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## Prediction of the edaphic factors influence upon the copper and cobalt accumulation in two metallophytes using copper and cobalt speciation in soils

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3

4 **Prediction of the edaphic factors influence upon the copper and cobalt accumulation in**  
5 **two metallophytes using copper and cobalt speciation in soils**

6

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27 **Key-words** chemical speciation, copper, cobalt, hyperaccumulation, metal availability, WHAM 6.

28

29 **Abstract**

30 **Background and Aims** Among the unique flora on copper and cobalt rich soils, some species are able to  
31 hyperaccumulate the Cu and Co in their shoots, however, the unexplained high variations of Cu and Co  
32 concentrations in shoots have been highlighted. A good comprehension of the Cu and Co accumulation  
33 variations would go through a characterization of the Cu and Co speciation in soils. We examined the  
34 covariations of Cu and Co speciation in soils and Cu and Co concentrations in plants.

35 **Methods** Plant samples of two species and soil samples (n=146) were collected in seven pedogeochemically  
36 contrasted sites. Cu and Co speciation in soils was modeled by WHAM 6.0.

37 **Results** Variation in copper accumulation in plant shoots were mostly influenced by Cu adsorbed by the Mn and  
38 Fe oxides fractions, whereas Co accumulation variations were strongly influenced by Co free and Co adsorbed  
39 by the OM and Fe fractions.

40 **Conclusions** Availability of Cu and Co seems to be species-specific and is not explained only by the free Cu and  
41 Co content in the soil solution, but also strongly by the part linked to colloidal fractions. Availability of Cu and  
42 Co is a complex mechanism, closely related to all the biogeochemical processes which occur in the rhizosphere.  
43 Future work should perform experiments in controlled conditions to examine the soil parameters that influence  
44 the Cu and Co availability.  
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48

49 **Abbreviations**

50

51 MnOX = manganese oxides

52 FeOx = iron oxides

53 HM = humic material

54 OM = organic matter

55 -MnOx = bound to manganese oxides

56 -FeOx = bound to iron oxides

57 -OM = bound to organic matter

58 SD = standard deviation

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63

## 64 **Introduction**

65           Soils contaminated by metals represent an important constraint for vegetation. Only a few species  
66 tolerate soil metal concentrations up to 1,000 times higher than the concentration usually found in normal soils  
67 (Ernst 1974; Reeves and Baker 2000). Some of these, called hyperaccumulators, are able to concentrate metal in  
68 their shoot up to extremely high concentrations (Baker 1981; Macnair 2003; Krämer 2010; Rascio and Navari-  
69 Izzo 2011). For two decades, hyperaccumulator species have attracted much attention because of their potential  
70 use as a phytoextraction strategy. According to the recent literature, species would be considered as Cu and Co  
71 hyperaccumulators when the metal concentration in shoots exceeds  $300 \text{ mg.kg}^{-1}$ , without toxicity symptoms and  
72 growth inhibition (Krämer 2010; Van der Ent et al. 2013). Although Macnair (2003) suggests that Cu  
73 hyperaccumulation has not yet been observed in control conditions, Küpper et al. (2009) found that *Crassula*  
74 *helmsii* growing in hydroponic conditions could accumulate more than  $9,000 \text{ mg.kg}^{-1}$  in shoot when the nutrient  
75 solution was enriched with less than  $1 \text{ mg.kg}^{-1} \text{ Cu}^{2+}$ . Only a few species around the world (mostly  
76 Brassicaceae), are considered as constitutive hyperaccumulators. These species show that all populations have  
77 very high foliar metal concentrations irrespective of metal concentration in soil, including *Noccaea caerulescens*  
78 (Dechamps et al. 2007), *Arabidopsis halleri* (Frerot et al. 2010) and *Gomphrena claussenii* (Villafort Carvalho et  
79 al. 2013) (zinc and cadmium hyperaccumulators); as well as several *Alyssum* species (nickel hyperaccumulator)  
80 (Brooks and Radford 1978; Brooks et al. 1979; Broadley et al. 2001; Hanikenne and Nouet 2011). Most of  
81 metallophytes are defined as facultative hyperaccumulators, which means that populations or individuals are  
82 hyperaccumulators and some are not (Pollard et al. 2002).

83           Plants which accumulate Cu and Co in their shoots are rare and most of them occur in Katanga (Dem.  
84 Rep of Congo) (Reeves and Baker 2000; Reeves 2006). In this region of South Central Africa, a species-rich  
85 vegetation is associated to natural outcrops of Cu and Co enriched bedrocks, including cuprophytes (Duvigneaud  
86 1958; Duvigneaud and Denaeyer-De Smet 1963; Ernst 1974; Ernst 1990; Faucon et al. 2012a; Ilunga wa Ilunga  
87 et al. 2013) and cobaltophytes (Duvigneaud 1959; Faucon et al. 2010; Saad et al. 2012; Seleck et al. 2013).  
88 Ecology and evolution of copper and cobalt tolerance and accumulation in this vegetation is still misunderstood,  
89 due to high inter- and intraspecific variations of the Cu and Co shoot concentrations. Unusually high shoot Cu  
90 and Co concentrations reported in earlier studies may result from surface contamination by soil particles and,  
91 therefore, plant materials from metal-rich soils should be washed prior to analysis (Faucon et al. 2007; Van der  
92 Ent et al. 2013). Copper in shoots ranges from 80 to  $1,400 \text{ mg.kg}^{-1}$  and from 330 to  $1,200 \text{ mg.kg}^{-1}$  for  
93 *Crepidiorhapon perennis* and *Anisopappus chinensis*, respectively (Faucon et al. 2007; Faucon et al. 2009a). In

