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Metal mixture toxicity to aquatic biota in laboratory experiments; application of the WHAM-F_{TOX} model

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ABSTRACT

The WHAM-F_{TOX} model describes the combined toxic effects of protons and metal cations towards aquatic organisms through the toxicity function (F_{TOX}), a linear combination of the products of organism-bound cation and a toxic potency coefficient (α_i) for each cation. Organism-bound, metabolically-active, cation is quantified by the proxy variable, amount bound by humic acid (HA), as predicted by the WHAM chemical speciation model. We compared published measured accumulations of metals by living organisms (bacteria, algae, invertebrates) in different solutions, with WHAM predictions of metal binding to humic acid in the same solutions. After adjustment for differences in binding site density, the predictions were in reasonable line with observations (for logarithmic variables, r^2 = 0.89, root mean squared deviation = 0.44), supporting the use of HA binding as a proxy. Calculated loadings of H^+, Al, Cu, Zn, Cd, Pb and UO_2 were used to fit observed toxic effects in 11 published mixture toxicity experiments involving bacteria, macrophytes, invertebrates and fish. Overall, WHAM-F_{TOX} gave slightly better fits than a conventional additive model based on solution concentrations. From the derived values of α_i, the toxicity of bound cations can tentatively be ranked in the order: H < Al < (Zn ~ Cu ~ Pb ~ UO2) < Cd. The WHAM-F_{TOX} analysis indicates much narrower ranges of differences amongst individual organisms in metal toxicity tests than was previously thought. The model potentially provides a means to encapsulate knowledge contained within laboratory data, thereby permitting its application to field situations.

Key words: aquatic organisms, chemical speciation, metals, toxicity, WHAM, WHAM-F_{TOX}

INTRODUCTION

The Biotic Ligand Model (BLM, Paquin et al., 2002) was developed to explain how water chemistry (pH, DOC, hardness etc) affects toxicity, initially for single metals. The essential idea is to replace metal concentration in solution as the expression of toxic exposure, by the occupancy of a key (biotic) ligand, the reactions of which are described with conventional coordination chemistry. Account can thus be taken of the ever-present competition reactions, in which toxic and non-toxic cations, including H^+, compete for binding to ligands, not only the biotic ligand but also those present in solution, in particular dissolved organic matter. The aim of the BLM was to make risk assessment more scientific, compared to the use of a single standard concentration, or perhaps hardness-dependent values. This aim has largely been achieved, changing how we think about metal toxicity in aquatic and terrestrial ecosystems. Furthermore, there have been several efforts to use the BLM to account for the toxic effects of metal mixtures (Playle, 2004; Hatano & Shoji, 2008; Kamo & Nagai, 2008; Jho et al., 2011), each based on the assumption that the different toxic metals share the same biotic ligand. Hatano & Shoji (2008) fitted the model to data for the toxicity of Cu and Cd to *Lemna paucicostata*, at different pH values, and obtained far better agreement with observations than could be achieved with a conventional model based on LC_{50} toxic units, and ignoring pH variations.

The WHAM-F_{TOX} model (Stockdale et al., 2010) provides a different way of describing metal toxicity, while retaining the idea that exposure depends on the interactions of metals and protons with the organism. Instead of postulating a specific biotic ligand through which metal toxicity is mediated, WHAM-F_{TOX} expresses exposure of the organism to toxic metals by the overall, non-specific, accumulation of cations at the reversible binding sites present within the organism or on its surface. Such sites exist due to the presence of weak-acid groups in different biomolecules (e.g. proteins, polysaccharides, lipids, nucleic acids, fatty acids), and their occupancy depends upon the competitive interactions of toxic and non-toxic metals and protons, assuming them to be in equilibrium with the surrounding solution. The binding ligands could, in principle, include one or more specific biotic ligands but the majority of them will not be associated directly with the toxic response. The model then assigns a unique, purely empirical, toxicity coefficient to each cation, which describes the extent to which the bound cation is toxic. Total toxicity is then determined by the sum of the products of amounts bound and the toxicity coefficients. The chemical interactions and toxic effects are thus formally separated, unlike in the BLM where the equilibrium constants for binding at the

biotic ligand reflect not only the chemical strength of binding, but also toxic strength (Playle, 2004).

An advantage of the WHAM-F_{TOX} approach is that the need to fit the model to organism-bound metal data is avoided, by assuming that metal accumulation by living organisms can be estimated with a pre-existing chemical speciation model, i.e. WHAM, using cation binding by humic acid (HA) as a proxy. Evidence to justify this assumption comes from field data (Tipping et al., 2008; Stockdale et al., 2010), although as yet it must be regarded as incomplete. But the idea is worth pursuing in order to avoid an inordinate amount of new experimental work and associated modelling to quantify cation accumulation by living organisms (cf. Borgmann et al., 2008). If cation accumulation can reliably be estimated *a priori*, then relatively few parameters are needed to fit toxicity data. The use of cation binding to HA, calculated with WHAM, to express metal exposure produced a good description of the toxicity of copper towards duckweed in laboratory experiments (Antunes et al., 2012).

In previous work with the WHAM-F_{TOX}, we focused only on field data, firstly using stream macroinvertebrate species richness, at *c.* 400 sites affected by abandoned metal mines and acid deposition, as the toxic response variable for fitting (Stockdale et al., 2010). The same version of the model was used to evaluate acidification recovery (Stockdale et al., 2013a), and a version has been produced to describe lake zooplankton diversity (Stockdale et al., 2013b). Analysis of stream mesocosm data (Iwasaki et al., 2013) further supports the use of calculated binding to HA as a measure of exposure. However, under circumstances pertaining in the field and in mesocosms, non-chemical factors (e.g. discharge variation, suspended sediment, competition, predation, food web structure) also affect the measured variables, hampering the precise and unequivocal attribution of toxicity. Therefore the principal aim of the present work was to test the ability of the model to fit toxicity data obtained in controlled laboratory experiments. Before addressing toxicity however, we first tested the other aspect of the model, i.e. its appropriateness as a proxy for cation accumulation by living organisms. The longer-term goal of this work is to produce a model that can be parameterised with the abundant laboratory data that describe metal toxicity, in order to make use of the fundamental knowledge to understand and predict toxic effects of metals in the field.

2. Methods

2.1. Modelling chemical speciation with WHAM

In this work we used WHAM (Tipping, 1994) incorporating Humic Ion-Binding Model VII (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+}, Cd^{2+} etc.) and their first hydrolysis products ($AlOH^{2+}$, $CuOH^{+}$, $CdOH^{+}$ 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.

If dissolved organic carbon (DOC) was present in the solutions considered here, we took complexation into account by assuming dissolved organic matter (DOM) to be 50% carbon, with 65% of sites active with respect to cation binding, represented by FA (Tipping et al., 2008). For example, a DOC concentration of 5 mg L^{-1} corresponds to a FA concentration of 6.5 mg L^{-1} for modelling. For waters from the field, we estimated Fe(III) concentrations with the empirical equation of Lofts et al. (2008), suitably modified for Humic Binding Model VII.

We calculated the equilibrium binding of the metals to HA by assuming it to be present at a very low concentration, insufficient to affect the bulk speciation, and finding ν_i values (mol/gHA). To match the values of ν_i to observed accumulations of metal by living organisms, we defined the equivalent HA per gram of organism dry weight, E_{HA} (g g^{-1}). The value of E_{HA} would be 1.0 if the organism's site content per gram were equal to that of HA,

but is expected usually to be less than 1.0 because living organisms generally have fewer exposed sites than does HA.

2.2. Fitting toxicity data with WHAM-F_{TOX}

For the toxicity model, it is assumed that each organism possesses binding sites that have the same properties as those of HA, and it is the fractional occupancy of these sites that measures exposure to cations, not the absolute amount of metal per unit weight of organism. Different species exposed to the same solution have the same ν_i values but differ in absolute body burdens (mol g dry weight^{-1}) if their values of E_{HA} differ. Thus, because only relative binding is needed, the model simply uses the calculated ν_i values for HA as the measure of exposure. This means that toxicity parameters for different organisms are directly comparable.

The toxicity function is defined by the equation;

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

in which α_i is the toxicity coefficient of cation *i*. The toxic response (TR), on a scale from zero to unity, depends upon lower and upper thresholds of F_{Tox} according to the following definitions;

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

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

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

For each data set, the object of the fitting was to minimise the sum of the squared differences between observed and calculated toxic response (luminescence, survival, growth rate or filtration rate). To fit the model, the values of α_i, $F_{TOX,LT}$ and $F_{TOX,UT}$ could in principle be optimised by fitting the model to the available toxicity data. Since the toxicity coefficients are only relative numbers, the value of α_H can be set to the same value in all cases, and unity is chosen for convenience.

