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

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

3

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9 **Abstract**

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

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

27 **Key words**

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

29 **1 Introduction**

30 Mineral mining is increasing due to global economic growth and consequent demand for the metals
31 (Pokhrel and Dubey 2013). While the demand for most metals has steadily increased in the last
32 decade, number, grade and quality of new ore deposits have declined (Brierley 2008, Brierley and
33 Brierley 2013). The bio-hydrometallurgy i.e. biomining is particularly suitable technically and
34 economically for processing lower grade and complex polymetallic mineral assemblages (Brierley
35 2008, Brierley and Brierley 2013). Biomining simplifies the enrichment process of sulphide ores as
36 microbes are used to oxidize insoluble metal sulphides to soluble metal sulphates, which are then
37 extracted from leachates (Morin et al. 2008). Biomining also has lower process temperatures, lower
38 energy costs and smaller carbon footprints than conventional mining (Morin et al. 2008). Thus,
39 biomining has been perceived as a more environmentally benign approach than conventional mining
40 processes (Morin et al. 2008, Johnson 2014, 2015). The contamination from conventional mining
41 include CO₂ emissions, SO₂ emissions and acid mine drainage (AMD) (Dold 2008). Although
42 biomining consumes less energy, it is likely to cause similar releases of salts, heavy metals and acid
43 mine drainage (AMD) to the environment as processing with other techniques such as floatation
44 (Dold and Weible 2013). As the utilisation of biomining is likely to increase (Brierley 2008, Brierley
45 and Brierley 2013), and novel approaches such as *in situ* biomining are developed (Johnson 2015,
46 Morin et al. 2008) the environmental impacts of biomining, particularly, on water resources, should
47 be studied.

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

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

71

72

73 **Materials and methods**

74 **2.1 Study site**

75 The water samples for this study were collected from two watersheds that receive effluents from
76 the Talvivaara multimetal mine in Sotkamo, Finland. The mine uses bioheapleaching to recover
77 metals from low-grade ore. The Talvivaara deposit is hosted by metamorphosed black shales (black
78 schists) and contains 300 million metric tons (Mt) of low-grade ore averaging 0.26 percent Ni, 0.14
79 percent Cu, and 0.53 percent Zn (Loukola-Ruskeenniemi, et al. 1996). The main products of
80 Talvivaara are a mixed nickel cobalt sulphide, copper sulphide and zinc sulphide (Riekkola-Vanhanen
81 2010). In Talvivaara, an environmental accident happened in November 2012 affecting waterways of
82 Oulujärvi and Vuoksi. The wall of a storage pond ruptured and acidic waste water containing
83 raffinate from enrichment process leaked to the nearby waterways (Onnettomuustutkintakeskus,
84 2014). According to the report of Finnish Environmental institute, the main contaminants were Ni,
85 Zn, Cd, Al, U and salting due to sulphate (Kauppi et al. 2013). The accident also caused fluctuating
86 pH, as the waste water was acidic and the accident treatment included occasional neutralisation of
87 waters with lime addition (Kauppi et al. 2013).

88 **2.2 Water samples and chemical analyses**

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

96 meter above the lake bottom with a Limnos sampler. The lake water sampling was designed to study
97 concurrent effects of metals and salts in the lakes hypolimnion. Representative one litre water
98 samples for the chemical analyses were taken at the same sampling site as the samples for the
99 exposures. To measure the total and dissolved concentrations of elements two parallel 100 ml sub-
100 samples were taken. Both of the 100 ml sub-samples were preserved with 0.5 ml of HNO₃ (100441,
101 Suprapur 65%, Merck, Germany), and the sample representing dissolved concentration was filtered
102 (GD/XP, 0.45 µm, Whatman) in the field.

