He, Erkai; Baas, Jan; van Gestel, Cornelis A.M. 2015. **Interaction between nickel and cobalt toxicity in Enchytraeus crypticus is due to competitive uptake**. *Environmental Toxicology and Chemistry*, 34 (2). 328-337. 10.1002/etc.2802

Environmental Toxicology **Environmental Toxicology and Chemistry**
DOI 10.1002/etc.2802

INTERACTION BETWEEN NICKEL AND COBALT TOXICITY IN *ENCHYTRAEUS CRYPTICUS* IS DUE TO COMPETITIVE UPTAKE

Running title: Dynamic toxicity of Ni and Co mixtures

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Additional Supporting Information may be found in the online version of this article.

Submitted 1 October 2014; Returned for Revision 5 November 2014; Accepted 5 November 2014

Abstract: Uptake and toxicity of Ni-Co mixtures in *Enchytraeus crypticus* were determined after 4, 7, 10 and 14 d exposure. Generally, body concentrations of Ni and Co increased with increasing exposure concentrations. Ni body concentration was significantly reduced in the presence of Co, while Ni only marginally affected Co uptake. When expressed as free ion activities, individual toxicity of Ni and Co increased with time, with LC50s decreasing from 78.3 and 511 µM at 4 d to 40.4 and 393 µM at 14 d, respectively. When expressed as body concentrations, $LC50_{BodyNi}$ remained constant with time whilst $LC50_{BodyCo}$ increased during the first 7 d but remained stable afterwards. As identified by the MIXTOX model, interactions between Ni and Co were mainly antagonistic when based on free ion activities, however, no interaction was observed when based on body concentrations. A process-based model, incorporating exposure time to analyse the mechanisms underlying the dynamic mixture toxicity confirmed the differences in toxicokinetics of the 2 metals. The author's findings suggest that body concentrations, which incorporate bioaccumulation processes, are time-independent and can act as a more constant indicator of metal toxicity. The observed antagonism was mainly caused by competition between Co and Ni for binding sites and subsequent inhibition of Ni uptake. This competitive interaction occurred at the uptake level (toxicokinetics), but not at the target level (toxicodynamics).

Keywords: Mixture toxicology, Bioaccumulation, Metal speciation, Toxicokinetics, Toxicodynamics

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INTRODUCTION

Risk assessment of metals is usually based on toxicity data of single metals. As contamination in the environment rarely occurs as single metals but rather concerns (complex) mixtures of varying composition, this approach may have little environmental relevance [1]. Multiple metals may interact with each other, which leads to more-than-additive (synergism) or less-than-additive (antagonism) effects [2]. Hence, a risk assessment that ignores the possibility of joint action of metals is likely to underestimate or overestimate the actual risks. To meet future regulatory demands and ensuring adequate risk assessment [3], it is necessary to develop simple and efficient approaches for modelling metal mixture toxicity.

There are 2 most widely used models for predicting the effects of mixtures from the individual components: concentration addition (CA) and independent action (IA). The CA model assumes that mixture components have a similar mode of action, while IA assumes that the components have dissimilar modes of action [2,4]. The CA model usually estimates a higher combined effect than the IA model and therefore represents the worst-case scenario for mixture response [5]. In a risk assessment context, CA is therefore a more conservative choice when it is difficult to identify the mode of action of mixture components. It should be noted that both the CA model and the IA model in their standard form do not consider mixture interactions in estimating mixture toxicity. However, mixture components may interact at various levels: (1) exposure level, (2) uptake level, (3) target level [6,7]. Identifying interactions at relevant levels will therefore help to explain differences in interaction patterns that occur between different exposure media and between different test organisms.

The investigation of joint effects of mixtures on the basis of both external concentrations and body concentrations can contribute to a better understanding of the mechanism of

interactions at different levels. Body metal concentration has been shown to be a better indicator than external exposure concentration for predicting single metal toxicity to organisms [8-10]. Compared to external exposure concentrations, body concentration avoids the effect of environmental factors on metal accumulation [11]. In the present study, it is envisaged that body concentration of each mixture component may also serve as a useful indicator of mixture effects.

The importance of time in determining uptake and toxicity of single metals in organisms has been widely reported [8,12]. Generally, with the increase of time the amount of metal accumulated in an organism increases and toxic effects occur when the critical body threshold is reached [9,13]. Uptake and elimination rates differ for each metal [14,15]. The different kinetics of metals cause a time-dependent composition of the internal metal mixtures in exposed organisms, and subsequently the joint toxicities of metal mixtures are also time-related [16]. Spehar and Fiandt [17] found that the joint action of metals for fathead minnows was more than additive in an acute toxicity test, but less than additive in a chronic test. Baas et al. [18] investigated the toxicity of binary metals mixtures to *Folsomia candida*, observing that the interactions between, for instance, Cu and Cd changed over time when based on the CA (or IA) model. Therefore, the evaluation of mixture toxicity should take exposure time into account. However, at present, most models developed for predicting mixture toxicity of metals are based on a fixed exposure time without considering the impact of time on toxic interactions of metals [19,20].

Ni and/or Co pollution in the environment mainly resulted from the burning of fossil fuels, spreading of sewage sludge and manure, and mining activities [21]. Elevated levels of Ni and/or Co can cause harmful effects on the environment and human health. As Ni and Co are frequently encountered together in the environment, assessment of their joint effects is extremely

relevant [22]. The toxic effects of single Ni and Co on soil organisms have been well studied [8,23,24], but the binary mixture toxicity of them has rarely been investigated.

The present study aims to determine time-dependent mixture toxicity of Ni and Co to *Enchytraeus crypticus*, to quantify the mixture interactions at different exposure times with the MIXTOX model, and to describe the dynamics of mixture toxicity with a process-based model. Two research questions will be addressed: 1) do the interaction patterns of Ni and Co vary with time?; and 2) do the interaction patterns differ from each other when exposure is expressed on the basis of free ion activities or body concentrations?

MATERIALS AND METHODS

Test organism

Enchytraeids play an important role in the functioning of terrestrial ecosystems and are sensitive to chemical stressors [25]. *Enchytraeus crypticus* (Enchytraeidae; Oligochaeta; Annelida) was used as test organism in the present study. They were cultured in a climate room at 16 °C, with 75% relative humidity and in complete darkness. The animals were fed twice a week with a mixture of oat meal, dried baker's yeast, yolk powder, and fish oil. Adults were used, which could be distinguished by white spots in the clitellum region and with a length of approximately 1 cm.

Test medium

A quartz sand-solution system was used to avoid the disturbance of complex soil processes and to enable better control of metal exposure and speciation in the toxicity tests. The quartz sand was pre-treated following the method of He and Van Gestel [8] to remove all the organic matter, carbonates, and reactive Fe and Mn components. All used chemicals were of reagent grade (Sigma-Aldrich; > 99%). A basic solution composed of 0.2 mM Ca^{2+}, 0.05 mM

Mg^{2+}, 2.0 mM Na^{+} and 0.078 mM K^{+} was used as the control. Stock solutions of $NiCl_2$ and $CoCl_2$ were prepared by adding different amount of $NiCl_2 \cdot 6H_2O$ and $CoCl_2 \cdot 6H_2O$ to the basic solution. Test solutions with Ni alone, Co alone, or a mixture of Ni and Co were prepared by adding different volumes of stock solution to basic solution. All test solutions were adjusted to pH 6.0 (5.95-6.05) by using 0.75 g/L MOPS (3-[N morpholino] propane sulfonic acid) (AppliChem; >99%), 0.75 mg/L MES (2-[N-morpholino] ethane sulfonic acid) (Sigma-Aldrich; >99%) and 0.1 M NaOH when necessary.