94 the same study, for Co, both species had shoot concentrations that ranged from 61 to 1,105 mg.kg<sup>-1</sup> and from 933  
95 to 1,948 mg.kg<sup>-1</sup>, respectively. Such variations have a genetic origin for Cu, especially due to genetic  
96 differentiations among populations, as demonstrated by cultivation in uniform conditions (Faucon et al. 2012b).  
97 However, high inter- and intra-population phenotypic variations in Cu and Co shoot concentration suggest a  
98 diversity of accumulation responses and an influence of edaphic parameters upon Cu and Co accumulation by  
99 plants. A good understanding of the Cu and Co accumulation variations would go through an accurate  
100 characterization of the soil-root interface properties and mechanisms, controlling metal availability. The  
101 influence of soil upon metal accumulation by plants can be studied in experimental conditions, by using a metal  
102 contamination gradient in the substrate, but this approach has limits and can be criticized due to differences with  
103 the in nature soil properties (e.g. Van der Ent et al. 2013). In such an approach, Cu hyperaccumulation is poorly  
104 expressed (Morrison et al. 1979, 1981; Chipeng et al. 2010; Faucon et al. 2012) and variations could not be  
105 distinguished from substrate differences (Escarre et al. 2013). Another approach could identify the relationship  
106 between metal concentrations in soils and in shoots by studying populations from pedogeochemically contrasted  
107 sites. Ecological studies have commonly used total metal concentrations or extractable metal concentrations  
108 determined at a fixed pH value (AcNH<sub>4</sub>EDTA 1M pH=4.65) (Brun et al. 1998; Faucon et al. 2009). In this way,  
109 pH variations are not considered, while it highly influences metal mobility (Alloway 1995; McLaren and  
110 Crawford 1973; Kabata-Pendias and Pendias 2001; Chaignon et al. 2002; Faucon et al. 2011). It is important to  
111 include soil pH, due to the direct impact of the Cu and Co speciation upon the Cu and Co uptake by plant  
112 (Poschenreider et al. 2001; Krishnamurti and Naidu 2002; Chaignon et al. 2002).

113 By considering soil pH, organic matter content and total concentrations of several elements in soil,  
114 chemical speciation modeling appears to be an interesting tool to investigate the relationships between soil  
115 properties and metal accumulation in plants. Using speciation calculation codes including chemical constants  
116 and equilibrium, these methods can assess metal fractionation in soils. Windermere Humic Aqueous Model  
117 (WHAM) (Tipping 1998; Pourret et al. *submitted*) can be used to calculate metal fractionation, and thus, estimate  
118 the bound to MnOx, FeOx and OM metal concentrations, as well as the free metal concentration (i.e. ionic form)  
119 in a soil sample. To understand Cu and Co accumulation variations, inclusion of the essential soil parameters  
120 controlling Cu and Co mobility in soils is necessary: pH, redox potential, OM quality and quantity, oxides, clays,  
121 sulphides and carbonates (Kabata-Pendias and Pendias 2001). These factors may partly control Cu and Co  
122 availability, by influencing the equilibrium between total and available metal concentration in soils. However,  
123 predicting the availability of metals, especially in contaminated environments, is still very difficult (Hinsinger

124 and Courchesne 2008). Key role of root-induced processes on soil constituents' mobility (Harter and Naidu  
125 2001; Hinsinger 2001; Adriano et al. 2004; Houben and Sonnet 2012) as well as rhizosphere chemistry and  
126 microbiology (Wenzel 2009; Alford et al. 2010) increases the complexity of metal availability. Mobility and  
127 availability of metals at the soil root-interface need to be investigated and to date, there is no universal method to  
128 estimate the metal availability to plants and soil organisms (Nolan et al. 2003). It has however been established  
129 that the metal concentration in the soil solution would be the only fraction directly available for plant uptake  
130 (Fageria et al. 1991; Marschner 1995; Whitehead 2000).

131 In the present study, speciation modeling is used to investigate the relationships between Cu and Co  
132 accumulation by plants and Cu and Co chemical forms in soil. Aims were to (i) examine variations of Cu and Co  
133 speciation in soils and Cu and Co concentrations among plant populations, (ii) determine which edaphic factors  
134 and Cu and Co fractions influence the Cu and Co accumulation in plants, and (iii) compare the responses for two  
135 different species. Several hypotheses can be suggested: (i) variations in the Cu and Co speciation would explain  
136 the Cu and Co shoot concentration variations; (ii) the mobile Cu and Co concentration in soils would explain  
137 accumulation variations.

138

## 139 **Materials and methods**

140

### 141 *Plant populations*

142

143 Two model species have been selected in the study. *Crepidiorhapon tenuis* (S. Moore) Fischer (Linderniaceae)  
144 is a pseudometallophyte, Cu and Co hyperaccumulator species (Faucon et al. 2009). It is an annual species  
145 colonizing recently disturbed mine deposits. *Anisopappus chinensis* (L.) Hook.f. & Arn. (Asteraceae) is also a  
146 pseudometallophyte. It is a short-lived perennial found in relatively closed steppic savanna on stabilised  
147 contaminated substrates (Ilunga wa ilunga et al. 2013; Seleck et al. 2013). Large variations of Cu and Co  
148 concentrations in shoots of this species have already been highlighted (Faucon et al. 2007). Four populations of  
149 both species were selected in different sites, in the Katanga region (Dem. Rep. of Congo), as described in Table  
150 1. Two populations of *C. tenuis* were sampled on the anthropogenically disturbed sites Ruashi (Ru)  
151 (recolonization of mine deposits) and Vallée Karavia (VK) (Cu-Co atmospheric fall-out contamination) and two  
152 naturally occurring populations in the Cu-Co hills locally disturbed by reworked substrate (artisanal mining):  
153 Niamumenda (Nm) and Kalabi (Ka). The four populations of *A. chinensis* have been sampled on two natural Cu-

154 Co undisturbed hills from the Tenke-Fungurume region: Fungurume5 (F5) and Goma2 (G2), and two disturbed  
155 Cu-Co hills in the Lubumbashi region: Mine de l'Etoile (E) and Niamumenda (Nm).