2.3. Conventional toxicity model

For comparison with the outputs of WHAM-F_{TOX} modelling, a conventional toxic unit approach was applied to the datasets, assuming additivity of toxic responses. This entailed fitting the dataset to a standard logistic dose–response curve:

$$TR = TR_0 / (1 + TU^{\beta}) \quad (5)$$

where TR is the toxic reponse, TR_0 is the control response and β is a slope parameter. The term TU quantifies the 'toxic units' for a given exposure:

$$TU = \sum[X_i] / EC_{50}(i) \quad (6)$$

where $[X_i]$ is the dissolved concentration of toxicant *i* in the exposure and $EC_{50}(i)$ is the dissolved concentration of metal *i* causing a 50% toxic effect. The model, referred to as CTU, was fitted to each entire dataset by optimisation of the parameters β and $EC_{50}(i)$.

3. Results

3.1. Accumulation of metals by living organisms

The data summarised in Table 1 were used to compare the accumulation of metals by living organisms with binding by HA. Results of Yee & Fein (2001) and Borrock & Fein (2005) show how metal binding to bacteria varied with pH, via "adsorption edges", and also as a function of ionic strength. The data were predicted reasonably well with WHAM (Figure 1), after calibration by adjustment of E_{HA}. Calculated binding closely follows the pH dependence of observed binding, approximates the dependence on ionic strength, and accounts for the relative binding of Cd and Pb. There are quite similar values of E_{HA} of 0.66 and 0.84 for *B. subtilis* and *E. coli* respectively, based on bacterial dry weight. A much lower E_{HA} of 0.060 for *P. putida* arises because Borrock & Fein (2005) chose to express their results in terms of wet weight, noting that this is about five times the dry weight, which implies an E_{HA} value of 0.30 on a dry weight basis.

The results of Hassler & Wilkinson (2003) for Zn accumulation at the external surface of the alga *Chlorella kesslerii* (Figure 2) cover a wide range of $[Zn^{2+}]$. As noted by these authors, the slope of the log-log plot is well below unity, implying a high degree of binding site heterogeneity. This is captured by WHAM (Figure 2), although the model is unable to predict the complex nature of the binding curve. We could not derive a precise value of E_{HA} in this case because the experimental results were expressed in terms of moles Zn sorbed per unit area, but by assuming a dry weight bulk density of 0.1 g cm^{-3} for the algae, which are spherical cells of radius 1.8 μm, we obtain $E_{HA} = 0.032$.

Borgmann and co-workers (Borgmann et al., 1993, 2004; Norwood, 2007) performed laboratory studies on the binding of metals to *H. azteca*, and as shown in Figure 3(a), the model gives fairly good correlations with their results after calibration ($E_{HA} = 0.044$). Field measurements on the same organism, exposed in cages to mine-contaminated streamwaters, were best accounted for with $E_{HA} = 0.11$ (Figure 3(b)), which is 2.5 times the value derived from the laboratory data. Here, there are some systematic deviations for individual metals, although the overall prediction is fair. Binding to the zebra mussel (Kraak et al., 1994; Figure 4) yielded $E_{HA} = 0.017$. Binding to the invertebrates (Figures 3 and 4) covers the behaviour of many metals, although the results refer only to neutral pH values, and shows that the order of binding is essentially as expected, taking into account the different solution concentrations of the metals. There do not seem to be any consistently anomalous metals among those that

214 are known to be toxic, although for *H. azteca*, the binding of Cd that is predicted tends to be
215 low relative to the other metals, this being seen in both laboratory (Figure 3a) and field
216 (Figure 3b).

217 Linear regression of the logarithms of all 467 observed and calculated data pairs, including
218 calibration with E_{HA}, yields an intercept insignificantly ($p > 0.05$) different from zero, and
219 with the intercept fixed at zero the slope is 0.99, with $r^2 = 0.89$, and a root-mean-squared-
220 deviation of 0.44, equivalent to a factor of 2.75. Taking the present results and those for
221 stream bryophytes and macroinvertebrates, the values of E_{HA} tend to fall as organism size
222 increases or surface area falls, with bacteria and bryophytes having values ~ 0.5, stream
223 insects and Hyalella ~ 0.1, and the larger mussel ~0.02. The low E_{HA} for the alga may be
224 explained at least partly by accumulation only at external sites (Hassler & Wilkinson, 2003).

225 *3.2. Fitting toxicity data*

226 If both $F_{TOX,LT}$ and $F_{TOX,UT}$, together with the α_i values for metallic cations, were allowed to
227 vary for each data set, the average root-mean-squared deviation (RMSD) in toxic effect over
228 the 11 data sets was 11.5%. However it was found in most cases that many different sets,
229 some physically unrealistic, could fit the data nearly as well. Therefore we sought a means to
230 reduce the number of adjustable parameters. All 11 data sets were fitted by assuming that the
231 same values of $F_{TOX,LT}$ and $F_{TOX,UT}$ applied to each, while permitting the values of α_i to be
232 adjusted for each data set. This resulted in $F_{TOX,LT}$ and $F_{TOX,UT}$ values of 2.22 and 6.01
233 respectively, and an average root-mean-squared deviation (RMSD) in toxic effect over the 11
234 data sets of 12.9%. Then to fit individual data sets, the mid-point value of $F_{TOX,LT}$ and
235 $F_{TOX,UT}$, equivalent to 50% toxic effect, was fixed at the average value of 4.12, but the
236 difference between $F_{TOX,LT}$ and $F_{TOX,UT}$ was optimised, along with α_i. The average RMSD
237 was then reduced to 12.0%, not much greater than the value obtained when $F_{TOX,LT}$ and
238 $F_{TOX,UT}$ were allowed free variation. Table 3 summarises the fitting results obtained with the
239 fixed mid-point value.

240 Example results are displayed in Figures 5 to 8. Ideally, the model should, within
241 experimental error, condense all data points onto a single line in each case, defined by the
242 upper and lower thresholds ($F_{TOX,LT}$ and $F_{TOX,UT}$). There should also be minimal bias
243 between predictions and observations, and this clearly applies to the plots in Figures 7 and 8.
244 The results of Jho et al. (2011) in Figure 5 show that Pb toxicity is predicted to be less than
245 observed at high Ca, and for mixture the model predicts somewhat higher toxicity than is

observed. Also the Charles et al. (2006) data for duckweed in Figure 6(a) show that the model expects somewhat more toxicity from the mixture than is observed.

Only for three metals, Cu, Zn and Cd, are several values of α_i obtained (Table 3), permitting differences to be tested, and it is found that the mean α_{Cu} and α_{Zn} are not significantly different ($p > 0.05$) whereas both differ significantly from the mean α_{Cd} ($p < 0.05$).

The average RMSD obtained with the CTU model (Table 4) is slightly greater than that with WHAM-F_{TOX}, 13.4% vs. 13.0%, not including the Hickie et al. (1993) data, and the average r^2 slightly lower (0.81 vs 0.85) indicating that WHAM-F_{TOX} is slightly superior for these data sets. The models can also be compared in terms of the ranges of the x-axis (Figure 9), plotted equivalently in terms of the logarithm of the variable that combines exposure and toxicity for each model. Fitting a logistic dose-response curve to the combined data yields a factor of 2.2 for the range of F_{TOX} covering 5 to 95% of the toxic effect, whereas the factor is ten times greater (21.6) for the range of CTU toxic units.

4. Discussion

4.1. Accumulation of cations by living organisms

The results in Figures 1 to 4 show that the observed binding or accumulation of metals by living organisms approximately follows the expectations of competitive binding by an array of binding sites based on weak acids, broadly similar to those in humic acid. This reinforces previous results for the accumulation behaviours in the field of stream bryophytes (Tipping et al., 2008) and macroinvertebrates (Stockdale et al., 2010).

Although the WHAM predictions produce strong parallels with observed accumulation, it would be incorrect to conclude that the living organism body burdens are in fact controlled simply by quasi-equilibrium chemical reactions. Interactions at the external surfaces of organisms exposed to the bathing solution might reasonably be expected to follow chemistry, as long as the organism does not affect its immediate environment (for example at fish gills the exchange of potentially-buffering molecules notably CO_2 and NH_3 will regulate pH; Evans et al., 2005). But metals accumulated within an organism must be in contact with solutions of different composition to the external one. A more mechanistically-correct model would include biodynamic concepts; thus Luoma & Rainbow (2005) and Rainbow (2007) highlighted the importance of the dynamic uptake and removal of metals, as well as storage in metal-rich granules and metallothioneins, and internal regulation of the levels of essential metals such as Cu and Zn. It seems undeniable, however, that water chemistry exerts a strong control on the steady-state levels of metals accumulated by living organisms, and that this phenomenon must have implications for toxicity.

