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1 **Modelling metal accumulation using humic acid as a surrogate for plant roots**

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23 **ABSTRACT**

24 Metal accumulation in roots was modelled with WHAM VII using humic acid (HA)
25 as a surrogate for root surface. Metal accumulation was simulated as a function of
26 computed metal binding to HA, with a correction term (E_{HA}) to account for the
27 differences in binding site density between HA and root surface. The approach was
28 able to model metal accumulation in roots to within one order of magnitude for 95%
29 of the data points. Total concentrations of Mn in roots of *Vigna unguiculata*, total
30 concentrations of Ni, Zn, Cu and Cd in roots of *Pisum sativum*, as well as internalized
31 concentrations of Cd, Ni, Pb and Zn in roots of *Lolium perenne*, were significantly
32 correlated to the computed metal binding to HA. The method was less successful at
33 modelling metal accumulation at low concentrations and in soil experiments.
34 Measured concentrations of Cu internalized in *L. perenne* roots were not related to Cu
35 binding to HA modelled and deviated from the predictions by over one order of
36 magnitude. The results indicate that metal uptake by roots may under certain
37 conditions be influenced by conditional physiological processes that are cannot
38 simulated by geochemical equilibrium. Processes occurring in chronic exposure of
39 plants grown in soil to metals at low concentrations complicate the relationship
40 between computed metal binding to HA and measured metal accumulation in roots.

41 *Keywords: root uptake, metal, modelling, WHAM, affinity.*

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47 **1. Introduction**

48 Consumption of vegetables is one of the most important sources for metal
49 accumulation in humans (Cohen et al., 1998; Swartjes et al., 2007). Considerable
50 amounts of metals can be accumulated in vegetables, subsequently entering the
51 human food chain thus posing potential risks to human health (Redjala et al., 2009).
52 According to Lijzen et al. (2001), exposure via vegetable consumption should be
53 considered in human risk assessment for all metals. So far, vegetable consumption has
54 been included in various exposure models in different countries, e.g., CLEA, RBCA
55 Tool Kit, and CSOIL (Carlton and Swartjes, 2007; Brand et al., 2007; DEFRA and
56 EA, 2002; Conor et al., 2007). In such models, metal concentrations in edible parts of
57 vegetables are usually estimated from empirical bioconcentration factors or regression
58 equations, while soil properties are hardly taken into account or only via rather
59 simplified approaches (Swartjes et al., 2007). These empirical methods provide no
60 understanding of underlying processes determining metal accumulation in edible parts
61 of vegetables. In most models, root uptake is not assessed explicitly, although it has
62 been demonstrated to be the most important route for the accumulation of many
63 metals (Smolders, 2001; Malecka et al., 2008). Investigation of metal uptake by plant
64 roots is the first step toward a better understanding of the relationship between human
65 exposure to metals via vegetable consumption and contamination in soil and a
66 mechanistically based modelling approach.

67 The plant root surface is a heterogeneous mixture of functional groups that are
68 potential binding sites for metals and protons (Cox et al., 1999; Fein et al., 1997;
69 Tiemann et al., 1999; Parsons et al., 2002; Gardea-Torresdey et al., 2002; Ginn et al.,
70 2008). According to Ke and Rayson (1992), it is impossible to adequately simulate
71 ion sorption to the plant root surface without considering specific binding. In addition,

72 the negative charge at the root surface creates an electrical potential (Wagatsuma and
73 Akiba, 1989; Kinraide, 1998). Both the electrostatic interactions and chemical
74 heterogeneity of biological surfaces influence metal root uptake (Kinraide, 2001;
75 Lindberg et al., 2004). These two characteristics of plant root surfaces have also been
76 observed in humic acids (Milne et al., 1995). Humic acids possess a heterogeneous
77 mixture of metal-binding groups, largely carboxylic and phenolic acids (Antunes et
78 al., 2012) with smaller amounts of N- and S-based groups, which display strong
79 affinities for metals. Furthermore, the ratio of 1:2 between the site densities of
80 phenolic and carboxylic groups assumed in WHAM (Tipping, 1998) is within the
81 range reported for root cell walls of different species such as lupine, wheat, and pea,
82 i.e., from 0.49:1 to 1:1 (Meychik and Yermakov, 2001). Because of these similarities,
83 humic acids in humic ion-binding models have been considered a surrogate for
84 biological surfaces such as root cell walls in estimating metal bioaccumulation
85 (Antunes et al., 2012; Tipping et al., 2008; Iwasaki et al., 2013; Tipping and Lofts,
86 2013). The relevance of using metal binding to HA to represent metal
87 bioaccumulation at biological surfaces is attributable to the nature of metal cation
88 sorption onto biological surfaces (Postma et al., 2005; Antunes et al., 2007).
89 Accordingly, competition among cations, which is considered when computing metal
90 binding to HA, is taken into account in predicting metal accumulation in plant roots.
91 In addition, the available set of binding constants in WHAM facilitates wide
92 application for estimating root uptake of metals in different species. Because of the
93 promising results from using this approach to estimate metal accumulation in
94 bryophytes and metal toxicity to duckweed and macroinvertebrates (Antunes et al.,
95 2012; Tipping et al., 2008; Iwasaki et al., 2013), we aimed to evaluate its potential for
96 modelling metal accumulation in roots of vascular plants. Such root accumulation

97 models might ultimately lead to improved models for predicting human exposure in
98 risk assessment.

99 In previous studies, binding sites of humic acids have been used to represent the
100 binding sites on reactive surfaces of various organisms (Antunes et al., 2012; Tipping
101 et al., 2008; Iwasaki et al., 2013; Tipping and Lofts, 2013). However, the
102 concentration of metals in roots available for transport to other parts of vegetables
103 depends not only on metal binding to sites at the root surface. After being adsorbed to
104 the root surface, metals might be transported into root cells (internalization)
105 (Campbell et al., 2002; Kalis et al., 2007). Only metals bound to physiologically
106 active sites will be internalized (Campbell et al., 2002). The objective of the present
107 study is to assess the applicability of WHAM with its default binding constants to
108 model both internalized and total (including surface-bound and internalized) metal
109 concentrations in plant roots based on data in the literature. If validated, the use of
110 available binding constants in WHAM allows the WHAM-HA model to be applicable
111 to different species. This initial investigation may provide a basis for further studies
112 on application of the WHAM-HA model for estimating metal accumulation in roots,
113 and hence its applicability in exposure assessment models.

114 **2. Methods**

115 *2.1. Data sets*

116 Our simulations of total and internalised metal concentrations in plant roots used
117 data provided by Kopittke et al. (2011), Wu (2007), and Kalis (2006). In the
118 hydroponic studies of Kopittke et al. (2011) and Wu (2007), total metal
119 concentrations in roots exposed to metals in solutions were measured. Effects of
120 major cations, but not organic matter (no humic acid was added to the nutrient

121 solution), were taken into account in determining metal accumulation in plant roots.
122 Kopittke et al. (2011) investigated total concentrations of Mn in roots of *Vigna*
123 *unguiculata*. The exposure solutions contained varying concentrations of Al (0-10
124 mM), Ca (0-20 mM), Mg (0-15 mM), and Na (0-20 mM) (Kopittke et al., 2011). Wu
125 (2007) examined total concentrations of Cd, Cu, Ni, and Zn in roots of *Pisum sativum*
126 following exposure to binary mixtures of these metals with Ca in the range from 0-2
127 mM (i.e., Cd–Ca; Cu–Ca; Ni–Ca; and Zn–Ca). Kalis (2006) measured the
128 concentrations of Cd, Cu, Ni, Pb, and Zn that are metabolically taken up (internalized)
129 by roots of *Lolium perenne* exposed to mixtures of these metals in pot experiments. In
130 the experiments, the plants were grown on the soil directly taken from the field, rather
131 than spiking soil with metal salts. The concentration of dissolved organic carbon
132 (0.01M CaCl₂ extraction) in soil varied from 8 to 16 mg/L. Further information on the
133 data sets, e.g., pH and exposure duration, is given in Table 1.

134 2.2. Bioaccumulation modelling

135 In the present study, WHAM VII was used for modelling metal accumulation in
136 plant roots (Tipping, 1994; Tipping et al., 2011). In WHAM, metal sorption to humic
137 substances is simulated by using a structured formulation of discrete, chemically-
138 plausible, binding sites for protons and metals. This allows the creation of regular
139 arrays of bidentate and tridentate binding sites for metals. Metal aquo ions compete
140 with their first hydrolysis products and protons as well as other metals for binding
141 sites. Together with the intrinsic metal binding strength to uncharged molecules,
142 electrostatic effects, which depend on molecular charge and ionic strength, were taken
143 into account in simulating metal binding to HA. The model is parameterised using
144 literature datasets on proton and metal binding to humic and fulvic acids, to derive
145 ‘best average’ default binding constants.

