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1 Does specific parameterization of WHAM improve the prediction of copper
2 competitive binding and toxicity on plant roots?

3

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19

20 Abstract

21 We aimed at assessing whether the binding and rhizotoxicity of metal cations such as copper that exhibit high
22 affinity for plant roots could be adequately predicted using the Windermere Humic Aqueous Model (WHAM)
23 default parameterization. Accordingly, we first compared the ability of the default parameterization of WHAM
24 and a specific parameterization for terrestrial higher plants (WHAM-THP) to model the competitive binding of
25 copper on wheat (*Triticum aestivum* L.) and tomato (*Solanum lycopersicum* L.) roots. Secondly, in an external
26 dataset, we evaluated the ability of WHAM-THP to predict the copper concentration and toxicity to pea (*Pisum*
27 *sativum* L.) roots relative to WHAM. WHAM-THP estimates generated a slightly better fit for the competitive
28 binding of copper on wheat and tomato roots (\log_{10} of the root-mean-square error, $RMSE = 0.15$) than WHAM
29 estimates ($RMSE = 0.24$). WHAM-THP estimates slightly better fitted the copper concentration in pea roots
30 ($RMSE \leq 0.49$) than WHAM estimates ($RMSE \leq 0.67$) at low copper exposure and $pH \leq 5$. However, WHAM-
31 THP did not at all improve the prediction of copper toxicity to pea roots ($RMSE = 13\%$ as also for WHAM). We
32 thus conclude that, although the default parameterization of WHAM does not neatly predict the binding of metal
33 cations on roots, it could however be used with confidence in predictive ecotoxicology for terrestrial higher
34 plants without any specific parameterization.

36 Keywords

37 Biotic ligand model; Complexation; Humic substances; Phytotoxicity; Trace element

39 Highlights

- 40 • WHAM-THP is a specific parameterization of WHAM for terrestrial higher plants
- 41 • WHAM-THP much improves the prediction of root acidic properties
- 42 • WHAM-THP only slightly improves the prediction of root copper competitive binding
- 43 • WHAM-THP does not improve the prediction of copper rhizotoxicity

44

1. Introduction

Models designed to predict the ecotoxicity of metal cations at the organism level are based on the description of the competitive binding of metal cations to biotic ligands borne to the outer surface of the organism (Di Toro et al. 2001). These models seem particularly promising for modelling the toxicity of metal cations to plant roots, i.e. rhizotoxicity. It was recently suggested that the primary mechanism driving the rhizotoxicity of metal cations is their binding strength to biotic ligands borne by root surfaces (Kopittke et al. 2014). The model's ability to accurately describe metal cation binding on plant roots is therefore a crucial issue.

Accordingly, advanced geochemical models initially designed to model metal cation binding on humic substances have started to be applied in predictive ecotoxicology studies over the past decade (Plette et al. 1996; Tipping et al. 2008). The Windermere Humic Aqueous Model (WHAM) has particularly been used to adequately model the binding and toxicity of metal cations to aquatic and terrestrial biota (Tipping et al. 2008; Antunes et al. 2012; Tipping and Lofts 2013; Qiu et al. 2015 and 2016).

The humic acid (HA) in WHAM was used as a surrogate of biotic ligands (Tipping et al. 2008; Antunes et al. 2012). The amount of metal cations bound to biotic ligands was predicted with WHAM by numerically optimizing the equivalent mass of HA per gram of organism (Tipping and Lofts 2013). The difference between the measurement and the WHAM prediction of the amount of metal cations bound to biotic ligands was hypothetically attributed only to the difference in the density of binding sites between the organism and the HA in WHAM. This hypothesis however overlooks the fact that the binding affinity for metal cations may also differ between organisms and HA in WHAM.

For terrestrial higher plants, a recent investigation showed that WHAM was able to satisfactorily model cadmium (Cd), nickel (Ni), and zinc (Zn) concentrations in pea (*Pisum sativum* L.) roots, but that it overestimated the copper (Cu) concentration in roots (Le et al. 2015). These authors concluded that the default parameterization of HA in WHAM may be not applicable for metal cations having a high affinity for natural organic matter such as Cu.

We recently characterized Cu binding on wheat (*Triticum aestivum* L.) and tomato (*Solanum lycopersicum* L.) roots by combining X-ray absorption spectroscopy with the specific parameterization of a two HA model based on WHAM (Guigues et al. 2016). We showed that Cu binding on wheat and tomato roots was driven by two types of sites, i.e. low- and high-affinity sites respectively corresponding to carboxylic and nitrogen (N) functional groups. As the high-affinity sites parameterized in WHAM presumably correspond to phenolic groups

75 rather than N functional groups, this finding suggested that specific parameterization of WHAM would be
76 necessary for terrestrial higher plants.

77 Accordingly, we aimed at assessing whether Cu root binding and rhizotoxicity could be adequately
78 predicted with the WHAM default parameterization or if tailored parameterization would be necessary. We first
79 compared the ability of WHAM parameterized with default settings (hereafter referred to as WHAM) and
80 WHAM specifically parameterized for terrestrial higher plants (hereafter referred to as WHAM-THP) to model
81 Cu competitive binding on roots. Wheat and tomato were chosen as model species to be respectively
82 representative of monocots and dicots. Secondly, we evaluated, on an external dataset, the ability of WHAM-
83 THP to predict the Cu concentration in roots and its rhizotoxicity relative to WHAM predictions.

84

85

86 2. Experimental approach

87

88 We used only analytical grade reagents unless otherwise stated.

89

90 2.1. Plant growth and root recovery

91 Wheat (cv. Premio) and tomato (cv. Moneymaker) were germinated for 7 days in darkness and then grown
92 for 14 days in hydroponic conditions in a growth chamber under the following climatic conditions (day/night):
93 25/20°C, 75/70% relative humidity and 16/8 h with a photon flux density of 450 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ during the
94 day (see Guigues et al. 2014 for details). At harvest, roots were separated from shoots, blotted with paper towels,
95 subdivided into homogenous subsamples, and stored frozen.

96 After thawing, roots were rinsed with 1 mM $\text{Ca}(\text{NO}_3)_2$ to eliminate vacuolar compounds released due to
97 membrane leakage during freezing. Roots were stirred in HNO_3 solution (trace analysis grade) at pH 3 for 1 h to
98 remove highly bound or precipitated cations (e.g. Fe and Al) and rinsed twice with ultrapure water (18.2 M Ω)
99 for 30 min. Roots were finally oven-dried at 50°C to constant mass.

100 The plant root material obtained (hereafter referred to as roots) was metabolically inactive. This metabolic
101 inactivity prevented the physiologically-driven absorption of Cu in root cells and enabled us to study only Cu
102 binding onto root surfaces. Roots contained cell walls and plasma membranes, i.e. the two compartments which
103 give roots their cation binding properties (Guigues et al. 2014). Root pretreatment may have partly impacted
104 their binding properties in comparison with live plant roots. However, there is to our knowledge no other reliable

105 analytical technique to distinguish binding from absorption of metal cations in the several dozens of samples
106 necessary to test a model for practical ecotoxicological applications.

