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Systematic analysis of freshwater metal toxicity with WHAM-F_{TOX}

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Highlights

- We sought chemical and biological trends in metal toxicity data, using the WHAM-F_{TOX} potency parameter $\alpha_{M,max}$.
- There were no consistent differences in $\alpha_{M,max}$ among invertebrates, plants and vertebrates.
- There were significant differences in $\alpha_{M,max}$ among species, but greater within-species variability.
- Values of $\alpha_{M,max}$ depended strongly upon Pearson's hardness-softness categories.

Abstract

We used the WHAM chemical speciation model and the WHAM-F_{TOX} toxicity model to analyse the published results of laboratory toxicity experiments covering 52 different freshwater biological test

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species and 24 different metals, a total of 2037 determinations of EC_{50} with accompanying data on solution composition. The key extracted parameter was α_M, the parameter in WHAM-F_{TOX} that characterises the toxic potency of a metal on the basis of its estimated metabolically active body burden. For 16 data sets applying to metal-test species pairs with appreciable variations in solution composition, values of EC_{50} back-calculated from averaged values of α_M showed significantly ($p<0.001$) less deviation from the measured EC_{50} values than did the simple average EC_{50}, confirming that the modelling calculations could account for some of the dependence of toxicity on chemical speciation. Data for different exposure times permitted a simple parameterisation of temporal effects, enabling values of $\alpha_{M,max}$ (values at infinite exposure time) to be obtained, and the effects of different exposure times to be factored out for further analysis. Comparison of averaged values of $\alpha_{M,max}$ for different metals showed little difference among major taxa (invertebrates, plants, and vertebrates). For Cd, Cu, Ni and Zn (the four metals with most data) there were significant differences among $\alpha_{M,max}$ values for different species, but within-species variabilities were greater. Reasonably similar species sensitivity distributions of standardised $\alpha_{M,max}$ applied to Cd, Cu, Ni and Zn. The average values, over all species, of $\alpha_{M,max}$ increased in the order Al < lanthanides < Zn ~ UO_2 < Ni ~ Cu < Pb < Cd < Ag. Considering all the $\alpha_{M,max}$ values, there was a strong dependence ($r^2 = 0.56$, $p < 0.001$) on Pearson's hardness-softness categories, and a slightly stronger relationship ($r^2 = 0.59$) if ionic radius was included in the statistical model, indicating that softer, larger cations are the most effective toxicants.

Key words: Chemical speciation; Meta-analysis; Metals; Toxicity; WHAM; WHAM-FTOX

1. Introduction

The toxicity of cationic metals towards aquatic organisms depends strongly upon solution chemical speciation (Luoma, 1983; Campbell, 1995), and this has led to the development of models to quantify the dependence of toxicity on solution chemistry. Pre-eminent among these is the Biotic Ligand Model (BLM), first described in full by DiToro et al. (2001) and Paquin et al. (2002), and recently reviewed by Ardestani et al. (2015). Over the past twenty years, the BLM has been applied to numerous toxicity data sets, usually with the aim of producing a practical means to take water chemistry into account when conducting risk analysis for individual metals (e.g. Peters et al., 2009). A related but distinct model, WHAM-F_{TOX} (Stockdale et al., 2010; Tipping & Lofts, 2013), has a more ecological purpose, the explanation of field results, including metal mixture effects. Developments to date of both models have mostly involved their applications to individual laboratory data sets, with little attempt to combine results for different biological test species and different metals to explore underlying

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relationships. Neither has chemical speciation-based toxicity modelling been used to relate the toxicity of metals to their physico-chemical characteristics, as has been done with EC_{50} values, under standardised conditions (Khangarot & Ray, 1989; Walker et al., 2007; Kinraide, 2009). Here we report an attempt to bring together chemical and biological trends in freshwater toxicity data, by using WHAM-F_{TOX} to analyse the published results of laboratory toxicity experiments with a range of metals and biological test species.

The WHAM-F_{TOX} model assumes that exposure to metals is proportional to the amount of metal bound by weak-acid coordination sites on or in the organism, in equilibrium with the surrounding medium. The fractional occupancy of sites reflects metal bioavailability from the surrounding medium, akin to the use of metal body burdens as a measure of contamination (Rainbow, 2007; Borgmann et al., 2008; Maclean et al., 1996; Adams et al., 2010; Wang, 2013). The toxic response is given by the product of the fractional occupancy and a toxicity parameter α_M which is specific to the metal and to the biological species in question. Thus the toxic effect of a metal, or the proton, arises from two factors, binding site occupancy and toxic potency. The model assumes that the products of site occupancy and α_M for each metal can be added together to give the overall toxic effect. As yet, the exact mechanism of toxicity is not specified; α_M is an empirical measure, optimised to match experimental (Tipping & Lofts, 2013, 2015) or field (Stockdale et al., 2010, 2014) data. If a single metal is present, the toxic effect is simply due to the metal and the proton (always present), but mixtures of metals are readily combined, taking competition (antagonism) into account (Tipping & Lofts, 2013, 2015).

The naming of the WHAM-F_{TOX} model arises from the assumption that metal accumulation by living organisms can be estimated with a pre-existing chemical speciation model, i.e. WHAM (Tipping et al., 2011), using cation binding by humic acid (HA) as a proxy. In other words, the weak-acid groups in different biomolecules (e.g. proteins, polysaccharides, lipids, nucleic acids, fatty acids), are assumed to be adequately represented by those of natural (non-living) organic matter. Evidence that this approximation is valid for metal accumulation by a variety of living organisms comes from Stockdale et al. (2010) and Tipping & Lofts (2013). Although it is very much an approximation, the significant advantages of the approach are that (a) competition, and hence mixture effects, are readily taken into account, and (b) much additional effort, to measure and then model interactions with living organisms exposed to different metal-bearing solutions, is avoided.

So far, the analysis of laboratory toxicity data with WHAM-F_{TOX} has focused on results with mixtures, from which 38 separate estimates of α_M have been made, covering 7 metals and 13 test species (Tipping & Lofts, 2015). While the exercise has been useful to explore the ability of the model to explain mixture effects, the results are too few to permit a wider analysis. Therefore we extended the

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model parameterisation by fitting data collated from single metal toxicity studies, a total of 2036 separate determinations of EC_{50} with accompanying solution data, extracted from published papers, from which the key WHAM-F_{TOX} parameter α_M could be calculated for each metal-test species pair. The results referred to experiments on 52 different species (25 of which had four or more data points) and 24 metals (although 11 were lanthanides with similar toxic properties). We used the derived α_M values to address a number of questions, as follows. (1) Does the model consistently account for variability in toxic response (expressed as EC_{50}), arising from variations in solution chemical speciation? (2) Can temporal variability in α_M be parameterised and quantified? (3) Does α_M for a given metal differ in any consistent or systematic way among large taxonomic units (invertebrates, plants, vertebrates) or species? (4) Are there quantitative relationships between α_M values and the chemical properties of the metals?

