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TESTING WHAM-F_{TOX} WITH LABORATORY TOXICITY DATA FOR MIXTURES OF METALS (Cu, Zn, Cd, Ag, Pb)

E. TIPPING* & S. LOFTS

Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg, Lancaster LA1 4AP, United Kingdom

Correspondence to: Professor Edward Tipping
Centre for Ecology and Hydrology
Lancaster Environment Centre
Bailrigg
Lancaster
LA1 4AP
United Kingdom

E-mail et@ceh.ac.uk

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ABSTRACT

The WHAM-F_{TOX} model describes cation toxicity to aquatic organisms in terms of (a) accumulation by the organism of metabolically-active protons and metals at reversible binding sites, and (b) differing toxic potencies of the bound cations. Cation accumulation (ν_i in mol g^{-1}) is estimated through calculations with the WHAM chemical speciation model by assuming that organism binding sites can be represented by those of humic acid. Toxicity coefficients (α_i) are combined with ν_i to obtain the variable F_{TOX} (= $\Sigma\ \alpha_i\nu_i$) which, between lower and upper thresholds ($F_{TOX,LT}$, $F_{TOX,UT}$), is linearly related to toxic effect. Values of α_i, $F_{TOX,LT}$ and $F_{TOX,LT}$ are obtained by fitting toxicity data. Reasonable fits (72% of variance in toxic effect explained overall) were obtained for four large metal mixture acute toxicity experiments involving daphnids (Cu, Zn, Cd), lettuce (Cu, Zn, Ag) and trout (Zn, Cd, Pb). Strong non-additive effects, most apparent in results for tests involving Cd, could be explained approximately by purely chemical competition for metal accumulation. Tentative interpretation of parameter values obtained from these and other experimental data suggests the following order of bound cation toxicity: H < Al < (Cu Zn Pb UO_2) < (Cd Ag). Another trend is a strong increase in Cd toxicity relative to that of Zn, as organism complexity increases (from bacteria to fish).

Note to the editor and reviewers: This is one of 11 manuscripts under consideration for an ET&C Special Section on Metal Mixtures. The Section includes an introduction, a technical background, a comparative evaluation of multiple modeling approaches, a lessons-learned manuscript, and seven manuscripts on specific modeling and interpretation approaches. While each manuscript should be able to stand alone, the individual manuscripts are interrelated and cross-reference each other. If another cross-referenced, submitted manuscript is essential to complete the review of the present manuscript, please request the other manuscript from the Corresponding Guest Editor, copying the handling editor. The Corresponding Guest Editor for the series is Eric Van Genderen (evangenderen@zinc.org). Any unpublished material provided to assist your review must also be treated in confidence.

INTRODUCTION

Hitherto, quantification of metal toxicity to aquatic and soil organisms has been based almost entirely on the results of experiments involving single metals, even when bioavailability has been taken into account using either the Biotic Ligand Model (BLM) [1] or simpler pH-dependent descriptions [2]. The information obtained in this way has been used in environmental risk assessment to define concentrations of individual metals above which unacceptable toxic effects would be expected in the field. This approach is pragmatic, sensibly exploiting a large body of available knowledge, obtained from laboratory toxicity testing, to protect the natural environment. When it comes to mixtures, single metal standards based on conventional toxicity endpoints have been combined using the Cumulative Criterion Unit [3] to generate combined quality standards. This approach will give conservative risk assessments if mixture effects are less-than-additive, but there would be underestimation of risk if more-than-additive effects occur.

Risk assessment on the basis of laboratory experiments involves the definition of maximum acceptable concentrations. It is also desirable to be able to interpret observed field effects, to test whether effects projected from laboratory data actually occur in the field, to confirm and justify quality standards, to understand the extents of effects, and to evaluate the feasibility and success of remediation methods (e.g. clean up of mines and contaminated land). This definitely needs models that take into account both environmental chemistry and mixture toxicity, and has prompted efforts to adapt the BLM for application to mixtures, by theoretical [4], data fitting [5-7] and field work [8]. Further work with the BLM is reported in this volume [9-12]. An alternative approach is the WHAM-F_{TOX} model [13,14].

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 will not be associated directly with the toxic response. The model then assigns a toxicity coefficient to each cation, which quantifies 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.

To apply this concept quantitatively, it is assumed 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. In other words, the array of HA binding sites postulated in WHAM is assumed to provide an acceptable representation of the sites possessed by organisms, taking into account differences in total site contents per unit dry weight. This is at least a reasonable first approximation, given that humic substances are formed from by the partial decomposition of living tissue, albeit mainly from plants. Results showing strong correlations between observed metal contents of living organisms, in both field and laboratory studies, and predicted metal binding to HA have been reported by Tipping et al. [15], Stockdale et al. [13] and Tipping & Lofts [14]. These correlations are taken to justify the modelling approach, although it is recognised that the modelled bound metals constitute a "metabolically-active body burden", whereas body burdens determined by chemical analysis may in some cases also include metal that has been rendered metabolically inactive, e.g. by strong binding to metallothioneins or incorporation into precipitated granules [16], which means that precise agreements are not necessarily expected.

We have already applied WHAM-F_{TOX} to 11 published laboratory data sets describing metal mixture toxicity to aquatic organisms, with promising results [14]. However, those data sets did not present major difficulties for the model because the solution chemical conditions were quite limited, and indeed the fits with WHAM-F_{TOX} were only slightly superior to those with a conventional toxicity model based on total concentrations in solution, and assuming additive toxic effects. In the work reported here, we applied WHAM-F_{TOX} and the

conventional model to four more comprehensive data sets (Table 1) that were anticipated to provide sterner tests, gathered as part of the "Metal Mixture Modeling Evaluation (MMME) Project" [Table 2, 17]. In particular, these additional data cover conditions under which metals would be expected to compete significantly for the postulated reversible binding sites on the organisms, which we expect to be accounted for by WHAM-F_{TOX} but not by the purely additive solution-based model.

METHODS

Modelling chemical speciation and accumulation in living organisms

We used WHAM [18] incorporating Humic Ion-Binding Model VII [19]. 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 [18]. The inorganic reactions in this database are restricted to monomeric complexes of metals. The effects of ionic strength on the inorganic reactions are accounted for with 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, and 65% of the DOM to be active with respect to cation binding, represented by FA [15]. For example, a DOC concentration of 5 mg L^{-1} corresponds to an FA concentration of 6.5 mg L^{-1} for modelling. For toxicity experiments in which natural waters were used to prepare the test solutions, dissolved Fe(III) activities were estimated with the empirical equation of Lofts et al. [20], re-parameterised for Humic Ion-Binding Model VII.

We calculated the equilibrium binding of protons and 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^{-1}). The ν_i are the combined contributions from coordinative binding and accumulation as counterions in the diffuse layer surrounding the (invariably negatively) charged humic molecules.

Fitting toxicity data

In WHAM-F_{TOX}, 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, rather than the absolute amount of metal per unit weight of organism. 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;

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

in which α_i is the toxicity coefficient of cation *i*. Thus the model assumes strictly additive toxicity, when exposure is expressed in terms of accumulated or bound metal. 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. 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. In previous work [14] we constrained the values of $F_{TOX,LT}$ and $F_{TOX,UT}$ such that their average was the same (4.12) for each data set, because the data were insufficient to permit both parameters to be estimated. We continued with this constraint in the present study (see Discussion). In practice, we optimised $F_{TOX,LT}$, so that $F_{TOX,UT}$ is equal to 8.24 - $F_{TOX,LT}$.