146 In our assessment, we used WHAM to calculate metal accumulation in roots
147 considering the HA as an analogue for roots. Metal binding to HA was modelled
148 using the default Model VII parameters. This method was chosen because only small
149 variations in binding constants among plant species have been reported in the
150 literature (Fein et al., 1997; Gin et al., 2008; Kaulbach et al., 2005; Naeem et al.,
151 2006). For example, the logarithm of the binding constant of Cd for the carboxylic
152 group on the membrane surface of bacteria, fungi, and plants was in the range 3.3–3.5.
153 The cell walls of algae, maize, soybeans, and higher plants have been shown to have
154 similar titration curves (Allan and Jarrell, 1989; Dufey et al., 1985; Morvan et al.,
155 1979). These observations suggest that metal ion sorption to biological surfaces of
156 different species can be reasonably predicted by a single set of proton and metal
157 binding constants (Ginn et al., 2008). Our method was further supported by results in
158 the study of Tipping et al. (2008) that binding constants of metals for functional
159 groups obtained in the optimisation were comparable to the default values for humic
160 and fulvic acids in WHAM.

161 Metal binding to HA was modelled by WHAM using inputs available in the studies
162 of Kopittke et al. (2011), Wu (2007), and Kalis (2006), i.e., pH, temperature, and
163 concentrations of trace metals, Al^{3+} , and alkali (Na^+ and K^+) and alkaline earth (Ca^{2+}
164 and Mg^{2+}) metals (Table 1). In the hydroponic experiments by Kopittke et al. (2011)
165 and Wu (2007), pH and cation concentrations were controlled and these values were
166 used as inputs to the model. In the pot experiments by Kalis (2006), the pH and free
167 metal concentrations was measured in the pore water. These measurements were used
168 as inputs to WHAM for estimating metal binding to HA. As such, bias in evaluating
169 effects of organic matter and precipitation on characterisation of reactive species of
170 metals can be avoided. In the studies of Kopittke et al. (2011) and Wu (2007),

171 concentrations of free metal ions were not measured and total metal concentrations
172 were therefore used as inputs to WHAM for estimating metal binding to HA.

173 The equilibrium binding of metals to HA, was calculated using a very small
174 concentration of HA, $1.0 \cdot 10^{-15}$ g/L and CO₂ pressure of 390 ppm (Iwasaki et al.,
175 2013). Differences between metal binding to HA and metal accumulation in plant
176 roots were attributed to the difference in the density of the groups between plant roots
177 and HA. Accordingly, the equivalent HA per gram dry weight (DW) or fresh weight

178 (FW) of roots, (denoted as E_{HA} , $\frac{\text{mol/g DW}}{\text{mol/g HA}} = \text{g HA/g DW}$ or $\frac{\text{mol/g FW}}{\text{mol/g HA}} = \text{g HA/g FW}$), was

179 used to account for the differences between the amounts of metals bound to HA and
180 the total or internalized metal concentrations in roots (Tipping and Lofts, 2013). We
181 expect E_{HA} values to usually be less than unity, as organisms typically have fewer
182 binding sites per unit mass than HA. Additionally where metal accumulation is
183 expressed in terms of root fresh weight, we would expect E_{HA} to be lower than if root
184 dry weight were used.

185 The value of E_{HA} represents an apparent relationship between the modelled amount
186 of metals bound to HA and the measured total or internalized metal concentrations in
187 roots. If E_{HA} is smaller than unity, there are fewer sites per gram of roots than per
188 gram of HA. On being determined from the relationship between amounts of metals
189 bound to HA modelled by WHAM and metal concentrations measured in roots, the
190 E_{HA} value may indicate the comparison between the density of function groups on HA
191 and the total density of both active and inert sites in roots. In principle, the difference
192 in the E_{HA} values reflects variability in the site density on the root surface between
193 species. Similarly, when E_{HA} accounts for the differences between modelled metal
194 concentrations bound to HA and measured internalized concentrations in roots, its

195 value may reflect the density of active sites in plant roots in comparison with the site
196 density of HA. However, a number of factors may affect the characteristics and
197 density of metal-binding sites on root surface. These should be considered in
198 interpreting the relationship between the computed metal binding to HA and the
199 measured metal accumulation in roots. This issue is described in more detail in the
200 *Discussion* section.

201 2.3. Assessment of the predictive potential of the WHAM-HA model

202 The performance of the WHAM-HA method in modelling metal accumulation in
203 roots was evaluated by comparing modelled (total or internalized) root concentrations
204 with the corresponding measured concentrations using different means of statistical
205 parameters. The coefficient of determination (r^2) was used to indicate the potential of
206 the approach to explain the observed variation. The relationship between the modelled
207 and measured concentrations was represented by the p -value. In addition, the
208 deviations between modelled and measured concentrations were expressed by mean
209 absolute error (MAE) and root mean square error (RMSE). In the assessment based on
210 data reported by Kalis (2006), the predictive potential was assessed for all metals
211 simultaneously because the data were generated in mixture exposures.

212 3. Results

213 Significant relationships were found between concentrations of Mn bound to HA
214 modelled with WHAM and total concentrations of Mn in roots of cowpea *V.*
215 *unguiculata*, measured at varying concentrations of Al^{3+} , Ca^{2+} , H^+ , Mg^{2+} , and Na^+ (n
216 = 120; $p < 0.00001$; Fig. 1). Approximately 81% of the variation in total Mn
217 concentrations in cowpea roots could be explained by modelled Mn binding to HA (r^2
218 = 0.81; Fig. 1). Total Mn concentrations in the roots were best described with an E_{HA}

219 of 0.0034 g/g FW (Table 2). The ratio between the root fresh weight and the root dry
220 weight varies from two to 14 (Sadeghipour and Aghaei, 2013; Khan and Khan, 1996).
221 Consequently, a ratio of eight was used to calculate the HA-equivalent site density of
222 cowpea roots on a dry weight basis, resulting in a value of 0.027 g/g DW. Generally,
223 total Mn concentrations in the roots calculated with the modelled value of E_{HA} were
224 within one order of magnitude of measured concentrations, except at the lowest
225 exposure level (Fig. 2; Table 3), where Mn binding to the roots was underestimated.
226 The variations in modelled Mn concentrations in cowpea roots were larger than the
227 variations in measurements (Fig. 2).

228 Computed metal binding to HA in binary mixtures of Cd, Cu, Ni, and Zn with Ca
229 was significantly correlated with the total concentration of these metals in roots of pea
230 *P. sativum* L. measured by Wu (2007), giving an optimal E_{HA} value of 0.044 g/g DW
231 ($n = 360$; $p < 0.0001$; $r^2 = 0.54$; Fig. 3). For all these metals, total concentrations in
232 pea roots modelled were generally within one order of magnitude of the
233 measurements (Fig. 4; Table 3). At the narrow range of Ca concentrations in exposure
234 solutions (0 – 2 mM), differences of less than one order of magnitude were found
235 between the estimates and the measurements of Ca concentrations in roots of *P.*
236 *sativum* (Fig. 4; Table 3). The largest deviations between modelled and measured
237 concentrations were observed for Cu, especially for lower accumulated concentrations
238 ($< 10^{-5}$ mol/g DW) (Fig. 4). For Zn, the deviations between the measurements and the
239 calculations by the WHAM-HA model increased with decreasing root concentrations,
240 similar to the observation for Mn accumulation in *V. unguiculata* roots (Fig. 4). The
241 potential of the WHAM-HA model to explain variations in metal accumulation in
242 roots of *P. sativum* was metal-specific (Table 3). About 80–92% of the variations in
243 total root concentrations of Cd, Ni, and Zn measured could be explained by the

244 modelled binding of these metals to HA (Fig. 3). Total concentrations of Ca modelled
245 with the common value of E_{HA} showed the least deviation from the measurements
246 although only 16% of the variations in Ca concentrations in roots could be explained
247 by the computed metal binding to HA (RMSE = 0.21).

248 Based on the soil experiments by Kalis (2006), in general, metal binding to HA
249 was significantly related to internalized concentrations in roots of *L. perenne* (Table
250 3). However, the strength of the correlation was metal-specific. Significant and strong
251 relationships were found between metal binding to HA computed and internalized
252 metal concentrations measured in roots of *L. perenne* for Cd ($n = 10$; $p < 0.00001$; r^2
253 = 0.96) and Zn ($n = 10$; $p < 0.00001$; $r^2 = 0.97$) (Fig. 5). A weaker correlation was
254 observed for Ni ($n = 10$; $p < 0.005$; $r^2 = 0.69$), whereas no significant relationship was
255 seen for Cu ($n = 10$; $p = 0.40$; $r^2 = 0.06$) and Pb ($n = 10$; $p = 0.16$; $r^2 = 0.22$) (Fig. 5).
256 Unifying the binding of these metals to HA and their internalized concentrations in
257 roots gave an E_{HA} value of 0.26 (g/g DW) (Table 2). Internalised metal concentrations
258 modelled were within one order of magnitude of the measurements, except for Cu
259 (Fig. 6 and Table 3). The internalized concentration of Cu was overestimated,
260 deviating from the measurements by more than one order of magnitude (Fig. 6).