Toxicity test

The mixture experiment consisted of 3 simultaneous treatment series (i.e. Ni alone, Co alone, and mixtures of Ni and Co). The concentrations of added Ni ranged from 0.2 to 12.8 mg/L, and the concentrations of added Co from 3 to 96 mg/L. The detailed design of concentrations of mixture combinations can be seen in **Figure S1** (Supplemental Data). Some combinations of the 2 metals at their highest concentrations were excluded because in our preliminary studies the test animals never survived combined exposure to these high concentrations. Toxicity tests with *E. crypticus* were conducted with 4 exposure times (4, 7, 10 and 14 d) and 3 replicates for each treatment and exposure time. As metal toxicity varies with time, the test concentrations in the mixtures were slightly different at different time intervals. Ten adults were exposed in 100 mL glass jars filled with 20.0 g pre-treated quartz sand and 5.4 mL test solution. The sand and the test solution were equilibrated for 1 d before introducing the animals. The experiments were incubated at 20 °C with a cycle of 12h light: 12h dark. The jars were weighted twice a week and water evaporation was compensated by adding deionized water. Animals were not fed during the experiment. Mortality was checked after different

exposure times. Surviving animals were collected, washed with deionized water and frozen at -18°C for further analysis of body metal concentrations.

Chemical analysis

The initial concentrations of Ni and Co in the test solutions were analyzed by flame atomic absorption spectrophotometry (AAS; Perkin Elmer AAnalyst 100). The frozen animals were freeze dried for at least 24 h, weighed individually on a microbalance, and digested in a 7:1 mixture of concentrated HNO_3 (Mallbaker Ultrex Ultra Pure, 65%) and $HClO_4$ (Mallbaker Ultrex Ultra Pure, 70%). Body concentrations of Ni and Co were measured by graphite furnace AAS (Perkin Elmer 1100B). DOLT-4 was used as certified reference material for quality control; the measured Ni and Co concentrations were always within 15% of the certified values.

Data analysis

Free ion activities of Ni and Co in the test solutions were calculated using the Windermere Humic Aqueous Model (WHAM VII) [26]. The measured pH values and concentrations of Ni, Co, Ca, Mg, Na and K were used as input parameters. The median lethal concentrations (LC50) with 95% confidence intervals (95% CI) of single Ni and Co based on free ion activities and body metal concentrations were calculated using the trimmed Spearman-Karber method [27].

Model description

Concentration-addition model (CA). Both Ni and Co are divalent cations in solutions with almost the same molecular weight. It is assumed that they may have a similar mode of action because of structural electronic similarities. The CA model was therefore used to predict the toxicity of Ni and Co mixtures.

$$MT = \sum TU_{Xi} = \sum_{i=1}^{n} \frac{c_i}{EC_{Xi}} \quad (1)$$

where c_i is the concentration of component *i* in the mixture, EC_{Xi} is the concentration of component *i* causing a certain effect X when applied alone, TU_{Xi} is the dimensionless toxic unit that quantifies the relative contribution of the individual component *i* to the toxicity of the mixtures; Mixture toxicity (MT) is regarded as the sum of the toxic units of the individual metals. In the present study, binary mixtures of Ni and Co were investigated and LC50 for mortality was selected as the endpoint, so Equation 1 can be rewritten as:

$$MT = \frac{c_{Ni}}{\text{LC50 of single Ni}} + \frac{c_{Co}}{\text{LC50 of single Co}} \quad (2)$$

When using free metal ion activity as the expression of exposure, c_{Ni} and c_{Co} are the free ion activities of Ni and Co in the test solutions (μM), and LC50 (μM) is the free ion activity of Ni and Co causing 50% mortality of *E. crypticus* when applied singly (denoted as LC50{Ni^{2+}} and LC50{Co^{2+}}, respectively). When using body metal concentration as the expression of exposure, c_{Ni} and c_{Co} are the body concentrations of Ni and Co in the organism (μM/kg dry body weight), and LC50 (μM/kg) is the dry body concentration of Ni and Co causing 50% mortality of *E. crypticus* when applied singly (denoted as $LC50_{BodyNi}$ and $LC50_{BodyCo}$, respectively).

A logistic dose-response model was used to correlate the survival of *E. crypticus* to the calculated MT after different exposure times.

$$S = \frac{S_{max}}{1+(\frac{MT}{MT_{50}})^b} \quad (3)$$

where *S* is the number of surviving *E. crypticus*, S_{max} the number of survivors in the control, *b* the slope parameter, and MT_{50} the MT level causing 50% mortality. The parameters were estimated

by fitting Equation 3 to the data for each exposure time separately, using the nonlinear regression module in SPSS 19.0.

MIXTOX model. The MIXTOX model is basically a more elaborate version of the standard CA model that allows using additional parameters to quantify possible deviations (i.e, synergism, antagonism, dose-ratio or dose-level dependent synergism/antagonism) from the standard model, following the method of Jonker et al. [28]. Extra parameters are introduced into the model using a stepwise approach to describe deviations (see Jonker et al. [28] for details). The model was fitted to the data using the method of maximum likelihood while minimizing the sum of the squared residuals. The statistical significance of the improvement in fit from the extended parameters was obtained through chi-square (χ^2) tests. The interpretations of the extra parameters can be found in Jonker et al. [28].

Process-based model. A process-based model was used for better understanding the dynamics of the effects of the mixture by taking into account the processes of uptake and elimination [18]. The main difference with the CA (or IA model) is that the entire time course of the toxic effects of the mixture is incorporated within 1 model. In this model, it was assumed that when the internal concentration exceeds a certain threshold, the probability to die starts to deviate from that of the control. For both metals in single and mixture exposures, 3 time-independent parameters were estimated to describe the dynamic effect: a toxicological threshold below which no effects occur, no effect concentration (NEC) (mM), and which is a measure for the toxic potency of the compounds, the killing rate ($mM^{-1}\ d^{-1}$) and a kinetic parameter, the elimination rate (d^{-1}). In addition the model included the control or blank mortality rate (d^{-1}) to correct for control mortality, and an interaction parameter for the mixtures.

RESULTS

Body concentrations of mixtures of Ni and Co

Body concentrations of one metal in the presence of the other metal after different exposure times are shown in **Figures 1 and 2**. Generally, increasing exposure concentrations of Ni and Co increased their uptake by *E. crypticus*. At different time points, the body concentration of Ni was significantly and negatively correlated with the increase of Co concentrations ($p<0.01$) (**Figure 1**). For example, at a Ni exposure concentration of 27.3 µM, body Ni concentration decreased from 177 to 51.6, 147 to 92.8, 153 to 80.8 and 151 to 55.3 µM/kg after 4, 7, 10 and 14 d, respectively, when Co concentration increased from 0 to 814 µM. In contrast, the addition of Ni did not significantly affect the uptake of Co at the different exposure times ($p>0.05$) (**Figure 2**). These findings suggest a strong interaction effect of Co on Ni during the uptake phase.

