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Article

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A systematic evaluation of chronic metal mixture toxicity to three species and implications for risk assessment

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ABSTRACT

Metal contamination generally occurs as mixtures. However, it is yet unresolved how to address metal mixtures in risk assessment. Therefore, using consistent methodologies, we have set up experiments to identify which mixture model applies best at low level effects, i.e. the independent action (IA) or concentration addition (CA) reference model. Toxicity of metal mixtures (Ni, Zn, Cu, Cd, and Pb) to *Daphnia magna, Ceriodaphnia dubia*, *and Hordeum vulgare* was investigated in different waters or soils, totaling 30 different experiments. Some mixtures of different metals, each individually causing <10% inhibition, yielded much larger inhibition (up to 66%) when dosed in combination. In general, IA was most accurate in predicting mixture toxicity, while CA was most conservative. At low effect levels important in risk assessments, CA overestimated mixture toxicity to daphnids and *H. vulgare* on average with a factor 1.4 to 3.6. Observed mixture interactions could be related to bioavailability, or by competition interactions either for binding sites of dissolved organic carbon or for biotic ligand sites. Our study suggests that the current metal-by-metal approach in risk evaluations may not be conservative enough for metal mixtures.

Introduction

Metal contamination in the environment occurs generally as mixtures of metals. However, environmental risk evaluation and derivation of environmental quality standards for metals in soil and water are mainly based on metal-by-metal assessments. As a consequence, these assessments implicitly assume that the risk of mixtures is related to the risk of the most toxic metal. A mixture of different metals can, however, have a larger effect than each metal individually, because the effects of each metal add up or because metals can even interact and act synergistically. In the European Union, however, it has been anticipated that future environmental risk assessment procedures for metals will require the consideration of mixture effects.[1]

For risk assessment purposes, it is crucial that the effect of a mixture on an organism can be predicted from the known effect of each single component in that mixture. In mixture toxicity studies, two contrasting concepts are most commonly used to predict mixture effects; independent action (IA) and concentration addition (CA). Conceptually, these models are based on different assumptions related to the modes of action (MoA) of a substance. The CA model is used when two or more chemicals have a similar MoA, e.g. they target the same enzyme. CA is based on the dilution-principle and assumes that any component of a mixture can be replaced by an equi-effective concentration of another component, without altering the overall effect of the mixture.[2] The concept can be mathematically expressed for a mixture of n individual chemicals as:

$$\sum TU_{ECx} = \sum_{i=1}^{n} \frac{Ci}{ECx_i} = 1 \qquad (1)$$

where $\sum TU_{ECx}$ (the mixture dose) is the sum of toxic units (TU) expressed relative to the x% effect concentration (ECx). c_i is the concentration of component i in the mixture and ECx_i is the

x% effect concentration, i.e. the concentration of component i in the mixture that yields x% effect in a single substance exposure. If for a mixture that causes x% effect, the $\sum TU_{ECx}$ equals 1, then CA holds.

IA is used when two or more chemicals have different MoA.[3] IA is embedded in the statistical theory of independent random events. It is assumed that the susceptibilities of individual organisms to each of the chemicals in the mixture are statistically independent. Therefore, the predicted relative effect ($RE_{mix,pred}$) of a mixture with n chemicals can be calculated by multiplication of the non-effects of the individual substances in the mixture (Eq. 2).[4]

$$RE_{mix,pred} = 100\% - \left(\prod_{i=1}^{n}[100\% - RE(c_i)]\right) \quad (2)$$

with $RE(c_i)$ the relative effect (ranging between 0% and 100%, with 100% resulting in full inhibition) of the individual component i at a concentration of c_i.

Both CA and IA depart from the idea that substances do not interact at target sites, also described as ‘additivity’. However, this assumption is not always fulfilled because substances can enhance or diminish each other’s toxicity, i.e. substances may interact when combined in a mixture. If the observed mixture effect is larger (smaller) than expected based on the reference model, the mixture acts synergistically or ‘more than additive’ (antagonistically or ‘less than additive’).[5,6]

Toxicity studies with metal mixtures have shown that mixture effects are difficult to predict as all potential outcomes have been observed.[7,8] A review of 191 studies with aquatic species showed that 43% of the mixture effects were antagonistic, 27% non–interactive and 29% synergistic.[7] The interactions vary with species,[9] metal combinations and doses.[7] In addition, it has been shown that interactions can be contradictory across different experiments,[10] or that interactions can be strongly concentration-dependent.[10,11] Although plenty of metal mixture studies have been conducted, many of the published studies suffer from statistical design issues due to non-simultaneous testing of single and combined effects, resulting in high probabilities of drawing false conclusions about the predicting ability of the CA model.[12] In addition, most of these studies deal with acute toxicity at relatively high doses, which is less relevant for environmental risk assessment. Mixture effects and mixture interactions are predictably different in chronic tests than in acute test, since the latter do not fully account for metal interactions taking place during longer term detoxification. Current reviews have concluded that there is yet insufficient knowledge about the validity of the mixture reference models for chronic metal mixture toxicity at low effect doses to allow the integration of metal mixture toxicity in risk assessment frameworks.[13,14] The unresolved environmental questions remain how mixture toxicity should be incorporated in risk assessment and if CA or IA is the most accurate model and which one is conservative at low effect levels.

To address the latter issue, we set up a project to investigate toxicity of metal mixtures (Ni, Zn, Cu, Cd, and Pb) using a similar methodology on three different species, i.e. tests with *Hordeum vulgare* (growth inhibition experiments in solutions and soils),[15-18] *Daphnia magna* (reproductive toxicity),[19] and *Ceriodaphnia dubia* (reproductive toxicity).[20, 21] Many of these tests showed that metals interact in their effects on organisms when supplied as a mixture, or, in other words, that perfect CA or IA is often violated. In addition, the interactions vary with the considered reference model,[19-20] species,[21] metal combination,[15,21], water chemistry of the medium,[17,18,20] metal concentration ratio,[20] expression of the dose,[18,21] and the concentration of the individual metals in the mixture.[19] Because each of these studies on their own represented a relatively limited number of experiments, no general conclusions about chronic metal mixture effects could be derived for environmental risk assessment applications from any individual study. Therefore, we performed in the present study a systematic analysis of all data obtained in our project (and published in these studies). The general objectives of this study were to collate all the mixture data and summarize it into generalities that can be used in risk assessment.

