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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 CO_2 emissions, SO_2 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 HNO_3 (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 HNO_3 (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 K_2CrO_7

137 in concentrations 0, 0.25, 0.5, 1, 1.5 and 2 mg/l. In addition, Ni ($NiCl_2$ × 6H2O, 106717, Merck, grade

138 ACS) in concentrations 0, 1,2,3,6, and 10 mg/l and Cd ($CdCl_2$Hydrate, 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 µg/l, and Cd in

152 concentrations 7.9, 33, and 88 µg/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 persevered 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 EC_{50}-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 $NiCl_2$ 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 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

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 EC_{50} value of the positive control

226 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

227 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.*

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 EC_{50} of Cd in the present study was 0.47 mg/l (SD 0.47). For cadmium the ECOTOX database

230 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*

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 EC_{50} 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 μg/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 μg/l) as the other three samples (0.1 μg/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 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.

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 µg/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.

349 **Acknowledgements**

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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
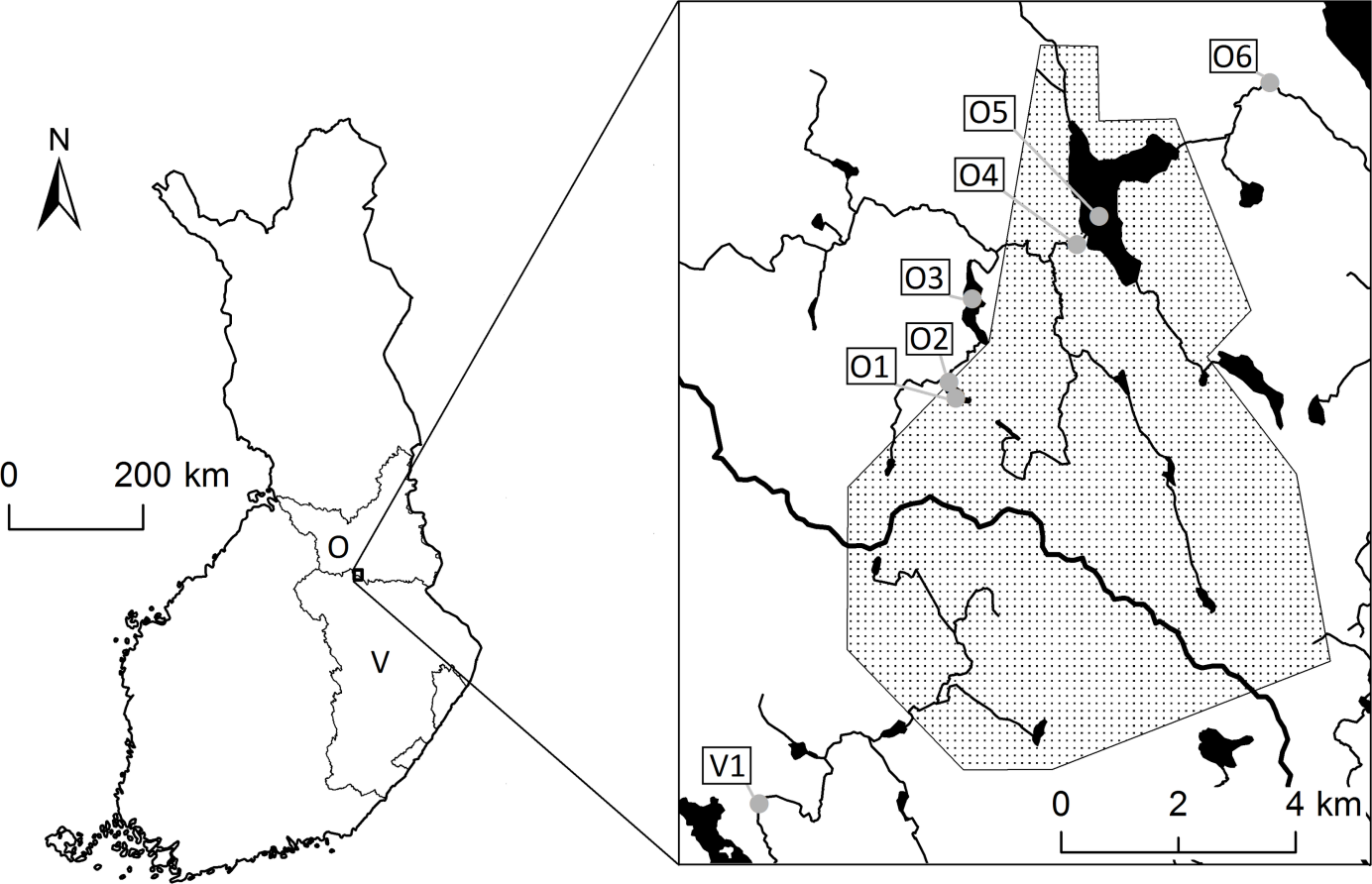
Monooxygenase
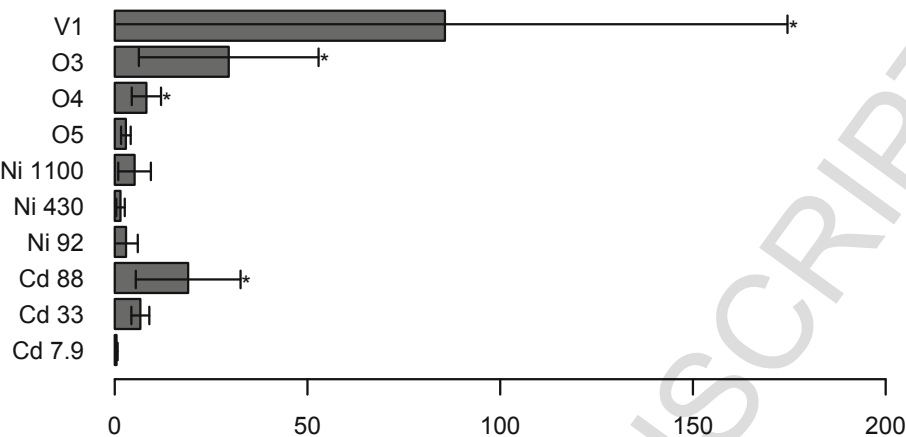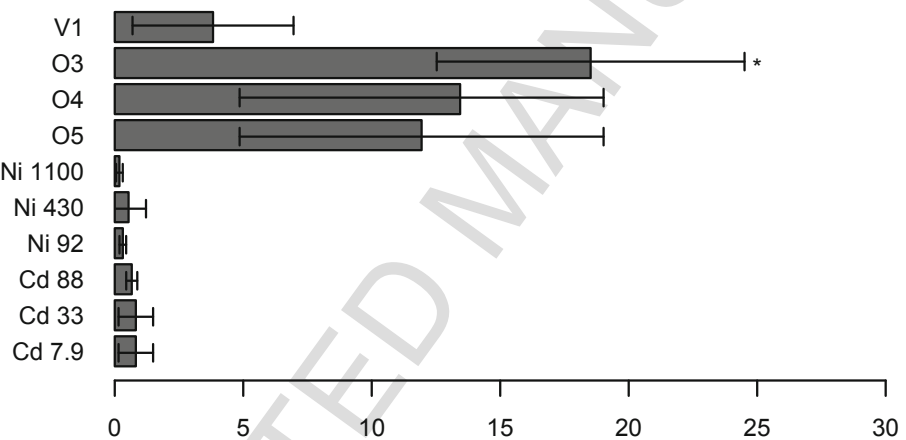
Vitellogenin−SOD

