Modeling Rhizotoxicity and Uptake of Zn and Co Singly and in Binary Mixture in Wheat in Terms of the Cell Membrane Surface Electrical Potential

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Ⓢ *Supporting Information*

ABSTRACT: The usually negative, but variable electrical potential (ψ_0) at the cell membrane (CM) surface influences the surface activities of free ions and the electrical driving force for the transport of ions across the CM. The rhizotoxic effects and uptake of Zn^{2+} and Co^{2+} singly and in binary mixture in wheat (*Triticum aestivum* L.) at three pH values (4.5, 5.5, or 6.1) were examined in terms of the free ion activities of Zn^{2+}, Co^{2+}, and H^+ at the CM surface (these ions are denoted $\{M^{n+}\}_0$). Toxicity and uptake of Zn^{2+} or Co^{2+} singly to roots were better correlated with $\{M^{2+}\}_0$ than with their bulk-phase activities. Studies of toxicant interactions using the electrostatic approach and a response-multiplication model for toxicant mixtures indicated that $\{Co^{2+}\}_0$ significantly enhanced the toxicity of $\{Zn^{2+}\}_0$, but $\{Zn^{2+}\}_0$ did not significantly affect the toxicity of $\{Co^{2+}\}_0$. $\{H^+\}_0$ substantially enhanced the toxicity of both metal ions. Taking ψ_0 into account improved the correspondence (denoted r^2) between observed and predicted uptake of both Zn^{2+} and Co^{2+}, and each inhibited the uptake of the other. Results showed that r^2 increased from 0.776 to 0.936 for Zn uptake and improved from 0.805 to 0.951 for Co uptake. Thus electrostatic models for metal toxicity and uptake proved superior to models incorporating only bulk-phase activities of ions.
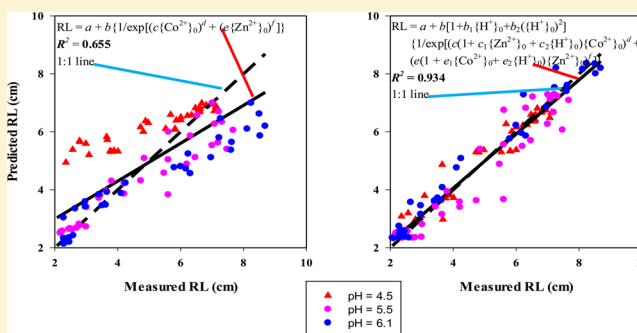
■ INTRODUCTION

Metal contamination has received increasing attention due to its adverse effects and ubiquitous existence.[1] Contamination rarely occurs as single metals, and situations with multiple contaminants have increasingly attracted more and more concern.[2] Metal toxicity and uptake assessments based on the effects of combined toxicants will be more realistic than assessments based upon single toxicant.[3] Several studies have been conducted to investigate the toxicity of metal mixtures to certain plant species.[4−7] Exposure of cucumber (*Cucumis sativus*) to binary combinations of Cu^{2+}, Cd^{2+}, and Pb^{2+} resulted in interactions known as antagonistic, additive, and synergistic, and the bioaccumulation of Cu^{2+} in shoots was influenced by Cd^{2+} and/or Pb^{2+} in mixtures.[4] Compared to the uptake of single metals, combined Cd^{2+} and Zn^{2+} in soil resulted in a decrease in Zn^{2+} uptake but an increase in Cd^{2+} uptake by soybeans (*Glycine max*).[7] Although the combined toxicity of Zn^{2+} with other metals (such as Cd^{2+}, Cu^{2+}, and Pb^{2+}) has been reported,[3,7] the rhizospheric toxicity of Zn^{2+} and Co^{2+} mixtures is less known. Zn^{2+} is an essential micronutrient, which is toxic at elevated concentrations.[8] Co^{2+} is a

nonessential element for higher plants, and at high concentrations, Co^{2+} is toxic to most plant species.[9]

Over the past decade, models have been developed to assess the toxic effect of single metals in the environment. These include the free ion activity model (FIAM),[10] the biotic ligand model (BLM),[11] and the electrostatic toxicity model (ETM).[12] The BLM, as an extension of the FIAM, proposes that the toxicity of metals depends on the binding of metal ions to an assumed biotic ligand that can be affected by site-specific competition.[1,2,13] The ETM focuses on the electrical potential (ψ_0) at the cell membrane (CM) surface, which affects the toxicity and uptake by influencing (i) the distribution of metal ions between the exterior CM surfaces and the bulk solution and (ii) the electrical driving force upon ions across the CM.[12] Previous studies have applied this electrostatic approach to assess the toxicity and uptake of single metals[14−16] but rarely for assessing the toxicity and uptake of metal mixtures.

Usually, the toxicity assessment for multiple toxicants is based on dose addition (DA) and response addition (RA) models.[17] DA is applied for mixtures in which toxicants exert effects by similar mechanisms at similar sites of action. In contrast, in the RA model, toxicants are supposed to act by different mechanisms at different sites.[18,19] In either case, the toxicants may or may not act interactively (one enhancing or reducing the effect of the other). Verification of interaction can be problematic.[20−22] In the present study, we aimed to (i) examine the toxicity of Zn^{2+} and Co^{2+} singly and in binary mixture, giving particular consideration to the activities of metal ions at the CM surface, (ii) incorporate electrostatic theory in the evaluation of rhizotoxicity of Zn^{2+} and Co^{2+} in mixtures, and (iii) model the uptake of Zn^{2+} or Co^{2+} by plant roots, based on the previously developed electrostatic uptake model.

MATERIALS AND METHODS

Preparation of Test Solutions. In all the experiments, the salts used were analytical grade, and double deionized water was used throughout. All test media contained 0.25 mM $CaCl_2$, were buffered with 2.0 mM MES, and were adjusted to pH values of 4.5, 5.5, or 6.1 with NaOH or HNO_3. For each treatment, different concentrations of $ZnCl_2$ and $Co(NO_3)_2$ were added to the test media. The anions (Cl^- and NO_3^-) contribute so little (<1.0 mV) to the ψ_0 variation that their influence was assumed to be negligible.[23] All media were prepared 1 d before use to obtain near-equilibrium solutions. The pH values and concentrations of Zn and Co in the solutions were all remeasured before each test. The metal concentrations of the culture solutions and digestion solutions of wheat roots were determined by flame atomic absorption spectrometry (F-AAS; Hitachi Z-2000).