The first question addressed in this paper is identifying if metal mixture effects are relevant, i.e. identifying whether significant mixture effects (defined as effects resulting in more than 10% inhibition) occur for mixtures where each of the individual metals are present below their 10% effect concentration (EC10). The related scientific questions are to determine which of both reference models (CA or IA) generally applies across species and media and to investigate whether the commonly suggested CA model is conservative at low effect concentrations. The second question was whether bioavailability can explain observed interactions, i.e. if there are competition effects, either on binding sites in the medium (dissolved organic matter; particulates) or on biotic ligand (BL) sites.

Methods

Description of data: Only peer-reviewed studies were included in the analysis. Invertebrates (*D. magna* & *C. dubia*: 21d and 7d reproductive toxicity, respectively)[19-21] and a higher plant (*H. vulgare*: 4d or 14d growth inhibition)[15-18] were exposed to various mixtures of metals (Cu, Ni, Zn, Cd, and Pb) in water (natural and reconstituted) and/or soils (Table 1: Table S1). Details of the designs and tests are given in the corresponding references.[15-21] The experimental work focused on (chronic) exposure at low effect metal doses to increase the environmental relevance of the work. The 4d-barley root elongation test is an acute test. However, it has been shown that this test is in general more sensitive to metal toxicity than a 21d-tomato yield test.[22] In all experiments, the dose-response relationship of single metals was investigated simultaneously with the toxicity of the metal mixtures, to avoid any possible bias in subsequent data interpretation due to temporal sensitivity variations.[12]

Table 1. Overview of mixture combinations included in the collation of metal mixture toxicity experiments.

Species	Mixture combination	Number of experiments	Number of treatments
Ceriodaphnia dubia[20,21]	Ni-Zn, Pb-Zn, Ni-Pb, Ni-Zn-Pb	10	185
Daphnia magna[19]	Ni-Zn	2	66
***Hordeum vulgare* (solution)**[15,16,17]	Cu-Zn, Cu-Cd, Ni-Zn, Cd-Zn, Ni-Cd, Cu-Cd-Zn, Ni-Zn-Cd, Cu-Ni-Cd-Zn	15	225
***Hordeum vulgare* (soil)**[18]	Cu-Zn	3	54

Speciation calculations Because free ion activities are more representative for metal toxicity than total or dissolved concentrations, we calculated chemical speciation of the metals in the test water solutions or soil solutions based on measured concentrations with WHAM VII[23] for *C. dubia,* and *D. magna* and with WHAM VI[24] for *H. vulgare*. Specific assumptions used for speciation modelling can be found elsewhere for daphnids,[20,21] and barley.[18] Results of the analyses based on free ion activities are given in the main paper, while for daphnids those based on dissolved concentrations are also given in Supplemental Data. For barley, calculated free ion activities in solutions were within 95% of measured total dissolved metal concentrations, therefore analysis were only conducted on free ion activities.

Individual metal effects First, responses were expressed as relative effects (%), i.e. the effect expressed relative to the control of the respective experiment, using Eq. 3. Then, for every experiment separately, dose-response curves were fitted to the individual metal exposure data, using a two parameter log-logistic concentration response model (Eq. 4)

$$RE_{j,k} = 100\% - RR_{j,k} = 100\% - 100\% \times \frac{R_{j,k}}{R_{con,k}} \quad (3)$$

$$RE_{Me_i,k} = 100\% \times \left(1 - \left(\frac{1}{1+\left(\frac{x_{Me_i,k}}{EC50_{Me_i,k}}\right)^{\beta_{Me_i,k}}}\right)\right) \quad (4)$$

In equation 3, $RE_{j,k}$ is the relative effect of treatment *j* in experiment *k* (%), $RR_{j,k}$ is the relative response of treatment *j* in experiment *k* (%). $R_{j,k}$ is the response in treatment *j* in experiment *k* (number of juveniles for daphnids, and root elongation for barley) and $R_{con,k}$ is the response in the control treatment of experiment *k*. In Equation 4, $RE_{Mei,k}$ is the predicted individual relative effect of metal *i* in experiment *k* (%), $x_{\mathrm{Mei,\,k}}$ is the total metal concentration, dissolved concentration or free ion activity of metal *i* in experiment *k*. $EC50_{Mei,k}$ is the fitted 50% effective concentration of metal *i* in experiment *k*. $\beta_{Mei,k}$ is the fitted slope parameter of the dose response curve of metal *i* in experiment *k*. The estimated $EC50_{Mei,k}$ and $\beta_{Mei,k}$ for all experiments are summarized in Supplemental Data 2 (Table S2 and Table S3 for barley and daphnids, respectively).

Importance of metal mixture toxicity The importance of metal mixture toxicity was investigated by comparing the observed mixture effect in each treatment with the individual effect of the most toxic metal in that treatment. The latter effect was predicted from the fitted dose-response curves of the individual metals (Eq. 4). Mixture effects in a treatment were defined to be significant if more than 10% inhibition was observed and if the most toxic metal in that treatment would result in less than 10% inhibition when applied individually.