Individual toxicity of Ni and Co after different exposure times

The dose-response relationships for the effects of Ni and Co on the survival of *E. crypticus* after different exposure times are shown in **Figure 3**, using 2 expressions of exposure (free ion activities and body concentrations). Generally, mortality increased with increasing free ion activity and body concentration of each metal tested. When expressed as free ion activities, LC50{Ni^{2+}} decreased gradually from 78.3 at 4 d to 40.4 µM at 14 d, and the final LC50{Ni^{2+}} was not reached after 14 d exposure. LC50{Co^{2+}} were reduced from 511 at 4 d to 393 µM at 14 d, the toxicity of Co almost reached steady state after 10 d exposure, with an LC50{Co^{2+}} of 401 µM (**Table 1**). Interestingly, the slope of the dose-response curve for Ni became much steeper after 14 d compared to 4 d. When expressed as body concentrations, $LC50_{BodyNi}$ remained almost constant with the increase of time, being 341, 383, 330 and 341 µM/kg after 4, 7, 10 and 14 d, respectively, and slope of the dose-response curves showed little difference for different

exposure times. $LC50_{BodyCo}$ increased from 2155 after 4 d to 3184 µM/kg after 7 d, and then levelled off to values of 3840 and 3591 µM/kg after 10 and 14 d, respectively (**Table 1**). Ni was more toxic than Co to *E. crypticus*, with LC50s after different exposure times differing a factor of 7 to 10 when based on free ion activities and a factor of 6 to 10 when expressed on the basis of body concentrations.

Toxicity of mixtures of Ni and Co after different exposure time

In the mixture solutions of Ni and Co, the free ion activities of one metal were not significantly affected by the presence of the other metal (Supplemental Data, **Figure S2**). The observed toxic effects of Ni-Co mixtures after different exposure times are plotted against MT based on both free ion activities (**Figure 4**) and body metal concentrations (**Figure 5**). Generally, the survival rate of *E. crypticus* significantly decreased with increasing MT ($p<0.01$). On the basis of free ion activity, the model fit improved significantly from 4 d to 7 d, with R^2 values increasing from 0.69 to 0.84. After 7 d no further improvement of the fits was observed, with R^2 of 0.82 at 10 d and 0.81 at 14 d (**Table 2**). The estimated MT_{50} (95% CI) were 1.19 (1.09-1.29), 1.13 (1.07-1.19), 1.18 (1.13-1.22) and 1.16 (1.11-1.20) after 4, 7, 10 and 14 d, respectively (**Figure 4**). With the increase of exposure time, no significant changes in model fit were observed when based on body metal concentration, with R^2 varying between 0.50 and 0.58 at the different exposure times (**Table 2**). The estimated MT_{50} (95% CI) were 1.02 (0.893-1.15), 0.845 (0.729-0.929), 0.980 (0.880-1.08), 0.880 (0.744-1.02) after 4, 7, 10 and 14 d, respectively (**Figure 5**).

The mixture data were further analysed using the MIXTOX model to quantify the deviation from concentration addition. The estimated value of the interaction parameters (*a* and *b*), the goodness of fitting (R^2), and significance test results ($p(\chi^2)$) are shown in **Table 2**. When

based on free metal ion activity, the MIXTOX model showed that the interaction between Ni and Co after different exposure times was mainly antagonistic, with the value of parameter *a* being positive. The inclusion of parameter a (2.46) at 4 d significantly improved the model fit, with $p(\chi^2)<0.05$. For 7 and 10 d, the best model fit was obtained when including a second parameter b_{DR} to describe a dose ratio-dependent deviation. The estimated values of b_{DR} were positive, being 1.32 at 7 d and 2.12 at 10 d, indicating that a decreased joint effect was due mostly to Co. For 14 d exposure, the extension of the CA model with a second parameter b_{DL} (-5.86) to describe dose level-dependent dependence provided the best description of the data. This revealed the interaction pattern was antagonism and the magnitude of antagonism was dose-level dependent. However, on the basis of body metal concentrations, for the treatments at all 4 exposure times, the deviation from additivity was not significant ($p(\chi^2) >0.05$).

The survival data of *E. crypticus* exposed to Ni and Co at different time points were fitted using the process-based model for both single and mixture exposure. The estimated parameters are shown in **Table 3**. For the single metal exposures, control mortality rate was rather low, 8.0×10^{-4} and 6.5×10^{-3} d^{-1} for Ni and Co, respectively. The estimated NEC for Ni and Co were 0.036 and 0.37 mM, respectively, indicating that *E. crypticus* is approximately 10 times more sensitive to Ni than to Co. The killing rate of Ni (10.2 $mM^{-1}d^{-1}$) was almost 3 times higher than that of Co (3.2 $mM^{-1}d^{-1}$). The elimination rates of Ni and Co were similar, with values of 1.66 d^{-1} for Ni and 1.20 d^{-1} for Co. For the mixtures of Ni and Co, the killing rate of each metal was lower than that for the single exposures, being 3.63 $mM^{-1}d^{-1}$ for Ni and 1.43 $mM^{-1}d^{-1}$ for Co (i.e. ≈ 3-fold differences). The elimination rate of Ni decreased from 1.66 to 0.70 d^{-1} in the presence of Co, while the elimination rate of Co was not affected by the presence of Ni. No significant interaction was found in the mixtures.

DISCUSSION

Single toxicity

When applied singly, Ni was more toxic to *E. crypticus* than Co, with the individual LC50 and NEC of Ni approximately 10 times lower than that of Co. The slope of the dose-response curve of single Ni after 14 d exposure was much steeper than that after 4 d exposure, while the slope of Co was rather constant (**Figure 3**). In addition, it took a longer time for Ni toxicity to reach steady state than for Co toxicity. This indicates that Co is a faster acting toxicant than Ni. A number of studies have investigated the single toxicity of Ni and Co to aquatic and soil organisms. For instance, Griffitt et al. [29] found that 48h LC50 values of Ni and Co were 1.48 and 9.72 mg/L, respectively, for *Daphnia pulex*, and 19.6 and 94.7 mg/L, respectively, for *Ceriodaphnia dubia*. Their study showed that Ni is approximately 5 times more toxic than Co after 48h. These results are consistent with our findings. The present study showed that the toxicity of Ni and Co increased with exposure time when based on free ion activities. Previous studies reported that toxicity of Ni to *F. candida* and *E. crypticus* increased over time and almost reached steady states after 49 d and 21 d exposure, respectively [8,16]. Bioaccumulation of a metal is the net result of uptake, distribution and elimination processes in an organism during exposure. The body concentrations of metals increase over time until steady state is reached between influx and efflux [30]. Toxic effects are induced when body metal concentration exceeds a critical level. Noteworthy, when single toxicity of Ni and Co was based on body concentrations, the $LC50_{BodyNi}$ was almost constant with time and the $LC50_{BodyCo}$ also remained constant from 7 d exposure onwards. He and Van Gestel [8] reported that the LC50 for the toxicity of Ni to *E. crypticus* expressed as body concentrations was approximately constant and independent of exposure time, with a value of 285 μM/kg, which was comparable with the result

obtained in the present study (330-383 µM/kg). This suggests that body concentration is a better indicator of toxicity than free ion activity and that both Ni and Co in *E. crypticus* comply with the concept of Critical Body Residues [9].

