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Bernard CLEMENT (bernard.clement@entpe.fr)
CNRS UMR5023: Laboratoire d'Ecologie des Hydrosystemes Naturels et Anthropises
Vincent FELIX
Ecole Nationale des Travaux publics de l'Etat
Valentin BERTRAND
ENTPE: Ecole Nationale des Travaux publics de l'Etat

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Bernard CLEMENT*, Vincent FELIX, Valentin BERTRAND
Univ Lyon, Université Claude Bernard Lyon 1, CNRS, ENTPE, UMR5023, LEHNA, F-69518, Vaulx-en-Velin, France

* corresponding author; tel: 33(0)472047062; mobile: 33(0)666918798; bernard.clement@entpe.fr

Abstract

For the prediction of metals mixture ecotoxicity, the BLM approach is promising since it evaluates the amount of metals accumulated on the biotic ligand on the basis of water chemistry, i.e. species (major cations) competing with metals, and related toxicity. Based on previous work by Farley et al. 2015 (MMME research project), this study aimed at modelling toxicity of Cd:Cu mixtures (0:1 - 1:1 - 1:0 - 1:2 - 1:3 - 2:1 - 3:1 - 4:1 - 5:1 - 6:1) to the crustacean *Daphnia magna* (48h immobilization tests) and the microalga *Pseudokirchneriella subcapitata* (72h growth inhibition tests). The USGS model was chosen, assuming additivity of effects and accumulation of metals on a single site. The assumption that EDTA could contribute to toxicity through metals complexing was also tested, and potential effects due to reduction of ions Ca^{2+} absorption by metals were considered. Modelling started with parameter values of Farley et al. 2015 and some of these parameters were adjusted to fit modelled data on observed data. The results show that toxicity can be correctly predicted for the microalgae and that the hypothesis of additivity is verified. For daphnids, the prediction was roughly correct, but taking into account CuEDTA led to more realistic parameter values close to that reported by Farley et al. 2015. However, It seems that, for daphnids responses, metals interact either antagonistically or synergistically depending on the Cu:Cd ratio. Furthermore, synergy could not be explained by additional effects linked to a reduction of Ca absorption since this reduction, mainly due to Cd, increased inversely to synergy. Finally, the USGS model applied to our data was able to predict Cu:Cd mixture toxicity to microalgae and daphnids, giving rise to estimated EC50s roughly reflecting EC50s calculated from observed toxicity.

Keywords

cadmium – copper – daphnid – microalgae – mixture – BLM – bioavailability – EDTA

Introduction

Aquatic ecosystems are most often contaminated with mixtures of contaminants, among which metals emitted from human activities. However, environmental regulations and risk assessment procedures generally define standards on a metal-by-metal basis (Meyer et al. 2015). Though a variety of models exist to describe how metals interact in mixtures and impair aquatic organisms (Jho et al. 2011), predicting metals mixture toxicity still presents difficulties. One limitation of the previous approaches is due to the fact that metal mixture toxicity was generally modeled by considering addition of toxicity based on total dissolved concentrations (Meyer et al. 2015),

with non-consideration of bioavailable fractions. Some progress has been done recently based on the BLM approach which was first developed for single metals. This model accounts (1) for metal ion speciation through binding to dissolved organic matter and inorganic ligands (HCO_3^-, CO_3^{2-}, and Cl^-, …), and (2) competition of free metal ions with cations and protons (Ca^{2+}, Na^+, H^+) on biotic ligand sites to predict the toxicity of a metal (Jho et al. 2011, Farley et al. 2015, Meyer et al. 2015). A strong assumption, based on numerous observations, is that toxicity can be attributed to free metal ions (Di Toro et al. 2001; Paquin et al. 2000). BLMs have been extended to metal mixtures a few years ago, with previous works by Balistrieri et al. 2014, 2015, Santore et al. 2015, Iwasaki et al. 2015, and the comprehensive study of Farley et al. 2015 who tested 4 models within the frame of the Metal Mixture Modeling Evaluation (MMME) project. All models aim at modeling and interpreting effects of metal mixtures based on bioavailability (Meyer et al. 2015). In these models, metals exhibit either similar joint action (with toxicity expressed in terms of concentration addition of metal accumulation on the biotic ligand) or independent joint action (with toxicity expressed in terms of a multiplicative function of the responses to the individual metals) (Farley and Meyer 2015).

The present work aims at modeling results of daphnids and microalgae bioassays on a Cu:Cd mixture on the basis of one of the four MMME models, the USGS model. Cu and Cd are widespread heavy metals discharged by activities such as pigments manufacturing, mining, smelting, and electroplating (Hatano and Shoji 2008).The USGS model expresses toxicity as a function of the fractional coverage of accumulated metals on a single site, and metals are assumed to exhibit different potencies when bound to the biotic ligand. In addition, we tested the role of EDTA in the bioavailability of Cu. As a matter of fact, in our medium (Clément et al. 2013), EDTA is used as Fe-EDTA to keep Fe^{3+} in solution and set it available to micro-algae and daphnids. Though EDTA has often been shown to decrease toxicity by complexing metals and thus reducing concentrations of free metal ions supposed to be the available forms of metals (Guilhermino et al. 1997), some authors showed that reduction of toxicity by EDTA is not systematic (Sillanpää and Oikari, 1996, Rodea-Palomares et al. 2009), and that EDTA complexes might take part to toxicity (Morel, 1983; Tubbing et al. 1994; Campbell et al. 2002). Finally, we also analyzed the concomitant effect of metal accumulation on Ca^{2+} absorption. As a matter of fact, Cd toxicity would be partly due to impairment of calcium absorption by aquatic organisms (Santore et al. 2002). Several studies have stated that Cd is a significant antagonist of ions Ca^{2+} absorption in fish gills (Verbost et al. 1987, 1988, 1989; Hollis et al. 2000; Niyogi and Wood 2004). Other studies have shown competition between Ca^{2+} and Cd^{2+} for D. magna (Tan and Wang, 2008) and D. pulex (Clifford and McGeer 2010).

Materials and methods

Bioassays

72h-algal bioassays were carried out in conditions adapted from the ISO standard (ISO 2012). Algae (*Pseudokirchneriella subcapitata,* CCAP 278/4) were cultured as recommended by standard methods (ISO 2012). Axenic cultures were maintained by weekly transferring a few mL of an exponentially growing culture in fresh medium sterilized by autoclaving. The microalgae were exposed in 48-well plates filled with exposure medium used in previous microcosm assays (Clément et al. 2013, 2014). This synthetic medium presented the following

characteristics : pH 8.0, hardness 60 mg $CaCO_3$ L^{-1}, alkalinity 120 mg $CaCO_3$ L^{-1}, conductivity 290 µS cm^{-1}, phosphorous 100 µg L^{-1}, nitrogen 1308 µg L^{-1}, $FeEDTANa_26H_2O$ 109.4 µg L^{-1}, oligo-elements and vitamins of M4 medium (Elendt and Bias 1990). In bioassays, test medium was not sterilized. Light intensity was 5000 lux 16h per day. $Cd(NO_3)_2$ and $CuCl_2$ were used as metallic salts in stock solutions of 1 mg L^{-1}. Test solutions were prepared by diluting stock solutions in 50-mL borosilicate-glass volumetric flasks and left 24h at 20 °C before use for ensuring equilibrium of chemical kinetics (Rodea-Palomares et al. 2009). Microalgae from an exponentially-growing culture (age: 5-7 days, density: 4 to $6x10^6$ cells mL^{-1}) were added to these equilibrated solutions to obtain 20000 cells mL^{-1}, then 2-mL wells were filled with these suspensions. For each bioassay, Cd and Cu were tested alone and as mixtures (1 to 3 simultaneously), which enabled to limit variability within bioassay (Barata et al. 2006; De Laender et al., 2009). The following concentrations of Cd were tested: 0 ; 10 ; 20 ; 40 ; 60 ; 80 ; 100 ; 120 ; 140 ; 160 µg L^{-1}. For Cu the concentrations were: 0 ; 10 ; 20 ; 30 ; 40 ; 50 ; 60 ; 80 ; 100 ; 120 µg L^{-1}. There were 4 replicates per concentration. The duration of bioassays was 72h. At the end of bioassays, 100 µL were sampled from each well for algal counting using a particle counter (Beckman Coulter®, Z1 model) to determine algal density in each well. Mean final algal density was then calculated for each treatment and a growth rate for each treatment was calculated as follows:

$$\mu = \frac{\ln(mean\ final\ algal\ density) - \ln(initial\ algal\ density)}{3} \quad (1)$$

Then, a growth inhibition rate for each treatment was calculated as follows:

$$\%inhib = \frac{(\mu_{control} - \mu_{treatment})}{\mu_{control}} \quad (2)$$

The following mixtures Cd : Cu were tested several times : 0:1 - 1:1 - 1:0 - 1:2 - 1:3 - 2:1 - 3:1 - 4:1 - 5:1. 12 algal bioassays for the mixtures, 9 for the assays on Cu alone and 9 for the assays of Cd alone were exploited.