Predictive performance of mixture models The adherence of chronic metal mixture toxicity to either CA or IA is crucial for the incorporation of metal mixture toxicity in future risk assessments. Previously, it has been advocated to use *a priori* knowledge of the MoA of the mixture constituents to apply either CA or IA in environmental mixture risk assessments.[25] However, the MoAs of metals are often complex and not always fully understood.[26] Moreover, certain MoA may be shared between metals (e.g. formation of reactive oxygen species), while others are unique to a metal (e.g. disruption of the homeostasis of a specific cation). In addition, MoA of metals may be different between species. As a consequence, the choice for the application of either CA or IA based on the presumed mode of action of the components of the mixture is almost impossible. In the present study, the mixture data were, therefore, analyzed to compare the accuracy of both existing mixture reference models (CA and IA) over the complete dose response of the mixture. Both concepts assume that the effect of a mixture on an organism can be predicted from the known effects of each single component in the mixture.

Hence, the mixture effects ($RE_{mix,pred}$) were predicted with both mixture models using the parameters of the fitted dose-response curves of the individual metal exposures ($EC50_{Mei,k}$ and $\beta_{Mei,k}$ calculated with Eq. 4 and reported in Supplemental Data 2), i.e. with Eq. 5 for CA and Eq. 6 for IA.[27]

$$\sum_{i=1}^{n} \frac{x_{Me_i,k}}{EC50_{Me_i,k} \times \left(\frac{RE_{mix,pred,k}}{100-RE_{mix,pred,k}}\right)^{\frac{1}{\beta_{Me_i,k}}}} = 1 \qquad (5)$$

$$RE_{mix,pred,k} = 100 \times \left[1 - \prod_{i=1}^{n} \left(\frac{1}{1+\left(\frac{x_{Me_i,k}}{EC50_{Me_i,k}}\right)^{\beta_{Me_i,k}}}\right)\right] \qquad (6)$$

Where, $RE_{mix,pred,k}$ is the predicted mixture effect (%) relative to the control in experiment *k*. The non-linear equation of CA (Eq. 5) was solved for $RE_{mix,pred}$ using the generalized reduced gradient iterative solver function in Excel 2010. Predictions were made for every experiment separately, using the dose-response parameters of each specific experiment. The performance of CA versus IA was evaluated by comparing the predicted RE_{mix} to the observed RE_{mix}, using the root mean squared error (RMSE).

Protectiveness of CA at low effect sizes In a last step, the accuracy of the CA model at low effect sizes was evaluated. The CA model is often adopted in risk assessment due to its simplicity.[28] However, it is therefore crucial that the CA model is protective at the low effect levels important for risk assessment, i.e. the EC10-level in European risk assessment frameworks. This was evaluated by estimating the deviations from additivity relative to CA using the TU-approach at the EC10 level. Concentrations of all metals in the mixture were first expressed as TU relative to their EC10 using Eq. 1. $\sum TU_{EC10}$ is then defined as the sum of toxic units of all metals. If the CA model is conservative at low effect sizes (i.e. at or below the 10% effect size) than the observed mixture effect should be lower than 10% for mixture treatments in which $\sum TU_{EC10}<1$. To evaluate the CA model at these low effect sizes, we then fitted for every experiment a 2-parameter log-logistic dose response curve (Eq. 5; see Supplemental data 3) to the dose response data of the mixture treatments in that experiment with the mixture dose expressed as $\sum TU_{EC10}$. This fitting was done in Statistica 7 and yielded an estimated $EC10_{\sum TUEC10}$ for each experiment separately.

$$RE_{mix,k} = 100 - \left(\frac{100}{1+\frac{1}{9}\left(\frac{\sum TU_{EC10,k}}{EC10_{\sum TU_{EC10,k}}}\right)^{\beta_k}} \right) \quad (7)$$

In Eq. 7, the $RE_{mix,k}$ is the relative mixture effect (%; relative to the control) in experiment *k*, predicted with the fitted dose-response curve for experiment *k* as a function of the mixture dose $\sum TU_{EC10}$. The $EC10_{\sum TUEC10}$ can be regarded as a measure of deviation from the CA reference model at low effect sizes (i.e. 10% effect). Theoretically, if CA holds, the $EC10_{\sum TUEC10}$ is equal to 1. When $EC10_{\sum TUEC10}<1$, CA tends to underestimate mixture toxicity effects (i.e. trend towards synergism), while a $EC10_{\sum TUEC10}>1$ means that CA tends to overestimate mixture effects (i.e. trend towards antagonism). An $EC10_{\sum TUEC10}$ was estimated for each mixture experiment separately, i.e. *C. dubia* (*n*=10 experiments), *D. magna* (*n*=2), and solution culture *H. vulgare* (*n*=10). Only these experiments were selected that included points with low mixture effects (<20 % effect). To evaluate the overall protectiveness of CA, a mean and median $EC10_{\sum TUEC10}$ was calculated for every species. The distribution of mixture interaction for *H. vulgare* and *C. dubia* were visualised as observed cumulative distribution plots.

Results & Discussion

Importance of metal mixture toxicity

Presently, the environmental risk assessment of metals and the derivation of environmental quality standards in most regions, such as the European Union, are performed on a metal-by-metal basis.[29] However, in the environment organisms mostly encounter multi-metal contaminations. Hence, metal-by-metal environmental risk assessment procedures are only conservative if metal mixture effects are not larger than the individual effect of the most toxic metal in the mixture. Our data clearly show that this condition is not met. Indeed, if the entire concentration response range of the investigated metal mixtures was considered, observed effects in 62% to 73% of the mixture treatments were larger than the effect of their most toxic metal when tested in isolation, the range representing the three different species (Figure 1; Table S4). We acknowledge that mixture treatments combining metals below their individual 10% effect levels are considered to be more relevant for risk assessment (all points at the left side of the vertical dashed lines in Figure 1).[29] Yet, even in that group, still 26% to 72% of the mixture treatments caused more than 10% mixture effect, even when all metals in a mixture caused less than 10% effect individually (points in the red shaded area in Figure 1). The latter indicates that combining metals below their individual EC10 can result in significant mixture effects, with a 90-percentile inhibition of 41% for *H. vulgare*, 24% for *C. dubia,* 17% for *D. magna*, and inhibitions reaching up to 66% (Figure 1). At the other side of the spectrum, when all metals in a mixture caused less than 10% effect individually, a stimulation effect of more than 10% was observed in 16% to 37% of the mixture treatments (points in the blue shaded area in Figure 1), with stimulations reaching up to 37%. On average for all species and tests, there was 6% inhibition in the zone where each metal caused <10% inhibition individually.

