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The use of invertebrate body burdens to predict ecological effects of metal mixtures in mining-impacted waters

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Abstract

The present study investigated whether invertebrate body burdens can be used to predict metal-induced effects on aquatic invertebrate communities. Total dissolved metal levels and four invertebrate taxa (*Leuctra* sp., Simuliidae, *Rhithrogena* sp. and Perlodidae) were sampled in 36 headwater streams located in the north-west part of England. Using the River Invertebrate Prediction and Classification System (RIVPACS) taxonomic completeness of invertebrate communities was assessed. Quantile regression was used to relate invertebrate body burdens to a maximum (90^{th} quantile) ecological response, both for all metals separately and in mixtures. Significant relations between Cu, Zn and Pb burdens in *Leuctra* sp. (Zn, Pb), Simuliidae (Zn, Pb), *Rhithrogena* sp. (Cu, Zn, Cu + Zn) and Perlodidae (Zn) and both taxonomic completeness (O/E taxa) and Biological Monitoring Working Party index scores (O/E BMWP) were observed. Corresponding the obtained Cu-Zn mixture model an acceptable impact of 5% change in taxonomic completeness is expected at *Rhithrogena* sp. body burdens of 1.9 µmol Cu g^{-1} in case of low Zn bioavailability (*Rhithrogena* sp. body burden of 2.9 µmol Zn g^{-1}), which will drop to 0.30 µmol g^{-1} in case of higher Zn bioavailability (body burden of 72.6 µmol Zn g^{-1}). For Zn, 5% change in taxonomic completeness is expected at *Rhithrogena* sp. body burdens of 76.4 µmol Zn g^{-1} in case of low Cu bioavailability (body burden of 0.19 µmol Cu g^{-1}), which will drop to 6.6 µmol Zn g^{-1} at higher Cu bioavailability (body burden of 1.74 µmol Cu g^{-1}). Overall, the present study concludes that invertebrate body burdens can be used to 1) predict metal-induced ecological effects and 2) to derive critical burdens for the protection of aquatic invertebrate communities.

Keywords: metal toxicity; body burdens; macroinvertebrate community; quantile regression; RIVPACS

1 Introduction

Metal pollution in aquatic ecosystems still poses a severe environmental problem worldwide and managing ecological effects of affected streams remains an important challenge (Luoma and Rainbow, 2008). Elevated levels of trace metals can have a negative impact on macroinvertebrate communities, primarily resulting in the loss of certain metal sensitive taxa such as heptageniid mayflies (Ephemeroptera) and caddisflies (Trichoptera) (Clements et al., 2000 and De Jonge et al., 2008). Therefore it is crucial that environmental risk regulators have the right tools, which estimate impacts of metal pollution in a biologically-relevant way, and which are mainly based on ecological observations (Luoma and Rainbow, 2008 and Luoma et al., 2010).

Metal bioavailability and toxicity from solution generally depends on the activity of the free metal ion, which is dominated by its chemical speciation (binding to dissolved organic carbon (DOC), abiotic ligands and effect of pH), and is largely influenced by other cations (e.g. $Ca2+$, $Mg2+$ and $Na+$) and $H+$ ions competing with trace metals for uptake at biological membranes (Hare and Tessier, 1996 and Hare and Tessier, 1998). Since in many cases metal pollution will not occur as the only stressor, it is difficult to demonstrate the contribution of trace metals to observed ecological effects under natural field conditions. Moreover, most often trace metals occur in mixtures of different concentrations, in which metals can interfere with each other both for uptake and at the site of toxic action (Norwood et al., 2003 and Borgmann et al., 2008). The latter hampers easy discrimination between single and mixture effects of metals in the field. Recently important attempts have been proposed in order to assess field effects of metal mixtures, e.g. the Chronic Criterion Accumulation Ratio (CCAR)

(Schmidt et al., 2010) and the WHAM-FTOX model (Stockdale et al., 2010). The latter concept describes toxicity of proton-metal mixtures to aquatic invertebrates in natural streams and is based on chemical speciation using the Windermere Humic Aqueous Model (WHAM) (Tipping, 1994 and Tipping, 1998) to predict the metabolically-active metal accumulated by the organism (Stockdale et al., 2010).

Another way to assess ecological effects of trace metals, taking into account bioavailability aspects, is by using invertebrate body burdens (Luoma et al., 2010 and Adams et al., 2011). The quantification of invertebrate body burdens represents an integrated and ecologically-relevant image of metal bioavailability and allows less physico-chemical measurements compared to monitoring in environmental compartments such as surface water and sediment (Norwood et al., 2007, Luoma et al., 2010 and De Jonge et al., 2012). Recently, a growing body of evidence has observed significant relations between body burdens in the caddisfly Hydropsyche sp. and metal-induced ecological effects such as a decrease in mayfly abundance or invertebrate taxa richness (David, 2003, Cain et al., 2004, Sola et al., 2004, Luoma et al., 2010 and Rainbow et al., 2012). Comparable relations have been found for the mayflies Rhithrogena sp. and Drunella sp., Chironomidae midge larvae and the zebra mussel Dreissena polymorpha in diverse freshwater ecosystems (Schmidt et al., 2011 and De Jonge et al., 2012). Schmidt et al. (2011) used quantile regression models based on Zn accumulation in both Rhithrogena sp. and Drunella sp. to describe changes in maximum (90th quantile) population density. Approaches such as the latter make it possible to predict ecological effects of metal toxicity using invertebrate body burdens, whereas effects of both biotic and abiotic confounding factors are minimized. However, to date these concepts have been only applied to a very limited number of species, trace metals and river systems, while in most cases only single metal exposure and toxicity was assumed (Luoma et al., 2010 and Schmidt et al., 2011). Information regarding relations between invertebrate body burdens and community effects is crucial in order to accurately monitor and predict ecological impacts of metal pollution in freshwater ecosystems. Moreover it has been suggested that invertebrate body burdens can be useful indicators of exposure and effects of metal mixtures (Norwood et al., 2007 and Borgmann et al., 2008).

The main objective of the present study was first to relate Ni, Cu, Zn, Cd, Pb and Al body burdens in the aquatic invertebrates Leuctra sp., Simuliidae, Rhithrogena sp. and Perlodidae to observed metal-induced ecological effects. Secondly, to derive critical body burdens, predicting adverse effects of metal mixtures on invertebrate communities.

2 Material and methods

2.1 Sampling and ecological impact assessment

In total 36 headwater streams of the Lake District, Ribbledale, Swaledale and the Howgill Fells, which are all located in the north-west part of England (Table S1), were sampled as part of an extended field survey (Bass et al., 2008). Some of these sites have been strongly metal contaminated from discharge of nearby abandoned mining sites while others have been affected by acid deposition. Maximum stream width was 10 m and dissolved oxygen levels were near saturation at all sites. Samples for the determination of water chemistry were taken on four occasions (March 6–8, March 20–22, April 3–5 and April 17–19, 2006). Separate samples were taken for major solutes (one-liter, high-density polyethylene bottles), for pH (glass bottles with a ground glass stopper, completely filled) and trace metals (500 cm3 acid-washed polyethylene bottles). All samples were kept in cool boxes at 4 °C during transport to the laboratory, where they were kept cool and dark.

