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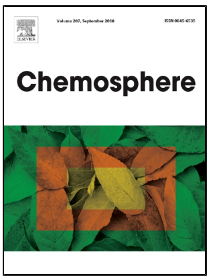

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Toxicity of biomining effluents to *Daphnia magna*: Acute toxicity and transcriptomic biomarkers

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Toxicity of biomining effluents to *Daphnia magna*: Acute toxicity and transcriptomic biomarkers

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Abstract

Increasing metal consumption is driving the introduction of new techniques such as biomining to exploit low grade ores. The biomining impacts notably aquatic ecosystems, yet, the applicability of ecotoxicological tests to study the complex mixture effects of mining waters is insufficiently understood. The aim of the present work was to test if transcriptomic biomarkers are suitable and sensitive for the ecotoxicity assessment of biomining affected waters. The study site had been affected by a multimetal biomine, and the studied water samples formed a concentration gradient of contamination downstream from the biomining site. Cadmium and nickel were used as positive controls in the toxicity tests. Selected transcriptomic biomarkers, previously shown to be differentially regulated by metals, were used to evaluate the ecotoxicity of the water samples. Parallel samples were used to compare the transcriptomic biomarkers with the conventional acute *D. magna* toxicity test. In the acute test, one sample was acutely toxic to *D. magna,* when pH was adjusted according to the standard, whereas, in the native pH, three samples caused total immobility. Monooxygenase was up-regulated by the highest concentration of Cd in control samples and three of the water samples. Vtg-SOD was up-regulated by one of the water samples, and

catalase by the second highest concentration of Cd. The results show that transcriptomic biomarkers in *D. magna* can be used as sensitive bioindicators for metal mixture toxicity assessment in complex environmental water samples.

Key words

Metals, mixture toxicity, mining effluent, bioleaching, sub-lethal, mRNA

1 Introduction

Mineral mining is increasing due to global economic growth and consequent demand for the metals (Pokhrel and Dubey 2013). While the demand for most metals has steadily increased in the last decade, number, grade and quality of new ore deposits have declined (Brierley 2008, Brierley and Brierley 2013). The bio-hydrometallurgy i.e. biomining is particularly suitable technically and economically for processing lower grade and complex polymetallic mineral assemblages (Brierley 2008, Brierley and Brierley 2013). Biomining simplifies the enrichment process of sulphide ores as microbes are used to oxidize insoluble metal sulphides to soluble metal sulphates, which are then extracted from leachates (Morin et al. 2008). Biomining also has lower process temperatures, lower energy costs and smaller carbon footprints than conventional mining (Morin et al. 2008). Thus, biomining has been perceived as a more environmentally benign approach than conventional mining processes (Morin et al. 2008, Johnson 2014, 2015). The contamination from conventional mining include CO_2 emissions, SO_2 emissions and acid mine drainage (AMD) (Dold 2008). Although biomining consumes less energy, it is likely to cause similar releases of salts, heavy metals and acid mine drainage (AMD) to the environment as processing with other techniques such as floatation (Dold and Weible 2013). As the utilisation of biomining is likely to increase (Brierley 2008, Brierley and Brierley 2013), and novel approaches such as *in situ* biomining are developed (Johnson 2015, Morin et al. 2008) the environmental impacts of biomining, particularly, on water resources, should be studied.

Biochemical biomarkers have been proposed as sensitive tools for risk assessment (Jemec 2010). Biomarkers can be used for understanding the mechanism of toxic action, for screening of unknown pollutants in the environment (i.e. stressor identification), and for detecting early signs of chronic toxicity (Snell et al. 2003). Biochemical biomarkers are generally considered to be more sensitive to stressors than whole-organism responses are (Jemec 2010). Effect based tools such as toxicogenomics can be used for monitoring water quality, and for identifying either priority groups of pollutants or potentially affected biological targets (Brack et al. 2015). Toxicogenomics in *D. magna* have been shown as a useful tool for environmental monitoring of a copper mining contaminated site (Poynton et al. 2008).

The aim of the present study was to test if transcriptomic biomarkers are suitable and sensitive for the ecotoxicity assessment of biomining affected waters. The study site was polluted by a multimetal mine that uses bioleaching. The studied water samples formed a concentration gradient of contamination downstream from the biomining site. The water flea *Daphnia magna* was selected as a test species as it is a widely studied species, which is known to be sensitive to metal contamination and metal mixture contamination (Okamoto et al. 2015, Yim et al. 2006). *D. magna* represents also an important trophic level in an aquatic food chain. Selected transcriptomic biomarkers were studied and compared to the conventional acute toxicity test with *D. magna*. First, preliminary testing was conducted with a set of known biomarker genes that had shown differential expression upon metal exposure (Poynton et al. 2007, Kim et al. 2010). Monooxygenase (*mox*), vitellogenin superoxide dismutase (*vtg-sod*) and catalase (*cat*) showed promising results and were selected for this study. Secondly, the observed changes in the expression of biomarker genes were compared against the background variables including physicochemical water characteristics, to reveal the underlying causes.

