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1 **Relating metal exposure and chemical speciation to trace metal**

2 **accumulation in aquatic insects under natural field conditions**

3 Maarten De Jonge[a], Stephen Lofts[b], Lieven Bervoets[a], Ronny Blust[a]

4 *[a]Department of Biology, Systemic Physiological and Ecotoxicological Research group (SPHERE), University of*

5 *Antwerp, Groenenborgerlaan 171, B-2020 Antwerp, Belguim*

6 *[b]NERC Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg,*

7 *Lancaster LA1 4AP, United Kingdom*

8 * Corresponding author. Tel.: +32 3 265 3533: fax: +32 3 265 3497. E-mail address:

9 maarten.dejonge@uantwerpen.be (M. De Jonge).

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26 **Abstract**

27 The present study investigated to what extent measured dissolved metal concentrations,

28 WHAM-predicted free metal ion activity and modulating water chemistry factors can predict

29 Ni, Cu, Zn, Cd and Pb accumulation in various aquatic insects under natural field conditions.

30 Total dissolved concentrations and accumulated metal levels in four taxa (*Leuctra* sp.,

31 Simuliidae, *Rhithrogena* sp. and Perlodidae) were determined and free metal ion activities

32 were calculated in 36 headwater streams located in the north-west part of England. Observed

33 invertebrate body burdens were strongly related to free metal ion activities and competition

34 among cations for uptake in the biota. Taking into account competitive effects generally

35 provided better fits than considering uptake as a function of total dissolved metal levels or the

36 free ion alone. Due to the critical importance and large range in pH (4.09 to 8.33), the H^+ ion

37 activity was the most dominant factor influencing metal accumulation. Adding the influence

38 of Na^+ on Cu^{2+} accumulation improved the model goodness of fit for both *Rhithrogena* sp.

39 and Perlodidae. Effects of hardness ions on metal accumulation were limited, indicating the

40 minor influence of Ca^{2+} and Mg^{2+} on metal accumulation in soft-water streams (0.01 to 0.94

41 mM Ca; 0.02 to 0.39 mM Mg). DOC levels (ranging from 0.6 to 8.9 mg L^{-1}) significantly

42 affected Cu body burdens, however not the accumulation of the other metals.

43 Our results suggest that 1) uptake and accumulation of free metal ions is most dominantly

44 influenced by competition of free H^+ ions in low-hardness headwaters and 2) invertebrate

45 body burdens in natural waters can be predicted based on the free metal ion activity using

46 speciation modeling and effects of H^+ competition.

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48 ***Keywords***: Metal pollution; Metal speciation; Invertebrate body burdens; Windermere Humic

49 Aqueous Model (WHAM); Biotic Ligand Model (BLM)

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50   **1 Introduction**

51

52   Metal bioavailability and toxicity from water-borne exposure generally depends on the

53   activity of the free metal ion, which is controlled by chemical speciation processes (binding to

54   dissolved organic carbon (DOC), inorganic ligands (e.g. Cl^-, OH^-, CO_3^{2-}) and the effect of

55   pH), and is largely influenced by other cations (e.g. Na^+, Ca^{2+}, Mg^{2+}) and H^+ ions competing

56   with trace metals for uptake at particular biological uptake sites (e.g. ion-channels and other

57   transporters in the gills of aquatic organisms) (Hare and Tessier, 1996, 1998; Bervoets and

58   Blust, 2000). The latter concepts are integrated in the Free Ion Activity Model (FIAM), which

59   states that the activity of the free metal ion is a good predictor of both metal availability and

60   toxicity to aquatic organisms (Campbell, 1995), and has been used as the main rationale for

61   the construction of the Biotic Ligand Model (BLM) in order to predict water-borne metal

62   toxicity (Paquin et al., 2002; Niyogi and Wood, 2004).

63   Over the last decades efforts have been made to determine and predict trace metal speciation

64   in natural waters. This has led to the construction of chemical speciation models such as the

65   Windermere Humic Aqueous Model (WHAM), which enables calculation of the free metal

66   ion concentration and activity in solution, based on water chemistry measurements and

67   equilibrium binding interactions (e.g. pH, temperature, Ca^{2+}, Mg^{2+}, DOC) (Tipping, 1994,

68   1998; Tipping et al., 1998). Although WHAM is currently incorporated in the BLM (Paquin

69   et al. 2002; Niyogi and Wood, 2004), the speciation model has been rarely used to predict

70   metal accumulation in aquatic insects under natural conditions (Hare and Tessier, 1996, 1998;

71   Croteau et al., 1998; Ponton and Hare, 2009; Stockdale et al. 2010). Since water chemistry

72   and exposure scenarios can largely vary compared to conditions in the laboratory, relations

73   between metal exposure, chemical speciation and invertebrate body burdens taking into

74   account the influence of major ions and other metals at biological uptake sites should be

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75   assessed directly in the field. Recently, studies of Stockdale et al. (2010) and Tipping and

76   Lofts (2013) were able to model metal levels in field-collected aquatic invertebrates using

77   WHAM, considering organisms as humic acids, which corresponded well with measured

78   body burdens. Since accumulated metal levels represent a time-integrated and ecologically-

79   relevant measure of metal exposure and bioavailability, body burdens have gained increasing

80   attention in biomonitoring studies during recent years (Hare and Tessier, 1996; Adams et al.,

81   2011; De Jonge et al., 2013).

82   The aim of the current study was first to evaluate influences of chemical speciation and water

83   chemistry (pH, DOC and major cation effects) on insect body burdens under natural field

84   conditions, and secondly to evaluate whether WHAM-predicted free metal ion activity and

85   other modulation factors could accurately predict the observed body burdens.

86

87   **2 Material and methods**

88   **2.1 Study area and sampling design**

89

90   In total 36 headwater streams of the Lake District, Ribbledale, Swaledale and the Howgill

91   Fells, which are all located in the north-west part of England (table S1), were sampled as part

92   of an extended field survey (Bass et al., 2008). Some of these sites have been severely metal

93   contaminated from discharge of nearby abandoned mining sites. Samples for the

94   determination of water chemistry were taken on four occasions (March 6-8, March 20-22,

95   April 3-5 and April 17-19 2006). Separate samples were taken for major solutes (one-litre,

96   high-density polyethylene bottles), for pH (glass bottles with a ground glass stopper,

97   completely filled) and trace metals (500 cm³ acid-washed polyethylene bottles). All samples

98   were kept in cool boxes at 4 °C during transport to the laboratory, where they were kept cool

99   and dark.

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2.2 Water chemistry analysis and chemical speciation

102

Within one day after collecting, samples were analysed for pH using a glass electrode while taking care to avoid de-gassing of the samples. Total concentrations of Na, Mg, Al, K, Ca, Mn and Fe were measured after one week using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). Chloride (Cl), nitrate (NO_3-N) and sulphate (SO_4-S) were determined by ion chromatography; alkalinity was measured by Gran titration and dissolved organic carbon (DOC) by combustion. Ammonia-N (NH_4-N), phosphorus (P) and silica (SiO_2) were determined colorimetrically and suspended particulate matter (SPM) was determined gravimetrically. Water samples intended for trace metal analysis were filtered over a 0.45 μm polypropylene filter, acidified with 1% nitric acid (HNO_3; 69%) and total levels of Ni, Cu, Zn, Cd and Pb were quantified using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS). All analyses made use of International Quality Control standards, with verification by the Proficiency Testing scheme. Furthermore, a Certified Reference Material (CRM) was used in the determination of trace metals in surface waters.

Free ion activity (FIA) calculations of the measured trace metals were performed using the Windermere Humic Aqueous Model (WHAM) (Tipping, 1994), incorporating Humic Ion-Binding Model VI (Tipping, 1998). To calculate the chemical speciation, the concentrations of Na, K, Ca, Mg, Cl, NO_3, SO_4 and concentrations of filterable trace metals (Ni, Cu, Zn, Cd and Pb) were assumed to represent truly dissolved components (i.e. the inorganic ionic species and complexes and/or metals bound to dissolved organic matter (DOM)). The filterable fraction may also include some metal in association with mineral and organic colloids, however these species are neglected in the present analysis. The cation-binding properties of DOM were expressed in terms of isolated fulvic acid, which is thought to be the

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125 most active DOM fraction in natural waters (Vincent et al., 2001). DOM concentrations were

126 estimated based on measured DOC, assuming DOM to be 50% carbon and that 65% of the

127 DOM behaves like isolated FA and is thus active regarding cation binding (Tipping et al.,

128 2008). Ionic strength effects on the inorganic reactions were taken into account using the

129 extended Debye-Hückel equation.

