Mine Water Environ (2018) 37:88–97
DOI 10.1007/s10230-017-0472-2

TECHNICAL ARTICLE

Toxicological Analysis of Acid Mine Drainage by Water Quality and Land Use Bioassays

Soledad Chamorro[1] · Carlos Barata[2] · Benjamín Piña[2] · Marta Casado[2] · Alex Schwarz[3] · Katia Sáez[4] · Gladys Vidal[1]

Received: 20 November 2016 / Accepted: 5 June 2017 / Published online: 10 June 2017

Abstract AMD toxicity was evaluated using water quality and land use bioassays. In particular, we determined concentrations for lethal and sublethal effects as part of the risk assessment process. *Lactuca sativa* (lettuce), *Raphanus sativus* (radish), and *Triticum aestivum* (wheat) were used to predict phytotoxic effects caused by metals, as oxidative stress inhibits germination. The results, expressed as the fraction of AMD resulting in 50% lethality (LC50) at 144 h, showed that wheat was more tolerant (LC50 = 62%) than radish (LC50 = 17.0%) or lettuce (LC50 = 21%). The AMD was found to be very toxic to *Daphnia magna* (cladoceran) and *Danio rerio* (zebrafish) embryos, two of the best model organisms in aquatic ecology and ecotoxicology (LC50 <1%). However, when EDTA was used to chelate the metals, the animal toxicity effects was essentially eliminated, pointing to metal content as the main driver of toxicity. Analysis of two molecular biomarkers for an organic pollutant's toxicity, *cyp19a1b* and *cyp1a*, showed no activation of either gene, further indicating that the toxicity was mainly associated with the metals. The results also suggest that the environmental risk associated with the AMD could be largely mitigated by relatively cheap and simple measures to reduce metal mobility, like liming.

Keywords Environmental impact · Mining industry · Acids wastes · Bioindicators

Introduction

Acid mine drainage (AMD) is one of the most significant environmental challenges facing the mining industry worldwide. Water infiltrating through metal sulphide minerals, effluents of mineral processing plants, and seepage from tailing dams becomes acidic, allowing metals to be transported in their most soluble form (Sheoran and Sheoran 2006), with extremely adverse environmental effects for aquatic ecosystems (Yim et al. 2006). In particular in Chile; more than 95% of the country's mines contain porphyry copper deposits (Obreque-Contreras et al. 2015) and their exploitation has generated millions of tons of sterile wastes and large AMD releases.

High concentrations of metals can affect freshwater species, including microalgae, microcrustaceans, and fish (Ibemenuga 2013; Lopes et al. 1999). An ecotoxicological assessment of mine water usually starts by assessing the concentrations of metals (e.g. Fe, Mn, Cu, As, Ba, Cd, Zn, Ni, and Pb) (Rügner et al. 2006). However, AMD has numerous interactive factors, i.e. potentially toxic elements can react with each other and form non-hazardous precipitates (Lopes et al. 1999). Metal distribution, speciation, and bioavailability in sediments and the water column can have deep impact by AMD toxicity. So, the environmental effects could be evaluated by biological assays (Yim et al. 2006).

✉ Gladys Vidal
glvidal@udec.cl

[1] Engineering and Environmental Biotechnology Group, Environmental Science Faculty and Center EULA-Chile, University of Concepción, Concepción, Chile

[2] Department of Environmental Chemistry, IDAEA-CSIC, Barcelona, Spain

[3] Hydraulic and Environmental Laboratory, Civil Engineering Faculty, University of Concepción, Concepción, Chile

[4] Department of Statistics, Faculty of Mathematics and Physical Sciences, University of Concepción, Concepción, Chile

Daphnia species (sp) have been used in standard toxicity tests for many years due to their high sensitivity, manageability, and high parthenogenetic reproduction rate, which ensures a uniform response (Chamorro et al. 2010, 2016). Furthermore, these species plays an important role in the aquatic food chain (Khan et al. 2014; Reichwaldt et al. 2013). *Daphnia sp* are non-selective filter-feeders, unable to differentiate the quality of its consumed food (Chamorro et al. 2010, 2016). Thus, the process of feeding has a direct influence on the physiological performance in terms of growth, metabolism, and reproduction (Allen et al. 1995). Small freshwater fish species *Danio rerio* (zebrafish) have been widely utilized as a vertebrate model organism in different areas of research, including drug screening and toxicology (He et al. 2014; Hill et al. 2005), due to the small size, low housing cost, short embryonic development, high fertility, and the transparency of their externally differentiating embryos. Zebrafish has been extensively used in environmental monitoring of animal and human health. For example, induction of the cytochrome P450 (*cyp1a*) enzyme is used to indicate exposure of fish to dioxin-like compounds (Spitsbergen and Kent 2003), as an expression of *cyp19a1b* is a marker for exposure to estrogens, even in embryos (Hao et al. 2013).