Materials and methods

2.1 Study site

The water samples for this study were collected from two watersheds that receive effluents from the Talvivaara multimetal mine in Sotkamo, Finland. The mine uses bioheapleaching to recover metals from low-grade ore. The Talvivaara deposit is hosted by metamorphosed black shales (black schists) and contains 300 million metric tons (Mt) of low-grade ore averaging 0.26 percent Ni, 0.14 percent Cu, and 0.53 percent Zn (Loukola-Ruskeenniemi, et al. 1996). The main products of Talvivaara are a mixed nickel cobalt sulphide, copper sulphide and zinc sulphide (Riekkola-Vanhanen 2010). In Talvivaara, an environmental accident happened in November 2012 affecting waterways of Oulujärvi and Vuoksi. The wall of a storage pond ruptured and acidic waste water containing raffinate from enrichment process leaked to the nearby waterways (Onnettomuustutkintakeskus, 2014). According to the report of Finnish Environmental institute, the main contaminants were Ni, Zn, Cd, Al, U and salting due to sulphate (Kauppi et al. 2013). The accident also caused fluctuating pH, as the waste water was acidic and the accident treatment included occasional neutralisation of waters with lime addition (Kauppi et al. 2013).

2.2 Water samples and chemical analyses

As a part of a larger monitoring survey of the effects of the spill, eight water samples were collected by a consulting company and Kainuu Centre for Economic Development, Transport and the Environment between February and March within 11 days from the rivers and lakes affected the most three months after the accident (see the map in Figure 1). Seven of the samples were from the watershed of Oulujärvi and one (River Lumijoki) from the watershed of Vuoksi (Figure 1). River water samples were taken 0.5-1.0 meter below water surface into 10-20 l plastic containers, rinsed with the water at the site and stored at 4 °C until exposures. The lake water samples were taken one

meter above the lake bottom with a Limnos sampler. The lake water sampling was designed to study concurrent effects of metals and salts in the lakes hypolimnion. Representative one litre water samples for the chemical analyses were taken at the same sampling site as the samples for the exposures. To measure the total and dissolved concentrations of elements two parallel 100 ml sub-samples were taken. Both of the 100 ml sub-samples were preserved with 0.5 ml of HNO_3 (100441, Suprapur 65%, Merck, Germany), and the sample representing dissolved concentration was filtered (GD/XP, 0.45 µm, Whatman) in the field.

Concentration of Al, As, Ba, Ca, K, Mg, Na, S, Sr and Ti were analysed with ICP-OES following standard SFS-EN ISO 11885:2009. Cd, Co, Cr,Cu, Ni, Pb, Sb, Se, Zn, U and V were analysed with ICP-MS according to SFS-EN ISO 17294-1:2006 and 17294-2:2005. Fe and Mn were measured with IRIS Intrepid II XSP (Thermo Scientific). For the total concentration analyses the samples were microwave digested with HNO_3 (ISO 15587- 2:2002). Analyses were performed in accredited laboratories (EN ISO/IEC 17025) of the Finnish Environmental institute (FINAS T003, K054) and Nablabs Ltd. (FINAS T111, T142). The measurements were done according to standards (standard number given in brackets): Oxygen concentration at field (SFS-EN 25813:1996), pH (SFS-EN ISO 10523-2012), conductivity with temperature compensation to 25 °C (SFS-EN 27888-1994), solids content (SFS-EN 872:2005), total hardness (SFS 3003:1987), fluoride (SFS-EN-ISO 10304-1:2009), and sulphate (SFS-EN10304-2009).

2.3 Acute toxicity assays

The 24-hour acute toxicity assays were performed according to the ISO standard (6341:2012). The *Daphnia magna* neonates originated from dormant eggs (MicroBioTests Inc., Belgium), which were cultivated in the laboratory for several generations prior to deployment in the toxicity assays. To preserve the environmental relevance of the samples they were pre-treated as little as possible prior to the acute toxicity testing (e.g. samples were not diluted). The samples were vacuum filtrated (Whatman 25-mm GD/XP syringe filter, pore size 0.45 µm) to remove the particulate material.

Samples were tested both in original and in adjusted pH. The toxicity assay was replicated five times for each of the samples. Each replicate contained five *Daphnia magna* neonates (< 24 h) in 10 ml volume, which contained 9 ml of the sample and 1 ml of the ISO test water (ISO 6341:2012). ISO test water addition was due to neonate transfer, which was done with an automated pipette using 200 µl of ISO test water per neonate. Therefore, the final sample concentration in the test flask was 90%. The sample concentrations were corrected according to the dilution factor for the calculations of the effective concentrations (EC-values). Mobility of the neonates was used as the end point. The assay was carried out at 20°C (±0.2), with a light rhythm of 8:16 h (dark: light), and light intensity < 1000 lux.

Each sample was tested both in native pH and with pH adjusted to 6.5 (±0.2) with 0.1 M NaOH which was assumed to be close to the natural pH of the test waters as typical Finnish freshwaters and yet within the pH tolerance of *D. magna* (Seco et al., 2003). No buffers were used to avoid chelation of metals. Two control treatments in different pH (6.5 and 7.0) of ISO test water were used (Table 3). At the beginning and at the end of each assay pH (PHM220 LAB, Radiometer, Copenhagen) and oxygen (HI9635, Hanna Instruments) were measured to ensure that they fulfil the criteria of ISO 6341:2012. The validity of the acute toxicity test was assured with a positive reference control test using K_2CrO_7 in concentrations 0, 0.25, 0.5, 1, 1.5 and 2 mg/l. In addition, Ni ($NiCl_2$ × 6H2O, 106717, Merck, grade ACS) in concentrations 0, 1,2,3,6, and 10 mg/l and Cd ($CdCl_2$Hydrate, 529575, Aldrich, 99.995%) in concentrations 0, 0.1, 0.2, 0.3, 0.6, and 1.25 mg/l were used as positive controls in the test. The effective concentrations (EC) 50% were calculated using neonate mobility as the end point.