103 Concentration of Al, As, Ba, Ca, K, Mg, Na, S, Sr and Ti were analysed with ICP-OES following
104 standard SFS-EN ISO 11885:2009. Cd, Co, Cr, Cu, Ni, Pb, Sb, Se, Zn, U and V were analysed with ICP-
105 MS according to SFS-EN ISO 17294-1:2006 and 17294-2:2005. Fe and Mn were measured with IRIS
106 Intrepid II XSP (Thermo Scientific). For the total concentration analyses the samples were microwave
107 digested with HNO₃ (ISO 15587- 2:2002). Analyses were performed in accredited laboratories (EN
108 ISO/IEC 17025) of the Finnish Environmental institute (FINAS T003, K054) and Nablabs Ltd. (FINAS
109 T111, T142). The measurements were done according to standards (standard number given in
110 brackets): Oxygen concentration at field (SFS-EN 25813:1996), pH (SFS-EN ISO 10523-2012),
111 conductivity with temperature compensation to 25 °C (SFS-EN 27888-1994), solids content (SFS-EN
112 872:2005), total hardness (SFS 3003:1987), fluoride (SFS-EN-ISO 10304-1:2009), and sulphate (SFS-
113 EN10304-2009).

114 **2.3 Acute toxicity assays**

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

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

130 Each sample was tested both in native pH and with pH adjusted to 6.5 (±0.2) with 0.1 M NaOH which
131 was assumed to be close to the natural pH of the test waters as typical Finnish freshwaters and yet
132 within the pH tolerance of *D. magna* (Seco et al., 2003). No buffers were used to avoid chelation of
133 metals. Two control treatments in different pH (6.5 and 7.0) of ISO test water were used (Table 3). At
134 the beginning and at the end of each assay pH (PHM220 LAB, Radiometer, Copenhagen) and oxygen
135 (HI9635, Hanna Instruments) were measured to ensure that they fulfil the criteria of ISO 6341:2012.
136 The validity of the acute toxicity test was assured with a positive reference control test using K₂CrO₇
137 in concentrations 0, 0.25, 0.5, 1, 1.5 and 2 mg/l. In addition, Ni (NiCl₂ × 6H₂O, 106717, Merck, grade
138 ACS) in concentrations 0, 1, 2, 3, 6, and 10 mg/l and Cd (CdCl₂Hydrate, 529575, Aldrich, 99.995%) in
139 concentrations 0, 0.1, 0.2, 0.3, 0.6, and 1.25 mg/l were used as positive controls in the test. The
140 effective concentrations (EC) 50% were calculated using neonate mobility as the end point.

141 **2.4 Quantitative reverse transcription PCR (qRT-PCR)**

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

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

157 The primers were designed with Primer3 (version 4.0.0. at <http://primer3.wi.mit.edu/>) and checked
158 for specificity with Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primerblast/>). The features of
159 the primers are presented in Table 1. *gapdh* and *tubulin* were chosen as reference genes, because
160 they showed the smallest variance between treatments among the tested group of genes. Total RNA
161 was extracted from pools of 8-10 *D. magna* using Tri reagent (Molecular Research Center) following
162 the manufacturer's instructions. Extractions were performed on 3-4 independent biological
163 replicates. RNA concentration and purity were estimated with the NanoDrop 1000 (Thermo Fisher
164 Scientific), and RNA integrity verified with the Agilent 2100 BioAnalyzer (Agilent), using Eukaryote
165 total RNA 6000 nano kit (Agilent). RNA was preserved at -80°C prior to analyses. One microgram of
166 total RNA was DNase treated (DNase I, Fermentas), reverse transcribed to cDNA (iScript cDNA
167 Synthesis Kit, Bio-Rad, USA) and diluted 1+9 with nuclease-free water. One 25 μl qPCR reaction
168 consisted of 5 μl of the diluted cDNA, 1.5 μl each of forward and reverse primers (final concentration
169 300 nM), 4.5 μl sterile H_2O and 12.5 μl of iQ SYBR Green Supermix (Bio-Rad). The qPCR was run on a
170 CFX96 Real-Time PCR cycler (Bio-Rad). The protocol was 3 min at 95°C ; 40 cycles (10 s at 95°C , 30 s
171 at 58°C); 10 s at 95°C and melt curve from 55°C to 95°C . No template controls (water instead of