Sampling of the macroinvertebrate fauna and the assessment of ecological responses was carried out using the River Invertebrate Prediction and Classification System (RIVPACS) (Wright et al., 1984 and Clarke et al., 2003). This approach assesses ecological quality of rivers based on the macroinvertebrate community using selected reference sites, which are considered to have a good chemical and biological quality and to be representative for a particular river type (Clarke et al., 2003). The sampling approach comprises recovery of macroinvertebrates from all major habitats at a site and includes a three-minute kick sampling with a pond net followed by an one-minute hand search (Wright et al., 1984). At the laboratory each sample was carefully sorted through for macroinvertebrates, aiming to record all taxa present in the sample. The collected organisms were identified to family or genus level and abundances were recorded. Using the macroinvertebrate abundance data the Biological Monitoring Working Party (BMWP) score system was calculated. The BMWP uses the sensitivity of different macroinvertebrate groups to pollution in order to calculate scores ranging from 1 (very poor biological quality) to 100 or higher (best ecological quality) (Hawkes, 1998). Additionally, various physical, chemical and ecological characteristics (e.g. slope, surface geology, distance from source, altitude, mean substrate size, etc.) were determined of all sampling sites in order to generate RIVPACS predictions of the probability of macroinvertebrate taxa occurrence (expected taxa number) and index scores (expected BMWP score). Using the RIVPACS bioassessment system it was possible to standardize ecological observations and to account for site-specific differences in macroinvertebrate community composition, which were not caused by the presence of metal pollution (Wright et al., 1984 and Clarke et al., 2003). The latter was done by dividing the observed taxa number and BMWP index scores by site-specific RIVPACS expected values, resulting in ratios of the observed/predicted taxa (O/E taxa or taxonomic completeness) and observed/predicted BMWP score (O/E BMWP).

2.2 Water chemistry analysis and chemical speciation

Within one day after collection, samples were analyzed 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 (NO3-N) and sulphate (SO4-S) were determined by ion chromatography; alkalinity was measured by Gran titration and dissolved organic carbon (DOC) by combustion. Total (persulphate–perchloric acid-digestible), ammonia-N (NH4-N), phosphorus (P) and silica (SiO2) were determined colorimetrically and suspended particulate matter (SPM) was determined gravimetrically. Water samples initiated for trace metal analysis were filtered over a 0.45 μm polypropylene filter, acidified with 1% nitric acid (HNO3; 69%) and total levels of Ni, Cu, Zn, Cd, Pb and Al 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, 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). In calculating chemical speciation, the concentrations of Na, Mg, K, Ca, Cl, NO3, SO4 and concentrations of filterable trace metals (Ni, Cu, Zn, Cd and Pb) were assumed to represent truly dissolved components (i.e. 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 colloids, however these species are neglected in the present analysis. The cation-binding properties of DOM were expressed in terms of isolated fulvic acid (FA), which is thought to be the most active DOM fraction in natural waters (Vincent et al., 2001). DOM concentrations were estimated based on measured DOC, assuming DOM to be 50% carbon and that 65% of the DOM behaves like isolated FA and is thus active regarding cation binding (Tipping et al., 2008). Ionic strengths effects on the inorganic reactions were taken into account using the extended Debye–Hückel equation.

2.3 Determination of invertebrate body burdens

For the metal analysis individual or pooled organisms were collected as part of the invertebrate sampling mentioned above. Analysis of invertebrate tissue levels is confined to the taxa that were present in more than 18 of the 36 sampled sites, including *Leuctra* sp. (O. Plecoptera; 33 sites), Perlodidae (O. Plecoptera; 25 sites), Simuliidae (O. Diptera; 24 sites) and *Rhithrogena* sp. (O. Ephemeroptera; 18 sites). Both simuliid and perlodid families represent unidentified single species. All samples were put in 1.5 mL polypropylene sampling vials and stored at 4 °C during transport and −20 °C at the laboratory. For each 50 sample vials with invertebrates also 5 empty vials were included to be used as process blanks. Samples were dried until constant temperature at 60 °C in a laboratory furnace. Subsequently they were weighed on a Sartorius SE2 Ultra Micro balance (accuracy 0.1 μg) and transferred to acid-cleaned and pre-weighed 0.5 or 1.5 mL polypropylene vials. Invertebrate samples were microwave digested in a nitric acid–hydrogen peroxide (H2O2; 30%) solution (3:1, v/v) by a step-wise method in which samples were microwave treated for four times, each time increasing the microwave power by 10% (Blust et al., 1988). For each series of 50 samples also 5 blank samples were processed and 5 samples of invertebrate reference material (mussel

BCR-668) were included for quality control. After the digestion procedure the digest was diluted with ultra-pure water (Milli-Q) to obtain a solution of 5% acid and the vials were reweighed to accurately determine the final sample volume. Metals were analyzed using a quadrupole Inductively Coupled Plasma Mass Spectrometer (ICP-MS; Varian UltraMass 700, Victoria, Australia). Results of invertebrate body burdens from the present paper have been partly incorporated in the paper of Stockdale et al. (2010).

2.4 Data processing and statistical analysis

Prior to analysis, all data were tested for normality with the Shapiro–Wilk test and for equality of variances using the Levene's test. Analysis of Variance (ANOVA) with post hoc Tukey test was used to compare averages. Linear regression models were used to describe the relations between body burdens and total dissolved metals, WHAM-predicted free metal ions and WHAM-predicted metal ions considering the influence of H+ ions at biological uptake sites (Hare and Tessier, 1996, Hare and Tessier, 1998 and De Jonge et al., 2013) (see Fig. S1). The latter models were used to predict invertebrate body burdens at sites where certain taxa could not be sampled, and the results were used in the quantile regression analysis to enhance statistical power (see below). Detailed information regarding the above-mentioned models is described in the paper of De Jonge et al. (2013).

Quantile regression was used to relate invertebrate body burdens to both O/E taxa and O/E BMWP. This type of regression analysis permits estimation of the 90th quantile of ecological responses as a function of an environmental stressor and thus projects the maximum ecological response (Koenker and Bassett, 1978, Cade and Noon, 2003, Linton et al., 2007 and Crane et al., 2007). An advantage of using this maximal response is that it is less likely to be influenced by other factors (e.g. habitat, life history, food availability, pH and other contaminants), which may all constrain the maximal macroinvertebrate diversity at a certain sample site (Crane et al., 2007, Stockdale et al., 2010 and Schmidt et al., 2011). This contrasts with ordinary least squares regression which generally focuses on estimating changes in the mean response variable (Cade and Noon, 2003). The 90th quantile regression models ($\tau = 0.9$) were constructed considering either the metals separately and in multiple mixtures using the equation:

$$y = \sum_{i}^{n} \alpha_i x_i + \beta \qquad (1)$$

In which y is the observed ecological response (O/E taxa or O/E BMWP), x_i the measured invertebrate body burden of metal i, α_m the toxicity coefficient of metal i and β the intercept. Toxicity coefficients were expected to be negative since the basic assumption behind the analysis is that metals can only reduce toxicity. Quantile regression analysis was done using the quantreg package of the statistical software R (Koenker, 2005). Based on the significant constructed regression models critical body burdens representing percent change in taxonomic completeness and BMWP could be calculated. Percent reduction in ecological response (O/E taxa and O/E BMWP) was calculated as follows:

$$\% \text{ Reduction in } 90^{th} \text{ quantile} = (\alpha\, R_{ref}) - (R_{ref} - R_{all}) \qquad (2)$$

where α is the predicted % reduction in ecological response (1 for 0% to 0 for 100% reduction), R_{ref} is the 90th quantile of ecological response corresponding to invertebrate body burdens measured at background dissolved metal levels, which are 0.014 µM for Zn, 0.008 µM for Cu, 0.0002 µM for Cd and 0.0002 µM for Pb. The latter environmental levels are assumed to correspond with uncontaminated reference sites of comparable Colorado headwater streams (Church et al., 2009 and Schmidt et al., 2011). R_{all} is the 90th quantile of ecological response at all sites. Eq. (2) adjusts for differences in the 90th quantile of ecological responses between reference sites and all other sites (Linton et al., 2007).