2.4 Quantitative reverse transcription PCR (qRT-PCR)

To study the induction of selected biomarker genes with qPCR, 7-8 days old *D. magna* juveniles were pooled as one sample to obtain enough tissue for the transcriptomics. The juveniles were exposed to the environmental samples for 24 h. The water samples were filtrated (0.45 µm cellulose acetate, Whatman) and pH of the samples was adjusted with 0.1 M NaOH to 6.5 (±0.2) prior to exposure. The

ISO-test water was used as a control treatment. Four parallel exposure beakers were used for each sample. A beaker contained 25 juveniles in 250 ml of the tested water. At the end of the exposure the water samples were poured through a 100 µm net to collect the juveniles. Juveniles were immediately transferred into plastic micro-centrifuge tubes and flash-frozen in liquid nitrogen. Based on the results obtained from acute toxicity tests, aiming for a 10 % concentration of EC_{50} as the highest concentration, Ni was used in concentrations 92, 430, and 1100 µg/l, and Cd in concentrations 7.9, 33, and 88 µg/l. The Ni and Cd concentrations presented here are analysed with ICP-MS according to SFS-EN ISO 17294:05 in an accredited laboratory (Nablabs Ltd. FINAS T111, T142).The samples for metal analyses were taken from exposure water into 15 ml polypropylene tubes (Sarstedt Ltd.). To preserve metal samples the pH was adjusted below 2 with HNO_3 (Suprapure, Merck).

The primers were designed with Primer3 (version 4.0.0. at http://primer3.wi.mit.edu/) and checked for specificity with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primerblast/). The features of the primers are presented in Table 1. *gapdh* and *tubulin* were chosen as reference genes, because they showed the smallest variance between treatments among the tested group of genes. Total RNA was extracted from pools of 8-10 *D. magna* using Tri reagent (Molecular Research Center) following the manufacturer's instructions. Extractions were performed on 3-4 independent biological replicates. RNA concentration and purity were estimated with the NanoDrop 1000 (Thermo Fisher Scientific), and RNA integrity verified with the Agilent 2100 BioAnalyzer (Agilent), using Eukaryote total RNA 6000 nano kit (Agilent). RNA was persevered at -80°C prior to analyses. One microgram of total RNA was DNase treated (DNase I, Fermentas), reverse transcribed to cDNA (iScript cDNA Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water. One 25 µl qPCR reaction consisted of 5 µl of the diluted cDNA, 1.5 µl each of forward and reverse primers (final concentration 300 nM), 4.5 µl sterile H_2O and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad). The qPCR was run on a CFX96 Real-Time PCR cycler (Bio-Rad). The protocol was 3 min at 95 °C; 40 cycles (10 s at 95 °C, 30 s at 58 °C); 10 s at 95 °C and melt curve from 55 °C to 95 °C. No template controls (water instead of

cDNA) were run on each plate for each gene, and the CT values were always over 38. Melt curves showed a single peak, confirming the formation of only one PCR product. The expression of the target genes were calculated with the Bio-Rad CFX Manager software v. 3.1. In the procedure the Ct values of the target genes were normalized to the two reference genes, taking into account the efficiencies of the primers (Pfaffl, 2001). To compare multiple plates within the analysis, inter-run calibration was used.

2.5 Statistical analyses and database survey

For the positive controls (Ni, Cd) of acute toxicity assays the EC_{50}-values were calculated with log-logistic model using DRC-page of the statistical software R (Ritz and Streibig, 2005; R Development Core Team, 2008). Database survey on the toxicity of Ni and Cd was done using the ECOTOX database (U.S. EPA. http://cfpub.epa.gov/ecotox/, Visited 26.5.2014). The selection criteria were: test organism was *Daphnia magna*, effect was immobility (IMBL) or mortality (MORT) and observation duration was 1-2 days. The test chemicals looked for were $NiCl_2$ for Ni (n=15) and CdCl for Cd (n=154).

For the RT-qPCR assays the equality of variance of the log-transformed expression values were tested with Bartlett test. Null hypothesis was that the variances of the groups are equal and variance is homogenic when p-value is < 0.05. After log transformation all test groups showed homogenic variance ($p<0.05$ in Bartlett's test). Therefore, one way analysis of variance was made followed by multiple comparisons with pairwise t-test. Bonferroni p-value correction was used in analysing which groups were significantly different from the control group.

To find the possible predictors of observed toxicity from the background data, exploratory regression data analysis was performed for both the RT-qPCR assay and the acute toxicity assay. Regression of each of the background parameters against the response variables was checked. For the acute toxicity assay, number of the mobile individuals per treatment was used as response

196 variable. The acute toxicity data is binomial and, therefore, logistic regression analyses was used. For
197 the RT-qPCR assay log transformed expression values were used as response variables of the linear
198 regression analyses. The background data for the regression analysis comprised the elements
199 analysed from the water samples and the water chemistry parameters (pH, hardness, DOC,
200 conductivity, distance from mine and sulphate concentration). The background data had missing
201 values of dissolved metal concentrations due to protocol used in the monitoring programme that
202 was the source of the background data for the study. All the statistical analyses were performed
203 with R (R Development Core Team, 2008).