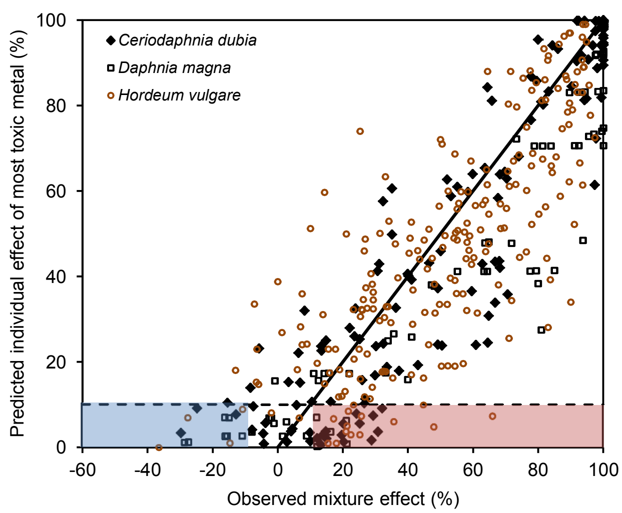

Figure 1. Predicted individual effect of the most toxic metal in the mixture (%) plotted against the observed mixture effect (%) for *Ceriodaphnia dubia* (diamonds), *Daphnia magna* (squares), and *Hordeum vulgare* (circles). The observed mixture effect was larger than the predicted individual effect of the most toxic metal for 64% of the data over the three species (points below the diagonal line). In 22% of the data the most toxic metal was present at a concentration causing on itself less than 10% (area below the horizontal dashed line), these are the situations of highest relevance for risk assessment. For 26% to 72% of these data, depending on the species, significant mixture effects occurred (red shaded area), i.e. predicted individual effect of the most toxic metal is smaller than 10%, while the observed mixture effect is larger than 10%. The blue box indicates the situations where the predicted individual effect of the most toxic metal is smaller than 10%, while the observed mixture effect is smaller than -10% (i.e. stimulation effect).

Prediction performance of the mixture reference models

The reproducibility of mixture toxicity by a reference model is crucial for future metal mixture risk assessment approaches. Therefore, we evaluated the prediction performance of two generally applied mixture reference models: IA and CA, both using the free ion activity in solution as the dose; soil data were excluded. Combining data of all performed mixture toxicity experiments per species, showed CA predicted metal mixture toxicity to *D. magna* slightly more accurately than IA. For *C. dubia* and *H. vulgare*, however, IA is a more accurate predictor of mixture toxicity than CA (lowest RMSE; Figure 2 and Figure S2). Interestingly, the performance of CA relative to IA for *H. vulgare* may be dependent on the metal mixture combination (Figure S2). Indeed, for *H. vulgare,* IA resulted in more accurate predictions than CA for all mixture combinations with Zn, while in the mixtures without Zn CA was (slightly) more accurate. It has previously been argued that IA and CA can be seen as two extremes of a prediction continuum between which the toxicity of mixtures of substances with not entirely similar or entirely dissimilar modes of action are expected to fall.[30] When Zn is present in the mixtures for *H. vulgare*, the IA becomes more accurate (Table S5; Figure S2). The number of metal mixture combinations evaluated for *D. magna* and *C. dubia* is too limited to evaluate any such tendency for these species.

Ni-Zn is the only mixture combination that has been evaluated for all three species. Comparison between CA and IA for this mixture, suggests that the goodness of fit of both mixture models might be dependent on the biological species (Table S5; Figure S3). For *D. magna*, CA predicts Ni-Zn mixture toxicity (slightly) better compared to IA, while for *H. vulgare* and *C. dubia* IA is clearly a better predictor of Ni-Zn mixture toxicity than CA. However, water chemistry can have an effect on the magnitude of observed mixture effect (see *section 'Multi-metal bioavailability effects'*). Because the medium differed between the toxicity tests of these three species, it is not clear whether these differences in goodness of fit between CA & IA are related to the species considered or to the chemistry of the test medium. Future research using consistent test designs (e.g. same test medium over different species) is needed to resolve whether mixture toxicity is dependent on species.

Although IA was for most mixtures the most accurate model, the CA model was generally more conservative than the IA model, i.e. for 90% of the mixture treatments the CA model resulted in more conservative predictions than the IA model. Overall, differences in predictions between the IA and CA model depend on the number of mixture components, their concentration ratio, and the steepness of dose-response curves of the individual components in the mixture.[31]