107

108 2.2. Potentiometric titration

109 Potentiometric titration of wheat and tomato roots were extensively described by Guigues et al. (2014).
110 Briefly, ca. 0.2 g (dry mass basis) of roots was placed in 100 ml of 10 mM KNO₃ under stirring and flushed with
111 ultra-pure nitrogen. During titration, the pH was first lowered to 2.5 with 0.2 M HNO₃ (trace analysis grade)
112 addition and was then increased step-by-step to 11.5 with the incremental addition of 0.1 M KOH (trace analysis
113 grade) either at a rate of 100 µl in the 2.5-3.5 and 10.5-11.5 pH ranges or at a rate of 20 µl in the 3.5-10.5 pH
114 range.

115

116 2.3. Copper sorption experiments

117 A dry mass of 10 (± 0.5) mg of wheat and tomato roots was shaken end-over-end for 24 h at 25°C in 25 ml
118 of solution with varying chemical conditions representative of acidic soil solutions and including the competitive
119 effect of some important major (H and Ca) and minor (Zn) metal cations (Tables S1 and S2). In experiment 1,
120 the initial total Cu concentration of the solution ranged from pCu_{in} 7.3 to 3.0 (pCu_{in} = -log₁₀[Cu]_{in}). The solution
121 ionic strength and pH was set at 30 mM with NaNO₃ and 4.7 (± 0.2), respectively.

122 In experiment 2, the solution ionic strength was set at 0.6 or 300 mM with NaNO₃. The initial total Cu
123 concentration was set at pCu_{in} 4.2, 5.2, or 6.2 and the pH was set at 4.7 (± 0.2).

124 In experiment 3, the solution pH was set at 4.1 (± 0.1) or 6.3 (± 0.1). The initial total Cu concentration was
125 set at pCu_{in} 4.2, 5.2, or 6.2. The ionic strength was set at 30 mM with NaNO₃.

126 In experiment 4, the initial total solution Ca concentration ranged from pCa_T 2.0 to 4.0. The initial total
127 solution Cu concentration, ionic strength and pH was set at pCu_{in} 6.3, 30 mM with NaNO₃, and 5.1 (± 0.4),
128 respectively.

129 In experiment 5, the initial total solution Zn concentration ranged from pZn_T 4.5 to 7.2. The initial total
130 solution Cu concentration, ionic strength and pH was set at pCu_{in} 6.3, 30 mM with NaNO₃, and 4.7 (± 0.1),
131 respectively.

132 The pH was buffered with 1 mM 2-(N-morpholino) ethanesulfonic acid and adjusted with NaOH or HNO₃
133 (trace analysis grade). Each sorption experiment was performed in duplicate. After a few minutes of root
134 sedimentation, the supernatant was collected for analysis. The copper concentration in the initial (pCu_{in}) and

135 final (i.e. at equilibrium, pCu_{eq}) solutions was determined by inductively coupled plasma mass spectrometry
 136 (ICP-MS, NexION 300X Perkin Elmer) to determine the amount of Cu bound to wheat and tomato roots. Blanks
 137 and certified reference material (EnviroMAT Drinking water EP-L-3 and groundwater ES-H-2) were included in
 138 the analyses. The measurement uncertainty was lower than 10%. This procedure was cross-validated with Cu
 139 measurements obtained on digested root samples (Guigues et al. 2016).

140

141

142 3. Modelling approach

143

144 3.1. Specific parameterization of WHAM

145 Experimental data were modelled using the humic ion-binding model included in WHAM VII. The
 146 formalism of this model was extensively described by Tipping (1998) and Tipping et al. (2011). Briefly, WHAM
 147 was designed to simulate the cation binding properties of humic substances depicted as a regular array of two
 148 types of binding sites. The density (L_{Hi} , $cmol_c.kg^{-1}$) of type-1 sites is arbitrarily set as twofold higher than the
 149 density of the type-2 sites (i.e. $L_{H1} = 2 \times L_{H2}$). Protons and metal cations compete for binding on type 1 and 2
 150 sites. Metal cations are able to form mono-, bi- and tri-dentate complexes.

151 Proton sorption to humic substances was characterized by two intrinsic proton dissociation constants (pKa_1
 152 and pKa_2) and two distribution terms (ΔpKa_1 and ΔpKa_2) for type 1 and 2 sites, respectively. Metal binding to
 153 humic substances was characterized by two intrinsic equilibrium constants ($K_{M,1}$ and $K_{M,2}$) for type 1 and 2 sites,
 154 respectively, and one heterogeneity parameter ($\Delta LK2_M$). The parameter $K_{M,2}$ was calculated from $K_{M,1}$, pKa_1 , and
 155 pKa_2 as follows (Tipping et al. 2011):

$$156 \log K_{M,2} = \log K_{M,1} \times \frac{pKa_2}{pKa_1} \quad \text{Eq. 1}$$

157 Electrostatic effects are accounted for in WHAM by approximating the diffuse layer/bulk solution system
 158 with a Donnan model. While WHAM can account for the complexation of the free ionic form and the first
 159 hydrolysis product of each metal, we only accounted for Cu^{2+} as a preliminary speciation calculation showed that
 160 Cu^{2+} represented > 95% of the total Cu in solution with $pH \leq 6.3$. However, at higher pH the presence of Cu
 161 hydroxide should be considered. The partial pressure of CO_2 was assumed to be that of the ambient atmosphere
 162 ($10^{-3.5}$ atm) and the temperature was set at 25 °C for the calculations.

163 To develop a predictive model for ecotoxicological assessment, we aimed at fitting the experimental data as
 164 accurately as possible, but with the lowest possible number and the most generic set of parameters for wheat and

165 tomato. Accordingly, we first simulated the root binding properties with a single HA having the default
166 parameterization (pK_{a_i} , ΔpK_{a_i} , $K_{M,i}$, $\Delta LK2_M$) used in WHAM (Tables 1 and 2). The HA concentration was set at
167 the concentration found in dry roots in batch solutions, i.e. 400 mg l⁻¹. The total density of binding sites on HA
168 was set according to the potentiometric titration of wheat and tomato roots reported by Guigues et al. (2014).

169 The ratio between the density of low- pK_a (type-1) and high- pK_a (type-2) sites (hereafter referred to as the
170 L/H- pK_a ratio) that is arbitrarily assigned at 2 in WHAM was out of line with the L/H- pK_a ratios of 0.4 and 0.9
171 experimentally determined for wheat and tomato roots, respectively (Guigues et al. 2014). In agreement, the
172 L/H- pK_a ratios on roots of dicots and monocots found in the literature were also lower than 2, i.e. ranging from
173 0.5 to 1.7, except for *Lupinus albus* L. that had a ratio of 3.8 (Meychik and Yermakov 1999 and 2001; Ginn et al.
174 2008; Wu and Hendershot 2009; see Table S3).