156

### 157 *Sampling and samples preparation*

158

159 In each site, 20 plants (whole shoot) and 20 soil samples from the rhizosphere of each plant (0-15 cm) were  
160 collected. Study populations were carefully delimited and sampling was carried out systematically across the  
161 sites, covering the soil heterogeneity in each site. Plants were collected at the same development stage. Sampling  
162 of *C. tenuis* occurred in April 2006 and 2007 and sampling for *A. chinensis* occurred during March and April  
163 2012. A total of 146 plant and soil samples were considered in this study.

164 After harvesting, plants were carefully brushed (whole shoots), washed with Alconox® 1% in demineralized  
165 water, dried at 60°C for 48 h (Faucon et al. 2007) and weighted. Soil samples were dried at room temperature,  
166 sieved (2 mm) and ground in a mill (RETSCH RM 200).

167

### 168 *Plant and soil analysis*

169

170 The *Crepidiorhopalon tenuis* samples were mineralized by dry ashing in a muffle furnace at 550°C for 12 h. Ash  
171 was dissolved in HCl. The samples were then analyzed by Inductively Coupled Plasma Optical Emission  
172 Spectrometry (ICP-OES) (Varian Vista MPX) to determine Cu-Co concentration in shoot. To determine Cu-Co  
173 concentration in shoot of *A. chinensis* two steps were performed. A mass of shoot were digested using a mixture  
174 of 8 mL HNO<sub>3</sub> and 2 mL HCl (Avula et al. 2010) with a low pressure mineralization (Lavilla et al. 2009).  
175 Vessels containing the mix were placed in a Mars 5 microwave (Microwave Accelerated Reaction System –  
176 CEM corporation, USA) according to the Avula et al. (2010) treatment. Then, Cu and Co concentrations in  
177 samples were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Scientific  
178 XSERIES2). Quantitative analyses were carried out by external calibration (eight points) by using mono- and  
179 multi-element standard solutions (Accu Trace Reference, USA). Indium was used as an internal standard at a  
180 concentration of 100 µg.L<sup>-1</sup> in order to correct for instrumental drift and matrix effects. The measurement bias  
181 for the determination of the concentration of Cu and Co was assessed by the analysis of the SRM1573a certified  
182 reference material (tomato leaves: Gills 1995). Typical uncertainties including all error sources are below ±5%  
183 for Cu and Co. The soil samples pH (water) was determined on a saturated soil-water paste and Organic Matter

184 (OM) content by loss on ignition (500°C for 8 h). Soil chemical analyses were performed by Acme Analytical  
185 Laboratories Ltd. (Vancouver Canada), accredited under ISO 9002. The considered analyzed elements were Co,  
186 Cu, Fe, Mn, Zn, K, Mg, Al, Ca. Soil samples were digested using a strong multi-acid method that dissolves most  
187 minerals. Then, 0.25 g split was heated in HNO<sub>3</sub>-HClO<sub>4</sub>-HF to fuming and taken to dryness. The residue was  
188 further dissolved in HCl and solutions were analyzed using ICP-MS.

189

#### 190 *Chemical speciation calculations*

191

192 WHAM 6 (version 6.0.10) was used to calculate Cu and Co speciation. Predictions for the equilibrium metal  
193 binding by environmental colloids made for the present study were done using the combined WHAM-SCAMP  
194 speciation code. WHAM-SCAMP is able to provide a full description of solid-solution speciation by  
195 incorporating two main codes: (i) the Windermere Humic Aqueous Model (WHAM) to calculate the equilibrium  
196 solution speciation (Tipping 1994), and (ii) the Surface Chemistry Assemblage Model for Particles (SCAMP) to  
197 calculate the binding of protons and metals by natural particulate matter (Lofts and Tipping 1998). The code for  
198 the WHAM model incorporates a number of submodels: Humic Ion-Binding Model 6 and a description of  
199 inorganic solution chemistry, cation exchange by clays, the precipitation of aluminium and iron oxyhydroxides,  
200 and adsorption-desorption of fulvic acids. The SCAMP model consists of three submodels: (i) Humic Ion-  
201 Binding Model 6, (ii) a SCM describing proton and metal binding to oxides (e.g., MnOx or FeOx), and (iii) a  
202 model describing the electrostatic exchange of cations on clays. Three binding phases were examined: MnOx,  
203 FeOx, and Humic Material (HM). The X-Ray diffraction revealed that MnOx and FeOx are respectively  
204 pyrolusite and a mix hematite-goethite. The concentration of HM were derived from the experimental OM  
205 measurements: 50% of the OM measured in the field samples was assumed to be HM, themselves being defined  
206 as 100% fulvic acid. Saturation index and mineral precipitation were not considered; which could be a limitation  
207 of this approach (Pourret et al. *submitted*).

208 Input data for the Cu and Co speciation determination were total concentrations of Cu, Co, Mg, Ca, Mn, Fe, K,  
209 Zn, pH of soils and OM content. A speciation calculation was achieved for the 160 soil samples. In this study,  
210 the term free was used to qualify the Cu and Co modeling output mobile fraction, considered as the ionic  
211 fraction. Sulphates and carbonates were not considered.