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:

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

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 $EC_{50}(i)$.

The toxicity calculations were performed with Microsoft Excel, using its SOLVER function to optimise parameter values.

Data sets

Data for the toxicity of Cu, Zn and Cd to *Daphnia magna* were from a comprehensive series of acute toxicity (48 hour survival) tests carried out by Meyer et al. [22], which is Index 4 in Van Genderen et al. [17]. Each experiment comprised 2, 3 or 4 separate dose-response series (up to 25 data in all), aimed a systematic examination of mixture toxicity effects, and with an unprecedented degree of replication. Combination of the chemical speciation outputs with the solubility products (25^{o}C) given by Grauer [23] indicated oversaturation of some of the test solutions with respect to the carbonates of both Zn (11% of the solutions) and Cd (21%), by up to a factor of 10. This raised the possibility that in some tests the organisms were exposed to smaller amounts of Zn and Cd than the measured "soluble" concentrations would suggest. However, the exact extents of saturation are 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 have higher solubility products than the better-ordered phases used to obtain the published solubility products. Therefore we did not attempt to take into account the possibility that metal precipitation affected toxic responses.

Data for the toxicity of Cu, Zn and Ag to *Lactuca sativa* (Index 9 in ref. 17) were published by Le et al. [7, 24], and refer to the effects of single metals and pairs of metals (Cu and Zn, Cu and Ag) on 96-hour root elongation. Free-ion metal concentrations were reported, which meant that WHAM did not have to be used for solution speciation, only to estimate accumulation by the plant.

Data for the toxicity of Zn, Cd and Pb to trout species (Index 6 in ref. 17) were published by Mebane et al. [25], and refer to 96-hour survival of juvenile cutthroat (*Oncorhynchus clarkii lewisi*) and rainbow (*Oncorhynchus mykiss*) trout. The test media

were natural waters from the South Fork Coeur d'Alene River watershed, Idaho, USA, amended with metals. We assumed dissolved Al to be present at a concentration of 0.25 μM (based on data of Balistrieri & Blank [27] and Mebane et al.[25]). Combination of the chemical speciation outputs with the solubility products (25°C) given by Grauer [23] indicated oversaturation with respect to $PbCO_3$(s) of 6% of the cutthroat trout test solutions and 7% of the rainbow trout solutions, by up to a factor of 10. However, for the reasons given above in relation to the results for *D. magna*, we did not attempt to take into account the possibility that metal precipitation affected toxic responses.

RESULTS

All the results presented here refer to chemical speciation modelling conducted with Model VII, but very similar results were obtained with Model VI [21]. The toxicity data sets and fitted toxicity parameters are summarised in Table 1.

Toxicity of Cu, Zn and Cd to Daphnia magna

Firstly we fitted the entire data set (870 data points) with optimisation of a single parameter set ($F_{TOX,LT}$, α_{Cu}, α_{Zn}, α_{Cd}). The model was able to explain 57% of the variance in % survival with an RMSD (root-mean-squared deviation) between observed and calculated survival of 29%. Comparison of observed and predicted values revealed that the greatest discrepancies arose in experiments involving cadmium, whereas the single parameter set gave consistent results for the copper-zinc experiments. Therefore, we next fitted all the data with universal values of $F_{TOX,LT}$, α_{Cu} and α_{Zn}, but with α_{Cd} optimised for each experiment. The use of the experiment-specific values of α_{Cd} appreciably improved the overall fit (r^2 = 0.74, RMSD = 22%), but produced considerable variation in α_{Cd} with values as low as zero and as high as 1200.

To progress further, i.e. to consider interactions between metals in individual experiments, we restricted the modelling analysis to the experiments that yielded the middle 50% of individual α_{Cd} values, thereby reducing the total number of data points to 542. When these data were fitted with four adjustable parameters ($F_{TOX,LT}$, α_{Cu}, α_{Zn}, α_{Cd}), the values of r^2 and RMSD were 0.65 and 25% respectively. Analysis of the same data with the CTU model yielded a poorer fit (Table 1).

To illustrate metal mixture effects, plots were made comparing the experimental data with predictions made by WHAM-F_{TOX} and CTU, both parameterised on the entire 542-point data set, i.e. the model predictions are not the best fits of individual experiments. Figures 2 and 3 show that the effects of Cu and Zn on each other's toxicity approximately follow the expectations of the WHAM-F_{TOX} model, which postulates interference via competitive chemical binding reactions. However, the calculated competition effects are minor, and consequently the CTU model, which ignores any effects of one metal on another, provides results that are nearly as good as those from WHAM-F_{TOX}. More extreme situations are found with Cu-Cd and Zn-Cd pairings (Figures 4 and 5), in which the less toxic metals (Cu and Zn) are observed to reduce Cd toxicity. Thus in several cases, increases in the

concentrations of Cu or Zn actually decrease the toxicity of the mixture over a substantial portion of the experimental range (bottom two panels of Figures 4 and 5). This phenomenon is approximately reproduced by WHAM-F_{TOX} because the chemical speciation model predicts that Cu and Zn can displace the more toxic Cd from organism binding sites. However, the CTU model cannot simulate this phenomenon.

WHAM-F_{TOX} outputs (ν_i and F_{TOX}) for two of the mixture toxicity experiments are shown in Figure 6, and compared with expected effects were there no competition. Considering the left-hand panels, it is seen that competition by Cu decreases the binding of Zn by about one-third, and of course the binding of Cu must be less than would occur in the absence of Zn. These competition effects diminish the total F_{TOX} by a modest amount, and so the predicted mixture toxicity differs relatively little from additive behaviour. The right-hand panels of Figure 6 show how the addition of only 0.25 mg L^{-1} Zn severely reduces Cd binding, sufficient to decrease the total F_{TOX}. Subsequent additions of Zn then cause total F_{TOX} to increase, but the predicted mixture toxicity is now substantially lower than expected for the case with no competition.

Toxicity of Cu, Zn and Ag to Lactuca sativa

The WHAM-F_{TOX} fit (Figure 7) gave $r^2 = 0.78$ and an RMSD of 14%. The derived values of α_{Cu}, α_{Zn} and α_{Ag} are 12.2, 3.5 and 2090 respectively, showing the very high toxicity of bound Ag.

Figure 8 compares observed and modelled results for some of the individual experiments. The model deals reasonably well with Cu-Zn mixtures (panels a and b), and predicts that bound Cu protects somewhat too strongly against Ag toxicity (panel c). The model predicts that Cu should cause an appreciable reduction in toxicity when added to solutions containing fixed free-ion concentrations of Ag, but the data are equivocal in this respect. This, and the fact that the CTU model gives a slightly better overall fit than WHAM-F_{TOX} for this data set (Table 1), suggests that any competition effects generated in the experimental data are fairly minor.

These data were also analysed by Le et al. [7, 24], using both the BLM and an extended empirical concentration-addition toxicity model. To apply the BLM, they assumed that Cu and Zn compete for binding to their respective biotic ligands, whereas Cu and Ag do not. The fitted BLM gave an r^2 of 0.65 for Cu-Zn mixtures and 0.69 for Cu-Ag mixtures. Their extended concentration-addition toxicity model included adjustable parameters

accounting for interactions among metals. For Cu-Zn mixtures they achieved $r^2 = 0.92$, and for Cu-Ag $r^2 = 0.80$.