Plant Toxicity Assays. The tested seeds of wheat (*Triticum Aestivum* L.Yangline16) were purchased from the Yangzhou Agricultural Academy of Sciences, China. Seeds were sterilized with 0.5% NaClO for 10 min and then washed with deionized water at least eight times to remove NaClO from the seed surfaces. The seeds were germinated on wet filter paper at 25 °C for 48 h. After germination, seedlings with root lengths between 1 and 2 cm were chosen for the experiments.

The 48 h root elongation tests were performed following ISO Guideline 11269-1 (1993) and EPA Guideline 850-4200 (1996). In the acute toxicity tests, six uniform seedlings (1−2 cm) were transferred into acid-washed polyethylene beakers containing 500 mL of test solution. After 48 h, the two longest roots of each seedling were measured, and the mean value for each treatment was calculated. After that, the roots of each treatment were submerged in 10 mM Na_4EDTA, whose pH had been adjusted in accordance with the treatment, for 10 min to remove the surface Zn^{2+} and Co^{2+}, and then the roots were washed twice with deionized water. The washed roots were cut from the seedlings, dried, and digested with 20 mL of 5 M pure HNO_3 for later measurement.

In a preliminary experiment, test solutions were set at pH 6.0 and contained 0.25 mM $CaCl_2$, buffered with 2.0 mM MES. Five Zn^{2+} concentrations (3.9−54 μM) or five Co^{2+} concentrations (7.4−112 μM) and one control (without Co^{2+} and Zn^{2+}) were added to derive the EA_{50} values for Zn^{2+} and Co^{2+}. EA_{50} values are the activities of Zn^{2+} or Co^{2+} that induce a 50% inhibition of root elongation, and smaller values for EA_{50} indicate greater sensitivity of the organism or greater toxicity of the metal. For the metal mixture experiments, the tested concentrations for each metal were 0, 0.3, 0.5, 1, 2, and 4 times

EA_{50} at three pH values (4.5, 5.5, or 6.1). The mixture experiments were carried out according to a full cross design (6 Zn^{2+} concentrations × 6 Co^{2+} concentrations × 3 pH values = 108 solutions). Three replicates were performed for each test solution.

Analysis of Root Elongation. Assessing the effects of toxicant mixtures usually begins with an assessment of effects induced by each toxicant in the mixture considered singly. See Supporting Information (SI) section S2 for more details on the theory of a toxicity-multiplication model, including equations that incorporate the interactions among H^+, Zn^{2+}, and Co^{2+}. In our study, we use the term "extrinsic" in reference to responses to ion activities in the bulk-phase medium, and the term "intrinsic" in reference to responses to ion activities at the CM surface.

The free metal ion activities of Zn^{2+} and Co^{2+} in the bulk media (denoted $\{Zn^{2+}\}_b$ and $\{Co^{2+}\}_b$) were calculated using the Visual Minteq 3.0 speciation software. In all test solutions, Zn^{2+} and Co^{2+} are the principal metallic species. The cell membrane surface potential (ψ_0) and $\{M^{2+}\}_0$ were calculated using a Gouy−Chapman−Stern (GCS) model (see SI section S1 for details). The updated computer program (SGCS) is available at http://www.uq.edu.au/agriculture/sgcs/.

The Electrostatic Uptake Model. To simulate Zn^{2+}/Co^{2+} uptake in wheat roots, a mathematical model generated from the classical Michaelis−Menten kinetics and electrostatic approach was introduced. Detailed description of the model, including equations, are given in SI section S3.

Statistics. All experiment data were listed in SI Table S1, and coefficients in equations were evaluated by regression analysis so that r^2 and the statistical significance of the coefficients could be determined. Regression analyses were conducted using SYSTAT 12 (Cranes Software International Ltd., India). Bulk-phase activities or CM-surface activities in regression analyses were expressed in μM units unless otherwise stated. No coefficients are reported where the 95% confidence interval encompassed zero unless they are so noted.

RESULTS

The Toxicity of Zn^{2+} or Co^{2+} Singly. Root growth was significantly inhibited by Zn^{2+} or Co^{2+} in bulk solution. For example, root growth decreased to 65% with 9.4 μM $\{Zn^{2+}\}_b$ or 18.4 μM $\{Co^{2+}\}_b$ in the test solution at pH 6.0. According to a dose−response relationship, $EA_{50}\{Zn^{2+}\}_b$ was 12.3 μM at pH 6.0; this was half the value of $EA_{50}\{Co^{2+}\}_b$ (25.0 μM) at pH 6.0. These data were similar to $EC_{50}[Zn^{2+}] = 12.4$ μM (i.e., the concentration of metal ion causing 50% root length inhibition) for wheat roots at pH 6.0 and the $EC_{50}[Co^{2+}] = 25.8$ μM for barley roots at pH 5.0.[14,24] In the range of pH from 6.1 to 4.5, increase in H^+ alleviated the toxicity of Zn^{2+} and Co^{2+} significantly. The 48 h $EA_{50}\{Zn^{2+}\}_b$ increased from 22.5 to 44.1 μM when pH decreased from 6.1 to 4.5. The 48 h $EA_{50}\{Co^{2+}\}_b$ increased from 34.0 to 90.6 μM (SI Table S2). Thus H^+ induced reductions in the extrinsic toxicity of the metals.