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

178 **2.5 Statistical analyses and database survey**

179 For the positive controls (Ni, Cd) of acute toxicity assays the EC₅₀-values were calculated with log-
180 logistic model using DRC-page of the statistical software R (Ritz and Streibig, 2005; R Development
181 Core Team, 2008). Database survey on the toxicity of Ni and Cd was done using the ECOTOX
182 database (U.S. EPA. <http://cfpub.epa.gov/ecotox/>, Visited 26.5.2014). The selection criteria were:
183 test organism was *Daphnia magna*, effect was immobility (IMBL) or mortality (MORT) and
184 observation duration was 1-2 days. The test chemicals looked for were NiCl₂ for Ni (n=15) and CdCl
185 for Cd (n=154).

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

192 To find the possible predictors of observed toxicity from the background data, exploratory
193 regression data analysis was performed for both the RT-qPCR assay and the acute toxicity assay.
194 Regression of each of the background parameters against the response variables was checked. For
195 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 SO₄ 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 SO₄, 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 SO₄ (Table 2). The sampling site

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

224 3.2 Acute toxicity

225 In the standardised acute *D. magna* assay (ISO 6341:2012), the EC₅₀ value of the positive control
226 K₂CrO₇ was 1.44 mg/l (SD 0.181), which is in the acceptable range (0.6–2.1 mg/l) of the standard (ISO
227 6341:2012). The EC₅₀ of Ni was 11.77 mg/l (SD 5.96), which is well in a range with the EC₅₀ for *D.*
228 *magna* values found in U.S. EPA ECOTOX database: mean 5.7 mg/l and range 0.74-17.3 mg/l (n=15).
229 The EC₅₀ of Cd in the present study was 0.47 mg/l (SD 0.47). For cadmium the ECOTOX database
230 survey gave a mean EC₅₀-value of 0.26 mg/l and range from 0.00027mg/l to 22.6 mg/l for *D. magna*
231 (n=154).

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

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

244 2003), and the total immobility of daphnids in samples V1, O2, and O3 is explained by their pH (<4)
245 being out of this tolerance range (Table 3). The effect of pH on metal solubility is another factor
246 explaining the obtained results. Most metals become more soluble as the pH gets lower (Weiner,
247 2008; Wang et al. 2016). Therefore, the increased toxicity compared to the pH adjusted samples can
248 be due to direct toxic effects of decreased pH to *D. magna*, due to increased solubility and
249 bioavailability of toxic metals, or both. The high toxicity in pH-adjusted sample O2 is most probably a
250 result of the presence of several toxic elements, high total hardness (49.3 mmol/l) and possibly
251 osmotic stress caused by high sulphate concentration (11000 mg/l) (Table 2, Table 3). The *D. magna*
252 EC₅₀ value for the acute toxicity of sulphate is in the range of 3300 mg/l (Yi et al., 2011). The high Ca
253 concentration observed in the samples can protect *D. magna* against metal toxicity, as intake of
254 metals such as Ni, Zn, Se, and Cd can then be decreased (Penttinen et al., 1995; Yu and Wang, 2002;
255 Komjarova and Blust, 2008).

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

262 **3.3 Changes in mRNA expression**

263 **3.3.1 Monooxygenase**

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

268 highest Cd concentration (33 $\mu\text{g/l}$) increased monooxygenase expression 6.6-fold, but this was not
269 statistically different from control (p-value=0.0761).

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

274 The induction of monooxygenase expression under exposure to Cd could explain why V1 sample
275 induced higher expression values than other environmental samples. The V1 sample had about twice
276 as high concentration of Cd (0.235 $\mu\text{g/l}$) as the other three samples (0.1 $\mu\text{g/l}$). In addition, Cd is the
277 only measured constituent that is present in the highest concentration in the V1 Sample (Table 1).