48h-daphnid bioassays were carried out in conditions adapted from the standard. Neonate daphnids were exposed in glass tubes containing 10 mL test solution and 10 individuals. Test solutions were prepared as for algal bioassays with the same synthetic medium aerated during at least 48h prior introduction of metals. Tested concentrations were: 0 ; 10 ; 20 ; 30 ; 40 ; 60 ; 80 ; 100 ; 120 ; 160 µg L^{-1} for Cd and 0 ; 2.5 ; 5 ; 7.5 ; 10 ; 12.5 ; 15 ; 20 ; 25 ; 30 ; 40 µg L^{-1} for Cu. There were 4 replicates per concentration. The duration of bioassays was 48h. At the end of bioassays, mobile daphnids were visually counted and an immobilization rate per treatment was calculated as follows:

$$\%immo = \frac{final\ of\ mobile\ daphnids - initial\ number\ of\ mobile\ daphnids}{initial\ number\ of\ mobile\ daphnids} \quad (3)$$

The following mixtures Cd : Cu were tested several times : 0:1 - 1:1 - 1:0 - 1:2 - 1:3 - 2:1 - 3:1 - 4:1 - 5:1. 26 daphnid bioassays for the mixtures, 12 for the assays on Cu alone and 11 for the assays of Cd alone were exploited.

Modelling

For modelling the effects of metals, ECx values (and 95% credible intervals) for the species of interest were estimated, based on 3-parameters log-logistic model using the MOSAIC web-interface for statistical analyses in ecotoxicology (Charles et al. 2018). MOSAIC is available at https://mosaic.univ-lyon1.fr/. The calculations within MOSAIC are based on the R package 'morse' (Baudrot et al. 2018) with a Bayesian framework.

Then the methodology proposed by Farley et al. 2015 was adapted to our data and tools. Chemical speciation of Cd and Cu in the water, alone or in mixtures, was carried out using PHREEQC (version 2), a computer program freely available on the web. We used the database MINTEQ+ which allows to take into account organic species such as EDTA present in the medium. The modelling was carried out with a solution composition based on the synthetic medium used, with known concentrations of salts and known pH. This led to determine the activities of Cu^{2+}, Cd^{2+}, Ca^{2+}, Mg^{2+}, Na^{+}, H^{+}. As mentioned above, our methodology was adapted from the USGS model based on one biotic ligand site. The stability constants for the binding of Cu^{2+}, Cd^{2+}, $CuOH^{+}$, Ca^{2+}, Mg^{2+}, Na^{+}, H^{+} were chosen from USGS model (Fairley et al. 2015), first excluding CuEDTA as a component of toxicity. The stability constants of $CuCO_3$ was added from De Schamphelaere et al. 2002. As a matter of fact, the pH of solution being 8.0, the activity of $CuCO_3$ must be taken into account (De Schamphelaere et al. 2003; Gopalapillai and Hale 2015). The logarithmic expressions of the constants are displayed in table 1.

Table 1. Values of stability constants for the binding of major ions and metal complexes on the biotic ligand

$logK_{CuBL}$	$logK_{CuOHBL}$	$logK_{CuCO3BL}$	$logK_{CdBL}$	$logK_{CaBL}$	$logK_{MgBL}$	$logK_{NaBL}$	$logK_{HBL}$
7.6	0.62	7.01	8.1	5	4.4	4	6.7

From activities calculated with PHREEQC and stability constants of table 1, f_{Cu} and f_{Cd} were calculated with equations (4) and (5):

$$f_{Cu} = \frac{K_{CuBL}\cdot(Cu^{2+})+K_{CuCO3BL}\cdot(CuCO3)+K_{CuOHBL}\cdot(CuOH^{+})}{\{1+K_{CuBL}\cdot(Cu^{2+})+K_{CuCO3BL}\cdot(CuCO3)+K_{CuOHBL}\cdot(CuOH^{+}) \\ +K_{CdBL}\cdot(Cd^{2+}) \\ +K_{CaBL}\cdot(Ca^{2+})+K_{MgBL}\cdot(Mg^{2+})+K_{NaBL}\cdot(Na^{+})+K_{HBL}\cdot(H^{+})\}} \quad (4)$$

$$f_{Cd} = \frac{K_{CdBL}\cdot(Cd^{2+})}{\{1+K_{CuBL}\cdot(Cu^{2+})+K_{CuCO3BL}\cdot(CuCO3)+K_{CuOHBL}\cdot(CuOH^{+}) \\ +K_{CdBL}\cdot(Cd^{2+}) \\ +K_{CaBL}\cdot(Ca^{2+})+K_{MgBL}\cdot(Mg^{2+})+K_{NaBL}\cdot(Na^{+})+K_{HBL}\cdot(H^{+})\}} \quad (5)$$

The total fraction f of toxic metal-biotic ligand complexes and the concentration of unoccupied biotic ligand sites $[BL^{-}]$ (mol L^{-1}) can be calculated as follows:

$$f = f_{Cu} + f_{Cd} = [BL-Cu^{2+}] + [BL-CuOH^{+}] + [BL-CuCO_3] + [BL-Cd^{2+}] \quad (6)$$

$$[BL^{-}] = \frac{[BL-]_T\cdot(1-f)}{\{1+K_{CaBL}\cdot(Ca^{2+})+K_{MgBL}\cdot(Mg^{2+})+K_{NaBL}\cdot(Na^{+})+K_{HBL}\cdot(H^{+})\}} \quad (7)$$

where $[BL-]_T$ is the total concentration of biotic ligands, equal to 1 within this model.

Given that (Di Toro et al. 2001):

$$[MiBL^{+}] = K_{MiBL} \cdot [M_i^{2+}] \cdot [BL^{-}] \quad (8)$$

with Mi = Cu or Cd for example

it is possible to calculate the concentrations of the complexes with biotic ligand [BL-Ca^{2+}], [BL-Mg^{2+}], [BL-H^{+}], [BL-Na^{+}], [BL-Cd^{2+}], [BL-Cu^{2+}], [BL-$CuOH^{+}$] and [BL-$CuCO_3$] from activities. As an example with Cu^{2+} (Playle et al. 1993, De Schamphelaere and Janssen 2002):

$$[BL - Cu^{2+}] = K_{CuBL} \cdot (Cu^{2+}) \cdot [BL^{-}] \quad (9)$$

Toxic response is determined in 2 steps. First, a Tox function is defined as:

$$Tox = \alpha_{Cu} f_{Cu} + \alpha_{Cd} f_{Cd} \quad (10)$$

where α_{Cu} and α_{Cd} are potency factors for the two metals accounting for different toxicities when bound to the biotic ligand. The Tox function expresses a concentration-addition type of approach.

The coefficients α_{Cu} and α_{Cd} of the USGS model are displayed in table 2.

Table 2. Coefficients α_{Cu}, α_{Cd}, and β_i parameters according to Farley et al. 2015

	Daphnia magna	*Pseudokirchneriella subcapitata*
α_{Cu}	4.78	4.78
α_{Cd}	1.91	1.91
β_1	6.59	1.01
β_2	9.09	1.89
β_3	1.03	1.07

The similarity of values for both organisms is due to the assumption made by Farley et al. 2015 that potency factors are intrinsic to metals and independent of the organism.

Second, a 3-parameter logit function was used to calculate biological response as a function of Tox

$$R = \frac{1}{\left(1+e^{-(\beta_1+\beta_2 log Tox)}\right)^{\beta_3}} \quad (11)$$

For the USGS model, LA_{50}, the accumulation on the biotic ligand at 50% effect, is expressed as (Farley et al. 2015):

$$LA_{50} = 10^{-(\frac{\beta_1}{\beta_2})} \quad (12)$$

When Tox is equal to LA_{50}, equation (12) leads to R = 0.5

For *Daphnia magna*, LA_{50}=0.188, the geometric mean of all LA_{50} values reported by Farley et al. 2015. For *Pseudokirchneriella subcapitata*, LA_{50}=0.292.

The β_i parameters of the USGS model are displayed in table 2. Farley et al. 2015 assume that these parameters are organism-specific.

To adjust the model to experimental data, we chose to set dissociation constants at values published by Farley et al. 2015 and reported in table 1, and to fit coefficients α_{Cu} and α_{Cd} and parameters β_i from data of single-metal bioassays. The SOLVER function of Excel was used to first determine α_{Cu} and α_{Cd} from bioassays on respectively Cu and Cd, with initial β_i values of the USGS model (table 2), then determine β_i values from data on single metals with the additional constraint LA_{50}=0.188 for daphnids and LA_{50}=0.292 for microalgae. The determination of the parameters with the SOLVER function was based on the least squares method which consisted in minimizing the sum of the squares of the differences between observed and modeled effects. The quality of fitting was evaluated through the Normalized Root-Mean Square Error (NRMSE):

$$NRMSE = \frac{1}{\bar{Y}}\sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_{obs,i} - y_{pred,i})^2} \quad (13)$$

The fitting was qualified as good for values of NRMSE < 0.5.