261 **4. Discussion**

262 The results of the present study indicate that the approach of using WHAM-
263 computed metal binding to HA to estimate metal concentrations in plant roots has
264 promise, when values of E_{HA} for specific plant species are available. The significant
265 relationship between computed metal binding to HA and metal accumulation in roots
266 allows for improvement of the predictive accuracy of metal uptake, although
267 deviations of larger than one order of magnitude between the estimates and the
268 measurements may be observed with default binding constants in WHAM. Previous

269 studies (e.g., Tipping et al., 2008) have demonstrated one method for improving the
270 performance of the WHAM-HA model by optimising binding constants. Being
271 statistically significantly related to total concentrations of metals measured in plant
272 roots, computed metal binding to HA is a potential predictor of metal accumulation in
273 plant roots. This is consistent with the findings of Cox et al. (1999) that metal
274 accumulation on bacterial surfaces could be simulated by normal chemical equilibria.
275 Metal sorption to biological surfaces is controlled by properties and affinity of the
276 groups (Fein et al., 1997; Ke and Rayson, 1992; Plette et al., 1996). Abiotic and biotic
277 binding constants have been related to fundamental chemical properties of metals
278 (Zhou et al., 2011; Kinraide and Yermiyahu, 2007).

279 The variations in the HA-equivalent site density of plant roots among different
280 species reflect the species-specific number of binding sites. In the present study, the
281 HA-equivalent site density of roots as expressed by E_{HA} for the two plant species
282 tested, i.e., *V. unguiculata* and *P. sativum*, varied by a factor of two. Larger variations
283 are expected when more species are investigated. The total fixed charge of plant roots,
284 which represents the density of sites for proton and metal binding, has been reported
285 to differ by more than one order of magnitude (Grignon and Sentenac, 1991; Wu and
286 Hendershot, 2009). However, the E_{HA} calculated is not species-specific because it
287 strongly depends on the input to WHAM. For example, the use of total metal
288 concentrations in water/soil or free metal activities in water/soil solution may result in
289 different values of E_{HA} because of the varying estimates of metal binding to HA. In
290 addition, the E_{HA} values calculated may reflect the influence of exposure conditions
291 on metal bioavailability. Chemical properties of the exposure hydroponic
292 solutions/soil solution affect the computed metal binding to HA by influencing the
293 amount of metals available for binding to HA and metal-binding characteristics of

294 root surface (Huang et al., 2013; Linderman and Davis, 2004). The former has been
295 well simulated by speciation models (Le and Hendriks, 2014; Groenenberg et al.,
296 2010) while the latter has been hardly considered in metal risk assessment, as
297 exemplified by our assessment of internalized metal concentrations in roots of *L.*
298 *perene*. In this assessment, effects of soil properties on metal mobilisation (Degryse et
299 al., 2009) were accounted for by using the measured free metal ion concentration as
300 inputs to WHAM. Among soil properties, organic matter plays an important role in
301 metal bioavailability in terrestrial systems (Allen and Janssen, 2006). In the
302 assessment based on data provided by Kalis (2006), effects of organic matter were
303 accounted for in terms of the influence on characterisation of the reactive species of
304 metals, but not the sorption of organic matter on biological surfaces (Verhey et al.,
305 2014; Slaveykova et al., 2003) or the potential uptake of complexed metals (Kalis et
306 al., 2006). Besides organic matter, metal bioavailability is influenced by pH. The
307 narrow range of pH set up in the experiments did not allow for detailed investigation
308 on effects of this parameter on root uptake. The variability in the E_{HA} values
309 calculated for *V. unguiculata* and *P. sativum* reflects not only show the differences in
310 the metal-binding characteristics of root surfaces between the species, but also
311 varying exposure conditions. Therefore, the applicability of the E_{HA} to various
312 conditions requires further validation.

313 For some metals, binding to HA modelled may not correlate with the internalized
314 concentration measured in plant roots grown on soil following long-term exposure.
315 The intercept of the relationship between metal binding to HA computed and
316 internalized concentrations in roots for some metals, (e.g., Cu and Pb in the present
317 study), may differ from that of others. The lack of a significant relationship between
318 amounts of Cu and Pb bound to HA and their internalized concentrations may be

319 partly due to the narrow range of concentrations investigated by Kalis (2006). Besides
320 this statistical reason, a number of conditional processes in response to chronic
321 exposure of plants grown in soil to metals may contribute to these results as well as
322 the lower predictive potential of the WHAM-HA model in this assessment than in
323 assessments for hydroponic cultures. For instance, the assumption in the WHAM-HA
324 model for predicting metal bioaccumulation, that the bulk speciation is not affected by
325 root uptake, may not hold in pot experiments because of the high density of roots in
326 pots. In such conditions, root exudation may have considerable effects on metal
327 mobility, and subsequently on root absorption (Degryse et al., 2008). For example,
328 organic acids exuded by roots of *L. perenne* exposed to Cu and Zn (Malinowski et al.,
329 2004; Wei-Hong et al., 2007) can influence metal speciation in soil, thus affecting
330 root uptake. Oxalate and citrate acids exuded following exposure to Mn were reported
331 to reduce the bioavailability of this metal (Mora et al., 2009). Root exudes affect
332 physiochemical properties of the rhizosphere, especially pH and redox potential
333 (Dong et al., 2007). In addition, organic acids in root exudates may chelate or form
334 complexes with ions (Dong et al., 2007; Hinsinger, 2001; Schwab et al., 2005),
335 thereby influencing metal bioavailability to plants in two different ways. On the one
336 hand, this effect of the organic acids on metal speciation reduces the concentration of
337 metals in the reactive species, therefore reducing metal toxicity. This has been
338 demonstrated by the results in the study of Parker et al. (2001) that malonate, malate,
339 and citrate alleviated Zn toxicity to *Triticum aestivum*. On the other hand, the
340 organically complexed metals can be taken up by plants in case of diffusion-limited
341 uptake of free ions, enhancing metal accumulation (Antunes and Hale, 2006; Degryse
342 et al., 2012). This mechanism may explain the major underestimation of internalized
343 concentrations of Cd, Cu, and Ni in roots of *L. perenne*. Malonate and malate have

344 been reported to increase Cu toxicity to *T. aestivum* (Parker et al., 2001), consistent
345 with the findings for other species like algae (Errecalde et al., 1998; Errecalde and
346 Campbell, 2001). Some additional factors should be considered in assessment for
347 chronic exposure. For example, for long-term exposure, plants have developed
348 different extracellular (e.g., root exudation as described above) and intracellular (i.e.,
349 increased binding to cell walls) mechanisms to detoxify metals (Mizra et al., 2014), as
350 is further discussed below.

351 Deviations between measured and modelled concentrations of metals internalized
352 in plant roots by the WHAM-HA model may be related to specific physiological
353 processes either limiting or enhancing metal ion entry to the cytoplasm, that are not
354 accountable for using a geochemical equilibrium approach. One example is
355 homeostasis, i.e., the enhancement of the uptake of essential metals in plants to
356 maintain essential tissue levels (Tsukamoto et al., 2006). This may, for example,
357 explain the tendency to underestimate low root Mn concentrations in the dataset of
358 Kopittke et al. (2011). Other processes that might influence internalized metal
359 concentrations include compartmentalization (i.e., the distribution of metals into cell
360 walls and cell membrane, cytoplasm, vacuoles, mitochondria, and peroxisomes),
361 sequestration in the apoplast, active transport, and uptake regulation (Malecka et al.,
362 2008; Wu and Hendershot, 2010; Millaleo et al., 2010). The specificity of these
363 processes among metals, as reported by Cataldo et al. (1983) and Hart et al. (1998)
364 could not be explained by a geochemical equilibrium approach. The largest deviation
365 as well as the insignificant correlation between the modelled and the measured
366 internalized concentrations of Cu in roots of *L. perenne* suggests an important role of
367 such processes in the internalization of this metal into the roots. Macro elements are
368 usually taken up by plants in higher quantities than trace elements (Stevovic et al.,

369 2010), potentially contributing to a different intercept of the relationship between Ca
370 binding to HA modelled and Ca concentrations measured in roots from the intercepts
371 for micro-elements. This explanation should be examined in further studies because of
372 the narrow range of Ca concentrations investigated. Despite this exception, Ca
373 concentrations modelled with the default binding constant in WHAM and the
374 common HA-equivalent site density of roots were within one order of magnitude of
375 the measurements.