2. Methods

2.1. Data assembly

The ECOTOX database (https://cfpub.epa.gov/ecotox/index.html) was searched using the "Advanced Database Query" option, to identify freshwater toxicity studies with sufficient solution chemistry data for speciation calculations, i.e. there were data (including definite zero values) at least for pH, DOC, Na and/or K, Cl, and Mg and/or Ca. We referred to the source references in order to extract the solution chemistry data, together with EC_{50} as the concentration-based endpoint, exposure duration, temperature, species scientific name and taxonomic group. We discounted data for aluminium and thorium at neutral pH, because of uncertainty about hydrolysis products. In the absence of parameters for WHAM (see below), we did not consider Pt toxicity data. Data were extracted from papers published by De Schamphelaere and colleagues (see Table S1 for references). The database published by Brix et al. (2017) was used without further checking. We ensured that the toxicity test solutions were free of metal-complexing ligands such as EDTA (ethylenediaminetetraacetic acid) or NTA (nitrilotriacetic acid), but we accepted data from solutions containing non-complexing buffers such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The final number of data lines, each with an individually-measured EC_{50}, was 2037, sourced from 70 published references.

The data are summarised in Table 1 and given in full in Table S1. They are not evenly spread; 76% are for Cu, 48% for fish, 42% for crustacea. The main metals are Ag, Cd, Cu, Ni, Pb and Zn. The results refer to 52 biological species, 72% of which are covered by the top five species; *Daphnia magna* (510), *Oncorhynchus mykiss* (280), *Pimephales promelas* (450), *Pseudokirchneriella subcapitata* (101) and *Ceriodaphnia dubia* (118). The data for vertebrates, mostly fish, refer to organisms in early life stages,

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those for invertebrates are for early stage and adult organisms. The toxicity endpoint for plants was growth (or growth inhibition), and for vertebrates it was survival (or mortality). For invertebrates, survival was by far the commonest endpoint (880 of 931 determinations), but some *Daphnia magna* results (51 of 488 determinations) referred to reproduction or growth.

In the following, we use the term "data set" to mean a collection of data obtained in a single study, and referring to results for a single test organism and metal. Studies with more than one metal or test species could therefore yield more than one data set.

2.2. Modelling chemical speciation with WHAM

The chemical speciation of each test solution was calculated from the published chemical composition (Table S1) using WHAM (Tipping, 1994) incorporating humic ion-binding model VII (Tipping et al., 2011). This modelling takes into account the competitive complexation of cations, including protons, major cations and potentially toxic metals, by organic and inorganic ligands, the reactions of the carbonate system, ionic strength etc. The following text describing the model is based on a previous paper published in this journal (Tipping & Lofts, 2013); for detailed information about the assumptions of the model and the construction of its database, see Tipping (1998, 2002) and Tipping et al. (2011). Model VII uses a structured formulation of discrete, chemically-plausible, binding sites for protons in humic and fulvic acids (HA, FA), in order to allow the creation of regular arrays of bidentate and tridentate binding sites for metals. Metal aquo ions (Al^{3+}, Cu^{2+}, Zn^{2+} etc.) and their first hydrolysis products ($AlOH^{2+}$, $CuOH^{+}$, $ZnOH^{+}$ etc.) compete with each other, and with protons, for binding. The same intrinsic equilibrium constant (K_{MA}) for binding to carboxyl or type A groups is assumed to apply to the aquo ion and its first hydrolysis product. The constant (K_{MB}) for binding to weaker acid groups is related to K_{MA}, and the contributions of rarer "soft" ligand atoms are factored in. The intrinsic equilibrium constants are modified by empirical electrostatic terms that take into account the attractive or repulsive interactions between ions and the charged macromolecule. The humic ion-binding model is combined with an inorganic speciation model, the species list and constants for which were given by Tipping (1994). The inorganic reactions in this database are restricted to monomeric complexes of metals. The effects of ionic strength on the inorganic reactions are taken into account using the extended Debye-Hückel equation. Temperature effects on reactions between inorganic species are taken into account using published or estimated enthalpy data, but in the absence of experimental information, reactions involving humic substances are assumed to be independent of temperature.

When natural dissolved organic carbon (DOC) was present in the test solutions, proton and metal complexation were taken into account by assuming dissolved organic matter (DOM) to be 50% carbon,

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with 65% of sites active with respect to cation binding, represented by fulvic acid, FA (Tipping et al., 2008). For example, a DOC concentration of 5 mg L^{-1} corresponds to a concentration of FA equal to 6.5 mg L^{-1} for modelling. When isolated FA or humic acid (HA) were present, reported DOC was converted to FA or HA by multiplying by 2.0, on the assumption that FA and HA are 50% carbon. In performing the speciation calculations, we assumed a CO_2 partial pressure of 0.0040 atm. We did not take into account possible competition by dissolved Al and Fe(III) species for metal binding by organic matter (see Tipping et al., 2002; Lofts et al., 2008), because of uncertainty about possible changes in Al and Fe(III) solubility controls caused by the filtration and storage of natural waters used for toxicity testing. Comparisons of speciation outputs calculated with and without solubility control by $Al(OH)_3$ and $Fe(OH)_3$ showed only minor differences in metal free ion concentrations and organic complexation.

For six of the metals studied (Cd, Co, Cu, Ni, Pb, Zn), the possible precipitation of carbonates was checked, using the speciation outputs compared with the solubility products given by Grauer (1999). Oversaturation was calculated for Cd (7% of solutions), Co (67%), Ni (26%), Pb (12%) and Zn (19%). However, as previously argued (Tipping & Lofts, 2015), whether precipitation really occurred is quite uncertain because the solutions are dilute and the times for precipitation to occur are fairly short, so any precipitates would likely be poorly crystalline and, therefore, would have higher solubility products than the better-ordered phases used to obtain the published solubility products. Moreover, the degrees of oversaturation were modest, rarely exceeding a factor of 10. Therefore, we did not attempt to take into account the possibility that metal precipitation affected toxic responses.

2.3. The WHAM-F_{TOX} model

The key variable of the model is the toxicity function, which was originally defined by Stockdale et al. (2010) as;

$$F_{TOX} = \sum \alpha_i \nu_i \quad (1)$$

where i refers to each toxic cation (metals and the proton), ν_i is the amount of toxic cation bound to HA (mmol g^{-1}), and α_i is the toxicity coefficient. For laboratory toxicity experiments, the toxic response (TR), on a scale from zero to unity, depends upon lower and upper thresholds (LT and UT) of F_{TOX} according to the following definitions;

$$F_{TOX} \le F_{TOX,LT} \qquad TR = 0 \quad (2)$$

$$F_{TOX,LT} < F_{TOX} < F_{TOX,UT} \qquad TR = (F_{TOX} - F_{TOX,LT}) / (F_{TOX,UT} - F_{TOX,LT}) \quad (3)$$

$$F_{TOX} \ge F_{TOX,UT} \qquad TR = 1 \quad (4)$$

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We realise that some confusion arises from the use of v_i, i.e. calculated binding to HA, as a measure of the fractional occupation of binding sites possessed by the biological organism. This confusion is exemplified by the description of WHAM-F_{TOX} in the review of Liu et al. (2017), in which the authors state that v_i is the amount of metal bound by the organism. This was not our intention; we only consider v_i to be *proportional* to the fractional occupancy of organism sites. Therefore it is preferable to use, instead of v_i, the variable Θ_i which we define as the amount of metal or proton bound divided by the number of cation-binding sites per gHA (5.1 mmol g^{-1}), i.e. $\Theta_i = v_i / n_{HA}$. The dimensionless variable Θ_i is the same for all cation-binding agents, i.e. HA and all the different biological species that might be of interest. Although the actual number of binding sites, e.g. in mmol g^{-1}, will vary among these cation-binding agents (see Tipping & Lofts, 2013), the value of Θ_i will be the same for all binding agents that are in equilibrium with the same solution of protons, metals, inorganic anions, DOM, etc. Therefore our revised version of equation (1) is:

$$F_{TOX} = \sum \alpha_i \Theta_i \quad (5)$$

The upshot of replacing equation (1) with equation (5) is that the absolute values of F_{TOX} all change by the same factor, which is $1/n_{HA}$. For example using equation (1) a value of F_{TOX} might be calculated as {(1 x 2) + (10 x 0.1)} mmol g^{-1} for a condition in which the HA binding of protons and a single metal were 2 and 0.1 mmol g^{-1} respectively, with α values of 1 (proton) and 10 (metal ion). This F_{TOX} would be 3.00 mmol g^{-1}. Using equation (5), F_{TOX} would be {(1 x 0.002)/n_{HA} + (10 x 0.0001)/n_{HA}}, i.e. 0.588. Equations (2) - (4) still apply after changing to equation (5). Numerical values of α_i are unaffected, and the parameter remains dimensionless. The variable F_{TOX} is now also dimensionless.