For incorporation of CuEDTA in the model regarding effects on *Daphnia magna*, since no value for $K_{CuEDTABL}$ was available we first added CuEDTA in the equations of speciation regarding the biotic ligand (equations (4) to (7)). Secondly, we determined $K_{CuEDTABL}$ with the SOLVER by fitting new modeled values to observations in the assays on Cu alone and α and β_i values from Farley et al. 2015 (table 2). Then, the SOLVER was again used to optimize α_{Cu} value and β_i values with data of Cu assays. With these values, α_{Cd} was adjusted to improve fitting of modeled to observed values (Cu being present in the test medium, changes were also expected on Cd assays).

The same method was used with CdEDTA but calculations led to a value of 0 indicating the absence of implication of CdEDTA in the toxicity of Cd. For microalgae, the same method led to a 0 value for $K_{CuEDTABL}$, indicating that this complex is not bioavailable to algae.

Once parameters α and β determined, the model was applied to all assays on Cu:Cd mixtures.

Isobolograms were plotted from metals accumulated on the biotic ligand and their LA50s (Meyer et al. 2015) and, by extension, from Tox functions and Tox50. When additivity is supposed (assumption of the USGS model) the following equation is verified:

$$TU_{Cu} + TU_{Cd} = \frac{[BL-Cu]_{Total}}{LA_{50Cu}} + \frac{[BL-Cd]}{LA_{50Cd}} = \frac{Tox_{Cu}}{Tox_{50Cu}} + \frac{Tox_{Cd}}{Tox_{50Cd}} = 1 \quad (14)$$

Results

The determination of $\log K_{CuEDTABL}$ led to a value of 7.12. The values found for α_{Cu}, α_{Cd}, and β_i parameters are displayed in table 3. For *P. subcapitata*, since considering CuEDTA was not successful, parameters are given without CuEDTA.

Table 3. Coefficients α_{Cu}, α_{Cd}, and β_i parameters found in this study

	Daphnia magna		*Pseudokirchneriella subcapitata*
	without CuEDTA	with CuEDTA	without CuEDTA
α_{Cu}	35.78	4.73	4.51
α_{Cd}	0.79	0.83	0.71
β_1	1.73	4.36	2.39
β_2	2.38	6.01	4.47
β_3	0.74	0.96	1.00

192

193 $CuCO_3$ constitutes the major form of Cu accumulated on BL for microalgae (96%) and daphnids when CuEDTA is
194 not considered (96 to 98%), Cu^{2+} represents respectively 4% and 2-4%, and CuOH+ is negligible (< 1.10^{-6} %). When
195 CuEDTA is taken into account in the toxicity to daphnids, CuEDTA becomes the major contributor (70 to 98%).

196 Figures 1 to 6 show observed and model-calculated responses of daphnids and microalgae exposed to Cu, Cd
197 alone and Cu:Cd mixtures. The values of the Normalized Root-Mean Square Error (NRMSE) indicated in the titles,
198 all lower than 0.5, underline acceptable or even good fits of modeled to observed data. For mixtures related to
199 daphnids effects where Cu dominates (Cu:Cd = 1:1, 2:1 and 3:1), the position of model curves suggests an
200 underestimation of toxicity, i.e. toxicity would be synergistic since the model is based on additivity. And this is
201 the case whatever the role of CuEDTA (figures 2 and 4). Furthermore, when considering CuEDTA in toxicity to
202 daphnids, it seems that this tendency concerns also the mixture Cu:Cd = 1:2.

203 Figure 7 shows isobolograms obtained for microalgae and daphnids. For algae (fig 7A), points are located close
204 to the additivity line, which suggests absence of interactions between copper and cadmium, hence additivity of
205 effects. For daphnids without CuEDTA (fig 7B), the results suggest antagonism for Cd-rich mixtures (Cu:Cd = 1:3
206 to 1:6) and synergism for Cu-rich mixtures (Cu:Cd = 1:2, 1:1, 2:1, 3:1). When assuming bioavailability of CuEDTA
207 (fig 7C), the interaction between Cu and Cd is more and more synergistic when copper becomes dominant in the
208 mixture.

209 Despite interactions, the plotting of EC50s calculated by the BLM versus observed EC50s (figure 8) shows a
210 relatively good correspondence.

211 Accumulation of Ca^{2+} and Tox (sum of Cu species and Cd^{2+} considered as toxic) on Biotic Ligand was calculated at
212 EC50s of various Cu:Cd mixtures (figure 8). It can be noted first that the more Cd in the mixture, the more
213 accumulation of metals on BL for the same effect (50%), and the less accumulation of Ca^{2+} on BL. This is
214 particularly the case for microalgae, but this can also be noted for daphnids. In both cases, the points are located
215 according to the ratio Cu:Cd. However, for mixtures where Cu is dominant (Cu:Cd = 1:0, 3:1, 2:1) and for equi-
216 mixture Cu:Cd (1:1), the reduction of Ca^{2+} on BL is almost negligible.

217

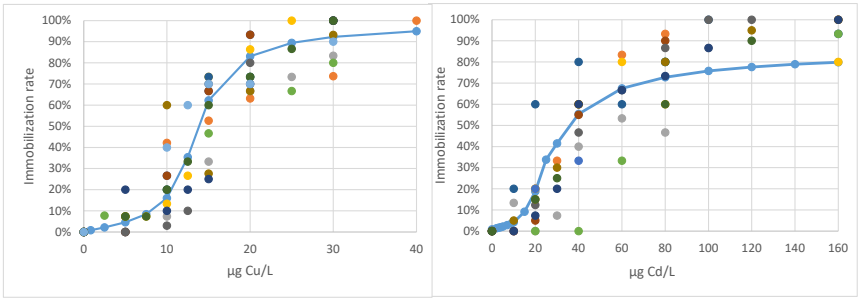


Figure 1. Observed and model-calculated (BLM without EDTA) responses of daphnids exposed to Cu alone (left) and Cd alone (right), after determination of alpha and beta values. NRMSE = 0.21 for Cu and 0.30 for Cd.

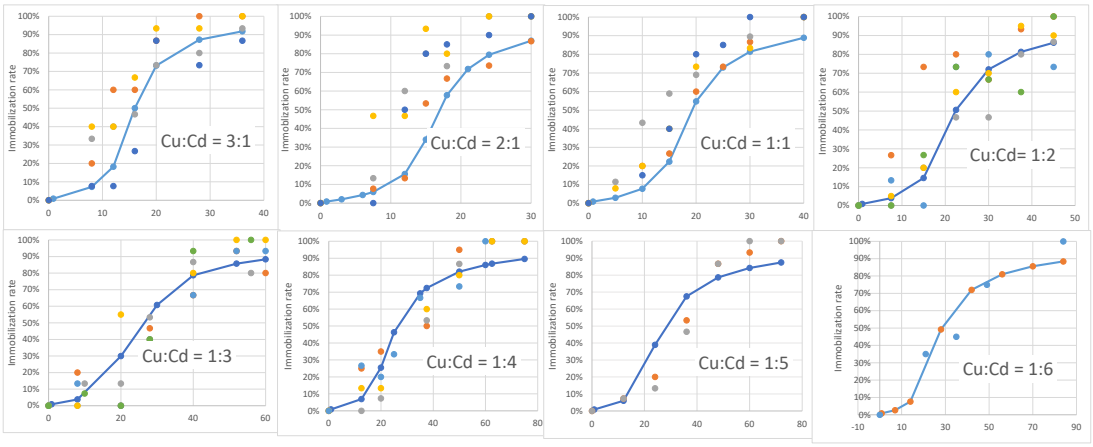


Figure 2. Observed and model-calculated (BLM without CuEDTA) responses of daphnids exposed to various mixtures of Cu:Cd (X axis : µg/L; Y axis : immobilization rate). NRMSE (all data) = 0.33.

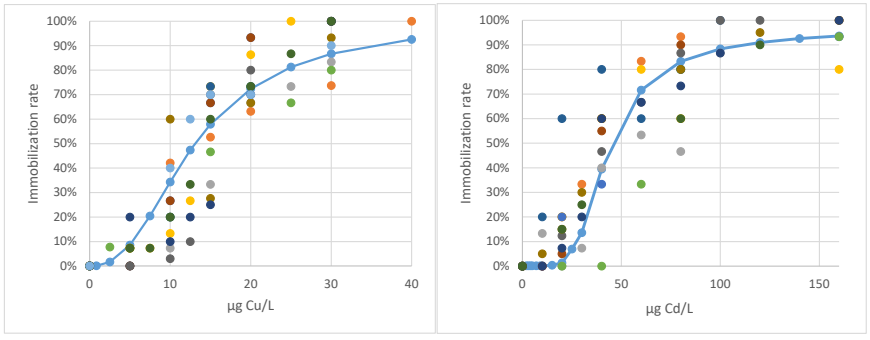
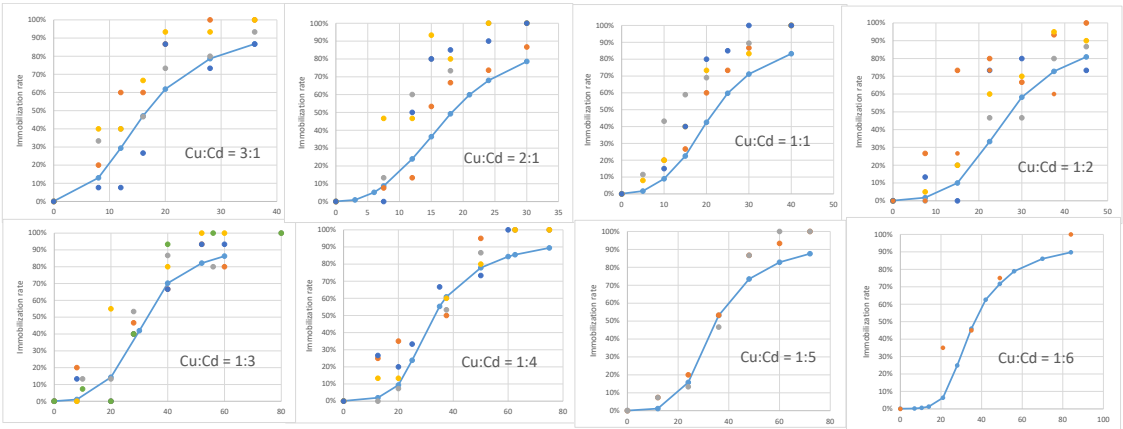