Furthermore, because the modelled cation accumulation involves only conventional reversible reactions, it is arguably a better index of toxic exposure than measured body burdens, which as noted above reflect more complicated processes. In this respect the modelled values might reasonably be considered proxies for steady-state "metabolically available" cations (cf. Rainbow, 2007). It is worth repeating here that WHAM-F_{TOX} does not use the absolute concentrations of reversibly-accumulated metals, but rather the fractional occupancy of the binding sites, so that although different values of E_{HA} are required to explain observed differences in metal accumulation among species, E_{HA} does not appear in the quantification of toxicity.

4.2. Toxicity parameters

The two types of parameter, thresholds and toxicity coefficients, used in WHAM-F_{TOX} are not uniquely defined by the data sets analysed here, because (a) the experimental conditions cover relatively small ranges, and (b) the experimental results tend to be quite noisy. Thus we were obliged to constrain the threshold parameter values in order to force unique data fits and parameter sets. This means that the physical meanings of the parameters cannot be reliably judged. It should also be recognised that the derived toxicity parameters may well be compensating for errors in the chemical speciation modelling, i.e. incorrect prediction of the actual accumulation of metals by the different organisms.

Because the thresholds were constrained, their values cannot be interpreted at this time. However, we can say that the values of α_i summarised in Table 3 show the toxicities of bound cations to fall approximately into three groups, namely (i) H^+ and Al, (ii) Cu, Zn, Pb and UO2, and (iii) Cd on its own. There is a hint that bound Cd toxic potency as quantified by α_{Cd} increases with taxonomic rank (i.e. from bacteria to plants to invertebrates). Many more data would be required to discern systematic inter-specific variations in the toxicity parameters.

4.3. Variations among individual organisms in toxicity tests

The dose in aquatic toxicity tests is conventionally expressed as a concentration, e.g. mg L^{-1} in aquatic toxicology and mg kg^{-1} in soil and sediment toxicology, which can be converted to toxic units by dividing by the LC_{50} (cf. Figure 9). Usually the x-axis is logarithmic, which suits the logistic model, and permits wide ranges of concentration to be displayed. The width of the range is usually at least one order of magnitude; for the data sets analysed with the CTU model in the present work, the logistic fit of all the data combined has a range from 5% to 95% of 21.6 fold (Section 3.2, Figure 9). Reasons for the x-axis ranges are contested (Newman & Ungar, 2002). It may be that individuals differ intrinsically, or more stochastic processes may operate such that there is always a range of physiological states at the start of the experiment, even amongst cloned cultures (Kooijman, 1996; Newman & Ungar, 2003). The 21.6-fold range obtained with the CTU model suggests a very wide range of differences amongst test individuals. However, the picture changes if the variation in F_{TOX} is considered, for which the present data cover a range of only 2.2-fold (Figure 9), implying much less variation amongst individuals. This reflects the different means of quantifying exposure.

The smaller range obtained with F_{TOX} arises largely from the way that cation binding varies with solution concentration. If we first consider a single (homogeneous) binding site, the Law of Mass Action demands that binding changes relatively less than the free metal concentration at high occupancies, i.e. as the Langmuir-type plot curves towards the upper limiting value. This would be the case with the BLM approach, so the x-axis range should be narrowest when the biotic ligand occupancy corresponding to 50% toxic effect is high. Thus, x-axis ranges of about 5-fold for the 5 to 95% toxicity range can be derived from data presented by De Schamphelaere & Janssen (2002), Heijerick et al. (2002) and Jho et al. (2011), for which the 50% effect biotic ligand occupancies were between 0.3 and 0.6. However, a much larger range of c. 100 fold is evident in the results of Hatano & Shoji (2008) with 50% effect biotic ligand occupancies of around 0.01. WHAM-F_{TOX} differs from the BLM in having an array of heterogeneous binding sites for cations, the effect of which is illustrated by the results in Figure 2, where the WHAM prediction shows that, at higher loadings of Zn, a change in dissolved metal of nearly four orders of magnitude is required to change the amount adsorbed by a factor of ten. Thus when exposure is quantified in terms of bound cations, i.e. through F_{TOX}, the toxic response occurs over a much narrower range than when solution concentrations are used (Figure 9).

4.4. Applications of WHAM-F_{TOX}

The model is purely empirical in its description of how metals and protons exert their subcellular effects, which are simply represented by the parameter α_i. However, it is mechanistically more sophisticated than most other models in using detailed chemical speciation to quantify exposure, importantly including competition effects, which appear able to account for mixture effects. And by using bound metal as the measure of exposure, the results suggest that individual test organisms are less variable in their responses than is apparent from solution exposure. Therefore, the WHAM-F_{TOX} approach provides new mechanistic insights, and should help to interpret the results of studies designed to elucidate subcellular toxicity mechanisms, especially those involving metal mixtures or pH dependence. The model could also contribute to the planning of experiments, for example by forecasting experimental conditions under which competition effects are especially noticeable.

As demonstrated here, WHAM-F_{TOX} can readily fit individual toxicity data sets within a coherent and consistent framework. It therefore has the potential to organise and encapsulate

353 laboratory knowledge, through a meta-analysis of the vast store of available laboratory
354 toxicity data, for both single metal and mixture toxicity. This activity might reveal
355 relationships and patterns among metals and test species, thereby providing new insights into
356 metal toxicity. Furthermore, the fully-parameterised version of WHAM-F_{TOX} would be
357 valuable in the interpretation and prediction of the toxic effects of metals and protons in the
358 field, either for more realistic risk assessment or understanding ecosystem community
359 response. In this way the model can contribute to the ultimate goal of aquatic toxicological
360 research.

361

362

5. Conclusions

This study has demonstrated that WHAM-F_{TOX} can be used to fit cation mixture toxicity data for aquatic organisms, making the model an alternative to conventional toxicity parameterisation and the Biotic Ligand Model. For the data sets considered in the present work, WHAM-F_{TOX} provides somewhat better fits than the CTU model overall, but the improvements are not great. This probably arises firstly because the analysed data refer to relatively small ranges of both pH and metal concentrations, and secondly because the measured toxicity responses are quite noisy, so they do not provide sufficiently stringent tests for proper model comparison. For example, data fitting is reasonably satisfactory if fully additive effects are assumed (CTU), or if there is potential antagonism due to competitive chemical binding reactions (WHAM-F_{TOX}). Further testing on more demanding data sets is clearly desirable. There is also a case for applying the models to large numbers of toxicity data sets, to attempt to rationalise toxicity knowledge within a coherent framework, and seek trends and patterns within the parameter values.

The following specific conclusions can be drawn.

a) WHAM-predicted binding of metals by humic acid provides a good guide to their accumulation in living organisms, and therefore a measure of exposure to toxic cations.

b) Laboratory mixture toxicity data can be adequately fitted via the variable F_{TOX}, which gives slightly better results than obtained with a conventional additive logistic toxicity model based on toxic units.

c) Toxic potency of bound metal increases in the approximate order H < Al < (Zn ~ Cu ~ Pb ~ UO_2) < Cd.

d) The overall range of F_{TOX} over which the toxic effect changes from 5 to 95% is about 10 times smaller than the range of toxicity units derived with a conventional model. Thus the WHAM-F_{TOX} analysis indicates much narrower ranges of differences amongst individual organisms in metal toxicity tests than was previously thought.

e) WHAM-F_{TOX} can contribute to the interpretation and design of mechanistic studies, and potentially provides a means to encapsulate the knowledge contained within laboratory data, thereby permitting its application to field situations.