In the WHAM-F_{TOX} model, values of Θ_i are assumed to be the same for all biological species exposed to a given solution, and variations in toxic response among species are attributed entirely to species-specific values of α_i. This picture differs from that of the BLM, which is usually parameterised for a single metal-test species pair, so that all the equilibrium constants for ion-binding by the BL, as well as the critical effect concentration (i.e. the occupancy of the BL corresponding to the observed effect) are species-specific.

2.4. Calculation of α_M from EC_{50} and solution composition

For each data line, we applied WHAM/Model VII to calculate the chemical speciation of the test solution, assuming the presence of a small concentration of HA (10^{-9} g L^{-1}), insufficient to affect the bulk speciation but yielding the cation loading of HA (v_i in mol gHA^{-1}) for toxicity modelling. Note that in these calculations the values of v_i depend, through chemical equilibria, upon the bulk solution concentrations of ions. The calculations follow conventional chemical equilibrium rules, which means

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that binding sites cannot be fully saturated (although in principle their occupancy by a particular ion can be very high). Another point to appreciate is that near-saturation of binding sites cannot occur simply by the solution metal concentration greatly exceeding the total concentration of HA binding sites; the extent of site occupation depends on binding affinity and competition by other ions, including H^+.

Values of v_i (mol gHA^{-1}) for the toxic metal and H^+ were converted to Θ_i by dividing by n_{HA}. For each value of EC_{50}, we thus obtained values of Θ_M (toxic metal) and Θ_H. In this work, values of Θ_M ranged from 0.00015 to 0.343, in experiments with Cd and Zn respectively.

We used the same constraint as in previous work (Tipping & Lofts, 2013, 2015) to fix the value of F_{TOX} at which TR= 0.5, i.e. $F_{TOX,0.5}$. Previously the modelling was done with equation (1) for which $F_{TOX,0.5}$ = 4.12 mmol g^{-1}. Here we used equation (5) and so $F_{TOX,0.5} = 4.12/n_{HA} = 0.808$. The value of α_M for the test metal is given by;

$$\alpha_M = (0.808 - \Theta_H) / \Theta_M \qquad (6)$$

For example, for $\Theta_H << 0.808$, which applies at neutral pH, then if $\Theta_M = 0.5$, $\alpha_M = 1.6$. This would apply to a weakly-toxic metal, requiring a high fractional occupancy of sites to exert the toxic effect. On the other hand, if the toxic effect could be generated at low binding, e.g. $\Theta_M = 0.001$, then a high α_M value of 808 would apply. Because we only used measured values of EC_{50}, the parameters $F_{TOX,LT}$ and $F_{TOX,UT}$ do not feature in the present work; their average values from model applications to 15 data sets covering a range of toxic responses (Tipping & Lofts, 2015) were 0.45 and 1.17 respectively, on the Θ scale introduced here.

Thus, for each value of EC_{50} we can obtain a value of α_M. The EC_{50} values depend upon solution composition, which means that in a series of tests with the same metal and organism in different solutions, a number of different EC_{50} values will be obtained. However, the WHAM-F_{TOX} model should take such chemical variation into account, and so ideally the derived α_M values should all be the same for each of the series of tests.

2.5. Variation of α with exposure time

We assumed that after exposure of the test organism to the potentially-toxic single metal solution, it takes time for the organism fully to equilibrate with the solution chemistry. This can be pictured as an extent of penetration into the organism, quantified by the expression $kt / (1 + kt)$, where t is time and k is a constant. Thus at time zero there is no toxic effect, while the full effect is achieved at $t = \infty$.

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A complication arises because before exposure to the potentially-toxic solution, the organism can be assumed to be in equilibrium with protons, which means that the $\Theta_H \alpha_H$ term in F_{TOX} (equation 5) already applies. In principle, the value of Θ_H then changes as metal equilibration takes place, due to competition between the metal cation(s) and H^+ for the binding sites. To calculate this over time would be difficult, and is not really necessary, since the change in bound H^+ will be small, and the value of α_H and hence of $\Theta_H \alpha_H$, is also relatively small. We therefore neglected this secondary effect, and assumed that only metal cation binding alters over time, according to the equation:

$$\alpha_M = \alpha_{M,max}\, k\mathrm{t} / (1 + k\mathrm{t}) \quad (7)$$

To explore temporal variability in α_M, we extracted data from studies in which the same or similar test conditions were used, but with different exposure times. They were fitted to equation (7) to derive a common value of k and a value of $\alpha_{M,max}$ (the value of α_M for long-term or chronic toxicity) for each data set. Standardised values of α_M were calculated as the ratio of α_M to $\alpha_{M,max}$.

2.6. Model evaluation

The model should account for variability in observed EC_{50}, due to variations in the chemical composition of the test medium. To test for this we identified 16 data sets (786 EC_{50} values) in which at least 20 EC_{50} values were reported for a range of solution compositions, with the same metal, test organism and exposure time. We only accepted data with non-zero values of natural DOC concentration.

For each data set we computed RMSD-null, the root-mean-squared deviation between each observed $\log_{10} EC_{50}$ and the mean $\log_{10} EC_{50}$. We also computed RMSD-α_M the root-mean-squared deviation between each observed $\log_{10} EC_{50}$ and the corresponding value of $\log_{10} EC_{50}$ obtained by back-calculating (using systematic trial-and error) the EC_{50} value from the average α_M for the data set. Log_{10} EC_{50} was used to avoid bias towards high EC_{50} values.

We also tested results against predictions obtained with the multiple linear regression (MLR) model introduced by Brix et al. (2017), which uses an equation of the form;

$$\log EC_{50} = a + b \log [\mathrm{DOC}] + c \log [\mathrm{hardness}] + d\ \mathrm{pH} \quad (8)$$

where square brackets indicate concentrations and hardness has units of mg $CaCO_3$ L^{-1}. Brix et al. (2017) reported that the MLR model provided a level of accuracy comparable to the BLM. We used it because it allowed the toxicity data to be analysed in a consistent way, i.e. with four adjustable parameters, whereas BLM applications have used different numbers of parameters, depending upon data availability and with the exercise of judgement by modellers. Brix et al. (2017) used natural logarithms in their work, we used $\log_{10}$ to be consistent with our other calculations. The choice of

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logarithm only affects the values of the regression coefficients, not the significance of fit, nor the back-calculation of EC_{50} from the MLR model. For each data set, RMSD-MLR was computed as the root-mean-squared deviation between each observed $\log_{10} EC_{50}$ and the corresponding value of $\log_{10} EC_{50}$ obtained from the parameterised MLR model.