Most metals are plant growth inhibitors, exerting various adverse effects leading to phytotoxic responses and to decreased yield and quality of agricultural crops (Yang et al. 2010). Phytotoxic effects caused by metals are related to oxidative stress. Concentrations between 1.5 and 10 mM of Cu and/or Zn inhibit germination and early growth of barley, rice, and wheat (Mahmood et al. 2007). Cu, Zn, and other elements interfere with cellular division, diminish respiration at the roots, reduce water intake, and alter the transport and metabolism of various essential nutrients (Casierra-posada and Cárdenas-Hernández 2007). As a consequence, inhibition in germination and in elongation of the radicle and epicotyl are determinant final points for assessment of AMD's phytotoxic effects (Bagur-González et al. 2011).

Although AMD can be treated at the mine, e.g. by neutralization using lime (Akcil and Koldas 2006) or by photocatalytic process (Yeber et al. 2009), these processes are never fully effective because sulfate concentrations are usually not affected. The aim of this was to evaluate the toxicity of AMD using water quality and land use bioassays.

Materials and Methods

Acid Mine Drainage Samples

AMD was collected from an active mining company in northern Chile. The area experiences a desertic climate, with rainfall limited to summer when it sometimes experiences "altiplanic winter" episodes of heavy rain. This area has a long history of mining activities, especially copper mining.

The samples were collected from the influent of the high sludge density plant on Dec 2015 in new 20 L plastic cans that had been rinsed twice with AMD. The samples were neither filtered nor acidified.

The metal content (Fe, Mn, Zn, Cu, and Al) in the AMD was analyzed by atomic absorption spectroscopy (AAS) (AAnalyst400, PerkinElmer). Sulphate content was quantified according to HACH, Sulfaver 4500E by Standard Methods (APHA 2005). Electrical conductivity, pH, and oxidation–reduction potentials (OPR) were measured using electrodes HQ40d (Hach) and Orion 370 Thermo. A 4 mg L^{-1} solution prepared by dilution of a 1000 mg L^{-1} (Cu $(NO_3)_2$ copper standard in 0.5 mol L^{-1} HNO_3, Merck. CertiPUR®), traceable to NIST, was used for AAS calibration. The metal concentrations were determined using the standard addition method (1000 mg L^{-1}. Merck, CertiPUR®). For each metal analysis, a calibration curve was made with at least five standards. The EC electrode was calibrated with 011007 1413 µS/cm EC/TDS and 011006 12.9 mS/cm EC/TDS standards. Standard 967961, $E = 220 \pm 3$ mV at 25 °C was used for ORP calibration. The pH measurements were calibrated using buffer Orion 910104, pH 4.01 ± 0.1 (H_2O, $C_6H_5O_4K$), 910107, pH 7.00 ± 0.1 (H_2O, Na_2HPO_4, KH_2PO_4, $C_4H_4BrNO_4$), and 910110, pH 10.01 ± 0.1 (H_2O, Na_2CO_3, $NaHCO_3$, $C_8H_8O_3$) traceable to NIST.

Daphnia magna Acute Toxicity Assay

Daphnia magna individuals were obtained from continuous cultures maintained in the Bioassay Laboratory, Faculty of Environmental Sciences and EULA-Chile at the University of Concepción. Stock cultures were maintained at 20 ± 2 °C with a photoperiod of 16 h light and 8 h dark (Xavier et al. 2005). Feeding regimen and make up of reconstituted water followed USEPA (1994). The composition of culture water consisted of $NaHCO_3$ (2.59 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (4.93 g L^{-1}), KCl (0.23 g L^{-1}), and $CaCl_2 \cdot 2H_2O$ (11.76 g L^{-1}) to 1 L. Hardness of the culture medium was maintained at 250 ± 25 mg L^{-1} and the pH ranged between 7.5 and 8.6 (NCh 2083 1999). The chamber water was changed every 48 h, which promote parthenogenesis population growth (Xavier et al. 2005). Bioassays were conducted at the same temperature and photoperiod conditions.

Acute toxicity was observed in neonates of *D. magna* (<24 h old) exposed to AMD at 24 and 48 h. Three concentrations (AMD dilutions) were evaluated. 0.1% v/v, 0.01% v/v, and 0.001% v/v denominated D1, D2, and D3, respectively, besides the control (the water culture medium

for *D. magna*). Four replicates of 25 mL (each one containing five organisms) were performed for each condition. At the end of each exposure, the concentration that generated a 50% mortality (LC50) of the *D. magna* population was determined.