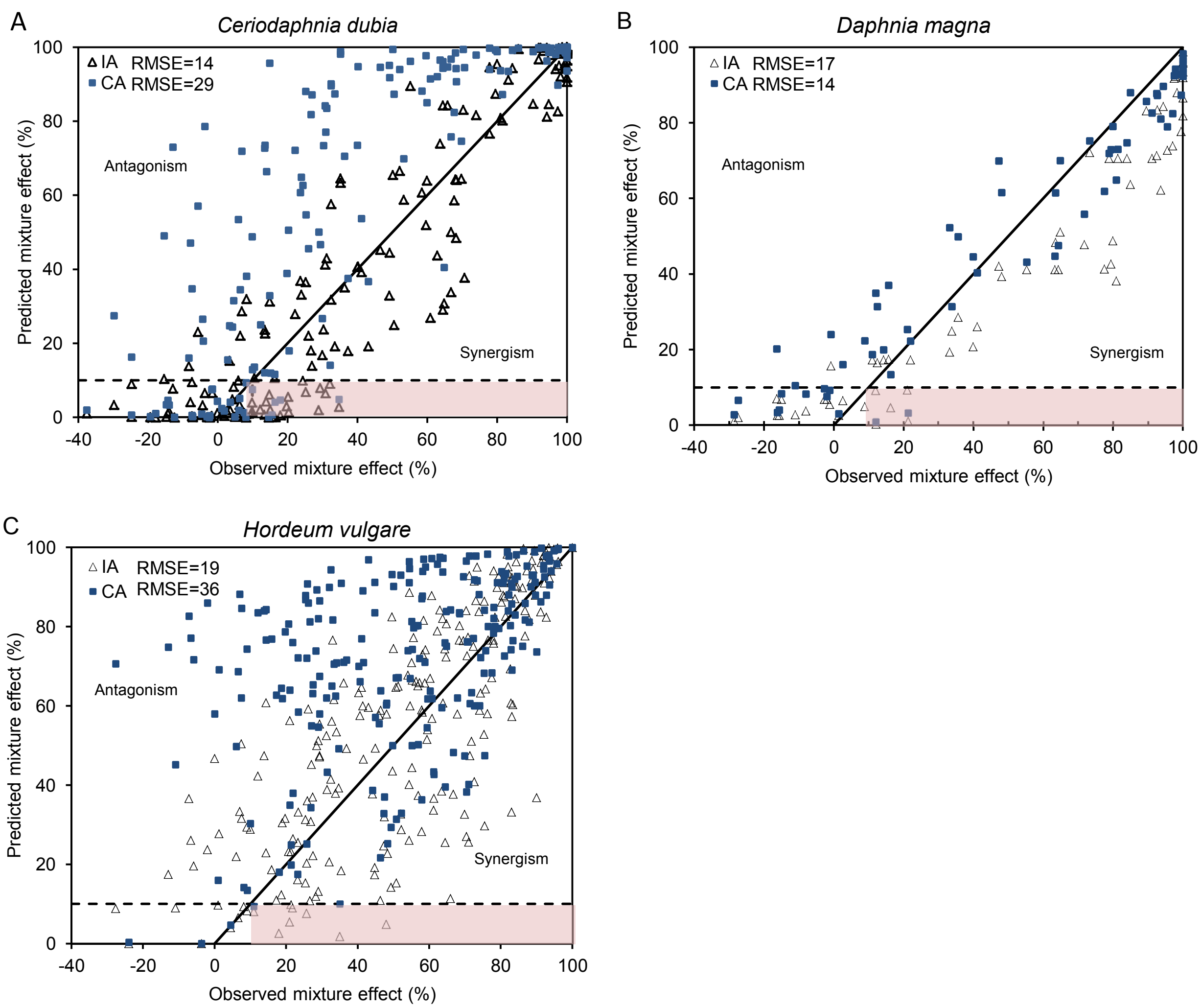

Figure 2. Predicted mixture effect (%) versus observed mixture effect using either the concentration addition (CA: Eq.5; blue filled squares) or independent action mixture reference model (IA: Eq. 6; open triangles) for *Ceriodaphnia dubia* (A), *Daphnia magna* (B), and *Hordeum vulgare* hydroponic experiments (C) The full line represents the perfect fit between the observed and predicted effects. Root mean square errors (RMSE) for both models are given. The red shaded area denote the situations where the predicted mixture effect is les than 10%, but the observed mixture effect is more than 10%. CA results in more conservative predictions of mixture toxicity. IA is the most accurate model for *C. dubia* and *H. vulgare*, while CA is slightly more accurate compared to IA for *D. magna*.

The global interactive effects relative to both reference models observed in the respective studies for daphnids and barley (assessed using the mixture toxicity evaluation method of Jonker et al.[5]) are summarized in the Supplemental Data 4 (Table S5). For barley, the observed interactive effects were generally the same relative to both reference models, while for daphnids the type of the interactive effect observed was dependent on the reference model. The latter can be explained based on the differences in the slope of the concentration response curves of barley and daphnids.[31] For low slopes (e.g. *H. vulgare* 5%-95% percentile of β_{Me2+}=0.77-3.94) predicted effects of IA and CA are relatively similar, while predictions of CA and IA deviate more from each other for steeper slopes (e.g. *C. dubia* 5%-95% percentile of β_{Me2+}=2.12-11.3).

In general, global additivity or antagonistic interactions relative to both CA and IA were observed for daphnids and barley (Table S6). In regulatory frameworks, mainly synergisms are of great concern, since occurrence of these type of interactions raises doubt on the conservativeness of the reference models applied in these frameworks. Among 17 metal mixture combinations tested, mixtures were additive in 6, antagonistic in 9 and synergistic in 1 case(s) using the IA model. With the CA model, mixtures were additive in 5 cases, and antagonistic in 11 cases, while no synergisms were observed. For *D. magna*, the synergistic deviations occurred only when the metals were combined at relatively high effect sizes.[19] This is in accordance with the study of Cedergreen,[34] wherein true synergistic deviations for mixtures of pesticides, metals and anti-fouling agents (based on mainly acute exposures) were relatively rarely observed when evaluated relative to the CA model and often occurred at high concentrations. This suggests that both reference models can serve as protective models to evaluate metal mixture toxicity to daphnids and higher plants at the concentrations relevant for risk assessment frameworks. For *H. vulgare,* mixture effects shifted towards antagonistic effects when Zn was present in the mixture, suggesting that Zn protects against metal mixture toxicity in plants. This protective effect of Zn might be linked to its role in maintaining the cell membrane stability or in oxidative stress regulation in plants, for example because Zn acts as a cofactor of superoxide dismutase.[32-33]