Toxicity of Zn, Cd and Pb to trout species

We fixed the value of α_{Al} at 2.1, the value obtained for rainbow trout from the data of Hickie et al. [27] by Tipping & Lofts [14]. However, contributions of Al to F_{TOX} were small. Some competition by Fe(III) (which is assumed not to be toxic) for metal binding was evident, most importantly towards Cd. The fitted WHAM-F_{TOX} model gave RMSD = 16% and $r^2 = 0.81$ for the cutthroat trout, but a poorer fit (RMSD = 24%, $r^2 = 0.64$) for the rainbow trout. The parameter values were similar for the two species (Table1), with particularly high values of α_{Cd}. Figure 9 shows WHAM-F_{TOX} fits to the two data sets.

The experiments were carried out less systematically then those for *D. magna* and *L. sativa*, and consequently lend themselves less well to plotting. However, Figure 10 compares calculated and observed survival as a function of Cd concentration for low and high Zn concentrations for the two trout species. The data show that Zn reduces toxicity for both species, and this is correctly forecast by WHAM-F_{TOX}, but not by the CTU model, which predicts greater toxicity at high Zn, because it cannot deal with competitive effects.

Compilation of data

Table 2 combines the four new parameter sets derived in the present work with data previously reported for laboratory toxicity data by Tipping & Lofts [14]. The derived parameters indicate that the toxicities (α values) of bound Zn and Pb do not vary greatly among test species, α_{Cu} varies by a factor of ten, and α_{Cd} varies over more than two orders of magnitude. The ratio α_{Cd}/α_{Zn} is not much more than unity for bacteria, about 10 for invertebrates, and about 100 for fish, which means that the relative toxicity of the two metals diverges with organism complexity. Overall, we can propose a tentative order of toxicity H < Al < (Cu Zn Pb UO_2) < (Cd Ag).

Tipping & Lofts [14] found that when exposure to toxic cations was expressed by F_{TOX} (i.e. accumulated cations) the overall range of values over which toxic effects change from 5% to 95% was 2.2-fold. Because the mean value of $F_{TOX,LT}$ and $F_{TOX,UT}$ is fixed, this range is effectively normalised. After normalising the CTU model results, the comparable range was much higher, 22-fold. The fact that the range depends upon the variable chosen to

316 express exposure was taken to indicate that variation among individual organisms in toxicity
317 tests is actually much less than might have been supposed. Tipping & Lofts [14] explained
318 this in terms of the variation of bound metal with solution concentration. The present results
319 are consistent with the previous ones, the combined data yielding a 5-95% toxic response
320 range of 3.7-fold with F_{TOX}, but 59-fold with the normalised CTU model.

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DISCUSSION

The WHAM-F_{TOX} model explains diverse data fairly successfully using a simple structure with few adjustable parameters. On average, the model explained 72% of the variance in the data considered here, with an average RMSD between observed and calculated toxic response of 20%. Some of the discrepancy must be due to the model's approximation of metal accumulation though its use of humic acid as a surrogate, and some perhaps to oversimplification of the relationship between organism-accumulated cations and toxic response. But the fitting of the four data sets of the present work was less than that obtained by Tipping & Lofts [14] for 11 different data sets (average RMSD 13%, average r^2 0.85). The poorer precision may partly reflect the greater ranges of experimental conditions covered by the present data sets, but must also result from noise in the experimental toxicity data. This is especially seen in the results of Meyer et al. [22] for *D. magna* (Index 4 in ref. 23) which were obtained by many replications or near-replications, show considerable variation in the apparent toxicity of Cd, and led us to remove a substantial part of the data set (328 points, 38% of the total) in order to conduct our analysis. The variation demonstrated by Meyer et al. [22] highlights the difficulties in obtaining reproducible results, which should be borne in mind when considering the results of the more usual, smaller scale, experiments. This means that parameters derived from the results of a single limited study cannot be taken as representative for that organism and the metals involved; they may better be regarded as a sample from a population of toxicity outcomes. Understanding the variability in toxicity experiment results is thus a pressing need.

A major motivation for carrying out this work within the MMME project was to ascertain the extents to which metals, or more generally cations, influence one another's toxicities. In WHAM-F_{TOX}, the postulated mechanism by which this occurs is chemical competition for the non-specific binding sites. The contribution of cations to the toxic effect is strictly additive, being based on bound cations, i.e. the "metabolically active body burden". Thus any interference among metals, toxic or non-toxic, is attributed to purely chemical effects. We recognise that this is likely an over-simplification, but in the absence of information about actual toxic receptor sites within organisms (see also below) it provides an approach that is readily implemented using available information about the chemical composition of the aquatic medium to which the organisms are exposed. How interference between metals can come about is illustrated in the simulations for Zn and Cd toxicity towards *D. magna* in Figure 6, which show how competition between metals with similar

chemical affinities but widely differing toxicities (α values), can lead to strong deviations from additivity. The same effect is also evident for the pairing of Cu and Cd (Figure 4), in the trout results of Figure 10, and is forecast for Ag-Cu competition in *L. sativa*, although less well supported by the experimental data (Figure 8, bottom right panel). For the present data, the superiority of WHAM-F_{TOX} over the CTU model arises chiefly from the inability of the latter to deal with these competitive effects. It is worth pointing out that, in general, the WHAM-F_{TOX} model does not recognise true single-metal toxicity because H^+ is ever-present so that even tests with a single added metal involve cation competition.

Even though WHAM-F_{TOX} has few adjustable parameters, it has not proved possible to define them very well with the data sets available thus far, since various combinations of parameter values yield similarly good fits. Therefore to constrain the fitting we resorted to fixing the average value of $F_{TOX,LT}$ and $F_{TOX,UT}$. The need to do this might be avoided if new data sets become available that cover ranges of pH, since this would constrain the relative α_i values, reducing the compensatory co-variance of α_i, $F_{TOX,LT}$ and $F_{TOX,UT}$. Although formally, the adjustable or potentially-adjustable parameters α_i, $F_{TOX,LT}$ and $F_{TOX,UT}$ are separate from the binding model (i.e. WHAM/Model VII), it must be borne in mind that the adjustments may compensate for inaccurate representation of exposure (accumulation by the organism) through the speciation calculations.

The above considerations demand circumspection in the interpretation of parameter values derived by the application of WHAM-F_{TOX} (Table 2), and the following discussion is offered with this in mind. Consideration of the α_i values suggests an approximate order of toxicity (see above), with bound Cd and Ag being especially toxic. Although the model does not specify how bound metals exert their toxic effect, the general picture is that the pool of non-specifically bound metal controls the supply of that metal to one or more key toxicity receptors, not in equilibrium or steady-state with the external solution. The high toxicities of accumulated Cd and Ag might therefore depend upon the presence of sulphur ligands in those receptors, since the two metals have appreciably higher affinities for S in organic compounds than do the other metals considered here [28]. If so, we would expect Cu(I), Hg(II) and CH_3Hg also to have high α values. However, relative binding affinities to S-containing example ligands would not readily explain the toxic mechanisms of Al, Cu, Zn, Pb and UO_2, since several metals usually considered non-toxic (Mg, Ca, Fe) have similar or higher affinities for S [28]. Therefore, from the WHAM-F_{TOX} results we can hypothesise that more than one type of receptor for metal toxicity must exist. The second implication of the

388 derived values of α_i is an apparent relationship of Cd toxicity to organism complexity, given
389 that the relative toxicity of Cd compared to Zn increases with order (see Results), and this
390 may prove to be informative in deciphering the chemical properties of toxic receptors.