Zebrafish Embryo Assays

Wild type zebrafish purchased from a local dealer were maintained at 27 ± 1.5 °C in a 12 h light, 12 h dark cycle using reconstituted water (Instant Ocean 90 mg L^{-1}; 0.58 M $CaSO_4{\cdot}2H_2O$ in reverse osmosis purified water) (Pelayo et al. 2011). Zebrafish embryos were collected following adult mating and maintained at the same conditions as adults. For the zebrafish embryo test (ZFET), fish were selected 24 h post-fertilization (hpf) and placed in each well in six-well plates (NUNC, Roskilde, Denmark) with 5 mL of AMD dilutions 0.1% v/v, 0.01% v/v, and 0.001% v/v, D1, D2, D3, respectively, with and without EDTA (E) 1 µM (D1 + E, D2 + E, D3 + E) and control. In addition, exposure to Cu(II) and Zn(II) solutions at 0.3 and 67 mg L^{-1}, respectively, was also evaluated.

Zebrafish embryos were observed daily (48–72 hpf) under a stereomicroscope (NIKON SMZ 1500) for modification endpoints such as egg coagulation, delayed hatching, and deformities (heart or yolk edema and tail malformation) according to Scholz et al. (2008). Six replicates (ten organisms) were performed for each treatment and control. For individual metals, three replicates were performed. After 48 and 72 hpf, the embryos were immediately frozen and kept at −80 °C until processed for reverse transcription polymerase chain reaction (qRT-PCR).

RNA Quantification by qRT-PCR

Total RNA was isolated from 30 frozen embryos using the Trizol reagent protocol (Invitrogen Life Technologies, Carlsbad, CA). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality-checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). The RNA was treated with DNAseI (Ambion, Austin, TX) to remove genomic DNA contamination, and retro-transcribed to cDNA using a First Strand cDNA synthesis kit (F. Hoffmann-La Roche, Basel Switzerland). Appropriate primers for *cyp1a, cyp19a1b* (target gene), and ppia2 (reference gene) (Morais et al. 2007) were designed and validated (Pelayo et al. 2011). PCR products (amplicons) were sequenced in an Applied Biosystems 3730 DNA analyzer and compared to the corresponding reference sequences at the NCBI server. Aliquots of 40 ng of total RNA were used to quantify specific transcripts in a LightCycler 480 real-time PCR system (F. Hoffmann-La Roche) using SYBR Green Mix (Takara Bio INC, Siga, Japan). PCR efficiency values for reference and tested genes were calculated using at least five serial 1:3 dilutions for each gene (Pfaffl 2001). Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated in triplicate). The Cp values for the target genes (*cyp1a, cyp19a1b*) were compared to the corresponding values for the reference gene (ppia2), according to Olivares et al. (2013).

Seed Germination Bioassay and Phytotoxicity Indicators

Seed germination, root growth, hypocotyl growth, and coleoptile growth tests were performed on *Triticum aestivum* (wheat), *Raphanus sativus* (radish), and *Lactuca sativa* (lettuce). Seeds of uniform size were exposed to five dilutions of AMD (6.25, 12.5, 25, 50, and 100% v/v). 5 mL of AMD were poured onto filter-paper disks covering the bottom of petri dishes with ten seeds and incubated in each dish at room temperature (20 °C) with light for 144 h. The 50% lethal concentration (144 h-LC50) was defined as the concentration that inhibited 50% of the seed germination. The sprouts were counted and their hypocotyl for *R. sativus* and L. *Sativa*. The coleoptile was measured in *T. aestivum*. Each bioassay was performed in triplicate (Villamar et al. 2014). The control consisted in distilled water. The percentages of germination inhibition (PGI) after 144 h of exposure were calculated as in Eq. 1.

$$\mathrm{PGI} = \frac{\mathrm{GC} - \mathrm{GS}}{\mathrm{GC}} \times 100. \tag{1}$$

in which GS is the number of seeds germinated in the AMD, GC represents the number of seeds germinated in the control, and PGI the percentage of ungerminated seeds. PGI values below 10% were considered indicative of non-toxicity, values between 10 and 25% corresponded to moderate toxicity, and PGI values above 25% indicated strongly toxic samples.