278 The regression analysis, however, does not support the major role of the Cd as the only cause of up-
279 regulation of mono-oxygenase in the water samples. Statistically most significant (p<0.001)
280 predictors for the monooxygenase induction were distance from the discharge source, pH, DOC, As,
281 Cd, Co, Cr, Cu, Pb, Mg, Mn, Ni, Zn, Fe and U concentrations. Of these predictors, the best fit
282 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
283 ($R^2 = 0.87$), pH ($R^2 = 0.86$), Cu ($R^2 = 0.85$) and Cd ($R^2=0.84$). The results presented here are based on
284 the total concentrations of elements. Overall, according to preliminary testing with data, the
285 dissolved concentration showed similar results, when there were enough samples (data not shown).

286 It must also be borne in mind that varying mixture effects, such as additivity, synergism, or
287 antagonism, may occur in metal mixtures.

288 The original pH of the water sample seems to have a significant effect on the mono-oxygenase
289 induction although the pH of the samples was adjusted before the qPCR exposure. This is an
290 expected result as the solubility of metals is strongly dependent on the water pH and therefore
291 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 Cannon 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 $\mu\text{g/l}$ 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 $\mu\text{g/l}$) 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 $\mu\text{g Cd/l}$ and a decreased induction 50 $\mu\text{g Cd/l}$. Poynton et al.

315 (2008) showed that several metal-biomarker genes lose specificity at high concentrations of Cd,
