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De Jonge, Maarten; Lofts, Stephen; Bervoets, Lieven; Blust, Ronny. 2014. **Relating metal exposure and chemical speciation to trace metal accumulation in aquatic insects under natural field conditions.** *Science of the Total Environment*, 496. 11-21. [10.1016/j.scitotenv.2014.07.023](https://doi.org/10.1016/j.scitotenv.2014.07.023)

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[10.1016/j.scitotenv.2014.07.023](https://doi.org/10.1016/j.scitotenv.2014.07.023)

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1 **Relating metal exposure and chemical speciation to trace metal**  
2 **accumulation in aquatic insects under natural field conditions**

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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<sup>+</sup> ion  
37 activity was the most dominant factor influencing metal accumulation. Adding the influence  
38 of Na<sup>+</sup> on Cu<sup>2+</sup> 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<sup>2+</sup> and Mg<sup>2+</sup> 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<sup>-1</sup>) 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<sup>+</sup> 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<sup>+</sup> competition.

47

48 **Keywords:** Metal pollution; Metal speciation; Invertebrate body burdens; Windermere Humic  
49 Aqueous Model (WHAM); Biotic Ligand Model (BLM)

## 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.  $\text{Cl}^-$ ,  $\text{OH}^-$ ,  $\text{CO}_3^{2-}$ ) and the effect of  
55 pH), and is largely influenced by other cations (e.g.  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ) and  $\text{H}^+$  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,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , 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

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, Ribblesdale, 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 cm<sup>3</sup> 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.

100

## 101 **2.2 Water chemistry analysis and chemical speciation**

102

103 Within one day after collecting, samples were analysed for pH using a glass electrode while  
104 taking care to avoid de-gassing of the samples. Total concentrations of Na, Mg, Al, K, Ca, Mn  
105 and Fe were measured after one week using Inductively Coupled Plasma - Optical Emission  
106 Spectrometry (ICP-OES). Chloride (Cl), nitrate (NO<sub>3</sub>-N) and sulphate (SO<sub>4</sub>-S) were  
107 determined by ion chromatography; alkalinity was measured by Gran titration and dissolved  
108 organic carbon (DOC) by combustion. Ammonia-N (NH<sub>4</sub>-N), phosphorus (P) and silica  
109 (SiO<sub>2</sub>) were determined colorimetrically and suspended particulate matter (SPM) was  
110 determined gravimetrically. Water samples intended for trace metal analysis were filtered  
111 over a 0.45 µm polypropylene filter, acidified with 1% nitric acid (HNO<sub>3</sub>; 69%) and total  
112 levels of Ni, Cu, Zn, Cd and Pb were quantified using Inductively Coupled Plasma - Mass  
113 Spectrometry (ICP-MS). All analyses made use of International Quality Control standards,  
114 with verification by the Proficiency Testing scheme. Furthermore, a Certified Reference  
115 Material (CRM) was used in the determination of trace metals in surface waters.

116 Free ion activity (FIA) calculations of the measured trace metals were performed using the  
117 Windermere Humic Aqueous Model (WHAM) (Tipping, 1994), incorporating Humic Ion-  
118 Binding Model VI (Tipping, 1998). To calculate the chemical speciation, the concentrations  
119 of Na, K, Ca, Mg, Cl, NO<sub>3</sub>, SO<sub>4</sub> and concentrations of filterable trace metals (Ni, Cu, Zn, Cd  
120 and Pb) were assumed to represent truly dissolved components (i.e. the inorganic ionic  
121 species and complexes and/or metals bound to dissolved organic matter (DOM)). The  
122 filterable fraction may also include some metal in association with mineral and organic  
123 colloids, however these species are neglected in the present analysis. The cation-binding  
124 properties of DOM were expressed in terms of isolated fulvic acid, which is thought to be the

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**

132

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<sub>3</sub> - hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 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 ( $[M]_{\text{organism}}$ ), 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

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

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

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

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

165

166 Where equation (1) is used if competition by hydrogen ions ( $\{H^+\}$ ) or other metal ions  
 167 ( $\{M^{2+}\}$ ) is not considered, (2) if competition by  $\{H^+\}$  alone is considered, (3) if competition  
 168 by  $\{M^{2+}\}$  alone is considered and (4) if competition by both  $\{H^+\}$  and  $\{M^{2+}\}$  is considered.  
 169  $F$  (which equals  $k \cdot K_M$ ) encloses a proportionality constant specific to the studied taxon ( $k$