175 To relax the L/H- pK_a ratio, we thus mimicked the root binding properties with two HA that were
176 specifically parameterized in WHAM-THP (Tables 1 and 2). The concentration of each HA was set at the
177 concentration established in dry roots in batch solutions, i.e. 400 mg l⁻¹. The total density of binding sites and the
178 distribution between low- and high-affinity sites was set for each HA according to the potentiometric titration of
179 wheat and tomato roots reported by Guigues et al. (2014). The first HA (HA_I) represented the low- pK_a sites
180 while the second HA (HA_{II}) represented the high- pK_a sites. We first parameterized pK_{a_1} , pK_{a_2} , ΔpK_{a_1} and
181 ΔpK_{a_2} for HA_I and HA_{II} to fit the experimental titration curves of wheat and tomato roots (Guigues et al. 2014).
182 We then parameterized $K_{Cu,1}$, $K_{Cu,2}$ and $\Delta LK2_{Cu}$ to fit the Cu sorption data from experiment 1. The ability of
183 WHAM-THP to account for ionic strength and pH effects on Cu binding was verified by predicting Cu sorption
184 data from experiments 2 and 3 without additional parameterization. Finally, the ability of WHAM-THP to
185 account for the competitive effect of Ca and Zn was assessed by parameterizing $K_{Ca,1}$, $K_{Ca,2}$, $\Delta LK2_{Ca}$, $K_{Zn,1}$, $K_{Zn,2}$
186 and $\Delta LK2_{Zn}$ to fit the Cu sorption data from experiments 4 and 5.

187

188 3.2. Application of WHAM to an external dataset

189 To evaluate the extent to which WHAM-THP improves the prediction of Cu accumulation into roots and Cu
190 rhizotoxicity in comparison with WHAM, we concomitantly applied the two models to the dataset obtained by
191 Wu and Hendershot (2010). These authors measured the Cu concentration in roots and the root length of pea
192 seedlings exposed to solutions with varying Cu (0 to 24.8 μ M) and Ca (0.04, 0.18, and 1.92 mM) concentrations
193 at pH 4, 5, or 6.

194 The prediction of root Cu concentration with WHAM and WHAM-THP was done by considering a factor of
 195 0.044 g HA.g⁻¹ DW to convert a quantity of Cu per g of HA in a quantity of Cu per g of dry roots, as described
 196 by Le et al. (2015) on the same dataset. The relative root elongation (*RRE*, %) was calculated from the root
 197 length (*RL*, mm):

$$198 \quad RRE = \frac{RL}{RL_{max}} \times 100 \quad \text{Eq. 2}$$

199 with RL_{max} being the maximal *RL* measured. The *RRE* was predicted with a log-logistic dose-response curve:

$$200 \quad RRE = \frac{100}{1 + \left(\frac{Ftox}{Ftox_{50}}\right)^\beta} \quad \text{Eq. 3}$$

201 and

$$202 \quad Ftox = \sum \alpha_i \times v_i = \alpha_H \times v_H + \alpha_{Cu} \times v_{Cu} \quad \text{Eq. 4}$$

203 with *Ftox* being the toxicity function of a mixture of metal cations (H and Cu herein) calculated by summing the
 204 products of the metal-specific toxicity coefficient (α_i) and the concentration of metal bound to HA (v_i , mol.g⁻¹
 205 ¹ HA) calculated with WHAM or WHAM-THP, $Ftox_{50}$ is the value of *Ftox* inducing a 50% reduction in the
 206 *RRE*, and β is a shape parameter. The α_H was set at 1 according to Tipping and Lofts (2013).

207

208 3.3. Parameter optimization

209 The quality of the fits obtained with WHAM and WHAM-THP were determined by calculating the root
 210 mean square error (*RMSE*) between the experimental data and model outputs (Table S4). *RMSE* was calculated
 211 from untransformed data for potentiometric titrations as some experimental data were negative. *RMSE* was
 212 calculated from log₁₀-transformed data for Cu sorption experiments to balance the weight of the highest values.
 213 The *RRE* was predicted by optimizing the α_{Cu} , $Ftox_{50}$, and β to minimize the *RMSE* between measured and
 214 predicted values.

215

216

217 4. Results and Discussion

218

219 4.1. WHAM-THP substantially improves the modeling of acidic properties of wheat and tomato roots

220 Despite the fact that the total density of binding sites has been fitted to the experimental data (Guigues et al.
 221 2014), WHAM estimates failed to fit the titration data for wheat and tomato ($RMSE = 12.7 \text{ cmol}_c.\text{kg}^{-1}$; Figure 1;
 222 Figure S1). WHAM estimates overestimated the density of binding sites observed experimentally over the whole

223 investigated pH range. The shape of the WHAM simulation curves also differed substantially from that of the
224 experimental data. The inadequacy of WHAM estimates to fit the experimental data was partly due to the L/H-
225 pK_a ratio arbitrarily assigned at 2 in WHAM (Table 1), which differs substantially from the L/H- pK_a ratios
226 determined experimentally in previously published studies, and particularly for wheat and tomato roots in our
227 study (see 3.1 for rationale).

228 Relaxing the L/H- pK_a ratio by using two HA but with the default parameterization of WHAM for the two
229 HA (e.g. not as with WHAM-THP for which the two HA were specifically parameterized) generally improved
230 the fit of the titration data for wheat and tomato ($RMSE = 4.7$ and $5.2 \text{ cmol}_c.\text{kg}^{-1}$), but this only satisfactorily
231 fitted the experimental data for tomato at $\text{pH} \leq 6$ (data not shown). The inadequacy of WHAM estimates to fit
232 the experimental data was thus also partly due to the default parameterization of proton dissociation (pK_a and
233 ΔpK_a) of HA in WHAM.

234 The acidic properties of plant roots are so different from those of HA that a specific parameterization of HA
235 for terrestrial higher plants in WHAM-THP is required (Table 1). By contrast with WHAM estimates, WHAM-
236 THP estimates accurately fitted the titration data for wheat and tomato ($RMSE = 1.6 \text{ cmol}_c.\text{kg}^{-1}$; Figure 1;
237 Figure S1).

238

239 4.2. WHAM-THP slightly improves the modelling of copper competitive binding on wheat and tomato roots

240 Over the five experiments of Cu sorption for wheat and tomato, WHAM estimates fitted 79% of the
241 experimental data within twofold (Figure 2A). However, WHAM estimates almost systematically overestimated
242 Cu binding by a factor 1.6 ($RMSE = 0.24$). Le et al. (2015) showed that the WHAM default parameterization also
243 overestimated the Cu concentration in pea roots. The overestimation increased as the Cu concentration in roots
244 decreased.

245 WHAM-THP estimates better fitted the experimental data ($RMSE = 0.15$) than WHAM estimates, with 91%
246 of the experimental data within twofold and without any systematic bias (Figure 2B). The comparison of the
247 results obtained with WHAM and WHAM-THP in each of the five Cu sorption experiments is detailed below in
248 sections 4.2.1 to 4.2.4. The specific parameterization of WHAM-THP (Tables 1 and 2) thus slightly improved
249 the goodness of fit for the modelling of Cu competitive binding relative to the WHAM default parameterization.
250 This suggests that WHAM-THP should also improve modelling of the concentration in roots and the
251 rhizotoxicity of Cu.

252

253 4.2.1. Experiment 1 on the copper binding affinity

254 For wheat, WHAM estimates overestimated Cu binding ($RMSE = 0.23$), with an increase in the
255 overestimation as the Cu concentration in solution increased (Figure S2A). By contrast, WHAM-THP estimates
256 neatly fitted ($RMSE = 0.04$) Cu binding on wheat roots throughout the investigated Cu concentration range.