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400 **References**

401 Antunes, P.M.C., Scornaienchi, M.L., Roshon, H.D., 2012. Copper toxicity to *Lemna minor*
402 modelled using humic acid as a surrogate for the plant root. Chemosphere 88, 389-
403 394.
404 Borgmann, U., Norwood, W.P., Clarke, C., 1993. Accumulation, regulation and toxicity of
405 copper, zinc, lead and mercury in *Hyalella azteca*. Hydrobiologia 259, 79-89.
406 Borgmann, U., Norwood, W.P., Dixon, D.G., 2004. Re-evaluation of metal bioaccumulation
407 and chronic toxicity in *Hyalella azteca* using saturation curves and the biotic ligand
408 model. Environmental Pollution 131, 469-484.
409 Borrok, D.M., Fein, J.B., 2005. The impact of ionic strength on the adsorption of protons, Pb,
410 Cd, and Sr onto the surfaces of Gram negative bacteria: testing non-electrostatic,
411 diffuse, and triple-layer models. J. Coll. Interface Sci. 286, 110–126
412 Charles, A.L., Markich, S.J., Ralph, P., 2006. Toxicity of uranium and copper individually
413 and in combination, to a tropical freshwater macrophyte (*Lemna aequinoctialis*).
414 Chemosphere 62, 1224-1233.
415 Couillard, Y., Grapentine, L.C., Borgmann, U., Doylea, P., Masson, S., 2008. The amphipod
416 *Hyalella azteca* as a biomonitor in field deployment studies for metal mining Environ.
417 Pollut. 156, 1314–1324.
418 De Schamphelaere, K.A.C., Janssen, C.R., 2002. A Biotic Ligand Model predicting acute
419 copper toxicity for *Daphnia magna*: the effects of calcium, magnesium, sodium,
420 potassium, and pH. Environ. Sci. Technol. 36, 48-54.
421 De Schamphelaere, K.A.C., Janssen, C.R., 2004. Effects of dissolved organic carbon
422 concentration and source, pH, and water hardness on chronic toxicity of copper to
423 *Daphnia magna*. Environ. Toxicol. Chem. 23, 1115–1122.
424 Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site
425 of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous
426 waste. Physiol. Rev 85, 97–177.
427 Hassler, C.S., Wilkinson, K.J., 2003. Failure of the biotic ligand and free-ion activity models
428 to explain zinc bioaccumulation by *Chlorella kesslerii*. Environ. Toxicol. Chem. 22,
429 620-626.
430 Hatano, A., Shoji, R., 2008. Toxicity of Copper and Cadmium in Combinations to Duckweed
431 Analyzed by the Biotic Ligand Model. Environ. Toxicol. 23, 372–378.
432 Heijerick, D.G., De Schamphelaere, K.A.C., Janssen, C.R., 2002. Predicting acute zinc
433 toxicity for *Daphnia magna* as a function of key water chemistry characteristics:

development and validation of a biotic ligand model. Environmental Toxicology and Chemistry, 21, 1309–1315.

Hickie, B.E., Hutchinson, N.J., Dixon, D.G., Hodson, P.V., 1993. Toxicity of trace metal mixtures to alevin rainbow trout (*Oncorhynchus mykiss*) and larval fathead minnow (*Pimephales promelas*) in soft, acidic water. Can. J. Fish. Aq. Sci. 50, 1348-1355.

Ivorra, N., Kraak, M.H.S., Admiraal, W., 1995. Use of lake water in testing copper toxicity to desmid species. Water Res. 29, 2113-2117.

Iwasaki, Y., Cadmus, P., Clements, W.H., 2013. Comparison of different predictors of exposure for modeling impacts of metal mixtures on macroinvertebrates in stream microcosms Aquat. Toxicol. 132–133, 151– 156

Jho, E.H., An, J., Nam, K., 2011. Extended biotic ligand model for prediction of mixture toxicity of Cd and Pb using single metal toxicity data. Environ. Toxicol. Chem. 30, 1697–1703.

Kooijman, S.A.L.M., 1996. Toxic effects as process perturbations. Chapter 2 In S.A.L.M. Kooijman & J.J.M. Bedaux The analysis of aquatic toxicity data. VU University Press, Amsterdam.

Kraak, M.H.S., Lavy, D., Schoon, H., Toussaint, M., Peeters, W.H.M., van Straalen, N.M., 1994. Ecotoxicity of mixtures of metals to the zebra mussel *Dreissena polymorpha*. Environ. Toxicol. Chem. 13, 109-114.

Lofts, S., Tipping, E., Hamilton-Taylor, J., 2008. The chemical speciation of Fe(III) in freshwaters. Aquat. Geochem. 14, 337-358.

Luoma, S.N., Rainbow, P.S. 2005. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environ. Sci. Technol. 39, 1921-1931.

Newman, M.C., Ungar, M.A., 2003. Fundamentals of Ecotoxicology, 2nd Edn. Lewis Publishers, Boca Raton.

Norwood, W.P., 2007. Metal mixture toxicity to *Hyalella azteca*: relationships to body concentrations. PhD Thesis, University of Waterloo, Canada.

Norwood, W.P., Borgmann, U., Dixon, D.G., Wallace, A., 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Human. Ecol. Risk Assess. 9, 795-811.

Paquin, P.R., Gorsuch, J.W., Apte, S., Batley, G.E., Bowles, K.C., Campbell, P.G.C., Delos, C.G., Di Toro, D.M., Dwyer, R.L., Galvez, F., Gensemer, R.W., Goss, G.G., Hogstrand, C., Janssen, C.R., McGeer, J.C., Naddy, R.B., Playle, R.C., Santore, R.C.,

467 Schneider, U., Stubblefield, W.A., Wood, C.M., Wua, K.B., 2002. The biotic ligand
468 model: a historical overview. Comp. Biochem. Physiol. Part C 133, 3-35.
469 Playle, R.C., 2004. Using multiple metal–gill binding models and the toxic unit concept to
470 help reconcile multiple-metal toxicity results. Aquatic Toxicology 67 (2004) 359–
471 370.
472 Rainbow, P.S. 2007. Trace metal bioaccumulation: models, metabolic activity and toxicity.
473 Environ. Internat. 33, 576-582.
474 Shaw, J.R., Dempsey, T.D., Chen, C.Y., Hamilton, J.W., Folt, C.L., 2006. Comparative toxicity
475 of cadmium, zinc and mixtures of cadmium and zinc to daphnids. Environ. Toxicol.
476 Chem. 25, 182-189.
477 Stockdale, A., Tipping, E., Lofts, S., Ormerod, S.J., Clements, W.H., Blust R., 2010. Toxicity
478 of proton-metal mixtures in the field: linking stream macroinvertebrate species diversity
479 to chemical speciation and bioavailability. Aquat. Toxicol. 100, 112-119.
480 Stockdale, A., Tipping, E., Fjellheim, A., Garmo, Ø.A., Hildrew, A.G., Lofts, S., Monteith,
481 D.T., Ormerod, S.J., Shilland, E.M., 2013a. Recovery of macroinvertebrate species
482 richness in acidified upland waters assessed with a field toxicity model. Ecol. Indicat.,
483 doi: 10.1016/j.ecolind.2011.11.002
484 Stockdale, A., Tipping, E., Lofts, S., Fott, J., Garmo, Ø.A., Hruska, J., Keller, B., Löfgren, S.,
485 Maberly, S.C., Majer, V., Nierzwicki-Bauer, S.A., Persson, G., Schartau, A-K.,
486 Thackeray, S.J., Valois, A., Vrba, J., Walseng, B., Yan, N., 2013b. Metal and proton
487 toxicity to lake zooplankton: a chemical speciation-based modelling approach.
488 Environmental Pollution submitted.
489 Tipping, E., 1994. WHAM - A chemical equilibrium model and computer code for waters,
490 sediments and soils incorporating a discrete-site / electrostatic model of ion-binding by
491 humic substances. Comp. Geosci. 20, 973-1023.
492 Tipping, E., 1998. Humic Ion-Binding Model VI: an improved description of ion-binding by
493 humic substances. Aquat. Geochem. 4, 3-48.
494 Tipping, E., 2002. Cation-Binding by Humic Substances. Cambridge University Press,
495 Cambridge.
496 Tipping, E., Vincent, C.D., Lawlor, A.J., Lofts, S., 2008. Metal accumulation by stream
497 bryophytes, related to chemical speciation. Environ. Pollut. 156, 936–943.
498 Tipping, E., Lofts, S., Sonke, J.E., 2011. Humic Ion-Binding Model VII: a revised
499 parameterisation of cation-binding by humic substances. Environ. Chem. 8, 225-235.

500 Vijver, M.G., Peijnenburg, W.J.G.M., De Snoo, G.R., 2010. Toxicological mixture models
501 are based on inadequate assumptions. Environ. Sci. Technol. 2010, 44, 4841–4842
502 Yee, N., Fein, J.B., 2001. Cd adsorption onto bacterial surfaces: a universal adsorption edge?
503 Geochim. Cosmochim. Acta 65, 2037–2042.