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

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

182

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

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  $\mu\text{M}$  (table 1). WHAM- predicted  
197 free Ni ion activities were significantly positively correlated with  $\{\text{H}^+\}$ ,  $\{\text{K}^+\}$ ,  $\{\text{Mg}^{2+}\}$  and all  
198 other trace metal ions (table 2). No significant correlation was observed between  $\{\text{Ni}^{2+}\}$  and  
199 both  $\{\text{Na}^+\}$  and  $\{\text{Ca}^{2+}\}$ . Nickel body burdens ranged from 0.003 (Perlodidae) to 0.68  $\mu\text{mol g}^{-1}$   
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  $\{\text{Ni}^{2+}\}$  poorly correlated to  $[\text{Ni}]_{\text{organism}}$  for all taxa.  
204 Conversely, better relations were found between  $\{\text{Ni}^{2+}\}$  considering the influence of  $\text{H}^+$  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  $3.37 \pm 1.17 \mu M$ , 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  $2.76 \mu M$  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  $\text{Ni}^{2+}$  ions for entry at  $\text{Mg}^{2+}$  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  $\text{H}^+$  ions, rather than  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  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 result 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  $\mu\text{M}$  (table 1). WHAM- predicted  
260 free Cu ion activities were significantly positively correlated with  $\{\text{H}^+\}$  and all other metal  
261 ions and negatively with  $\{\text{Ca}^{2+}\}$  (table 2). No significant correlations were observed between  
262  $\{\text{Cu}^{2+}\}$  and  $\{\text{Na}^+\}$ ,  $\{\text{K}^+\}$  and  $\{\text{Mg}^{2+}\}$ . Invertebrate Cu body burdens ranged from 0.18  
263 (Perlodidae) to 13.8  $\mu\text{mol g}^{-1}$  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  $\mu\text{mol g}^{-1}$  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  $\{\text{Cu}^{2+}\}$  to  $[\text{Cu}]_{\text{organism}}$  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  $\{\text{H}^+\}$  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  $\{\text{Cu}^{2+}\}$ . Adding the combined influence of  $\{\text{H}^+\}$  and  $\{\text{Na}^+\}$   
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  $\text{Na}^+$  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  $\text{Cu}^{2+}$  to the monovalent  $\text{Cu}^+$  at biological  
284 membranes (Rolf and Hediger, 1999), compete with  $\text{Na}^+$  for uptake in epithelial cells (via  
285 apical  $\text{Na}^+$  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  $\text{H}^+$  ions for  
287 binding at Cu uptake sites was more important compared to  $\text{Na}^+$  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  $\mu M$  (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  $\mu\text{mol g}^{-1}$  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  $\mu\text{mol g}^{-1}$  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  $\text{Cd}^{2+}$  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  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  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  $\{\text{K}^+\}$ ,  $\{\text{Mg}^{2+}\}$  and all other  
374 metal ions (table 2). No significant correlations were observed between  $\{\text{Cd}^{2+}\}$  and  $\{\text{H}^+\}$ ,  
375  $\{\text{Na}^+\}$  and  $\{\text{Ca}^{2+}\}$ . Cadmium body burdens ranged from 0.001 (Simuliidae) to  $0.304 \mu\text{mol g}^{-1}$   
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  $\{\text{Cd}^{2+}\}$  resulted in stronger models for *Rhithrogena* sp. ( $r^2 = 0.769$ )  
380 and Perlodidae ( $r^2 = 0.660$ ). Considering the influence of  $\{\text{H}^+\}$  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  $\{\text{Cd}^{2+}\}$ . Analogously, both Hare and Tessier (1996, 1998) and Croteau et  
384 al. (1998) demonstrated the major competitive influence of  $\text{H}^+$  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  $1.7 \mu\text{M}$  (Hare and Tessier, 1998) and  $0.8 \mu\text{M}$  (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  $2.61 \mu\text{M}$  to  $3.23 \mu\text{M}$ ).

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  $\mu M$  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  $\mu\text{M}$ ) was found to significantly inhibit Cd accumulation in the  
415 amphipod *Hyaella 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  $\{\text{H}^+\}$  and all other metal ions  
424 (table 2). No significant correlations were observed between  $\{\text{Pb}^{2+}\}$  and ion activities of the  
425 hardness ions. Lead body burdens ranged from 0.001 (Simuliidae) to 12.0  $\mu\text{mol g}^{-1}$  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  $\{\text{Pb}^{2+}\}$  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  $\{\text{H}^+\}$  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  $\{\text{Pb}^{2+}\}$  without pH influence on uptake sites. The latter results are in  
435 agreement with the FIAM (Campbell, 1995). A decreased Pb uptake due to  $\text{H}^+$  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,  $\text{Pb}^{2+}$  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

## 489 **4 Conclusions**

490

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

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

505

506

## 507 **Acknowledgement**

508

509 This research project was funded by the Environment Agency of England and Wales, the  
510 European Copper Institute, European Nickel Industry Association, International Cadmium  
511 Association, International Zinc Association (Europe), Rio Tinto and the Scottish Environment  
512 Protection Agency (Bass et al., 2008). We thank Edward Tipping and the CEH staff for  
513 collecting the invertebrate specimens in the field and performing the water chemistry analyses

514 - Nicholas Kneebone, Alan Lawlor, Jonathan Bass, Tracey Corbin, Sarah Thacker and Colin  
515 Vincent. Judith Voets and Irina Komjarova are acknowledged for the digestion and metal  
516 analyses of the invertebrate samples. Maarten De Jonge is funded by a post-doctoral research  
517 grant of the Research Foundation Flanders (FWO).

518

## 519 **References**

520

521 Adams, W. J., Blust, R., Borgmann, U., Brix, K. V., DeForest, D. K., Green, A. S., Meyer, J.  
522 S., McGeer, J. C., Paquin, P. R., Rainbow, P. S., and Wood, C. M. (2011) Utility of tissue  
523 residues for predicting effects of metals on aquatic organisms. *Integrated Environmental*  
524 *Assessment and Management* **7** (1), 75-98.

525

526 Bass, J. A. B., Blust, R., Clarke, R. T., Corbin, T. A., Davison, W., De Schamphelaere, K. A.  
527 C., Janssen, C. R., Kalis, E. J. J., Kelly, M. G., Kneebone, N. T., Lawlor, A. J., Lofts, S.,  
528 Temminghoff, E. J. M., Thacker, S. A., Tipping, E., Vincent, C. D., Warnken, K. W., and  
529 Zhang, H. Environmental Quality Standards for Trace Metals in the Aquatic Environment.  
530 2008. Bristol, UK, Environment Agency.

531

532 Bervoets, L. and Blust, R. (2000) Effects of pH on cadmium and zinc uptake by the midge  
533 larvae *Chironomus riparius*. *Aquatic Toxicology* **49** (1-2), 145-157.

534

535 Blust, R., Vanderlinden, A., Verheyen, E., and Decler, W. (1988) Evaluation of Microwave-  
536 Heating Digestion and Graphite-Furnace Atomic-Absorption Spectrometry with Continuum  
537 Source Background Correction for the Determination of Iron, Copper and Cadmium in Brine  
538 Shrimp. *Journal of Analytical Atomic Spectrometry* **3** (2), 387-393.

539

540 Buchwalter D. B., and Luoma S. N. (2005) Differences in dissolved cadmium and zinc uptake  
541 among stream insects: Mechanistic explanations. *Environmental Science and Technology* **39**,  
542 498-504.