376 The present study suggests that application of the WHAM-HA model to estimate
377 both total and internalized metal concentrations in roots has promise. Binding of
378 metals to active and inert sites occurs in a similar way, i.e., equilibrium reactions
379 between metals and functional groups (Kalis, 2006). With the potential for predicting
380 internalized metal concentrations in roots, WHAM-HA modelling may provide
381 reliable estimates of metals that are available for translocation into the edible parts of
382 plants. In addition, estimation of total root metal concentrations may be a reliable
383 indicator of metal toxicity as metals that are adsorbed to the root surface, but not
384 transported across the membrane, can also be toxic to plants (Campbell et al., 2002).
385 However, the metal-specific performance of the WHAM-HA model indicates that the
386 default set of binding constants in WHAM is not applicable to some metals. For
387 example, the overestimation of Cu accumulation in roots of *P. sativum* and of Pb
388 accumulation in roots of *L. perenne* may be related to the very strong affinity of these
389 metals for HA. Heterogeneity of metal-binding sites is accounted for in the WHAM-
390 HA model, but it does not allow for distinguishing metals bound to different sites,
391 which is important to metal bioavailability (Antunes et al., 2007; Postma et al., 2005;
392 Balistrieri and Mebane, 2014). The contribution of different binding sites, e.g., high-
393 and low-affinity, to total metal bioaccumulation has been reported on different

394 species, including plants (Antunes et al., 2007; Wu and Hendershot, 2009). Moreover,
395 the reactivity of low- or high-affinity binding sites depends on exposure
396 concentrations (Antunes et al., 2007).

397 Similar to the WHAM-HA model, metal binding to sites on sorption surfaces is
398 included in the electrostatic model for predicting metal accumulation in roots
399 (Kopittke et al., 2011; Wang et al., 2013; Kinraide and Yermiyahu, 2007). The model
400 considers the effect of the development of electrical potential at the root plasma
401 membrane surface on ion-binding strengths, and thus metal uptake by plants
402 (Kinraide et al., 1998; Kinraide, 2001). As the electrical potential at the plasma
403 membrane surface is influenced by ionic composition of the exposure solution
404 (Kinraide et al., 1998), the electrostatic model provides a more complete description
405 of the effects of environmental chemistry on metal uptake than would a non-
406 electrostatic model (Le et al., 2014). The WHAM model also takes electrostatic
407 effects on metal binding to humic substances into account. The electrostatic model
408 ($r^2 = 0.84$; Kopittke et al., 2011) was better than the WHAM-HA model ($r^2 = 0.81$;
409 Table 3) in explaining the variation in Mn uptake by roots of *V. unguiculata*. This
410 observation may be related to considerable changes in the electrical potential at the
411 plasma membrane surface. Moreover, further comparison should be implemented in
412 validation with independent data sets to obtain accurate assessment on the predictive
413 potential of these two models.

414 Results obtained with the WHAM-HA and electrostatic models indicate the
415 importance of including organisms in assessment systems, i.e., plasma membrane in
416 the former and HA-surrogate root surface in the latter, in predicting metal
417 bioavailability (Le, 2012). The WHAM-HA model shows promise for explaining
418 metal root uptake at relatively high exposure concentrations, suggesting that it might

419 be a reasonable tool for predicting uptake under contaminated conditions. However,
420 the above-mentioned factors should be considered in interpreting the relationship
421 between the computed metal binding to HA and the measured metal accumulation in
422 roots. Differences in the calculated E_{HA} among species do not necessarily reflect
423 differences in binding site densities only, but may also indicate the existence of non-
424 equilibrium uptake and loss processes. Additionally, the results suggest that
425 applicability of the WHAM-HA model under conditions of low metal uptake needs to
426 be further assessed because of its limited potential for estimating metal accumulation
427 in roots at low exposure levels. Such conditions, while not necessarily of the highest
428 relevance for risks of metal toxicity, are nonetheless important for assessment of
429 essential metal uptake under conditions of deficiency and site-specific assessment
430 when metal concentrations are usually considerably lower than the values in toxicity
431 tests. Overall, systematic studies of metal uptake over the widest possible range of
432 exposure levels and soil chemical conditions are required to test the WHAM-HA
433 approach fully.

434

435 **References**

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Tables

Table 1. Overview of studies used to investigate metal bioaccumulation

Experiments	Species	Metal	n	Exposure concentration (μM)	Exposure duration	pH	Input		Output	Studies
							Competing cation	Metal species		
Hydroponic cultures	<i>Vigna unguiculata</i>	Mn	120	0-1515	48 hours	4-6	Ca, Mg, Na, Al	Total concentration	Total root concentration	Kopittke et al. (2011)
Hydroponic cultures	<i>Pisum sativum</i>	Cu	45	0-25	48 hours	4-6	Ca	Total concentration	Total root concentration	Wu (2007)
		Ni	45	0-50						
		Zn	54	0-140						
		Cd	45	0-65						
Pot (field soil)	<i>Lolium perenne</i>	Cd Ni Pb Cu Zn	50		7 weeks	4-7.1	Al, Fe, Ca, K, Mg, Na	Free metal ion concentration measured in porewater	Internalized concentration	Kalis (2006)

Table 2. The equivalent site density of plant roots compared to the humic acid found in the present study and of other species (E_{HA} , g HA/g DW)

Organisms	Species	E_{HA} (g HA/g DW)	Source
Plant	<i>Vigna unguiculata</i>	0.014	Present study
	<i>Pisum sativum</i>	0.044	
	<i>Lolium perenne</i>	0.26	
Bacteria	<i>Pseudomonas putida</i>	0.30	Tipping and Lofts (Unpublished results)
	<i>Bacillus subtilis</i>	0.66	
	<i>Escherichia coli</i>	0.84	
Alga	<i>Chlorella kesslerii</i>	0.03	Tipping and Lofts (Unpublished results)
Amphipod	<i>Hyalella azteca</i>	0.044 ^a 0.11 ^b	
Mussel	<i>Dreissena polymorpha</i>	0.017	

* Calculated from the value of 0.0034 on the basis of fresh weight; ^aIn laboratory experiments; ^b In field studies.

Table 3. Statistical parameters (including mean absolute error MAE and root mean square error RMSE) comparing total and internalized concentrations in plant roots predicted by the WHAM-HA approach and measured concentrations

Statistical parameters	<i>Vigna unguiculata</i>		<i>Pisum sativum</i>				<i>Lolium perenne</i> *
	Mn	Cu	Ni	Zn	Cd	Ca	Cu, Ni, Zn, Cd, Pb
<i>n</i>	120	45	45	54	45	171	50
<i>p</i>	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	<0.001
<i>r</i> ²	0.81	0.62	0.92	0.80	0.87	0.16	0.022
MAE	0.76	0.58	0.33	0.20	0.37	0.16	0.71
RMSE	0.69	0.70	0.39	0.24	0.43	0.20	0.84

*Exposed to mixtures of Cu, Ni, Zn, Cd, and Pb in pot experiments

1 **Figure captions**

2 **Fig 1.** The relationship between total Mn concentrations in roots of cowpea *Vigna*
3 *unquiculata* measured by Kopittke et al. (2011) and contents of Mn bound humic acid
4 modelled by the WHAM. The line has a slope of unity, and an offset of -2.47,
5 yielding $E_{\text{HA}} = 0.0027$ g/g DW.

6 **Fig 2.** Comparison of the total concentrations of Mn in roots of cowpea *Vigna*
7 *unquiculata* L. measured by Kopittke et al. (2011) and the concentrations of Mn
8 sorbed by roots as predicted by the WHAM-HA model. The solid line represents the
9 1:1 ratio. The dashed lines represent a factor of one order of magnitude variations
10 above and below the 1:1 line.

11 **Fig 3.** The relationship between total metal concentrations in roots of pea *Pisum*
12 *sativum* measured in laboratory experiments by Wu (2007) and contents of metals
13 bound to humic acid (HA) modelled using WHAM. The line has a slope of unity, and
14 an offset of -1.36, yielding $E_{\text{HA}} = 0.044$ g/g DW.

15 **Fig 4.** Comparison of the total concentration of Cu, Ni, Zn, Cd, and Ca in roots of pea
16 *Pisum sativum* L. measured by Wu (2007) and the concentration modelled by the
17 WHAM-HA modelling. The solid line represents the 1:1 ratio. The dashed lines
18 represent a factor of one order of magnitude variations above and below the 1:1 line.