316 while at low concentration dose-response can be observed.

317 The expression values of catalase increased with increasing Ni both in the positive control and in the
318 exposures with the contaminated water samples. Also the expression values of the environmental
319 samples increased with increasing Ni content: O3 having the highest Ni concentration and
320 expression values and O4 having the lowest (Figure 2, Table 1). When exposed only to Ni (92-1100
321 $\mu\text{g/l}$), however, the expression values were lower than under exposure to the contaminated water
322 samples containing Ni in the same range, suggesting that Ni is not the only compound enhancing
323 transcription of catalase.

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

326 **3.2.4 Sum of the gene induction as predictor**

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

333 **4 Conclusions**

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

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

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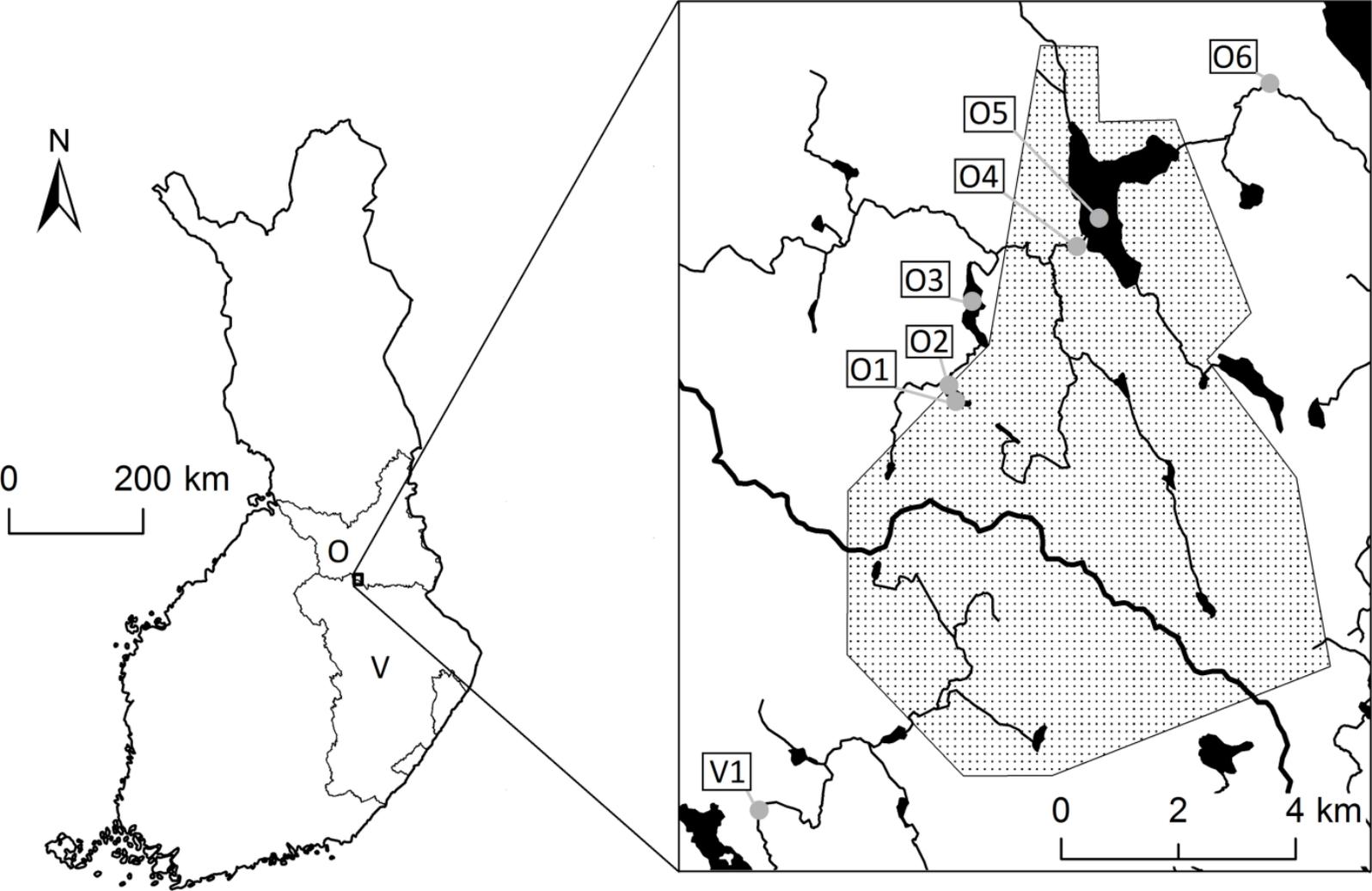
Figure 1: Location of the sampling sites in Oulujoki (O) and Vuoksi (V) watersheds in Finland. The Talvivaara mine district is indicated in grid-like fill, and lakes and streams in black. Map data: General and Topographic maps of Finland © National Land Survey of Finland 2018; Catchment Areas and National 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 *monoxygenase*, *vtg-sod* and *catalase*
- Transcriptomic biomarkers are more sensitive than acute toxicity tests