204 **3 Results & Discussion**

205 **3.1 Water characteristics**

206 The sampling site O2 (lake Salminen hypolimnion) was clearly the most impacted sampling site with
207 11 g/l of SO_4 and the highest concentrations of metals, including Ni, Cd, Cu, As, Al, Ba, Fe, Mg, Mn,
208 Zn, Pb, Co, Cr, U and V (Table 2). The concentrations of SO_4, Al, Mg, Mn, and Co showed a decreasing
209 gradient over distance from the sampling site O2 (Table 2). The sampling site O2 had been affected
210 by the biomining effluents already before the gypsum pond leak, and the hypolimnion had been
211 hypoxic due to high sulphate that had caused permanent stratification (Kauppi et al. 2013). Calcium
212 was the only compound that showed a full concentration gradient from the sampling site O1,
213 located closest to the mine, to site O5 (Table 2). This Ca probably originated from neutralisation of
214 acidic waters with lime addition.

215 The sampling site O4 showed higher concentrations of V, Co, Ni, Cu, and As than O3 (Table 2). This
216 may be caused by the fact that the sampling site O4 is at a place that receives water not only from
217 Kalliojärvi (O3) but also from Kuusijoki, which via Kuusilampi was connected to Härkäpuro, a stream
218 situated close to the secondary heaps and carrying waters from mine ponds. The sampling site O5
219 showed only slightly elevated concentrations of Mn, Mg, Co, Zn and SO_4 (Table 2). The sampling site

V1 situated at the Vuoksi waterway was characterised with having intermediate concentration of studied metals. In this sampling site, the Cu and Zn concentrations were lower than at O2 but higher than at O3. The Mn and Co concentrations were at a similar level as in O3, and Mg and Fe concentrations were lower than at O3 but higher than at O4 (Table 2).

3.2 Acute toxicity

In the standardised acute *D. magna* assay (ISO 6341:2012), the EC_{50} value of the positive control K_2CrO_7 was 1.44 mg/l (SD 0.181), which is in the acceptable range (0.6–2.1 mg/l) of the standard (ISO 6341:2012). The EC_{50} of Ni was 11.77 mg/l (SD 5.96), which is well in a range with the EC_{50} for *D. magna* values found in U.S. EPA ECOTOX database: mean 5.7 mg/l and range 0.74-17.3 mg/l (n=15). The EC_{50} of Cd in the present study was 0.47 mg/l (SD 0.47). For cadmium the ECOTOX database survey gave a mean EC_{50}-value of 0.26 mg/l and range from 0.00027mg/l to 22.6 mg/l for *D. magna* (n=154).

In the standardised assay with pH adjusted to 6.5±0.2, the water samples showed no clear toxicity, except the hypolimnion sample from Lake Salminen (O2), which is located close to the mine (Table 3). No statistically significant predictors for acute toxicity were found from the background data, with exploratory logistic regression analyses, when the pH of samples was adjusted to 6.5 (±0.2) ($p<0.05$). To see the effect of the pH adjustment, the water samples were also tested in their natural pH. Toxicity was observed in the samples taken close to the mine, i.e. V1, O2, O3 and O4 (Table 3).

When acute toxicity results were studied against the background data with exploratory logistic regression analyses, many of the background parameters showed dependency with the immobility. The p-value less than 0.001 was found for pH, total hardness, N, Ba, Cd, Co, Cu, Mg, Mn, Ni, Fe, Zn, and U. As the studied samples are mixtures, many of the sample characteristics can explain the observed toxicity. The water flea *D. magna* is sensitive to pH changes and water hardness (De Schamphelaere and Janssen, 2004). The pH tolerance of *D. magna* is from 5.5 to 10 (Seco et al.,

2003), and the total immobility of daphnids in samples V1, O2, and O3 is explained by their pH (<4) being out of this tolerance range (Table 3). The effect of pH on metal solubility is another factor explaining the obtained results. Most metals become more soluble as the pH gets lower (Weiner, 2008; Wang et al. 2016). Therefore, the increased toxicity compared to the pH adjusted samples can be due to direct toxic effects of decreased pH to *D. magna,* due to increased solubility and bioavailability of toxic metals, or both. The high toxicity in pH-adjusted sample O2 is most probably a result of the presence of several toxic elements, high total hardness (49.3 mmol/l) and possibly osmotic stress caused by high sulphate concentration (11000 mg/l) (Table 2, Table 3). The *D. magna* EC_{50} value for the acute toxicity of sulphate is in the range of 3300 mg/l (Yi et al., 2011). The high Ca concentration observed in the samples can protect *D. magna* against metal toxicity, as intake of metals such as Ni, Zn, Se, and Cd can then be decreased (Penttinen et al., 1995; Yu and Wang, 2002; Komjarova and Blust, 2008).

It can be concluded that the water samples taken near to the mine (V1, O2-O4) were toxic to *D. magna*, but the toxicity was detected only when the pH of the samples was not adjusted to 6.5±0.2 according to the standardized *D. magna* acute toxicity test (Table 3). However, an acute toxicity test does not give specific information on the factors causing the observed immobility. Therefore, it can be questioned whether the acute Daphnia assay is sensitive and specific enough to be used for assessing the risk posed by biomining activities to aquatic organisms.

3.3 Changes in mRNA expression

3.3.1 Monooxygenase

Cadmium as a positive control induced monooxygenase expression (Figure 2). This was expected as monooxygenase has been shown to be induced by Cd in *D. magna* (Connon et al., 2008, Poynton et al., 2008). The highest concentration used (88 µg/l Cd) increased expression of monooxygenase 19-fold and the change was significantly different from the control (p-value=0.0150). The second

highest Cd concentration (33 µg/l) increased monooxygenase expression 6.6-fold, but this was not statistically different from control (p-value=0.0761).