Additionally elongation root (RI), hypocotyl (HI), and coleoptile indexes were calculated, using Eqs. 2, 3, and 4 (Bagur-González et al. 2011). In statistical terms, they represent the normalized residual elongation of the germinated seeds per treatment.

$$\mathrm{RI} = \frac{\text{elong sample} - \text{elong control}}{\text{elong control}}, \tag{2}$$

$$\mathrm{HI} = \frac{\text{elong sample} - \text{elong control}}{\text{elong control}}, \tag{3}$$

$$\mathrm{CI} = \frac{\text{elong sample} - \text{elong control}}{\text{elong control}}. \tag{4}$$

The indices were designed so that their values could vary from −1 (maximum phytotoxicity) to 0. In addition, a reduction of 50% in the variable studied (RI or HI) relative to the blank control (RI50 or RE50) was considered a measurement of potential "chronic toxicity", as this relatively short test is considered an indicator of the potential long-term influence of pollutants. This enabled us to establish the following scale: (a) 0 to −0.25 low toxicity, (b) −0.25 to −0.5 moderate, (c) −0.5 to −0.75 high and (d) −0.75 to −1, very high. RE values >0 would indicate seed growth stimulation (hormesis).

Statistical Analysis

Seed germination, root presence, root growth, hypocotyl presence, and hypocotyl growth for *L. sativa* and *R. sativus*, and seed germination, root presence, root growth, coleoptile presence, coleoptile growth, and secondary root number for *T. aestivum* were analyzed by one-way ANOVA tests with subsampling plus ad hoc Tukey's test to statistically define different subsets of samples. For root numbers, presence/absence of seed and root were analyzed using generalized linear models. Later, contrast analysis statistics test was used. The critical p value for all experiments was 0.05. The statistical treatment of the data was performed using "R" software.

Results and Discussion

Physicochemical Characterization of AMD

The AMD generated at the Chilean copper mine had a pH of 4.0 and elevated EC values of 4.78 mS cm^{-1}. Such parameters are well correlated with other variables such as sulfate concentration (2000 mg L^{-1}) and can be used as a field assessment (Jiménez et al. 2009). The very high concentrations on Cu (372.15 mg L^{-1}), Zn (77.80 mg L^{-1}), and Mn (124.55 mg L^{-1}) are consistent with the literature (Wang et al. 2012) (see Table 1). In contrast, the analysis showed a low concentration of organic matter; 37.45 mg L^{-1} (see Table 1).

Table 1 pH, electrical conductivity, oxidation reduction potential, chemical oxygen demand, mainly metals and sulphate concentration in copper acid mine drainage

Metal/parameter	Mean ± SD
Fe (mg L^{-1})	0.393 ± 0.006
Mn (mg L^{-1})	124.55 ± 1.39
Zn (mg L^{-1})	77.80 ± 1.34
Cu (mg L^{-1})	372.15 ± 2.90
Al (mg L^{-1})	36.03 ± 0.15
pH	4.01 ± 0.02
Sulphate (mg L^{-1})	2300 ± 0
EC (mS cm^{-1})	4.78 ± 0.01
ORP (mV)	280.30 ± 5.90
COD (mg L^{-1})	37.45 ± 4.16

EC electrical conductivity, *OPR* oxidation reduction potential, *COD* chemical oxygen demand, *SD* standard deviation

Toxicity Assays

Animal Toxicity Assays

Acute lethal toxicity tests showed that the AMD was toxic to both the *D. magna* (LC50 <1%) (Table 2) and *Danio rerio* embryos (LC50 <0.08%) (Fig. 1). Both toxic effects were eliminated when the chelator (EDTA) was added to the medium, pointing to metal content as the main drivers of animal toxicity. Alterations observed in the zebrafish embryos development are depicted in Fig. 2. Embryos exposed to AMD dilution 0.1% v/v show enlarged yolk, and pigmentation was missing in those exposed to 0.01 and 0.001% v/v dilution. Exposure to Cu and Zn solutions caused no mortality at 48 or 72 hpf, but Cu (67 mg L^{-1}) induced embryo deformation and a two-fold increase in yolk volume in 100% of the embryos (Fig. 2).

Metals such as Cu can exert a strong effect on *D. magna*. Toxicity of Cu has been described as nominal with levels for acute exposure around 0.2 mg L^{-1} (Winner and Farrell 1976; Yim et al. 2006). Different studies with daphnids have shown AMD of various origins to be very highly toxic, mainly due to dissolved metals (see Table 2) (Martins et al. 2007; Martin et al. 2009; Rádic et al. 2014). Cu is one of the most toxic elements to aquatic species; at levels just above that needed for growth and reproduction, it can accumulate and cause irreversible harm to some species (Tierney et al. 2010).

Various studies about toxicity using Daphnia and zebrafish as aquatic test models to metals (Chen et al. 2014; Xiao et al. 2015), drugs, etc., enable us to understand symptoms induced by that toxicant (Wei et al. 2010). Although detailed pathways and mechanisms of the toxic effects have not been clearly elucidated, in the context of ecological risk, genomic tools and fingerprinting approaches offer an exceptional platform for this analysis (Kim et al. 2015).