257 For tomato, WHAM-THP estimates adequately fitted ($RMSE = 0.03$) Cu binding for $pCu_{in} > 4.5$ in solution
258 (i.e. $pCu_{eq} > 5$; Table S2) (Figure S2B). WHAM-THP estimates fitted Cu binding for $pCu_{in} > 4.5$ better than
259 WHAM estimates ($RMSE = 0.09$), which slightly but systematically overestimated the experimental data.
260 WHAM and WHAM-THP estimates showed a twofold deviation with the experimental data at $pCu_{in} \leq 4.5$. This
261 deviation increased with increasing Cu concentration. These high Cu concentrations (equivalent to $pCu_{eq} < 5$, i.e.
262 $Cu_{eq} > 10 \mu M$) however exceeded the concentration range that usually generates acute Cu rhizotoxicity (Kopittke
263 et al. 2010 and 2011). We thus did not attempt to specifically parameterize WHAM-THP to fit the experimental
264 data at $pCu_{in} \leq 4.5$.

265 The $\log K_{Cu,1}$ of HA_I sites in WHAM set at 2.4 by default was intermediate between the $\log K_{Cu,1}$ (equal to
266 2.2) and the $\log K_{Cu,2}$ (equal to 2.7) of HA_I sites specifically parameterized in WHAM-THP (Table 2). Similar to
267 the low- pKa sites of HA_I parameterized by default in WHAM that presumably correspond to carboxyl groups
268 (Tipping 1998), our recent spectroscopic analyses on wheat and tomato roots showed that the low- pKa sites
269 involved in Cu binding on wheat and tomato roots corresponded to carboxyl groups (Guigues et al. 2016).
270 Considering the identical nature of the binding sites, it is thus hard to attribute the better fits obtained with
271 WHAM-THP as compared to those obtained with WHAM to the specific parameterization of Cu binding affinity
272 of HA_I in WHAM-THP.

273 The $\log K_{Cu,1}$ (equal to 6.0) and the $\log K_{Cu,2}$ (equal to 6.7) of HA_{II} sites specifically parameterized in
274 WHAM-THP was about one order of magnitude higher than the $\log K_{Cu,2}$ of HA_I sites in WHAM set at 5.1 by
275 default (Table 2). While the high- pKa sites of HA parameterized by default in WHAM presumably correspond to
276 phenolic groups (Tipping 1998), our recent spectroscopic analyses showed that the high- pKa sites involved in
277 Cu binding on wheat and tomato roots corresponded to N functional groups (Guigues et al. 2016). Since N
278 functional groups have a higher affinity for Cu than phenolic groups (Fry et al. 2002), the involvement of N
279 functional groups in Cu binding on wheat and tomato roots supports the higher $\log K_{Cu}$ fitted for the high- pKa
280 sites of HA_{II} in WHAM-THP. The better fits obtained with WHAM-THP than with WHAM could thus be
281 attributed to the specific parameterization of Cu binding affinity of HA_{II} in WHAM-THP.

282 The heterogeneity parameters, i.e. $\Delta LK2_{Cu,I}$ in WHAM, set at 2.3 by default were much higher than the
283 $\Delta LK2_{Cu,I}$ and $\Delta LK2_{Cu,II}$ that were fitted to 0 in WHAM-THP (Table 2). This heterogeneity parameter accounts for
284 binding sites that occur at a low density and that exhibit a particularly high affinity for Cu (Tipping 1998). With
285 our dataset, increasing the $\Delta LK2_{Cu}$ from 0 to 3 led to overestimation of the binding of Cu at low concentration
286 (i.e. $pCu > 7$) by up to four orders of magnitude (data not shown). The much lower $\Delta LK2_{Cu}$ fitted in WHAM-
287 THP than in WHAM thus likely explains why WHAM estimates overestimated Cu binding on wheat and tomato
288 roots at low Cu concentration compared to WHAM-THP estimates.

289

290 4.2.2. Experiment 2 on the effect of ionic strength

291 The increase in ionic strength from 0.6 to 300 mM decreased the amount of Cu bound to wheat and tomato
292 roots by 43% on average (Figure S3). As commonly described in the literature, the higher the ionic strength, the
293 higher the tendency for major cations (for a negatively-charged sorbent such as roots) to accumulate in the
294 diffuse layer (Vidali et al. 2011). This accumulation of major cations thus masks the negative charges on root
295 surfaces and thus decreases the ability of binding sites to attract and bind metal cations such as Cu (Wang et al.
296 2011).

297 At the two ionic strengths, WHAM estimates better fitted the experimental data for tomato ($RMSE = 0.18$)
298 than for wheat ($RMSE = 0.38$) (Table S4). At 300 mM ionic strength, WHAM estimates more particularly
299 overestimated Cu binding on wheat and tomato roots, except at the highest Cu concentration (i.e. $pCu_{in} 4.2$).
300 WHAM-THP estimates better fitted the experimental data for the two ionic strengths ($RMSE = 0.11$ and 0.21 for
301 tomato and wheat, respectively) than WHAM estimates (Table S4). These results show that the specific
302 parameterization of WHAM-THP better accounted for the effect of the ionic strength on Cu binding on roots
303 than the default parameterization.

304

305 4.2.3. Experiment 3 on proton competition

306 The increase of pH from 4 to 6 boosted the amount of Cu bound to wheat and tomato roots by 41 and 16%
307 on average, respectively (Figure S4). As commonly described in the literature, an increase in pH increases the
308 dissociation of protons from negatively-charged surfaces such as roots, thus decreasing the competition between
309 protons and metal cations such as Cu for binding on roots (Ginn et al. 2008; Bulgariu and Bulgariu 2012).

310 At pH 6, WHAM and WHAM-THP estimates similarly overestimated Cu binding on wheat and tomato
311 roots ($RMSE = 0.19$ and 0.14 , respectively) (Figure S4). At pH 4, WHAM estimates overestimated Cu binding

312 on wheat and tomato roots ($RMSE = 0.45$ and 0.13 , respectively). In comparison, WHAM-THP estimates
313 underestimated Cu binding on wheat and tomato roots ($RMSE = 0.38$ and 0.20 , respectively).

314 In the 4 to 6 pH range, WHAM modelled a very weak competitive effect of protons and thus consistently
315 overestimated the amount of Cu bound on wheat and tomato roots (Figure S4). By contrast, WHAM-THP
316 accounted for a substantial competitive effect of protons and overestimated it. This led WHAM-THP to
317 overestimate the decrease in Cu bound to roots from pH 6 to 4. The WHAM-THP estimates closely fitted the
318 potentiometric data between pH 3.5 and 10.5 (Figure S1), which should theoretically enable us to describe the
319 increase in the proportion of the total binding sites available for metal binding as the pH increases. This indicated
320 that the binding site affinity for Cu decreased from pH 4 to 6 and was consequently underestimated at pH 4 and
321 overestimated at pH 6 in comparison with the initial optimization made at pH 5. Faced with the same issue, Wu
322 and Hendershot (2010) chose to lower the $\log K_{Cu}$ by approximately 1 unit to fit the Cu binding on pea roots from
323 pH 4 to 6. Overall, proton competition was the only effect for which WHAM-THP estimates ($RMSE = 0.31$ and
324 0.17 for wheat and tomato, respectively) did not improve the fit of the experimental data obtained with WHAM
325 estimates ($RMSE = 0.38$ and 0.18) (Table S4).