391 These and our previous results [14], together with those reported by others in
392 connection with the MMME project [9-12, 22, 25, 29-31], have improved our understanding
393 of mixture effects with cations, but further measurements and modelling are needed to
394 provide a full picture. Data on a wider range of metals, and at different pH values, are
395 desirable, and there is a need to follow the approach of Meyer et al. [22] in designing metal
396 mixture experiments to challenge the models. In turn, the models might productively be used
397 to design such experiments. In addition, there is a large body of single-metal toxicity data, for
398 example those compiled in the ECOTOX database [32] which could fruitfully be analysed to
399 help establish patterns and trends among metals and toxicity test species, including durations
400 of exposure. The relatively sparse empirical data base on mixture effects, and the
401 complexities of both chemical speciation and mechanisms of toxicity, mean that more
402 research is required before we can confidently predict mixture toxicity in the field.

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CONCLUSIONS

The WHAM-F_{TOX} model was able to provide reasonable fits of four large and challenging data sets for metal toxicity to a daphnid, lettuce, and fish. In three cases the fit was clearly superior to that obtained using a conventional additive model based on solution exposure. Lack of precision in fitting must partly be due to the over-simplifications of the model, but a major factor is noise in the experimental data.

Strong non-additive effects of metal mixtures were apparent in the data for tests involving Cd, the toxicity of which could be markedly reduced by Cu and, particularly, Zn. WHAM-F_{TOX} could explain this effect approximately, on the basis of purely chemical competition for the accumulation of metals by the organisms.

Tentative interpretation of parameter values obtained from these and other data suggests the following order of bound cation toxicity: H < Al < (Cu Zn Pb UO_2) < (Cd Ag). Another trend is a strong increase in Cd toxicity relative to that of Zn, as organism complexity increases (from bacteria to fish).

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517

518 Table 1. Summary of data and fitting *results. Italicised alpha values* for H and Al were fixed
519 or assumed. RMSD is the root-mean-squared deviation between observed and calculated
520 values, and the r^2 refers to comparison of observed and calculated values.

	Daphnia magna	*Lactuca sativa*	*Oncorhynchus clarkii lewisi*	*Oncorhynchus mykiss*
Index (ref. 17)	4	9	6	6
End point	48 hr survival	96 hr root elongation	96 hr survival	96 hr survival
n	542	238	162	207
temperature ^oC	18.9 – 25.4	15	8.1 – 12.1	6.8 – 11.1
pH	7.0 – 8.7	7.0	6.6 – 7.6	6.0 – 7.6
metals	Cu, Zn, Cd	Cu, Zn, Ag	Zn, Cd, Pb	Zn, Cd, Pb
DOC mg L^{-1}	0.2 – 4.2	0.5	0.2 – 0.7	0.6
Ca mg L^{-1}	12 – 16	115	3 - 26	2 - 20
WHAM-F_{TOX} parameters and fits				
$F_{TOX,LT}$	1.85	1.40	2.39	2.13
$F_{TOX,UT}$	6.39	6.84	5.85	6.11
α_H	*1.0*	*1.0*	*1.0*	*1.0*
α_{Al}	-	-	*2.1*	*2.1*
α_{Cu}	21.9	12.2	-	-
α_{Zn}	8.4	3.5	6.7	10.3
α_{Cd}	320	-	1790	2070
α_{Ag}	-	2090	-	-
α_{Pb}	-	-	6.7	4.6
RMSD (%)	25	14	17	24
r^2	0.65	0.78	0.81	0.64
CTU fits				
RMSD	26	14	25	26
r^2	0.59	0.80	0.56	0.58

521

522 Table 2. Summary of parameter values from this study and Tipping & Lofts [14].

test organism	toxic response	$F_{Tox,LT}$	$F_{Tox,UT}$	α_H	α_{Al}	α_{Cu}	α_{Zn}	α_{Cd}	α_{Ag}	α_{Pb}	α_{UO2}
Escherichia coli	luminescence inhibition 15 min	3.57	4.67	1.0		3.2	13.9	18.6			
Pseudomonas fluorescens	luminescence inhibition 15 min	3.29	4.95	1.0		4.0	14.0	23.3			
Vibrio fischeri	luminescence inhibition 5 min	2.45	5.79	1.0				3.8		4.1	
Lemna aequinoctialis	growth rate 96 hr	2.24	6.00	1.0		20.8					16.0
Lemna paucicostata	growth rate 96 hr	1.73	6.51	1.0		2.7		7.6			
Lactuca sativa	root elongation 96 hr	1.40	6.84	1.0		12.2	3.5		2091		
Ceriodaphnia dubia	survival 96 hr	1.90	6.34	1.0			5.8	65.3			
Daphnia ambigua	survival 96 hr	2.04	6.20	1.0			5.5	133.7			
Daphnia magna	survival 96 hr	3.03	5.21	1.0			4.6	27.0			
	survival 48 hr	1.94	6.30	1.0		21.2	9.0	314.4			
Daphnia pulex	survival 96 hr	1.79	6.45	1.0			6.5	63.8			
Dreissena polymorpha	filtration rate 48 hr	1.84	6.40	1.0		30.3	6.0	85.8			
Oncorhynchus mykiss	survival 144 hr	2.39	5.85	1.0	2.1	11.9	4.6				
	survival 96 hr	2.13	6.11	1.0			10.3	2070		4.6	
Oncorhynchus clarkii lewisi	survival 96 hr	2.39	5.85	1.0			6.7	1790		6.7	

523

524

525

Figure captions

Figure 1. WHAM-F_{TOX} fit of selected *D. magna* toxicity data (542 points; see the text for explanation).

Figure 2. Effects of Cu on Zn toxicity towards *D. magna*, pH 7.6 – 7.9, [DOC] 2.9 – 3.3 mg L^{-1} [22]. The top left panel shows the effect of Zn alone, the other three show the effects of Cu at fixed concentrations of Zn (mg L^{-1}). Solid and dashed lines are WHAM-F_{TOX} and CTU fits respectively to the entire data set. Note that the lines simply join discrete predicted points.

Figure 3. Effects of Zn on Cu toxicity towards *D. magna*, pH 7.4 – 7.9, [DOC] 3.0 – 3.1 mg L^{-1} [22]. The top left panel shows the effect of Cu alone, the other three show the effects of Zn at fixed concentrations of Cu (mg L^{-1}). Solid and dashed lines are WHAM-F_{TOX} and CTU fits respectively to the entire data set. Note that the lines simply join discrete predicted points.

Figure 4. Effects of Cu on Cd toxicity towards *D. magna*, pH 7.9 – 8.2, [DOC] 3.3 – 3.5 mg L^{-1} [22]. The top panels show the effect of Cd alone, the remainder show the effects of Cu at fixed concentrations of Cd (mg L^{-1}). Solid and dashed lines are WHAM-F_{TOX} and CTU fits respectively to the entire data set. Note that the lines simply join discrete predicted points.

Figure 5. Effects of Zn on Cd toxicity towards *D. magna*, pH 8.0 – 8.4, [DOC] 2.9 – 3.3 mg L^{-1} [22]. The top panels show the effect of Cd alone, the remainder show the effects of Zn at fixed concentrations of Cd (mg L^{-1}). Solid and dashed lines are WHAM-F_{TOX} and CTU fits respectively to the entire data set. Note that the lines simply join discrete predicted points.