Protectiveness of CA at low effect sizes

Because of its simplicity, the CA model has been proposed as a first tier evaluation method in risk assessment frameworks for mixtures.[28] Hence, it is crucial that the CA model is protective at the low effect sizes important for these regulatory frameworks. To evaluate the latter, mixture doses were expressed as sum of toxic unit expressed relative to the EC10 ($\sum TU_{EC10}$). Theoretically, if CA holds no more than 10% mixture effect should be observed in mixture treatments in which $\sum TU_{EC10}<1$. However, our results show that in solution mixtures for which $\sum TU_{EC10} <1.0$, the average relative effects ranged between -7% (*D. magna*) and 19% (*H. vulgare*) when using free ion activities as dose. In this region ($\sum TU_{EC10} <1.0$), CA would not be protective (i.e. effects exceed 10% inhibition) for 16% (*C. dubia; n*=31*),* 17% (*D. magna; n*=12), and 80% (solution exposures of *H. vulgare; n*=5) of the mixtures, again using the free ion activities as the dose (red shaded area in Figure 3). Alternatively, there were also a considerable number of mixture treatments with $\sum TU_{EC10} >1.0$, in which less than 10% mixture effect or even a stimulation was observed, i.e. CA would be overprotective in these situations (blue shaded area in Figure 3).

For soils, metal exposure is often expressed as total metal concentrations and in this case, in 39% of the mixture treatments in soils where CA predicted maximum 10% effect, observed mixtures effects were larger than 10% (blue squares in Figure S4.C). This frequency decreased, however, to 8% of the soil mixture treatments using free ion activities (filled circles in Figure 3). These findings highlight the importance of accounting for speciation, especially in soils.

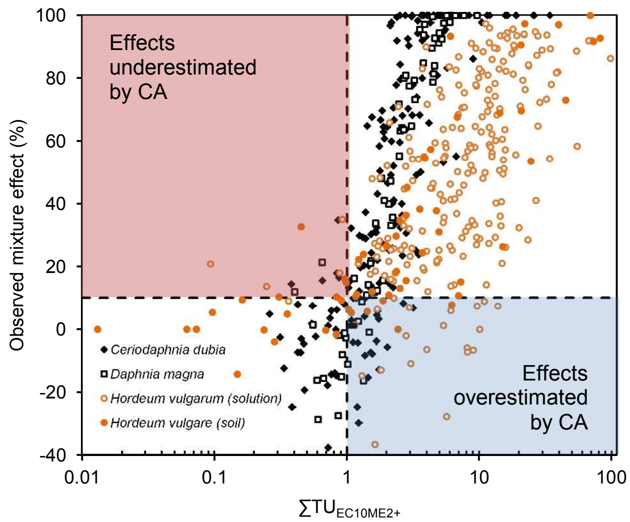

Figure 3. Observed mixture effects as a function of sum of toxic units, expressed relative to EC10, based on free ion activities ($\sum TU_{EC10Me2+}$) for *Ceriodaphnia dubia, Daphnia magna,* and *Hordeum vulgare*. The vertical dashed line denotes $\sum TU_{EC10Me2+}$=1, the horizontal dashed line the 10% observed effect and points at the intersection of these lines denoted perfect concentration addition at EC10. For 34% of the mixtures where $\sum TU_{EC10Me2+}<1$, CA would underestimate mixture effects (mixture effects>10%; red shaded area). For 54% of the mixtures where $\sum TU_{EC10Me2+}>1$, CA would overestimate mixture effects (mixture effects<10%; blue shaded area).

To investigate the protectiveness of CA at concentrations relevant for European riks assessment into more detail, we evaluated the degree of deviation from the CA model by calculating $EC10_{\sum TUEC10}$ for all mixture experiments, i.e. the mixture (expressed as $\sum TU_{EC10}$) at exactly 10% effect. If the mixture effect at the EC10 level follows the CA model, the $EC10_{\sum TUEC10}$ should be equal to 1. An $EC10_{\sum TUEC10}$ smaller than one indicates a trend towards synergistic interactions relative to the CA model, while values higher than one suggest a trend towards antagonistic interactions. In the present study, the $EC10_{\sum TUEC10}$ ranged between 0.95 and 8.06 for *H. vulgare*, between 1.09 and 2.73 for *C. dubia,* and between 1.38 and 1.40 for *D. magna*. It has been reported that the CA model mostly predicted 50% acutely lethal concentrations within a factor 2 for pesticide mixtures.[29] This observation was not confirmed here for metal mixtures at low effect levels, since the $EC10_{\sum TUEC10}$ values in 8 out of 10 experiments for *H. vulgare* and in 3 out of 10 experiments for *C. dubia* were higher than 2. We observed that the CA model overestimated toxicity on average with a factor 1.4 (*D. magna*), 1.6 (*C. dubia*) and 3.6 (*H. vulgare*) (Table 2). A synergistic $EC10_{\sum TUEC10}$, i.e. a value lower than 1, was only observed in one mixture experiment (barley; Figure 4.b; Table S5). Overall, these results suggest that the CA model is more conservative for chronic metal mixture toxicity to barley and daphnids at the 10% effect level, than for the acute toxicity of mixtures of pesticides, for which the median model deviation ratio has been reported to be equal to 1.[35]

It is predictable that metals interact since they compete for sorption, uptake in biota, translocation and detoxification or they might also have different MoA. Hence, the simplified concept of CA is unlikely to be generally valid. Nonetheless, this concept has been suggested to be used in mixture risk assessment frameworks because of its mathematical simplicity,[28] for example by summing the ratios of ambient concentrations to corresponding quality standards for different metals. For metals, this approach readily predicts the occurrence of ecological risk of metal mixtures close to or even below natural background concentrations in water and soil,[13] which already questions the validity of this approach at low exposure levels.