130

131 **2.3 Determination of invertebrate body burdens**

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133 Aquatic insect samples were taken using a pond net (April 17-19 2006). At the laboratory

134 each sample was carefully sorted through and the collected organisms were identified to

135 family or genus level. Not all taxa were equally well presented in the sample collection and

136 the current analysis is confined to the taxa that were present in more than 18 of the sampled

137 sites, including *Leuctra* sp. (O. Plecoptera; 33 sites), Perlodidae (O. Plecoptera; 25 sites),

138 Simuliidae (O. Diptera; 24 sites) and *Rhithrogena* sp. (O. Ephemeroptera; 18 sites). All

139 samples were placed into 1.5 mL polypropylene sampling vials and stored at -20 °C. For each

140 50 sample vials with invertebrates also 5 empty vials were included to be used as process

141 controls and reference material. Samples were dried until constant dry weight at 60 °C in a

142 laboratory furnace. Subsequently they were weighed on a Sartorius SE2 Ultra Micro balance

143 (accuracy 0.1 µg) and transferred to acid-cleaned and pre-weighed 0.5 or 1.5 mL

144 polypropylene vials.

145 Invertebrate samples were microwave digested in a HNO_3 - hydrogen peroxide (H_2O_2; 30%)

146 solution (3:1, v/v) by a step-wise method in which samples were microwave treated for four

147 times, each time increasing the microwave power by 10% (Blust et al., 1988). For each series

148 of 50 samples also 5 control samples were processed and 5 samples of invertebrate reference

149 material (mussel BCR-668) were included for quality control. After the digestion procedure

150 the digest was diluted with ultra-pure water (Milli-Q) to obtain a solution of 5% acid and the

151 vials were reweighed to accurately determine the final sample volume. Trace metal

152 concentrations in invertebrate tissue were analysed using a quadrupole Inductively Coupled

153 Plasma Mass Spectrometer (ICP-MS; Varian UltraMass 700, Victoria, Australia). Results

154 regarding invertebrate body burdens have been partly incorporated in the studies of Stockdale

155 et al. (2010) and De Jonge et al. (2013).

156

157 **2.4 Data treatment and statistical analysis**

158

159 According to the FIAM, metal concentrations in aquatic organisms ($[M]_{organism}$), can be

160 described by the activity of the free metal ion ($\{M^{2+}\}$) based on the following equations,

161 depending on competition between metal ions and other ions (e.g. H^+, Na^+, Ca^{2+}) for

162 biological uptake sites (Hare and Tessier, 1996, 1998; Croteau et al., 1998):

163

164 $$[M]_{organism} = F\{M^{2+}\} \qquad (1)$$

$$[M]_{organism} = \frac{F\{M^{2+}\}}{(\{H^+\} + K_a)} \qquad (2)$$

$$[M]_{organism} = \frac{F\{M^{2+}\}}{(1 + K_{M2}\{M2^{2+}\})} \qquad (3)$$

$$[M]_{organism} = \frac{F\{M^{2+}\}}{(\{H^+\} + K_a + K_a K_{M2}\{M2^{2+}\})} \qquad (4)$$

165

166 Where equation (1) is used if competition by hydrogen ions ($\{H^+\}$) or other metal ions

167 ($\{M2^{2+}\}$) is not considered, (2) if competition by $\{H^+\}$ alone is considered, (3) if competition

168 by $\{M2^{2+}\}$ alone is considered and (4) if competition by both $\{H^+\}$ and $\{M2^{2+}\}$ is considered.

169 F (which equals $k*K_M$) encloses a proportionality constant specific to the studied taxon (k

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170   value) and is related to the binding affinity of the metal ion M^{2+} for a biological uptake site

171   (K_M), K_a is a pseudo-equilibrium affinity constant for the reaction between H^+ and metal

172   uptake sites on biological membranes, and K_{M2} is a pseudo-equilibrium affinity constant for

173   the reaction between a competing metal ion $M2^{2+}$ with metal uptake sites on biological

174   membranes (Hare and Tessier, 1996, 1998; Croteau et al., 1998). Calculated K-values

175   encompass a variety of biological and geochemical processes influencing metal uptake and

176   accumulation in aquatic insects under natural field conditions. Linear regression analysis was

177   used to relate body burdens to total dissolved metals, WHAM-predicted free metal ion

178   activities and $\{M^{2+}\}$ considering the influence of $\{H^+\}$ and/or other metal ions at biological

179   uptake sites. Consequently, pseudo-equilibrium affinity constants K_a and K_{M2} could be

180   obtained by rewriting equations (2) and (3):

181

$$K_a = \frac{F\{M^{2+}\}}{[M]_{organism} - y_0} - \{H^+\} \tag{5}$$

182

$$K_{M2} = \frac{F\{M^{2+}\}}{[M]_{organism}\{M2^{2+}\} - y_0\{M2^{2+}\}} - \frac{1}{\{M2^{2+}\}} \tag{6}$$

183

184   Where F and y_0 of equation (5) and (6) are slope and intercept of the linear regressions from

185   equations (2) and (3) respectively.

186   Prior to statistical analysis, all data were log transformed in order to meet conditions of

187   normality and homogeneity of variances. Pearson correlations were used to determine

188   relations between total dissolved metal concentrations and WHAM-predicted free metal ion

189   activities. Overall, the significance level is represented as *: $p < 0.05$; **: $p < 0.01$; ***: $p <$

190   0.001. All statistical analyses were performed using the software package SigmaPlot version

191   11.0 (Systat Software Inc., San Jose, California, USA).

192

193   **3 Results and discussion**

194   **3.1 Effect of water chemistry on Ni body burdens**

195

196   Total dissolved Ni concentrations ranged from 0.002 to 1.29 μM (table 1). WHAM- predicted

197   free Ni ion activities were significantly positively correlated with $\{H^+\}$, $\{K^+\}$, $\{Mg^{2+}\}$ and all

198   other trace metal ions (table 2). No significant correlation was observed between $\{Ni^{2+}\}$ and

199   both $\{Na^+\}$ and $\{Ca^{2+}\}$. Nickel body burdens ranged from 0.003 (Perlodidae) to 0.68 μmol g^{-1}

200   dw (*Leuctra* sp.) (table 3). Ni body burdens in *Leuctra* sp., Simuliidae, *Rhithrogena* sp. and

201   Perlodidae were generally poorly correlated with total dissolved Ni levels (figure 1). Only for

202   Simuliidae a significant regression model (r^2 = 0.401; n = 24; $p<0.001$) was obtained.

203   Analogously, WHAM-predicted $\{Ni^{2+}\}$ poorly correlated to $[Ni]_{organism}$ for all taxa.

204   Conversely, better relations were found between $\{Ni^{2+}\}$ considering the influence of H^+ ions,

205   improving the model r^2 for both *Leuctra* sp. (r^2 from 0 to 0.146; n = 33; $p<0.05$) and

206   *Rhithrogena* sp. (r^2 from 0.156 to 0.209; n = 20; $p<0.05$).

207   In general, only little variation in Ni body burdens was explained in the selected insect taxa.

208   Metal concentrations can vary widely among sympatric species of insects of the same genus

209   (Martin et al., 2008) and even wider variations are possible among insect genera (e.g.

210   Buchwalter and Luoma, 2005; Martin et al., 2007). Most likely intra-family variability in

211   metal accumulation can explain the rather poor correlations between Ni exposure and

212   measured body burdens in the present study. Nevertheless for all other metals observed insect

213   body burdens could be much better explained using speciation modelling (higher r^2) (see 3.2

214   to 3.5).

215   Although observed model r^2 for Ni are generally low, our results follow the general principles

216   of both the FIAM (Campbell, 1995) and the BLM (Paquin et al., 2002), stating that water-

217   borne metal uptake and toxicity should be predominantly predicted by free ion activities in the

218   exposure medium rather than total dissolved metals, taking into account effects of H^+ and

219   other cations (Na^+, Ca^{2+}, Mg^{2+}…) competing with the free ion for binding to the biological

220   uptake site. Analogously, Ponton and Hare (2009) demonstrated that Ni levels in four

221   *Chaoborus* species of different lakes were best predicted by $\{Ni^{2+}\}/\{H^+\}$. The latter study

222   estimated a K_a of 3.37 ± 1.17 µM, which is slightly higher (maximum a factor two) compared

223   to K_a values calculated for Leuctra sp., Simuliidae and *Rhithrogena* sp. in the present study

224   ($K_a = 1.51$, 2.05 and 2.76 µM respectively) (table 4). Furthermore Ponton and Hare (2009)

225   observed a strong correlation between total dissolved Ni and *Chaoborus* body burdens, which

226   was explained by the fact that the majority of dissolved Ni was present as Ni^{2+}. In the present

227   study Ni accumulation in Simuliidae was also most strongly correlated with total dissolved Ni

228   ($r^2 = 0.401$; $n = 24$; $p<0.001$), which can be related to the fact that on average 44.6% (ranging

229   from 8.8% to 86.7%) of total dissolved Ni occurred as free metal ions (Pearson correlation

230   between total dissolved Ni and $\{Ni^{2+}\}$: $r = 0.864$; $n = 36$; $p<0.001$).