The monooxygenase was up-regulated under the exposure of the contaminated samples as well (Figure 2). The increased expression in three tested water samples was statistically different from the control treatment (V1: p-value=0.000033; O3: p-value=0.0027; O4: p-value=0.00787). The highest up-regulation was detected in the V1 sample, where it was 86-fold compared to the control.

The induction of monooxygenase expression under exposure to Cd could explain why V1 sample induced higher expression values than other environmental samples. The V1 sample had about twice as high concentration of Cd (0.235 µg/l) as the other three samples (0.1 µg/l). In addition, Cd is the only measured constituent that is present in the highest concentration in the V1 Sample (Table 1). The regression analysis, however, does not support the major role of the Cd as the only cause of up-regulation of mono-oxygenase in the water samples. Statistically most significant ($p<0.001$) predictors for the monooxygenase induction were distance from the discharge source, pH, DOC, As, Cd, Co, Cr, Cu Pb, Mg, Mn, Ni, Zn, Fe and U concentrations. Of these predictors, the best fit according to the adjusted R^2 were for Co (R^2=0.89), Ni (R^2 = 0.89), As (R^2 = 0.88), Mn (R^2 = 0.88), DOC (R^2 = 0.87), pH (R^2 = 0.86), Cu (R^2 = 0.85) and Cd (R^2=0.84). The results presented here are based on the total concentrations of elements. Overall, according to preliminary testing with data, the dissolved concentration showed similar results, when there were enough samples (data not shown). It must also be borne in mind that varying mixture effects, such as additivity, synergism, or antagonism, may occur in metal mixtures.

The original pH of the water sample seems to have a significant effect on the mono-oxygenase induction although the pH of the samples was adjusted before the qPCR exposure. This is an expected result as the solubility of metals is strongly dependent on the water pH and therefore affects the metal content of filtered samples used for exposure. It has been shown that low pH and

292 total organic carbon (TOC) increase metal solubility and therefore bioavailability in sulphite mineral
293 mining affected Nordic rivers (Gundersen & Steinnes, 2003).

294 Connon et al. (2008) linked the up-regulation of mono-oxygenase after 24 h Cd exposure to lowered
295 population growth rate after 9 days of exposure and speculated Cd to be an endocrine disruptor
296 affecting moulting. Up-regulation of mono-oxygenase could therefore correlate with reduced
297 offspring production.

298 **3.3.2 Vitellogenin superoxide dismutase**

299 Reproduction-related vitellogenin-superoxide dismutase (VTG-SOD) is a major component of yolk
300 proteins in *D. magna* (Kato et al. 2004). For VTG-SOD, none of the positive controls with Ni and Cd
301 were significantly different from the corresponding control treatments (ANOVA, p-value>0.05). This
302 is in contrast with previous studies, as down-regulation of VTG-SOD by Cd and Ni has been reported
303 in *D. magna* (Poynton et al., 2008; Vandenbrouck et al., 2009). Though all the environmental
304 samples seemed to up-regulate VTG-SOD, only the sample O3 indicated a statistically significant
305 difference compared to the control (p-value 0.028) (Figure 2). In the environmental samples, VTG-
306 SOD had significant ($P<0.01$) regression with total Al ($R^2=0.581$) and Ba ($R^2=0.5$) concentrations. Also,
307 hardness ($p<0.05$, $R^2=0.48$) weakly predicted the VTG-SOD induction.

308 **3.3.3 Catalase**

309 Catalase is an antioxidant enzyme catalysing hydrogen peroxide decomposition to water and oxygen,
310 and it protects organisms from reactive oxygen species (ROS). In the positive control assay with
311 cadmium, the concentration 33 µg/l Cd caused a statistically significantly different mRNA abundance
312 (fold change 1.6, p-value = 0.029) from zero concentration, but the higher concentration of Cd (88
313 µg/l) did not (Figure 2). A similar result has been obtained by Kim et al. (2010a) who observed the
314 maximum of catalase induction at 10 µg Cd/l and a decreased induction 50 µg Cd/l. Poynton et al.

(2008) showed that several metal-biomarker genes lose specificity at high concentrations of Cd, while at low concentration dose-response can be observed.

The expression values of catalase increased with increasing Ni both in the positive control and in the exposures with the contaminated water samples. Also the expression values of the environmental samples increased with increasing Ni content: O3 having the highest Ni concentration and expression values and O4 having the lowest (Figure 2, Table 1). When exposed only to Ni (92-1100 µg/l), however, the expression values were lower than under exposure to the contaminated water samples containing Ni in the same range, suggesting that Ni is not the only compound enhancing transcription of catalase.

For the catalase, only weakly dependent predictors were found. Ba, Ca and V concentrations had p-value less than 0.05 in regression analysis. However, the R^2 for all these were less than 0.34.

3.2.4 Sum of the gene induction as predictor

The statistically most significant ($p< 0.001$) predictors for sum of expression of all the three genes were the original pH of the water sample at the sampling site (R^2=0.49), and Cd (R^2=0.55). Weaker predictors ($p< 0.01$) were found for Co, Cr, Mn, Ni, Fe, Zn and V with R^2 being between 0.34-0.51. Some of the dissolved concentrations were not available in the original background data. Therefore, no preference between dissolved and total concentration as a predictor can be made based on the available data.

4 Conclusions

The present study showed that the standardized acute *D. magna* assay is not sufficient for toxicity assessment of water samples from biomining-impacted watercourses. The pH adjustment of the standardized assay diminished the acute toxicity of all but one of the water samples. Without pH adjustment, water samples taken up to 5.2 km from the mine caused severe acute toxicity to *D. magna*. We, therefore, recommend testing biomining-impacted waters in their native pH as well.