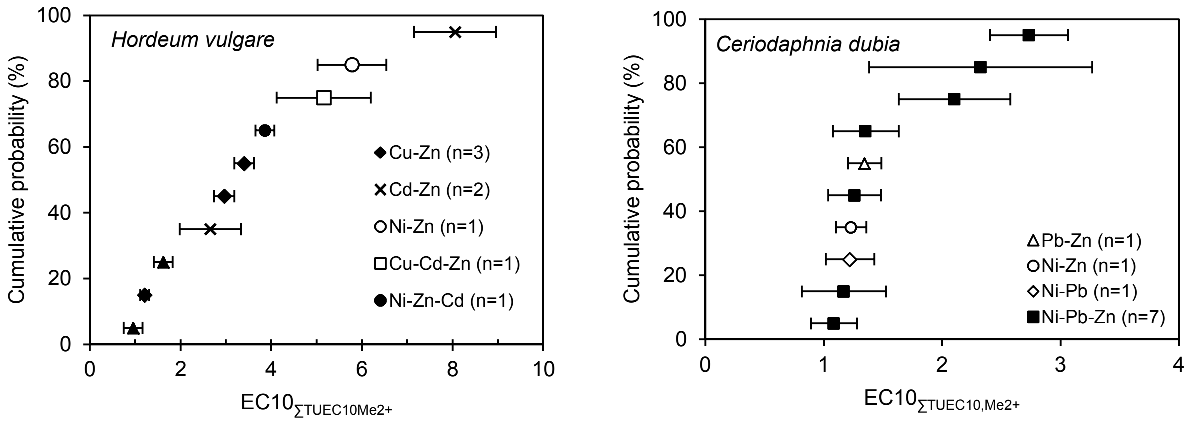

Figure 4. Distribution of mixture interactions for *Hordeum vulgare* (left panel) and *Ceriodaphnia dubia* (right panel). The figures represent the observed cumulative distribution of $EC10_{\sum TUEC10}$, i.e. the cumulative probability (%) of the $EC10_{\sum TUEC10}$ (Eq. 7; using free ion activities) in the chronic metal mixture experiments. The $EC10_{\sum TUEC10}$ represents the 10% effect concentration expressed as sum of toxic units relative to the EC10 and is therefore an expression of the degree of deviation from the CA reference model at low effect sizes (at ~10% effect). Data points are plotted at the Hazen plotting position. Each data point is the value derived from one experiment. Error bars denote the standard error on the estimated $EC10_{\sum TUEC10}$ (Eq. 7). $EC10_{\sum TUEC10}$ varied between 0.95 and 8.06 for *H. vulgare* and between 1.09 and 2.73 for *C. dubia*. $EC10_{\sum TUEC10}$ were generally higher than 1, indicating that the CA model tends to overestimate mixture toxicity.

Table 2. Median and mean 10% effect concentration expressed as toxic units ($EC10_{\sum TUEC10}$) for the metal mixture experiments with *Ceriodaphnia dubia, Daphnia magna,* and *Hordeum vulgare*

	Median $EC10_{\sum TUEC10Me2+}$	**Mean $EC10_{\sum TUEC10Me2+}$**	**Range $EC10_{\sum TUEC10Me2+}$**
***C. dubia* (*n*=10)**	1.30	1.58±0.58	1.09-2.73
***D. magna* (*n*=2)**	1.39	1.39±0.01	1.38-1.40
***H. vulgare* (*n*=10)**	3.18	3.57±2.24	0.95-8.06

Multi-metal bioavailability effects

In theory, interactions among metals can be related to bioavailability, i.e. to either the competition reactions for metal speciation in water or soil, or to the competition reactions at the biotic ligand (BL). The former are readily accounted for by using speciation measurements or calculations, while the latter can be indicated from antagonisms when the dose is expressed as free ion activity. In this project, it was found that a large part of the metal interactions was related to metal speciation in the medium.[17,18,20] For instance, in soil, mixture studies with Cu and Zn showed that the metal interactions varied largely with different expressions of the dose: based on total soil metal concentrations, synergism was found in soil samples with a medium and high cation exchange capacity (CEC) and antagonistic interactions at a low CEC soil (a sandy soil).[18] These synergisms were explained by competition reactions at soil binding sites, because antagonisms were found in all soils when expressing the dose as free ion activities (thus by accounting for speciation). Alternatively, some of the small antagonistic interactions occurring using free ion activities as dose could be explained by competition reactions at the receptor site.[17,18,20] Two examples of competition reactions at the BL are given below in Table 3, showing that antagonism among metal ions relative to CA decreases as concentrations of ions competing with toxic metals increase. The first example for *H. vulgare* shows that antagonistic interactions between Cu^{2+} and Zn^{2+} relative to CA were observed at low Ca level (0.4 mM), and that these interactions became smaller at higher Ca levels (10 mM), where Ca^{2+} outcompetes the Cu-Zn interactions. The second example for *C. dubia* shows that interactions among Ni^{2+}, Zn^{2+} and Pb^{2+} were observed at low H^+ activities (pH 8) and that these interactions became smaller at larger H^+ activities (pH 7). The above findings suggest that metal bioavailability models, such as BLMs, can be used to predict mixture toxicity data more accurately than CA or IA.

Table 3: Examples for *Hordeum vulgare* and *Ceriodaphnia dubia* showing that antagonism among metals ions decreases as the concentrations of ions competing with toxic metals increase.