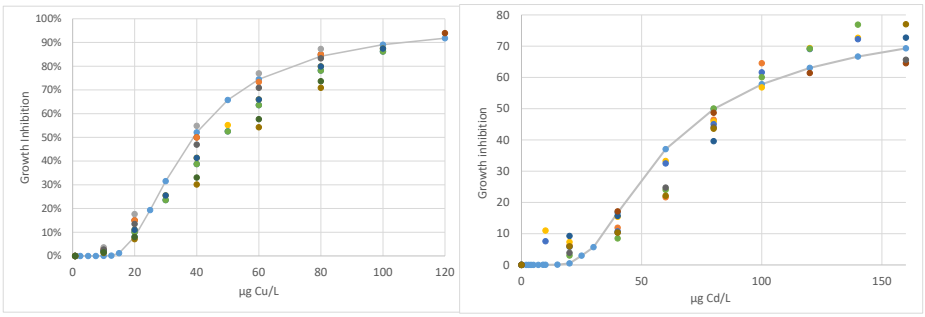
Figure 3. Observed and model-calculated (BLM with CuEDTA) responses of daphnids exposed to Cu alone (left) and Cd alone (right), after determination of alpha and beta values. NRMSE = 0.24 for Cu and 0.26 for Cd.



231

232 Figure 4. Observed and model-calculated (BLM with CuEDTA) responses of daphnids exposed to various

233 mixtures of Cu:Cd (X axis : µg/L; Y axis : immobilization rate). NRMSE (all data) = 0.35



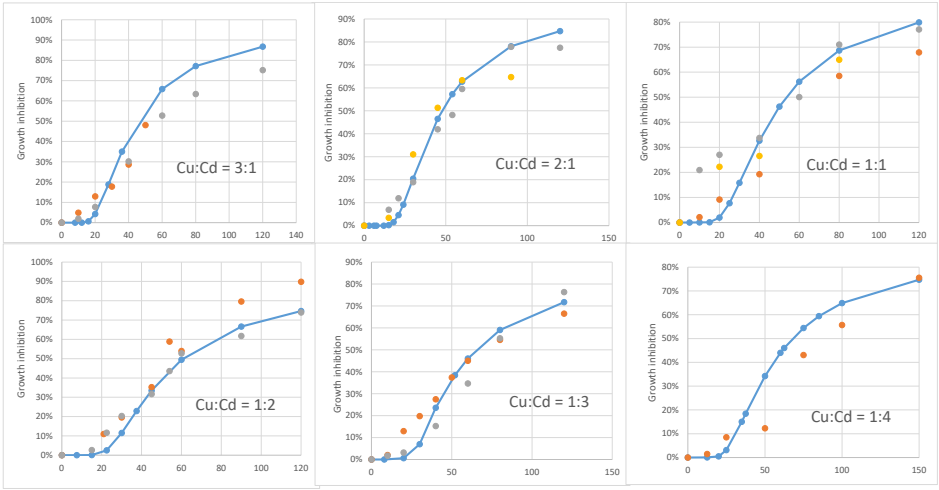
234

235 Figure 5. Observed and model-calculated (USGS) responses of microalgae exposed to Cu alone (left) and Cd

236 alone (right), after determination of alpha and beta values. NRMSE = 0.09 for Cu and 0.08 for Cd.

237

238



239

240 Figure 6. Observed and model-calculated (BLM) responses of microalgae exposed to various mixtures of Cu:Cd

241 (X axis : µg/L; Y axis : immobilization rate); NRMSE (all data) = 0.25

242

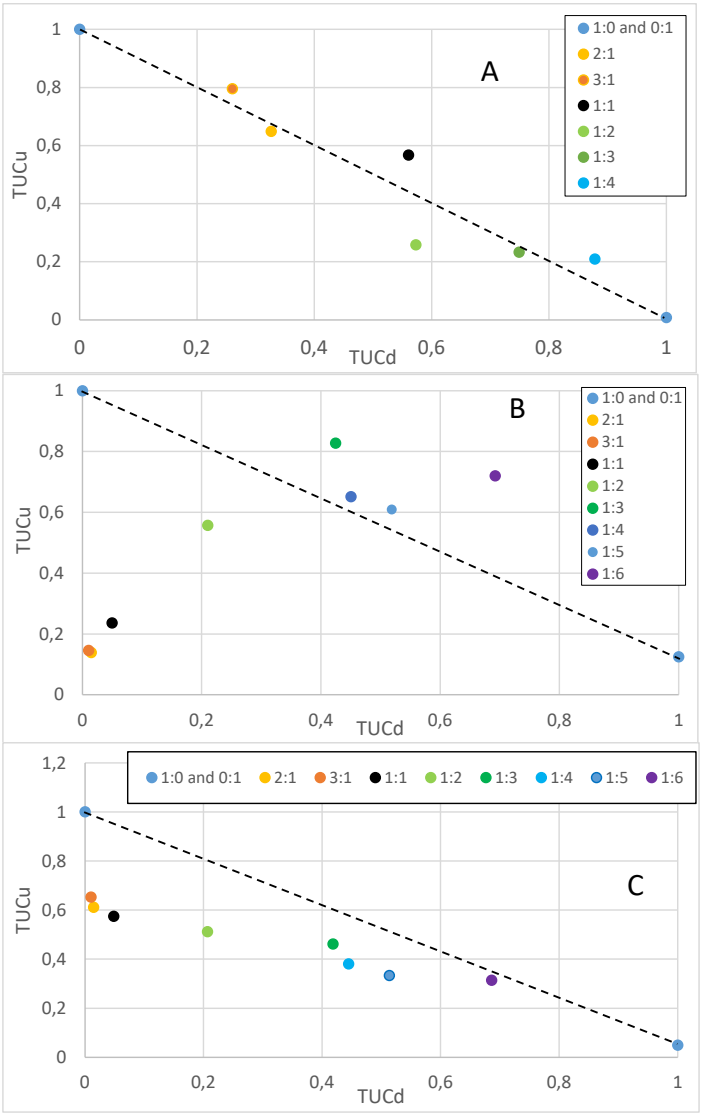


Figure 7. Isobolograms with Toxic Units calculated from TOX (legend refers to ratios Cu:Cd; A: microalgae; B: daphnids without CuEDTA; C: daphnids with CuEDTA)