Transcriptomic biomarkers were sensitive bioindicators that showed that even the pH-adjusted samples affected *D. magna*. Monooxygenase transcript abundance was a good indicator of the overall pollution in the studied sites, with a good correlation with the main components of the mining-affected waters and with the distance from the discharge source. Yet, there are limitations in the application of the transcriptomic biomarkers such as loss of differential expression at high concentrations. For example, the applicable concentration range for catalase induction was very narrow in our study. The study confirms that transcriptomic biomarkers could be used to detect elevated risk also in the complex environmental samples. Further research to develop the testing protocol and to select the most appropriate genes as biomarkers will be needed along with the knowledge on functional aspects of *D. magna* genome.

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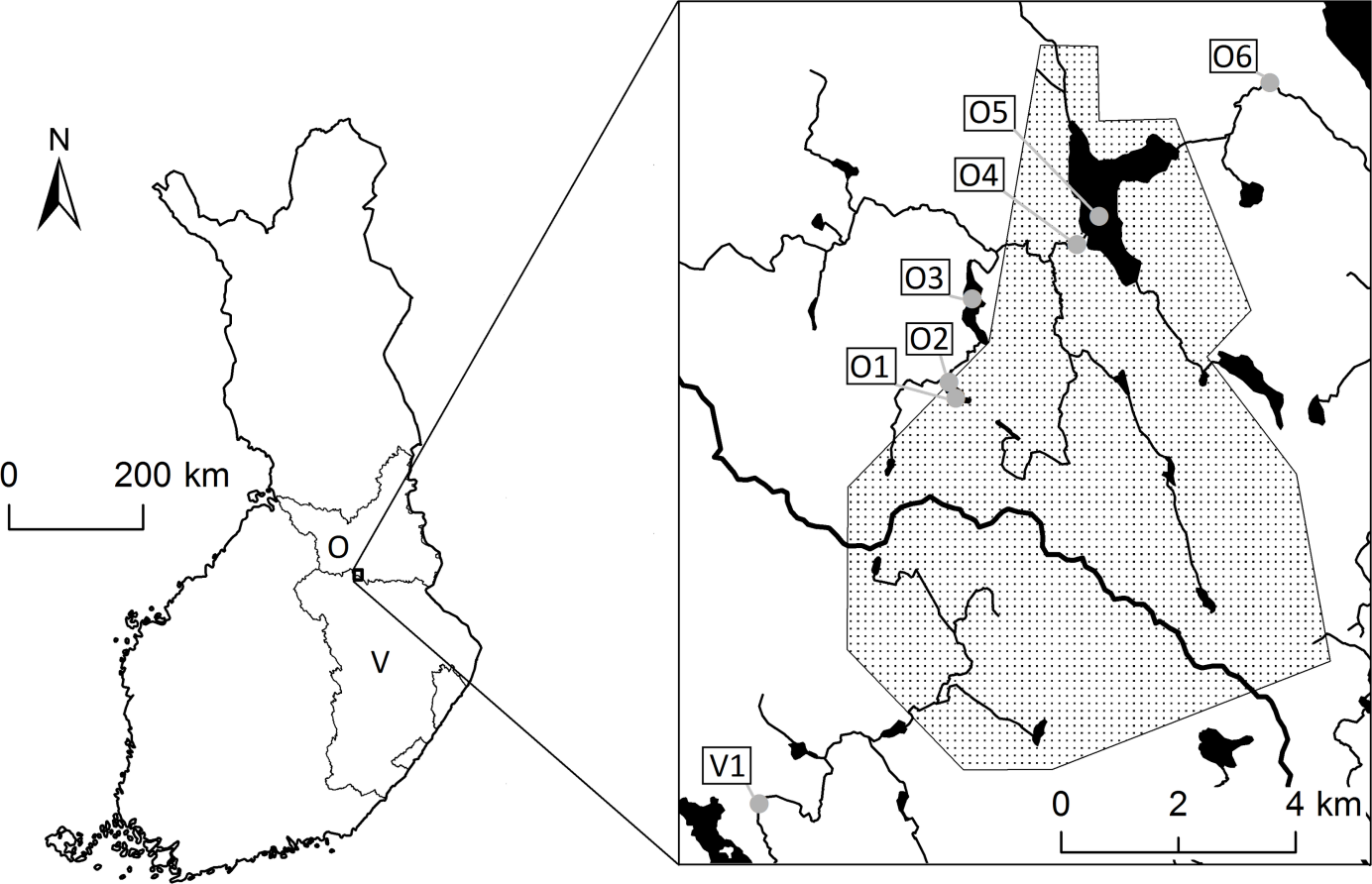
Figure 1: Location of the sampling sites in Oulujoki (O) and Vuoksi (V) watersheds in Finland. The Talvivaara mine district is indicated in grid-like fill, and lakes and streams in black. Map data: General and Topographic maps of Finland © National Land Survey of Finland 2018; Catchment Areas and National Database of Regional Land Use Plans © Finnish Environment Institute 2018. Maps were constructed with ArcGIS® v. 10.5.1 (ESRI Inc., Redlands, CA).

Figure 2: Gene expression normalised to control in D. magna exposed to the mining-affected water samples and positive controls of Ni (92-1100 µg/l) and Cd (7.9-88 µg/l). Statistical difference between a sample and corresponding control is denoted with an asterisk.