Mixture toxicity

In general, the CA model well described the mixture toxicity of Ni and Co on the basis of free ion activities. The explained variation increased from 69% after 4 d to 84% after 7 d, and then remained constant. This suggests that the steady state of Ni and Co accumulation in the mixtures was not yet reached after 4 d exposure. Accumulation (uptake and elimination) rates vary according to the chemical nature of the compound, and also the size and type of organisms tested [31]. The accumulation rate of Co was found to be higher than that of Ni in bivalve species [32]. The differences in toxicokinetics of each mixture component may become an uncertainty factor when considering mixture interactions at a fixed time point during the uptake phase. When using body metal concentration as the metric of exposure, the toxicokinetic process is included [33]. So the fit of the CA model did not change with time when mixture toxicity was related to body concentrations, even when interactions occurred in the uptake phase. However, only approximately 50% of the variation in the data was explained by the CA model when using body concentrations. Within an organism, metal exposure can be regulated by storing in inert forms and detoxification, suggesting that body concentration cannot fully represent the concentration at target sites [13]. Hence, the use of internal concentrations as indicator of toxicity still does not incorporate the toxicodynamic processes that quantitatively link the body concentration to the effect at the level of the individual organism over time [34]. In addition, unlike plants, the body concentration of metals can only be analysed in surviving animals, which may not directly reflect the concentration in the dead animals and subsequently reduce the model

performance. One evidence is that the tissue concentration showed to be the best predictor of toxic effects of As to plants, but was not predictive of the toxicity to earthworms [35].

Interaction patterns at different levels

In the present study, the existence of one metal cannot significantly affect the free ion activities of the other metal in the mixture solutions, indicating that no interaction occurred at the exposure level. The joint effect of Ni and Co after different exposure times was mainly antagonistic on the basis of free ion activities, and the fit of the MIXTOX model to the data was significantly improved when considering the deviations from concentration addition. Gikas [22] investigated the effects of Ni and Co on the microbial growth rate of activated sludge and found that mixture interactions shifted from synergism at relative low concentrations to antagonism at relative high concentrations. Whether mixture interactions are synergistic or antagonistic depends on whether one metal facilitates the uptake of the other or whether they compete for the same transport sites [36]. A possible explanation for the antagonistic interaction is that Ni and Co can compete with each other for uptake, thus resulting in less accumulation of either one or both metals. According to the concept of the Biotic Ligand Model (BLM), the coexisting cations can exert a protective effect by competing with metal ions for the binding sites on the surface of organisms and inhibiting the uptake of metals [37]. He et al. [38] provided evidence that Mg reduced the uptake of Ni through competition with Ni for the binding sites of *E. crypticus*. Lock et al. [24] reported that Mg also has a significant protective effect on the toxicity of Co to *Enchytraeus albidus*. Ni and Co are both divalent in solution and belong to the VIIIB group of the periodic table, having similar physicochemical properties. The competition for membrane binding sites and intracellular binding sites can occur for metals with similar ionic radii and

coordination geometry [39]. So, it is likely that Ni and Co share some common transport or target sites on the surface or inside the organisms.

There was no deviation from additivity on the basis of body concentrations. Weltje [6] reported that the toxic effects of Cd, Cu, Pb and Zn mixtures were mainly antagonistic using total soil concentrations and concentration additive behaviour was found when using metal concentrations in earthworm tissues. This finding is consistent with the result of the present study. The deviation from the CA model can result from toxicokinetic and toxicodynamic interactions among components in the mixture. Metals in solutions may interact at various levels including during uptake (toxicokinetics) and at target sites within an organism (toxicodynamics) [1,6]. Different conclusions on the interaction of metals can be drawn when using different expressions of exposure. The activities of Ni and Co in the exposure solution were not affected by the presence of each other, ruling out the interactions at the exposure level. The difference of the interaction patterns based on free ion activities and body concentrations suggests that the competitive interaction between Ni and Co mainly occurred during uptake, which affected toxicokinetics and subsequently the quantity available at the binding sites on the surface of the organisms. No competition at the target level, which may affect toxicodynamics and the concentration of metals on the target sites inside the organism, was observed.

In the presence of Co, the uptake of Ni was significantly reduced by 20-70%, while Ni did not exert appreciable effect on the uptake of Co (**Figure 1 and 2**), suggesting that Co acted as an antagonist and modified the bioaccumulation of Ni. In agreement with our results, Wang et al. [40] showed that the uptake of Zn and Co by plant roots was reduced in the presence of each other through a site competition mechanism. Franklin et al. [36] found that Cu inhibited the binding and cellular uptake of Zn, which resulted in decreased mixture toxicity to freshwater

algae (*Chlorella* sp.). These findings support our hypothesis that the antagonistic interaction between Ni and Co is caused by competition and subsequently reduced metal uptake. In the mixture solutions, Co concentrations were many-fold higher than Ni concentrations, this may explain why the uptake of Ni was reduced in the presence of Co and not vice-versa. Ni and Co interact with each other during the uptake process. This competition suggests a similar mode of action, providing a basis for assuming concentration-additive effects and supporting the selection of the CA model as a conservative choice for estimating the mixture toxicity of Ni and Co.

CONCLUSIONS

The present study determined the joint toxicity of Ni and Co to *Enchytraeus crypticus* and used 3 models (CA, MIXTOX and process-based model) to evaluate the toxic effects of Ni-Co mixtures at different exposure times. Body concentration was found to be a time-independent measure of metal toxicity and a certain exposure time is needed to reach steady state. Interaction between Ni and Co was mainly antagonistic on the basis of free ion activities, and concentration additive on the basis of body concentrations. Toxicity of the Ni and Co mixture was dominantly determined by interactions at the uptake level (toxicokinetics), but not at the target level (toxicodynamics). The present study provided insight into the mechanism of the interactive effect of binary Ni and Co mixtures on the survival of *E. crypticus* at different interaction levels. Further research is needed to obtain more insight into the mechanisms of the interaction between metals by applying more advanced metal speciation models (e.g. WHAM-F_{TOX}) and to investigate the mixture toxicity of metals in the context of soil ecosystems with more complex interactions.

SUPPLEMENTAL DATA

Figures S1–S2. (127 KB DOC).

Acknowledgment—E. He receives a PhD grant (No. 2011638012) from the China Scholarship Council. The authors would like to thank N. M. van Straalen for his suggestions to improve the manuscript.