543

544 Campbell, P. G. C. (1995) Interaction between trace metals and aquatic organisms: a critique  
545 of the free-ion activity model. In: Tessier, A. and Turner, D.R. (eds), *Metal speciation and*  
546 *bioavailability in aquatic systems*. 45-102, John Wiley, New York.

547

548 Cain, D. J., Croteau M. N., and Luoma S. N. (2011) Bioaccumulation dynamics and exposure  
549 routes of Cd and Cu among species of aquatic mayflies. *Environmental Toxicology and*  
550 *Chemistry* **30**, 2532-2541.

551

552 Croisetière, L., Hare, L., and Tessier, A. (2006) A field experiment to determine the relative  
553 importance of prey and water as sources of As, Cd, Co, Cu, Pb, and Zn for the aquatic  
554 invertebrate *Sialis velata*. *Environmental Science and Technology* **40** (3), 873-879.

555

556 Croteau, M.-N., Hare, L., and Tessier, A. (1998) Refining and testing a trace metal  
557 biomonitor (Chaoborus) in highly acidic lakes. *Environmental Science and Technology* **32**  
558 (9), 1348-1353.  
559

560 De Jonge, M., Blust, R., and Bervoets, L. (2010) The relation between Acid Volatile Sulfides  
561 (AVS) and metal accumulation in aquatic invertebrates: Implications of feeding behavior and  
562 ecology. *Environmental Pollution* **158** (5), 1381-1391.  
563

564 De Jonge, M., Tipping, E., Lofts, S., Bervoets, L., and Blust, R. (2013) The use of  
565 invertebrate body burdens to predict ecological effects of metal mixtures in mining-impacted  
566 waters. *Aquatic Toxicology* **142-143**, 294-302.  
567

568 Deleebeeck, N. M. E., De Schamphelaere, K. A. C., Heijerick, D. G., Bossuyt, B. T. A., and  
569 Janssen, C. R. (2008a) The acute toxicity of nickel to *Daphnia magna*: Predictive capacity of  
570 bioavailability models in artificial and natural waters. *Ecotoxicology and Environmental*  
571 *Safety* **70** (1), 67-78.  
572

573 Deleebeeck, N. M. E., De Schamphelaere, K. A. C., and Janssen, C. R. (2008b) A novel  
574 method for predicting chronic nickel bioavailability and toxicity to *Daphnia magna* in  
575 artificial and natural waters. *Environmental Toxicology and Chemistry* **27** (10), 2097-2107.  
576

577 De Schamphelaere, K. A. C., and Janssen, C. R. (2002) A biotic ligand model predicting  
578 acute copper toxicity for *Daphnia magna*: The effects of calcium, magnesium, sodium,  
579 potassium and pH. *Environmental Science and Technology* **36** (1), 48-54.  
580

581 De Schamphelaere, K. A. C., and Janssen, C. R. (2004) Development and field validation of a  
582 biotic ligand model predicting chronic copper toxicity to *Daphnia magna*. *Environmental*  
583 *Toxicology and Chemistry* **23** (6), 1365-1375.  
584

585 Grosell, M., and Wood, C. M. (2002) Copper uptake across rainbow trout gills: mechanisms  
586 of apical entry. *The Journal of Experimental Biology* **205** (8), 1179-1188.  
587

588 Grosell, M., Gerdes, R., and Brix, K. V. (2006) Influence of Ca, humic acid and pH on lead  
589 accumulation and toxicity in the fathead minnow during prolonged water-borne lead  
590 exposure. *Comparative Biochemistry and Physiology Part C* **143** (4), 473-483.  
591

592 Guo, L., Hunt, B. J., Santschi, P. H., and Ray, S. M. (2001) Effect of dissolved organic matter  
593 on the uptake of trace metals by American oysters. *Environmental Science and Technology* **35**  
594 (5), 885-893.  
595

596 Hare, L., and Tessier, A. (1996) Predicting animal cadmium concentrations in lakes. *Nature*  
597 **380** (6573), 430-432.  
598

599 Hare, L., and Tessier, A. (1998) The aquatic insect Chaoborus as a biomonitor of trace metals  
600 in lakes. *Limnology and Oceanography* **43** (8), 1850-1859.  
601

602 Heijerick, D. G., De Schamphelaere, K. A. C., and Janssen, C. R. (2002) Predicting acute zinc  
603 toxicity for *Daphnia magna* as a function of key water chemistry characteristics:



604 Development and validation of a Biotic Ligand Model. *Environmental Toxicology and*  
605 *Chemistry* **21**, 1309-1315.

606

607 Heijerick, D. G., De Schamphelaere, K. A. C., Van Sprang, P. A., and Janssen, C. R. (2005)  
608 Development of a chronic zinc biotic ligand model for *Daphnia magna*. *Ecotoxicology and*  
609 *Environmental Safety* **62** (1), 1-10.

610

611 Hyne, R. V. Pablo, F., Julli, M., and Markich, S. J. (2005) Influence of water chemistry on the  
612 acute toxicity of copper and zinc to the cladoceran *Ceriodaphnia dubia*. *Environmental*  
613 *Toxicology and Chemistry* **24** (7), 1667-1675.

614

615 Iwasaki, Y., Cadmus, P. and Clements, W. H. (2013) Comparison of different predictors of  
616 exposure for modeling impacts of metal mixtures on macroinvertebrates in stream  
617 microcosms. *Aquatic Toxicology* **132-133**, 151-156.

618

619 Keithly, J., Brooker, J. A., DeForest, D. K., Wu, B. K., and Brix, K. V. (2004) Acute and  
620 chronic toxicity of nickel to a cladoceran (*Ceriodaphnia dubia*) and an amphipod (*Hyaella*  
621 *azteca*). *Environmental Toxicology and Chemistry* **23** (3), 691-696.

622

623 Komjarova, I., and Blust, R. (2008) Multi-metal interactions between Cd, Cu, Ni, Pb and Zn  
624 in water flea *Daphnia magna*, a stable isotope experiment. *Aquatic Toxicology* **90**, 138-144.