Next, we sought to elucidate ψ_0 effects, so we calculated the $EA_{50}\{M^{2+}\}_0$ (i.e., the activity of Zn^{2+} or Co^{2+} at the external surface of the CM causing 50% reduction). These values for 48 h $EA_{50}\{M^{2+}\}_0$ reflected the intrinsic sensitivity of roots to metals. Interestingly, the intrinsic sensitivity to Zn^{2+} increased significantly with increasing H^+: the 48 h $EA_{50}\{Zn^{2+}\}_0$ decreased from 1071 to 518 μM (SI Table S2). Similarly, the decreased ψ_0 negativity enhanced the intrinsic sensitivity to
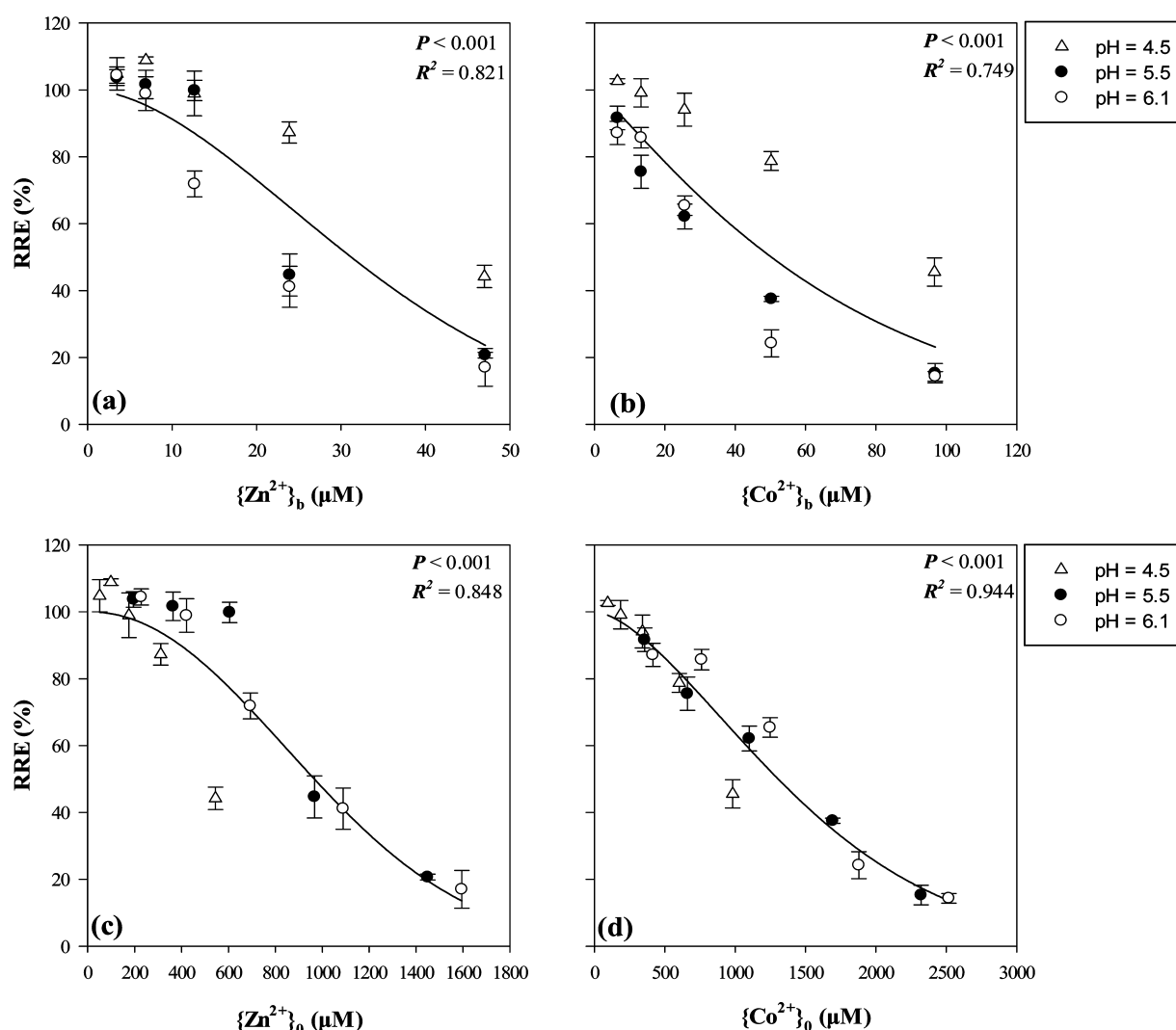
Figure 1. Relative root elongation of wheat seedlings exposed to Zn^{2+} or Co^{2+} in single-toxicant solutions at pH 4.5 ($\triangle$), 5.5 ($\bullet$), and 6.1 ($\circ$). Responses to ion activities in the bulk solution ($\{Zn^{2+}\}_b$ or $\{Co^{2+}\}_b$) (a,b) or to activities at the CM surface ($\{Zn^{2+}\}_0$ or $\{Co^{2+}\}_0$) (c,d).

Co^{2+}: the 48 h $EA_{50}\{Co^{2+}\}_0$ decreased from 1448 to 932 μM (SI Table S2). Thus reductions in pH caused contrasting results: decreases in extrinsic sensitivity and increases in intrinsic sensitivity.

Relative root elongation was fitted with SI eq 2, with Zn/Co intensity (T) variously expressed as bulk-phase activities ($\{T\}_b$) or CM-surface activities ($\{T\}_0$). Figure 1 presents the plots of *RRE* as a function of $\{T\}_b$ or $\{T\}_0$ together with the pH of the media. The correlation between *RRE* and $\{Zn^{2+}\}_b$ or $\{Zn^{2+}\}_0$ yielded r^2 of 0.821 and 0.848, respectively. When applied to $\{Co^{2+}\}_b$ or $\{Co^{2+}\}_0$, $r^2 = 0.749$ and 0.944, respectively (Figure 1). These results indicated that $\{Zn^{2+}\}_0$ and $\{Co^{2+}\}_0$ were more appropriate for assessing the toxicities of Zn^{2+} and Co^{2+} singly to wheat root growth than their bulk-phase activities.

The Toxicity of Zn^{2+}-Co^{2+} Mixtures. Extrinsic toxicities of $Zn^{2+}-Co^{2+}$ mixtures were assessed by a multiplicative function (eq 10, SI Table S3), initially without expansion of coefficients b, c, or e. The coefficients were determined by regression analysis and are listed in SI Table S3; $r^2 = 0.880$ (Figure 2a). When the intrinsic toxicities of $Zn^{2+}-Co^{2+}$ mixtures were similarly assessed by a multiplicative function (eq 11, SI Table S3), fitting the data into the equation produced four

nonsignificant coefficients among the six coefficients; $r^2 = 0.655$ (Figure 2c).