504 Table 1. Summary of datasets for metal accumulation by living organisms.

505

Organism	Species	Metals	Conditions	References
bacteria	*Pseudomonas putida*	Sr Cd Pb	laboratory 4 hours pH 3.5-8.6, ranges of [$NaClO_4$]	Borrok & Fein 2005
	Bacillus subtilis *Escherichia coli*	Cd	laboratory 2 hours pH 2-10, ranges of [$NaNO_3$]	Yee & Fein 2001
alga	*Chlorella kesslerii*	Zn	laboratory 25-60 minutes pH 7, ionic strength 1.8-3.2 mM	Hassler & Wilkinson 2003
amphipod	*Hyalella azteca*	Mn Co Ni Cu Zn Cd Hg Pb	laboratory 4-10 weeks Lake Ontario water and 1:10 dilutions pH ~ 8, [DOC] 1-2 mg L^{-1}	Borgmann et al. 1993, 2004 Norwood 2007
		Be Al Mn Fe Co Ni Cu Zn Cd Ba Pb U	mine-affected stream waters, 17 days pH 6.2-7.1, [DOC] 14-19 mg L^{-1}	Couillard et al. 2008
mussel	*Dreissena polymorpha*	Cu Zn Cd	laboratory 48 hours; lake water, pH 7.9, [DOC] 7 mg L^{-1}	Kraak et al. 1994 Ivorra et al. 1995 De Schamphelaere & Janssen 2004

506

507

508 Table 2. Summary of metal mixture toxicity data sets.

509

test organism	toxic response	pH	[Ca] mM	[DOC] mg L^{-1}	metals	reference
Escherichia coli	luminescence inhibition 15 min	5.5	0.0	0	Cu Zn Cd	Preston et al. 2000
Pseudomonas fluorescens	luminescence inhibition 15 min	5.5	0.0	0	Cu Zn Cd	Preston et al. 2000
Vibrio fischeri	luminescence inhibition 5 min	6.7 - 7.1[a]	0.025, 2.5, 25	0	Cd Pb	Jho et al. 2011
Lemna aequinoctialis	growth rate 96 hours	6.5	1	0	Cu UO_2	Charles et al. 2006
Lemna paucicostata	growth rate 96 hours	4.1 - 7.5	0.49	0	Cu Cd	Hatano & Shoji 2008
Ceriodaphnia dubia	survival 96 hr	7.4	0.025	0	Zn Cd	Shaw et al. 2006
Daphnia ambigua	survival 96 hr	7.4	0.025	0	Zn Cd	Shaw et al. 2006
Daphnia magna	survival 96 hr	7.4	0.025	0	Zn Cd	Shaw et al. 2006
Daphnia pulex	survival 96 hr	7.4	0.025	0	Zn Cd	Shaw et al. 2006
Dreissena polymorpha	filtration rate 48 hr	7.9	1.5	7	Cu Zn Cd	Kraak et al. 1994
Oncorhynchus mykiss	survival 144 hr	4.3 - 5.8	0.0625	2	Al Cu Zn	Hickie et al. 1993

510

511

512 [a] calculated from data in the paper, assuming pCO_2 to be at the atmospheric level.

513

514 Table 3. Results of fitting WHAM-F_{TOX} to the toxicity data sets of Table 2. The value of α_H is fixed at 1.0, and the mean of $F_{TOX,LT}$ and $F_{TOX,UT}$
515 is 4.12 in all cases (see the text).
516

test organism	$F_{Tox,LT}$	$F_{Tox,UT}$	α_H	α_{Al}	α_{Cu}	α_{Zn}	α_{Cd}	α_{Pb}	α_{UO2}	RMSD	r^2
Escherichia coli	3.57	4.67	1.0		3.2	13.9	18.6			19	0.68
Pseudomonas fluorescens	3.29	4.95	1.0		4.0	14.0	23.3			10	0.89
Vibrio fischeri	2.45	5.79	1.0				3.8	4.1		15	0.81
Lemna aequinoctialis	2.24	6.00	1.0		20.8				16.0	8	0.96
Lemna paucicostata	1.73	6.51	1.0		2.7		7.6			18	0.76
Ceriodaphnia dubia	1.90	6.34	1.0			5.8	65.3			16	0.77
Daphnia ambigua	2.04	6.20	1.0			5.5	133.7			7	0.96
Daphnia magna	3.03	5.21	1.0			4.6	27.0			14	0.84
Daphnia pulex	1.79	6.45	1.0			6.5	63.8			11	0.88
Dreissena polymorpha	1.84	6.40	1.0		30.3	6.0	85.8			11	0.92
Oncorhynchus mykiss	2.39	5.85	1.0	2.1	11.9	4.6				5	(-)

517

518 Table 4. Results from application of the Conventional Toxic Units (CTU) model. The EC_{50} values are in mg L^{-1}.

test organism	β	EC_{50}Cu	EC_{50}Zn	EC_{50}Cd	EC_{50}Pb	EC_{50}UO2	RMSD	r^2
Escherichia coli	2.77	2.36	0.33	1.09			16	0.79
Pseudomonas fluorescens	2.99	0.75	0.35	0.68			6	0.97
Vibrio fischeri	1.37			109	2.50		18	0.72
Lemna aequinoctialis	1.60	0.017				0.81	3	0.97
Lemna paucicostata	0.89	2.13		1.56			17	0.81
Ceriodaphnia dubia	1.55		0.39	0.05			22	0.58
Daphnia ambigua	1.96		0.56	0.02			14	0.83
Daphnia magna	3.20		0.78	0.15			13	0.85
Daphnia pulex	1.26		0.32	0.05			16	0.69
Dreissena polymorpha	2.25	0.044	4.13	0.23			10	0.93

519

520 **Figure captions**

521 Figure 1. Sorption of Sr, Cd and Pb by bacteria. The points in panels (a) - (c) are measured
522 data of Borrok & Fein (2005), those in panel (d) are from Yee & Fein (2001), the lines are fits
523 with WHAM/Model VII. Panel (a) results for Cd; open circles, I = 0.01 M, *P. putida* 10 g L^{-1};
524 closed circles, I = 0.5 M, *P. putida* 10 g L^{-1}; open squares, I = 0.1 M, *P. putida* 3 g L^{-1}.
525 Panel (b) results for Pb; open circles, I = 0.01 M, *P. putida* 1 g L^{-1}; closed circles, I = 0.5 M,
526 *P. putida* 1 g L^{-1}; open squares, I = 0.1 M, *P. putida* 3 g L^{-1}. Panel (c) results for Sr, pH 6.3,
527 *P. putida* 6 g L^{-1} (open diamonds); Cd, pH 5.9, *P. putida* 3 g L^{-1} (closed triangles); Pb, pH
528 5.5, *P. putida* 1 g L^{-1} (open triangles). Panel (d) Cd sorption by *Bacillus subtilis* (open
529 circles) and *Escherichia coli* (closed circles).

530 Figure 2. Binding of zinc to the surface of *Chlorella kesslerii.* The points are the data of
531 Hassler & Wilkinson (2003), converted to mol g^{-1} as described in the text. The line is the
532 WHAM/Model VII fit with E_{HA} = 0.032.

533 Figure 3. Accumulation of metals by *Hyalella azteca* in (a) the laboratory (Borgmann et al.
534 1993, 2004; Norwood, 2007) and (b) the field (Couillard et al., 2008), compared with
535 calculated binding by humic acid in equilibrium with the same solutions. The lines have
536 slopes of unity, and offsets of 1.36 (a) and 0.96 (b), yielding E_{HA} values of 0.044 and 0.11.

537 Figure 4. Accumulation of metals by the mussel *Dreissena polymorpha* measured by Kraak
538 et al. (1993), compared to predicted binding by humic acid. The line has a slope of unity, and
539 an offset of 1.78, yielding E_{HA} = 0.017.

540 Figure 5. Toxicity of Cd, Pb and their mixtures to *Vibrio fischeri* (Jho et al., 2011) fitted
541 with WHAM-F_{TOX}. Upper panel, Cd only; middle panel Pb only; bottom panel, Cd and Pb
542 combined. The symbols indicate low (open), medium (grey) and high (black) concentrations
543 of Ca. Note that the fits refer to the entire data set, not separately to the data of individual
544 panels.

545 Figure 6. Toxicity towards duckweed species fitted with WHAM-F_{TOX}. Panel (a); toxicity
546 of Cu, UO_2 and their mixtures to *Lemna aequinoctialis* (Charles et al., 2006). Panel (b):
547 toxicity of Cu, Cd and their mixtures to *Lemna paucicostata* (Hatano & Shoji, 2008).

548 Figure 7. Toxicity of Zn, Cd and their mixtures to daphnids (Shaw et al., 2006) fitted with
549 WHAM-F_{TOX}. Panel (a) *Ceriodaphnia dubia*, (b) *Daphnia ambigua*, (c) *Daphnia magna*, (d)
550 *Daphnia pulex.*

551 Figure 8. Toxicity of mixtures of Cu, Zn and Cd to the zebra mussel *Dreissena polymorpha*
552 (Kraak et al., 1993) fitted with WHAM-F_{TOX}.