326

327 4.2.4. Experiments 4 and 5 on calcium and zinc competition

328 While the Ca concentration range exceeded that of Cu by more than four orders of magnitude, Ca only had a
329 weak competitive effect on Cu binding on roots (Figure S5). For wheat, the amount of Cu bound to roots
330 decreased by only 15% as the Ca concentration increased. For tomato, the amount of Cu bound to roots did not
331 significantly decrease as the Ca concentration increased. The competitive effect of Zn on Cu binding on wheat
332 and tomato roots was also weak, but it was higher than the competitive effect of Ca (Figure S5). The amount of
333 Cu bound to roots decreased by 30% and 18% for wheat and tomato, respectively, as the Zn concentration
334 increased.

335 When determined for a given species (monocots or dicots) under similar experimental conditions for Cu, Ca,
336 and Zn, the $\log K_{Cu}$ in roots was reported to be higher than the $\log K_{Zn}$ by 1.9 to 3.4 orders of magnitude (Vulkan
337 et al. 2004; Wang et al. 2010 and 2012; Le et al. 2013) and were reported to be higher than the $\log K_{Ca}$ by 0.9 to
338 4.4 orders of magnitude (Cheng and Allen 2001; Luo et al. 2008; Wu and Hendershot 2010; Wang et al. 2012).
339 The $\log K_{Zn}$ also remained higher than the $\log K_{Ca}$ by 1 to 2 orders of magnitude (Wang et al. 2010; Kinraide
340 2009). The literature thus supports our findings, which showed that the competitive effect of Ca and Zn on Cu
341 binding on wheat and tomato roots was weak, but that Zn was a stronger competitor than Ca.

342 WHAM modelled almost no competitive effect of Ca and Zn and hence overestimated Cu binding on roots
343 in the presence of Ca or Zn for wheat ($RMSE=0.28$) and tomato ($RMSE=0.12$) (Figure S5; Table S4). In
344 contrast, WHAM-THP estimates correctly fitted ($RMSE=0.03-0.04$) the competitive effect of Ca and Zn on Cu
345 binding on wheat and tomato roots (Figure S5). To obtain this good fit, the $\log K_{Ca}$ and $\log K_{Zn}$ of HA_{II} sites in
346 WHAM-THP was increased by 2 orders of magnitude compared to the default $\log K_{Ca}$ and $\log K_{Zn}$ of type-2 sites
347 in WHAM (Table 2). However, the difference between the corresponding $\log K$ for Cu and Ca ($\Delta=1.0-1.2$ for
348 HA_I and $\Delta=2.0-2.2$ for HA_{II}) or for Cu and Zn ($\Delta=0.2$ for HA_I and $\Delta=0$ for HA_{II}) in WHAM-THP remained
349 approximately the same as in WHAM (Table 2). This suggests that the better fits obtained with WHAM-THP
350 than with WHAM could be attributed to the specific parameterization of the acidic properties of wheat and
351 tomato roots rather than to the specific parameterization of the binding affinity of Cu, Ca, and Zn.

352

353 4.3. WHAM-THP only slightly improves the prediction of copper concentration in pea roots

354 WHAM and WHAM-THP estimates exhibited the same goodness of fit for the Cu concentration in roots
355 when considering the whole dataset of Wu and Hendershot (2010) ($RMSE=0.69$ and 0.70 , respectively), with
356 almost all data points predicted within one order of magnitude. The deviation between root Cu concentrations
357 measured and those modelled with WHAM increased as the root Cu concentration decreased (Figure 3),
358 indicating that WHAM estimates tends to overestimate root Cu concentration at low Cu exposure levels.

359 It is noteworthy that the prediction of the root Cu concentration with WHAM could be improved
360 ($RMSE=0.56$) by setting the $\Delta LK2$ at 0 for Ca and Cu (data not shown). This suggests that the deviation
361 observed with WHAM estimates at low root Cu concentration was mainly due to a too strong contribution of low
362 density sites exhibiting a particularly high affinity for metal cations in the default parameterization of WHAM.
363 In WHAM-THP, the $\Delta LK2$ has been set at 0 for Ca and Cu. Accordingly, WHAM-THP estimates better fitted
364 the root Cu concentration than WHAM estimates for root Cu concentrations approximately lower than 10^5
365 mol.g^{-1} at pH 4 and $5 \cdot 10^{-6} \text{mol.g}^{-1}$ at pH 5 (Figure 3A and B).

366 WHAM estimates were very sensitive to pH as the $RMSE$ between measured and modelled data increased
367 from 0.42 at pH 4 to 0.90 at pH 6 (Figure 3). WHAM-THP estimates were less sensitive to pH than WHAM
368 estimates at pH 4 and 5 as the $RMSE$ between measured and modelled data were 0.33 and 0.49, respectively
369 (Figure 3A and B). However, at pH 6, WHAM-THP estimates deviated more from the measured data than
370 WHAM estimates (Figure 3C). This was in agreement with the results obtained in the Cu sorption experiment 3,

371 which showed that WHAM-THP estimates tended to overestimate the proton competition in comparison with
372 WHAM estimates (see 4.2.3; Figure S4).

373

374 4.4. WHAM-THP does not improve the prediction of copper rhizotoxicity

375 Estimates of WHAM and WHAM-THP combined with F_{tox} fitted very similarly the measured RRE
376 ($R^2 = 0.73$ and $RMSE = 13\%$; Figure 4A and B). The F_{tox} values calculated with WHAM-THP were closely
377 correlated with those of WHAM ($R^2 = 0.94$) although the F_{tox} values calculated with WHAM-THP were about
378 twofold higher. As the \bullet_{Cu} optimized in WHAM and WHAM-THP simulations was very similar (3.3 and 3.6,
379 respectively), the difference between the F_{tox} values calculated with WHAM and WHAM-THP comes,
380 according to Eq. 4, from the stronger complexation calculated with WHAM-THP (i.e. higher ν_{Cu} and ν_H) than
381 that calculated with WHAM.

382 Accordingly, WHAM and WHAM-THP estimates combined with F_{tox} predicted very similar RRE
383 ($R^2 = 0.97$; Figure 4C), even at low root Cu concentration for which WHAM-THP better predicted the root Cu
384 concentration than WHAM (data not shown). This may have been due to the fact that the prediction of RRE with
385 F_{tox} values involved the optimization independently for WHAM and WHAM-THP of three supplementary
386 parameters, i.e. F_{tox50} , β , and α_{Cu} . This supplemental optimization may enable correction of the small deviation
387 observed between WHAM and WHAM-THP estimates of Cu concentration in roots so as to finally achieve
388 similar RRE predictions with WHAM and WHAM-THP.

389 Although WHAM-THP slightly improved the prediction of Cu binding on roots (see 4.2) and the prediction
390 of Cu concentration in roots at low root Cu concentration (see 4.3) relative to WHAM, WHAM-THP hence did
391 not at all improve the prediction of Cu rhizotoxicity. Considering this result and how time-consuming it would
392 be to complete the parameterization of WHAM-THP for all metal cations of interest, we conclude that, although
393 the default parameterization of WHAM does not neatly predict the binding of metal cations on roots, it could be
394 used with a good level of confidence in predictive ecotoxicology for terrestrial higher plants without any specific
395 parameterization.