Next, the intrinsic toxicity was assessed according to SI eq 12, and coefficients were expanded according to the principle of electrostatic theory as shown in SI Table S4. The extended expressions for coefficients b, c, and e were used to further assess the influences of $\{H^+\}_0$ and possible interactions between $\{Zn^{2+}\}_0$ and $\{Co^{2+}\}_0$. The 95% confidence interval of the strength coefficient c_1 (-1.900×10^{-5}) encompassed zero, indicating no significant effects of surface Zn^{2+} on the toxicity of Co^{2+}. In contrast, the statistical significance of strength coefficient e_1 (4.420×10^{-4}) indicated an enhancement of $\{Zn^{2+}\}_0$ toxicity by $\{Co^{2+}\}_0$. The significance of coefficients b_1 and b_2 (SI Table S4) reflected a small inhibition by $\{H^+\}_0$ in solutions. Figure 2d presents the linear regression between the measured and predicted *RL* values, yielding $r^2 = 0.895$. Additionally, strength coefficients c and e were further extended to investigate interactions among Zn^{2+}, Co^{2+}, and H^+ through converting SI eq 12 into SI eq 13 (SI Table S5). Results showed that both c_2 (6.140×10^{-3}) and e_2 (1.520×10^{-2}) were significant, indicating that enhancing effect of H^+ on the intrinsic toxicities of the metal ions in mixtures as well as in single-toxicant solutions as noted above. The insignificant
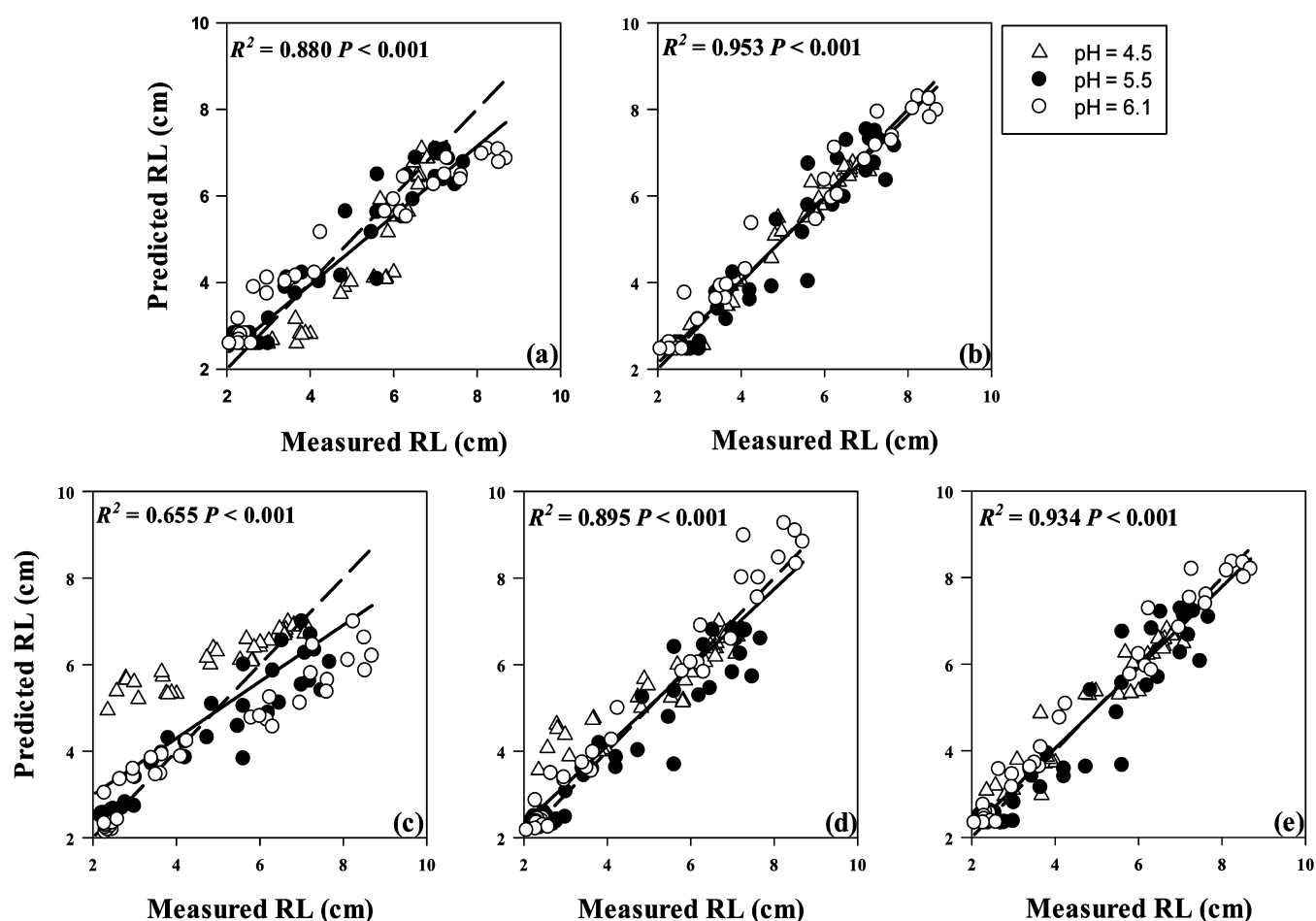
Figure 2. Comparison of measured and predicted values for root growth exposed to Zn^{2+} and Co^{2+} either singly or in binary mixtures expressed as the activities of Zn^{2+} or Co^{2+} in the bulk solution ($\{Zn^{2+}\}_b$ or $\{Co^{2+}\}_b$) (a,b) or to activities at the CM surface ($\{Zn^{2+}\}_0$ or $\{Co^{2+}\}_0$) (c−e). In (a) and (c), data were analyzed using SI eqs 10 and 11, while in (b) the data were analyzed using SI eq 14, extended by the interactions among H^+, Zn^{2+}, and Co^{2+}. In (d), the data were analyzed using SI eq 12 (i.e., extended by the interactions between Zn^{2+}, and Co^{2+}). For (e), the data were analyzed using SI eq 13 (i.e., incorporating the interactions among H^+, Zn^{2+}, and Co^{2+} into the model). The solid lines show the linear regression, and the dotted lines show the 1:1 correspondence. All coefficients are given in SI Tables S3, S4, S5, and S6.

impact of $\{Zn^{2+}\}_0$ on the toxicity of $\{Co^{2+}\}_0$ was reflected by the nonsignificant strength coefficient c_1 (7.000×10^{-5}). In contrast, the statistically significant value of e_1 (6.900×10^{-4}) indicated that $\{Co^{2+}\}_0$ enhanced the toxicity of $\{Zn^{2+}\}_0$. Incorporation of $\{H^+\}_0$ into the strength coefficients of metals improved the r^2 from 0.895 to 0.934 (Figure 2e).