Highlights

- Biomining affected waters show sub-lethal toxicity to *Daphnia magna*
- Water samples up-regulated *monooxygenase*, *vtg-sod* and *catalase*
- Transcriptomic biomarkers are more sensitive than acute toxicity tests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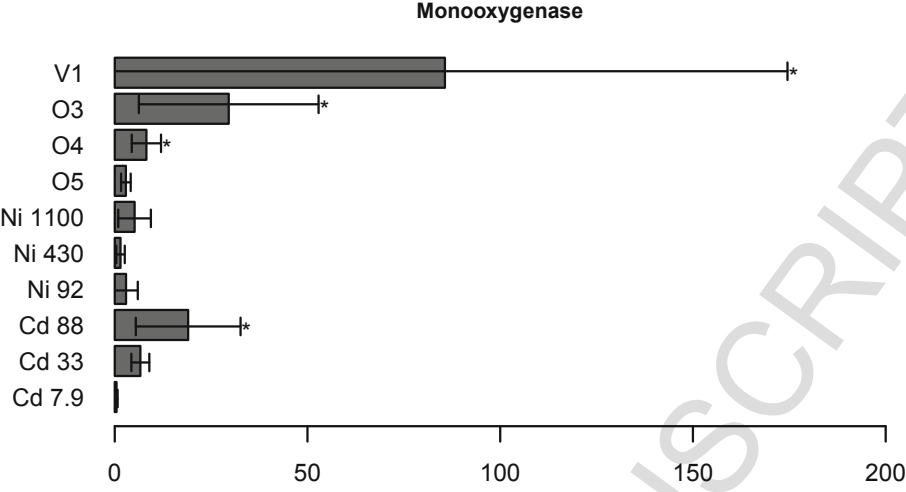

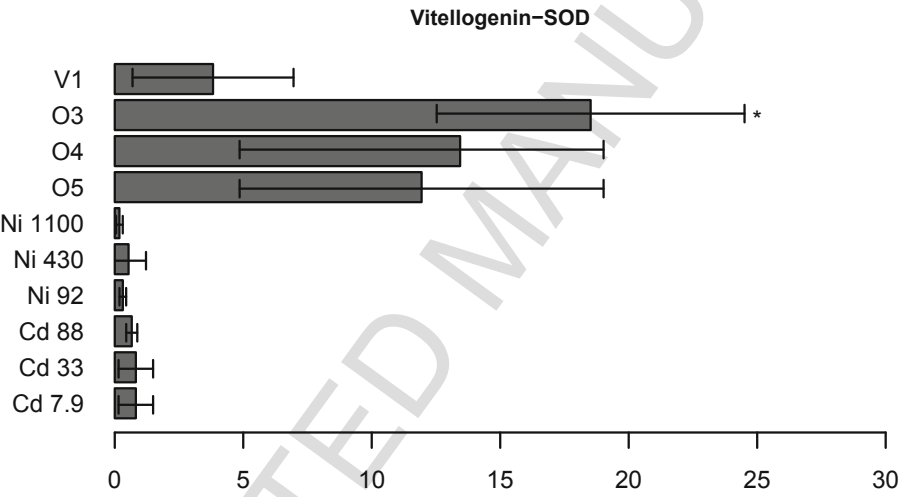

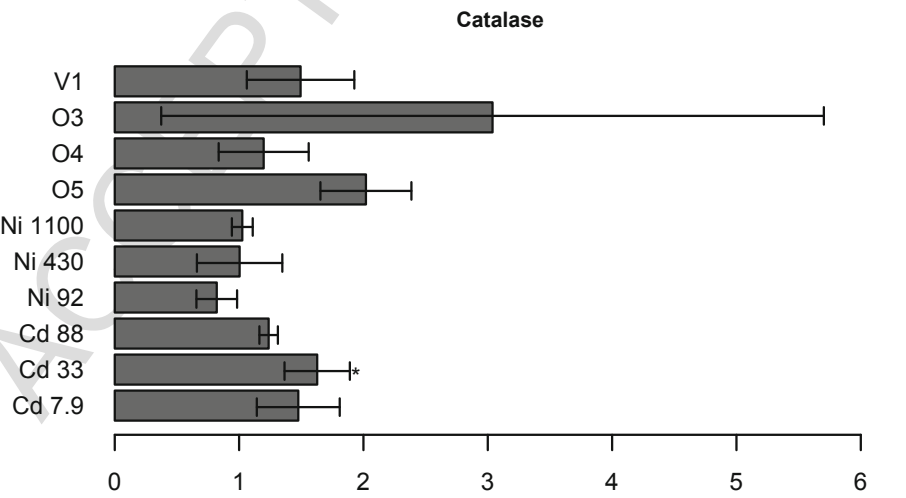

Table 1. Primers used in the qPCR.

Gene name	Symbol	GenBank accession no.	wFleaBase EST no.	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency	Amplicon size (bp)
*alpha-tubulin**	*tbl*		WFes0007807	tggaggtggtgacgact	ccaagtcgacaaagacagca	103.1	89
glyceraldehyde-3-phosphate dehydrogenase	*gadph*	AJ292555		gtcttcagtgaacgagaccc	gcatgggccttttcaagagt	101.2	104
catalase	*cat*	GQ389639		ccccaaagactatttcgccg	gtaggagaaaagacgcccct	98.4	112
monooxygenase	*mox*	DV437798		acacgtctatcccgcatcat	taccaagtaccgagccgttt	103.4	102
vitellogenin-superoxide dismutase	*vtg-sod*	AB252738		caagtacaacgagatgcccg	atgtagggacccaaccagtg	102.8	108

*Heckmann et al. BMC Genomics. 2006; 7: 175.