19 **Fig 5.** The relationship between internalized metal concentrations in roots of grass
20 *Lolium perenne* measured in pot experiments by Kalis (2006) and contents of metals
21 bound to humic acid (HA) modelled by WHAM. The line has a slope of unity, and an
22 offset of -0.43, yielding $E_{\text{HA}} = 0.37$ g/g DW.

23 **Fig 6.** Comparison of internalized concentrations of Cu, Cd, Ni, Zn, and Pb in roots of
24 *Lolium perenne* measured by Kalis (2006) and concentrations predicted by the

25 WHAM-HA model. The solid line represents the 1:1 ratio. The dashed lines represent
26 a factor of one order of magnitude variations above and below the 1:1 line.

27
28

Figures

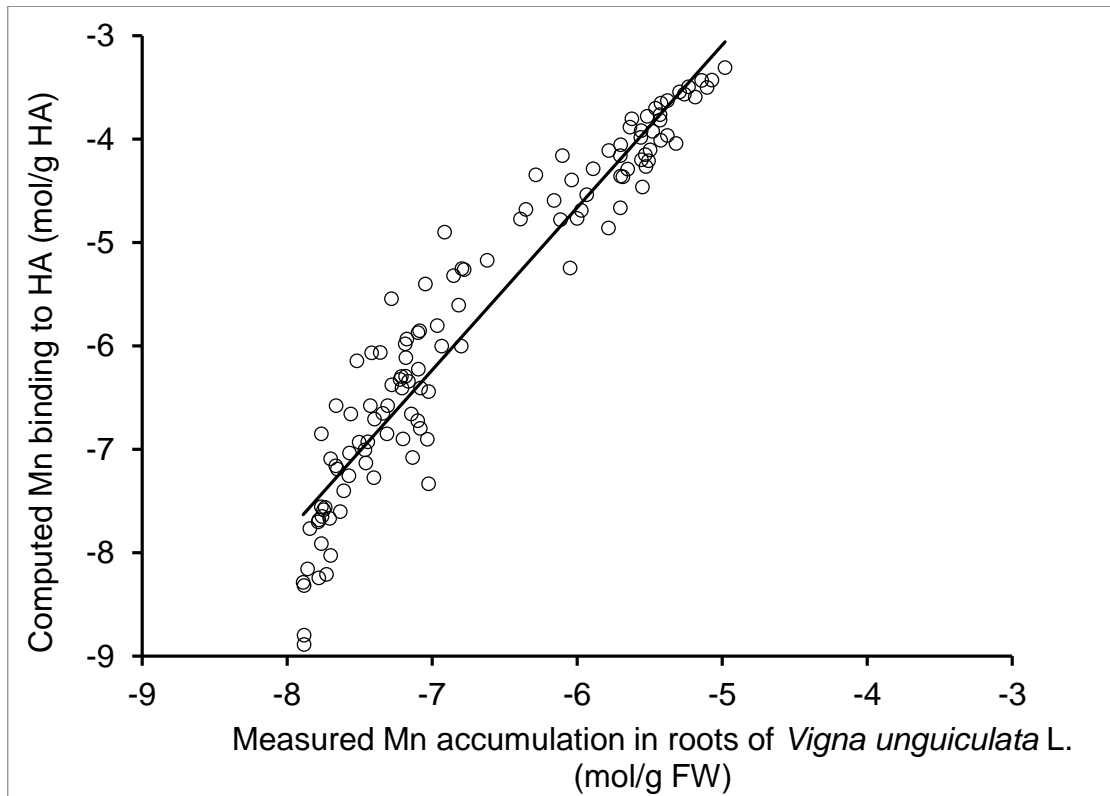


Fig. 1.