Figure 6. WHAM-F_{TOX} outputs (ν_i and F_{TOX}) for Cu-Zn and Zn-Cd toxicity experiments with *D. magna* [22]. The left panels refer to experiments with 0.33 mg Zn L^{-1} and varying Cu concentration, pH 7.9 and [DOC] 3.2 mg L^{-1}. The right panels refer to 0.023 mg Cd L^{-1} and varying Zn concentration, pH 8.3 and [DOC] = 3.2 mg L^{-1}. In the bottom panel, the values of total F_{TOX} include the contribution form H^+, and the horizontal lines show $F_{TOX\text{-}LT}$ and $F_{TOX\text{-}UT}$.

555 Figure 7. WHAM-F_{TOX} fit of the data of Le et al. [24,25] for Cu, Zn and Ag toxicity
556 to *Lactuca sativa*.

557 Figure 8. Observed (points) and fitted metal mixture toxicity to *Lactuca sativa*., data
558 of Le et al [7, 24]. In each case the (constant) free-ion concentration of one of the
559 metals increases in the order open circles < closed circles < open squares. Panel (a),
560 fixed metal Cu, free-ion concentrations 1.3, 37, 190 nM. Panel (b), fixed metal Zn,
561 free-ion concentrations 1.0, 42, 120 μM. Panel (c), fixed metal Cu, free-ion
562 concentrations 0.12, 100, 400 nM. Panel (d), fixed metal Ag, free-ion concentrations
563 18, 150, 210 nM.

564 Figure 9. WHAM-F_{TOX} fits of the data of Mebane et al. [25] for Zn, Cd and Pb
565 toxicity to trout species.

566 Figure 10. Variation of toxic response by trout species towards Cd, at low (open
567 symbols, solid lines) and high (filled symbols, dashed lines) concentrations of Zn.
568 The fits refer to the entire Mebane et al. [25] datasets. For cutthroat trout, the high Zn
569 concentration ranged from 49 to 905 μg L^{-1}, for rainbow trout it was 83.5 μg L^{-1}.
570 Concentrations of Pb were at or near the limits of detection in all cases.

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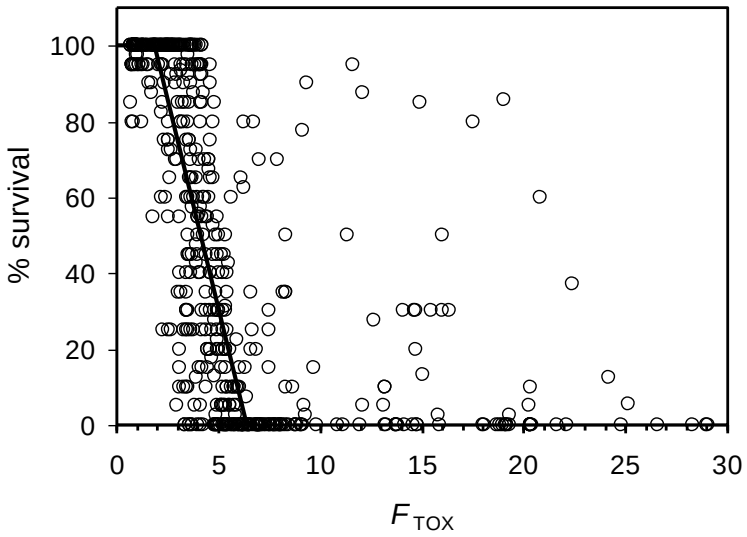


Figure 1.

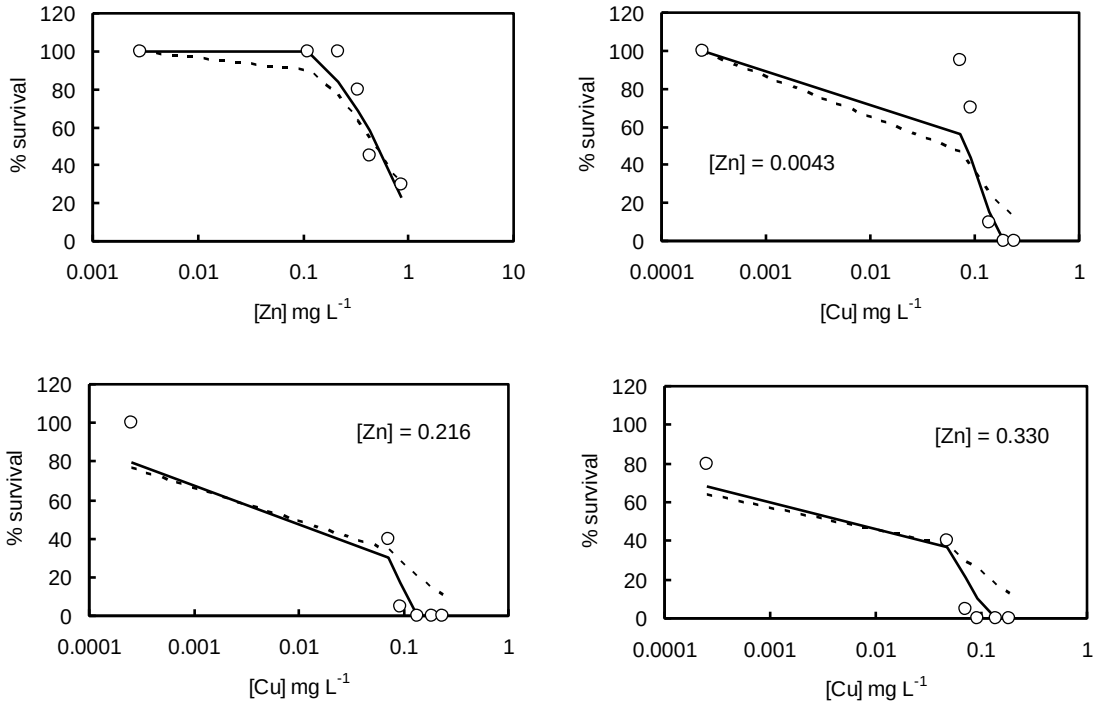
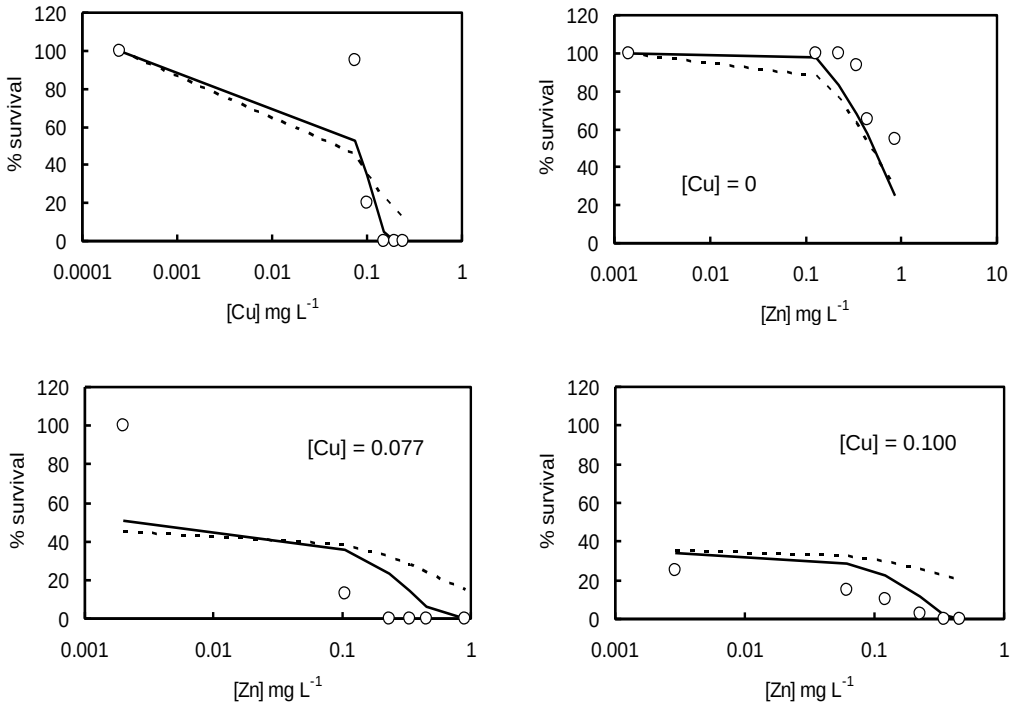