231   Furthermore several other ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+}) were tested as

232   possible competitors for Ni^{2+} uptake, however none of these models appeared to be significant

233   for any of the taxa. Consistent with our findings both Deleebeeck et al. (2008a) and

234   Komjarova and Blust (2009a) did not observe effects of increasing Na^+ concentrations on Ni

235   uptake in the waterflea *Daphnia magna* under laboratory conditions. Conversely, with our

236   results, Komjarova and Blust (2009a) observed a decreased Ni uptake at Ca^{2+} concentrations

237   which were however considerably higher (from 0.1 mM to 2.5 mM Ca) compared to the ones

238   measured in the natural waters of the present study (from 0.01 to 0.94 mM Ca). Deleebeeck et

239   al (2008a,b) observed decreased chronic toxicity in *D. magna* with increasing Ca^{2+} and Mg^{2+}

240   levels, which were also more elevated (0.25 mM to 3.0 mM for both Ca and Mg) compared to

241   the ones in the present study (0.02 mM to 0.39 mM Mg). In fact, Mg is known to compete

242   with Ni^{2+} ions for entry at Mg^{2+} uptake sites since both ions have similar dehydrated ionic

243   radii and share similar transporters (Deleebeeck et al. 2008a,b; Niyogi et al., 2014).

244   Nevertheless, our results indicate that in natural streams with lower water hardness (thus low

245   Ca and Mg levels) but varying pH, Ni uptake and accumulation will be dominantly influenced

246   by H^+ ions, rather than Ca^{2+} or Mg^{2+} levels. Moreover, in the studies of Komjarova and Blust

247   (2009a) and Deleebeeck et al. (2008a,b) Daphnids were not acclimatized to elevated Ca levels

248   prior to exposure. Therefore, Ca acclimatization/adaptation in the insects of the sampled

249   streams may results in the disappearance of hardness effects on Ni accumulation under natural

250   field conditions. Furthermore Keithly et al. (2004) observed a mitigating effect of both Ca and

251   Mg on acute Ni toxicity in *Ceriodaphnia dubia*, however not for chronic toxicity, suggesting

252   that Ca and Mg-dependent pathways are only involved in Ni uptake at relatively high

253   concentrations.

254   No significant relations were observed between DOC levels and Ni body burdens in any of

255   the sampled invertebrate taxa.

256

257   **3.2 Effect of water chemistry on Cu body burdens**

258

259   Total dissolved Cu concentrations ranged from 0.003 to 0.15 µM (table 1). WHAM- predicted

260   free Cu ion activities were significantly positively correlated with $\{H^+\}$ and all other metal

261   ions and negatively with $\{Ca^{2+}\}$ (table 2). No significant correlations were observed between

262      $\{Cu^{2+}\}$ and $\{Na^+\}$, $\{K^+\}$ and $\{Mg^{2+}\}$. Invertebrate Cu body burdens ranged from 0.18

263   (Perlodidae) to 13.8 µmol g^{-1} dw (Simuliidae) (table 3). Copper is an essential element (e.g.

264   present in a variety of enzymes in aquatic insects) and therefore Cu body burdens can be

265 homeostatically regulated within a certain environmental range (Rainbow, 2002). Following

266 our results, Cu seems to be regulated at body burdens ranging from 0.17 (*Rhithrogena* sp.) to

267 1.76 µmol g^{-1} dw (*Leuctra* sp.), until regulation breaks down and body burdens increase with

268 environmental levels (figure 2).

269 Significant relations were observed between total dissolved [Cu] and body burdens in *Leuctra*

270 sp. (r^2 = 0.190; n = 33; $p<0.05$), Simuliidae (r^2 = 0.780; n = 24; $p<0.001$) and Perlodidae (r^2 =

271 0.388; n = 25; $p<0.001$) (figure 2). Relating WHAM-predicted {Cu^{2+}} to [Cu]$_{organism}$ largely

272 improved the regression models for *Rhithrogena* sp. (r^2 from 0.060, $p>0.05$ to 0.373, $p<0.01$)

273 and Perlodidae (r^2 from 0.388, $p<0.001$ to 0.696, $p<0.001$), but resulted in less accurate

274 models for *Leuctra* sp. (r^2 from 0.190, $p<0.01$ to 0.146, $p<0.05$) and Simuliidae (r^2 from

275 0.780, $p<0.001$ to 0.493, $p<0.001$). Considering the influence of {H$^+$} at uptake sites for Cu

276 resulted in much stronger and highly significant (all $p<0.001$) regression models for *Leuctra*

277 sp. (r^2 = 0.349), Simuliidae (r^2 = 0.885) and *Rhithrogena* sp. (r^2 = 0.456), compared to the

278 models using dissolved [Cu] and {Cu^{2+}}. Adding the combined influence of {H$^+$} and {Na$^+$}

279 resulted in the improvement of the models for *Rhithrogena* sp. (r^2 = 0.490) and Perlodidae (r^2

280 = 0.796), which were all highly significant (all $p<0.001$). It has been shown that increasing

281 Na$^+$ levels decrease both the uptake and toxicity of Cu to a wide range of aquatic organisms

282 (Paquin et al. 2002; De Schamphelaere and Janssen, 2002, 2004; Niyogi and Wood, 2004).

283 Copper ions, which are reduced from the divalent Cu^{2+} to the monovalent Cu$^+$ at biological

284 membranes (Rolfs and Hediger, 1999), compete with Na$^+$ for uptake in epithelial cells (via

285 apical Na$^+$ channels) (Grosell and Wood, 2002). Nevertheless, in the present study K_a was

286 much higher compared to K_{Na} for all taxa (table 4), implying that the influence of H$^+$ ions for

287 binding at Cu uptake sites was more important compared to Na$^+$ under these natural

288 conditions. This is not surprisingly since Grosell and Wood (2002) showed that Cu uptake

289 through Na-channels was only important at very low Cu concentrations (< 40 nM).

290   Regression models for *Leuctra* sp. and Simuliidae were less strong after including the effect

291   of $\{Na^+\}$, compared to $\{H^+\}$ alone. Several studies demonstrated decreased Cu toxicity with

292   increasing pH in both *D. magna* and *C. dubia* under laboratory conditions (De Schamphelaere

293   and Janssen, 2002, 2004; Hyne et al., 2005) in the pH range 5.50 to 7.92, which is smaller

294   compared to the pH range in the waters of the present study (pH range 4.09 to 8.33).

295   Adding effects of K^+, Ca^{2+}, Mg^{2+}, Cd^{2+} and Zn^{2+} (both singular and in combination with H^+)

296   resulted in significant (all $p<0.05$) regression models for all taxa (results not shown), however

297   these models were weaker compared to the ones using pH and Na^+ normalizations.

298   Only for Cu a significant negative correlation between DOC and insect body burdens for all

299   taxa was observed (figure 3A). Since WHAM-predicted $\{Cu^{2+}\}$ were also significantly

300   negative correlated to DOC levels (figure 3B), we can assume that DOC decreased Cu uptake

301   in the aquatic insects by decreasing the Cu^{2+} activity. The latter illustrates the high affinity of

302   Cu ions to bind with DOC and thus the importance of DOC to reduce Cu accumulation and

303   toxicity in natural waters (De Schamphelaere and Janssen, 2004; Niyogi and Wood, 2004).

304   Nevertheless, increasing DOC levels may also result in increasing levels of particulate

305   organic matter (POM) by coating of particles, which have a high affinity for trace metals. The

306   study of Guo et al. (2001) showed an enhanced metal uptake in filter-feeding bivalves at

307   higher DOC levels (5 and 10 ppm). Also in the present study the relation between DOC and

308   Cu body burdens was attenuated at DOC levels around 2.5 ppm, suggesting possible metal

309   uptake via POM ingestion at elevated DOC levels.

310

311   **3.3 Effect of water chemistry on Zn body burdens**

312

313   Total dissolved Zn concentrations ranged from 0.017 to 168 μM (table 1). WHAM-predicted

314   Zn ion activities were significantly positively correlated with $\{K^+\}$, $\{Mg^{2+}\}$ and all other trace

315   metal ions (table 2). No significant correlations were observed between $\{Zn^{2+}\}$ and $\{H^+\}$,

316     $\{Na^+\}$ and $\{Ca^{2+}\}$. Zinc body burdens ranged from 1.96 (Simuliidae) to 85.7 µmol g^{-1} dw

317   (*Leuctra* sp.) (table 3). Like Cu, Zn is an essential element (e.g. key component of the enzyme

318   carbonic anhydrase) (Rainbow, 2002). In the present study evidence of homeostatic Zn

319   regulation was observed with constant body burdens ranging from 1.97 (Simuliidae) to 6.13

320   µmol g^{-1} dw (*Rhithrogena* sp.) (figure 4).