3 Results

3.1 Water chemistry and invertebrate body burdens

Water chemistry variables measured in the present study cover a wide range of pH (4.09 - 8.33), DOC (0.6 - 8.9 mg L^{-1}) and major cations including Na (0.11 - 0.97 mM), Mg (0.02 - 0.39 mM) and Ca (0.01 - 0.94 mM) (table 1). Total dissolved metal concentrations varied largely between waters and very high levels were observed for Zn (0.017 - 168 mM), Cd (0.027 - 171 nM) and Pb (0.242 - 754 nM). Levels of total dissolved metals were all significantly positive correlated with free metal ion concentrations (Ni: r = 0.864; Cu: r = 0542; Zn: r = 0.983; Cd: r = 0.981; Pb: r = 0.656; Al: r = 0.990; n = 36; all p< 0.001).
Metal body burdens ranged from 0.02 µmol g^{-1} (Simuliidae) to 0.68 µmol g^{-1} (*Leuctra* sp.) for Ni, from 0.18 µmol g^{-1} (Perlodidae) to 13.8 µmol g^{-1} (Simuliidae) for Cu, from 1.69 µmol g^{-1} (Simuliidae) to 85.7 µmol g^{-1} (*Leuctra* sp.) for Zn, from 0.001 µmol g^{-1} (Simuliidae) to 0.304 µmol g^{-1} (*Rhithrogena* sp.) for Cd, from 0.001 µmol g^{-1} (Perlodidae) to 12.0 µmol g^{-1} (*Leuctra* sp.) for Pb and from 0.15 µmol g^{-1} (Perlodidae) to 640 µmol g^{-1} (Simuliidae) for Al (table 2). Invertebrate Ni burdens between taxa were generally poorly correlated, except between Simuliidae and *Rhithrogena* sp. (r = 0.694; p < 0.001; n = 16). For Cu and Cd, invertebrate body burdens were all significantly co-correlated, with r-values being at least above 0.540 for Cu and above 0.775 for Cd. Regarding Zn and Pb, significant co-correlations between invertebrate body burdens were observed (r-values above 0.819 for Zn and above 0.435 for Pb) except between Perlodidae and both Simuliidae and *Rhithrogena* sp.

3.2 Ecological endpoints and relations with invertebrate body burdens

Taxonomic completeness (O/E Taxa) ranged from 0.32 to 1.16, which corresponds to 5 and 22 taxa observed (Table S1). O/E BMWP ranged from 0.41 to 1.24, corresponding to BMWP metric scores of 43 and 153 respectively.

A significant relation representing change in maximum (90^{th} quantile) taxonomic completeness as a function of accumulated Cu was observed for *Rhithrogena* sp. (Fig. 1), resulting in a toxicity coefficient (α_{Cu}) of -0.11 (table 3). Furthermore significant negative relations were observed between changes in maximum taxonomic completeness and Zn accumulation in *Leuctra* sp. (t = -2.31, n = 33, p<0.05), Simuliidae (t = -2.26, n = 33, p<0.05), *Rhithrogena* sp. (t = -2.49, n = 33, p<0.05) and Perlodidae (t = -2.35, n = 33, p<0.05) (figure 2, table 3). Toxicity coefficients (α_{Zn}) ranged from -0.002 for *Rhithrogena* sp. to -0.02 for Simuliidae and Perlodidae. A significant 90^{th} quantile mixture model was obtained for Cu and Zn burdens in *Rhithrogena* sp. (α_{Cu} = -0.09, t_{Cu} = -2.05, p_{Cu} = 0.04; α_{Zn} = -0.002, t_{Zn} = -3.03, p_{Zn}= 0.004; n = 33) (table 3).

Regarding BMWP, significant 90^{th} quantile regression models presenting change in maximum O/E BMWP as a function of accumulated Zn were observed for Simuliidae (t = -3.66, n = 33, p<0.001), *Rhithrogena* sp. (t = -2.05, n = 33, p<0.05) and Perlodidae (t = -2.24, n = 33, p<0.05) (figure 3) with α_{Zn} ranging from 0.001 for *Rhithrogena* sp. to 0.007 for Perloddiae. Significant quantile regression models depicting change in O/E BMWP as a function of Pb accumulation were obtained for both *Leuctra* sp. (t = -2.22, n = 33, p<0.05) and Simuliidae (t = -2.06, n = 33, p<0.05) (figure 4) with α_{Pb} = 0.01 for Simuliidae and α_{Pb} = 0.02 for *Leuctra* sp. (table 3). No significant mixture effect between Zn and Pb, or any other metal, on O/E BMWP scores was observed. Regarding Ni, Cd and Al body burdens, no significant 90^{th} quantile regression models were obtained for any of the studied taxa, neither for taxonomic completeness nor for BMWP (table 3).

Using the above-mentioned quantile regression models, critical invertebrate body burdens, corresponding to maximum taxonomic completeness and O/E BMWP, could be calculated (table 4). Consequently, accumulated Cu concentrations in *Rhithrogena* sp. corresponding to taxonomic completeness associated with 0%, 5%, 20%, 50% and 100% decrease are 0.62, 1.1, 2.6, 5.5 and 10.0 µmol g^{-1} Cu. Adding the influence of Zn, critical Cu levels in *Rhithrogena* sp. decreased according to the level of Zn present in the environment (table 5). For example Cu burdens calculated from the mixture (Cu + Zn) quantile regression model were higher than the ones calculated from the model using Cu alone at low Zn concentrations (2.9 µmol g^{-1}), while burdens of 0.48 µmol g^{-1} Cu, at which no change in taxonomic completeness is expected from the model using Cu alone, will result in a 20% decrease at high Zn body burdens (145 µmol g^{-1}).

Critical Zn body burdens corresponding to observed changes in taxonomic completeness could be calculated for all taxa and ranged from 4.4 to 25.0 µmol g^{-1} for 0%, from 7.0 to 50 µmol g^{-1} for 5%, from 14.8 to 128 µmol g^{-1} for 20%, from 30.3 to 283 µmol g^{-1} for 50% and from 56.0 to 540 µmol g^{-1} for 100% decrease (table 4). Critical tissue levels are very comparable between Simuliidae and Perlodidae, while they differ a factor 2 and 10 for both *Leuctra* sp. and *Rhithrogena* sp. respectively. Adding the influence of Cu, critical Zn tissue levels in *Rhithrogena* sp. decreased according to the level of Cu present in the environment (table 5). For example *Rhithrogena* sp. tissue levels of 50.5 µmol g^{-1} Zn will result in 0% change in taxonomic completeness at low accumulated Cu concentrations (0.19 µmol g^{-1}), while the same Zn level will result in a taxonomic decrease between 20% and 50% at high Cu tissue levels (3.52 µmol g^{-1}).

Critical Zn body burdens corresponding to observed changes in O/E BMWP were generally higher compared to the ones for O/E taxa, however the same trends were observed (table 4). Critical accumulated Pb levels corresponding to 0%, 5%, 20%, 50% and 100% decrease in

O/E BMWP could be calculated for both *Leuctra* sp. and Simuliidae and ranged from 2.6 to 4.7 µmol g^{-1} for 0%, from 3.9 to 7.3 µmol g^{-1} for 5%, from 7.9 to 15.2 µmol g^{-1} for 20%, from 15.7 to 31.0 µmol g^{-1} for 50% and from 28.8 to 57.0 µmol g^{-1} for 100% decrease.