% survival
[Zn] mg L-1
[Zn] = 0.0043
[Cu] mg L-1
[Zn] = 0.216
[Cu] mg L-1
[Zn] = 0.330
[Cu] mg L-1

Figure 2.



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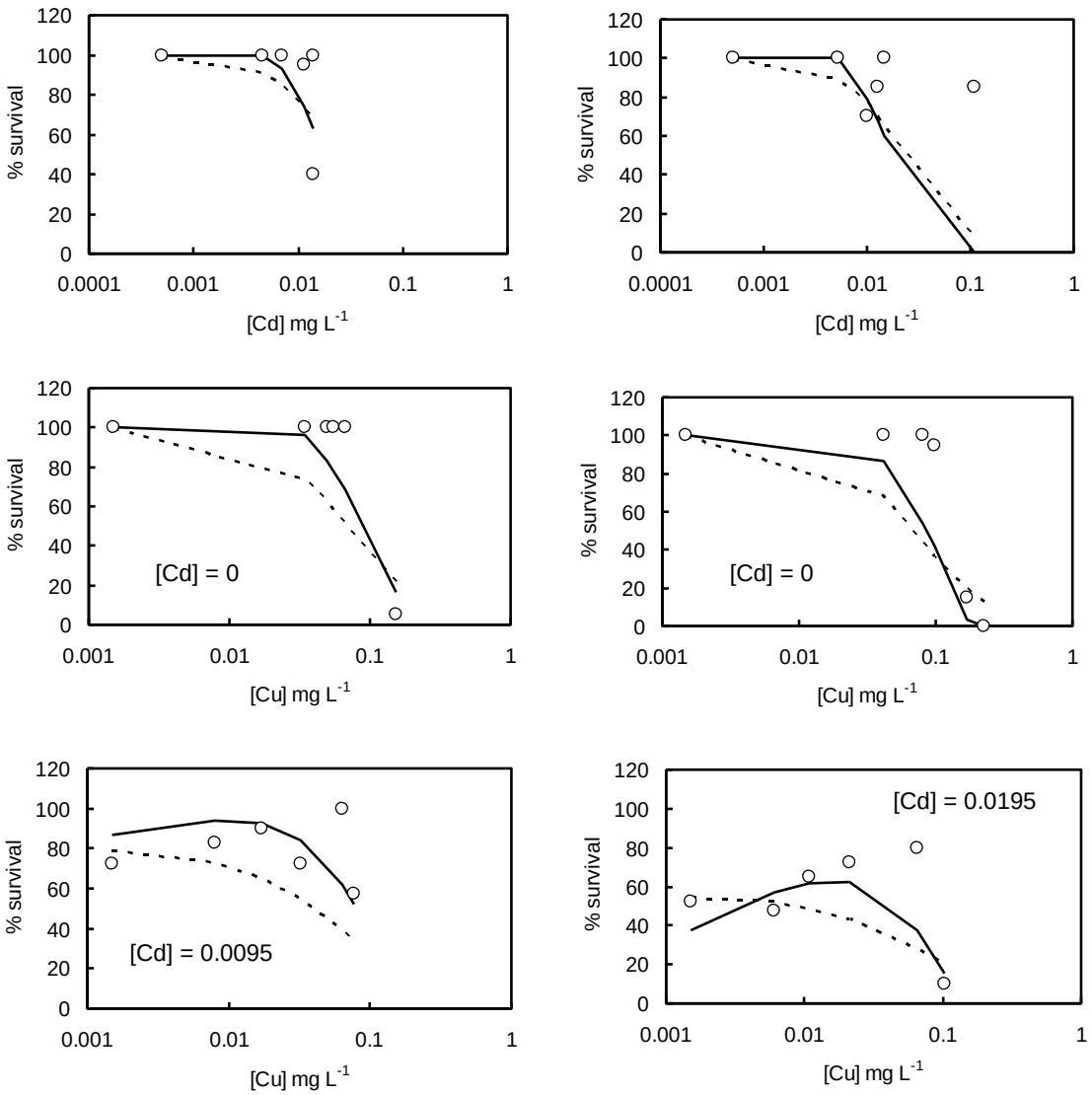

Figure 4.