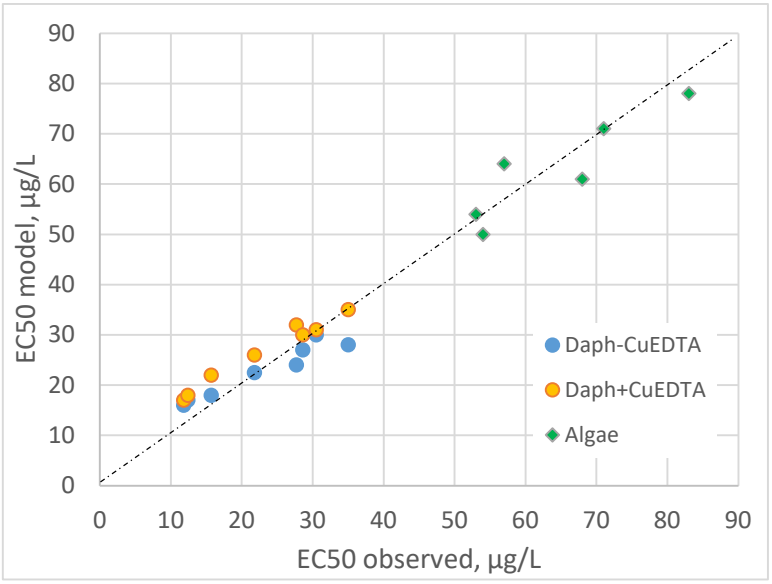


Figure 8. EC50s calculated by the model versus observed EC50s

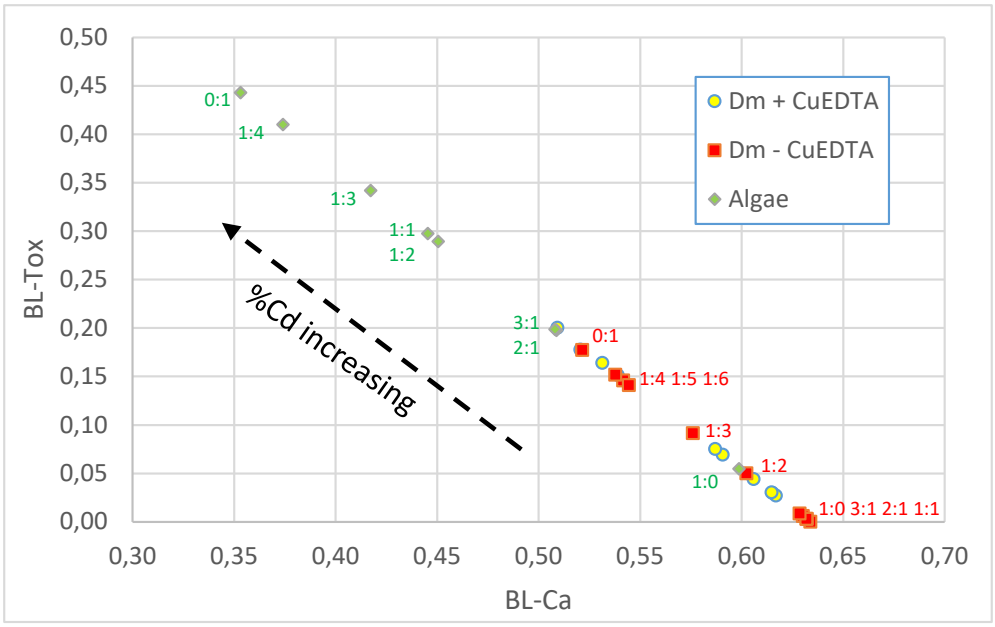


Figure 9. BL-Tox versus BL-Ca at EC50s of mixtures, for daphnids (with and without CuEDTA) and microalgae. Cu:Cd ratios are noted for Dm – CuEDTA and Algae.

Discussion

The present study aimed at increasing knowledge on interactions between cadmium and copper in mixtures applied to *Daphnia magna* and *Pseudokirchneriella subcapitata* in a synthetic medium containing EDTA.

Taking into account various chemical species in the toxicity of Cu, i.e. Cu^{2+}, $CuOH^{+}$ and $CuCO_3$, we showed that $CuCO_3$,due to a pH of 8.0, was by far the major species accumulated on the Biotic Ligand, for *D. magna* as well as *P. subcapitata*, except when CuEDTA was considered and became the major species accumulated on BL. Note that these results do not mean that, among Cu species, $CuCO_3$ or CuEDTA are the most toxic, because accumulation on BL is not equivalent to toxicity since this one is related to toxic potency.

The application of the BLM adapted from Farley et al. 2015 led to satisfying results at first sight. As a matter of fact, values of the Normalized Root-Mean Square Error (NRMSE) remained under 0.5, confirming rather good fits of modelled to observed data as shown on plots. However, the high value found for α_{Cu}, the potency factor for copper when CuEDTA was not taken into account, suggests that CuEDTA should be considered. And, indeed, considering CuEDTA in copper toxicity led to a value for α_{Cu} which is exactly that provided by Farley et al. 2015, with other values (α_{Cd}, β_1, β_2 and β_3) close to that provided by the same authors. An attempt to take into account CdEDTA in toxicity to *D. magna* led to a KCdEDTA-BL null, which allowed to discard this assumption. For microalgae, Campbell et al. 2002 have suggested with Morel 1983 that ternary metal complexes, such as EDTA-M-X-cell where M is the metal and X-cell the biological site (biotic ligand), might form at the algal cell surface, giving rise to M-X-cell, hence toxicity of M. This is corroborated by Tubbing et al. 1994 observations: despite absence of free Cu^{2+} ions due to chelation with EDTA in the medium, inhibition of *Selenastrum capricornutum* growth was noticed. However, taking into account CuEDTA for microalgae did not improve the results.

Though modelling based on BLM gave roughly good results, it appeared that interactions between metals affected the adjustment for daphnids, as clearly shown by isobolograms. Whereas the model, based on additivity assumption, represents quite well the observations for microalgae, the results for daphnids show antagonism or synergism depending on the ratio Cu:Cd and the role considered for CuEDTA. It should however been noted, and this is an argument for the CuEDTA hypothesis, that the evolution of these interactions with Cu:Cd ratio seems to be more coherent when considering CuEDTA (figure 7). Indeed, incorporating CuEDTA in Cu toxicity tended to reduce discrepancy of interactions between Cu and Cd. In addition, the fact that single metals and mixtures tests were systematically performed concurrently allows to avoid misleading interpretations of interactions (Meyer et al. 2015). Such interactions between Cu and Cd have been reported by Barata et al. 2006 for daphnid immobilization tests. These authors found less than additive effects, i.e. antagonistic, of Cu:Cd mixtures, whereas our observations tend to favor synergistic interactions increasing with the proportion of Cu in the mixture. Meyer et al. 2015b have studied the effects of a Cu:Cd mixture on daphnids in a medium containing fulvic acid. They found antagonism between metals for some ratios and explained this by competition between these metals for accumulation on BL. On the other hand, Meyer et al. 2015b have noted synergism or antagonism for Cu:Cd mixtures with metals concentrations lower than their EC50 and concluded that interactions were rather synergistic. Care should nevertheless be brought when comparing different studies with different media, as denoted by very different EC50s and toxicity ranges reported by Meyer et al. 2016 compared to ours (13.5 and 103 µg.L versus 43 and 14 µg/L for Cd and Cu respectively). In our case, synergism might have been explained by an additional effect depending on metals accumulation on BL, such as reduction of Ca^{2+}. However, the increasing synergism observed with increasing proportion of Cu in the mixture and the consequently increasing

accumulation of Ca^{2+} on BL allows to reject this hypothesis, the reason of interactions between Cu and Cd toxicity on daphnids stands thus unsolved in the conditions of our bioassays with the BLM. According to Farley and Meyer 2015 “a model that includes multiple types of biotic ligands may be needed to properly describe competition of metals and predict less than additive toxicity”. However, in an ecotoxicological risk assessment perspective, the relatively good correspondence of EC50s calculated from bioassays data (observed EC50s) and EC50s extracted from the BLM, as displayed on figure 8, shows that the BLM could be used without excessive uncertainty to infer toxicity values of Cu:Cd mixtures from that of metals alone.

Conclusion

The tested assumptions of this study were that (1) the BLM adapted from Farley et al. 2015 should be appropriate to describe our data of daphnids and microalgae bioassays on Cu:Cd mixtures, and (2) CuEDTA could be considered as a species contributing to Cu toxicity. Our results show that the first assumption is verified for microalgae test whereas the model does not completely describe observed data for daphnids, due to interactions of synergy and antagonism for which this model, based on additivity, is not conceived. Integrating CuEDTA as a species taking part to toxicity with $CuOH^+$, $CuCO_3$ and Cu^{2+} helps improving the realism of the model parameters, but it does not solve the question of interactions between Cu and Cd, which are still observed though they appear more easily related to Cu:Cd ratio and which this study did not succeed to explain. A specific study of the effect of EDTA on mixture Cu:Cd toxicity would be necessary to confirm or infirm the role of CuEDTA. In a first approach, it can nevertheless be stated that the BLM studied in this study could be used to infer toxicity values for mixtures of copper and cadmium.

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Ethical Approval

The authors declare that they submit original work as a whole, not published elsewhere, that the results are not falsified, and that proper acknowledgements to other works have been given.

Consent to Participate

All listed authors are volunteer to participate to this publication.

Consent to Publish

All listed authors have approved the manuscript before submission.

Authors Contributions

All authors contributed to the study conception and design. Experimental work and preliminary data analyses were carried out by Valentin BERTRAND and Bernard CLEMENT. Modelling was done by Vincent FELIX and Bernard CLEMENT. The first draft of the manuscript was written by Bernard CLEMENT and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

Data are available upon request from the first author.