553 Figure 9. Normalised log-linear plots of toxic response for the WHAM-FTOX and CTU
554 models, showing all data. Note that the Hickie et al. (1993) data are absent from the right-
555 hand plot.

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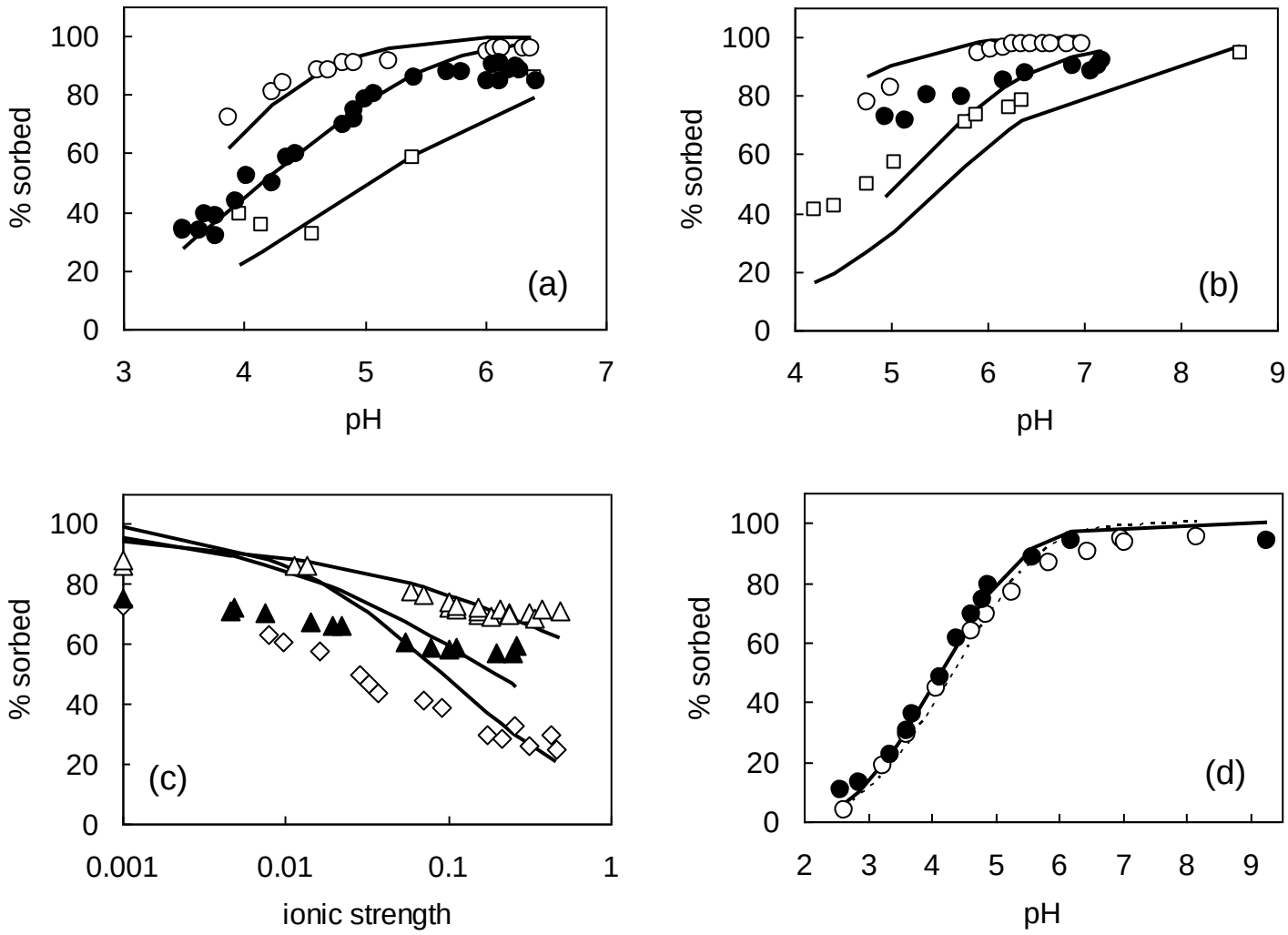

Figure 1.

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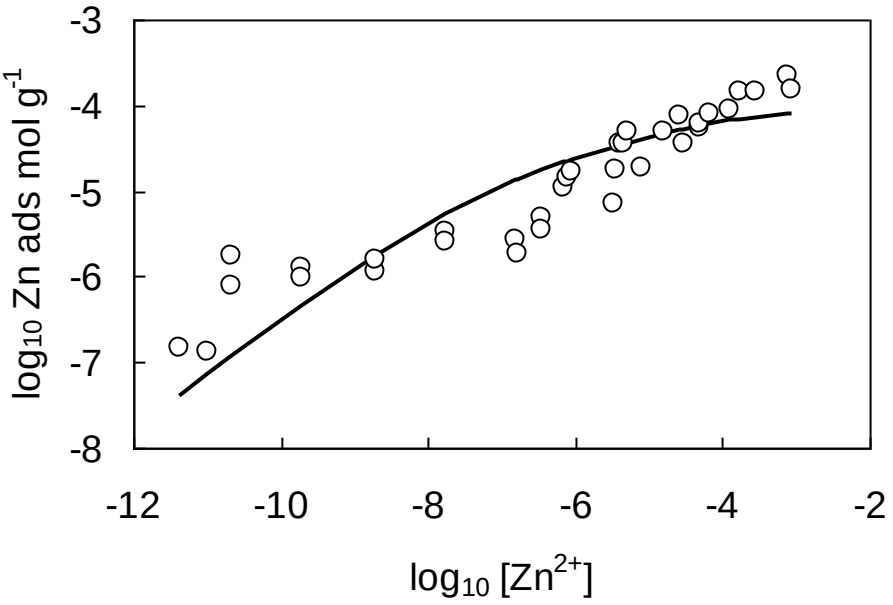

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581 Figure 2.

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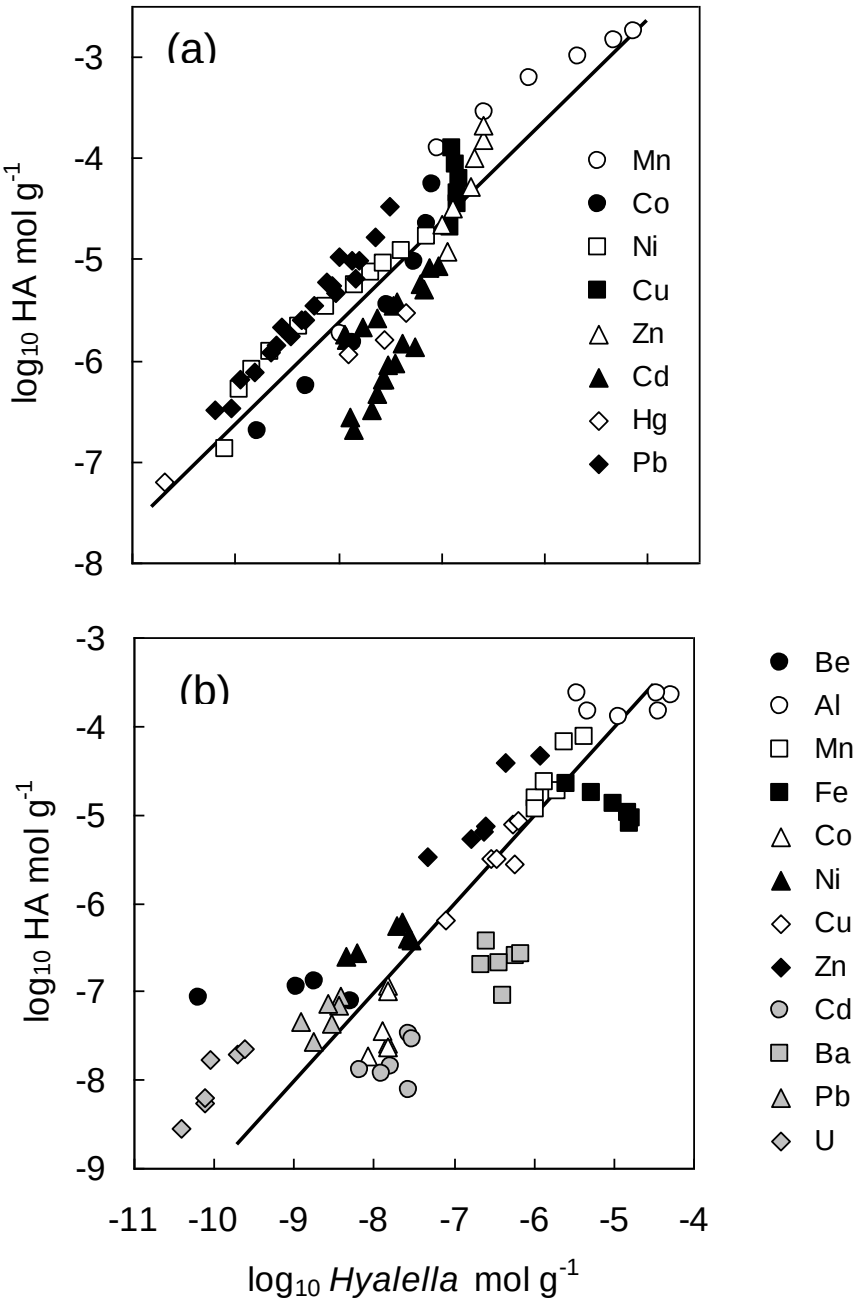
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Figure 3.