REFERENCES

1. Vijver MG, Peijnenburg WJGM, De Snoo GR. 2010. Toxicological mixture models are based on inadequate assumptions. *Environ Sci Technol*, 44: 4841-4842.
2. Van Gestel CAM, Jonker M, Kammenga JE, Laskowski R, Svendsen C. (Eds.). 2011. *Mixture toxicity: linking approaches from ecological and human toxicology*. SETAC press, Society of Environmental Toxicology and Chemistry, Pensacola 2011.
3. Kortenkamp A, Backhaus T, Faust M. 2009. State of the art review of mixture toxicity. Report to the Commission of the European Union (Directorate General for the Environment), 22 December.
4. Altenburger R, Walter H, Grote M. 2004. What contributes to the combined effect of a complex mixture? *Environ Sci Technol*, 38: 6353-6362.
5. Boedeker W, Drescher K, Altenburger R, Faust M, Grimme LH. 1993. Combined effects of toxicants: the need and soundness of assessment approaches in ecotoxicology. *Sci Total Environ*, 134: 931-939.

6. Weltje L. 1998. Mixture toxicity and tissue interactions of Cd, Cu, Pb and Zn in earthworms (Oligochaeta) in laboratory and field soils: A critical evaluation of data. *Chemosphere*, 36: 2643-2660.
7. Qiu H, Vijver MG, Peijnenburg WJGM. 2011. Interactions of cadmium and zinc impact their toxicity to the earthworm *Aporrectodea caliginosa*. *Environ Toxicol Chem*, 30: 2084-2093.
8. He E, Van Gestel CAM. 2013. Toxicokinetics and toxicodynamics of nickel in *Enchytraeus crypticus*. *Environ Toxicol Chem*, 32: 1835-1841.
9. McCarty LS, Mackay D. 1993. Enhancing ecotoxicological modeling and assessment. Body residues and modes of toxic action. *Environ Sci Technol*, 27: 1718-1728.
10. Borgmann U, Norwood WP, Dixon DG. 2008. Modelling bioaccumulation and toxicity of metal mixtures. *Hum Ecol Risk Assess*, 14: 266-289.
11. De Schamphelaere KAC, Stauber JL, Wilde KL, Markich SJ, Brown PL, Franklin NM, Janssen CR. 2005. Toward a biotic ligand model for freshwater green algae: surface-bound and internal copper are better predictors of toxicity than free Cu^{2+}-ion activity when pH is varied. *Environ Sci Technol*, 3: 2067-2072.
12. Crommentuijn T, Doodeman CJAM., Doornekamp A, Van Der Pol JJC, Van Gestel CAM, Bedaux JJM. 1994. Lethal body concentrations and accumulation patterns determine time-dependent toxicity of cadmium in soil arthropods. *Environ Toxicol Chem*, 13: 1781-1789.
13. Vijver MG, Van Gestel CAM, Lanno RP, Van Straalen NM, Peijnenburg WJGM. 2004. Internal metal sequestration and its ecotoxicological relevance: a review. *Environ Sci Technol*, 38: 4705-4712.

14. Spurgeon DJ, Hopkin SP. 1999. Comparisons of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. Appl Soil Ecol, 11: 227-243.
15. Lock K, Janssen CR. 2001. Zinc and cadmium body burdens in terrestrial oligochaetes: Use and significance in environmental risk assessment. *Environ Toxicol Chem*, 20: 2067-2072.
16. Broerse M, Van Gestel CAM. 2010. Mixture effects of nickel and chlorpyrifos on *Folsomia candida* (Collembola) explained from development of toxicity in time. *Chemosphere*, 79: 953-957.
17. Spehar RL, Fiandt JT. 1986. Acute and chronic effects of water quality criteria-based metal mixtures on three aquatic species. *Environ Toxicol Chem*, 5: 917-931.
18. Baas J, Van Houte BPP, Van Gestel CAM, Kooijman SALM. 2007. Modeling the effects of binary mixtures on survival in time. *Environ Toxicol Chem*, 26: 1320-1327.
19. Kamo M, Nagai T. 2008. An application of the biotic ligand model to predict the toxic effects of metal mixtures. *Environ Toxicol Chem*, 27: 1479-1487.
20. Le TTY, Vijver MG, Hendriks AJ, Peijnenburg WJGM. 2013. Modeling toxicity of binary metal mixtures (Cu^{2+}–Ag^{+}, Cu^{2+}–Zn^{2+}) to lettuce, *Lactuca sativa*, with the biotic ligand model. *Environ Toxicol Chem*, 32: 137-143.
21. Liber K, Doig LE, White-Sobey SL. 2011. Toxicity of uranium, molybdenum, nickel, and arsenic to *Hyalella azteca* and *Chironomus dilutus* in water-only and spiked-sediment toxicity tests. *Ecotox Environ Safe*, 74: 1171-1179.
22. Gikas P. 2007. Kinetic responses of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)): An isobolographic approach. *J Hazard Mater*, 143: 246-256.

23. Lock K, Janssen CR. 2002. Ecotoxicity of nickel to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. *Chemosphere*, 46: 197-200.
24. Lock K, De Schamphelaere KAC, Becaus S, Criel P, Van Eeckhout H, Janssen CR. 2006. Development and validation of an acute biotic ligand model (BLM) predicting cobalt toxicity in soil to the potworm *Enchytraeus albidus*. *Soil Biol Biochem*, 38: 1924-1932.
25. Didden W, Römbke J. 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotox Environ Safe*, 50(1): 25-43.
26. Lofts S, Tipping E. 2011. Assessing WHAM/Model VII against field measurements of free metal ion concentrations: model performance and the role of uncertainty in parameters and inputs. *Environ Chem*, 8: 501-516.
27. Hamilton MA, Russo RC, Thurston RV. 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ Sci Technol*, 11: 714-719.
28. Jonker MJ, Svendsen C, Bedaux JJM, Bongers M, Kammenga JE. 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environ Toxicol Chem*, 24: 2701-2713.
29. Griffitt RJ, Luo J, Gao J, Bonzongo JC, Barber DS. 2008. Effects of particle composition and species on toxicity of metallic nanomaterials in aquatic organisms. *Environ Toxicol Chem*, 27: 1972-1978.
30. Harrahy EA, Clements WH. 1997. Toxicity and bioaccumulation of a mixture of heavy metals in *Chironomus tentans* (Diptera: Chironomidae) in synthetic sediment. *Environ Toxicol Chem*, 16, 317-327.

31. Slaveykova VI, Wilkinson KJ. 2005. Predicting the bioavailability of metals and metal complexes: critical review of the biotic ligand model. *Environ Chem*, 2: 9-24.
32. Markich SJ, Brown PL, Jeffree RA. 2001. Divalent metal accumulation in freshwater bivalves: an inverse relationship with metal phosphate solubility. *Sci Total Environ*, 275: 27-41.
33. Ashauer R, Escher BI. 2010. Advantages of toxicokinetic and toxicodynamic modelling in aquatic ecotoxicology and risk assessment. *J Environ Monitor*, 12: 2056-2061.
34. Jager T, Albert C, Preuss TG, Ashauer R. 2011. General unified threshold model of survival - a toxicokinetic-toxicodynamic framework for ecotoxicology. *Environ Sci Technol*, 45: 2529-2540.
35. García-Gómez C, Esteban E, Sánchez-Pardo B, Fernández M. 2014. Assessing the ecotoxicological effects of long-term contaminated mine soils on plants and earthworms: relevance of soil (total and available) and body concentrations. *Ecotoxicology*, 23, 1195-1209.
36. Franklin NM, Stauber JL, Lim RP, Petocz P. 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella sp.*): The effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. *Environ Toxicol Chem*, 21: 2412-2422.
37. Di Toro DM, Allen HE, Bergman HL, Meyer JS, Paquin PR, Santore RC. 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. *Environ Toxicol Chem*, 20: 2383-2396.
38. He E, Qiu H, Van Gestel CAM. 2014. Modelling uptake and toxicity of nickel in solution to *Enchytraeus crypticus* with biotic ligand model theory. *Environ Pollut*, 188: 17-26.
39. Sunda WG, Huntsman SA. 1998. Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. *Sci Total Environ*, 219: 165-181.