4. Discussion

The present study observed significant negative relations between maximal (90th quantile) ecological responses and accumulated Cu, Zn and Pb levels in *Leuctra* sp., Simuliidae, *Rhithrogena* sp. and Perlodidae. Our findings add to a growing body of evidence suggesting the use of invertebrate body burdens as an indicator of ecological effects of metal toxicity (Luoma et al., 2010, Adams et al., 2011, Schmidt et al., 2011, Rainbow et al., 2012 and De Jonge et al., 2012). Invertebrate body burdens represents a more integrated and ecologically-relevant image of metal bioavailability in the aquatic environment, compared to concentrations in surface water or sediment (Norwood et al., 2007, Luoma et al., 2010 and De Jonge et al., 2012).

Critical body burdens corresponding to maximum taxonomic completeness (expressed as % decrease) could be derived, which can be used to predict ecological effects of metal toxicity. Based on the relations between *Rhithrogena* sp. Cu burdens and taxon richness observed in the present study, 10.0 µmol g^{-1} Cu (636 µg g^{-1} Cu) is predicted to result in the disappearance of all invertebrate taxa. Luoma et al. (2010) noted the complete disappearance of mayfly communities and impoverished macroinvertebrate taxa richness (only 10 taxa remaining) at accumulated Cu concentrations of 15.7 µmol g−1 (998 µg g−1 Cu) in larvae of the caddisfly *Hydropsyche* sp. Analogously, studies of David (2003) and Rainbow et al. (2012) both observed severely impoverished mayfly communities, consisting of 1 and 0 mayfly taxa respectively, at body burdens of 20.5 and 18.9 μmol g−1 Cu (1303 and 1200 μg g−1 Cu) in *Hydropsyche* sp. In the present study predicted critical Cu burdens in *Rhithrogena* sp., corresponding to the disappearance of invertebrate communities, are slightly lower (maximum a factor two) compared to *Hydropsyche* sp. body burdens observed in literature. The latter may be primarily explained by possibly lower Cu accumulation by *Rhithrogena* sp. compared to *Hydropsyche* sp., which is known to be a strong Cu accumulator (Luoma et al., 2010 and Rainbow et al., 2012). Therefore identified critical body burdens strictly apply to the taxon investigated and cannot be extrapolated between different taxa. Secondly, a strong effect of elevated Zn bioavailability on taxonomic completeness was observed using the mixture model, implicating that Cu is not the only pollutant affecting macroinvertebrate community composition and assessing Cu alone will overestimate its ecological impact at elevated Zn levels in the environment.

Critical body burdens based on the relations between accumulated Zn levels and taxonomic completeness and corresponding to changes in macroinvertebrate assemblages were generally comparable for *Leuctra* sp., Simuliidae and Perlodidae, however were much higher for *Rhithrogena* sp. (maximum a factor 9). Sola et al. (2004) observed a 50% decrease in macroinvertebrate taxa richness and density, compared to low-contaminated reference sites, which were associated with 49.1 μmol g^{-1} Zn (3210 μg g^{-1} Zn) in *Hydropsyche* sp. larvae. The latter results are in good agreement with critical body burdens in *Leuctra* sp., Simuliidae and Perlodidae corresponding with a 50% decrease in taxonomic completeness (58.6, 30.3 and 31.3 μmol g^{-1} Zn or 3831, 1981 and 2046 μg g^{-1} Zn respectively), that were obtained in the present study. Rainbow et al. (2012) observed decreased mayfly abundances at 9.73 μmol g^{-1} Zn (636 μg g^{-1} Zn) in *Hydropsyche siltalai* of UK streams, however still some heptageniid and ephemerellid mayflies were present. Schmidt et al. (2011) derived critical body burdens of 4.08 μmol g^{-1} Zn (267 μg g^{-1} Zn) in *Rhithrogena* sp. corresponding to a 20% decrease in population density. In the present study a maximal decrease of 20% in O/E taxa ratio corresponds to *Rhithrogena* sp. body burdens of 128 μmol g^{-1} Zn (8369 μg g^{-1} Zn) in the case where Zn was considered to be the only stressor. However, according to the mixture model obtained for *Rhithrogena* sp. also Cu affects invertebrate communities simultaneously with Zn. According to the latter model, a 20% decrease in taxonomic completeness should be expected at tissue concentrations of 3.52 μmol g^{-1} Cu (224 μg g^{-1} Cu) and 4.20 μmol g^{-1} Zn (275 μg g^{-1} Zn) in *Rhithrogena* sp., which is in good agreement with the Zn body burdens for *Rhithrogena* sp. observed by Schmidt et al. (2011). The latter demonstrates the importance of taking into account multiple metals when assessing ecological effects of metal toxicity. Corresponding the obtained Cu–Zn mixture model an acceptable impact of 5% change in taxonomic completeness is expected at *Rhithrogena* sp. Zn burdens of 76.4 μmol g^{-1} (4995 μg g^{-1} Zn) in case of low Cu bioavailability (*Rhithrogena* sp. Cu body burden of 0.19 μmol g^{-1} or 12.1 μg g^{-1}), which will drop to 6.6 μmol g^{-1} Zn (432 μg g^{-1} Zn) in case of higher Cu bioavailability (*Rhithrogena* sp. Cu body burden of 1.74 μmol g–1 or 111 μg g^{-1}). Similarly for Cu, an acceptable impact of 5% change in taxonomic completeness is expected at *Rhithrogena* sp. Cu burdens of 1.9 μmol g^{-1} (121 μg g^{-1} Cu) in case of low Zn bioavailability (*Rhithrogena* sp. Zn body burden of 2.9 μmol g^{-1} dw or 190 μg g^{-1}), which will drop to 0.30 μmol g^{-1} Cu (19.1 μg g^{-1} Cu) in case of higher Zn bioavailability (*Rhithrogena* sp. Zn body burden of 72.6 μmol g^{-1} dw or 4747 μg g^{-1}). Corresponding the obtained Cu–Zn mixture model critical body burdens cannot be applied when effects of metal mixtures are considered. In fact, metal uptake and bioaccumulation can be strongly influenced by the presence of other metals due to competitive, anti-competitive and non-competitive inhibitions, resulting in invertebrate body burdens which will vary in function of other metals present (Norwood et al., 2007 and Borgmann et al., 2008). Therefore, combining invertebrate body burdens in mixture models, as was done in the present study, can be a valuable approach to predict ecological effects of metal toxicity, taking into account multiple metals in mixtures.