625

626 Komjarova, I., and Blust, R. (2009a) Effect of Na, Ca and pH on simultaneous uptake of Cd,  
627 Cu, Ni, Pb, and Zn in the water flea *Daphnia magna* measured using stable isotopes. *Aquatic*  
628 *Toxicology* **94** (2), 81-86.

629

630 Komjarova, I., and Blust, R. (2009b) Effects of Na, Ca, and pH on the simultaneous uptake of  
631 Cd, Cu, Ni, Pb and Zn in the zebrafish *Danio rerio*: A stable isotope experiment.  
632 *Environmental Science and Technology* **43** (20), 7958-7963.

633

634 Luoma, S. N., and Rainbow, P. S. (2005) Why is metal bioaccumulation so variable?  
635 Biodynamics as a unifying concept. *Environmental Science & Technology* **39** (7), 1921-1931.

636

637 Mager, E. M., Brix, K. V., Gerdes, R. M., Ryan, A. C., and Grosell, M. (2011) Effects of  
638 water chemistry on the chronic toxicity of lead to the cladoceran *Ceriodaphnia dubia*.  
639 *Ecotoxicology and Environmental Safety* **74** (3), 238-243.

640

641 Martin, C. A., Cain, D. J., Luoma, S. N., and Buchwalter, D. B. (2007) Cadmium  
642 ecophysiology in seven stonefly (Plecoptera) species: Delineating sources and estimating  
643 susceptibility. *Environmental Science and Technology* **41**, 7171-7177.

644

645 Martin, C. A., Proulx, I, and Hare, L. (2008) Explaining metal concentrations in sympatric  
646 *Chironomus* species. *Limnology and Oceanography* **53**, 411-419.

647

648 Munger, C., and Hare, L. (1997) Relative importance of water and food as cadmium sources  
649 to an aquatic insect (*Chaoborus punctipennis*): Implications for predicting Cd  
650 bioaccumulation in nature. *Environmental Science and Technology* **31**, 891-895.

651

652 Niyogi, S., and Wood, C. M. (2004) Biotic Ligand Model, a flexible tool for developing site-  
653 specific water quality guidelines for metals. *Environmental Science and Technology* **38** (23),  
654 6177-6192.

655  
656 Niyogi, S., Brix, K. V., and Grosell M. (2014) Effects of chronic waterborne nickel exposure  
657 on growth, ion homeostasis, acid-base balance, and nickel uptake in the freshwater pulmonate  
658 snail, *Lymnaea stagnalis*. *Aquatic Toxicology* **150**, 36-44.

659  
660 Norwood, W. P., Borgmann, U., Dixon, D. G. and Wallace, A. (2003) Effects of metal  
661 mixtures on aquatic biota: a review. *Human and Ecological Risk Assessment* **9**, 795-811.

662  
663 Norwood, W. P., Borgmann, U., and Dixon, D. G. (2007) Interactive effects of metals in  
664 mixtures on bioaccumulation in the amphipod *Hyalella azteca*. *Aquatic Toxicology* **84**, 255-  
267.

665  
666 Paquin, P. R., Gorsuch, J. W., Apte, S., Batley, G. E., Bowles, K. C., Campbell, P. G. C.,  
667 Delos, C. G., Di Toro, D. M., Dwyer, R. L., Galvez, F., Gensemer, R. W., Goss, G. G.,  
668 Hogstrand, C., Janssen, C. R., Mcgeer, J. C., Naddy, R. B., Playle, R. C., Santore, R. C.,  
669 Schneider, U., Stubblefield, W. A., Wood, C. M., and Wu, K. B. (2002) The biotic ligand  
670 model: a historical overview. *Comparative Biochemistry and Physiology C-Toxicology &*  
671 *Pharmacology* **133** (1-2), 3-35.

672  
673 Ponton, D. E., and Hare, L. (2009) Assessment of Nickel Contamination in Lakes Using the  
674 Phantom Midge Chaoborus As a Biomonitor. *Environmental Science and Technology* **43** (17),  
675 6529-6534.

676  
677 Poteat, M. D., Diaz, M., and Buchwalter, D. B. (2012) Divalent metal (Ca, Cd, Mn, Zn)  
678 uptake and interactions in the aquatic insect *Hydropsyche sparna*. *The Journal of*  
679 *Experimental Biology* **215**, 1575-1583.

680  
681 Poteat, M. D., and Buchwalter, D. B. (2014) Calcium uptake in aquatic insects: Influences of  
682 phylogeny and metals (Cd and Zn). *The Journal of Experimental Biology* **217**, 1180-1186.

683  
684 Qiu, A., Shayeghi, M, and Hogstrand, C. (2005) Molecular cloning and functional  
685 characterization of a high-affinity zinc importer (Dr ZIP1) from zebrafish (*Danio rerio*).  
686 *Biochemical Journal* **388**, 745-754.

687  
688 Rainbow, P. S. (2002) Trace metal concentrations in aquatic invertebrates: why and so what?  
689 *Environmental Pollution* **120** (3), 497-507.

690  
691 Rainbow, P. S. and Black, W. H. (2005) Cadmium, zinc and the uptake of calcium by two  
692 crabs, *Carcinus maenas* and *Eriocheir sinensis*. *Aquatic Toxicology* **72** (1-2), 45-65.

693  
694 Rogers, J. T., and Wood, C. M. (2004) Characterization of branchial lead-calcium interaction  
695 in the freshwater rainbow trout *Oncorhynchus mykiss*. *The Journal of Experimental Biology*  
696 **207** (5), 813-825.

697  
698 Rolfs, A., and Hediger, M. A. (1999) Metal ion transporters in mammals: structure, function  
and pathological implications. *Journal of Physiology* **518** (1), 1-12.

699  
700 Stockdale, A., Tipping, E., Lofts, S., Ormerod, S. J., Clements, W. H., and Blust, R. (2010)  
701 Toxicity of proton-metal mixtures in the field: Linking stream macroinvertebrate species  
702 diversity to chemical speciation and bioavailability. *Aquatic Toxicology* **100**, 112-119.