396

397

398 **Supporting information**

399

400 Supporting information (Tables S1 to S4; Figures S1 to S5) can be found at [http:...](http://...)

401

402

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404

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408

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485

486 **Figure captions**

487

488 **Fig. 1.** Measured versus modelled acidic properties (expressed in charge Q corrected by the initial charge Q_0) of
489 wheat (crosses) and tomato (circles) roots. Green and red symbols correspond to data modelled with the
490 Windermere Humic Aqueous Model either parameterized by default (WHAM) or specifically parameterized
491 with two humic acids for terrestrial higher plants (WHAM-THP), respectively. The root mean square errors
492 ($RMSE$) pool data for wheat and tomato ($n = 176$). The solid line refers to 1:1 line and dashed lines refer to a
493 factor ± 2 .

494

495 **Fig. 2.** Measured versus modelled binding of copper (Cu) to wheat (crosses) and tomato (circles) roots. Green
496 and red symbols correspond to data modelled with the Windermere Humic Aqueous Model either parameterized
497 by default (WHAM, A) or specifically parameterized with two humic acids for terrestrial higher plants (WHAM-
498 THP, B), respectively. The root mean square errors ($RMSE$) pool data for wheat and tomato in the Cu sorption
499 experiments 1 to 5 ($n = 183$). The solid line refers to 1:1 line and dashed lines refer to a factor ± 2 .

500

501 **Fig. 3.** Measured versus modelled copper (Cu) concentration in pea roots exposed to solutions at pH 4 (A), 5 (B),
502 and 6 (C) with varying calcium (0.04, 0.18, and 1.92 mM) and Cu (0 to 24.8 μM) concentrations. Green and red
503 symbols correspond to data modelled with the Windermere Humic Aqueous Model either parameterized by
504 default (WHAM) or specifically parameterized with two humic acids for terrestrial higher plants (WHAM-THP),
505 respectively. The deviation between measured and modelled data is expressed as the root mean square errors
506 ($RMSE$). The solid line refers to 1:1 line and dashed lines refer to a factor ± 10 .

507

508 **Fig. 4.** Relative root elongation (RRE) measured for pea as a function of the exposure to toxic cations (proton
509 and copper) in solutions at pH 4, 5 or, 6 and with varying calcium (0.04, 0.18, and 1.92 mM) and copper (0 to
510 24.8 μM) concentrations (A and B). The proton and copper toxicity is calculated by combining a toxicity
511 function (F_{tox} , see 3.2 for rationale) with the Windermere Humic Aqueous Model either parameterized by
512 default (WHAM, green circles, A) or specifically parameterized with two humic acids for terrestrial higher
513 plants (WHAM-THP, red circles, B). The solid line refers to the RRE predicted with WHAM (A) or WHAM-
514 THP (B) and dashed lines refer to a deviation of $\pm 10\%$. The deviation between measured and modelled data is

515 expressed as the root mean square errors (*RMSE*). Relative root elongation predicted with WHAM-THP versus
516 *RRE* predicted with WHAM (C). The solid line refers to 1:1 line.

ACCEPTED MANUSCRIPT

1 Does specific parameterization of WHAM improve the prediction of copper
2 competitive binding and toxicity on plant roots?

3

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14

15 Tables = 2

16

17 **Tables**

18

19 **Table 1**

20 Proton dissociation constants (pK_{a_i}) and distribution terms (ΔpK_{a_i}) of wheat and tomato roots as parameterized in the Windermere Humic Aqueous Model by default
 21 (WHAM) for one humic acid (HA) and specifically parameterized for terrestrial higher plants (WHAM-THP) for two HA. Total site densities (L_{H_i} , $\text{cmol}_c\cdot\text{kg}^{-1}$) were
 22 determined experimentally by Guigues et al. (2014).

23

		HA _I						HA _{II}						$L_{H,total}$
		Type 1			Type 2			Type 1			Type 2			
		L_{H1}	pK_{a1}	ΔpK_{a1}	L_{H2}	pK_{a2}	ΔpK_{a2}	L_{H1}	pK_{a1}	ΔpK_{a1}	L_{H2}	pK_{a2}	ΔpK_{a2}	
WHAM	Wheat	24.4			12.2			–	–	–	–	–	–	
	Tomato	64.9	4.1	2.6	32.5	8.3	3.1							97.4
WHAM-THP	Wheat	6.3			3.3			18.0			9.0			36.6
	Tomato	31.3	4.2	1.5	15.7	5.2	2.0	33.6	9.8	0	16.8	8.8	1.5	97.4

24

25

26 **Table 2**

27 Intrinsic equilibrium constants ($K_{M,i}$) and heterogeneity parameters ($\Delta LK2_{M,i}$) of copper (Cu), calcium (Ca) and
 28 zinc (Zn) binding on wheat and tomato roots as parameterized in the Windermere Humic Aqueous Model by
 29 default (WHAM) for one humic acid (HA) and specifically parameterized for terrestrial higher plants (WHAM-
 30 THP) for two HA.

31

		HA _I			HA _{II}		
		Type 1		Type 2	Type 1		Type 2
		Log $K_{M,I}$	Log $K_{M,2}$	$\Delta LK2_{M,I}$	Log $K_{M,I}$	Log $K_{M,2}$	$\Delta LK2_{M,II}$
Cu	WHAM	2.4	5.1	2.3	–	–	–
	WHAM-THP	2.2	2.7	0	6.7	6.0	0
Ca	WHAM	1.3	2.3	0	–	–	–
	WHAM-THP	1.2	1.5	0	4.5	4.0	0
Zn	WHAM	1.9	4.1	1.3	–	–	–
	WHAM-THP	2.0	2.5	0	6.7	6.0	0