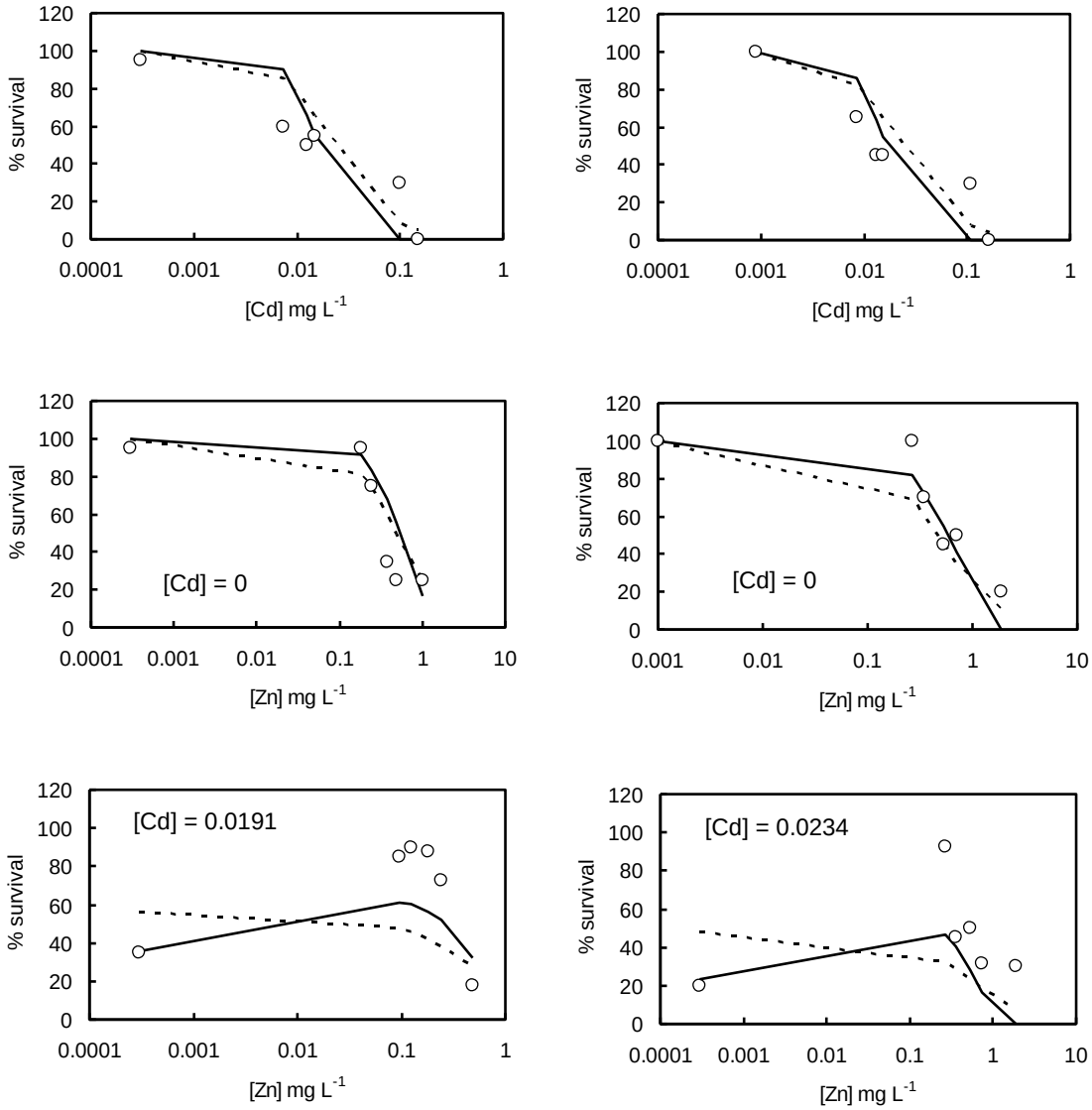

Figure 5.

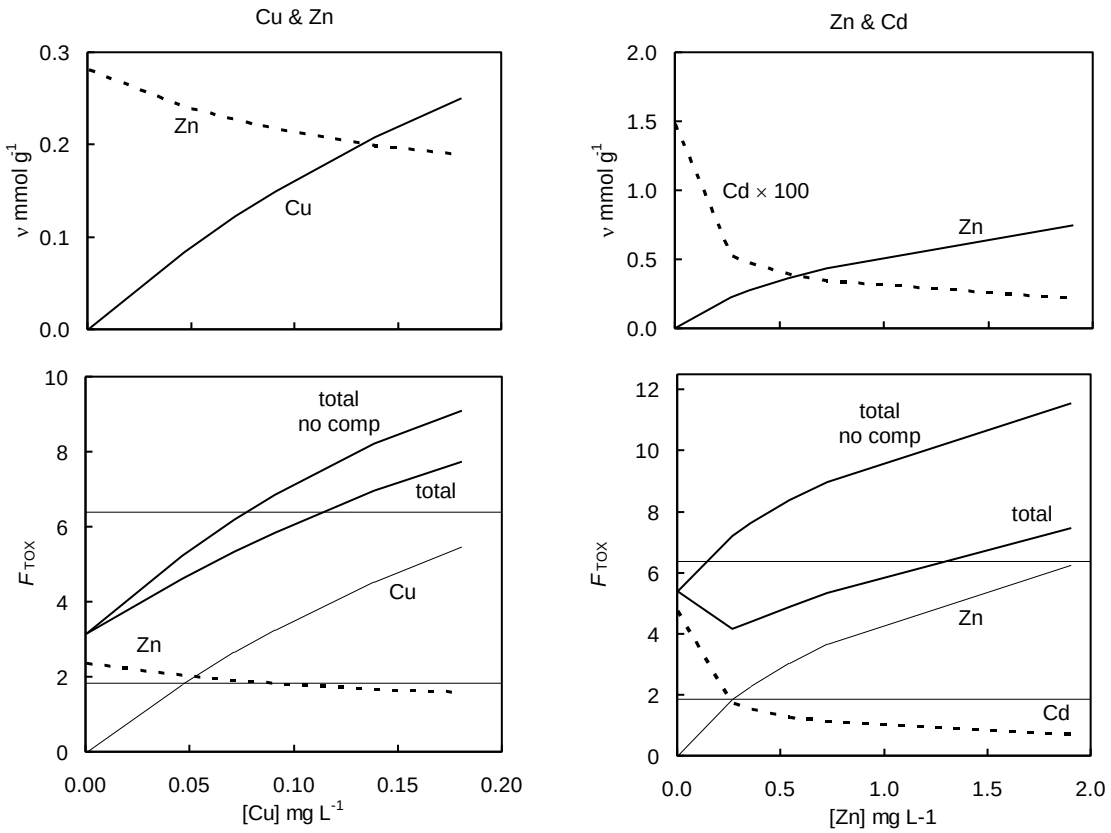
Cu & Zn
Zn & Cd
ν mmol g-1
Zn
Cu
Cd × 100
total
no comp
total
Cu
Zn
Cd
FTOX
[Cu] mg L-1
[Zn] mg L-1

Figure 6.

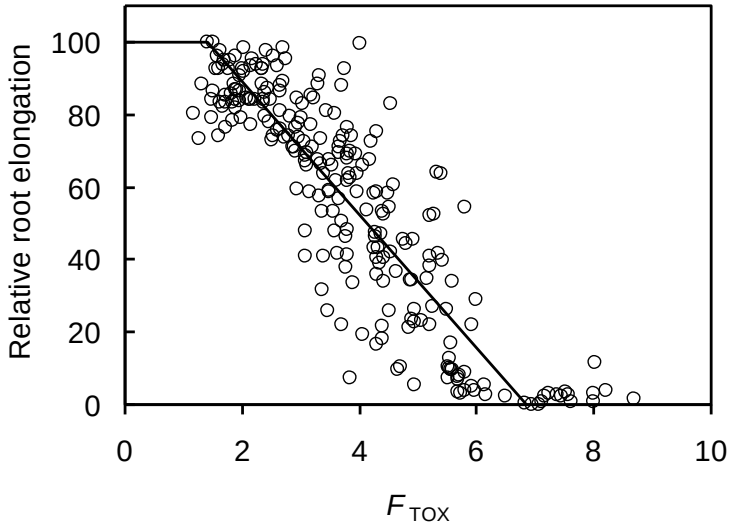

Figure 7.