The coordinated activation of different stress mechanisms is a fundamental element of the overall response to pollutants and to other potentially deleterious external inputs (Jaumot et al. 2015). In order to evaluate the effects of the AMD at the molecular level, we measured the activation of two genes: *cyp19a1b* (brain aromatase, responsive to estrogens) and *cyp1a* (the genetic counterpart of the EROD test) in fish embryos. Results show no activation of either gene, further indicating that the toxic effects of AMD

Table 2 Toxicity level of acid mine drainage (AMD) and metals on *Daphnia* species

Species	Samples	Concentration						Effect	References
Daphnia magna	Untreated AMD	100%						24 h—EC50 = 1% 48 h—EC50 = 0.21% 48 h—immobility = 0.06%	Radić et al. (2014)
	Soil water-leachable extract	As (%)	Cd (%)	Cu (%)	Ni (%)	Pb (%)	Zn (%)		Alvarenga et al. (2012)
	Soil A	2	4	0.5	4	0.3	1	48 h—EC50 = 26% v/v	
	Soil B	8	9	1	2	0.1	3	48 h—C50 = 13% v/v	
	Soil C	9	8	7	11	0.2	26	48 h—EC50 = 2% v/v	
	AMD sediment	0.02, 0.04, 0.06, 0.08 gAMD sediment g total sediment^{-1}						24 h—0% hatching success	Aluma et al. (2011)
	AMD sediment	100%						48 h—LC50 = 35–63%	Soucek et al. (2000)
	AMD water column (imput in the watershead)	100%						48 h—LC50 = 27–69%	
	Untreated AMD	0.1, 0.01, 0.001%						48 h—LC50 = <1% v/v	In this study
Daphnia longispina	AMD	100%						48 h—LT50 = 3%	Martin et al. (2007)
Ceriodaphnia dubia	Cu	1.0–25.5 µg L^{-1}						48 h—LC50 = 18 µg L^{-1}	Cooper et al. (2009)
								48 h—LC50 = 208.8 µg L^{-1}	
	Pb	<0.1–17.9 µg L^{-1}						48 h—LC50 = 73.5 µg L^{-1}	
Daphnia carinata								48 h—LC50 = 37.3 µg L^{-1}	
	Zn	2–216.4 µg L^{-1}						48 h—LC50 = 444.0 µg L^{-1}	
								48 h—LC50 = 339.8 µg L^{-1}	

AMD acid mine drainage, *EC* effective concentration, *LC50* lethal concentration that generated mortality of 50% of pupulation, *LT50* median lethal time that generated mortality of 50% of pupulation

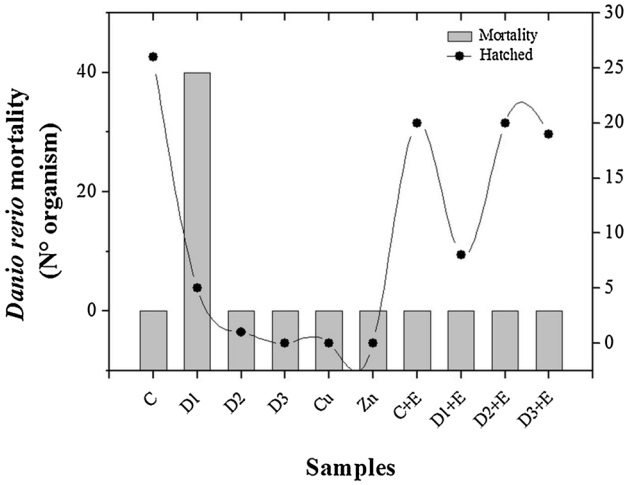

Fig. 1 Mortality and hatching in 72 hpf of *Danio rerio* exposed to acid mine drainage (AMD) concentrations: *C* control (Daphnia water medium), *D1* 0.1% v/v AMD, *D2* 0.01% v/v AMD; *D3* 0.001% v/v AMD; Cu (0.3 mg L^{-1}); Zn (67 mg L^{-1}); *C* + *E* control + EDTA 1 µM; *D1* + *E* 0.1%v/v AMD + EDTA 1 µM; *D2* 0.01% v/v AMD + EDTA 1 µM; *D3* 0.001% v/v AMD + EDTA 1 µM

were mainly related to metals (Fig. 3). In this sense, Truter et al. (2014) evaluated the effect of AMD-treated (95% neutralized AMD) in *Tilapia oreochromis* over the expression of thyroid receptor (*trα*), *trβ*, androgen receptor (*ar1*), *ar2*, glucocorticoid receptor (*gr1*), gr2, mineralocorticoid receptor (*mr*) and aromatase (*cyp19a1b*) quantified in juvenile fish after 48 h of exposure. Pronounced alterations in gene expression (*trα, trβ, gr1, gr2, ar1*, and *mr*) were associated with the higher concentrations of certain metals, which is evidence of endocrine disruptive activity associated with neutralized AMD, and also points to the metal content as the main toxic agent.