Since Ni, Cd, Pb nor Al body burdens significantly contributed to the observed change in taxonomic completeness, it can be assumed that in case of the present study, in which the headwaters are most dominantly affected by Cu and Zn, most elevated *Rhithrogena* sp. burdens (0.19 μmol g^{-1} or 11.2 μg g^{-1} for Ni, 0.30 μmol g^{-1} or 33.6 μg g^{-1} for Cd, 1.78 μmol g^{-1} or 368 μg g^{-1} for Pb and 40.5 μmol g^{-1} or 1093 μg g^{-1} for Al) represent safe levels for the macroinvertebrate community. On the other hand, critical body burdens based on the relation between Pb accumulation and O/E BMWP could be derived for both *Leuctra* sp. and Simuliidae. Rainbow et al. (2012) observed low mayfly abundances and the extinction of heptageniid and ephemerellid families associated with 2.13 μmol g^{-1} Pb (441 μg g^{-1} Pb) in *Hydropsyche siltalai*. The results of the present study indicate that a maximal O/E BMWP of 0.84, corresponding to a 20% decrease, is expected at body burdens of 7.9 and 15.2 μmol g^{-1} Pb (1635 and 3146 μg g^{-1} Pb) in *Leuctra* sp. and Simuliidae respectively. The latter critical concentrations are much higher (maximum a factor 7) compared to observed *H. siltalai* body burdens in the study of Rainbow et al. (2012). Although no significant mixture model of Pb and Zn for any of the studied invertebrate taxa was obtained, high Zn levels measured in the headwaters of the present study will have undoubtedly influenced observed BMWP scores and thus increased predicted invertebrate body burdens. Therefore, critical Pb burdens derived in the present study should be interpreted with great caution.

Following the relations observed in the present study, invertebrate communities were most dominantly affected by bioavailable Cu and Zn concentrations. The study of Stockdale et al. (2010), which partly used the same dataset, similarly observed contributions of WHAMmodeled metabolically available Cu and Zn levels, besides the influence of pH and Al, to be most significant in explaining toxic effects on the invertebrate community, expressed as the linear toxicity function F_{TOX}. These findings were due to the high concentrations of both Cu and Zn in some of the sampled mining-affected headwaters. The WHAM-F_{TOX} model was found to be a plausible model to describe both proton and metal mixture toxicity based on water chemistry and speciation concepts (Stockdale et al., 2010).

The present study used metrics of invertebrate taxa richness which are generally considered to be sensitive indicators of metal pollution (Hickey and Clements, 1998 and Clements, 2004). Nevertheless some studies observed that these metrics remain less sensitive compared to measurements of population densities (e.g. mayfly abundance) (Clements et al., 2000 and Clark and Clements, 2006). However in order to assess effects of metal pollution on aquatic communities it is crucial that natural variation in invertebrate taxa composition, which can be quite variable and depends on various biotic, abiotic and geomorphological characteristics, is taking into account. The present study accounts for this natural variation in two separate ways. First, using the RIVPACS bioassessment system, which includes the expected number of invertebrate taxa based on chemical, geomorphological and ecological measurements, site-specific differences in habitat characteristics can be better taken into account compared to metrics which are based on observed population and community metrics without normalization (Wright et al., 1984 and Clarke et al., 2003). Secondly, using quantile regression maximal responses of taxonomic completeness, which are generally less influenced by other non-modeled factors affecting invertebrate taxa richness, can be formulated as a function of invertebrate body burdens, providing an estimate of the ecological response in cases were metals are the only stressor (Linton et al., 2007, Crane et al., 2007, Stockdale et al., 2010 and Schmidt et al., 2011). The latter justifies the approach used in the current study to derive critical invertebrate body burdens based on observed relations between accumulated metal levels and changes in taxonomic richness.

5. Conclusions

Invertebrate body burdens can be used to (1) predict metal-induced ecological effects and (2) to derive critical burdens for the protection of aquatic invertebrate communities. Critical invertebrate body burdens corresponding to an acceptable 5% decrease in taxonomic completeness could be derived for Cu and Zn, resulting in protective burdens which can however change in function of increasing Cu/Zn bioavailability in the case of *Rhithrogena* sp. Using both the RIVPACS bioassessment system together with 90th quantile regression analysis, the present study was able to propose a method for deriving invertebrate body burdens predicting metal-induced community effects, which were based on ecological observations in the field and can be applied for metal mixtures. Without doubt, such biologically-based tools are of great importance in assessing ecological impacts of metal pollution on aquatic ecosystems and deserve increased scientific attention in the near future.

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The use of invertebrate body burdens to predict ecological effects of metal mixtures in mining-impacted waters

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Table 1: Range of water chemistry (means of four determinations) of all sample sites (*n*=36).

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
NO_3-N	$mg\ L^{-1}$	0.03	0.8
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*	0.1	1.22
Al	*µM*	0.04	58
Mn	*µM*	0.04	20
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: Ni, Cu, Zn, Cd and Pb tissue levels in the collected invertebrate taxa. Minimum - maximum, geometric mean and median values (in µmol g^{-1} dw) of all sample sites (*n*=14-33, depending on the taxa) are presented.

		Ni	**Cu**	**Zn**	**Cd**	**Pb**	**Al**
Leuctra sp.	Min.	0.03	0.34	2.79	0.002	0.02	14.3
n = 33	Max.	0.68	9.10	85.7	0.210	12.0	362
	Geo. mean	0.11	0.79	6.69	0.012	0.53	37.3
	Median	0.11	0.67	4.54	0.010	0.79	31.8
Simuliidae	Min.	0.02	0.20	1.96	0.001	0.01	5.67
n = 24	Max.	0.27	13.8	21.1	0.141	11.7	640
	Geo. mean	0.10	0.61	5.00	0.013	0.55	38.3
	Median	0.11	0.42	4.68	0.011	0.47	33.0
Rhithrogena sp.	Min.	0.03	0.19	2.85	0.015	0.01	4.41
n = 20	Max.	0.19	1.74	72.6	0.304	1.78	40.5
	Geo. mean	0.07	0.41	18.0	0.079	0.22	12.6
	Median	0.07	0.35	23.2	0.072	0.25	12.1
Perlodidae	Min.	0.003	0.18	2.49	0.001	0.001	0.15
n = 25	Max.	0.10	6.28	45.8	0.136	2.82	108
	Geo. mean	0.03	0.62	6.46	0.011	0.14	4.33
	Median	0.04	0.56	5.15	0.012	0.16	5.22

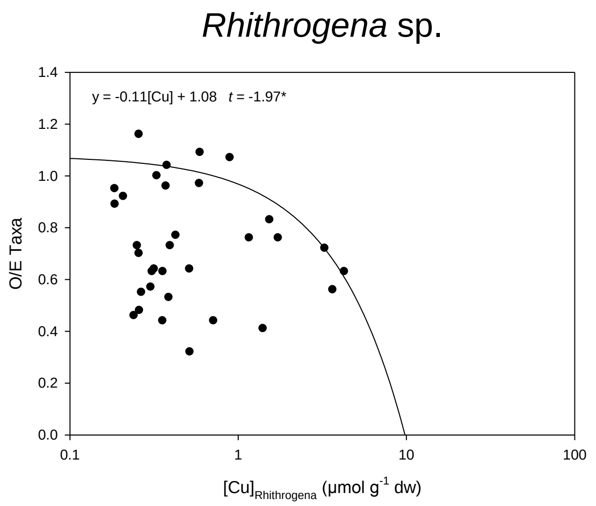

Figure 1: Ninetieth regression quantiles representing change in maximum O/E taxa as a function of accumulated Cu in *Rhithrogena* sp. Quantile regression equation (n = 33) and t-value is given. The significance level is presented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