212

#### 213 *Statistical analysis*



214

215 Descriptive statistics were performed on total soil analysis and normality of data and homogeneity of variances  
216 were verified. One way ANOVA (Analysis of Variance) tested differences in Cu and Co concentrations in shoots  
217 among populations and differences in Cu and Co fractionation among sites. Significance was defined and  
218 represented as follow: \*\*\*:  $p < 0.001$ , \*\*:  $p < 0.01$ , \*:  $p < 0.05$ , NS=non-significant. Post-hoc multiple comparisons  
219 (Tukey HSD) have been applied to compare populations from each other and Cu-Co fractions resulting from  
220 speciation modeling from each other. The relationship between element concentration in plants and edaphic  
221 factors (concentrations of Cu, Co, Mn, Fe, Mg, Ca, OM (%), pH and the binding phases from speciation  
222 modeling results) were characterized with Pearson correlations.

223

## 224 **Results**

225

### 226 *Cu and Co fractionation in soils*

227

228 Results of Cu and Co fractionation are presented in Table 2. Total Cu and Co concentrations showed differences  
229 between sites ( $F_{(6,147)} = 7.65$ ,  $p < 0.001$  and  $F_{(6,147)} = 14.26$ ,  $p < 0.001$ , respectively). There were significant  
230 differences between Cu fractions for each site (Tab. 2). For Cu, the two largest fractions were Cu bound to OM  
231 (Cu-OM) and to FeOx (Cu-FeOx). The free Cu concentration varied from 0.7% to 18.8% of the total Cu  
232 concentration. Copper was mainly bound to FeOx and OM. Significant differences for each Cu fraction existed  
233 between sites (Tab. 2). The highest concentrations of Cu-FeOx were found in Nm and E (69% of total Cu) where  
234 mean total Fe concentration reached 48,500 mg.kg<sup>-1</sup> and 42,300 mg.kg<sup>-1</sup>, respectively. Samples from VK were  
235 characterized by low total Fe and Mn contents (respectively mean = 19,610 ± 9,654 mg.kg<sup>-1</sup>; and mean = 65 ± 37  
236 mg.kg<sup>-1</sup>) and high OM content (mean = 9.38% ± 4.2%). At this site, the fraction of Cu bound to FeOx was the  
237 lowest while the fraction of Cu bound to OM was the highest (respectively mean = 15% and 81.8% of total Cu).  
238 There were significant differences between Co fractions for each site (except samples from E) (Tab. 2). Cobalt  
239 was predominantly free and bound to MnOx (Co-MnOx) in soils. The free Co fraction was higher than the free  
240 Cu one for each site (meanly from 28.1 to 69.4% of total Co). Significant differences for each Co fraction  
241 existed between sites. Nm was characterized by the highest Mn content in soil (mean = 5,700 mg.kg<sup>-1</sup>). At this  
242 site, the Co-MnOx concentration was the highest (mean = 71.1% of total Co). At VK, site where Mn

243 concentration was the lowest, Co was mainly bound to OM (meanly 53.8% of total Co). At this site, the Co-  
244 MnOx concentration was the lowest (mean = 5.8% of total Co).

245

#### 246 *Cu and Co accumulation in plants*

247

248 Results of Cu and Co concentrations in shoots showed large variations between species and populations  
249 (Fig. 1). For Cu, 22 of 79 samples of *A. chinensis* and 25 of 67 samples of *C. tenuis* accumulated more than 300  
250 mg.kg<sup>-1</sup> (range: 4 - 2,821 mg.kg<sup>-1</sup> and 34 - 2,524 mg.kg<sup>-1</sup> for *A. chinensis* and *C. tenuis*, respectively). For Co, 26  
251 of 79 samples of *A. chinensis* and 2 of 67 samples of *C. tenuis* accumulated more than 300 mg.kg<sup>-1</sup> (range: 3 -  
252 1,334 mg.kg<sup>-1</sup> and 8 - 605 mg.kg<sup>-1</sup> for *A. chinensis* and *C. tenuis*, respectively).

253 Large Cu and Co accumulation variations existed among populations of both species (for Cu,  $F_{(7,145)} =$   
254 7.1,  $p < 0.001$ ; for Co,  $F_{(7,145)} = 17.6$ ,  $p < 0.001$ ). Population F5 of *A. chinensis* had the highest Co shoot  
255 concentration (mean = 1,089 ± 768 mg.kg<sup>-1</sup>) ( $F_{(7,145)} = 19.2$ ,  $p < 0.001$ ). Strikingly low concentrations of Co in  
256 plant shoots (<20 mg.kg<sup>-1</sup>) were found in the Nm site for both species. On the contrary, that site yielded the  
257 highest shoot Cu accumulation for both species (*A. chinensis*, range: 104 - 1,335 mg.kg<sup>-1</sup>, mean = 455 mg.kg<sup>-1</sup>  
258 and *C. tenuis*, range: 84 - 2,525 mg.kg<sup>-1</sup>, mean = 673 mg.kg<sup>-1</sup>) ( $F_{(7,145)} = 125.4$ ,  $p < 0.001$ ).