Seed Germination Bioassay

Germination is one of the most important processes in vegetal development and growth, since stabilization of cultures under stressful conditions depends on it (Chen et al. 2016). Germination starts with imbibition or water absorption by the seed, and finalizes with the elongation

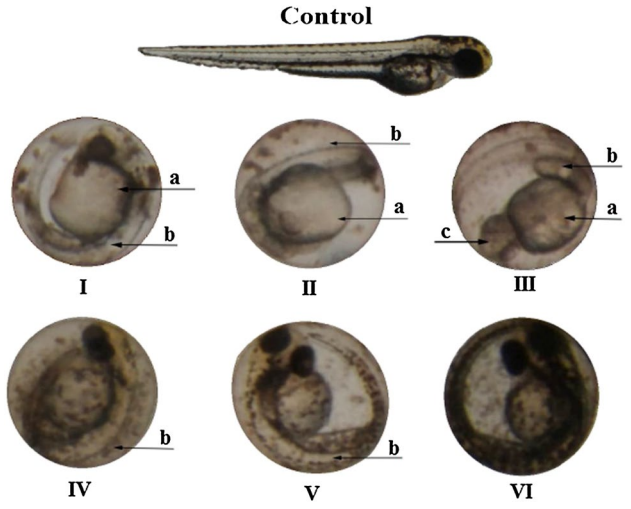

Fig. 2 Microscopy pictures of embryos and larvae in *Danio rerio* at different stages of development exposed to acid mine drainage (AMD). *Control* example of development (hatching) at 72hpf. **I** D1 (0.1% v/v AMD)—48 hpf; **II**: D2 (0.01% v/v AMD)—48hpf; **III** Cu- (0.3 mg L^{-1}) – 48hpf; **IV** D1 (0.1% v/v AMD)—72hpf; **V** D2 (0.01% v/v AMD)—72hpf; **VI**: D3 (0.001% v/v AMD)—72hpf. **IV**–**V** showed delays in hatching; *a* yolk sac edema, *b* embryos showed different degrees of hypopigmentation in muscle; *c* deformation during head development

of the embryonic axis, mainly from the root. Due to this fact, and as final points for assessment of the phytotoxic effects, inhibition in germination and in elongation of the root and epicotyl or hypocothyl was determined (Bagur-González et al. 2011). The results, expressed as the fraction of AMD content resulting in 50% lethality at 144 h (LC50) showed that wheat was more tolerant (LC50 = 62%) than radish (LC50 = 17%) or lettuce (LC50 = 21%).

Table 3 shows the root growth index (RI) of *T. aestivum, R sativus*, and *L. sativa*, exposed to AMD. Wheat showed higher tolerance to AMD than radish and lettuce, the latter displaying no elongation of the radicular at concentrations starting at 50%. The PGI index in *T. aestivum* and *R. raphanus* were not statistically different when they were exposed to a concentration of 6.25% v/v. Lettuce showed statistical differences with all tested concentrations. Specifically, lettuce germination was inhibited at AMD concentrations greater than 50% v/v.

In general, AMD strongly affected RI values, which ranged from 0.52 to 0.94. *R. sativus* (6.25% v/v) in particular was adversely affected, compared to *T. aestivum* at the same concentration. The index CI shows moderate toxicity measured in the coleoptide dimension when *T. aestivum* was exposed to 6.25–12.5% v/v of the AMD. However, when the AMD concentration exceeded 25% v/v, the toxicity of the *T. aestivum* was greater than for *R. sativus*.

Specific metals can alter cellular organelles such as the plasma membrane, cell wall, and the nucleus, and more specifically, can affect DNA (Casierra-Posada 2002). For example, Al reportedly interferes with cellular division at the roots, increases cellular wall rigidity via the crosslinking of pectins, diminishes respiration at the roots, modifies the structure and functioning of the plasma membranes, reduces water intake, and interferes with transport and metabolism of various essential nutrients (Casierra-Posada and Cárdenas-Hernández 2007). Mn is involved in secondary metabolism (e.g. lignin and flavonoid synthesis; Chen et al. 2016), while Zn is an essential micronutrient for animals, plants, and microorganisms (Dhankhar et al. 2012). Nevertheless, in excess, it accumulates in plant tissues, which causes physiological alterations and growth inhibition by oxidative damage to the membranes, and sometime