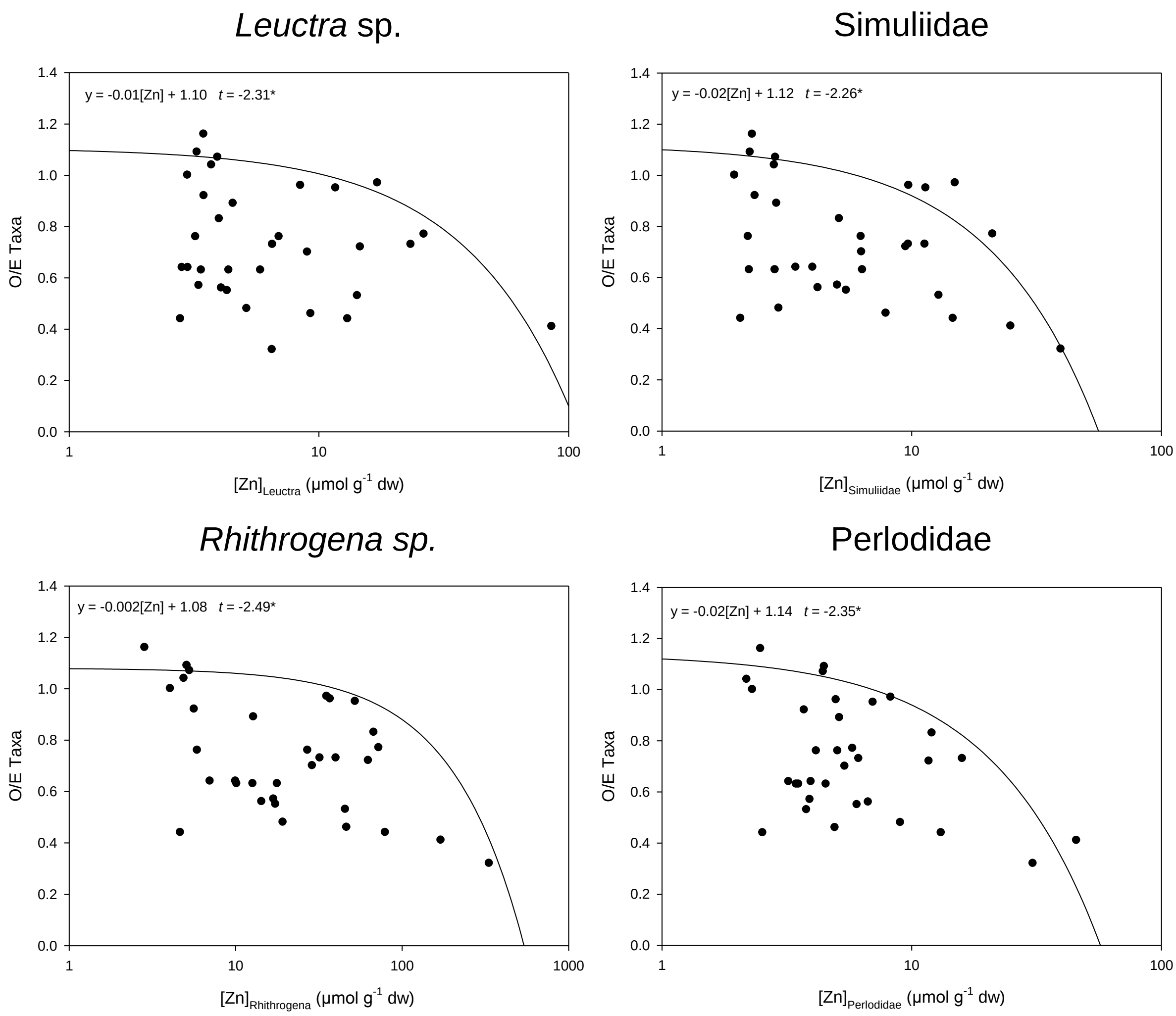

Figure 2: Ninetieth regression quantiles representing change in maximum O/E taxa as a function of accumulated Zn in *Leuctra* sp., Simuliidae, *Rhithrogena* sp. and Perlodidae. Quantile regression equations (n = 33) and t-values are given. The significance level is presented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

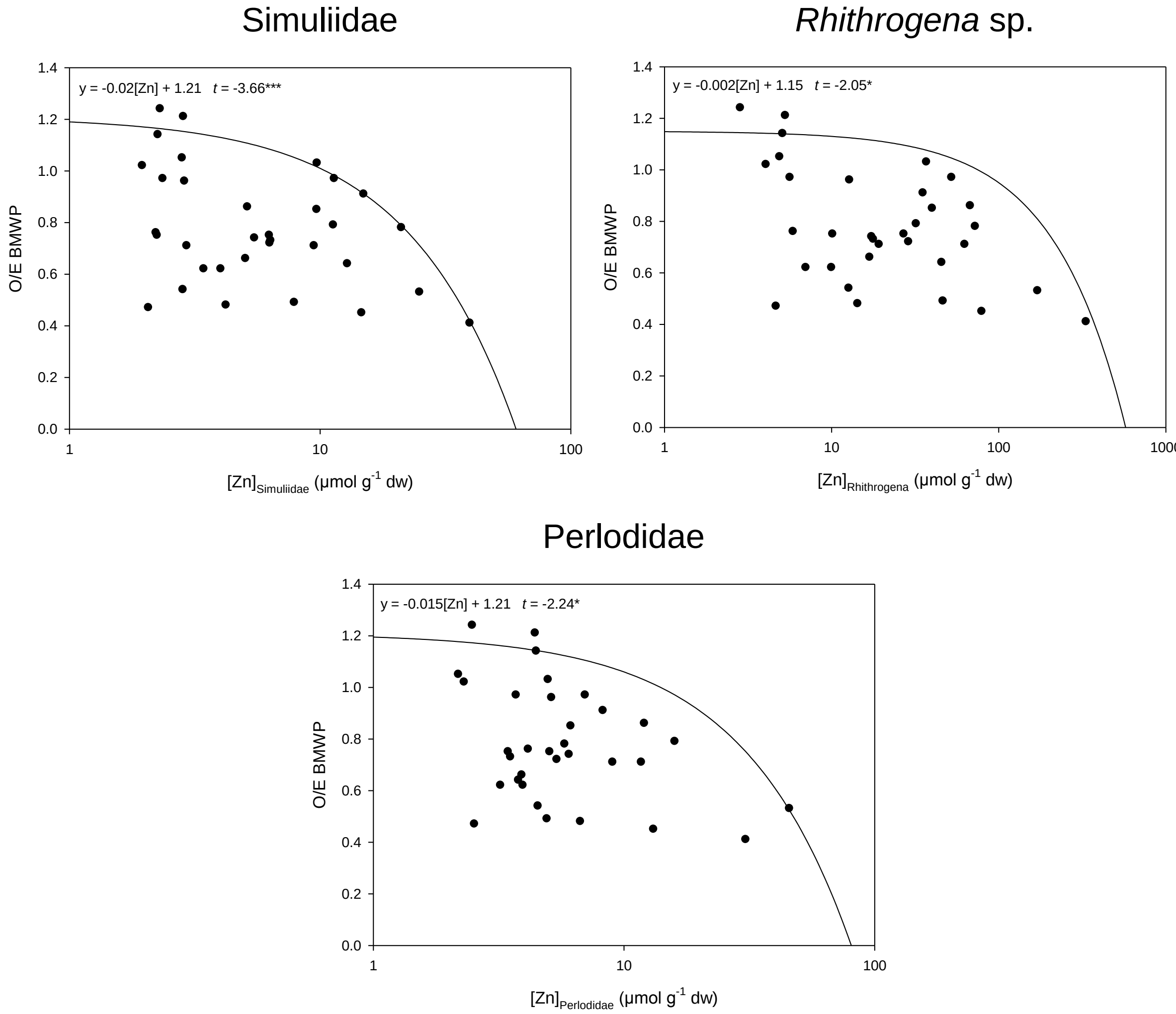

Figure 3: Ninetieth regression quantiles representing change in maximum O/E BMWP as a function of accumulated Zn in Simuliidae, *Rhithrogena* sp. and Perlodidae. Quantile regression equations (n = 33) and t-values are given. The significance level is presented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