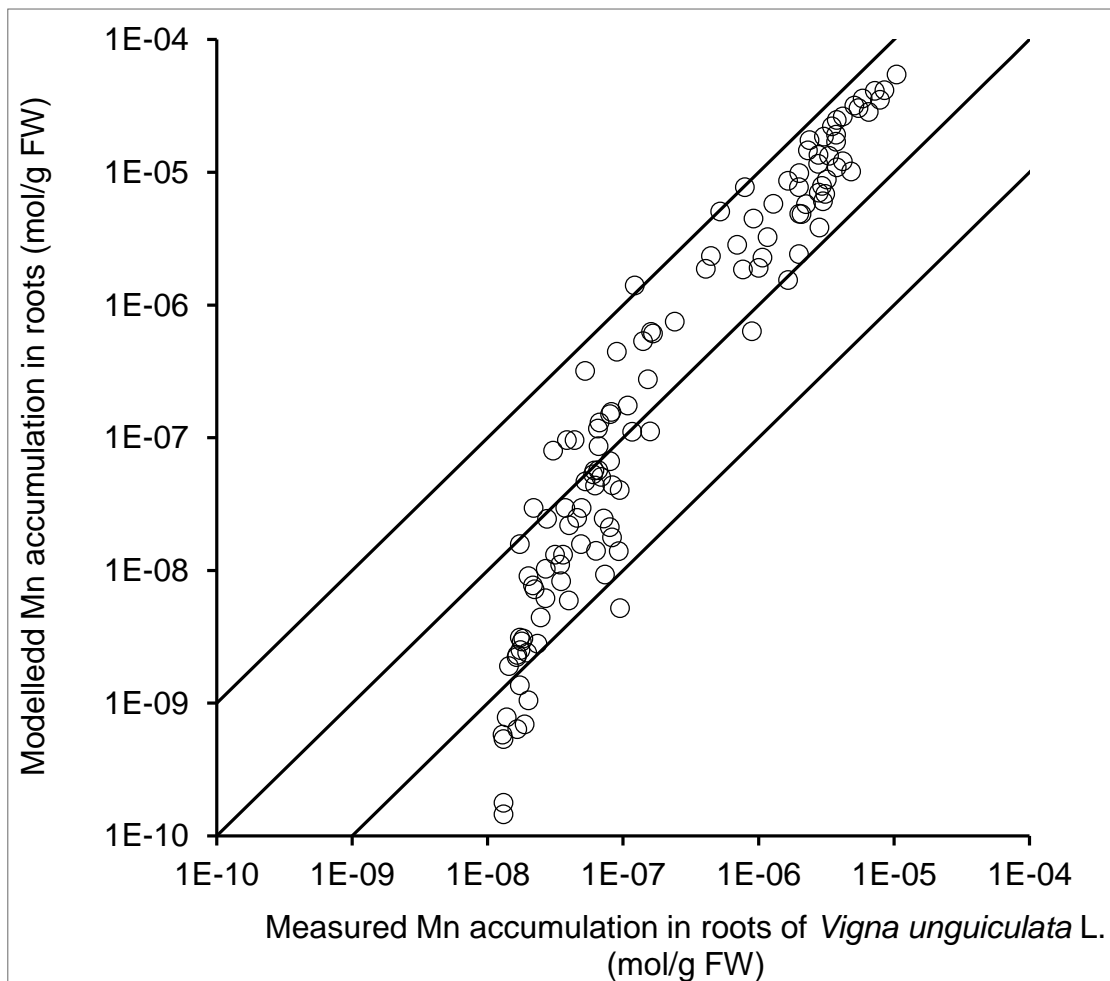


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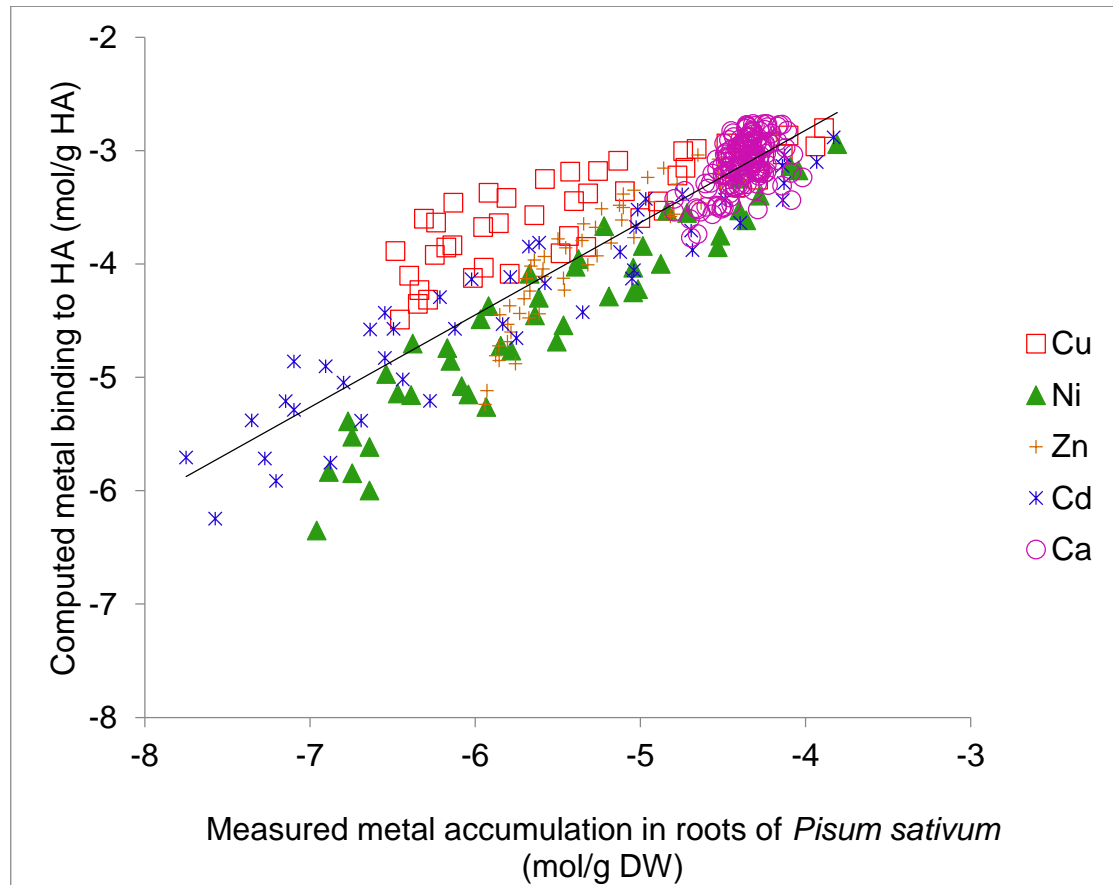