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Figures

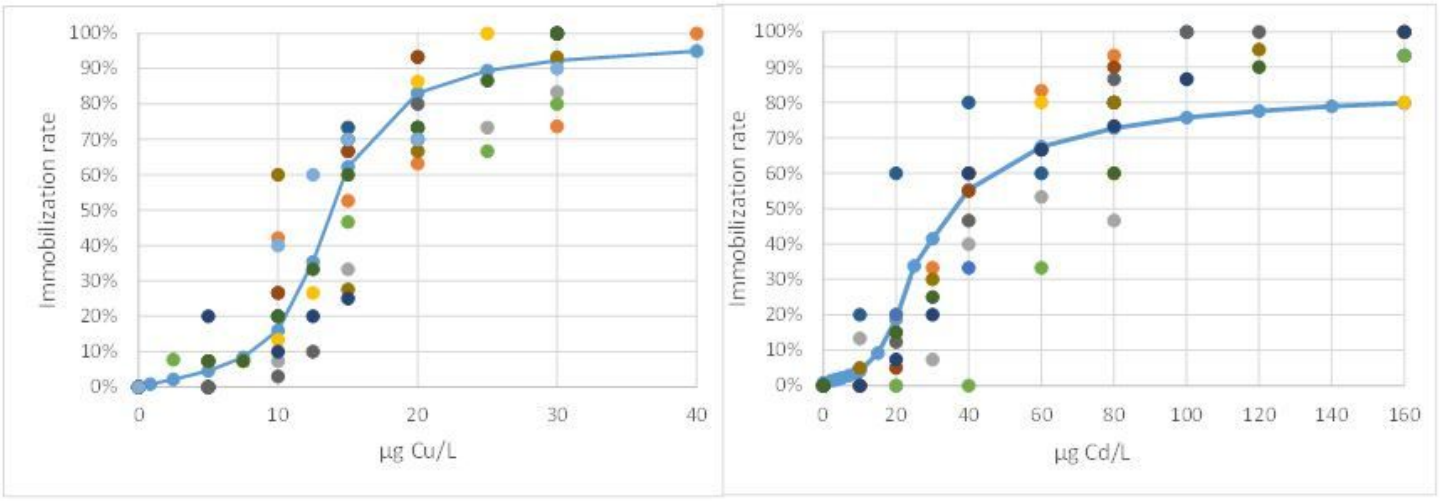

Figure 1

Observed and model-calculated (BLM without EDTA) responses of daphnids exposed to Cu alone (left) and Cd alone (right), after determination of alpha and beta values. NRMSE = 0.21 for Cu and 0.30 for Cd.

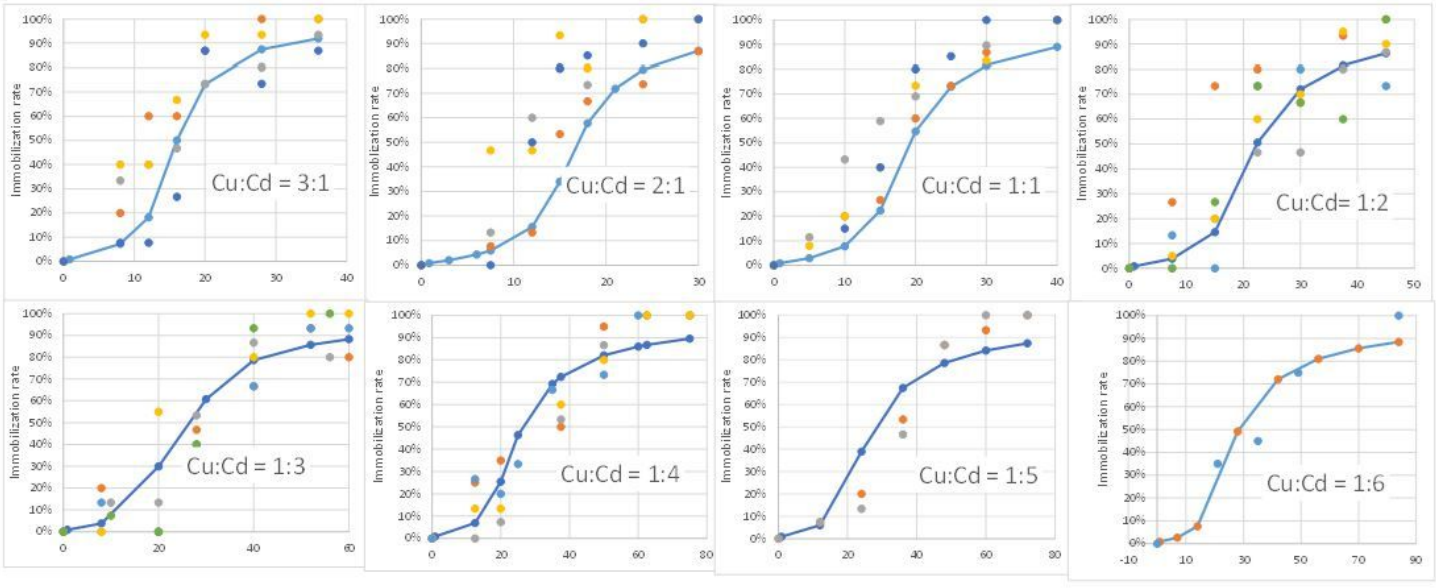

Figure 2

Observed and model-calculated (BLM without CuEDTA) responses of daphnids exposed to various mixtures of Cu:Cd (X axis : µg/L; Y axis : immobilization rate). NRMSE (all data) = 0.33.

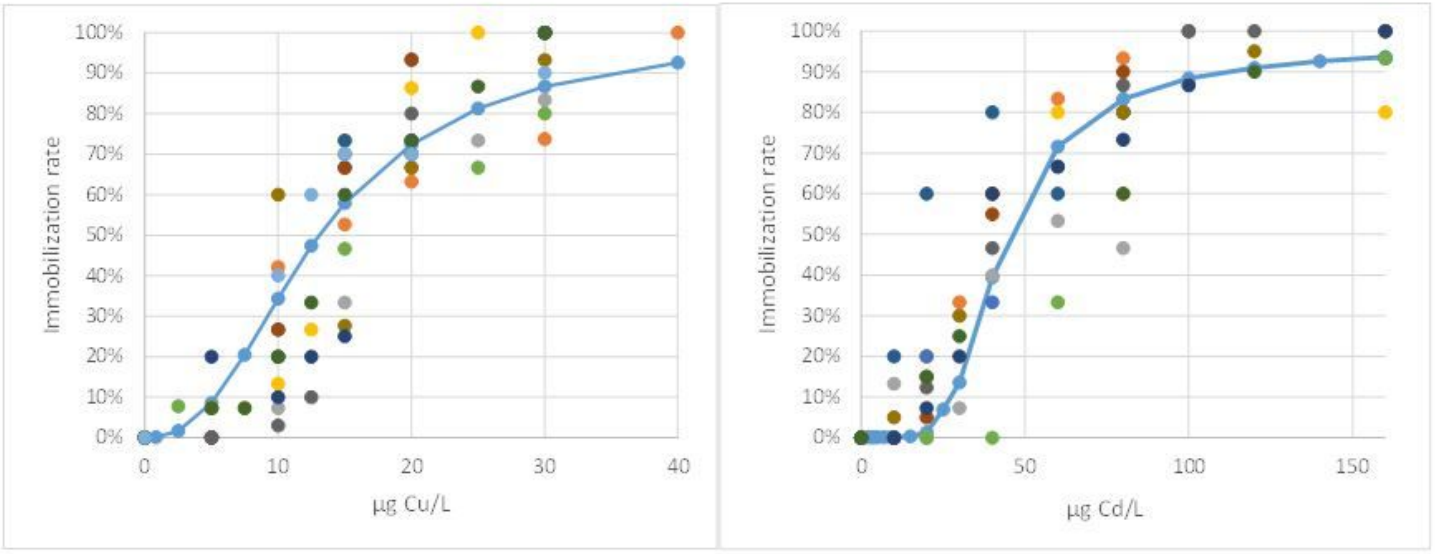

Figure 3

Observed and model-calculated (BLM with CuEDTA) responses of daphnids exposed to Cu alone (left) and Cd alone (right), after determination of alpha and beta values. NRMSE = 0.24 for Cu and 0.26 for Cd.

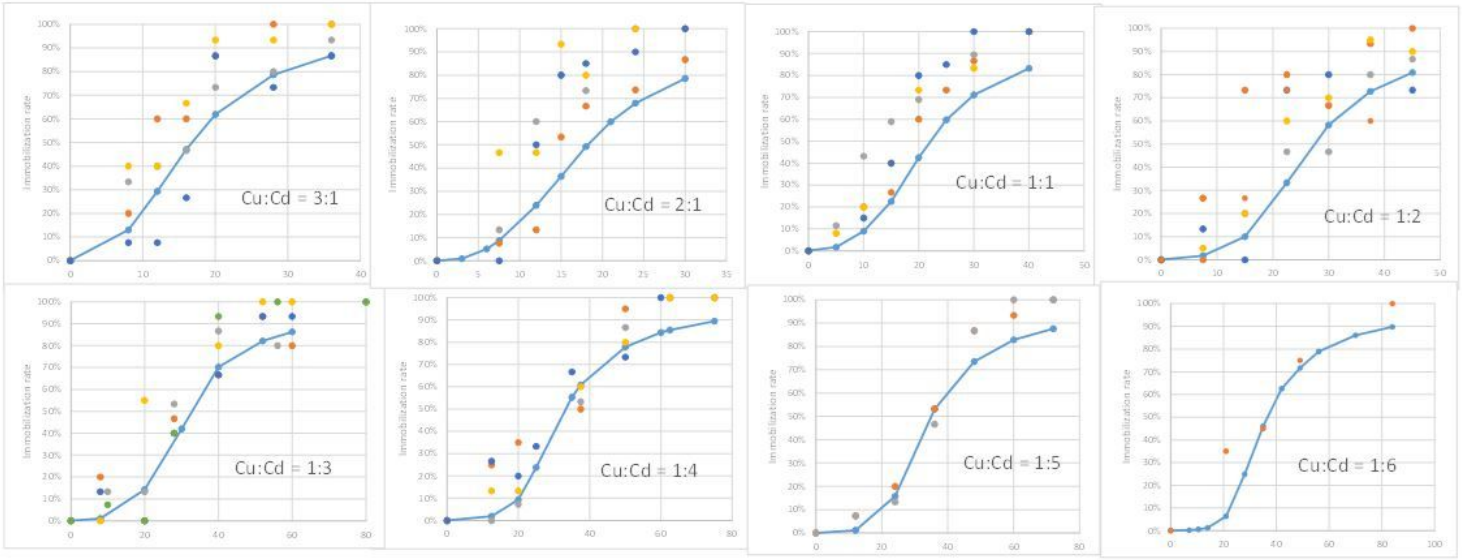

Figure 4

. Observed and model-calculated (BLM with CuEDTA) responses of daphnids exposed to various mixtures of Cu:Cd (X axis : µg/L; Y axis : immobilization rate). NRMSE (all data) = 0.35