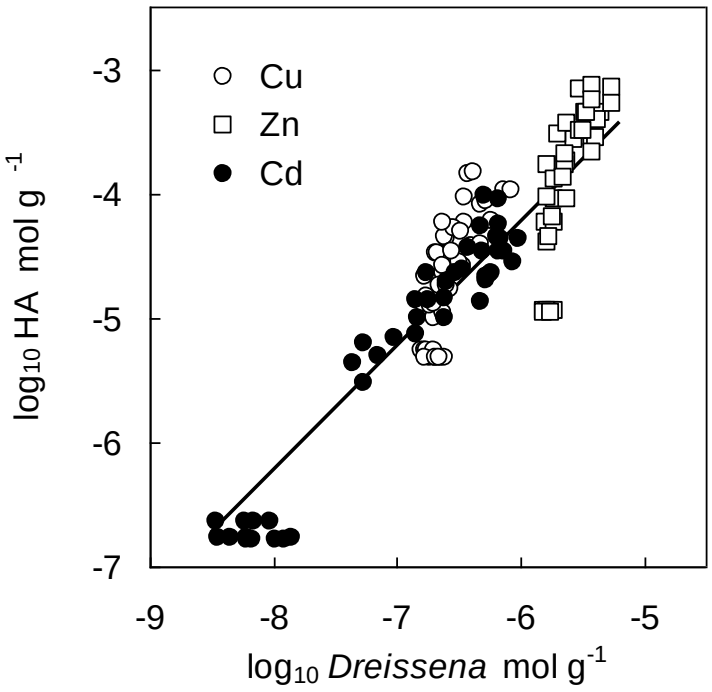

Figure 4.

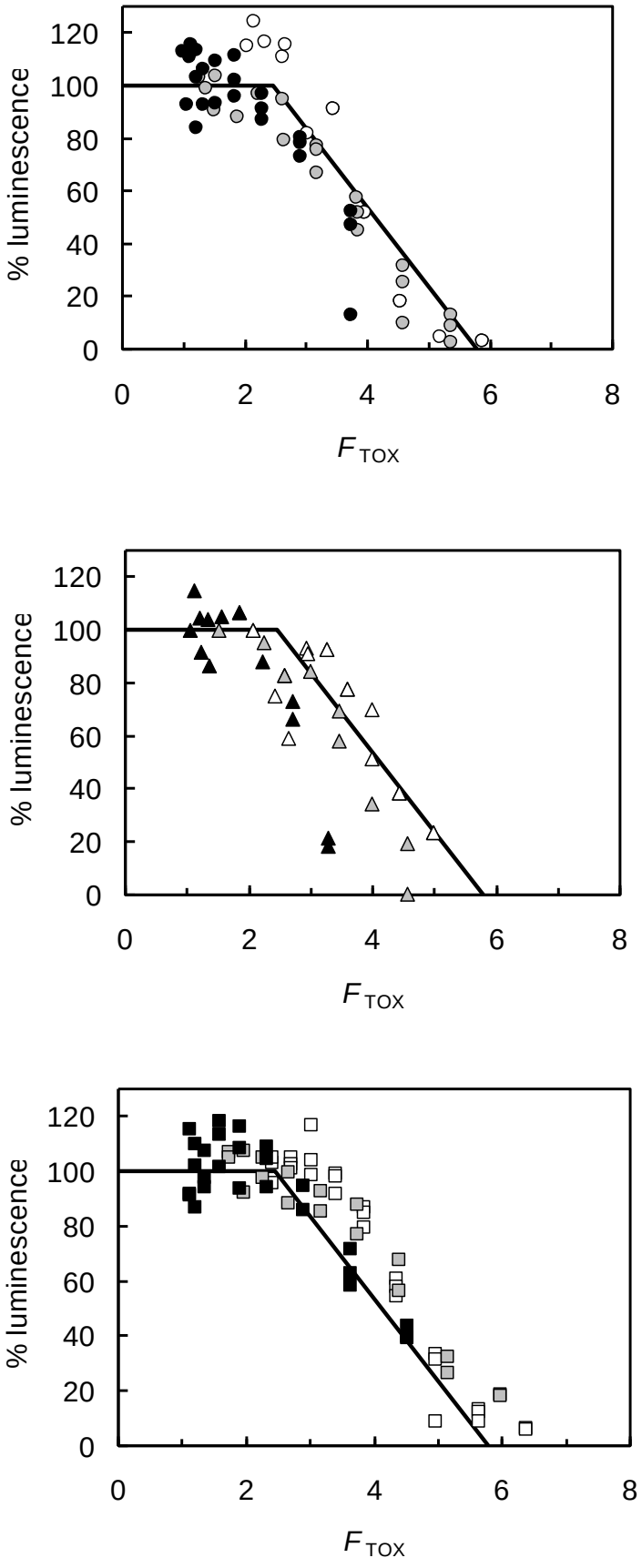
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Figure 5.

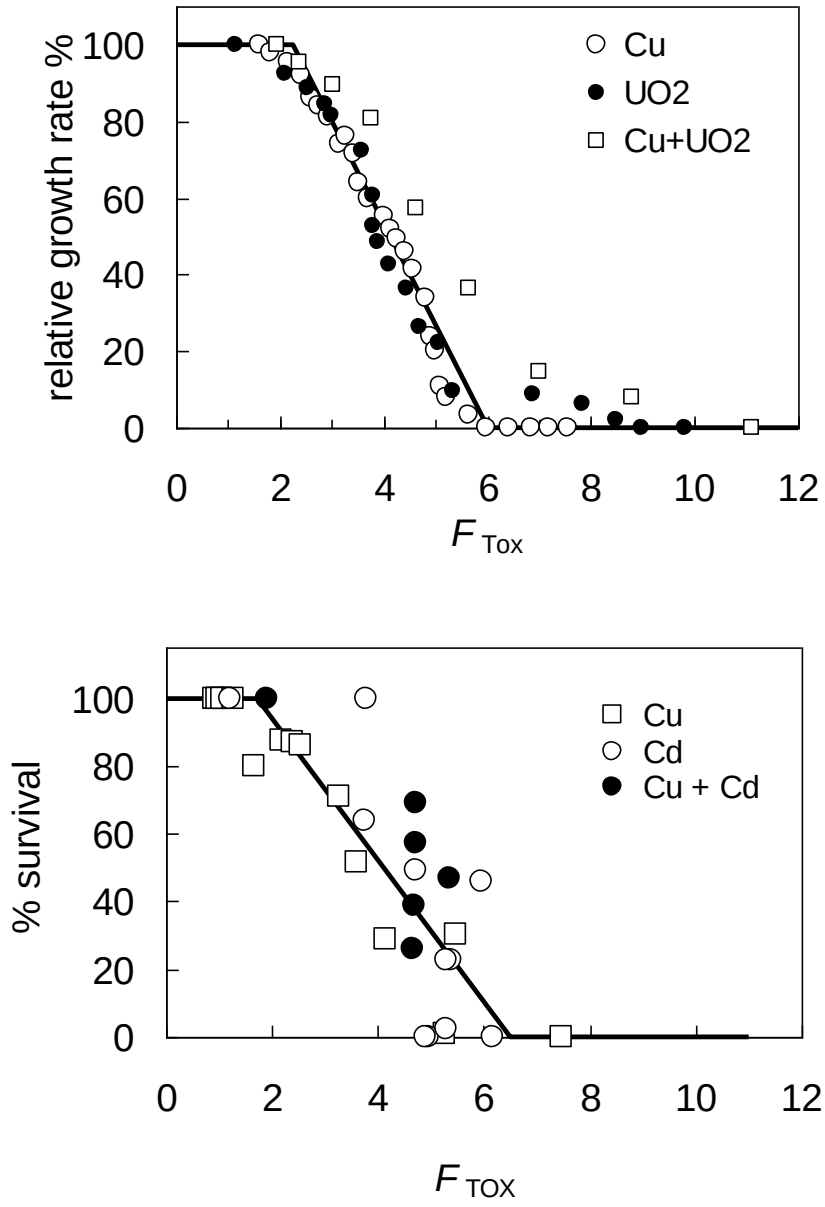
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Figure 6.