Moreover, the extrinsic toxicities and interaction patterns were evaluated by SI eq 14, where coefficients b, c, and e were expanded to include the influences from H^+, Zn^{2+}, and Co^{2+} (SI eq 14). As shown in SI Table S6, results showed that c_1 (5.609×10^{-2}) and c_2 (6.330×10^{-2}) were both significantly positive values in the denominators, indicating that a site-competition mechanism, other than the electrostatic effect, might play an important role in alleviating the toxicity of $\{Co^{2+}\}_0$ by $\{Zn^{2+}\}_0$ and $\{H^+\}_0$, respectively. Furthermore, the strength coefficient e_1 (9.760×10^{-3}) was significant and indicated that $\{Co^{2+}\}_b$ enhanced the toxicity of $\{Zn^{2+}\}_b$. Both the values of c_2 (6.330×10^{-2}) and e_2 (-1.415×10^{-2}) indicated ameliorative effects of H^+ on the extrinsic toxicities of the metal ions in mixtures as well as in single-toxicant solutions as noted above. Predicted RL was plotted against measured RL in Figure 2b.

Modeling Uptake of Zn^{2+} and Co^{2+} in Binary Mixtures. Co^{2+} reduced Zn^{2+} uptake by roots at three pH values. For example, at pH 4.5, root Zn concentration was 0.75 mg g^{-1}

RDW (root dry weight) after 48 h exposure to 3.47 μM $\{Zn^{2+}\}_b$ in solution without Co^{2+}. The Zn^{2+} uptake was reduced to 0.32 mg g^{-1} RDW as $\{Co^{2+}\}_b$ increased up to 96.1 μM in solution (Figure 3a, SI Table S1). Similarly, the presence of Zn^{2+} reduced the uptake of Co^{2+}. For instance, addition of 48.1 μM $\{Zn^{2+}\}_b$ reduced root Co concentration from 0.83 to 0.43 mg g^{-1} RDW after 48 h exposure to 6.51 μM $\{Co^{2+}\}_b$ (Figure 3b, SI Table S1). In addition, increases in pH from 4.5 to 6.1 enhanced the Zn^{2+} or/and Co^{2+} uptake. In our three pH treatments, decreases in H^+ increased Zn^{2+} and Co^{2+} uptake in Zn^{2+}−Co^{2+} mixtures (Figure 3, SI Table S1). For instance, Zn^{2+} uptake increased from 0.75 to 1.33 mg g^{-1} RDW when exposed to 3.47 μM $\{Zn^{2+}\}_b$ (Figure 3a,c); at 6.53 μM $\{Co^{2+}\}_b$, Co^{2+} uptake increased 1.7 times from 0.83 to 1.39 mg g^{-1} RDW (Figure 3b,d).

Uptake of root Zn^{2+} or Co^{2+} concentrations could be interpreted in terms of ψ_0 and its effect on uptake of these ions.[12] For example, Figure 3 illustrates that the uptake of Zn^{2+} both singly and in binary mixture as a function of $\{Zn^{2+}\}_b$ yielded $r^2 = 0.776$ (Figure 3a, eq 7 in SI section S3). Substitution of $\{Zn^{2+}\}_0$ for $\{Zn^{2+}\}_b$ improved r^2 to 0.820 (Figure 3c, SI eq 7). The addition of ψ_0 to the model further increased r^2 to 0.855 (Figure 4a, eq 8 in SI section S3). Similarly, ψ_0 also played an important role in Co^{2+} uptake and
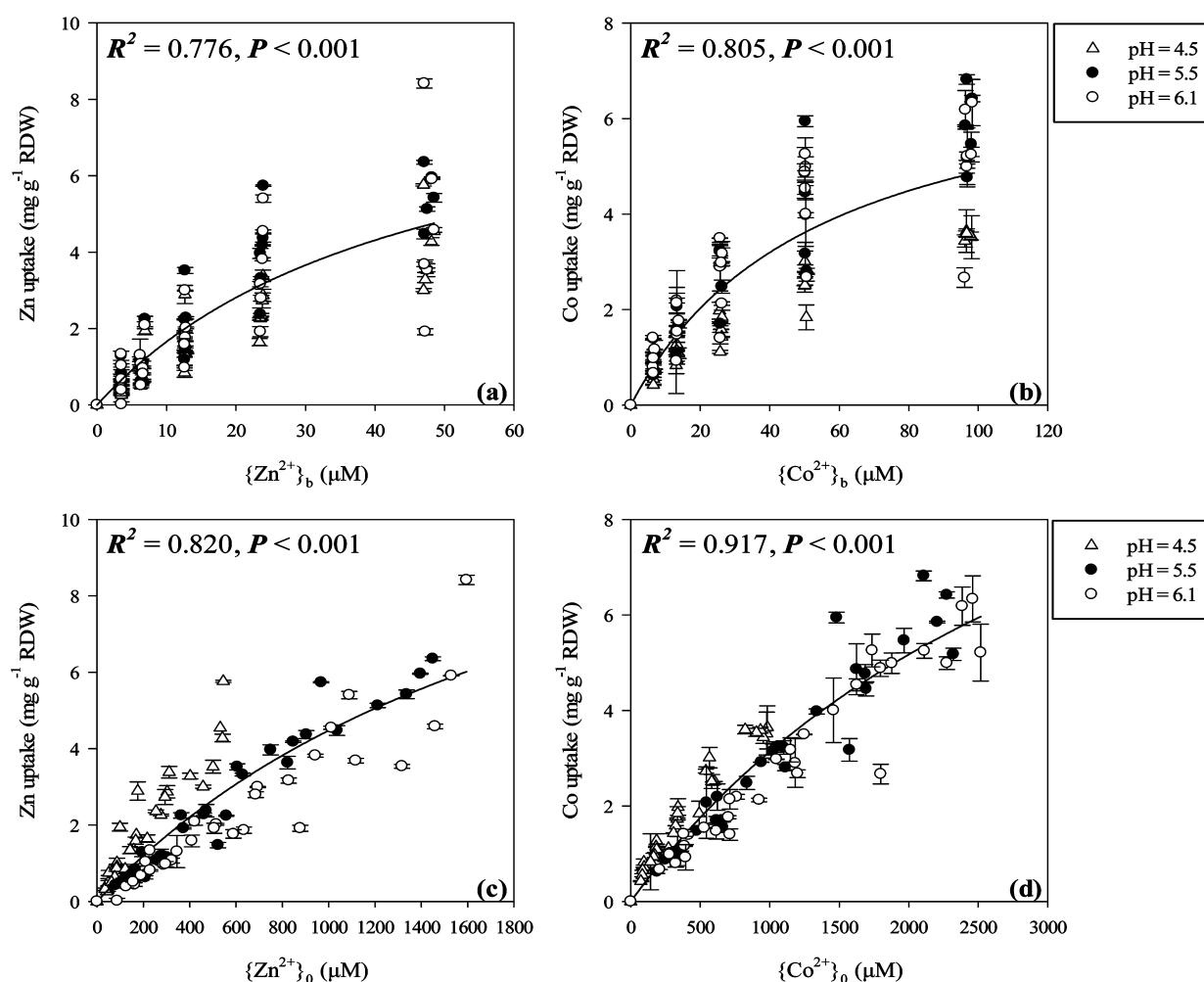
Figure 3. Uptake of Zn^{2+} and Co^{2+} in wheat seedling roots based on the activities of Zn^{2+} and Co^{2+} in the bulk-phase medium ($\{M^{2+}\}_b$) (a,b) or at the CM surface ($\{M^{2+}\}_0$) (c,d). The data were taken from both single and binary mixtures of metals at pH 4.5 ($\triangle$), 5.5 ($\bullet$), and 6.1 ($\bigcirc$). Drawn lines present the fitted equation $J_{uptake} = a\{M^{2+}\}/(K_m + \{M^{2+}\})$ (SI eq 7 in section S3).