Catalase
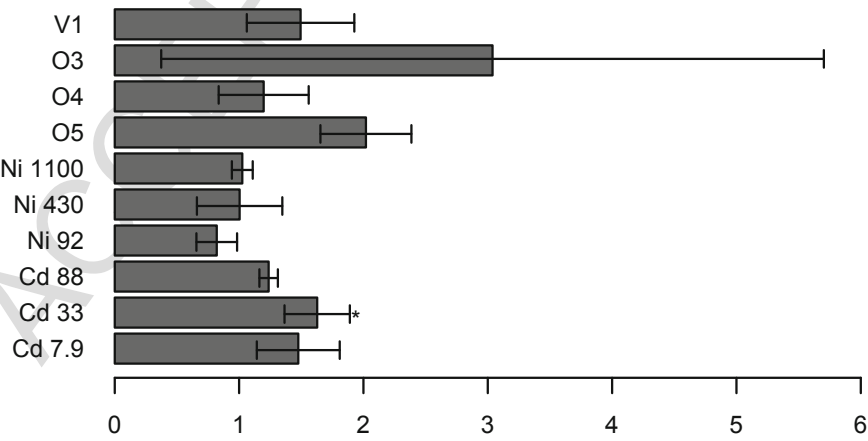
Table 1. Primers used in the qPCR.

Gene name	Symbol	GenBank accession no.	wFleaBase EST no.	Forward primer (5'-3')	Reverse primer (5'-3')	Efficiency	Amplicon size (bp)
*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