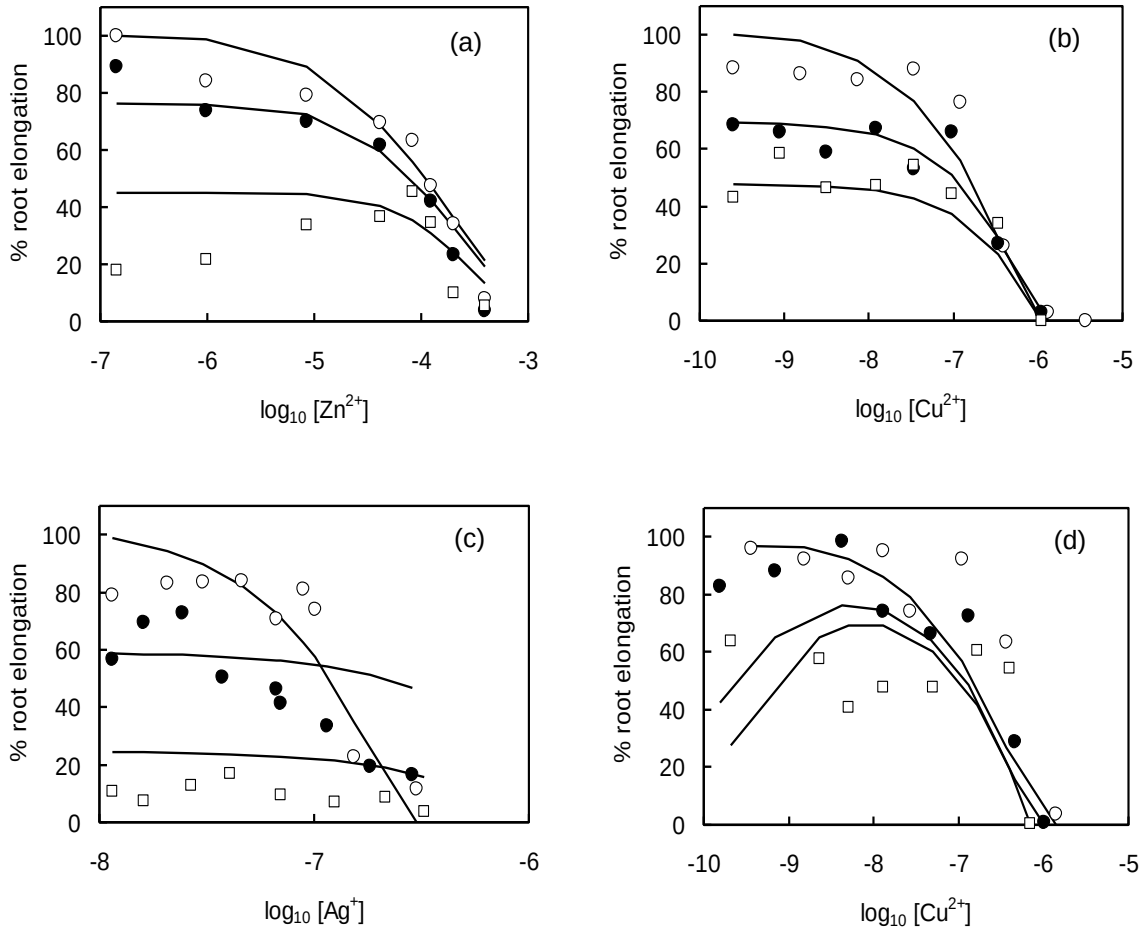
(a)
% root elongation
log$_{10}$ [Zn^{2+}]
(b)
% root elongation
log$_{10}$ [Cu^{2+}]
(c)
% root elongation
log$_{10}$ [Ag^{+}]
(d)
% root elongation
log$_{10}$ [Cu^{2+}]

Figure 8.

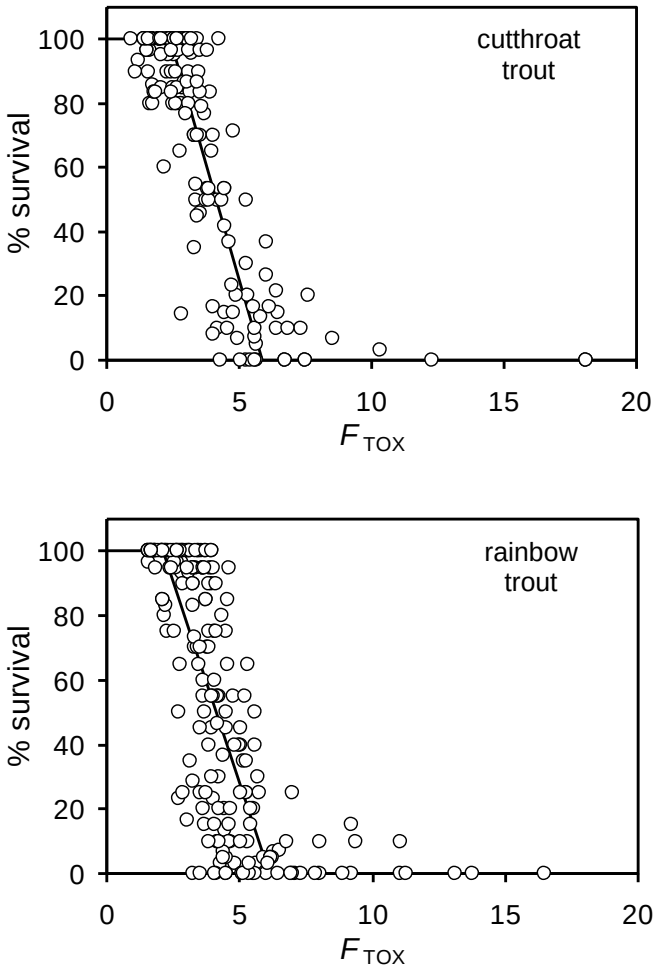
cutthroat trout
% survival
100
80
60
40
20
0
0
5
10
15
20
F_{TOX}
rainbow trout
% survival
100
80
60
40
20
0
0
5
10
15
20
F_{TOX}

Figure 9.

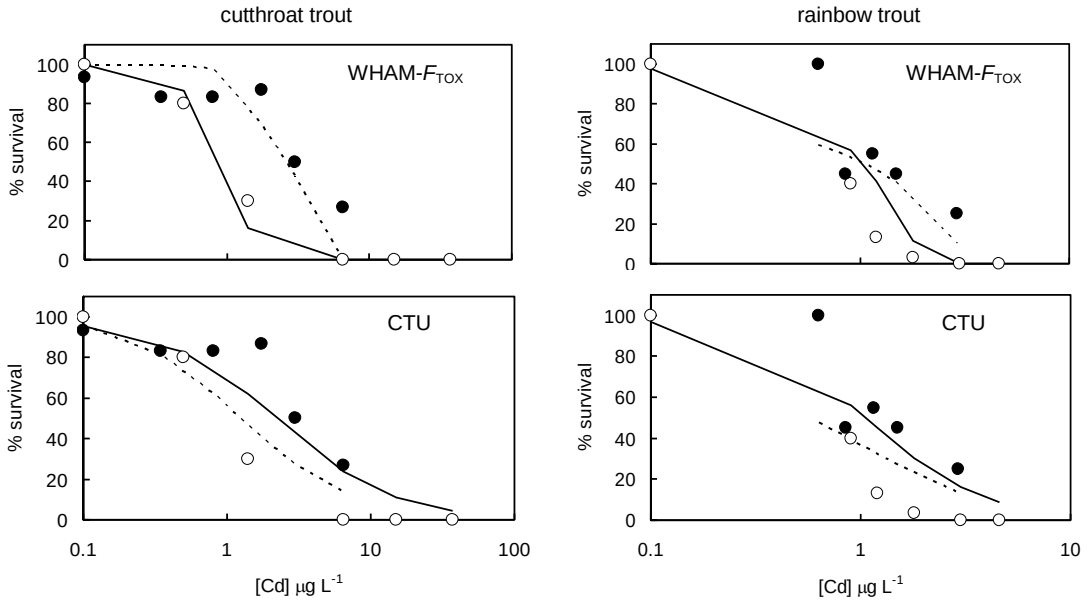
cutthroat trout
rainbow trout
WHAM-F_{TOX}
CTU
% survival
[Cd] μg L^{-1}
0
20
40
60
80
100
0.1
1
10
100

Figure 10.