321   Highly significant (all $p<0.001$) relations were observed between total dissolved levels and Zn

322   body burdens in all invertebrate taxa ($r^2 = 0.572$ for *Leuctra* sp.; $r^2 = 0.705$ for Simuliidae; r^2

323     $= 0.862$ for *Rhithrogena* sp.; $r^2 = 0.601$ for Perlodidae) (figure 4). Using WHAM-predicted

324     $\{Zn^{2+}\}$ did not improve the models based on dissolved [Zn], however models remained highly

325   significant (all $p<0.001$) for all taxa. The strong influence of total dissolved [Zn] on

326   invertebrate body burdens can be explained by the fact that in the sampled river waters of the

327   present study, 53.4% (ranging from 11.6 to 92.0%) of the total dissolved Zn occurred as free

328   metal ions, while only 13.8% was bound to DOC. Moreover, Zn speciation in natural waters

329   is generally less subject to variation, compared to free ion activities of Cu and Pb, which can

330   vary largely with both pH and DOC (Tipping et al., 1998; Vincent et al., 2001). No significant

331   relations between DOC levels and Zn body burdens were observed in the present study.

332   Considering the influence of $\{H^+\}$ on Zn uptake sites improved the regression model for

333   *Leuctra* sp. ($r^2 = 0.745$; n = 33; $p<0.001$), compared to the ones using dissolved [Zn] and

334     $\{Zn^{2+}\}$, but did not improve the goodness of fit for all other taxa ($r^2 = 0.468$ for Simuliidae; r^2

335     $= 0.745$ for *Rhithrogena* sp.; $r^2 = 0.584$ for Perlodidae). For instance Bervoets and Blust

336   (2000) demonstrated a decreased Zn uptake with increasing H^+ ions in the midge larvae

337   *Chironomus riparius* over a pH range of 5.5 to 10.0.

338   The influence of $\{Ca^{2+}\}$ on Zn uptake sites resulted in significant (all $p<0.05$) regression

339   models for all taxa ($r^2 = 0.171$ for *Leuctra* sp.; $r^2 = 0.197$ for Simuliidae; $r^2 = 0.443$ for

340   *Rhithrogena* sp.; r^2 = 0.385 for Perlodidae), which were however less strong compared to the

341   ones using total dissolved [Zn] and $\{Zn^{2+}\}/\{H^+\}$. Studies of Poteat et al. (2012) and Poteat

342   and Buchwalter (2014) analogously reported the lack of strong Ca interactions with Zn uptake

343   in aquatic insects. Nevertheless, it has been repeatedly demonstrated that Ca^{2+} and Zn^{2+} ions

344   share similar transport mechanisms and that Ca^{2+} ions lower Zn uptake and mitigate toxicity

345   in aquatic organisms (Paquin et al. 2002; Niyogi and Wood, 2004; Heijerick et al., 2005).

346   Komjarova and Blust (2009a) demonstrated a decrease of Zn uptake in *D. magna*, however

347   only at Ca concentrations which were much higher (2.5 mM Ca) compared to the levels found

348   in natural waters of the present study (from 0.01 mM to 0.94 mM Ca). The authors of the

349   latter study suggested that depending on the concentrations and binding characteristics of both

350   cations, the competitive effect of Ca may be limited at low environmental Ca concentrations

351   (Komjarova and Blust, 2009a). Furthermore, Ca acclimatization under natural conditions

352   might explain the limited Ca^{2+} influence on Zn uptake and accumulation in the rivers included

353   in the present study. It has been observed that the influence of Ca on Zn toxicity is much

354   higher under short-term conditions compared to chronic scenarios. For example studies of

355   Heijerick et al. (2002, 2005) experimentally showed that Ca reduced Zn toxicity in *D. magna*

356   with a factor 6.3 after 2 days (Heijerick et al., 2002) compared to a factor 1.8 after 21 days

357   (Heijerick et al., 2005). In addition to Ca channels, Zn can also be taken up by Zip proteins,

358   which appear to be Zn specific and are thus not influenced by competition of Ca (Qiu et al.,

359   2005).

360   Adding the effect of $\{Cd^{2+}\}$ on Zn uptake sites did not result in significant regression models

361   for any of the taxa. The latter is possibly due to the strong correlation between $\{Zn^{2+}\}$ and

362     $\{Cd^{2+}\}$ (r = 0.967; n = 36; p<0.001), which prevents revelation of any possible Cd effect on

363   Zn uptake and accumulation. Furthermore only little influence of $\{Cd^{2+}\}$ on Zn uptake was

364   expected since total dissolved Cd concentrations were much lower compared to Zn (on

365 average a factor 500). Nevertheless the competitive effect of Cd^{2+} on both Zn uptake and

366 toxicity has been documented (Norwood et al., 2003; Komjarova and Blust, 2008), which can

367 be explained by the fact that both Cd^{2+} and Zn^{2+} interact with Ca channels in epithelial cells

368 resulting in competitive inhibition (Van Ginneken et al., 1999).

369

370 **3.4 Effect of water chemistry on Cd body burdens**

371

372 Total dissolved Cd concentrations ranged from 0.027 to 171 nM (table 1). WHAM-predicted

373 Cd ion activities were significantly positively correlated with $\{K^+\}$, $\{Mg^{2+}\}$ and all other

374 metal ions (table 2). No significant correlations were observed between $\{Cd^{2+}\}$ and $\{H^+\}$,

375 $\{Na^+\}$ and $\{Ca^{2+}\}$. Cadmium body burdens ranged from 0.001 (Simuliidae) to 0.304 μmol g^{-1}

376 dw (*Rhithrogena* sp.) (table 3). Significant relations between total dissolved [Cd] and

377 invertebrate body burdens were observed for all taxa ($r^2 = 0.560$ for *Leuctra* sp.; $r^2 = 0.657$ for

378 Simuliidae; $r^2 = 0.761$ for *Rhithrogena* sp.; $r^2 = 0.647$ for Perlodidae; all $p<0.001$) (figure 5).

379 Using WHAM-predicted $\{Cd^{2+}\}$ resulted in stronger models for *Rhithrogena* sp. ($r^2 = 0.769$)

380 and Perlodidae ($r^2 = 0.660$). Considering the influence of $\{H^+\}$ at Cd uptake sites resulted in

381 highly significant models (all $p<0.001$) which were much stronger for *Leuctra* sp. ($r^2 =$

382 0.854), Simuliidae ($r^2 = 0.717$) and Perlodidae ($r^2 = 0.727$), compared to the ones using

383 dissolved [Cd] and $\{Cd^{2+}\}$. Analogously, both Hare and Tessier (1996, 1998) and Croteau et

384 al. (1998) demonstrated the major competitive influence of H^+ at Cd uptake sites in larvae of

385 the phantom midge *Chaoborus punctipennis* under natural field conditions. The latter studies

386 obtained K_a values for *Chaoborus* of 1.7 μM (Hare and Tessier, 1998) and 0.8 μM (Croteau et

387 al., 1998) respectively, which are generally lower (maximum a factor 4) compared to the ones

388 estimated in the present study (ranging from 2.61 μM to 3.23 μM).

389   Influences of $\{Ca^{2+}\}$ on Cd uptake sites, both with and without additional pH effect, resulted

390   in significant (all $p<0.05$) regression models for *Leuctra* sp. (Ca^{2+} only: $r^2 = 0.165$; $Ca^{2+} + H^+$:

391    $r^2 = 0.193$), *Rhithrogena* sp. (Ca^{2+} only: $r^2 = 0.197$; $Ca^{2+} + H^+$: $r^2 = 0.541$) and Perlodidae ($r^2 =$

392   0.431 for both models), which were however less strong compared to the ones using total

393   dissolved [Cd] and $\{Cd^{2+}\}/\{H^+\}$. Similarly, Poteat and Buchwalter (2014) observed only little

394   Ca influences on Cd uptake in aquatic insects (mayflies and caddisflies). Also Croteau et al.

395   (1998) observed no influence of Ca levels on Cd uptake and accumulation in various

396   *Chaoborus* species which could be explained by the negative correlation between H^+ and

397   Ca^{2+}, similarly found in the present study, and which hampers discrimination of Ca effects on

398   metal accumulation in both datasets (table 2). Nevertheless, competitive effects of Ca^{2+} on Cd

399   uptake sites could be expected, since Cd^{2+} ions can be taken up via Ca channels as both ions

400   have almost similar ionic radii (Cd: 0.92 Å; Ca: 0.94 Å) (Rainbow and Black, 2005).