increased r^2 from 0.805 (Figure 3b, SI eq 7) to 0.935 (Figure 4b, SI eq 8).

The coexistence of H^+, Zn^{2+} (or Co^{2+}) in test solutions is likely to affect the toxicity and uptake of Co^{2+} (or Zn^{2+}) through competition at the CM surface or other mechanisms. Fitting all data from Zn–Co systems (i.e., both single and binary mixtures) to eq 9 in SI section S3, a linear regression was found between the predicted and measured values; $r^2 = 0.936$ for the Zn^{2+} uptake in roots with $a = 30.99$, $b = 0.0129$, $c = -2.88 \times 10^{-4}$, and $K_m = 10^{3.19}$ (Figure 4c, SI Table S7). The negative value of c indicated that the presence of Co^{2+} inhibited Zn^{2+} uptake, and the positive value of b showed that a decrease in the negativity of ψ_0 enhanced the Zn^{2+} uptake. The r^2 for root Zn^{2+} uptake was improved from 0.855 (Figure 4a, SI eq 8) to 0.936 (Figure 4c, eq 9 in SI section S3). Similarly, we observed an r^2 of 0.951 (Figure 4d, SI eq 9) for Co^{2+} uptake, with $a = 21.34$, $b = 0.0092$, $c = -2.39 \times 10^{-4}$, and $K_m = 10^{3.44}$ (Figure 4d, SI Table S7). The negative value of c indicated that Co^{2+} uptake was suppressed by Zn^{2+}, and the r^2 for prediction improved from 0.935 (Figure 4b, SI eq 8) to 0.951 (Figure 4d, SI eq 9).

DISCUSSION

A number of studies have demonstrated that toxicity of Zn^{2+} and Co^{2+}, taken singly, is more closely related to their activities at the CM surface than in the bulk-phase medium.[14,25] Our observation of the ameliorative effect of H^+ on the extrinsic toxicity of Zn^{2+} or Co^{2+}, demonstrated by increased $EA_{50}\{Zn^{2+}\}_b$ and $EA_{50}\{Co^{2+}\}_b$, has been reported previously.[24,26] The BLM model proposes that the addition of cations to the growth solution alleviated the metal toxicity mainly due to a site-competition mechanism. However, the alleviation effect of added H^+ in test solutions could be better explained by changes in $\{Zn^{2+}\}_0/\{Co^{2+}\}_0$ rather than $\{Zn^{2+}\}_b/\{Co^{2+}\}_b$ (see a better correlation of RRE vs $\{Zn^{2+}\}_0/\{Co^{2+}\}_0$ in Figure 1). The addition of H^+ in root bathing media decreased the negativity of ψ_0 (SI Table S1), and this caused a decrease in $\{Zn^{2+}\}_0$ and $\{Co^{2+}\}_0$. The addition of cations (H^+, Zn^{2+}, and Co^{2+}) did not alleviate Zn^{2+}/Co^{2+} toxicity by an ionic strength effect. Similar results also have been reported by Kopittke et al.,[15] where the alleviation of Cu^{2+} toxicity by added cations (Ca^{2+}, Mg^{2+}, etc.) was attributed to a nonspecific reduction in the negativity of ψ_0, resulting in a decreased $\{Cu^{2+}\}_0$. However, an opposite effect of H^+ on intrinsic sensitivity as manifested by decreases in $EA_{50}\{Zn^{2+}\}_0$ and $EA_{50}\{Co^{2+}\}_0$ was observed. Similar phenomenon of the increased intrinsic metal toxicity
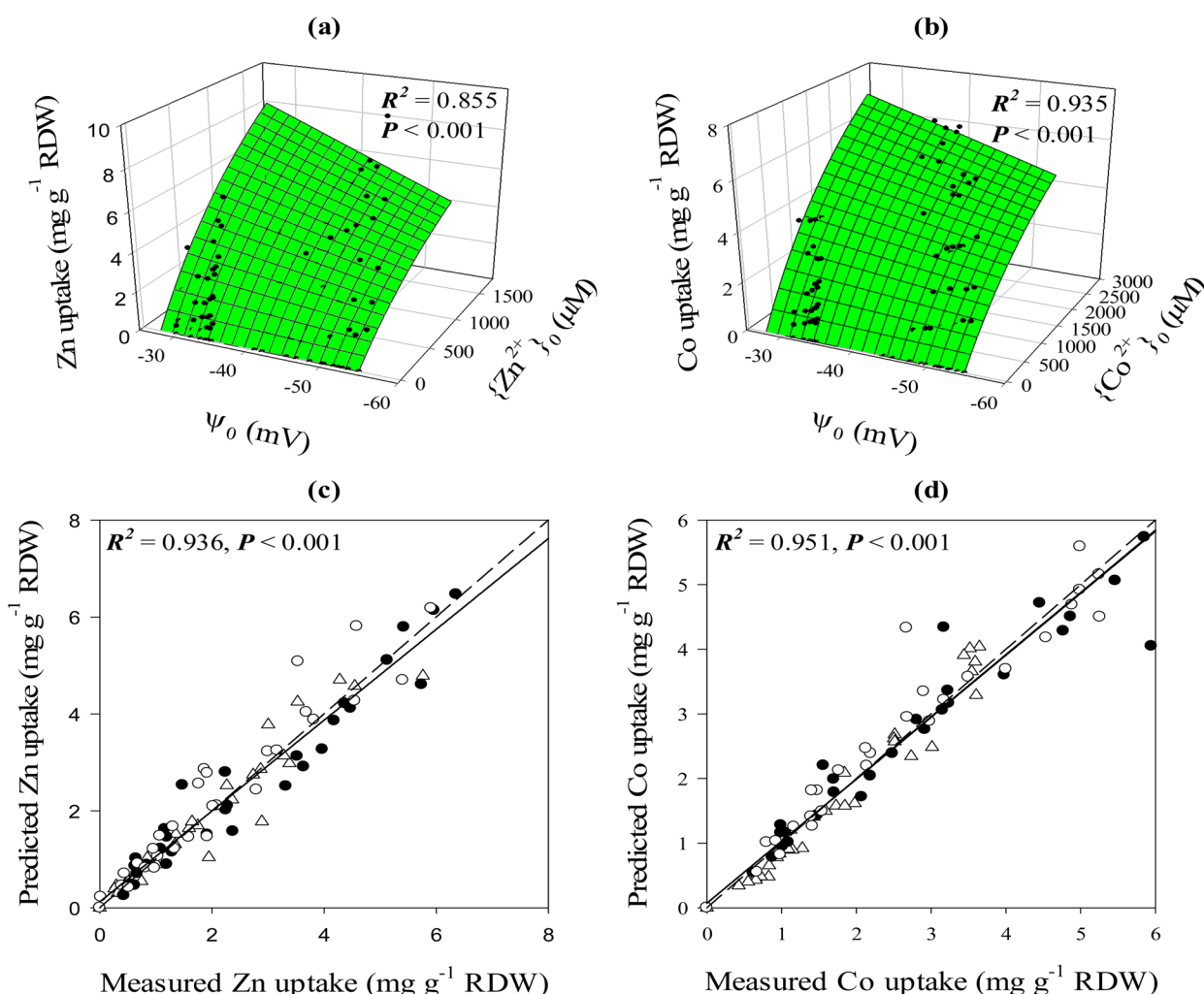
(a)