703 Tipping, E. (1994) Wham - A Chemical-Equilibrium Model and Computer Code for Waters,  
704 Sediments, and Soils Incorporating A Discrete Site Electrostatic Model of Ion-Binding by  
705 Humic Substances. *Computers & Geosciences* **20** (6), 973-1023.  
706

707 Tipping, E. (1998) Humic Ion-Binding Model VI: An Improved Description of the  
708 Interactions of Protons and Metal Ions with Humic Substances. *Aquatic Geochemistry* **4** (1),  
709 3-47.  
710

711 Tipping, E., Lofts, S., and Lawlor, A. J. (1998) Modelling the chemical speciation of trace  
712 metals in the surface waters of the Humber system. *Science of the Total Environment* **210** (1-  
713 6), 63-77.  
714

715 Tipping, E., Vincent, C. D., Lawlor, A. J., and Lofts, S. (2008) Metal accumulation by stream  
716 bryophytes, related to chemical speciation. *Environmental Pollution* **156** (3), 936-943.  
717

718 Tipping, E., and Lofts, S. (2013) Metal mixture toxicity to aquatic biota in laboratory  
719 experiments: Application of the WHAM-FTOX model. *Aquatic Toxicology* **142-143**, 114-  
720 122.  
721

722 Van Ginneken, L., Chowdhury, M. J., and Blust, R. (1999) Bioavailability of cadmium and  
723 zinc to the common carp, *Cyprinus carpio*, in complexing environments: a test for the validity  
724 of the free ion activity model. *Environmental Toxicology and Chemistry* **18** (10), 2295-2304.  
725

726 Vincent, C. D., Lawlor, A. J., and Tipping, E. (2001) Accumulation of Al, Mn, Fe, Cu, Zn, Cd  
727 and Pb by the bryophyte *Scapania undulata* in three upland waters of different pH.  
728 *Environmental Pollution* **114** (1), 93-100.  
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747 **Tables**

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

Variable	Unit	Min.	Max.
Temperature	$^{\circ}C$	4.55	7.38
pH	-	4.09	8.33
Cond	$\mu s\ cm^{-1}$	28	320
SiO <sub>2</sub>	$mg\ L^{-1}$	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 <sub>4</sub> -N	$\mu g\ L^{-1}$	< 5	53.0
NO <sub>3</sub> -N	$mg\ L^{-1}$	0.03	0.80
SO <sub>4</sub> -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	$\mu M$	0.04	58.0
Mn	$\mu M$	0.04	20.0
Fe	$\mu M$	0.12	9.04
Ni	$\mu M$	0.002	1.29
Cu	$\mu M$	0.003	0.15
Zn	$\mu M$	0.017	168
Cd	$nM$	0.027	171
Pb	$nM$	0.242	754

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754 **Table 2:** Pearson correlations between WHAM-predicted free metal ion activities ( $n = 36$ ).755  $r$ -values and significance level are presented; \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