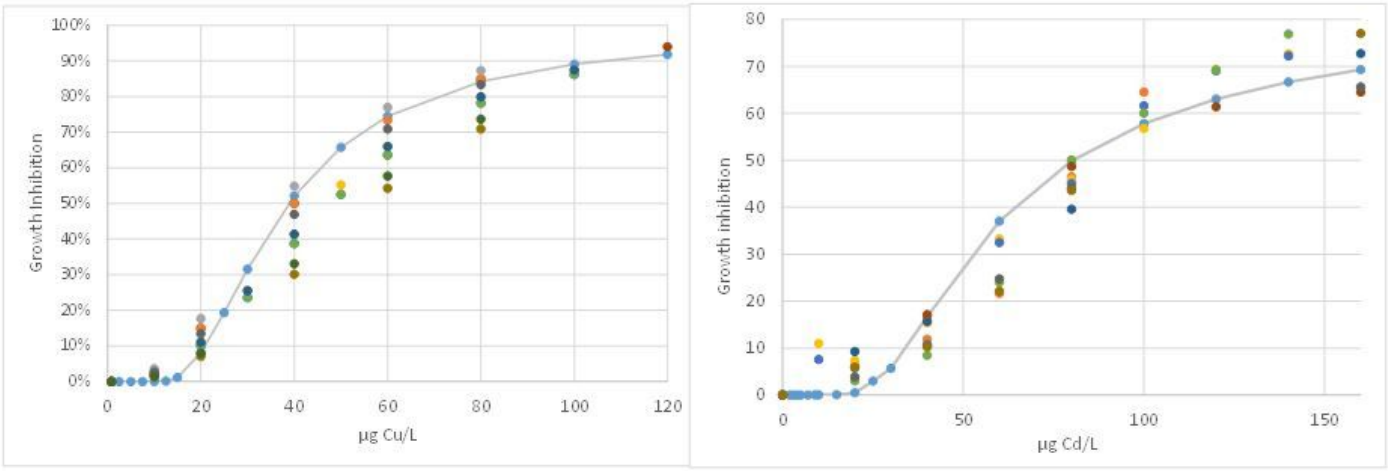

Figure 5

Observed and model-calculated (USGS) responses of microalgae exposed to Cu alone (left) and Cd alone (right), after determination of alpha and beta values. NRMSE = 0.09 for Cu and 0.08 for Cd.

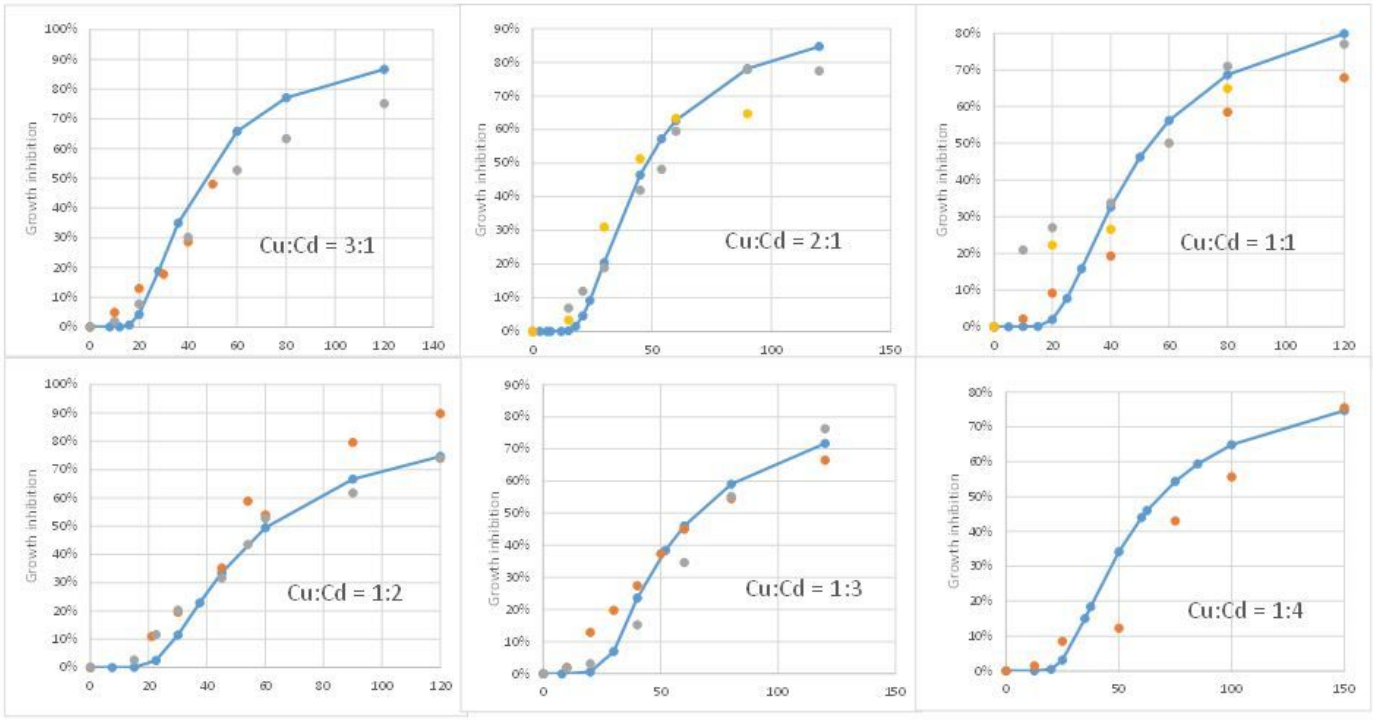

Figure 6

Observed and model-calculated (BLM) responses of microalgae exposed to various mixtures of Cu:Cd (X axis : µg/L; Y axis : immobilization rate); NRMSE (all data) = 0.25