Predictions of the WHAM-F_{TOX} and MLR models were made with Akaike information content (AIC) tests, by applying the equation

$$\Delta AIC = n \times \ln (SS\text{-}MLR/SS\text{-}\alpha_M) + 2\Delta DF \quad (9)$$

where n is the number of data, SS-MLR is the sum of squared residuals between observed and MLR-predicted $\log_{10} EC_{50}$, SS-α_M is the equivalent sum of squared residuals from WHAM-F_{TOX}, and ΔDF is the difference in the degrees of freedom, or number of model parameters (here, ΔDF = 6). A positive value of ΔAIC means that the MLR model is superior. See https://www.graphpad.com/guides/prism/7/curve-fitting/embim5.gif.

Regression analyses, t-tests and analyses-of-variance (ANOVA) were performed with Microsoft Excel.

3. Results

3.1. Accounting for variability in toxic response with WHAM-F_{TOX}

For 15 of the 16 studies suitable for model evaluation (Table S2), RMSD-α_M was lower than RMSD-null, with average values over all data sets of 0.226 and 0.404 respectively. These averages are significantly different (t-test, $p < 0.001$), indicating that WHAM-F_{TOX} accounts for some of the variation in solution composition.

We calculated how many of the predicted EC_{50} values were within a factor of two of the measured EC_{50}, this procedure being a widely-used measure of the success or failure of models of bioavailability and toxicity. The method has been used to evaluate BLMs (e.g. Paquin et al. 2002; Deschamphelaere et al. 2002, 2003; Villavicencio et al., 2011) and the models generally give more than 90% of predictions within a factor of two. With the α_M approach, on average 82% of the predicted EC_{50} values fell within a factor of two of the measured value, whereas with the null method, the result was only 52%, confirming that WHAM-F_{TOX} reduces variability.

The WHAM-F_{TOX} results were also compared with the results of applying the MLR model, equation (8), which has four parameters for fitting a sufficiently large data set. The average RMSD-MLR was 0.163, which was significantly ($p<0.001$) lower than both RMSD-null and RMSD-α_M. With the MLR model, 93% of the calculated EC_{50} values were within a factor of two of the observed values. We further evaluated

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the two models using the AIC method (equation 9), and found that for 13 of the 16 data sets, the MLR model was superior to WHAM-F_{TOX}, whereas in three cases WHAM-F_{TOX} performed better (Table S2). The WHAM-F_{TOX} and MLR model predictions of $\log_{10}EC_{50}$ are compared with measured values in scatter plots in Figure S1.

Because WHAM-F_{TOX} reduces variability in modelled EC_{50}, compared with the null model (see above), it is evidently capable of taking into account at least some of the variability in EC_{50} that is due to variations in solution chemistry. Therefore we are justified in proceeding to extract values of α_M from measured EC_{50} values for different metal-species pairs, to analyse them in terms of variations with exposure time, major taxa, and species, and to explore their dependence on metal physico-chemical characteristics.

3.2. Variation of α_M with exposure time

The data base (Table S1) yielded 14 suitable data sets that could be fitted to equation (7). Of these, 12 were for fish, taken from the studies of Besser et al. (2007), Cacela et al. (1996), Galvez & Wood (2002), Hansen et al. (2002), Welsh (1996) and Welsh et al. (1998, 2008), and two were for *Daphnia magna* (De Schamphelaere et al., 2005; Villavicencio et al., 2011). The 578 observations covered four different metals (Ag, Cd, Cu, Zn), with time-periods of up to 28 days. A highly significant ($p<0.001$) fit was obtained (Figure 1). The value of k was 0.770 d^{-1}, which means that for a one-day exposure the ratio of α_M to $\alpha_{M,max}$ is 0.44, for a four-day exposure it is 0.75. We assumed that this relationship was universal and converted all individual values of α_M to $\alpha_{M,max}$ (Table S1). The values of $\alpha_{M,max}$ were (of course) greater than those of α_M, the increases ranging from 6% (5 percentile) to 65% (95 percentile) with a median increase of 32%. The derived $\alpha_{M,max}$ values (included in Table S1) permit toxicity results for different exposure times to be analysed all together.

3.3. Systematic errors in speciation-toxicity modelling

As already noted, values of $\alpha_{M,max}$ should ideally be the same for all solutions in a toxicity experiment, but the results described in Section 3.1 show that, although WHAM-F_{TOX} reduces data dispersion, variability in derived α_M (and $\alpha_{M,max}$) remains. To examine whether this variability is systematic, we regressed $\log_{10}$ $\alpha_{M,max}$ against $\log_{10}$ [hardness], pH, and $\log_{10}$ [DOC], for 12 metal-test species pairs (Table S1). We used $\log_{10}$ values, except for pH, to avoid bias towards high values. Of the 36 bivariate relationships with sufficient data for analysis, 24 showed a significant trend (Table S3), indicating that systematic variability does indeed occur. However, the trends were split almost equally with respect to the signs of their slopes, precluding the application of simple universal adjustments to the model, that might produce general improvements.

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3.4. Variation of $\alpha_{M,max}$ among major taxa and species

Before considering average results for major taxa and individual species, the data were examined for possible dependences on toxicity endpoint; only for *Daphnia magna* were comparative results available. The average $\alpha_{Cu,max}$ obtained from the 405 experiments with survival as the endpoint was 39.4, significantly ($p < 0.001$) greater than the average of 23.3 obtained from the 37 experiments with reproduction as the endpoint. The average $\alpha_{Zn,max}$ values with survival (32 experiments) or reproduction or growth rate as endpoints (14 experiments) were 15.8 and 15.7 respectively, and did not differ significantly. The small numbers of experiments with non-survival endpoints, and the differing results between Cu and Zn, mean that drawing definite conclusions from these findings cannot be justified. Therefore we continued the analysis without distinguishing results for different endpoints.

We calculated average $\alpha_{M,max}$ values for the three major taxa (phyla or sub-phyla) for those metals with a sufficient number (taken to be six or more) of observations (Table 2). The values of $\alpha_{Ag,max}$ and $\alpha_{Cd,max}$ were consistently greater than $\alpha_{M,max}$ values for the other metals, and values of $\alpha_{Pb,max}$ were intermediate, although relatively few and subject to large standard deviations. The tendency of average $\alpha_{M,max}$ values for different metals to follow the same sequence within the different major taxa is illustrated in Figure 2.

Comparisons among taxa did not show any consistent differences. Different results were obtained for the three metals with sufficient data to compare the three taxa statistically. Thus, $\alpha_{Cu,max}$ for invertebrates was significantly ($p < 0.001$) greater than the values for plants and vertebrates, which did not differ significantly, while the values of $\alpha_{Ni,max}$ differed across the three taxa (vertebrates < invertebrates, $p < 0.01$; vertebrates < plants, $p < 0.001$; invertebrates < plants, $p < 0.05$), and the values of $\alpha_{Zn,max}$ did not differ among taxa.

Table 3 shows averaged values of $\alpha_{M,max}$ for metal-species pairs. The grand averages for each metal over all species showed a similar, but expanded, sequence to that found for major taxa (Table 2, Figure 2), increasing in the order Al < Ln < Zn~UO_2 < Ni ~ Cu << Pb < Cd < Ag. However, appreciable differences among species were evident from Table 3; considering the six metals with results for more than one species, the $\alpha_{M,max}$ values showed ranges across species of less than two-fold (Ag but only two species) to 55-fold (Cd), with intermediate ranges for Cu, Ni, Pb and Zn.