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

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

		Ni	Cu	Zn	Cd	Pb
<i>Leuctra</i> sp. $n = 33$	Min.	0.03	0.34	2.79	0.002	0.02
	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 $n = 24$	Min.	0.02	0.20	1.96	0.001	0.01
	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
<i>Rhithrogena</i> sp. $n = 20$	Min.	0.03	0.19	2.85	0.015	0.01
	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 $n = 25$	Min.	0.003	0.18	2.49	0.001	0.001
	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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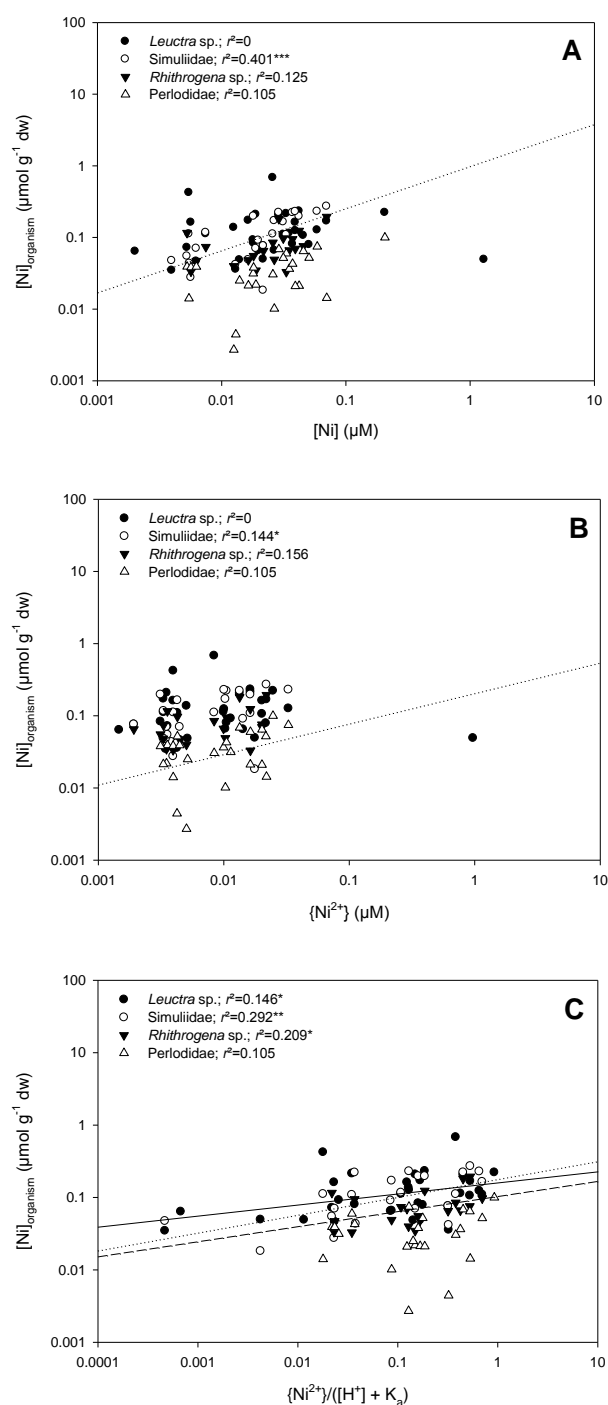
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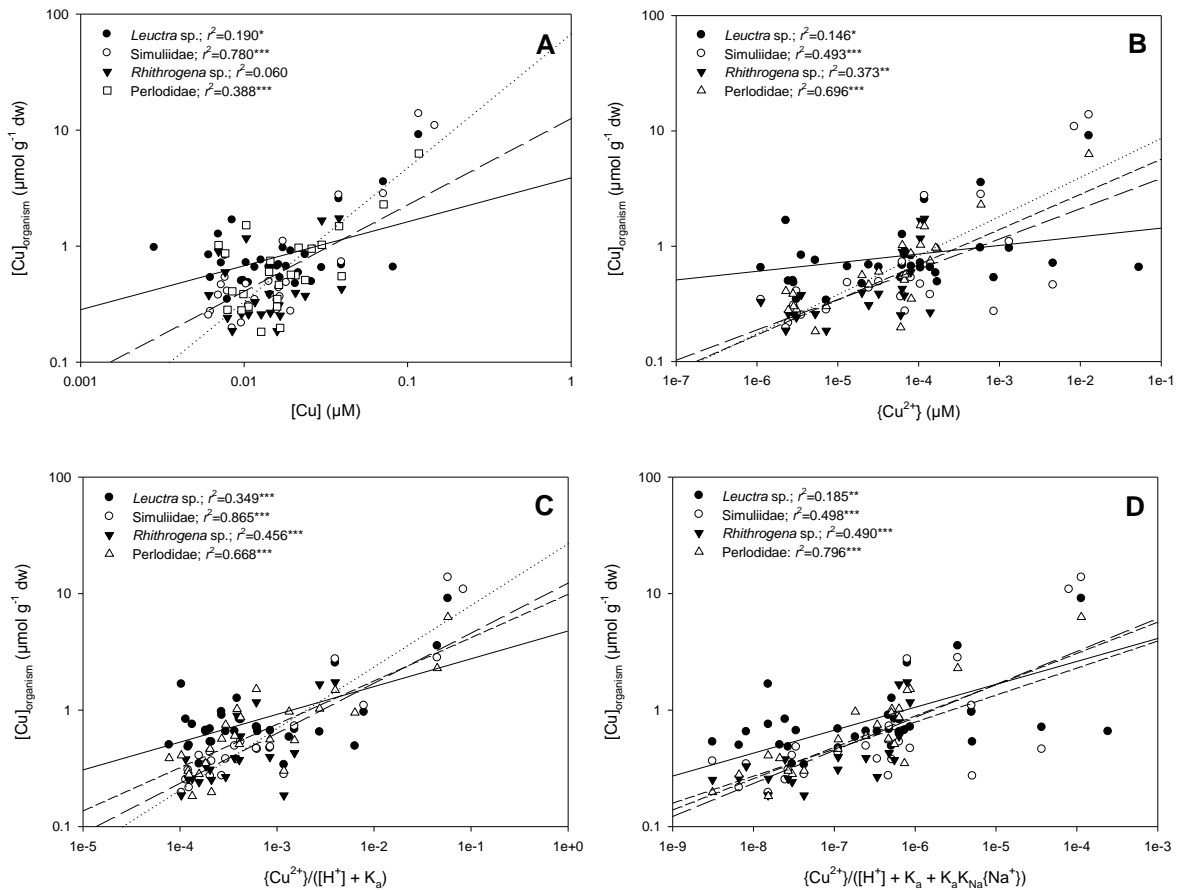
**Table 4:** Calculated pseudoequilibrium affinity constants ( $K$ ) for the reactions of metal and hydrogen ions with biological uptake sites in *Leuctra* sp., Simuliidae, *Rhithrogena* sp. and Perlodidae. Median values (in  $\mu\text{M}$ ) are presented. Values were calculated for H ( $K_a$ ) using equation (5) and for Na and Ca ( $K_{Na}$  and  $K_{Ca}$ ) using equation (6). N.s.: No  $K$  values could be calculated since the constructed model was not significant.

		<i>Leuctra</i> sp.	Simuliidae	<i>Rhithrogena</i> 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