259

#### 260 *Relationships between edaphic factors and Cu and Co accumulation in shoot*

261

262 Correlations between soil fractions and shoot accumulation were element- and species- dependent (Tab.  
263 3, Fig. 2), however, some general patterns emerged. Correlations between total Cu soil content and shoot Cu  
264 concentration were positive for *C. tenuis* ( $r = 0.51$ ,  $p < 0.001$ ) and non-significant for *A. chinensis* (Fig. 2a). Total  
265 Co soil content was positively correlated with shoot Co concentration for *A. chinensis* and *C. tenuis* ( $r = 0.77$ ,  
266  $p < 0.001$  and  $r = 0.45$ ,  $p < 0.001$ , respectively) (Fig. 2b). No correlations occurred between pH and shoot Co  
267 concentration for both species. On the contrary, pH was positively correlated with Cu concentration in shoots for  
268 *C. tenuis* ( $r = 0.40$ ,  $p < 0.001$ ) but not significant for *A. chinensis*. Organic matter soil content was negatively  
269 correlated with shoot Cu concentration for both species. Positive correlation have been observed between OM  
270 soil content and shoot Co concentration for *A. chinensis* ( $r = 0.57$ ,  $p < 0.001$ ) but not significant for *C. tenuis*. Shoot  
271 Cu concentration was correlated with total Mn soil content for *A. chinensis* and *C. tenuis* ( $r = 0.51$ ,  $p < 0.001$  and  
272  $r = 0.65$ ,  $p < 0.001$ , respectively). However, the strong negative correlation between total Mn soil content and shoot

273 Co concentration for *A. chinensis* ( $r = -0.65$ ,  $p < 0.001$ ) was not verified for *C. tenuis*. A positive correlation was  
274 established between total Fe soil content and shoot Cu concentration for *A. chinensis* ( $r = 0.42$ ,  $p < 0.001$ ), but not  
275 for *C. tenuis*. A negative correlation occurred between total Fe soil content and Co concentration in shoot for *A.*  
276 *chinensis* ( $r = 0.5$ ,  $p < 0.001$ ). This relationship was not verified for *C. tenuis*.

277 A positive correlation was observed between free Cu concentration and shoot Cu concentration for *C.*  
278 *tenuis* ( $r = 0.50$ ,  $p < 0.001$ ), but not for *A. chinensis* ( $r = 0.49$ ,  $p < 0.05$ ) (Fig. 2c). Free Co concentration was  
279 positively correlated with shoot Co concentration for *A. chinensis* and *C. tenuis* ( $r = 0.8$ ,  $p < 0.001$  and  $r = 0.57$ ,  
280  $p < 0.001$ , respectively) (Fig. 2d). For Cu, the highest correlation was observed between Cu bound to MnOx (Cu-  
281 MnOx) soil concentration and shoot Cu concentration for *A. chinensis* and *C. tenuis* ( $r = 0.39$ ,  $p < 0.001$  and  
282  $r = 0.76$ ,  $p < 0.001$ , respectively) (Fig. 2e). However, Cu-FeOx soil concentration was also positively correlated  
283 with shoot Cu concentration for both species. This latter concentration was negatively correlated with shoot Co  
284 concentration for *A. chinensis*. For Co, the highest correlation was observed between Co bound to OM (Co-OM)  
285 soil concentration and shoot Co concentration for *A. chinensis* and *C. tenuis* ( $r = 0.9$ ,  $p < 0.001$  and  $r = 0.64$ ,  
286  $p < 0.001$ , respectively) (Fig. 2f). A positive correlation was also observed between Co bound to FeOx (Co-FeOx)  
287 soil concentration and shoot Co concentration for both species.

288

## 289 **Discussion**

290

### 291 *Cu and Co speciation in soils*

292

293 Copper appeared to have a strong affinity for FeOx and OM which would explain the low free Cu  
294 concentrations in soils compared to other Cu fractions. In normal soils, copper co-adsorbs with Mn and Fe  
295 oxides (McLaren and Crawford 1973). Copper was fixed by OM when total Fe content was low (VK). An  
296 increase in the total Fe content (E, Nm, Ka) resulted in an increase of the Cu-FeOx concentration. This suggests  
297 that Fe oxides are potentially major competitors of OM for Cu speciation.

298 Our results showed that cobalt had a particular affinity with Mn oxides (Tab. 2). This is in agreement  
299 with the literature which highlights that cobalt is adsorbed at the surface of Mn oxides (Childs 1975; Li et al.  
300 2004; Tontgavee et al. 2005; Luo et al. 2010). Sites with the lowest Mn soil content exhibited major Co free and  
301 Co-OM fractions, suggesting an important role of Mn in the Co speciation.

302 Samples from VK, with a high OM content compared to other sites and the lowest total Mn  
303 concentration in soils showed the highest Co-OM concentration. Organic matter appears to be a major  
304 competitor of Mn oxides for Co speciation (Collins and Kinsela 2010, Collins and Kinsela 2011). Free Co  
305 concentration is higher, in percentage, than free Cu concentration, relative to the other Co or Cu fractions (Tab.  
306 2). This could be explained by the stronger capacity to complex with colloids for Cu, compared to Co. Indeed,  
307 the sequence of complex stability of transition metals, known as the Irving Williams series, is typically  
308  $\text{Co}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+} < \text{Zn}^{2+}$  (Stumm and Morgan 1996). Organic matter content seems to have a strong influence upon  
309 metal fractionation, which confirms results obtained by Pourret et al. (2007). It can be assumed that mobile and  
310 non-mobile Cu and Co fractions vary among sites and seem to be closely related to the MnOx and FeOx  
311 concentrations, the OM content and the soil pH. High variability of Cu and Co speciation in soils has been  
312 observed, which would involve variations of the Cu and Co availability.