401   Moreover, Ca^{2+} has been observed to reduce Cd uptake and toxicity in various aquatic

402   organisms (Niyogi and Wood, 2004; Rainbow and Black, 2005; Komjarova and Blust,

403   2009a). Komjarova and Blust (2009a) observed a large decrease in Cd uptake rate in *D.*

404   *magna* under laboratory conditions using much higher Ca levels (up to 2.5 mM Ca) compared

405   to the ones measured in the present study (0.11 to 0.97 mM Ca). Therefore, the low influence

406   of Ca^{2+} on Cd uptake sites might be also due to the low Ca levels in the natural waters of the

407   present study. In addition, differences in Ca-needs between Crustaceans (e.g. *D. magna*) and

408   aquatic insects might explain the large difference in Ca influence on Cd accumulation (and

409   other metal body burdens), which is observed between the present study using aquatic insects

410   and studies using the test species *D. magna*.

411   Adding the effect of $\{Zn^{2+}\}$ on Cd uptake sites did not result in significant regression models

412   for any of the studied taxa. Similarly, increasing Zn levels (from 0.1 to 1.25 μM Zn) did not

413   influence Cd uptake rates in *D. magna* after 96 h in the study of Komjarova and Blust (2008).

414   Conversely, Zn (on average 2.4 μM) was found to significantly inhibit Cd accumulation in the

415   amphipod *Hyalella azteca* after 28 d exposure to a mixture of 10 different metals (Norwood et

416   al., 2007).

417   No significant relations were observed between DOC concentrations and Cd body burdens in

418   any of the sampled insect taxa.

419

420   **3.5 Effect of water chemistry on Pb body burdens**

421

422   Total dissolved Pb concentrations ranged from 0.242 to 754 nM (table 1). WHAM-estimated

423   Pb ion activities were significantly positively correlated with $\{H^+\}$ and all other metal ions

424   (table 2). No significant correlations were observed between $\{Pb^{2+}\}$ and ion activities of the

425   hardness ions. Lead body burdens ranged from 0.001 (Simuliidae) to 12.0 μmol g^{-1} dw

426   (*Leuctra* sp.) (table 3). Significant relations were observed between total dissolved [Pb] and

427   invertebrate body burdens for all taxa ($r^2 = 0.761$ for *Leuctra* sp.; $r^2 = 0.610$ for Simuliidae; r^2

428    $= 0.808$ for *Rhithrogena* sp.; $r^2 = 0.473$ for Perlodidae) (figure 6). Using WHAM-predicted

429    $\{Pb^{2+}\}$ resulted in much weaker regression models for all taxa ($r^2 = 0.191$ for *Leuctra* sp.; $r^2 =$

430   0.253 for Simuliidae; $r^2 = 0.516$ for *Rhithrogena* sp.; $r^2 = 0.354$ for Perlodidae), which were

431   however all significant (all $p<0.01$). Considering the influence of $\{H^+\}$ at Pb uptake sites

432   resulted in highly significant models (all $p<0.001$) for all taxa, which were stronger for

433   *Leuctra* sp. ($r^2 = 0.768$) and Perlodidae ($r^2 = 0.630$) in comparison with the ones using either

434   dissolved [Pb] or $\{Pb^{2+}\}$ without pH influence on uptake sites. The latter results are in

435   agreement with the FIAM (Campbell, 1995). A decreased Pb uptake due to H^+ competition

436   has been observed for both zebrafish (*Danio rerio*) (Komjarova and Blust, 2009b) and fathead

437   minnow (*Pimephales promelas*) (Grosell et al., 2006). Nevertheless, Pb^{2+} ions can vary

438   largely with pH in natural waters (Tipping et al., 1998; Vincent et al., 2001) and this may

439 result in increased $\{Pb^{2+}\}$ (and thus increased uptake and toxicity) under acidic conditions

440 (Grosell et al., 2006; Komjarova and Blust, 2009a; Mager et al., 2011).

441 Adding the effect of $\{Na^+\}$ at Pb uptake sites resulted in significant models for all taxa ($r^2 =$

442 0.137 for *Leuctra* sp.; $r^2 = 0.126$ for Simuliidae; $r^2 = 0.462$ for *Rhithrogena* sp.; $r^2 = 0.281$ for

443 Perlodidae; all $p<0.05$) (results not shown). Considering the influence of $\{Ca^{2+}\}$, significant

444 models were only observed for *Rhithrogena* sp. ($r^2 = 0.336$) and Perlodidae ($r^2 = 0.170$; both

445 $p<0.05$) (results not shown). Recent studies have revealed that Pb^{2+} ions are taken up through

446 voltage-independent Ca channels in the gills of the rainbow trout, similar to the entry of Ca^{2+}

447 (Rogers and Wood, 2004; Niyogi and Wood, 2004). Furthermore the protective effect of Ca^{2+}

448 on both Pb uptake (Komjarova and Blust, 2009a,b; Grosell et al., 2006) and toxicity (Grosell

449 et al., 2006) has been demonstrated experimentally for *D. magna*, *D. rerio* and *P. promelas*.

450 Nevertheless increased water hardness did not protect *C. dubia* to chronic Pb toxicity in the

451 study of Mager et al. (2011). The low influence of Ca^{2+} on Pb uptake sites as observed in the

452 present study may be partly explained by the relative small range of Ca levels measured in the

453 sampled soft-water streams. Pb body burdens in Simuliidae and *Rhithrogena* sp. were most

454 dominantly related to total dissolved [Pb], rather than WHAM-predicted $\{Pb^{2+}\}$.

455 Although DOC concentrations significantly influenced $\{Pb^{2+}\}$ ($r = -0.427$; $p=0.009$; $n=36$)

456 (table 2), no significant relations were observed between DOC and Pb body burdens in any of

457 the sampled insect taxa.

458

459 **3.6 Dietary influences on metal accumulation**

460

461 In the present study WHAM-predicted free ion activities together with modulating water

462 chemistry variables could explain a large amount of the observed variation in metal body

463 burdens (maximal 40% for Ni, 87% for Cu and Zn, 85% for Cd and 81% for Pb). Chemical

464 speciation modeling together with effects of water chemistry has been already successfully

465 applied to describe and model both Cd and Ni body burdens in various *Chaoborus* sp. larvae

466 under natural conditions (Hare and Tessier, 1996, 1998; Croteau et al., 1998; Ponton and

467 Hare, 2009). Recently, studies of Stockdale et al. (2010) and Tipping and Lofts (2013) were

468 able to model metal levels in field-collected aquatic invertebrates based on metal binding to

469 humic acids using WHAM, considering organisms as HA, which corresponded well with

470 measured body burdens. The same approach was used by Iwasaki et al. (2013) to estimate

471 impacts of metal mixtures on aquatic invertebrates in stream microcosms.

472 Although the latter studies support the general idea of quantifying invertebrate body burdens

473 based on free ion activities from waterborne exposure, the importance of diet on metal

474 accumulation should not be neglected. It is widely accepted that trace metal bioaccumulation

475 in aquatic insects is driven by both uptake from water and diet (see e.g. Munger and Hare,

476 1997; Croisetière et al., 2006; Martin et al., 2007; De Jonge et al., 2010; Cain et al., 2011).

477 Luoma and Rainbow (2005) highlighted the importance of dynamic metal uptake from both

478 water and food, together with internal regulation, storage and elimination, in their concept of

479 biodynamic modelling. The study of De Jonge et al. (2010) revealed that Cd accumulation in

480 the midge larvae *Chironomus* sp. was explained for 98% by a regression model using

481 sediment-bound Cd concentrations. In the study of Croisetière et al. (2006) As, Cd, Co, Cu

482 and Zn accumulation in the alderfly *sialis velata* was mainly controlled (94%) by levels in

483 prey organisms. Also in the present study dietary metal uptake from prey, periphyton and/or

484 SPM might have contributed to observed metal body burdens. Nevertheless, our results

485 indicate that water chemistry plays a major role in controlling the steady-state of metals

486 accumulated by aquatic insects, either directly via uptake of free metal ions, or indirectly if

487 metals are taken up via food.