(b)

(c)

(d)

Figure 4. Predicted uptake of Zn^{2+} and Co^{2+} in wheat seedling roots based on the electrostatic uptake model. The data were taken from both single and binary mixtures at pH 4.5 ($\triangle$), 5.5 ($\bullet$), and 6.1 ($\circ$). Drawn grids were generated by the equation $J_{uptake} = a(1 + b\psi_0)\{M^{2+}\}_0/(K_m + \{M^{2+}\}_0)$ (eq 8 in SI section S3) for (a) and (b), and predicted uptake was computed by the equation $J_{uptake} = a(1 + b\psi_0)\{M_1^{2+}\}_0(1 + c\{M_2^{2+}\}_0)/(K_m + \{M_1^{2+}\}_0)$ (eq 9 in SI section S3) in (c) and (d). In (c) and (d), the solid line is the linear regression, and the dotted line is the 1:1 correspondence. All coefficients are given in SI Table S7.

(denoted $EA_{50}\{M\}_0$) was also reported by Wang et al.[23] Wang et al. proposed that $EA_{50}\{Cu^{2+}\}_0$ declined as $\{Ca^{2+}\}_b$ increased, and reasons are attributed to the decreased competition from cations (Mg^{2+}, H^+, etc.) at the CM surface.[23] A slight enhancement of intrinsic Cu^{2+} toxicity by Ca^{2+} also had been noted previously.[27] In our study, the addition of H^+-induced increases of $EA_{50}\{Zn^{2+}\}_0$ and $EA_{50}\{Co^{2+}\}_0$ may be due to the reduced competition from $\{Ca^{2+}\}_0$.

When electrostatic models incorporated the interactions among Zn^{2+}, Co^{2+}, and H^+ at the CM surface, the model prediction of the root length was improved as demonstrated by increased r^2 values from 0.655 to 0.934 (Figure 2c–e). Similarly, model predictions improved when interactions among Zn^{2+}, Co^{2+}, and H^+ were incorporated into extrinsic models, where r^2 values increased from 0.880 to 0.953 ((Figure 2a,b).

The toxicity of toxicant mixtures can often be described closely by one or more assumed models of combined toxicant effects.[17,20] Within each model, toxicants may or may not appear to interact. Specifically, one toxicant may appear to enhance the toxicity of another (synergism) or reduce the toxicity of another (antagonism), or toxicants may appear not

to interact with one another.[21] According to the response-multiplication model developed in SI section S2, $\{Co^{2+}\}_0$ appeared to enhanced the toxicity of $\{Zn^{2+}\}_0$, $\{Zn^{2+}\}_0$ did not affect the toxicity of $\{Co^{2+}\}_0$ significantly, and $\{H^+\}_0$ enhanced substantially the toxicity of both metal ions. To some extent, the appearance of $\{Co^{2+}\}_0$ enhancement of $\{Zn^{2+}\}_0$ toxicity in our study was confirmed by $\{Co^{2+}\}_b$ enhancement of $\{Zn^{2+}\}_b$ toxicity. Without an actual intrinsic enhancement, an extrinsic enhancement would not occur. Instead, bulk-phase Zn^{2+} would alleviate $\{Co^{2+}\}_b$ toxicity as other cations did (Ca^{2+}, Mg^{2+}, H^+, etc.).[14] In contrast with the response-multiplication model, the toxicant-addition model gave conflicting results with regard to interaction for the metal ions, but $\{H^+\}_0$ appeared to always enhance the toxicity of the metal ions as we found in assessing the Zn/Co toxicity singly.