40. Wang Y-M, Kinraide TB, Wang P, Zhou D-M, Hao X-Z. 2013. Modeling rhizotoxicity and uptake of Zn and Co singly and in binary mixture in wheat in terms of the cell membrane surface electrical potential. *Environ Sci Technol*, 47: 2831-2838.

Figure 1. Body Ni concentrations in *Enchytraeus crypticus* under the influence of Co after different times of exposure to different Ni concentrations in solutions embedded in an inert sand matrix. See Figure S1 for test design.

Figure 2. Body Co concentrations in *Enchytraeus crypticus* under the influence of Ni after different times of exposure to different Co concentrations in solutions embedded in an inert sand matrix. See Figure S1 for test design.

Figure 3. Effects of Ni and Co on the survival of *Enchytraeus crypticus* exposed for 4, 7, 10 and 14 d to solutions embedded in an inert sand matrix. Ni and Co exposure levels are expressed as free ion activities (A, B) and body metal concentrations in the surviving enchytraeids (C, D).

Figure 4. The relationship between the survival of *Enchytraeus crypticus* after 4 (A), 7 (B), 10 (C) and 14 (D) d exposure and binary mixture concentrations of Ni and Co expressed as the sum of toxic units (MT) based on metal free ion activities. The data points represent the observed values; the solid line shows the fit of a logistic dose-response model. See Figure S1 for test design.

Figure 5. The relationship between the survival of *Enchytraeus crypticus* after 44 (A), 7 (B), 10 (C) and 14 (D) d exposure and binary mixture concentrations of Ni and Co expressed as the sum of toxic units (MT) based on body metal concentrations. The data points represent the observed values; the solid line shows the fit of a logistic dose-response model. See Figure S1 for test design.

Table 1. Median lethal concentrations (LC50) for the toxicity of single Ni and single Co to *Enchytraeus crypticus* at different exposure times in solutions embedded in an inert quartz sand matrix. Exposures were expressed as free ion activities and body metal concentrations in surviving animals, respectively. The 95% confidence intervals are given in between brackets.

Time	LC50{Ni^{2+}}	LC50{Co^{2+}}	$LC50_{BodyNi}$	$LC50_{BodyCo}$
(d)	(µM)	(µM)	(µM/kg)	(µM/kg)
4	78.3 (68.8-87.9)	511 (489-534)	341 (301-381)	2155 (1718-2592)
7	65.2 (63.1-67.3)	444 (423-465)	383 (371-394)	3184 (2842-3527)
10	45.0 (42.9-47.2)	401 (389-413)	330 (305-356)	3840 (3337-4342)
14	40.4 (38.7-42.1)	393 (370-416)	341 (313-368)	3591 (3162-4091)

Table 2. Mixture toxicity of Ni and Co to *Enchytraeus crypticus* after different times of exposure to test solutions embedded in an inert sand matrix. See **Figure S1** for test design. The table summarizes the parameter values obtained by fitting the Concentration Addition (CA) module of the MIXTOX model [28]. CA is concentration addition; DR is dose ratio-dependent deviation from concentration addition; DL is dose level-dependent deviation from the concentration addition. The R^2 value indicates the goodness of fit; a and b are the parameters of the deviation functions; $p(\chi^2)$ is the statistic outcome of the χ^2 test ($p<0.05$, significant difference).

Time (d)		Free ion activity based					Body concentration based	
		R^2	a	b_{DR}/b_{DL}	p (χ^2)	pattern	R^2	pattern
4	CA	0.69					0.53	
	Deviation	0.86	2.46		2.1×10^{-18}	Antagonism		No deviation
7	CA	0.84					0.56	
	Deviation	0.90	0.16	1.32	0.027	DR		No deviation
10	CA	0.82					0.58	
	Deviation	0.91	0.18	2.12	1.5×10^{-5}	DR		No deviation
14	CA	0.81					0.50	
	Deviation	0.90	0.14	-5.86	0.033	DL		No deviation

Table 3. The estimated parameters for the blank mortality rate, no-effect concentration (NEC), killing rate, and elimination rate for the mixture toxicity of Ni and Co to *Enchytraeus crypticus* after different exposure time exposed to test solutions embedded in an inert sand matrix, using the mixture toxicity model of Baas et al. [18]. SD is standard deviation.

	Single		Mixture	
	Ni (±SD)	Co (±SD)	Ni	Co
Blank mortality rate (d^{-1})	8.0×10^{-4} ($\pm8\times10^{-4}$)	6.5×10^{-3} ($\pm2\times10^{-3}$)	0.004	
NEC (mM)	0.036 (±0.003)	0.37 (±0.007)	0.043	0.40
Killing rate ($mM^{-1}d^{-1}$)	10.2 (±2.3)	3.20 (±0.85)	3.63	1.43
Elimination rate (d^{-1})	1.66 (±0.98)	1.20 (±0.34)	0.70	1.10