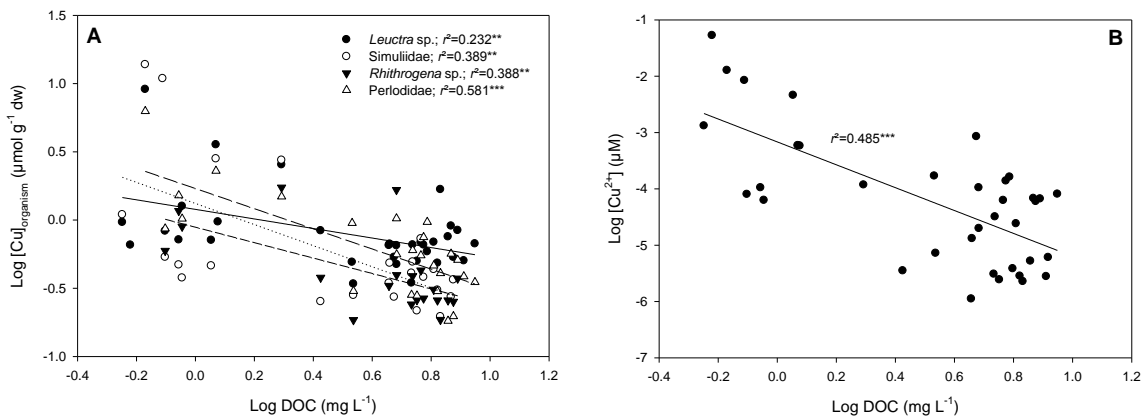
## Figures



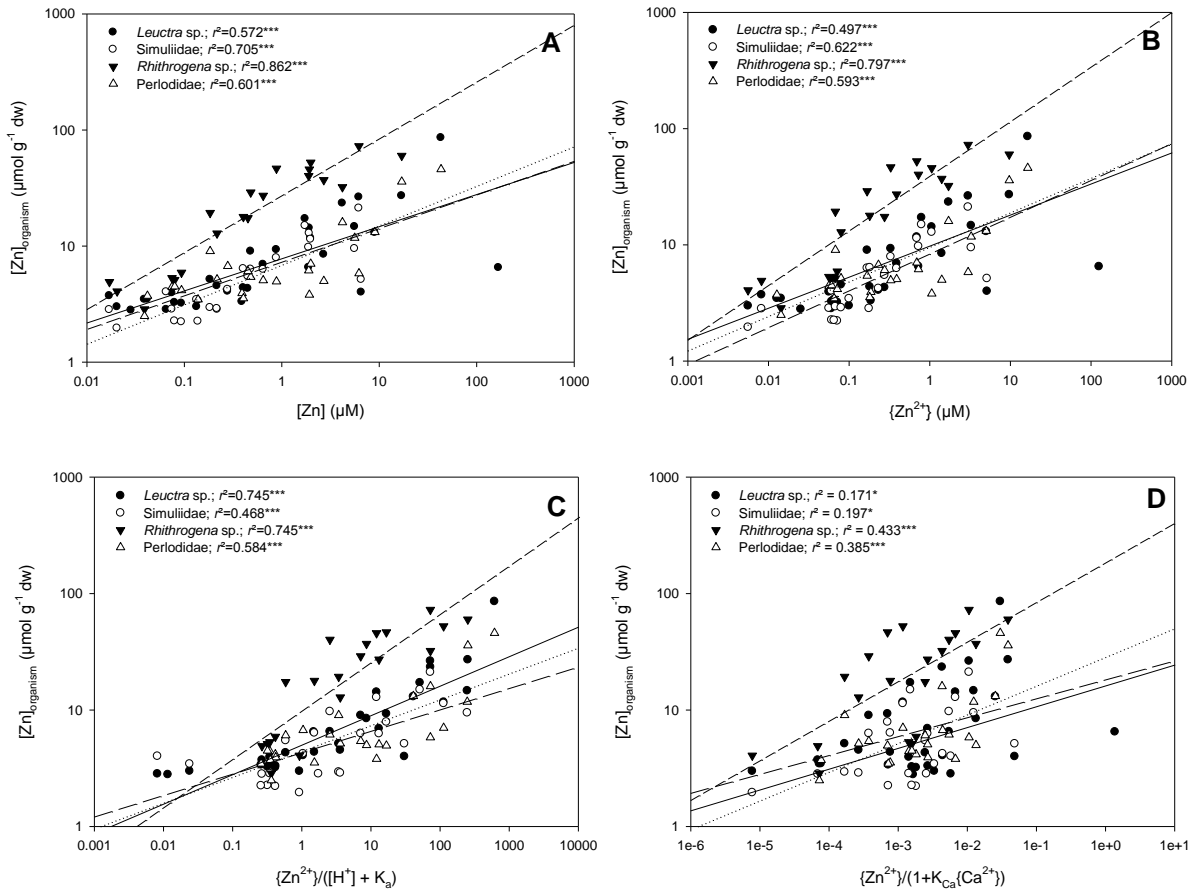
**Figure 1:** Relationships between Ni concentrations in *Leuctra* sp. (full circles; solid line;  $n = 33$ ), Simuliidae (open circles; dotted line;  $n = 24$ ), *Rhithrogena* sp. (full triangles; short dash;  $n = 20$ ) and Perlodidae (open triangles; long dash;  $n = 25$ ) and total dissolved Ni concentrations (A), WHAM-predicted free  $\text{Ni}^{2+}$  ion activity following equation (1) (B) and WHAM-predicted  $\{\text{Ni}^{2+}\}$ , considering the influence of  $[\text{H}^+]$  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<sup>2+</sup> ion activity following equation (1) (B), WHAM-predicted {Cu<sup>2+</sup>}, considering the influence of [H<sup>+</sup>] following equation (2)(C) and WHAM-predicted {Cu<sup>2+</sup>}, considering the combined influence of [H<sup>+</sup>] and {Na<sup>+</sup>} 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  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ .

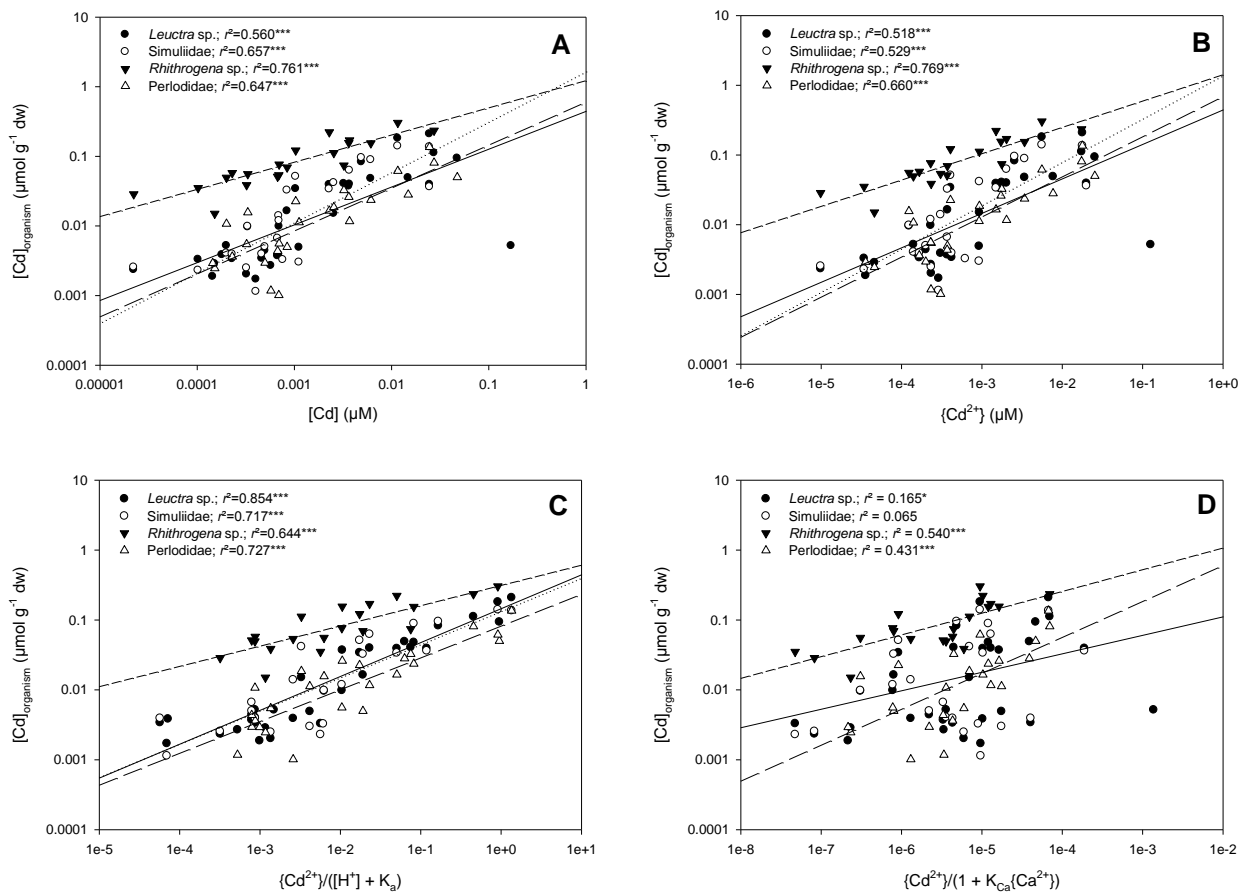


**Figure 3:** Relationships between Cu body burdens in *Leuctra* sp. (full circles; solid line;  $n = 33$ ), Simuliidae (open circles; dotted line;  $n = 24$ ), *Rhithrogena* sp. (full triangles; short dash;  $n = 20$ ) and Perlodidae (open triangles; long dash;  $n = 25$ ) and dissolved organic carbon (DOC) in surface water (A); Relationship between WHAM-predicted Cu<sup>2+</sup> and DOC (B). The amount of variation explained is given by the coefficient of determination (adjusted  $r^2$ ). The significance level is presented as  $*p < 0.05$ ;  $**p < 0.01$ ;  $***p < 0.001$ .

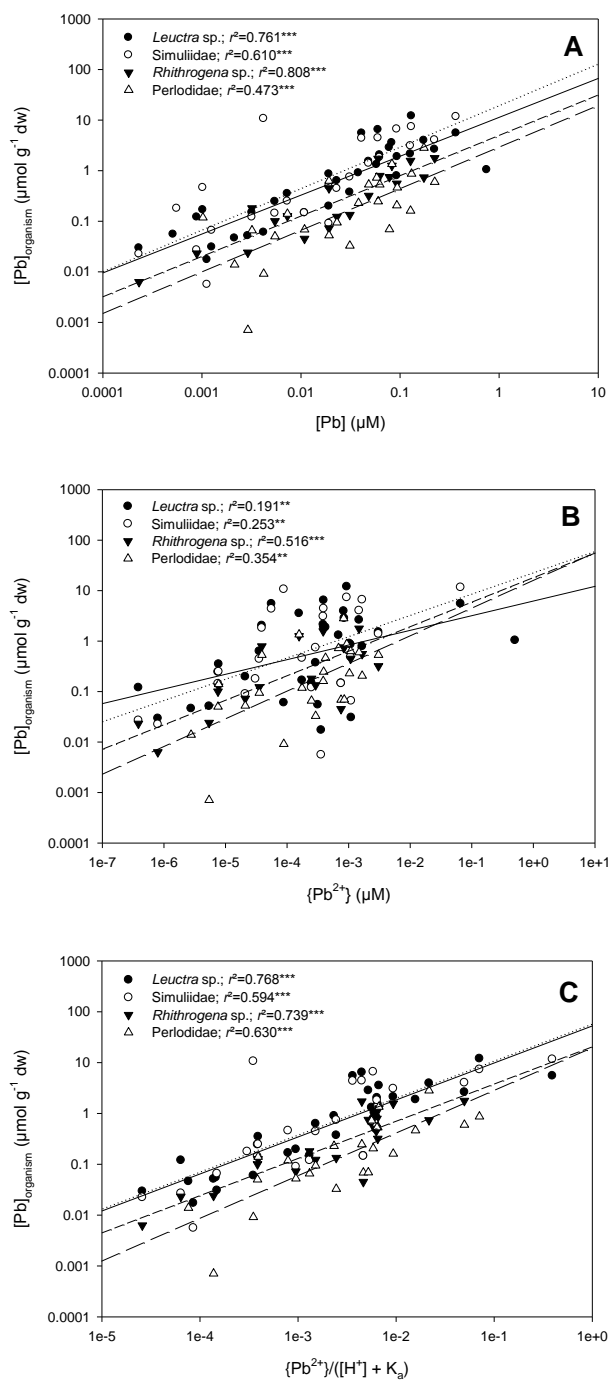


**Figure 4:** Relationships between Zn concentrations in *Leuctra* sp. (full circles; solid line;  $n = 33$ ), Simuliidae (open circles; dotted line;  $n = 24$ ), *Rhithrogena* sp. (full triangles; short dash;  $n = 20$ ) and Perlodidae (open triangles; long dash;  $n = 25$ ) and total dissolved Zn concentrations (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  $\text{Cd}^{2+}$  ion activity following equation (1) (B), WHAM-predicted  $\{\text{Cd}^{2+}\}$ , considering the influence of  $[\text{H}^+]$  following equation (2) (C) and WHAM-predicted  $\{\text{Cd}^{2+}\}$ , considering the influence of  $\{\text{Ca}^{2+}\}$  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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