability as sensitive markers of stress caused by environmental pollution.

As waters similar to AMD may pose a risk on the health of exposed human populations, the toxicity/genotoxicity bioassays should be used in AMD risk assessment together with physicochemical analysis. The combined CaO/electrochemical procedure proved to be highly efficient in removing toxicity and genotoxicity of AMD thus enabling safe discharge of such waters into environment.

Conflict of interest

Authors declare there are no potential or actual conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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