313

#### 314 *Variations of Cu and Co concentrations in shoots among populations*

315

316 Results confirm that *A. chinensis* and *C. tenuis* are facultative Cu and Co hyperaccumulators (Faucon et  
317 al. 2009), characterized by a high variation of Cu and Co shoot concentrations. For Cu and Co respectively, 17%  
318 and 21% of plants of *A. chinensis* and 17% and 2% of plants of *C. tenuis* have been recorded with a  
319 concentration in shoots of up to  $300 \text{ mg.kg}^{-1}$ , i.e. the hyperaccumulation threshold (Van der Ent et al. 2013),  
320 without showing any toxicity symptoms or growth inhibition.

321 The phenotypic accumulation variations would be associated with specific soil conditions.  
322 Interpopulation variations of Cu and Co accumulation in plant shoots would not only be explained by variations  
323 of the total and free Cu and Co soil content. The variation of edaphic factors, and especially the OM and total Fe  
324 content, could explain the Cu accumulation variability among populations of both species. The high Cu-OM and  
325 Cu-FeOx concentration seem to limit the Cu uptake in populations of both species (Fig. 1, Tab. 2). For Co, the  
326 high Co-MnOx concentration at Nm compared to the other Co fractions could explain the lowest Co  
327 accumulation observed for the two species (Fig. 1, Tab. 2). In this context, variations of Cu and Co accumulation  
328 among populations of *A. chinensis* and *C. tenuis* would correspond, not only to the genetic capacity to  
329 accumulate metal in shoots (Faucon et al. 2012), but also in part to edaphic context variations, especially Cu and  
330 Co speciation variations in the rhizosphere. Results showed the importance of relating Cu and Co concentrations

331 in shoots with Cu and Co fractions in soils, to explain the Cu and Co shoot concentration variations observed *in*  
332 *natura*.

333

334 *Relationships between Cu and Co speciation variations in soils and Cu and Co shoot accumulation variations*

335

336 Results confirm the strong synergistic influence of soil total Mn content on Cu accumulation in cuprophytes  
337 and its antagonist effect on Co accumulation as shown by Faucon et al. (2009). Interestingly, total Fe content in  
338 soils seems to be a positive predictor of Cu accumulation for *A. chinensis*, the opposite effect seen in regrass, a  
339 metal non-tolerant species (Li et al. 2004). The Cu mobility in soil is known to be negatively correlated with pH  
340 (Kabata-Pendias and Pendias 2001). Therefore, the positive correlation between Cu accumulation in *C. tenuis*  
341 and soil pH in this study may be surprising. However, the range in pH values in our study is relatively restricted  
342 for *C. tenuis* soils (from 4.4 to 6.2) and it also appears that the correlation is driven mostly by one site (VK)  
343 which has relatively low pH and relatively low Cu accumulation in plants.

344 The differences of Cu and Co accumulation in shoot plants with respect to the variability of Cu and Co  
345 speciation in soils and following edaphic factors, pH, OM, total Mn and total Fe contents exist between both  
346 species. These differences may also suggest species-specific mechanisms of the Cu and Co availability, uptake  
347 and transport in the plant, and confirms the complexity of soil-plant processes (Hinsinger and Courchesne 2008).

348 The results showed the influence of Cu and Co speciation on the variability of Cu and Co accumulation in  
349 both species (Tab. 3). Copper accumulation in plant shoots were positively influenced by Cu-MnOx and Cu-  
350 FeOx concentrations, whereas Co accumulation variations were strongly influenced by Co free and Co-OM and  
351 Co-FeOx concentrations. The Co-OM soil fraction seems to appear to be the most available Co fraction, in those  
352 soils for both species. Similarly, both Li et al. (2004) and McLaren et al. (1987) highlighted that organically-  
353 bound Co influenced Co availability to plants. Availability of Cu and Co does not seem to be strictly a result of  
354 the free Cu and Co concentrations in soils. Even if ionic species metal concentrations in soils would appear, by  
355 hypothesis, to be a good predictor of metal uptake concentration by plants, because of their direct availability  
356 (Fageria et al. 1991; Marschner 1995; Whitehead 2000), the bound to solid-phase fractions have to be considered  
357 for availability assessment. Indeed, some plants are able to mobilize and take up elements from the non-mobile  
358 solid fraction (Knight et al. 1997; Hinsinger et al. 2005). These significant influences of the solid phases upon  
359 the accumulation for both species could be explained by root-induced processes which could be responsible to  
360 pH changes (Marschner 1995; Hinsinger et al. 2003). Indeed, the soil solution acidification by root activity, may

361 causes desorption of metals (Marschner 1995; Harter and Naidu 2001; Hinsinger et al. 2003) and thereby,  
362 increase the ionic form metal concentration. This study confirms that the availability of metals is closely related  
363 to all the biogeochemical processes which occur in the rhizosphere. Estimate of the available metal concentration  
364 in soils seem to be complex but the present study is the first attempt of Cu and Co availability assessment in  
365 metalliferous soils. Undertaking a laboratory experiment which simulates the field conditions would help unravel  
366 some of this complexity. For this, both species could be cultivated in growth-chamber, on homogeneous soil  
367 contaminated by defined Cu and Co concentrations, in which chemical factors influencing Cu and Co  
368 availability (pH, OM, MnOx, FeOx) would vary in controlled conditions (Chaignon and Hinsinger 2003).  
369 However, as microbial activities would also influence the availability of chemical elements in soil (Hinsinger et  
370 al. 2005) and the patterns of metal accumulation in plant species (Fomina et al. 2005; Toler et al. 2005; Barzanti  
371 et al. 2007; Kabagale et al. 2010) incorporating microbial activities in an experimental setting to test their effect  
372 on the variation of Cu and Co accumulation in plants would prove fruitful. Perspectives would be to integrate the  
373 study of influence of microbial activities on the variation of Cu and Co accumulation in plants.