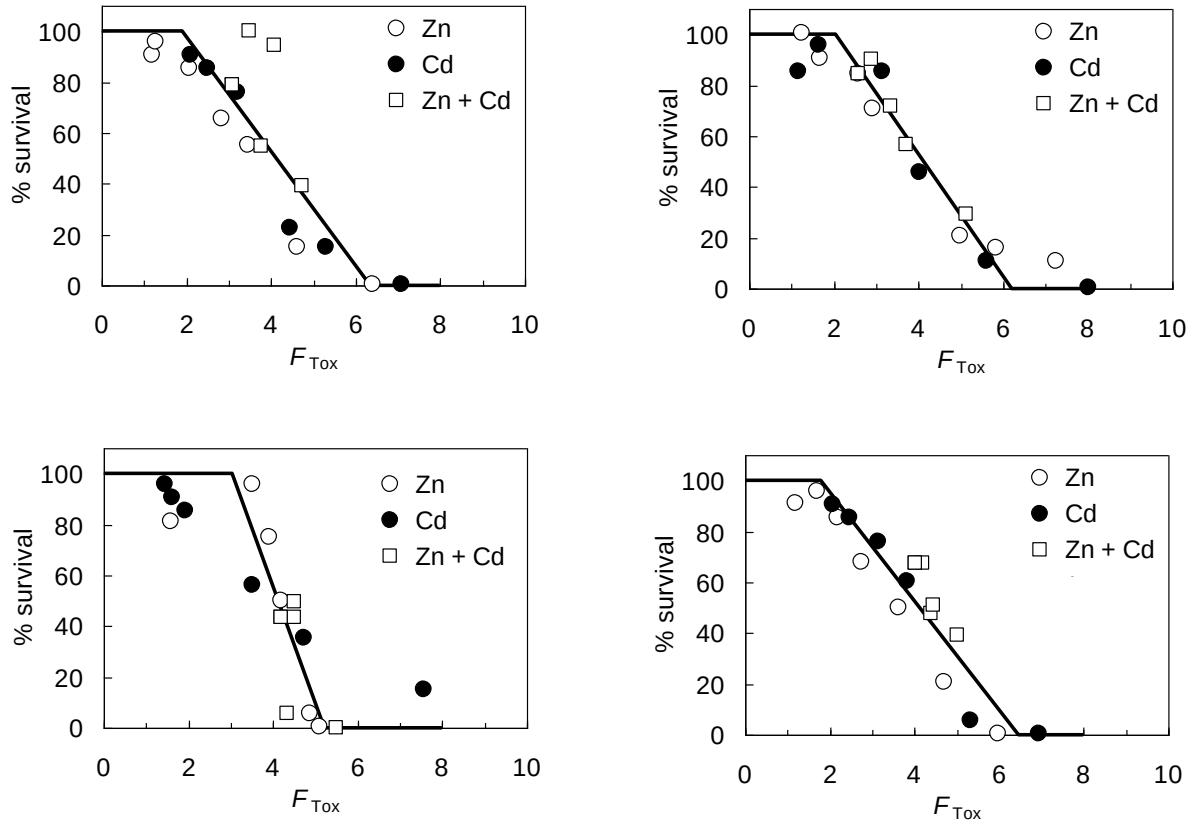

Figure 7.

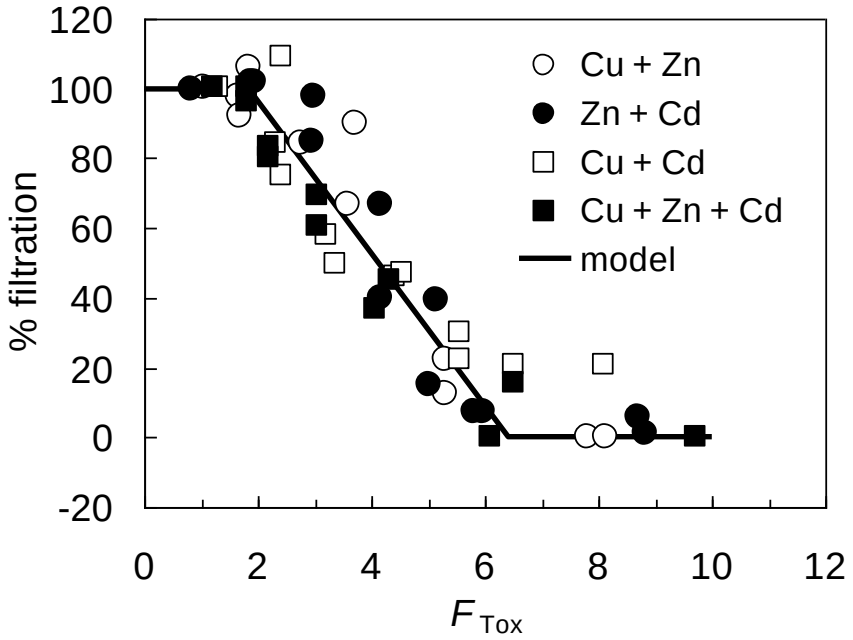
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Figure 8.

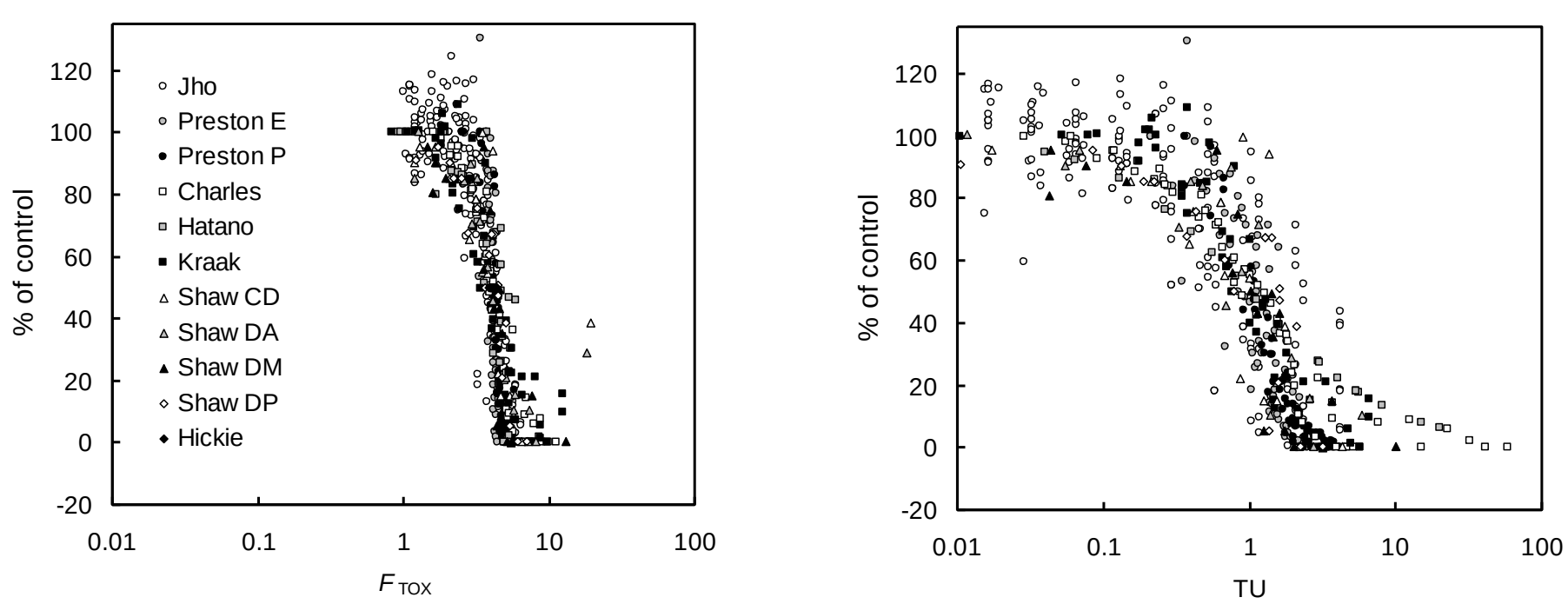
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Figure 9.