488

4 Conclusions

Strong relations were observed between insect body burdens and WHAM-calculated free metal ion activities and the competing ions for uptake, which generally provided superior fits compared to metal accumulation as a function of total dissolved metal levels or the free ion alone. Due to the large range in pH observed in the waters of the present study, the effect of H^+ ions on insect body burdens was most clearly revealed. In addition, the influence of Na^+ on Cu^{2+} uptake was observed for *Rhithrogena* sp. and Perlodidae. Furthermore Cu body burdens in all taxa were influenced by DOC levels, while no DOC effect was observed for any other metal. In contrast to general observations under laboratory conditions, effects of major hardness ions and other trace metals on metal accumulation were rather limited, indicating the minor influence of Ca^{2+}, Mg^{2+} or other trace elements on metal accumulation in natural soft-water streams and/or possible acclimatization/adaptation effects of aquatic insects under natural conditions.

Our results suggest that insect body burdens in natural waters can be predicted based on the free metal ion activity using speciation modeling and effects of H^+ competition.

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Tables

Table 1: Range of water chemistry variables (means of four determinations) of all sample sites ($n=36$) are presented.

Variable	Unit	Min.	Max.
Temperature	$°C$	4.55	7.38
pH	-	4.09	8.33
Cond	$\mu s\ cm^{-1}$	28	320
SiO_2	$mg\ L^{-1}$	0.85	9.76
Total P	$\mu g\ L^{-1}$	5.23	42.5
DOC	$mg\ L^{-1}$	0.6	8.9
SPM	$mg\ L^{-1}$	0.15	42.1
NH_4-N	$\mu g\ L^{-1}$	< 5	53.0
NO_3-N	$mg\ L^{-1}$	0.03	0.80
SO_4-S	$mg\ L^{-1}$	0.92	26.1
Alkalinity	$\mu eq\ L^{-1}$	< 1	2,010
Na	mM	0.11	0.97
Mg	mM	0.02	0.39
K	mM	0.003	0.05
Ca	mM	0.01	0.94
Cl	mM	0.10	1.22
Al	μM	0.04	58.0
Mn	μM	0.04	20.0
Fe	μM	0.12	9.04
Ni	μM	0.002	1.29
Cu	μM	0.003	0.15
Zn	μM	0.017	168
Cd	nM	0.027	171
Pb	nM	0.242	754

Table 2: Pearson correlations between WHAM-predicted free metal ion activities ($n = 36$). r-values and significance level are presented; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

	$\{Na^+\}$	$\{Mg^{2+}\}$	$\{K^+\}$	$\{Ca^{2+}\}$	$\{Ni^{2+}\}$	$\{Cu^{2+}\}$	$\{Zn^{2+}\}$	$\{Cd^{2+}\}$	$\{Pb^{2+}\}$
$[H^+]$	0.057	**-0.464****	**-0.476****	**-0.751*****	**0.443****	**0.706*****	0.150	0.131	**0.627*****
$\{Na^+\}$		-0.278	-0.027	-0.131	-0.055	-0.142	0.012	-0.059	0.026
$\{Mg^{2+}\}$			**0.780*****	**0.800*****	**0.363***	-0.153	**0.535*****	**0.524****	-0.013
$\{K^+\}$				**0.835*****	**0.363***	-0.264	**0.544*****	**0.486*****	-0.042
$\{Ca^{2+}\}$					0.116	**-0.530*****	0.279	0.251	-0.325
$\{Ni^{2+}\}$						**0.428****	**0.735*****	**0.654*****	**0.655*****
$\{Cu^{2+}\}$							**0.478*****	**0.543*****	**0.724*****
$\{Zn^{2+}\}$								**0.967*****	**0.706*****
$\{Cd^{2+}\}$									**0.705*****

756 **Table 3:** Ni, Cu, Zn, Cd and Pb invertebrate body burdens. Minimum - maximum, geometric mean and median
757 values (in µmol g^{-1} dw) of all sample sites are presented (*n*=20-33, depending on the taxa).

		Ni	Cu	Zn	Cd	Pb
Leuctra sp.	Min.	0.03	0.34	2.79	0.002	0.02
n = 33	Max.	0.68	9.10	85.7	0.210	12.0
	Geo. mean	0.11	0.79	6.69	0.012	0.53
	Median	0.11	0.67	4.54	0.010	0.79
Simuliidae	Min.	0.02	0.20	1.96	0.001	0.01
n = 24	Max.	0.27	13.8	21.1	0.141	11.7
	Geo. mean	0.10	0.61	5.00	0.013	0.55
	Median	0.11	0.42	4.68	0.011	0.47
Rhithrogena sp.	Min.	0.03	0.19	2.85	0.015	0.01
n = 20	Max.	0.19	1.74	72.6	0.304	1.78
	Geo. mean	0.07	0.41	18.0	0.079	0.22
	Median	0.07	0.35	23.2	0.072	0.25
Perlodidae	Min.	0.003	0.18	2.49	0.001	0.001
n = 25	Max.	0.10	6.28	45.8	0.136	2.82
	Geo. mean	0.03	0.62	6.46	0.011	0.14
	Median	0.04	0.56	5.15	0.012	0.16

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760 **Table 4:** Calculated pseudoequilibrium affinity constants (*K*) for the reactions of metal and hydrogen ions with
761 biological uptake sites in *Leuctra* sp., Simuliidae, *Rhithrogena* sp. and Perlodidae. Median values (in µM) are
762 presented. Values were calculated for H (K_a) using equation (5) and for Na and Ca (K_{Na} and K_{Ca}) using equation
763 (6). N.s.: No *K* values could be calculated since the constructed model was not significant.