374 Comprehension of the Cu and Co accumulation variations in plant shoots *in situ* and methods of  
375 phytoremediation of Cu and Co contaminated soils need therefore to be reassessed in the light of the present  
376 results.

377

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631 **Table 1 Location and habitat description of study sites**

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633 **Table 2 Cu/Co mean fractionation among seven sites (%) according to the total mean Cu/Co**  
634 **concentration in soil (mg.kg<sup>-1</sup>)**

635 F5: n= 20; G2: n =20; E: n=20; Nm: n=35; Ka: n=17; VK: n=16; Ru: n=18. \*\*\*: p<0.001, \*\*: p<0.01, \*: p<0.05,  
636 NS=non-significant. For columns, variables with the same letter above are not significantly different. For lines,  
637 Cu and Co fractions (considered separately) with the same letter below are not significantly different (results of  
638 post-hoc multiple comparison, Tukey HSD test). Free = ionic form, -MnOx = bound to manganese oxides, -  
639 FeOx = bound to iron oxides, -OM = bound to organic matter, SD= standard deviation

640

641 **Table 3 Correlation between soil factors and Cu and Co concentrations in shoots of *Anisopappus chinensis***  
642 **(n = 79) and *Crepidorhopalon tenuis* (n = 67): Pearson correlation coefficients using log-transformed data**

643 \*\*\*: p<0.001, \*\*: p<0.01, \*: p<0.05, NS=non-significant.

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691 **Table 1**

Site	Habitat description	Altitude (m)	Coordinates (GCSWGS84 DD)	Species sampled
Fungurume 5 (F5)	Natural Cu-Co hill not disturbed by mining. Sampled on grassland.	1,300	S 10,61777 E 26, 29112	<i>A. chinensis</i>
Goma 2 (G2)	Natural Cu-Co hill locally disturbed by mining. Sampled on grassland.	1,300	S 10,59966 E 26,13894	<i>A. chinensis</i>
Etoile (E)	Former natural Cu-Co hill completely disturbed by mining. Open pit. Sampled on remaining substratum.	1,280	S 11,63562 E 27,58449	<i>A. chinensis</i>
Niamumenda (Nm)	Natural Cu-Co hill locally disturbed by mining. Sampled on grassland locally disturbed.	1,340	S 11,60492 E 27,29400	<i>A. chinensis</i> <i>C. tenuis</i>
Kalabi (Ka)	Natural Cu-Co hill locally disturbed by mining. Open pit. Sampled on grassland locally disturbed.	1,200	S 10,78168 E 26,74053	<i>C. tenuis</i>
Vallée Karavia (VK)	Anthropogenic site: soil contaminated by atmospheric fallout from ore-smelter, moist.	1,230	S 11,67270 E 27,43091	<i>C. tenuis</i>
Ruashi (Ru)	Anthropic site: recolonization of Mine deposits. Sampled on remaining substratum.	1,300	S 11,62645 E 27,56328	<i>C. tenuis</i>