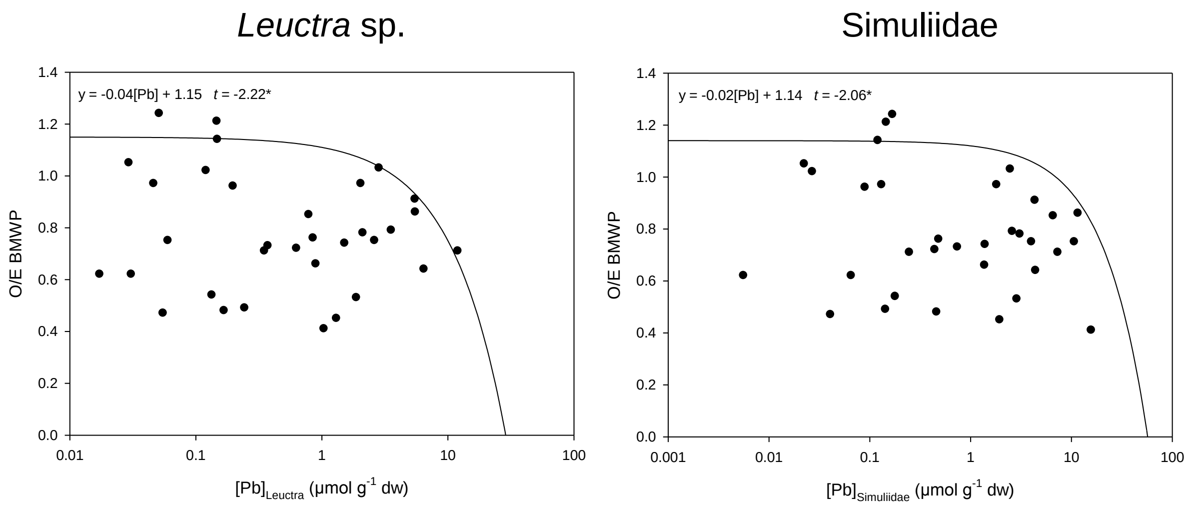

Figure 4: Ninetieth regression quantiles representing change in maximum O/E BMWP as a function of accumulated Pb in *Leuctra* sp. and Simuliidae. Quantile regression equations (n = 33) and t-values are given. The significance level is presented as $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Table 3: Overview of 90th quantile regression analysis based on single and combined invertebrate body burdens (n = 33). α: toxicity coefficient of the metal on the observed ecological response; SEM: Standard error of the mean; Mixture: The only significant metal mixture influencing observed ecological response was Zn + Cu. The significance level is presented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; NS: Not significant.

			O/E Taxa					**O/E BMWP**				
Stressor		**Taxon**	**α**	**SEM**	**Intercept**	**SEM**	***t* value**	**α**	**SEM**	**Intercept**	**SEM**	***t* value**
Single	Ni		NS					NS				
	Cu	*Rhithrogena* sp.	-0.11	0.05	1.08	0.08	-1.97*	NS				
	Zn	*Leuctra* sp.	-0.01	0.003	1.10	0.05	-2.31*	NS				
		Simuliidae	-0.02	0.007	1.12	0.06	-2.26*	-0.02	0.006	1.21	0.08	-3.66***
		Rhithrogena sp.	-0.002	0.001	1.08	0.04	-2.49*	-0.002	0.001	1.15	0.07	-2.05*
		Perlodidae	-0.02	0.007	1.14	0.07	-2.35*	-0.02	0.007	1.21	0.09	-2.24*
	Cd		NS					NS				
	Pb	*Leuctra* sp.	NS					-0.04	0.02	1.15	0.08	-2.22*
		Simuliidae	NS					-0.02	0.01	1.14	0.08	-2.06*
	Al		NS					NS				
Mixture	Zn	*Rhithrogena* sp.	-0.002	0.001	1.15	0.05	-3.03**	NS				
	Cu	*Rhithrogena* sp.	-0.09	0.05	1.15	0.05	-2.05*	NS				

Table 4: Critical invertebrate body burdens (in µmol g^{-1} dw) corresponding with a 0%, 5%, 20%, 50% and 100% decrease in O/E taxa and O/E BMWP. Tissue levels were calculated based on significant quantile regression models of single metals (figure 2, 3, 4 and 5). NS: No significant quantile regression models could be constructed.

		Cu	**Zn**				**Pb**	
	% Loss	***Rhithrogena* sp.**	***Leuctra* sp.**	**Simuliidae**	***Rhithrogena* sp.**	**Perlodidae**	***Leuctra* sp.**	**Simuliidae**
O/E Taxa	0	0.62	8.5	4.4	25.0	5.4	NS	NS
	5	1.1	15.0	7.0	49.9	8.0	NS	NS
	20	2.6	27.5	14.8	128	15.8	NS	NS
	50	5.5	58.6	30.3	283	31.3	NS	NS
	100	10.0	110	56.0	540	57.0	NS	NS
O/E BMWP	0	NS	NS	8.2	52.0	10.9	2.6	4.7
	5	NS	NS	10.8	78.2	14.4	3.9	7.3
	20	NS	NS	18.7	157	24.9	7.9	15.2
	50	NS	NS	34.4	314	45.8	15.7	31.0
	100	NS	NS	60.5	575	80.7	28.8	57.0

Table 5: Critical *Rhithrogena* sp. body burdens (in µmol g^{-1} dw) corresponding with a 0%, 20%, 50% and 100% decrease in O/E taxa based on 90^{th} quantile regression models using mixtures of Cu and Zn; O/E taxa = -0.09[Cu] - 0.002[Zn] + 1.15 (t_{Cu} = -2.05, p_{Cu} = 0.04; t_{Zn} = -3.03, p_{Zn}= 0.004). Each time tissue concentrations were derived for one metal while the other one was taken constant at both low and higher tissue levels. -: indicates that this tissue level of one metal is already too high to obtain the desired ecological response.

		Cu + Zn		**Zn + Cu**	
	% Loss	**[Zn]**	**[Cu]**	**[Cu]**	**[Zn]**
O/E Taxa	0	2.9	1.3	0.19	50.5
	5	2.9	1.9	0.19	76.4
	20	2.9	3.6	0.19	154
	50	2.9	7.2	0.19	309
	100	2.9	12.7	0.19	566
	0	72.6	-	1.7	-
	5	72.6	0.30	1.7	6.6
	20	72.6	2.1	1.7	84.3
	50	72.6	5.7	1.7	240
	100	72.6	11.2	1.7	497
	0	145	-	3.5	-
	5	145	-	3.5	-
	20	145	0.48	3.5	4.2
	50	145	4.1	3.5	160
	100	145	9.6	3.5	417