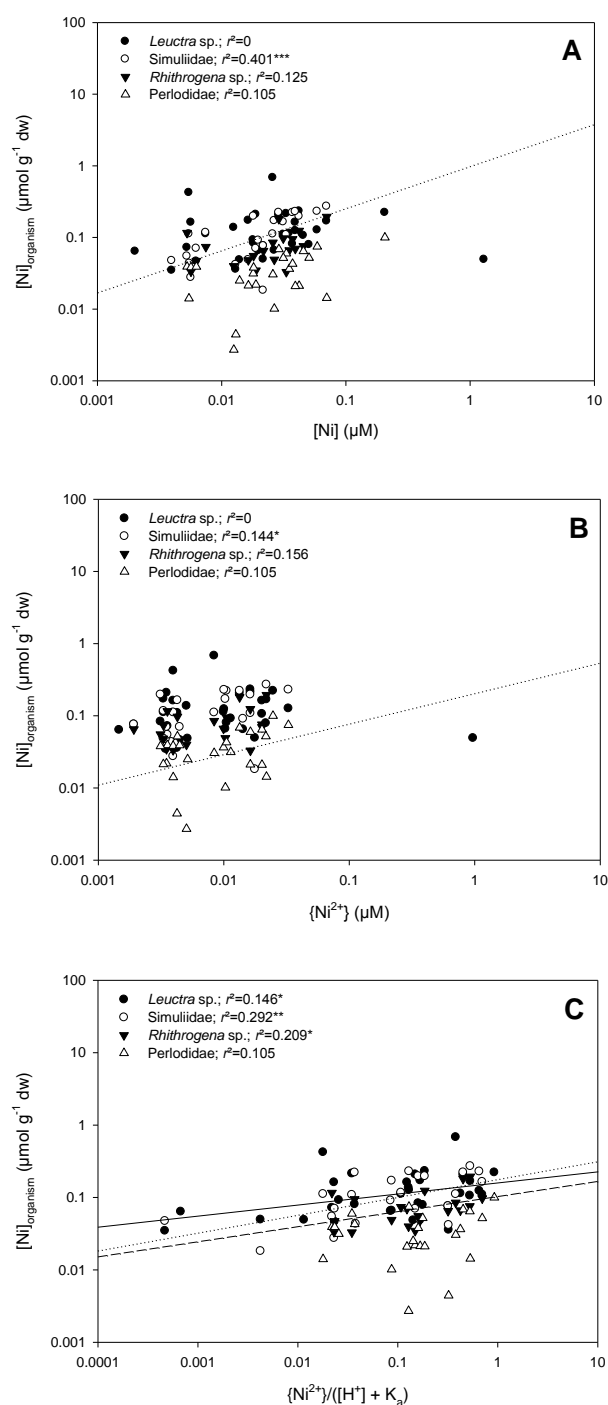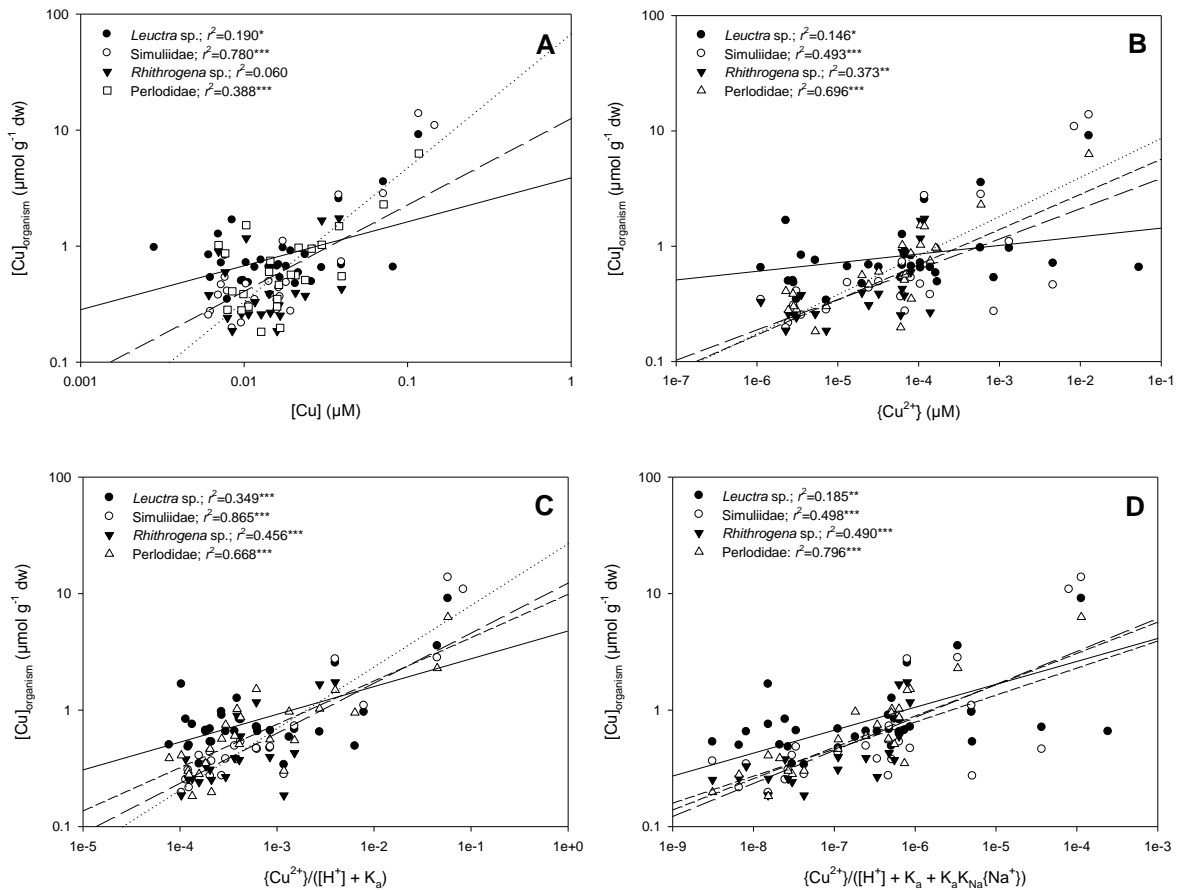|		*Leuctra* sp.	Simuliidae	*Rhithrogena* sp.	Perlodidae
Ni	K_a	1.51	2.05	2.76	N.s.
	K_{Na}	N.s.	N.s.	N.s.	N.s.
	K_{Ca}	N.s.	N.s.	N.s.	N.s.
Cu	K_a	2.49	2.86	2.88	2.65
	K_{Na}	0.47	0.41	0.40	0.44
	K_{Ca}	0.50	0.50	0.55	0.47
Zn	K_a	1.76	1.78	1.51	1.42
	K_{Na}	0.98	0.88	1.07	0.86
	K_{Ca}	1.10	1.01	1.23	1.07
Cd	K_a	2.61	3.06	3.23	2.83
	K_{Na}	0.49	0.51	0.45	0.53
	K_{Ca}	0.44	N.s.	0.66	0.58
Pb	K_a	2.69	2.85	3.02	2.76
	K_{Na}	0.46	0.41	0.43	0.44
	K_{Ca}	N.s.	N.s.	0.65	0.43

Figure 1: Relationships between Ni concentrations in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and total dissolved Ni concentrations (A), WHAM-predicted free Ni^{2+} ion activity following equation (1) (B) and WHAM-predicted $\{Ni^{2+}\}$, considering the influence of $[H^+]$ following equation (2) (C). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Figure 2: Relationships between Cu concentrations in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and total dissolved Cu concentrations (A), WHAM-predicted free Cu^{2+} ion activity following equation (1) (B), WHAM-predicted $\{Cu^{2+}\}$, considering the influence of $[H^+]$ following equation (2)(C) and WHAM-predicted $\{Cu^{2+}\}$, considering the combined influence of $[H^+]$ and $\{Na^+\}$ following equation (4) (D). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.
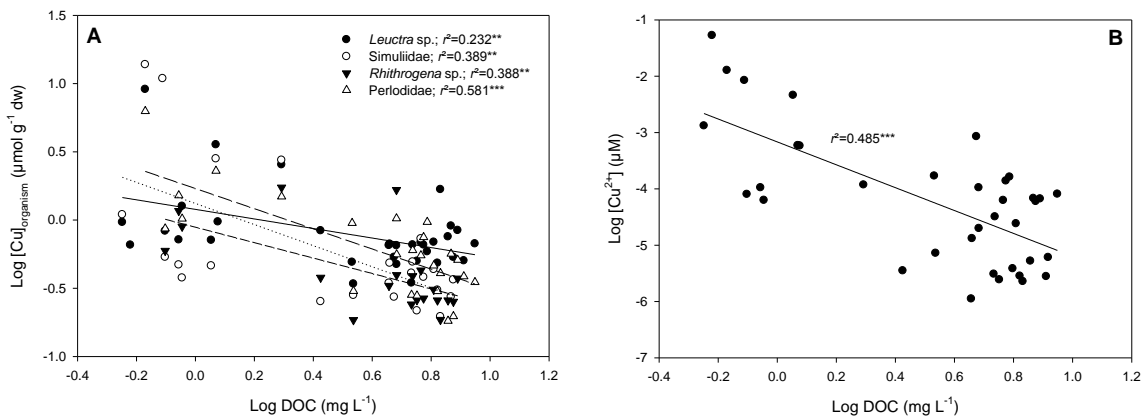