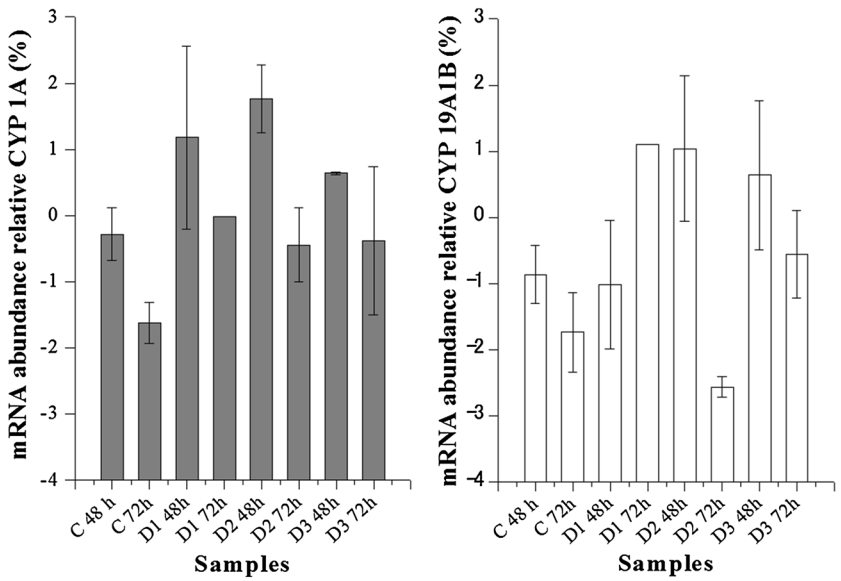

Fig. 3 mRNA expression relative of *cyp1a* and *cyp19a1b* in *Danio rerio* exposed to AMD concentrations. C: Control (Water medium of *Danio rerio*), *D1* 0.1% v/v AMD, *D2* 0.01% v/v AMD; *D3* 0.001% v/v AMD. Standard deviations were presented by *errors bars*

Table 3 Phytotoxic index for *Triticum aestivum, Raphanus sativus* and *Lactuca sativa* exposed to untreated acid mine drainage (AMD)

Specie/index	AMD (% v/v)	PIG (%)	RI	HI	CI
T. aestivum	6.25	0	−0.63	NA	−0.42
	12.5	23.3	−0.70	NA	−0.46
	25	63.3	−0.84	NA	−0.75
	50	46.7	−0.90	NA	−0.67
	100	50	−0.85	NA	−0.72
R. sativus	6.25	6.7	−0.52	0.72	NA
	12.5	50	−0.79	−0.81	NA
	25	66.7	−0.90	−0.84	NA
	50	80	−0.67	−0.83	NA
	100	100	UG	UG	NA
L. sativa	6.25	23.3	−0.90	−0.95	NA
	12.5	23.3	−0.94	−0.90	NA
	25	46.7	−0.94	−0.92	NA
	50	100	UG	UG	NA
	100	100	UG	UG	NA

AMD acid mine drainage, *PIG* percentage inhibition germination, *RI* index root growth, *HI* index hypocotyl growth, *CI* index coleoptile growth (only applied in *T. aestivum*), *UG* ungerminated, *N.A* not applicable

reaching toxic levels of 300 μg g^{-1} in plants (Barrameda-Medina et al. 2014).

The mechanisms involved in root growth inhibition are not well established. However, this phytotoxic effect is likely related to the ability of metals to accumulate and interact with specific sites located in the cell wall (e.g. pectins and hemicelluloses), in the plasma membrane (e.g. lipids or proteins), and in the cell nucleus (Ezaki et al. 2008; Horst et al. 2010).

Table 4 shows a statistical analysis of mean values of seed germination, root presence, root growth, hypocotyl presence, and hypocotyl growth measured in *L. sativa* and *R. sativus*. The seed germination and root growth of *L. sativa* were not significantly different from the control when exposed to AMD concentrations between 6.25–25% v/v. However, toxicity was evident in samples at concentrations greater than 50% v/v AMD. The elongation (exposed to 6.25–25% v/v of AMD) values were below 0.1 cm, compared to the control (2.0 cm). A significant reduction of hypocotyl presence was observed, even in the 12.5–25% v/v AMD samples, but the difference was less for hypocotyl growth, due to size uniformity. These results confirm the toxicity obtained by RI and HI.