Supporting information

Site	Site name	Grid reference	Area	Observed Taxa	Expected Taxa	O/E Taxa	Observed BMWP	Expected BMWP	O/E BMWP
LD1	Dell Beck	SD 285 985	Lake District	9	16	0.56	51	107	0.48
LD2	Levers Water Beck	SD 285 985	Lake District	10	16	0.63	57	105	0.54
LD3	Dale Head Gill	NY 242 006	Lake District	10	16	0.64	64	104	0.62
LD4	Mosedale Beck	NY 245 018	Lake District	7	16	0.44	49	105	0.47
LD5	Uzzicar stream	NY 236 218	Lake District	16	19	0.83	102	119	0.86
LD6	Newlands Beck above spoil	NY 229 181	Lake District	17	16	1.07	127	105	1.21
LD7	Newlands Beck below spoil	NY 231 186	Lake District	13	17	0.76	86	113	0.76
LD8	Eller Gill	NY 252 182	Lake District	22	20	1.09	151	133	1.14
LD9	Roughton Gill	NY 338 295	Lake District	16	21	0.76	104	139	0.75
LD10	Wood Head stream	NY 337 365	Lake District	16	22	0.72	105	148	0.71
LD11	Threlkeld stream	NY 322 255	Lake District	5	16	0.32	43	104	0.41
HS1	Long Gill	SD 796 823	Ribbledale	22	19	1.16	153	123	1.24
HS2	Slei Gill	NZ 018 021	Swaledale	20	21	0.95	132	136	0.97
HS3	Black Mires Gill	NY 993 036	Swaledale	18	19	0.97	102	112	0.91
HS4	Great Punchard Gill	NY 961 044	Swaledale	17	19	0.89	118	124	0.96
HS5	Hurr Gill	NZ 011 063	Swaledale	13	21	0.63	101	134	0.75
HS6	Uldale Beck	NY 815 037	Swaledale	10	16	0.64	64	104	0.62
HS7	Upper Scandale Beck	NY 742 028	Howgill Fells	20	20	1.00	131	129	1.02
HS8	Gais Gill	NY 716 011	Howgill Fells	17	16	1.04	113	108	1.05
HS9	Cluntering Gill	SD 712 800	Whernside	18	20	0.92	124	127	0.97
TD1	Brown Gill	NY 751 424	Tynedale	14	18	0.77	92	119	0.78
TD2	Rough Rigg stream	NY 823 343	Teesdale	9	16	0.57	69	104	0.66
TD3	Ashgill Head stream	NY 808 355	Teesdale	13	18	0.73	92	116	0.79
TD4	Reddycombe Sike	NY 808 333	Teesdale	9	20	0.46	63	128	0.49
TD5	Willyhole Sike	NY 814 333	Teesdale	13	19	0.70	86	120	0.72
TD6	Harwood Beck	NY 815 334	Teesdale	9	19	0.48	86	121	0.71
TD7	Langdon Beck	NY 849 334	Teesdale	12	19	0.63	89	122	0.73
TD8	Langdon Beck tributary	NY 848 336	Teesdale	10	18	0.55	88	119	0.74
TD9	Sedling Burn	NY 857 407	Weardale	10	19	0.53	78	122	0.64
TD10	Nenthead stream nr. 1	NY 782 435	Tynedale	8	19	0.41	66	125	0.53
TD11	Nenthead stream nr. 2	NY 784 434	Tynedale	9	20	0.44	60	133	0.45
TD13	Nentsberry stream	NY 765 446	Tynedale	15	16	0.96	107	104	1.03
TD16	Kilhope Burn	NY 830 429	Weardale	13	18	0.73	98	115	0.85

Table S1: Sample site information and ecological observations. Expected Taxa and BMWP scores were calculated using the River Invertebrate Prediction and Classification System (RIVPACS) (Wright et al., 1984).

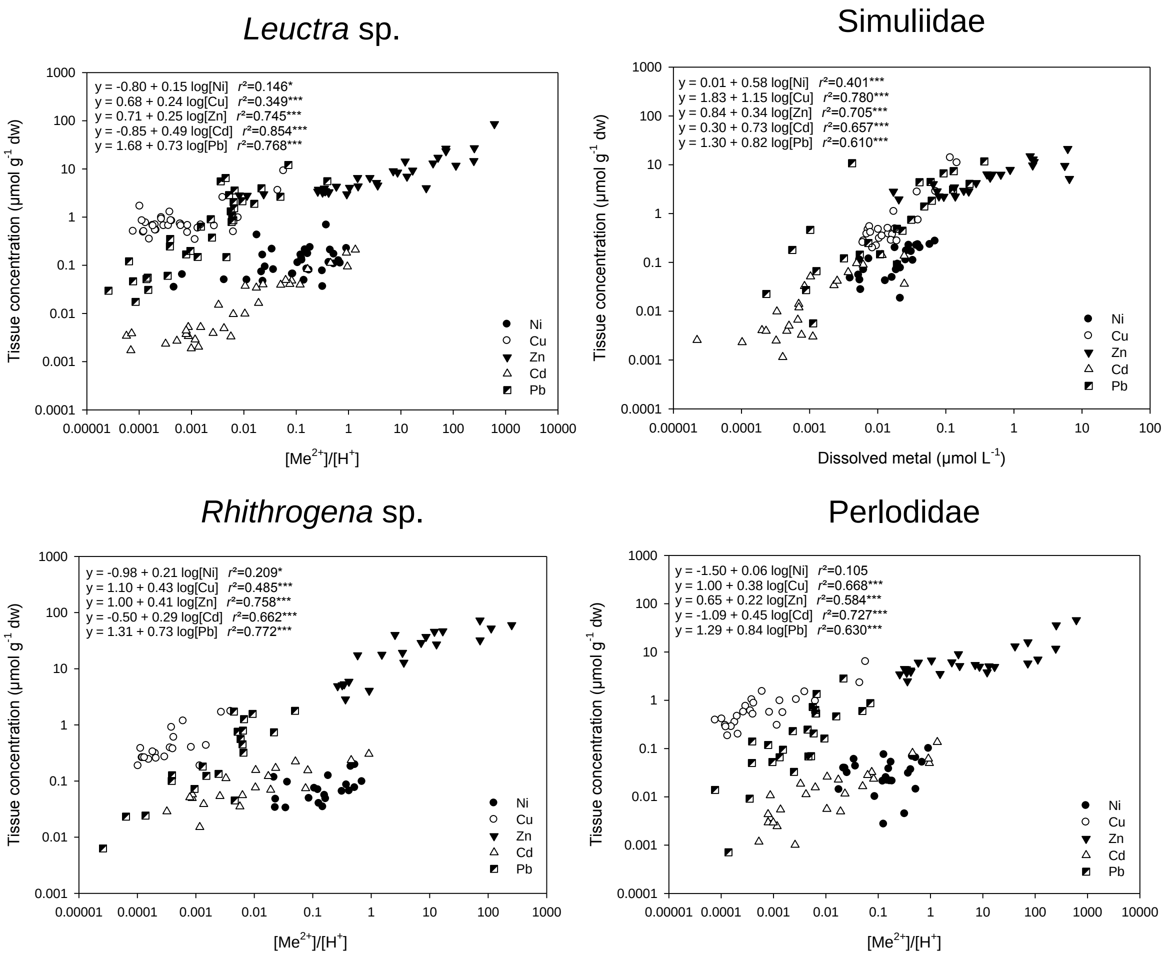

Figure S1: Relationships between body burdens in *Leuctra* sp. (n = 33), Simuliidae (n = 24), *Rhithrogena* sp. (n = 18) and Perlodidae (n = 25) and Ni, Cu, Zn, Cd and Pb levels in surface water. For Simuliidae, total dissolved metal concentrations most strongly explained the total variation in accumulated metal levels. For *Leuctra* sp., *Rhithrogena* sp. and Perlodidae, WHAM-predicted free metal ions, considering the influence of $[H^+]$ ions at biological uptake sites best predicted metal accumulation. Linear regression equations are presented and the amount of variation explained is given by the coefficient of determination (r^2). The significance level is presented as *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.