ACCEPTED MANUSCRIPT



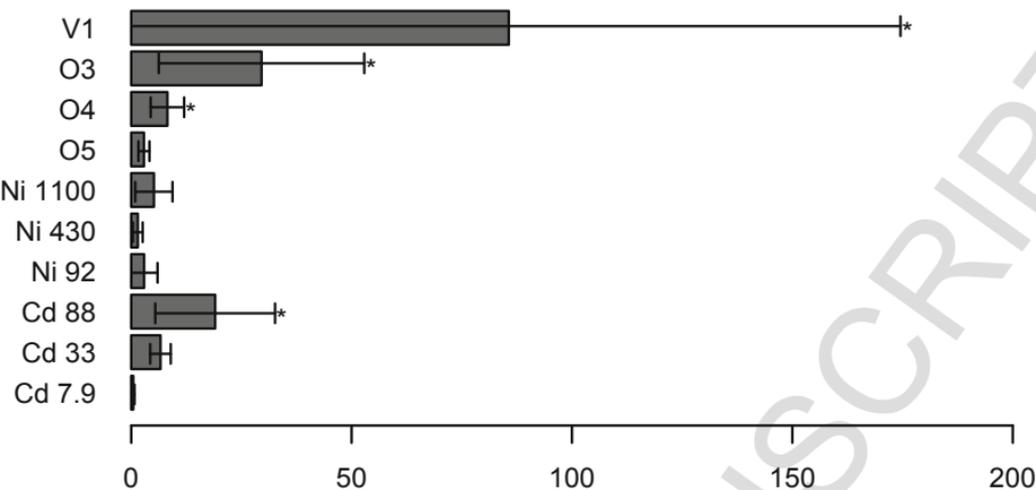
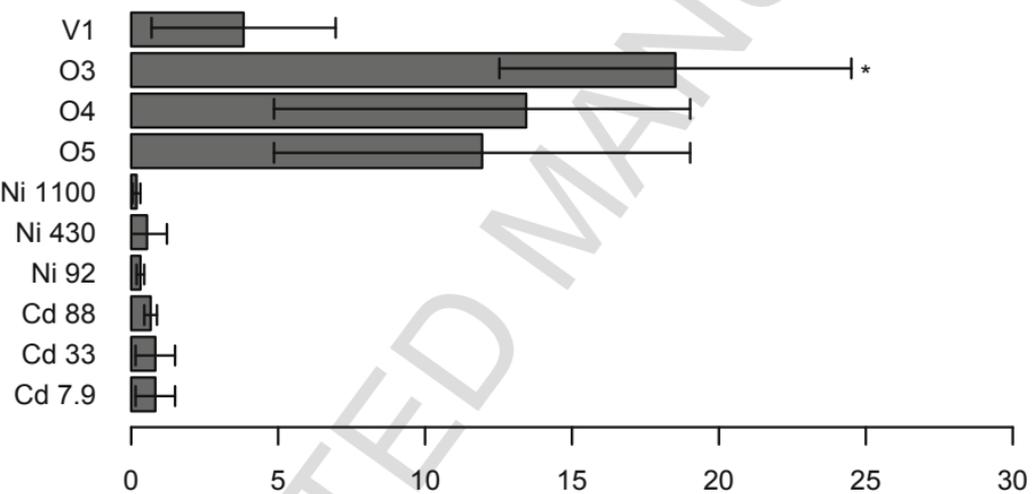
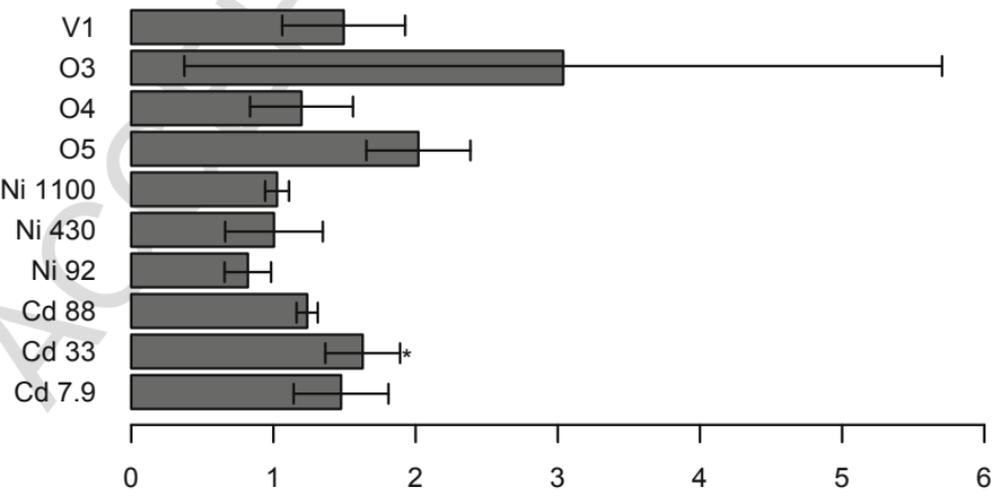
Monoxygenase**Vitellogenin-SOD****Catalase**

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)
<i>alpha-tubulin*</i>	<i>tbl</i>		WFes0007807	tggagtggtgacgact	ccaagtcgacaaagacagca	103.1	89
<i>glyceraldehyde- 3-phosphate dehydrogenase</i>	<i>gadph</i>	AJ292555		gtcttcagtgaacgagacc	gcatgggcctttcaagagt	101.2	104
<i>catalase</i>	<i>cat</i>	GQ389639		ccccaagactatttcgccg	gtaggagaaaagacgccct	98.4	112
<i>monooxygenase</i>	<i>mox</i>	DV437798		acagcttatcccgatcat	taccaagtaccgagccgttt	103.4	102
<i>vitellogenin- superoxide dismutase</i>	<i>vtg- sod</i>	AB252738		caagtacaacgagatgccg	atgtaggacccaaccagtg	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

	Unit	LOQ	Vuoksi waterway		R. Oulujoki waterway				
			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

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