Table 3: Acute toxicity of the mining affected water samples to neonate *D. magna* at 24 h.

Sample	pH*	Total Hardness (mmol/l)	Mobility 24h (%)	qRT-PCR-tested samples
Control	7.0	2.5	96	
	6.5	2.5	96	
V1	3.9	2.4	0	
	6.5		92	X
O1	10.3	9.1	96	
	6.4		100	
O2	3.1	49	0	
	6.4		4	
O3	3.4	14.1	0	
	6.6		84	X
O4	4.8	4.2	88	
	6.5		100	X
O5	6.4	2.8	96	X
O6	6.5		92	

* adjusted pH of the environmental samples in bold

			Vuoksi waterway	R. Oulujoki waterway					
	Unit	LOQ	R.Lumijoki (V1)	R. Viitapuro (O1)	L. Salminen(O2)	L. Kalliojärvi(O3)	R.Kalliojoki(O4)	L.Kolmisoppi (O5)	R. Tuhkajoki(O6)
Distance from the discharge[1]	km		3.3	1.8	2.1	3.6	5.2	5.8	9.3
Total depth[2, 3]	m		ca 0.8	ca. 0.5	7.7	5.1	1.0	13.1	ca 2.0
Sample temperature[2]	°C		0	0	4.6	5.9	-0.1	1.5	0
pH			4.7	–	3.7	4.8	5.4	5.9	6.1
Conductivity[2]	mS/cm		8.85	–	10.93	7.02	12.96	0.67	2.09
Alkalinity	mmol/l	0.02	<0.02	0.68	<0.02	<0.02	< 0.02	0.08	0.11
Total suspended solids	mg/l		12	8	33	20	9.8	6.8	1.3
Turbidity	FTU		12	2.1	120	40	7.4	5.6	1.4
Total hardness	mmol/l		2.4	9.1	49.3	14.1	4.2	2.3	1.2
DOC			6	–	–	–	4	–	20
SO_4	mg/l		465	3300	11000	4300	670	310	82
Al	mg/l	0.005	0.70/0.58	0.17/0.03	290/–	2.40/–	2.15/1.50	1.10/–	0.33/0.31
As	µg/l	0.05	0.22/0.13	–/<0.05	8.6/–	–/<0.05	0.14/0.11	–/<0.05	0.23/0.21
Ba	µg/l	0.5	–/11.87	–/46.0	47.0/–	47.0/–	17.37/15.87	20.0/–	–/14.35
Ca	mg/l	0.1	27.0/27.0	300/–	270	150	68.0/69.5	42.0/–	13.4/13.7
Cd	µg/l	0.01	0.24/0.27	<0.01	1.80/1.80	0.11/0.10	0.13/0.12	0.17/0.12	0.14/0.13
Co	µg/l	0.05	6.5/6.2	–/0.23	89/–	8.5/–	3.4/3.2	2.3/–	0.76/0.71
Cr	µg/l	0.2	0.5/0.4	–/0.27	21/–	0.3/–	0.4/<0.2	0.4/–	0.6/0.5
Cu	µg/l	0.1	10.0/7.8	–/<0.1	14/–	<0.1/–	3.4/2.1	1.1/–	5.6/3.0
Fe	mg/l	0.005	10.0/8.90	0.54/0.10	490/–	62/–	1.95/1.20	1.20/–	1.40/1.20
K	mg/l	0.1	1.4/1.5	13/-			2.3/2.2		0.9/0.9
Mg	mg/l	0.1	48.1/48.3	43/-	730/–	190/–	34.4/34.6	18/–	4.5/4.6
Mn	mg/l	0.001	26.0/26.0	0.96/–	380/–	30/–	9.60/9.55	5.2/–	0.37/0.37
N	mg/l	0.1	–	–	2.8/–	2.5/–	–	0.64/–	–
Na	µg/l	100	0.70/0.71	1.2/–	1.5/–	1.4/–	0.17/0.17	0.07/–	0.21/0.22
Ni	mg/l	0.0002	0.20/0.18	–/0.01	4.70/4.70	0.25/0.23	0.11/0.10	0.06/0.06	0.17/0.16
P	µg/l	50	–/<50	–	50/–	5/–	–/<50	10/–	–/<50
Pb	µg/l	0.05	0.24/0.16	–	–/2.70	–/<0.05	0.21/0.03	–/<0.05	0.37/0.30
Se	µg/l	0.2	<1.0/<1.0	–	–	–	<1.0/<1.0	–	<0.3/<0.2
Sr	µg/l	0.2	87.0/90.5	–	–	–	130/130	–	29.0/30.5
Ti	µg/l	1	2.45/<1.0	–	–	–	6.7/<1.0	–	4.1/2.9
U	µg/l	0.005	3.30/2.50	–/0.61	590/–	6.20/–	3.75/2.50	–/1.60	0.23/0.22
V	µg/l	0.05	0.51/0.30	–	1.2/–	<0.05/–	0.49/0.17	0.7/–	0.70/0.64
Zn	µg/l	0.5	110/99.5	–/<0.5	950/–	43.0/–	39.5/33.5	47/–	42.0/38.5

[1]Approximate distance as measured directly from the main discharge point to the sampling site [2]measured in the field at the time of the sampling [3]total depth at the lake sampling sites