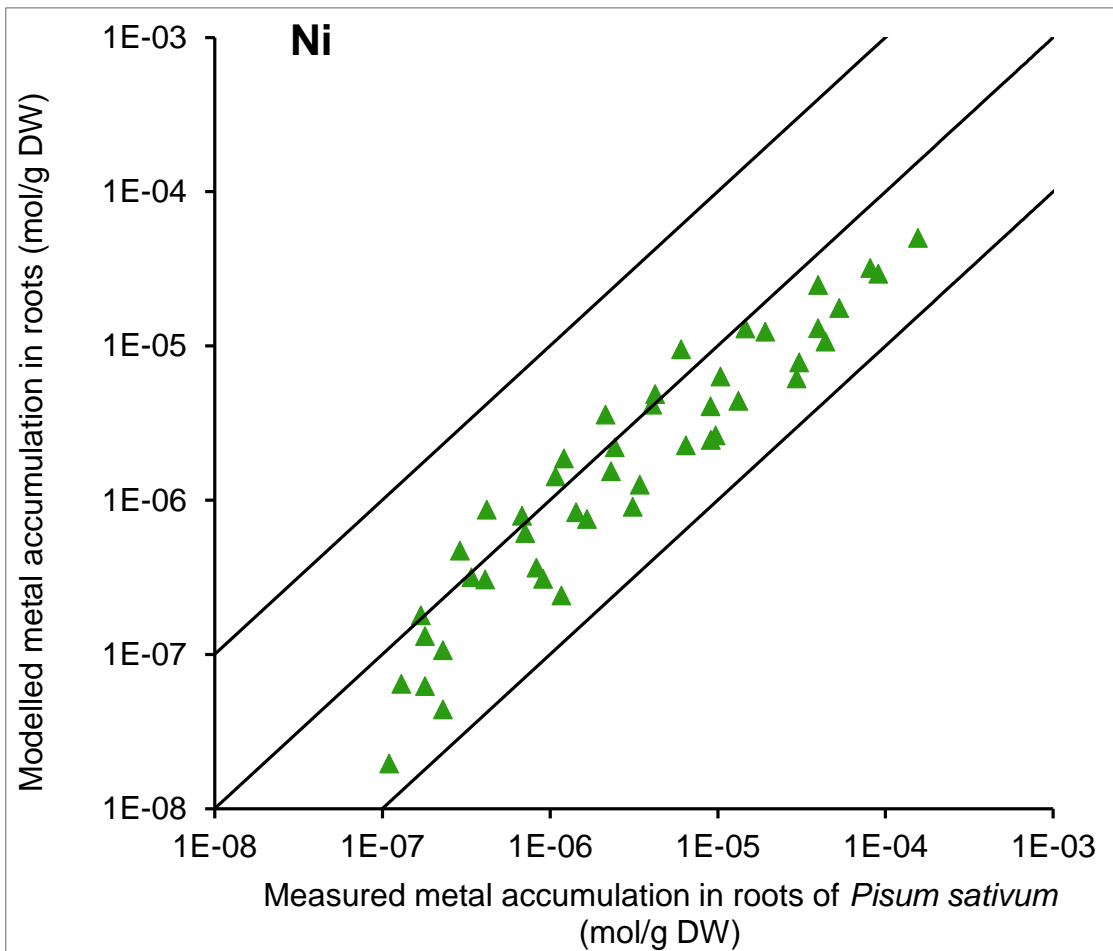
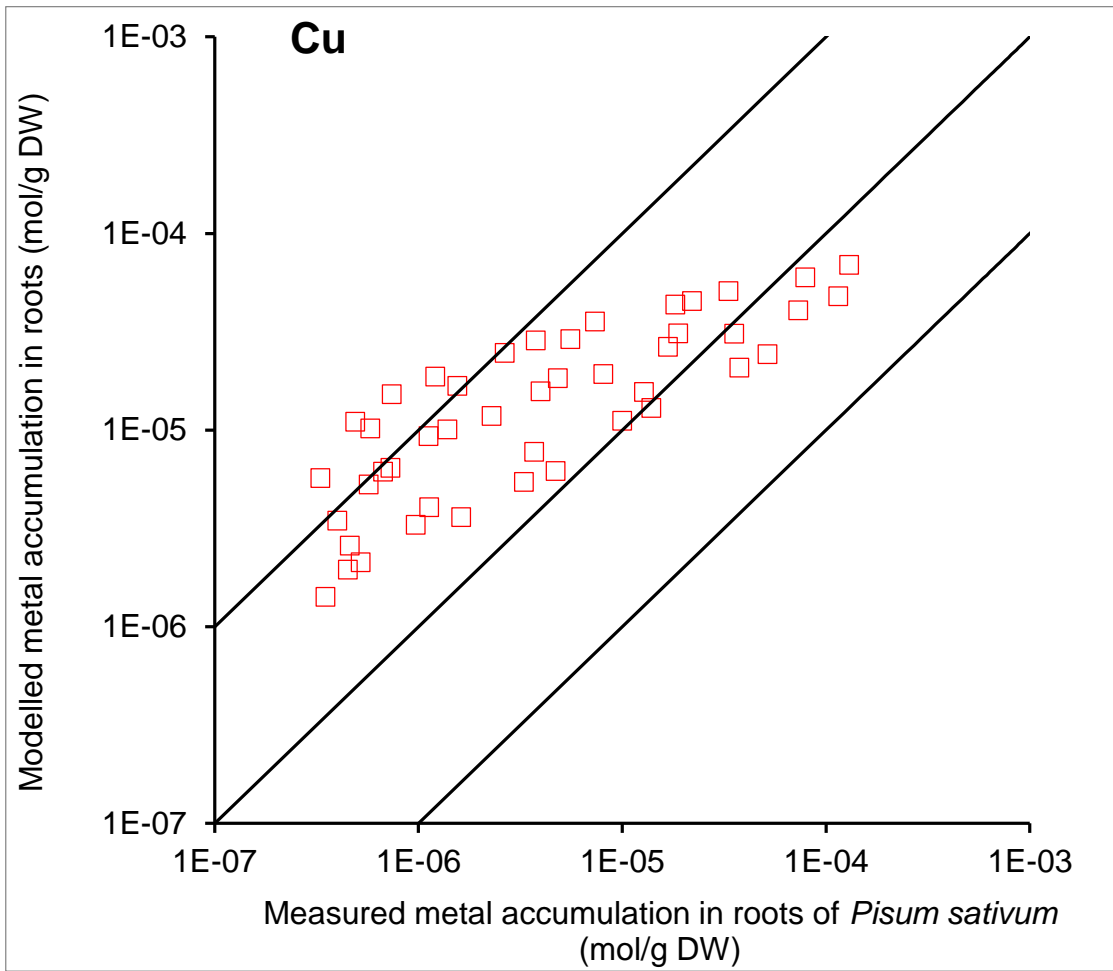
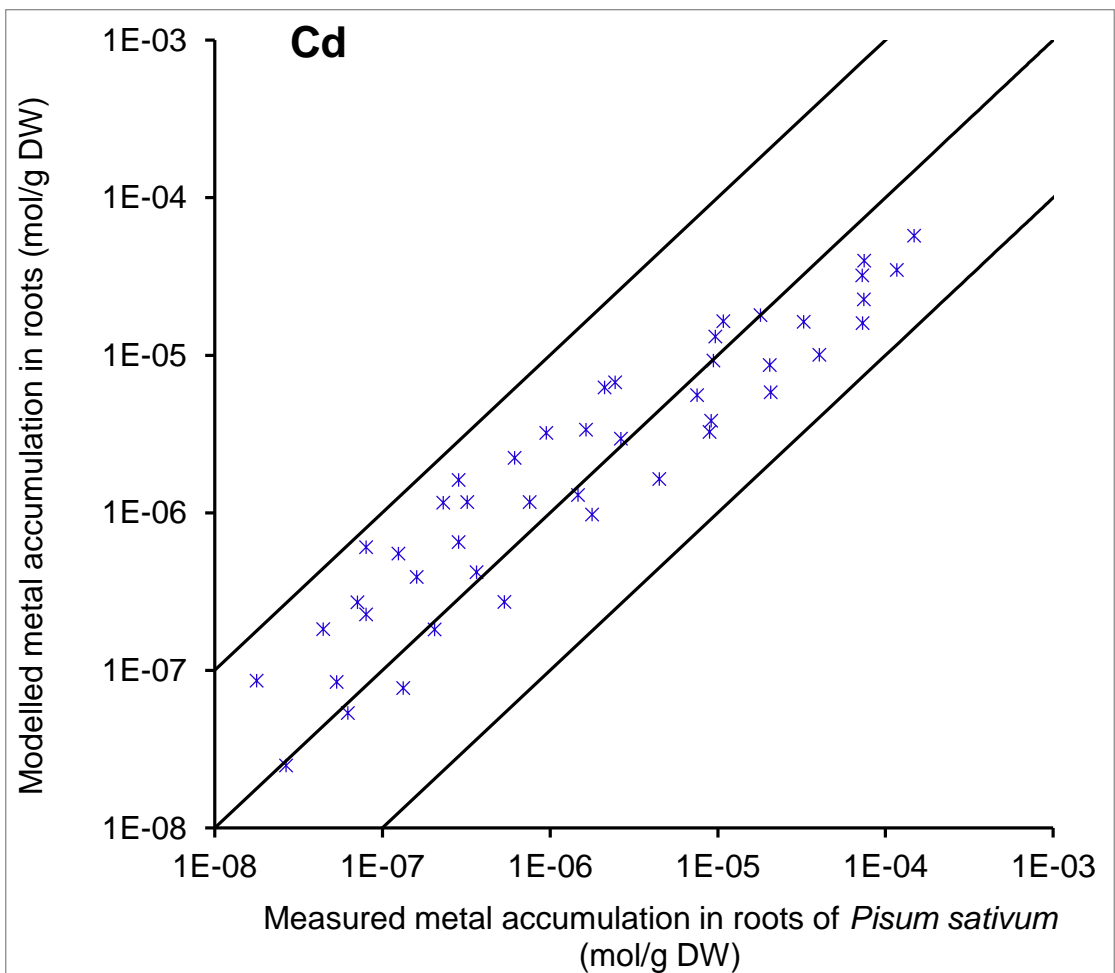
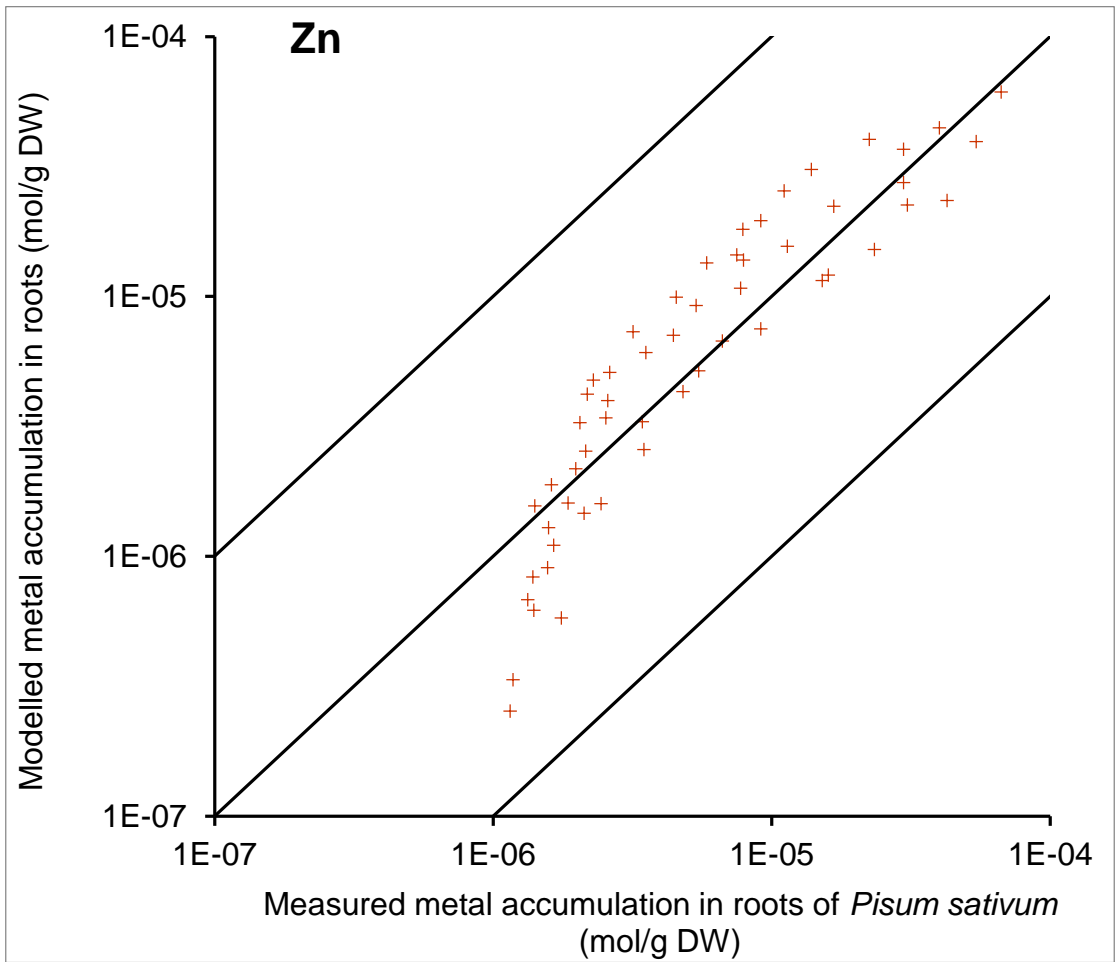