For four metals (Cd, Cu, Ni and Zn) there were sufficient data for ANOVA analysis (Table S4), which showed that species differences account for significant ($p < 0.001$) amounts of the total variance for the metal in question; 31, 20, 43 and 45% respectively for Cd (8 species), Cu (22 species), Ni (4 species)

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and Zn (7 species). Thus there were definitely differences among species, but most of the variance in each case was due to within-species variability.

To test whether a given test species is consistently sensitive, or insensitive, to different metals, we plotted values of $\alpha_{M,max}$ for one metal against those of another, by species, for cases where an average $\alpha_{M,max}$ was available for a pair of metals for four or more different species. This was possible in four cases, Cd vs Cu, Ni vs Cu, Zn vs Cu, and Cd vs Zn (Figure S2). In one case was there a significant relationship, $\alpha_{Zn,max}$ being positively correlated with $\alpha_{Cu,max}$ ($n = 7$, $r^2 = 0.70$, $p < 0.02$).

Species sensitivity distributions of $\alpha_{Cd,max}$, $\alpha_{Cu,max}$, $\alpha_{Ni,max}$ and $\alpha_{Zn,max}$, (Figure 3a) showed the distributions for Cu, Ni and Zn to be approximately log-normal, while that for Cd is distorted by the low value for *Danio rerio* (Table 3). However, if the $\alpha_{M,max}$ values were standardised, by dividing each one by the average for the metal in question, the shapes of the distributions, plotted on a linear scale, were quite similar (Figure 3b).

For six of the metal-species pairings of Table 3, the results obtained here were in fair agreement with previously-published values of α_M, obtained by Tipping & Lofts (2013, 2015) in the modelling of metal mixture effects (Table S5).

3.5. Dependence of $\alpha_{M,max}$ upon metal chemical character

Although there were variations among major taxa and species, there were no strong patterns that precluded lumping the data all together, by metal. Therefore variation of $\alpha_{M,max}$ with chemical character of the metals could be examined using all the data, while accepting substantial scatter.

Of the metals for which there were data for several species, Ag and Cd had appreciably higher $\alpha_{M,max}$ values than Cu, Ni and Zn (Tables 2 and 3), which suggests some relationship to the "softness" of the metal, in the terminology of Pearson (1963). Simply dividing all the metals into three softness categories, hard, intermediate and soft, led to the results of Table 4, showing highly significant ($p < 0.001$) differences in the mean values of log $\alpha_{M,max}$ for each hardness-softness category. The relative standard deviations in $\alpha_{M,max}$ were similar, at 2.05, 1.07, 0.78 for hard, intermediate and soft metals respectively. In terms of bound metal, the soft metals on average are 83 (780/9) times more toxically effective than the hard ones, and 22 (780/35 times more effective than those in the intermediate category.

Based on the idea that disruption of macromolecular structure is a likely metal toxicity mechanism (Tamas et al 2014), another possible factor with respect to toxicity and chemical character was ionic radius. Regression of $\log_{10}$ α_{max} for the different hardness-softness categories against ionic radius yielded evidence for this (Figure S3), with significant ($p < 0.001$) positive relationships found for hard

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and soft metals, while there was no relationship for intermediate metals. A predictive equation was derived from metal hardness-softness and ionic radius (Figure 4).

$$\log_{10} \alpha_{M,max} = p\ (\mathrm{HIS} + q\ \mathrm{IR}) \quad (10)$$

where HIS is 0, 1 or 2 for hard, intermediate or soft metals respectively, p =1.06 and q = 0.561. This relationship was somewhat better (r^2 = 0.577) than the same model but with q = 0, i.e. not including ionic radius (r^2 = 0.556).

Results for *Hyallela Azteca*, which cover many metals, show a similar trend to the whole data set, although the $\alpha_{M,max}$ values tend to be greater than average (Figure S4a). Values from previous mixture modelling (Tipping & Lofts, 2015) also show a similar trend (Figure S4b), but with $\alpha_{M,max}$ values tending to be lower than average.

4. Discussion

4.1. Empirical results

We set out to address four related questions about the performance of the WHAM-F_{TOX} model. These were essentially empirical questions, which can be asked whether or not the model is mechanistically correct or reasonable. In other words, they are to do with how well the model works. We had hoped to find data for different life stages, but nearly all the results for invertebrates and vertebrates are for early life stages, and so this could not be explored. Neither were there sufficient results for different toxicity endpoints to establish general trends across metals and test species.

4.1.1. Does the model consistently account for variability in toxic response (expressed as EC_{50}), arising from variations in solution chemical speciation?

The results for different studies involving a number of different solution compositions (Section 3.1, Table S2, Figure S1) show that WHAM-F_{TOX} takes into account some of the variation in toxic response due to differences in solution chemistry. Thus, using the average α_M obtained with WHAM-F_{TOX} to back-calculate EC_{50} values gave significantly better results than the null model using average measured EC_{50}. Investigations of $\alpha_{M,max}$ for individual species (Section 3.3) revealed systematic (although not consistent) errors in the model predictions, and these would probably account for the poorer performance of WHAM-F_{TOX} compared with the MLR model, and in all probability also the BLM.

This poorer performance is the price that must be paid for the generality of the WHAM-F_{TOX} approach, and highlights the different reasons for much BLM work compared to the goals of the present study.

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The BLMs (and the MLR approach; Brix et al. 2017) are primarily practical tools to assess water chemistry effects for single metal-single species responses, in connection with individual site assessments. Our ultimate goal with WHAM-F_{TOX} is a general description of field toxicity, which would be difficult with multi-parameter modelling for different species, but is facilitated by a single parameter (average $\alpha_{M,max}$) characterisation of toxic effect. The ability of WHAM-F_{TOX} to deal with metal mixtures is another advantage with respect to modelling field toxicity.

4.1.2. Can temporal variability in α_M be parameterised and quantified?

Parameterisation of equation (7) permits the exposure time of toxic exposure to be taken into account. For the same toxic effect to occur at different times (i.e. the same value of F_{TOX}), the values of Θ_i will thus have to vary, being larger at shorter times. The assumption that the same time dependency occurs with all metals and all test species is bold; our results are almost all for fish, there are no data for plants, and few for invertebrates. Therefore assuming a universal effect is a strong assumption, which certainly requires further testing. However, it is likely a useful first approximation. Support for our approach comes from the measurements by Feng et al. (2018) of Cd accumulation in *Danio rerio*, which showed that 50% of the maximum accumulation occurred within 24 hours, similar to our results (Figure 1).

We can attempt to relate the time dependence of toxicity derived here with acute-chronic ratios (ACRs) that are used to quantify toxicity variability in conventional parameterisations. These normally compare acute LC_{50} values with reproduction NOEC or LOEC values; ACRs in the range 10-28 have been reported for metals with aquatic species (Länge et al., 1998; Roex et al., 2000; Raimondo et al., 2007). For a two-day exposure (i.e. acute) to a single metal, compared to an "infinite" exposure, our parameterisation of equation (7) would give a ratio of Θ of 1.65. But the Θ value at infinite exposure refers to an EC_{50}, whereas NOEC or LOEC values would be smaller, by a factor of about two (Tipping & Lofts, 2015), so the ratio in Θ would be about 3.3. A more important reason for the difference between the ratio in Θ and the ACRs is that ranges of F_{TOX} are smaller than those of solution concentrations in dose-response plots; Tipping & Lofts (2013) showed that the 5%-95% range in solution concentration was about 10 times that of F_{TOX}. This would correspond to an ACR of about 30 for the present results, similar to the highest value from the literature range.