Additional analyses were performed with data restricted to those from the experiments at pH 6.1. At that pH, H^+ was not limiting and appeared to have no interactive effects with the metal ions. Thus four coefficients could be removed from the regression analyses (b_1, b_2, c_2, and e_2) (see eq 15 in SI Table S8). With those pH 6.1 data, the response-multiplication model also produced results consistent with $\{Co^{2+}\}_0$ enhancement of

$\{Zn^{2+}\}_0$ toxicity ($e_1 > 0$ was significant) and consistent with negligible effects of $\{Zn^{2+}\}_0$ upon $\{Co^{2+}\}_0$ toxicity ((c_1 was nonsignificant) (SI Table S8). Consequently, the consensus of all analyses is that $\{Co^{2+}\}_0$ significantly enhanced the toxicity of $\{Zn^{2+}\}_0$, but $\{Zn^{2+}\}_0$ did not significantly affect the toxicity of $\{Co^{2+}\}_0$.

We examined what interaction patterns could be observed from metal ion uptake. Competition between Zn^{2+} and Co^{2+} during the uptake processes has been reported in marine phytoplankton.[28] Dirilgen and Inel proposed that the accumulation of Zn^{2+} was stimulated by Co^{2+} and the accumulation of Co^{2+} was inhibited by Zn^{2+} in *Lemna minor* L..[6] Liu et al. also reported that when the concentration of Co^{2+} was 5 μM in culture solution, the uptake of toxic elements such as Mn^{2+}, Cu^{2+}, and Zn^{2+} was inhibited.[29]

Different from the conventional perspectives, Wang et al.[12,16] and Kinraide et al.[27,30] have put forward that electrostatic interactions at CM surface should account for much of the behavior of metal ion uptake and interaction. For example, in assessing the uptake of Cu^{2+} and $H_2AsO_4^-$ by wheat seedling roots, incorporation of ψ_0 into the classical Michaelis–Menten equation generated more accurate predictions.[12] In our study, we also confirmed that ψ_0 significantly influenced the uptake of Zn^{2+} or Co^{2+} in roots by the elevated r^2 values as shown in Figures 3 and 4. However, compared with predicting Co uptake, the magnitude of increase in r^2 values for modeling Zn uptake is less, probably due to Zn is an essential element for wheat growth in a low concentration.[8,31] Moreover, in studying Zn^{2+} uptake by root, the role of ψ_0 affected the $\{Zn^{2+}\}_0$ (r^2 increased from 0.776 to 0.820) is slightly greater than ψ_0 influenced the driving force for Zn^{2+} uptake (r^2 increased from 0.820 to 0.855).

When we extended the electrostatic uptake model designed for single metals (SI eq 8) by taking coexisting ions into consideration, the predicted values of Zn^{2+} or Co^{2+} uptake were significantly correlated with the measured values from experiments. There are two possible explanations responsible for this. First, in BLM model, cation–biotic ligand interactions are envisaged and used to predict metal binding at a biotic ligand site of action. The amount of binding is considered to be related to a toxicological response and later uptake.[13] This is reason for the alleviation of heavy metal (Cu^{2+}, Ni^{2+}) toxicity by nontoxic cations (e.g., Mg^{2+}, Ca^{2+}).[32] Second, electrostatic theory does not negate the existence of site-specific competition, but access to the site of competition is influenced by ψ_0. Furthermore, electrostatic theory does provide some alternative explanations of common transport phenomena such as substrate saturation, *cis*- and *trans*-inhibition, rectification, voltage gating, and shifts in voltage optima.[30] So the electrostatic models were developed for toxicity and uptake for metal mixtures. Metal toxicity involves at least three steps: bioavailability of a toxic metal, uptake, and reaction resulted from accumulation and metabolic activity of metal in organisms.[33] Each of these steps is potentially influenced by the electrical potential (ψ_0) at the cell membrane surface. Interactions at internal pathways could happen, as Collins et al. discovered for Co^{2+}, which existed mainly as complexes with citrate or malate in wheat and tomato plants.[9] Also Sarret et al. reported that in roots of *Arabidopsis halleri* grown on contaminated soil, Zn^{2+} was distributed among Zn^{2+}–malate, Zn^{2+}–citrate, and Zn^{2+}–phosphate.[31] Therefore Zn^{2+} and Co^{2+} could be complexed to similar functional groups, thereby affecting both their uptake and the metabolic activity of both metal ions in wheat seedlings roots.

Finally, the study demonstrated that the electrostatic approach can better predict the rhiztoxicity and uptake of Zn^{2+} and Co^{2+} in wheat roots than models that disregard electrostatic effects whether the metal toxicants are applied singly or as binary metal mixture. A principal determinant is the cell membrane (CM) surface electrical potential (ψ_0) which influences both the free ion activity at the CM surface and the electrical driving force across the CM. The electrostatic approach allows the computation of free ion activities at the CM surface. Once at the CM surface, ions such as Zn^{2+}, Co^{2+}, and H^+ interact, thereby engaging some of the principals of the biotic ligand model (BLM). Electrostatic models that incorporate ψ_0 and CM surface activities, rather than bulk-phase medium activities, predict values for toxicity and uptake that correlate very well ($r^2 > 0.900$) with measured values. However, results presented here also have some limitations. Experiments were short-term and were applied only to wheat seedlings, so we should try to apply the analytical methodology to long-term pot experiments in the future. Furthermore, multiple nonlinear regression analyses are often beset with ambiguities because of some unavoidable collinearity among multiple variables and the occasional model dependency of the appearance of interaction. Detailed consideration of these limitations is underway.

ASSOCIATED CONTENT

Supporting Information
Details of the theory of GCS model, the Weibull equation, and the electrostatic uptake model, the experimental data of our study, table of estimated $EA_{50}\{Zn^{2+}\}$ and $EA_{50}\{Co^{2+}\}$ values, parameter tables of toxicity of Zn–Co mixtures response to the ion activities in the bulk solutions, the mixture toxicity responses to ion activities at the CM surface, the interactions among H^+, Zn^{2+} and Co^{2+} interactions at the CM surface, and the interactions among H^+, Zn^{2+} and Co^{2+} interactions in the bulk solutions; modeling the uptake of $\{Zn^{2+}\}_0$ or $\{Co^{2+}\}_0$ by wheat seedling roots and the toxicity model for Zn^{2+} and Co^{2+} interactions at pH = 6.1. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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