Moreover, at AMD concentrations between 6.25 and 25% v/v, seed germination and root growth of the plants

Table 4 Analyses by statistical contrast of mean values of seed germination, root presence, root growth, hypocotyl presence and hypocotyl growth observed in *Lactuca sativa* and *Rapahnus sativus*

Specie/samples	Control	AMD concentration (% v/v)				
		6.25	12.5	25	50	100
Lactuca sativa						
Seed germination (%)	100a	76.67b	76.67b	53.33b	UG	UG
Root presence (%)	100a	13.33b	16.67b	6.67b	UG	UG
Root growth (cm)	2.04a	0.03b	0.01b	0.01b	UG	UG
Hypocotyl presence (%)	100a	76.67b	66.67bc	43.33c	UG	UG
Hypocotyl growth (cm)	0.54a	0.13b	0.22b	0.17b	UG	UG
Raphanus sativus						
Seed germination (%)	100a	93.33a	50b	33.33bc	20c	UG
Root presence (%)	100a	83.33b	46.67c	13.33d	6.67d	UG
Root growth (cm)	1.56a	0.66b	0.30b	0.26b	0.05b	UG
Hypocotyl presence (%)	100a	90b	50c	33.33cd	20d	UG
Hypocotyl growth (cm)	0.92a	0.74ab	0.53ab	0.46ab	0.44b	UG
Triticum aestivum						
Seed germination (%)	100a	100a	76.67b	53.33bc	53.33bc	50c
Root presence (%)	100a	86.67b	50c	26.67c	26.67c	33.33c
Root growth (cm)	5.19a	1.67b	0.75c	0.40c	0.24c	0.49c
Coleoptile presence (%)	100a	96.67a	73.33b	53.33b	53.33b	50b
Coleoptile growth (cm)	2.81a	1.56b	1.51b	0.70c	0.92c	0.76c
Secondary root (number)	2.10ab	2.63a	0.92b	1.12b	0.80b	1.33ab

For *Triticum aestivum* was detected seed germination, root presence, root growth, coleoptile presence, coleoptile growth and secondary root number exposed to acid mine drainage (AMD). a,b,c,d: Mean values follow by same letter are not significantly different ($p << 0.05$). So, "a" is for the major mean value, "b" for the one that follows, etc. In this table the analysis is per row

UG ungerminated

were not significantly different from the control. However, toxicity was evident in samples with concentrations exceeding 50% v/v AMD.

Seed germination of the *R. sativus* (93.3%) was not significantly different from the control. This suggests that *R. sativus* is more tolerant than *L. sativa* to AMD. Incremental toxicity was apparent, especially with the maximum AMD concentration, 100% v/v (20% germinated).

T. aestivum seed germination was similar to *R. sativus*, but exhibited total germination in 100% v/v AMD, unlike the other species in this study. There was no difference between 12.5 and 50% v/v AMD, or between 25 and 100% v/v. In particular, the root presence and growth were not significantly different at 12.5% v/v. Moreover, Coleoptile was present in all seeds exposed to 6.25% v/v AMD. There were no difference for the other samples (12.5 to 100% v/v), showing values of 73.3–50%, respectively. Concerning growth, the control averaged 2.81 cm elongation in contrast to 1.56 cm for 6.25% v/v AMD. A significant reduction of coleoptile length compared to the control was observed with 25% v/v AMD, with values less than 1 cm. The secondary root number was measured for *T. aestivum*. Seeds exposed to 6.25% v/v AMD had a greater root number, 2.6 cm, than the control (2.1 cm). The same effect was observed with 25 and 100% v/v AMD. This is probably an indication of a physiological ability to resist adverse conditions.

Previous studies have showed that metals in AMD, such as Cu, Hg, Zn, Cd, Co, Pb, and Ni reduce germination in lentil, radish, mustard, and rice seeds. It appears that seed coats are the main barriers to metals and prevent embryo contamination until the seed coat is torn apart by the germinating embryonic root (Munzuroglu and Geckil 2002; Srivastava et al. 2006). However, low pH and high EC might have contributed to inhibited growth at EC values over 4 mS cm^{-1} (Radić et al. 2014). Undiluted AMD and 50% v/v AMD were observed to cause acute toxicity in *Lemna minor* (plant), which was manifested in rapid (less than 24 h) bleaching of plant tissue (Radić et al. 2014).

This physicochemical and biological assessment suggests that the metals in AMD are largely responsible for AMD toxicity. The contaminants, fate, and transport of the metals, and the required treatment or remediation methods are all necessary components of a remediation strategy. In this sense, prediction and prevention of AMD are the best way to minimize its environmental impact. In the same way, information on copper mining activities are essential to generate legal monitoring frameworks in Chile and other countries.

Conclusions

AMD showed significant phytotoxicity, particularly for radish and lettuce, whereas wheat appeared to be relatively tolerant. It was also very toxic to *Daphnia magna* and *Danio rerio*. Animal toxicity was essentially eliminated by the presence of a metal chelator, EDTA. The *cyp19a1b* and *cyp1a* gene expression studies showed no changes in either gene, further indicating that the toxicity of AMD was mainly related to its metal content.

Acknowledgements This work was supported by CONICYT/FONDAP/15130015 Grants.

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