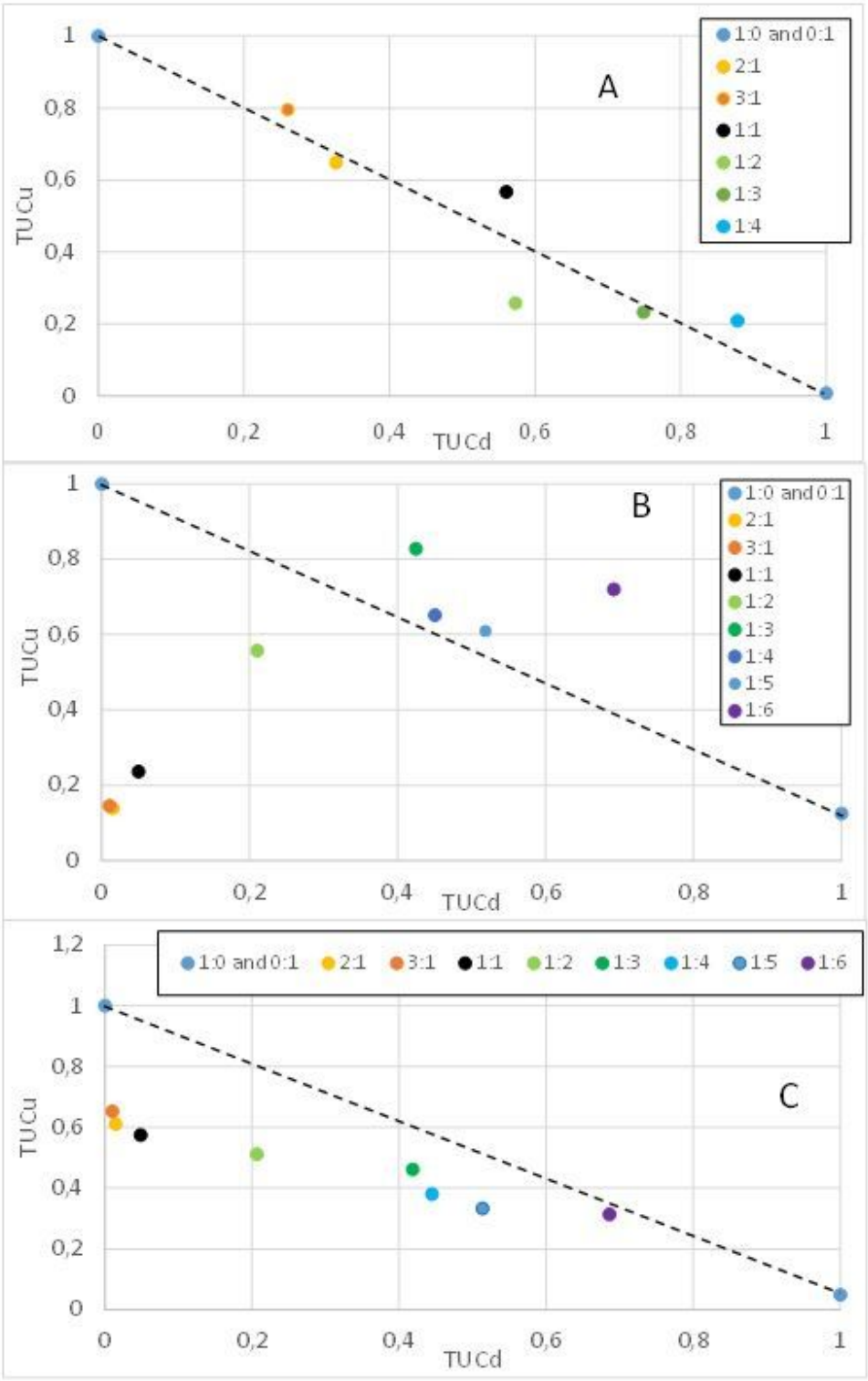

Figure 7

Isobolograms with Toxic Units calculated from TOX (legend refers to ratios Cu:Cd; A: microalgae; B: daphnids without CuEDTA; C: daphnids with CuEDTA)

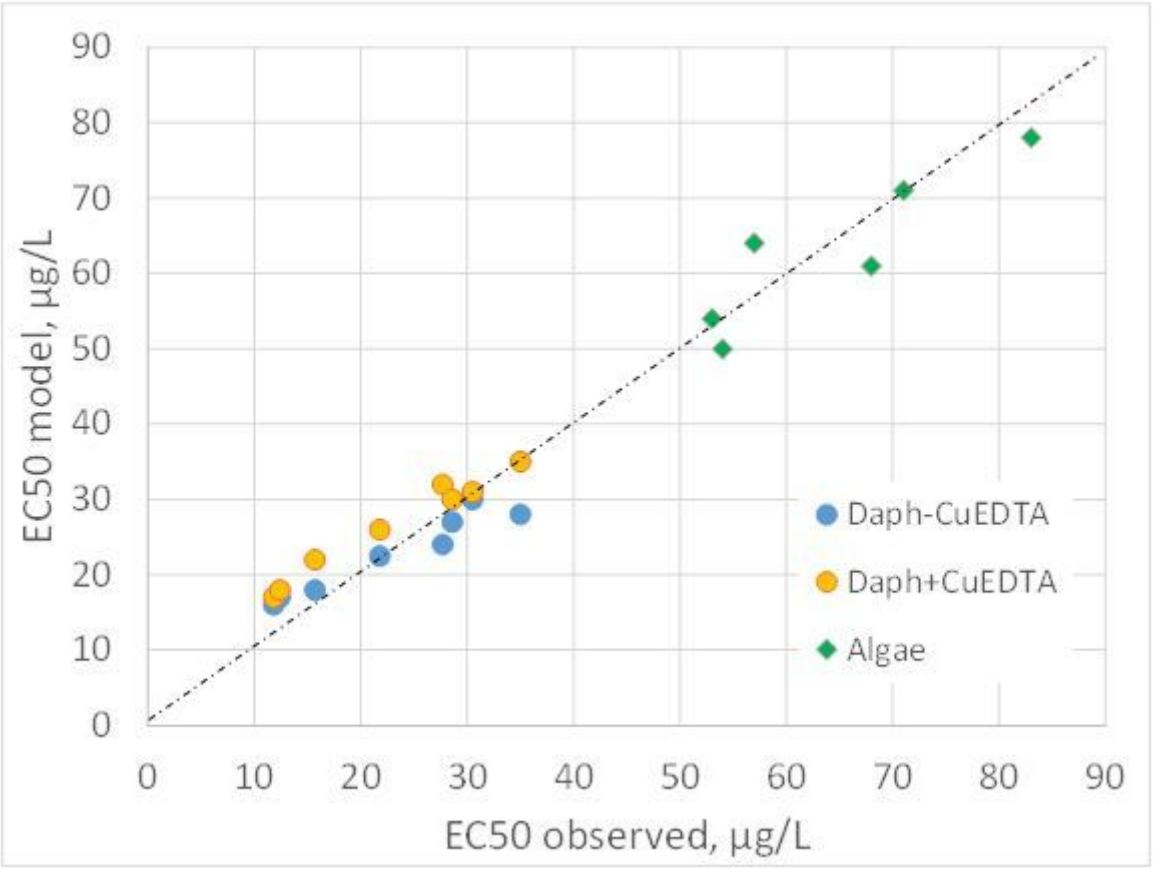

Figure 8

EC50s calculated by the model versus observed EC50s

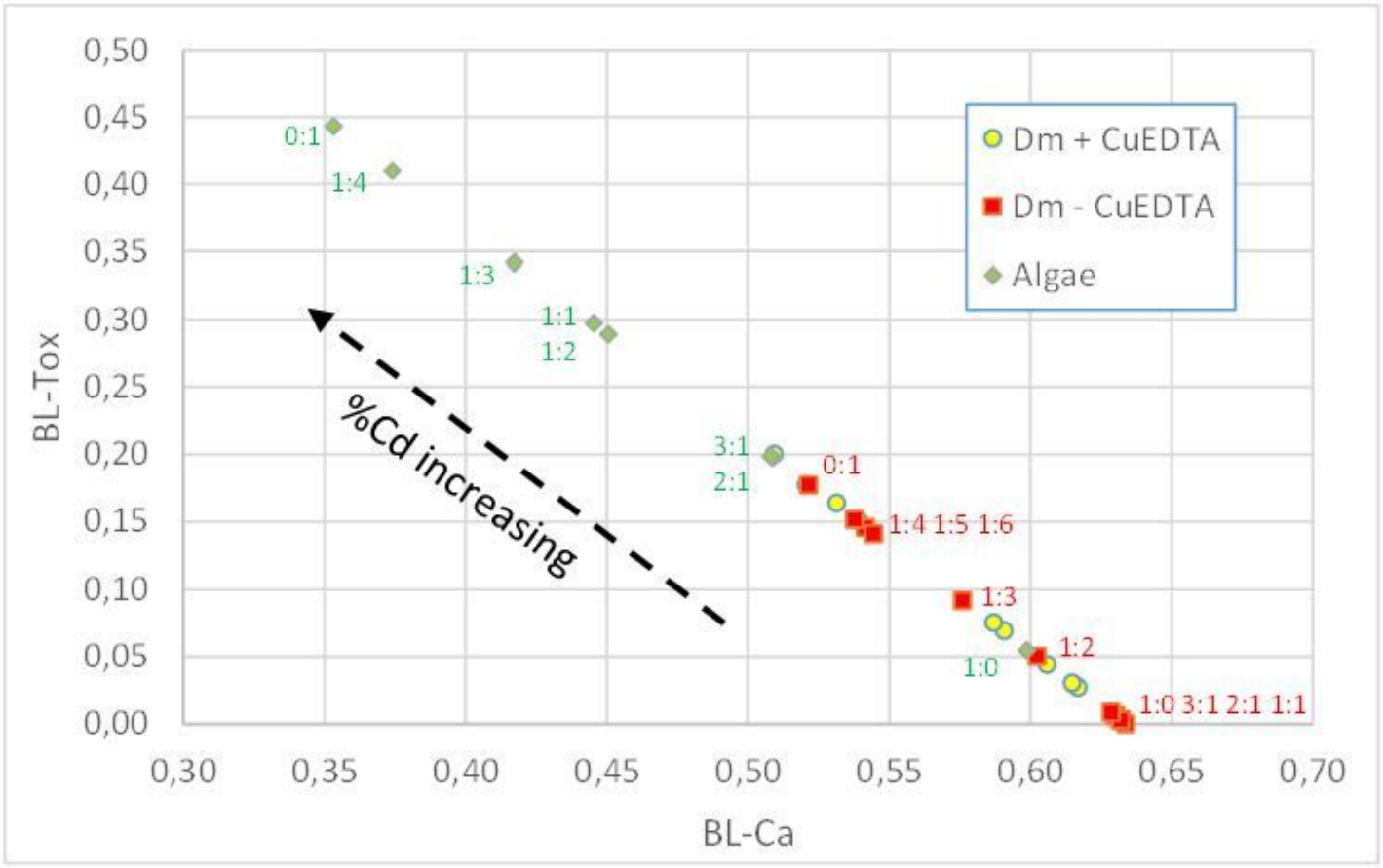

Figure 9

BL-Tox versus BL-Ca at EC50s of mixtures, for daphnids (with and without CuEDTA) and microalgae. Cu:Cd ratios are noted for Dm – CuEDTA and Algae.