We cannot claim that equation (7) provides a complete description of temporal effects, because it may not fully take into account long-term detoxification, due to the build up of induced metal-binding proteins (metallothioneins and phytochelatins) and metal-rich granules that occur in many organisms. Poteat & Buchwalter (2014) emphasised the lengthy periods (in some cases more than one year) required for aquatic insects to reach steady state with the surrounding aqueous medium.

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4.1.3. Does α_M for a given metal differ in any consistent or systematic way among large taxonomic units (invertebrates, plants, vertebrates) or species?

The results in Table 2 and Figure 2 show no clear systematic differences among the major taxa in their sensitivity to toxic metals. Generally, the average $\alpha_{M,max}$ values for invertebrates, plants, and vertebrates are similar for a given metal. In two of the three cases (Cu, Ni, Zn) with sufficient data for statistical testing, significant differences were found, but the sequence for Cu was different from that for Ni. In the third case (Zn) there were no significant differences among major taxa.

For Cd, Cu, Ni and Zn, significant differences in average $\alpha_{M,max}$ among test species were demonstrated by ANOVA analysis (Section 3.4, Table S4). However at least half of the variances in $\alpha_{M,max}$ arise from within-species variability. This will be partly due to imprecise modelling of solution speciation and toxicity by WHAM-F_{TOX}, and to analytical errors in input data. However, it must also be recognised that toxicity measurements are prone to considerable error; toxicity data are generally noisy and often not well-replicated (Hanson et al., 2017), and large variations have been reported even for the same laboratory, solution conditions and species (Meyer et al. 2015; Traudt et al. 2017).

Taking the results in Table 3 as a whole, a distinct pattern of sensitivity by species is elusive. There appears to be no straightforward ordering of species in terms of toxic responses to different metals. The only significant trend is found for Cu and Zn, which have the same order of sensitivity for the six common species with data for both metals (Figure S2), and there is a positive but insignificant trend with Ni and Cu.

Species sensitivity distributions in terms of $\alpha_{M,max}$ (Figure 3) show variability of an order of magnitude or more among metals (Cd, Cu, Ni, Zn), as also seen in the ranges of values in Table 3. After standardising the $\alpha_{M,max}$ values to enable comparisons across all four metals, the distributions show quite similar variability (Figure 3b).

4.1.4. Are there quantitative relationships between α_M values and the chemical properties of the metals?

There is a definite ordering of $\alpha_{M,max}$ values, according to their chemical characteristics, in particular their hardness-softness properties, with an additional contribution from ionic radius (Table 4, Figures 4 and S3). The findings here are novel in two ways. Firstly the results refer to the toxic potency of the metals, after correcting for chemical speciation and accumulation/exposure; in earlier analyses, chemical properties were compared with EC_{50} or similar variables (Khangarot & Ray, 1989; Walker et al., 2007; Kinraide, 2009). Secondly we have demonstrated a general behaviour over many biological test species, whereas the previous work focused on comparing metals for a single species.

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4.2. Mechanisms of toxicity

In earlier publications (Stockdale 2010, Tipping & Lofts, 2013, 2015) binding to "non-specific" sites on or in the organism were taken to be a measure of exposure to potentially-toxic cations, and their occupancy "controlled the supply of cations to one or more key toxicity receptors, not in equilibrium or steady state with the external solution" (Tipping & Lofts, 2015). This picture followed the BLM, the standard version of which involves interactions of toxic metal cations with a single key receptor - the biotic ligand - notably on fish gills (Playle et al 1993; Playle 1998; Wood et al 1999; Paquin et al 2002; Niyogi & Wood; 2004; Ardestani et al, 2015).

However, the idea of a single receptor can be questioned, given that a variety of metal interactions with cellular components are possible (Rainbow, 2002). Firstly, excess metals can coordinate to proteins, substituting for essential metals in enzymes, altering protein structure allosterically, and interfering with protein folding (Blundell & Jenkins, 1977; Tamás et al., 2014). Secondly, excess metals can increase the generation of free radicals and reactive oxygen species, and reduce the effectiveness of anti-oxidants such as glutathione (Strohs & Bagchi, 1995; Ercal et al., 2001). Thirdly metals may bind to nucleic acids (Anastassopoulou 2003). As well as interfering with ion regulation, toxic metals have other physiological effects. In fish, these include oxidative stress (McCrae et al., 2016; Pereira et al., 2016), neurological (Sonnack et al., 2015) and behavioural impairment (Nabinger et al), interference with embryo development (Kondera, 2016), and endrocrine stress (Alsop & Wood, 2011). In plants, metals are known to cause oxidative stress (Clemens, 2006; Emamverdian et al., 2015).

If multiple responses to excess metals, interacting at multiple sites, are accepted as a general description of toxic effects, then it seems reasonable to think of the WHAM-F_{TOX} variables Θ_H and Θ_M as direct quantifications of binding to sites (in both macromolecules and small molecules) that elicit toxic responses, rather than just expressions of exposure, as we originally suggested (see above). Of course, different sites will be associated with different degrees of toxic response, but one or a few sites may not be critical. If Θ_M covers binding to toxic sites, then α_M is a measure of the subsequent effect. Differences among metals therefore show how metals differ in their disruptive capabilities. For example, the large, soft metals Ag, Cd and Hg are the most effective (Figure 4), which might be due to their greater disruptive abilities once bound to macromolecules, and also to their greater abilities to block anti-oxidants. Small, hard metals (Al, Be) can be toxic, but more binding is required, hence α_{max} values are lower.

Considering all accumulated metal to induce toxic effects, rather than postulating an individual target site, is in line with the ideas of earlier workers (MacLean et al., 1996; Rainbow, 2002; Borgmann et al., 2008; Adams et al., 2010; Pentinnen et al.; 2011; Wang, 2013), bearing in mind the need to consider

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only metabolically available metal, i.e. stored detoxified metal is not included (Rainbow, 2002; Vijver et al, 2004). We suggest that the values of Θ_M calculated with WHAM-F_{TOX} are equivalent to the metabolically-available metal, expressed in terms of binding site occupancy. Previously we called the organism metal content calculated with WHAM-F_{TOX} the "metabolically active body burden" (Tipping & Lofts, 2015).

The modelling approach incorporating the metabolically active body burden assumes that all relevant cation binding sites possessed by the organism are in equilibrium with the external solution. Evidence to support this comes from a number of case studies in which measured metal body burdens were correlated with the metal loading of HA calculated with the WHAM speciation model (Tipping et al., 2008; Stockdale et al., 2010; Tipping & Lofts, 2013). However, several factors need to be acknowledged. First, HA is not an accurate representation of the molecules comprising living organisms, and even if it is a useful approximation, this will likely vary among species. Second, measured body burdens are likely to include any metal present in stored, detoxified forms, such as metallothionein complexes, phytochelatins, or insoluble metalliferous granules. Third, cytoplasmic solutions have different chemical compositions from the external solution, which implies that the distributions of cations between solution and intracellular constituents are not correctly modelled with WHAM. Therefore the use of WHAM to estimate metabolically active body burdens must be regarded as a fairly crude approximation, and this will have contributed to the considerable scatter in plots comparing measured and modelled body burdens (Tipping et al., 2008; Stockdale et al., 2010; Tipping & Lofts, 2013). It also means that the derived values of α_M presented here are imperfect measures of toxic effect, since they also reflect differences in binding properties among taxa or species.