Figure 3: Relationships between Cu body burdens in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and dissolved organic carbon (DOC) in surface water (A); Relationship between WHAM-predicted Cu^{2+} and DOC (B). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.
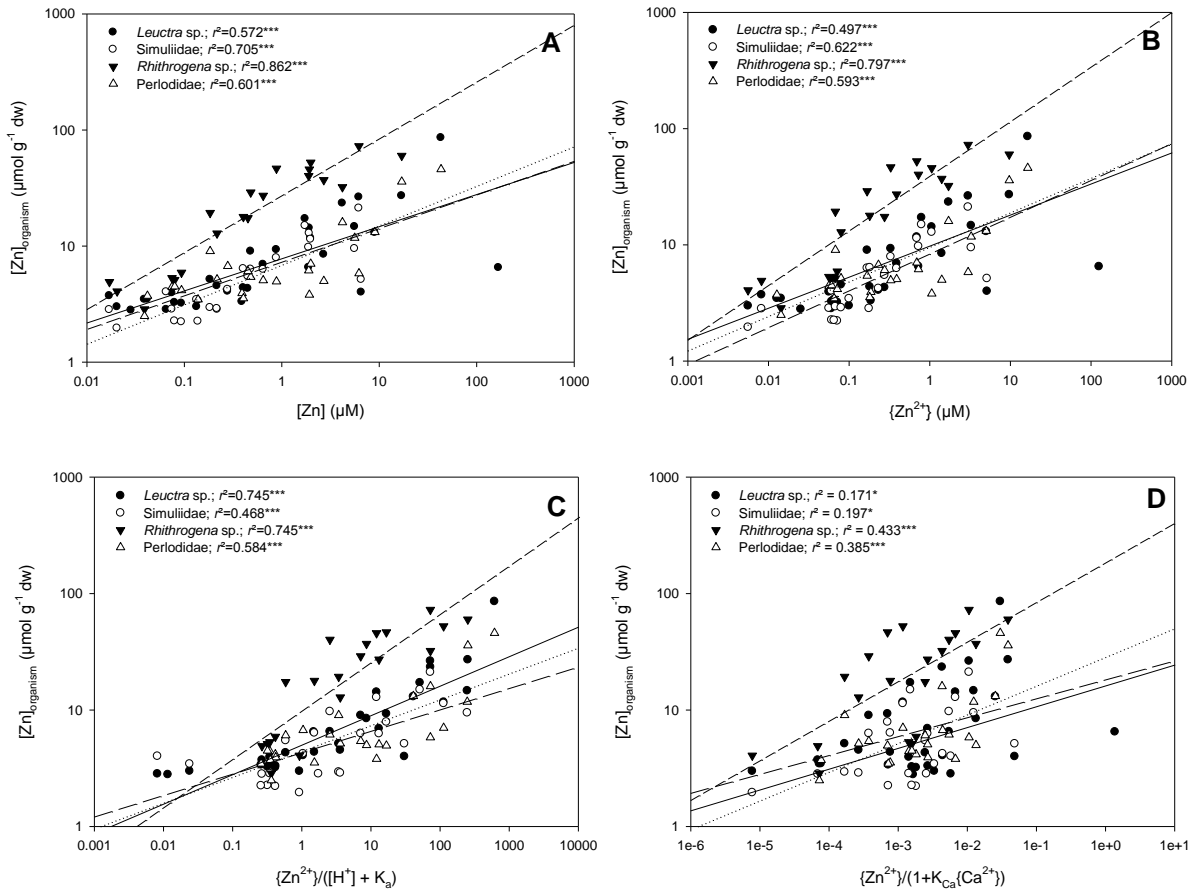
Figure 4: Relationships between Zn concentrations in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and total dissolved Zn concentrations (A), WHAM-predicted free Zn^{2+} ion activity following equation (1) (B), WHAM-predicted $\{Zn^{2+}\}$, considering the influence of $[H^+]$ following equation (2) (C) and WHAM-predicted $\{Zn^{2+}\}$, considering the influence of $\{Ca^{2+}\}$ following equation (3) (D). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.
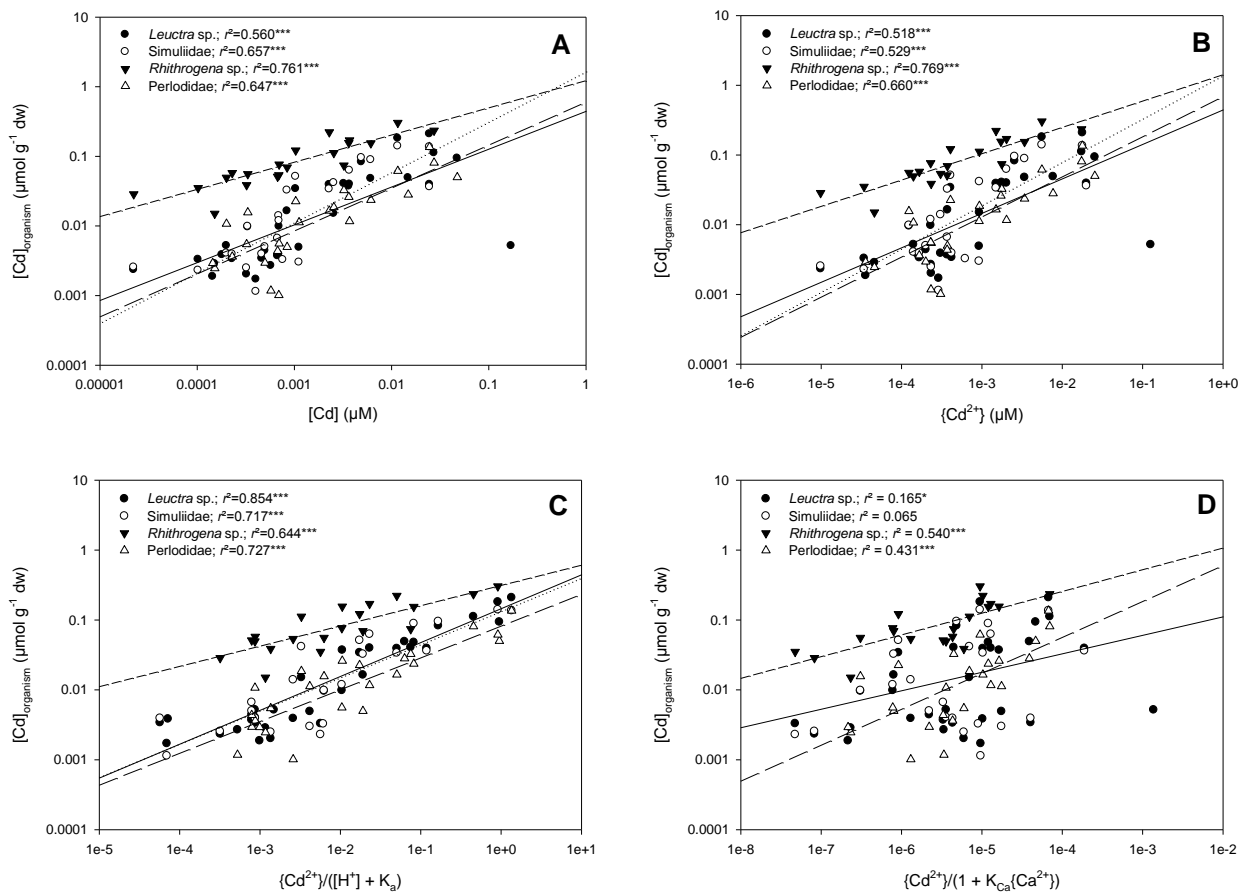
Figure 5: Relationships between Cd concentrations in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and total dissolved Cd concentrations (A), WHAM-predicted free Cd^{2+} ion activity following equation (1) (B), WHAM-predicted $\{Cd^{2+}\}$, considering the influence of $[H^+]$ following equation (2) (C) and WHAM-predicted $\{Cd^{2+}\}$, considering the influence of $\{Ca^{2+}\}$ following equation (3) (D). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.
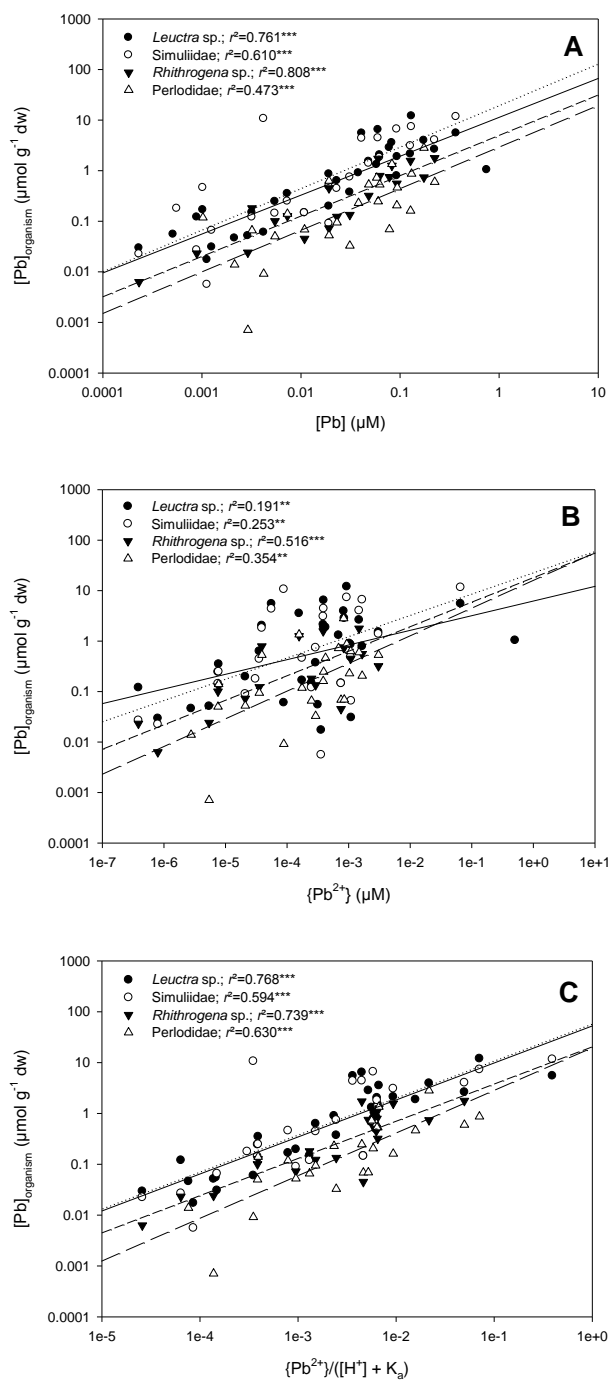
Figure 6: Relationships between Pb concentrations in *Leuctra* sp. (full circles; solid line; $n = 33$), Simuliidae (open circles; dotted line; $n = 24$), *Rhithrogena* sp. (full triangles; short dash; $n = 20$) and Perlodidae (open triangles; long dash; $n = 25$) and total dissolved Pb concentrations (A), WHAM-predicted free Pb^{2+} ion activity following equation (1) (B) and WHAM-predicted $\{Pb^{2+}\}$, considering the influence of $[H^+]$ following equation (2)(C). The amount of variation explained is given by the coefficient of determination (adjusted r^2). The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

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