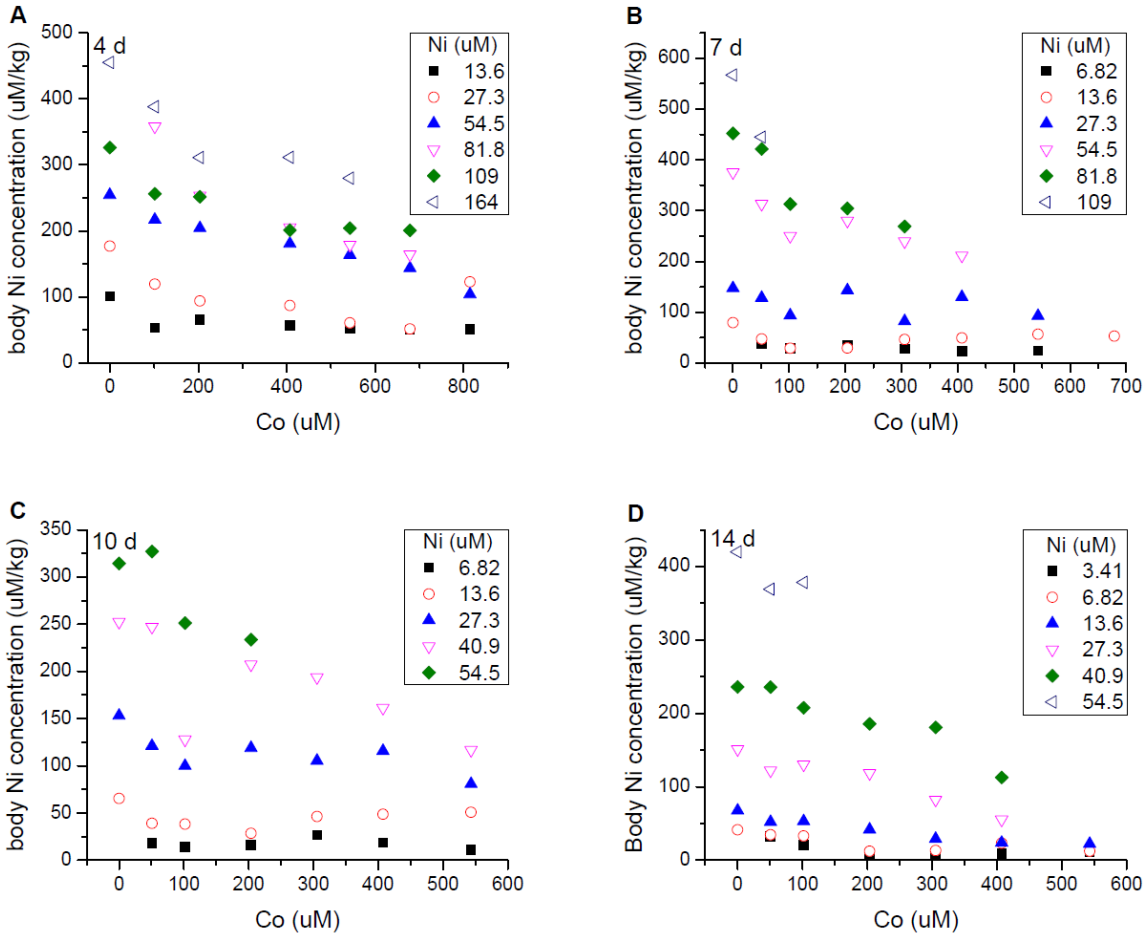

Figure 1

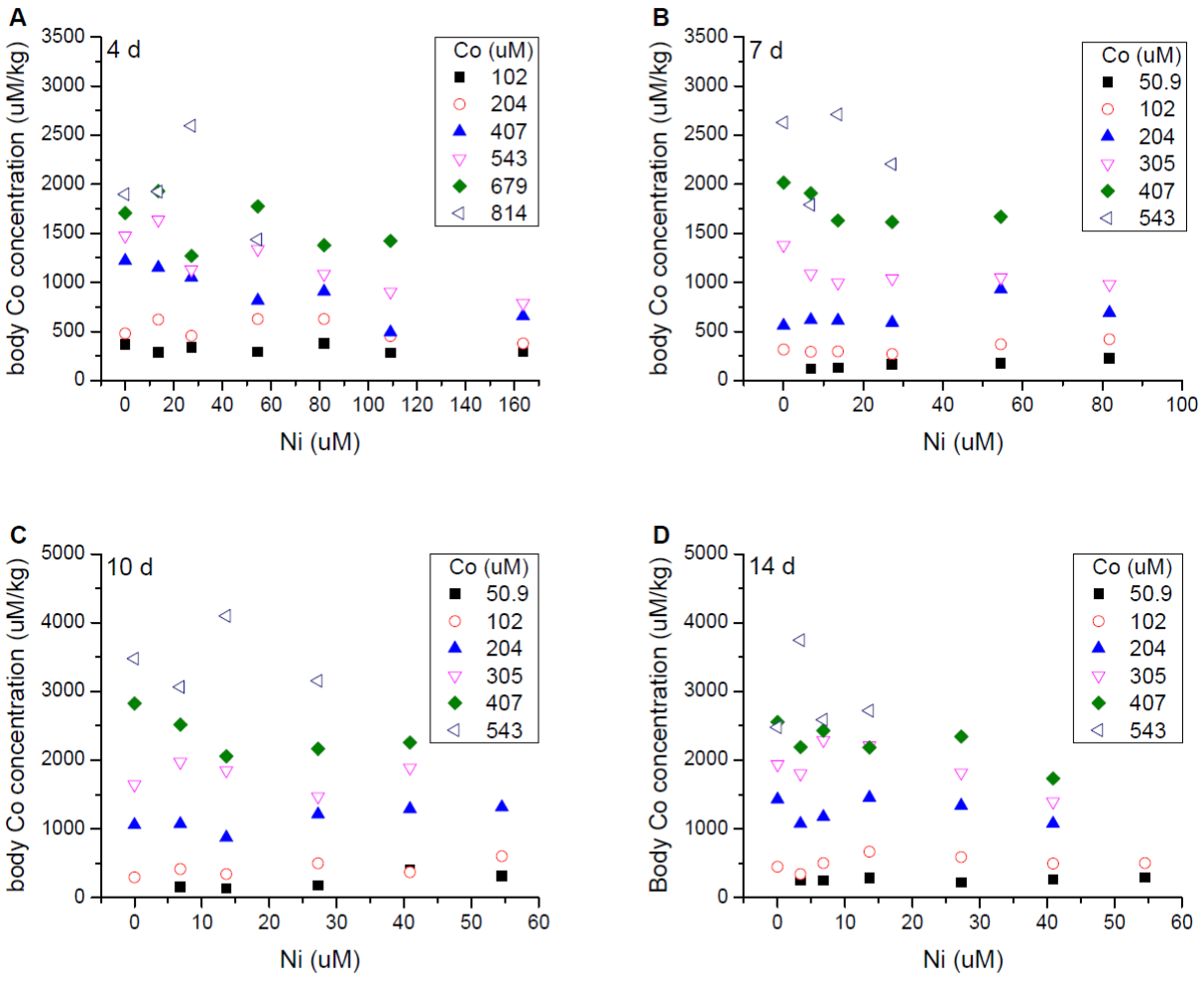

Figure 2

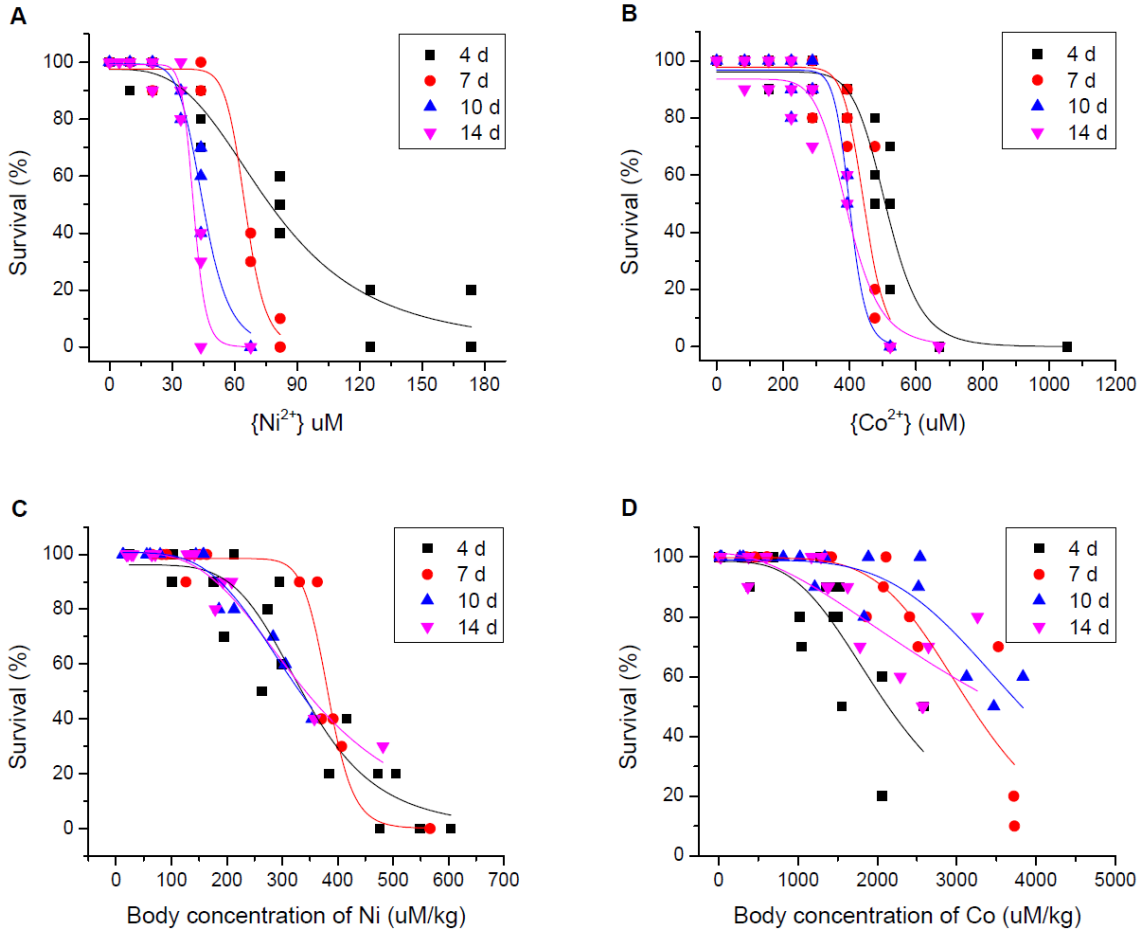