In the metabolically active body burden picture, the effect of time can be looked on as a penetration-loading effect. It can be envisaged that at short times the external parts of the organism are fully loaded, while internal ones are not, then over time all the sites become loaded (as much as is compatible with the solution chemistry) and maximum toxicity is achieved for that solution condition.

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5. Conclusions

This exercise has shown that metal toxicity data obtained from laboratory measurements with freshwater organisms can be partly rationalised within the concepts of the WHAM-F_{TOX} model. In terms of missing information, the results demonstrate the need for more data for metals other than Cu, and comparative toxicity experiments with several species exposed to the same metal-bearing solutions would help to improve understanding of species sensitivity. The model seems more consistent with multiple sites of toxic action than single key receptors. The most definite conclusions are as follows:

(a) The WHAM-F_{TOX} model partially accounts for variations in measured toxic effect (EC_{50}), by taking account of differences in solution chemical speciation and bioavailability.

(b) Temporal variation in responses to toxic metal exposure can be approximately quantified, permitting the estimation of the parameter $\alpha_{M,max}$, the toxic potency of accumulated metal at infinite time.

(c) Values of $\alpha_{M,max}$ for different metals show no clear or consistent differences among invertebrates, plants and vertebrates.

(d) For Cd, Cu, Ni and Zn, there are significant differences among $\alpha_{M,max}$ values for different species, but there is greater within-species variability.

(e) There is a strong relationship between $\alpha_{M,max}$ and metal chemical characteristics, i.e. Pearson's hardness-softness categories and ionic radius. The most potent metals, in terms of metabolically active body burden, are Ag, Cd and Hg; intermediately-potent metals include Cu, Ni and Zn; the least potent metals include Al, Be and UO_2.

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Table 1. Summary of data; the numbers indicate the number of EC_{50} values.

Metal	Inverte-brates	Plants	Verte-brates	Total
Ag	6		37	43
Al			7	7
Be	2			2
Cd	24		128	152
Ce	2			2
Co	6		3	9
Cu	743	49	751	1543
Dy	16			16
Er	2			2
Eu	2			2
Gd	2			2
Hg	4		1	5
Lu	2			2
Nd	2			2
Ni	27	39	13	79
Pb	11	18	4	33
Pr	2			2
Sc	2			2
Sm	2			2
Tb	2			2
UO_2	6			6
Y	2			2
Yb	2			2
Zn	62	9	47	118
Total	932	115	990	2037

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Table 2. Mean values of $\alpha_{M,max}$ for different metals and major taxa, for $n \geq 6$. The Ln row shows results for all lanthanides.

	Invertebrates			Plants			Vertebrates		
	n	mean	sd	*n*	mean	sd	*n*	mean	sd
Ag	*6*	2411	2015				*36*	973	354
Cd	*24*	376	490				*128*	729	396
Co	*6*	162	198						
Cu	*743*	42	28	*49*	26	17	*751*	28	27
Ni	*27*	26	32	*39*	43	28	*13*	6	1
Pb	*11*	182	166	*18*	116	125			
	6	44	48						
Zn	*62*	17	15	*9*	15	22	*47*	18	10
Ln	*36*	6	4						

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Table 3. Mean (bold) values of $\alpha_{M,max}$ by species, and standard deviations (italic) for $n \geq 4$. The Ln row collects results for all lanthanides.

Species	Ag		Al		Cd		Cu		Ni		Pb		UO_2		Zn		Ln	
Acipenser transmontanus							**45**	*30*										
Bufo americanus			**3**	*0*														
Ceratophyllum demersum							**14**	*6*										
Ceriodaphnia dubia					**274**	*75*	**53**	*26*	**77**	*7*					**61**	*28*		
Cottus bairdi					**630**	*295*	**39**	*24*							**26**	*9*		
Danio rerio					**17**	*4*	**17**	*5*										
Daphnia magna					**158**	*108*	**38**	*28*					**20**	*8*	**16**	*6*		
Daphnia obtusa							**36**	*11*										
Daphnia pulex							**50**	*14*										
Daphnia pulicaria							**108**	*47*	**14**	*3*								
Dugesia tigrina															**3**	*0*		
Etheostoma flabellare							**8**	*0*										
Etheostoma rubrum							**7**	*0*										
Hyalella azteca					**952**	*730*	**23**	*10*			**314**	*88*					**6**	*4*
Lampsilis siliquoidea							**34**	*11*										
Lumbriculus variegatus							**8**	*1*							**3**	*0*		
Lymnaea stagnalis							**37**	*10*										
Oncorhynchus clarki							**17**	*5*										
Oncorhynchus mykiss	**1206**	*283*			**869**	*282*	**26**	*16*							**18**	*10*		
Oncorhynchus tshawytscha							**18**	*4*										
Pimephales promelas	**896**	*351*			**785**	*392*	**33**	*35*	**6**	*1*								
Pseudokirchneriella subcapitata							**30**	*18*	**43**	*28*	**128**	*127*			**15**	*22*		
Pyrgulopsis idahoensis							**34**	*3*										
Salvelinus confluentus					**590**	*158*	**15**	*2*							**15**	*6*		
Villosa iris							**51**	*9*										
Mean	1051		3		534		32		35		221		20		19		6	
Min	896		3		17		7		6		128		20		3		6	
Max	1206		3		952		108		77		314		20		61		6	

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Table 4. Mean $\alpha_{M,max}$ values, classified according to the Pearson (1963) hardness-softness scheme. Hard metals comprise Al, Be, Sc, Y, Ln and UO_2), intermediate Co, Cu, Ni, Pb and Zn, soft Ag, Cd and Hg. Key: *n* = no. of values; sd = standard deviation; rsd = relative standard deviation. The mean values are significantly different (t-test) at $p < 0.001$.

metal type	*n*	mean	sd	rsd
hard	55	7	15	2.08
intermediate	1782	26	27	1.07
soft	200	550	454	0.82

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Figure captions

Figure 1. Time dependence of standardised α_M. The trend is highly significant, judged by plotting observed against calculated y-axis values (n = 578, $p < 0.001$; intercept not significant). One observation (21 days, standardised $\alpha_M = 3.88$) is not shown, although it was included in the analysis.

Figure 2. Mean $\alpha_{M,nax}$ for plants (filled circles) and invertebrates (open circles) vs mean $\alpha_{M,max}$ for invertebrates, plotted by metal. The data are from Table 2, which includes standard deviations. The 1:1 line is shown.

Figure 3. Species sensitivity distributions for Cd (closed circles), Cu (open circles), Ni (closed squares) and Zn (open squares). The x-axis values in panel (b) were obtained by dividing the $\alpha_{M,max}$ for each species by the mean value for each metal.

Figure 4. Observed $\log_{10} \alpha_{M,max}$ vs predicted values obtained with equation (10). The 1:1 line is shown.