Fig. 3.





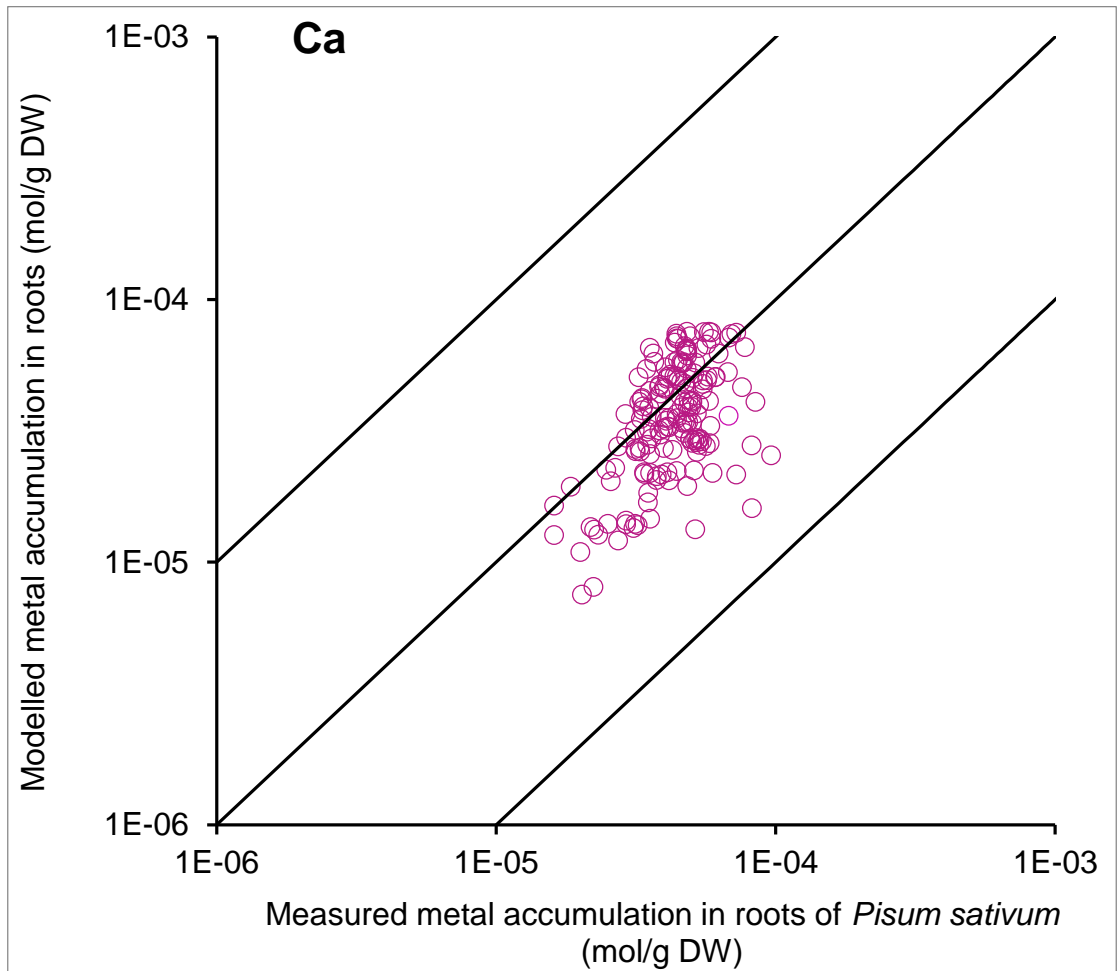


Fig. 4.

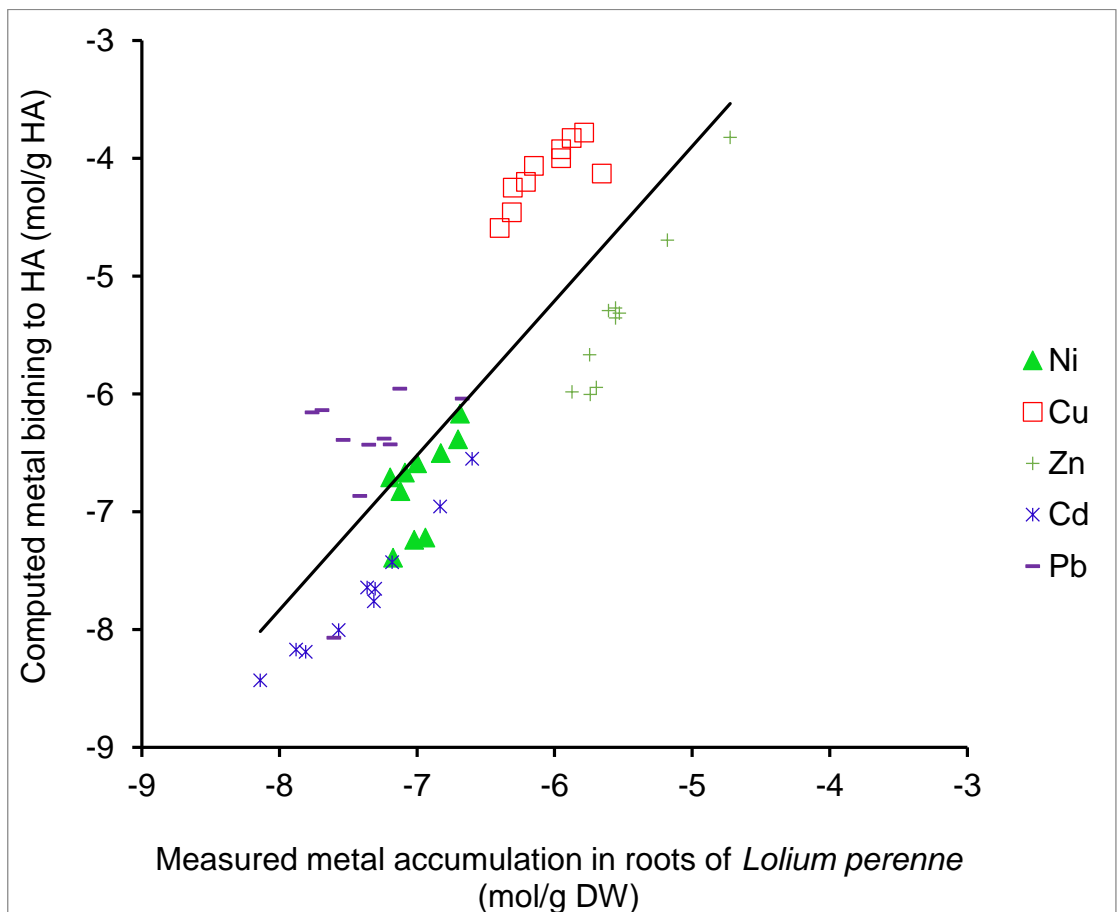


Fig. 5.

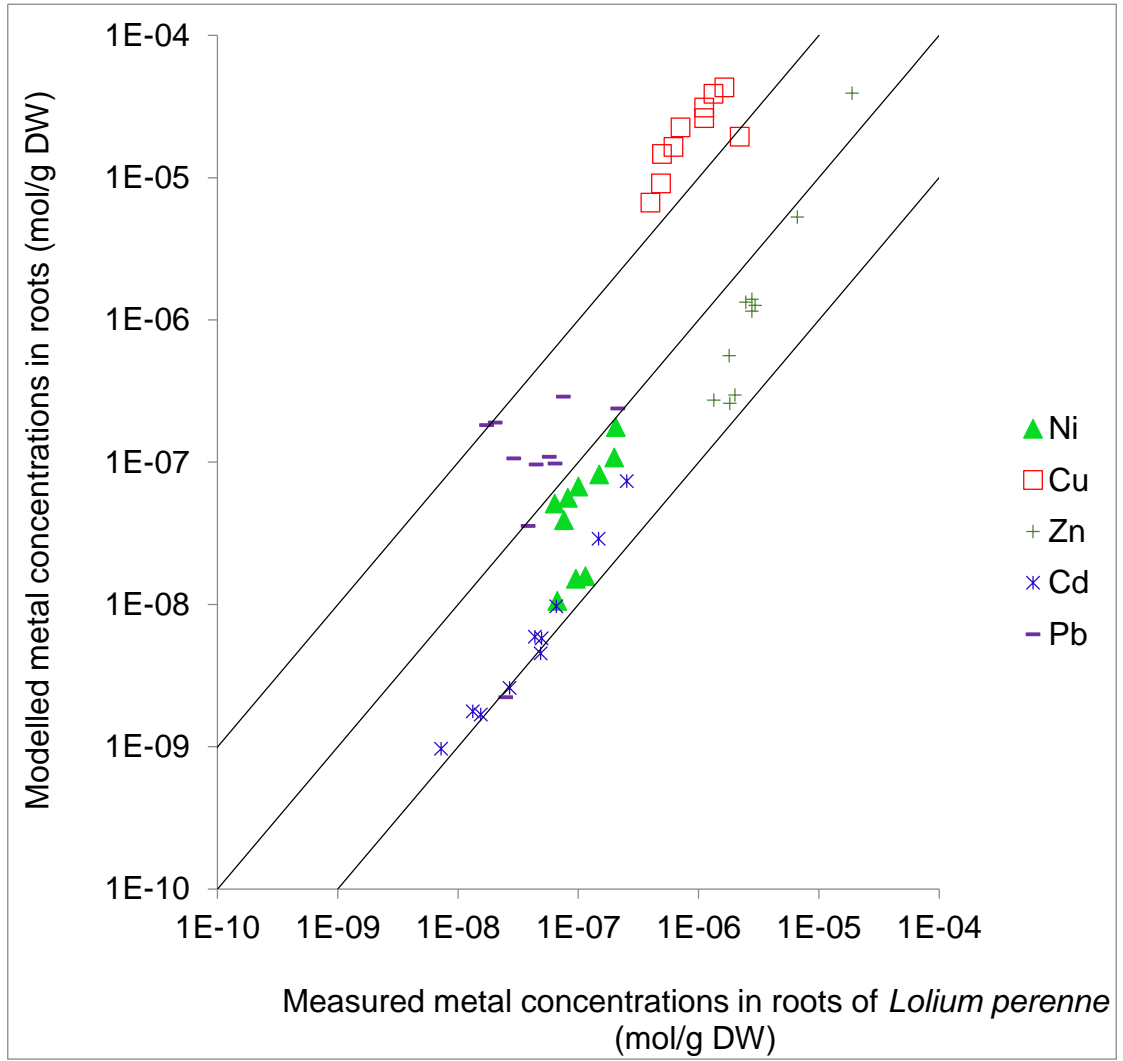


Fig. 6.