	Hordeum vulgare[a]			*Ceriodaphnia dubia*[b]		
	Cationic concentration	**Metals tested**	**$EC50_{\Sigma TUEC50}$**[c]	**Cationic concentration**	**Metals tested**	**$EC50_{\Sigma TUEC50}$**
Low cationic competition situation	Ca^{2+} 0.4 mM	Cu+Zn	1.70 [1.53-1.88]	H^+ 10^{-8}M	Ni+Zn+Pb	2.65 [2.31-3.04]
High cationic competition situation	Ca^{2+} 10 mM	Cu+Zn	1.14 * [1.02-1.25]	H^+ 10^{-7}M	Ni+Zn+Pb	1.77 * [1.04-2. 49]

[a] Data from [17]
[b] Data from [21]
[c] Toxicity thresholds ($EC50_{\Sigma TUEC50}$: i.e. 50% effect concentrations based on sum toxic units at EC50 level). An EC50 larger (smaller) than 1 indicates antagonism (synergism). 95% confidence intervals are reported between brackets. The asterisk indicates that the $EC50_{\Sigma TUEC50}$ of the low competition situation is significant ($p<0.05$) different from the high cationic competition situation, evaluated using the Wheeler ratio test.[39]

In general, bioavailability models account for metal speciation in the external medium, binding of a metal to a biological receptor and competition reactions at the receptor site. In these models, metal speciation is accounted for by calculating (e.g. using WHAM VII) the dose as free metal ion activities in solution. This dose expression is subsequently used in, for example, biotic ligand models (BLMs). The BLM is a bioavailability model that assumes that metal toxicity is proportional to the concentration of metals bound to the BL.[36] Other ions such as Ca^{2+}, Mg^{2+} and H^+ can reduce metal toxicity by competition with the trace metal for binding to the BL. In this way, the models can correct for the effects of water and soil solution composition (competition reactions) on toxicity. To model metal mixture toxicity under various water chemistries, single metal BLM can be combined or extended using the IA or CA reference model.[37] An alternative approach is the WHAM-F_{tox} model, where toxicity depends on the interactions of metals and protons with the organism at reversible binding sites, and these competitive chemical reactions can be represented by competitive binding to particulate humic acid (HA).[38] In our research project, it was shown that well calibrated bioavailability models for single metal exposure in combination with the selection of an appropriate mixture toxicity model (either CA or IA based) are important in predicting metal mixture toxicity under various water chemistries.[17,18,21] These bioavailability models provide superior mixture predictions to merely using total metal concentrations or free ion activities. These results are in agreement with the observations obtained in a recent metal mixture modeling evaluation project.[13,37] Our results highlight the importance of accounting for speciation in the exposure medium, especially in soils where competition reactions for soil binding sites can largely affect the observed interactions.[18] Multi-metal BLMs can explain antagonistic interactions between metals by competition reactions at the BL.[17,18] In contrast, conceptually, synergistic interactions cannot be explained by BLMs. However, interactions at the level of toxicity are not necessarily linked to competition interactions at the uptake sites, because metals may affect each other's transport and (de)toxification pathways inside the organisms in several manners.[16] Therefore, future research should also focus on explaining observed interactions from a mechanistic perspective.

Implications for risk assessment

In this study, it was shown that relevant significant mixture effects occur, i.e. mixtures of different metals each individually causing <10% inhibition, can result in much larger inhibition when dosed in combination, with inhibition reaching up to 66% (Figure 1). This even occurs under conditions where metals interact antagonistically, i.e. the degree of antagonism is not sufficient to overcome the effects of combined exposure. This means that the current metal-by-metal approach in risk evaluation may not be conservative enough for metal mixtures.

Here, it was shown that, in general, the IA reference model is the most accurate model, whereas the CA model is mostly more conservative than the IA model. In addition, the often proposed CA model was in most cases conservative at low effect concentrations and could, in principle, be used in risk assessment frameworks as a first line of evidence. Our data also suggested that the use of CA would generally be overprotective for higher plants and daphnids, with estimated species-specific average levels of overprotection of low level toxic effects ranging from factor 1.4 to factor 3.6. Ignorance of deviations from CA could result in inaccurate risk evaluations. Clearly, the knowledge about metal mixture toxicity and interactions is far from complete, neither in terms of mechanistic understanding nor in terms of performing mixtures risk assessment. From a risk assessment point of view, there is still a lot of work in the development of metal mixture risk assessment frameworks. Based on species distribution of $EC10_{\sum TUEC10}$ values scientifically more correct thresholds for CA-based methods could be obtained. We also suggest to incorporate validated chronic mixture toxicity models accounting for bioavailability in a tiered approach.

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Supporting Information.

Tables showing more detailed information about included data, interactions observed, concentration response parameters derived for the analysis and results of the analysis. Additional figures (Pdf).

Tables showing raw concentration response data (metal concentrations and responses) for barley and daphnids (Excel).

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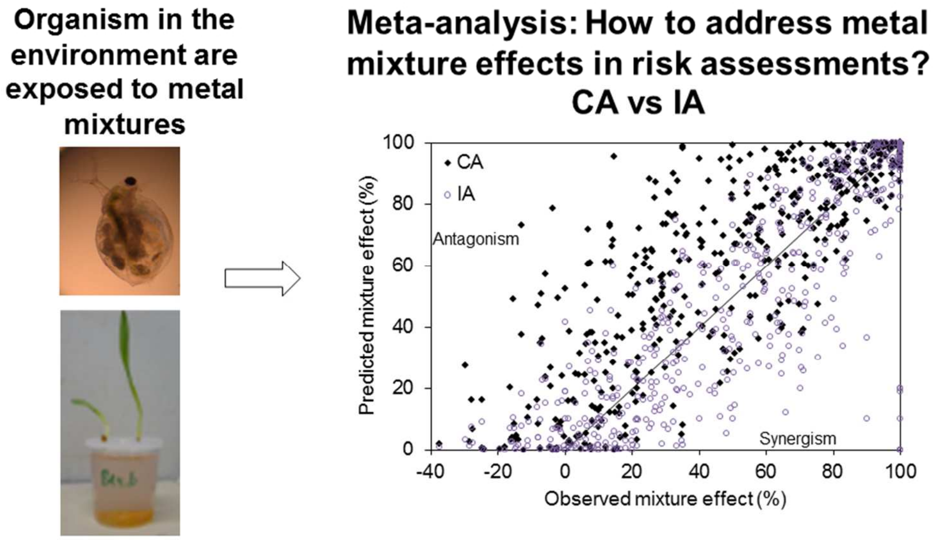

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