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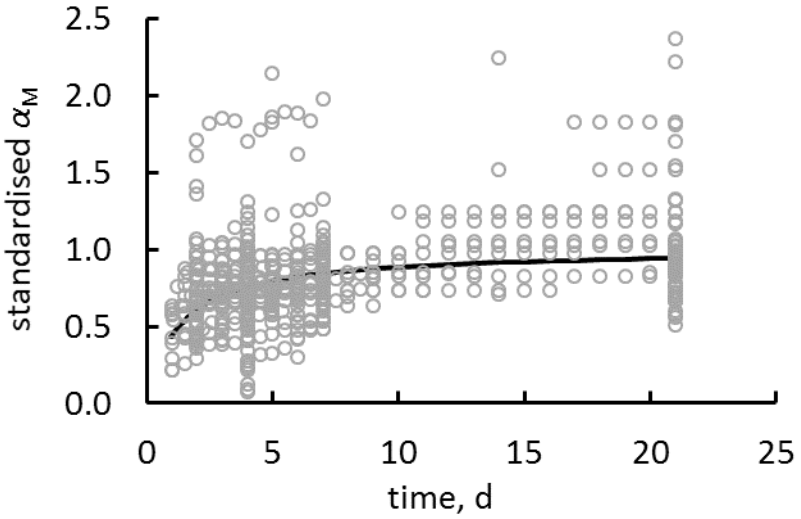

Figure 1.

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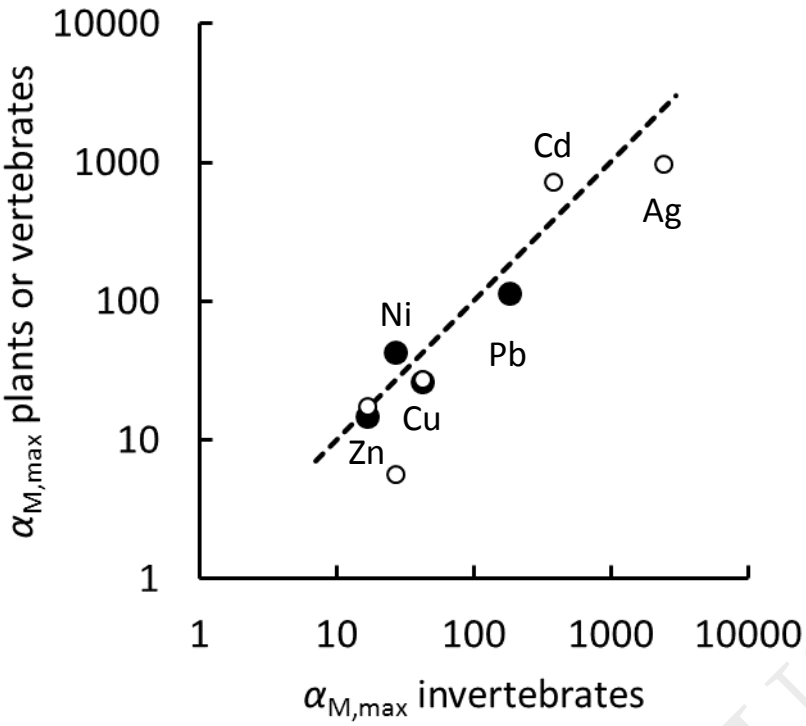

Figure 2.

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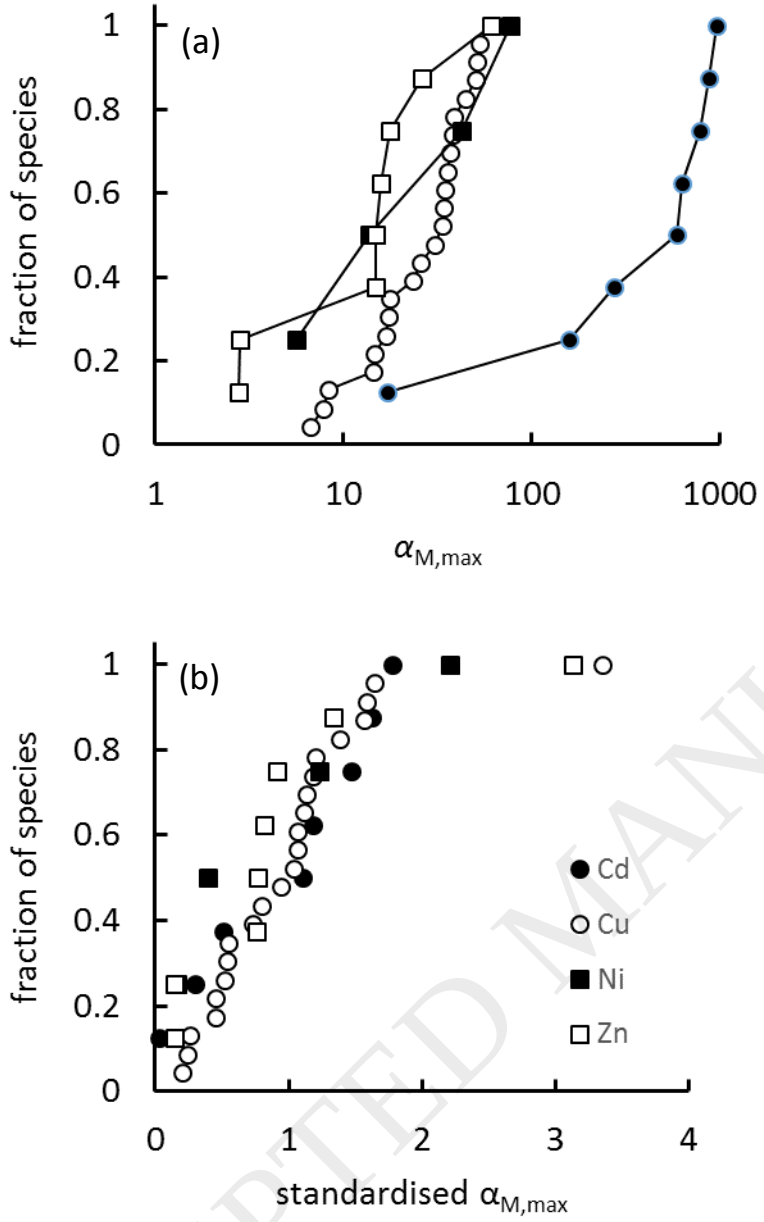

Figure 3.

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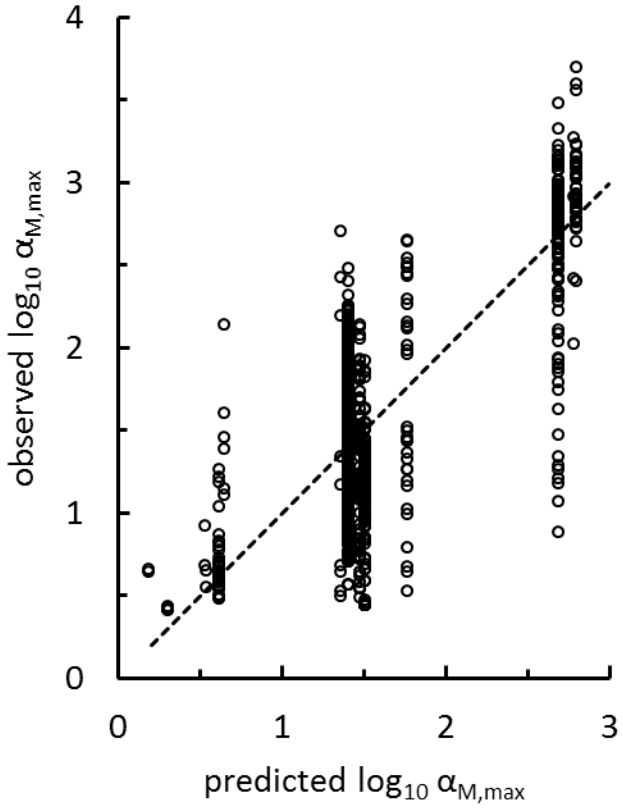

Figure 4.

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