Figure 3

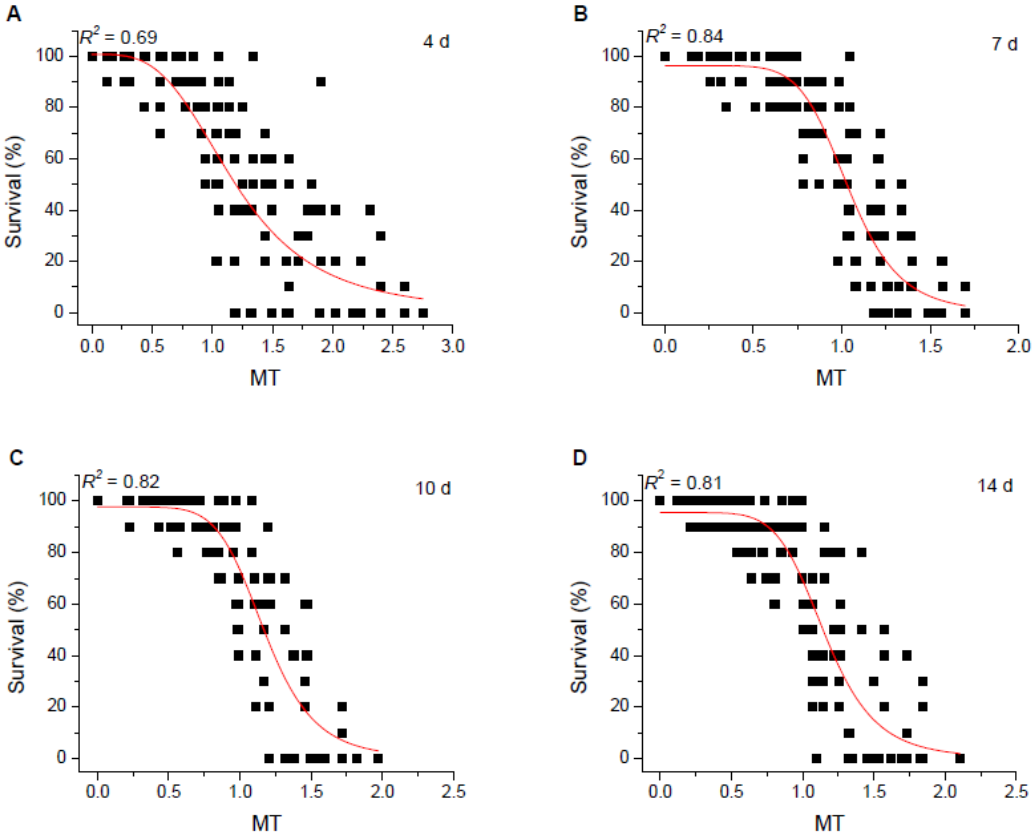

Figure 4

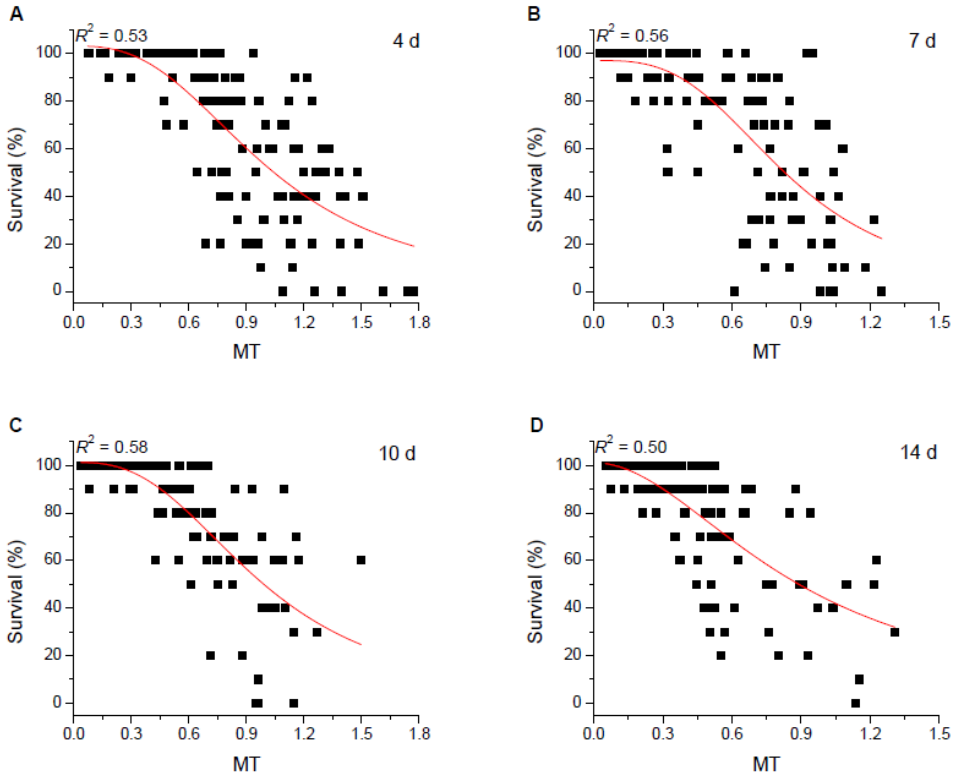

Figure 5