32

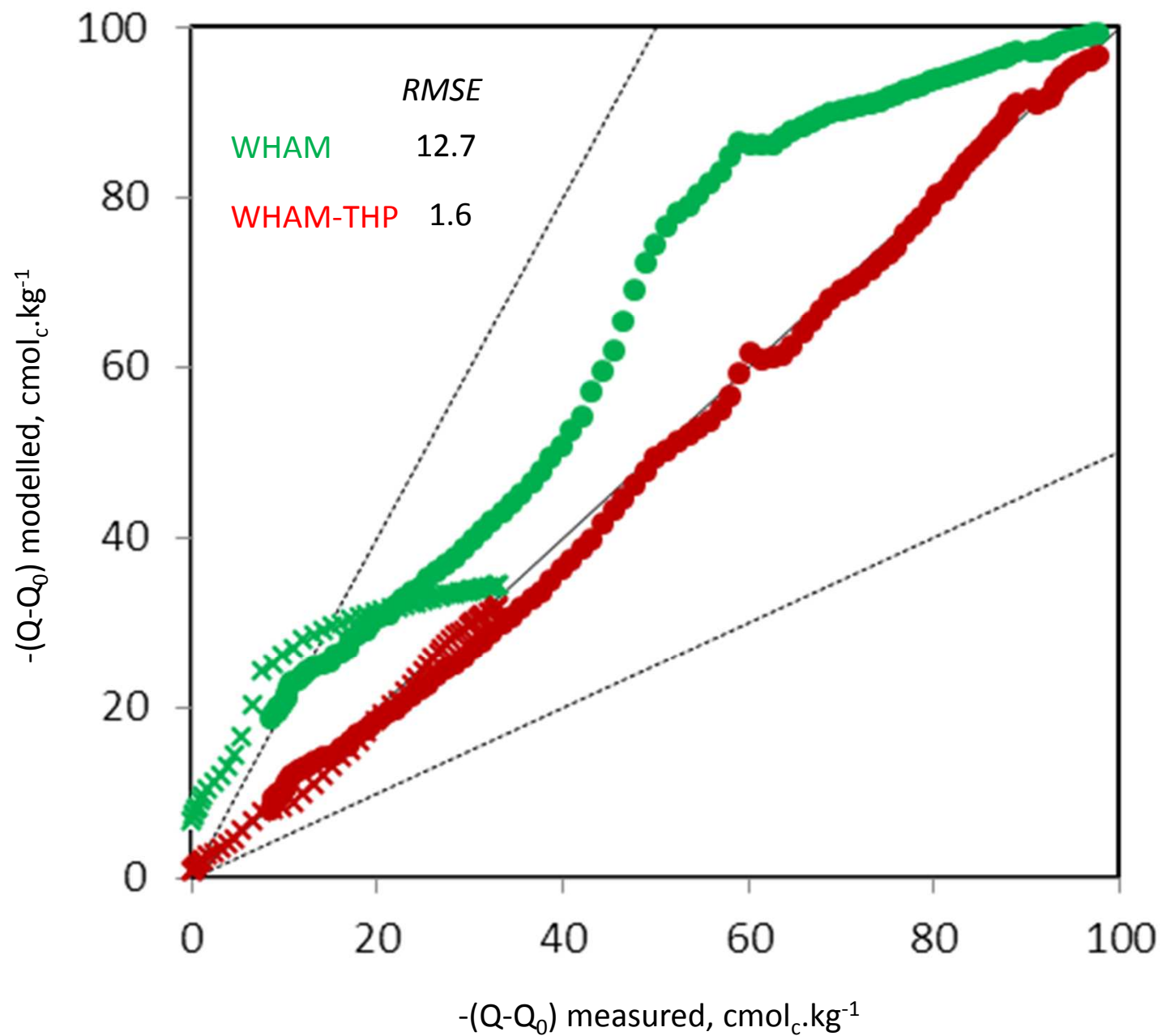


Fig. 1

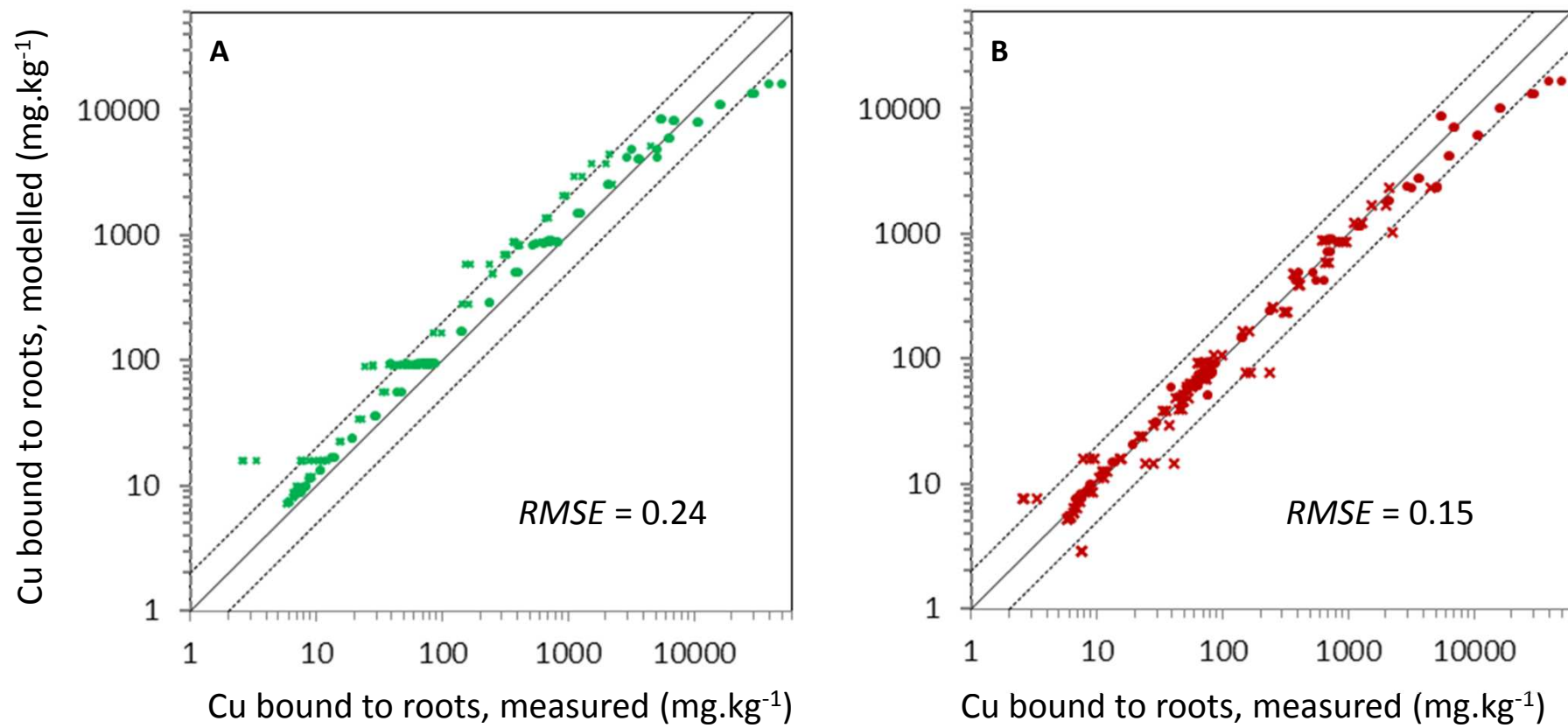


Fig. 2

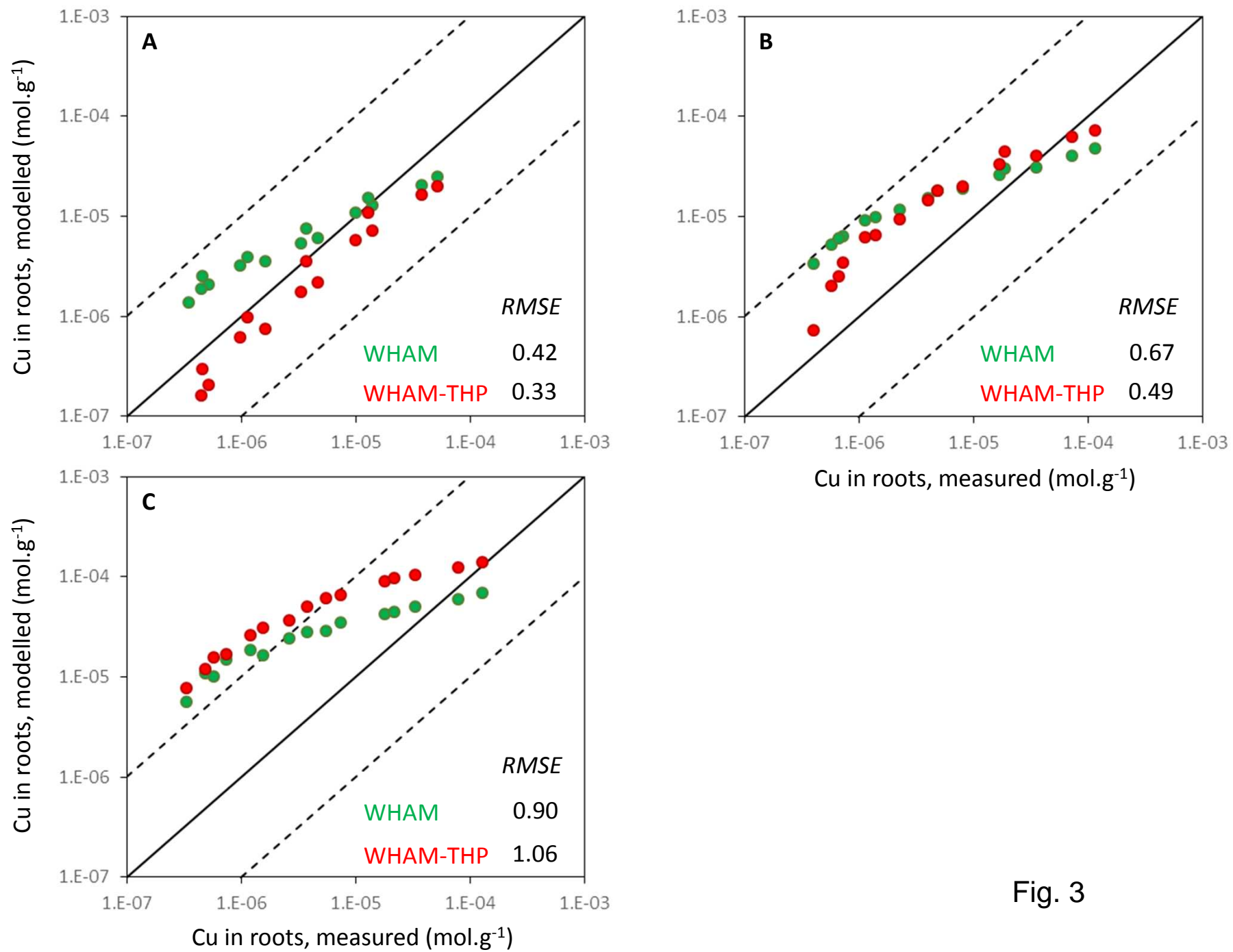


Fig. 3

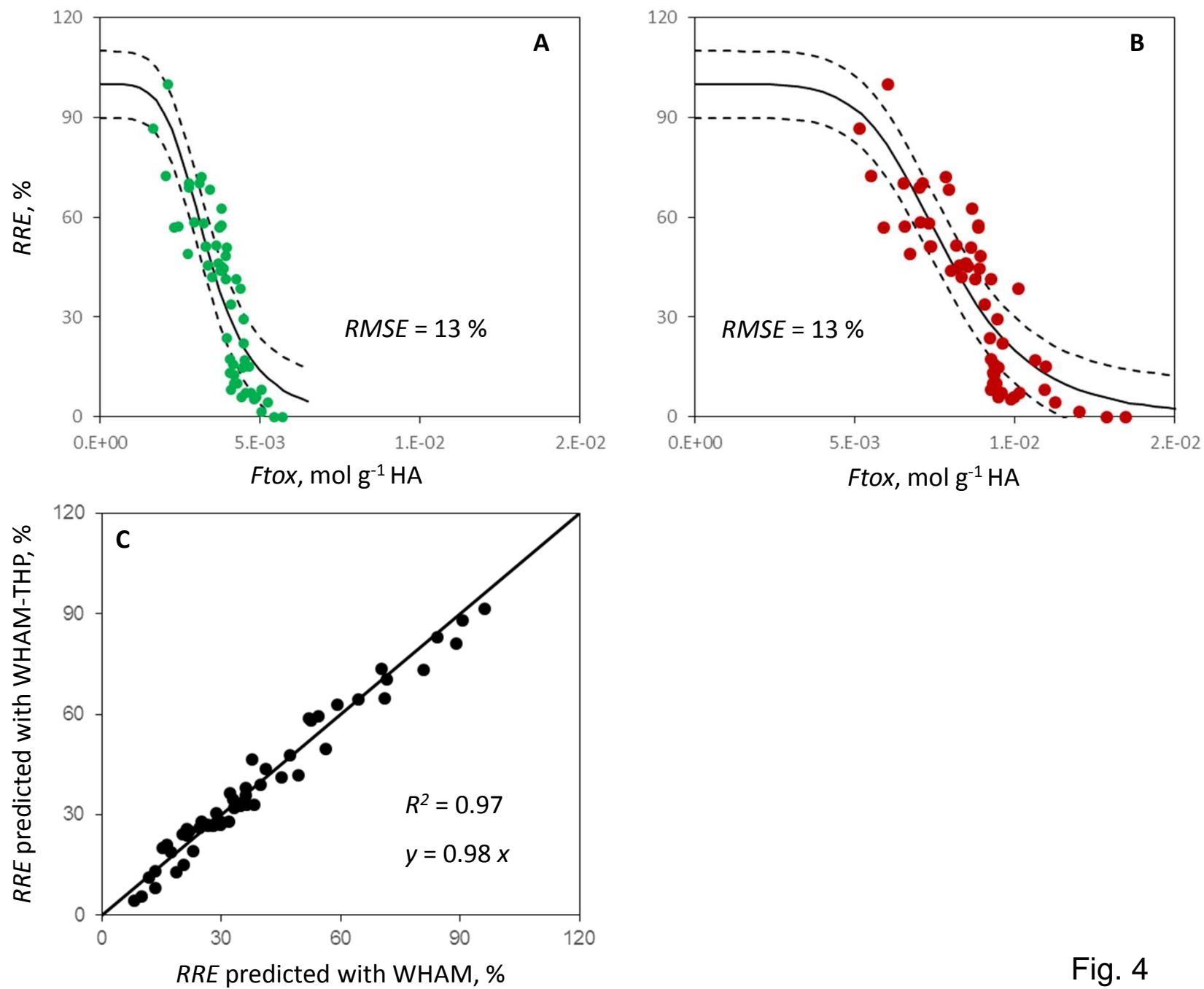


Fig. 4

1 Does specific parameterization of WHAM improve the prediction of copper
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3

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15

16

17 **Highlights**

- 18 • WHAM-THP is a specific parameterization of WHAM for terrestrial higher plants
- 19 • WHAM-THP much improves the prediction of root acidic properties
- 20 • WHAM-THP only slightly improves the prediction of root copper competitive binding
- 21 • WHAM-THP does not improve the prediction of copper rhizotoxicity

22

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