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**Table 2**

Sites	Cu						Co					
	Total (mg.kg <sup>-1</sup> )	Free (%)	-MnOx (%)	-FeOx (%)	-OM (%)	One-way ANOVA (among Cu fractions)	Total (mg.kg <sup>-1</sup> )	Free (%)	-MnOx (%)	-FeOx (%)	-OM (%)	One-way ANOVA (among Co fractions)
<b>F5</b> (SD)	18 298 <sup>a,b</sup> (13 285)	14 <sup>b</sup> <sub>a,b</sub>	0.3 <sup>b</sup> <sub>b</sub>	45.7 <sup>a,c</sup> <sub>a</sub>	39.9 <sup>a</sup> <sub>a</sub>	$F_{(3,75)} = 5.775$ *	3 451 <sup>a</sup> (2 614)	62.2 <sup>a</sup> <sub>a</sub>	11.3 <sup>c</sup> <sub>b</sub>	13.7 <sup>a,b</sup> <sub>b</sub>	12.8 <sup>a</sup> <sub>b</sub>	$F_{(3,75)} = 12.31$ ***
<b>G2</b> (SD)	27 793 <sup>b</sup> (17 117)	18.8 <sup>a</sup> <sub>a</sub>	1.1 <sup>b</sup> <sub>b</sub>	52.7 <sup>b,c</sup> <sub>a</sub>	27.4 <sup>a</sup> <sub>a</sub>	$F_{(3,79)} = 9.023$ ***	714 <sup>b</sup> (247)	61.2 <sup>b</sup> <sub>a</sub>	27.7 <sup>b,c</sup> <sub>b</sub>	4.5 <sup>b</sup> <sub>c</sub>	6.6 <sup>b,c</sup> <sub>c</sub>	$F_{(3,79)} = 38.87$ ***
<b>E</b> (SD)	12 566 <sup>a</sup> (12 502)	2.6 <sup>b</sup> <sub>a</sub>	0.4 <sup>b</sup> <sub>a</sub>	69.2 <sup>b,c</sup> <sub>b</sub>	27.8 <sup>b</sup> <sub>a</sub>	$F_{(3,79)} = 16.11$ ***	2 732 <sup>a</sup> (3 336)	28 <sup>a,b</sup> <sub>a</sub>	33.4 <sup>a</sup> <sub>a</sub>	30 <sup>a</sup> <sub>a</sub>	8.6 <sup>c</sup> <sub>a</sub>	$F_{(3,79)} = 2.027$ NS
<b>Nm</b> (SD)	20 833 <sup>a,b</sup> (17 899)	5.5 <sup>b</sup> <sub>a</sub>	4.8 <sup>a</sup> <sub>a</sub>	69.1 <sup>b</sup> <sub>b</sub>	20.6 <sup>b</sup> <sub>a</sub>	$F_{(3,136)} = 33.4$ ***	168 <sup>b</sup> (54)	23 <sup>b</sup> <sub>b</sub>	71.1 <sup>b,c</sup> <sub>c</sub>	3.3 <sup>b</sup> <sub>a</sub>	2.6 <sup>b</sup> <sub>a</sub>	$F_{(3,136)} = 97.02$ ***
<b>Ka</b> (SD)	18 624 <sup>a,b</sup> (13 392)	15.7 <sup>b</sup> <sub>a</sub>	0.8 <sup>b</sup> <sub>a</sub>	58.7 <sup>b,c</sup> <sub>b</sub>	24.9 <sup>b</sup> <sub>a</sub>	$F_{(3,71)} = 12.55$ ***	911 <sup>b</sup> (809)	69.4 <sup>b</sup> <sub>a</sub>	16.4 <sup>b,c</sup> <sub>b</sub>	4.8 <sup>b</sup> <sub>b</sub>	9.4 <sup>b,c</sup> <sub>b</sub>	$F_{(3,71)} = 16.47$ ***
<b>VK</b> (SD)	5 444 <sup>c</sup> (3 315)	3.1 <sup>b</sup> <sub>a</sub>	0.1 <sup>b</sup> <sub>a</sub>	15 <sup>a</sup> <sub>a</sub>	81.9 <sup>a,b</sup> <sub>b</sub>	$F_{(3,67)} = 38.62$ ***	79 <sup>b</sup> (39)	39.3 <sup>b</sup> <sub>a</sub>	5.8 <sup>b</sup> <sub>b</sub>	1 <sup>b</sup> <sub>b</sub>	53.9 <sup>b,c</sup> <sub>a</sub>	$F_{(3,67)} = 24.62$ ***
<b>Ru</b> (SD)	3 556 <sup>c</sup> (2 407)	0.7 <sup>b</sup> <sub>a</sub>	0.3 <sup>b</sup> <sub>a</sub>	36.9 <sup>a</sup> <sub>b</sub>	62 <sup>b</sup> <sub>b</sub>	$F_{(3,67)} = 20.62$ ***	657 <sup>b</sup> (677)	28.2 <sup>b</sup> <sub>a</sub>	38 <sup>c</sup> <sub>a</sub>	11.4 <sup>b</sup> <sub>a</sub>	22.5 <sup>c</sup> <sub>a</sub>	$F_{(3,67)} = 1.57$ ***
One-way ANOVA (among sites)	$F_{(6,147)} = 7.652$ ***	$F_{(6,147)} = 3.883$ *	$F_{(6,147)} = 14.43$ ***	$F_{(6,147)} = 10.66$ ***	$F_{(6,147)} = 11.18$ ***		$F_{(6,147)} = 14.26$ ***	$F_{(6,147)} = 8.684$ ***	$F_{(6,147)} = 14.34$ ***	$F_{(6,147)} = 5.025$ ***	$F_{(6,147)} = 16.47$ ***	

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756 Table 3

	<i>A. chinensis</i>		<i>C. tenuis</i>	
	Cu (shoot)	Co (shoot)	Cu (shoot)	Co (shoot)
[Cu] total	0.188 NS	- 0.192 NS	0.513 ***	0.092 NS
[Cu] free	0.111 NS	0.071 NS	0.497 ***	0.075 NS
[Cu-MnOx]	0.395 ***	- 0.535 ***	0.761 ***	- 0.195 NS
[Cu] free+ [Cu-MnOx]	0.207 NS	- 0.135 NS	0.566 ***	- 0.007 NS
[Cu-FeOx]	0.282 *	- 0.440 ***	0.603 ***	0.071 NS
[Cu] free + [Cu-FeOx]	0.204 NS	- 0.313 **	0.592 ***	0.085 NS
[Cu-OM]	-0.138 NS	0.404 ***	0.086 NS	0.295 *
[Cu] free + [Cu-OM]	-0.087 NS	0.314 **	0.232 NS	0.268 *
[Co] total	- 0.339 **	0.772 ***	0.245 *	0.475 ***
[Co] free	- 0.311 **	0.804 ***	0.219 NS	0.567 ***
[Co-MnOx]	-0.073 NS	0.280 *	0.452 ***	0.137 NS
[Co] free + [Co-MnOx]	-0.270 *	0.725 ***	0.374 **	0.392 **
[Co-FeOx]	-0.313 **	0.464 ***	0.055 NS	0.379 **
[Co] free + [Co-FeOx]	-0.376 ***	0.812 ***	0.183 NS	0.568 ***
[Co-OM]	-0.405 ***	0.899 ***	- 0.405 ***	0.644 ***
[Co] free + [Co-OM]	-0.333 **	0.836 ***	0.055 NS	0.638 ***
pH	-0.069 NS	- 0.101 NS	0.401 ***	0.202 NS
OM content	-0.336 **	0.567 ***	- 0.395 ***	0.219 NS
[Mn] total	0.507 ***	- 0.646 ***	0.652 ***	- 0.054 NS
[Fe] total	0.419 ***	- 0.554 ***	0.236 NS	- 0.053 NS
[Ca] total	-0.333 **	0.465 ***	- 0.443 **	0.244 *
[Mg] total	0.114 NS	- 0.390 **	0.530 ***	- 0.116 NS

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782 **Fig.1 Copper and cobalt concentrations accumulated in shoots of four populations of *Anisopappus***  
783 ***chinensis* and four populations of *Crepidorhopalon tenuis***

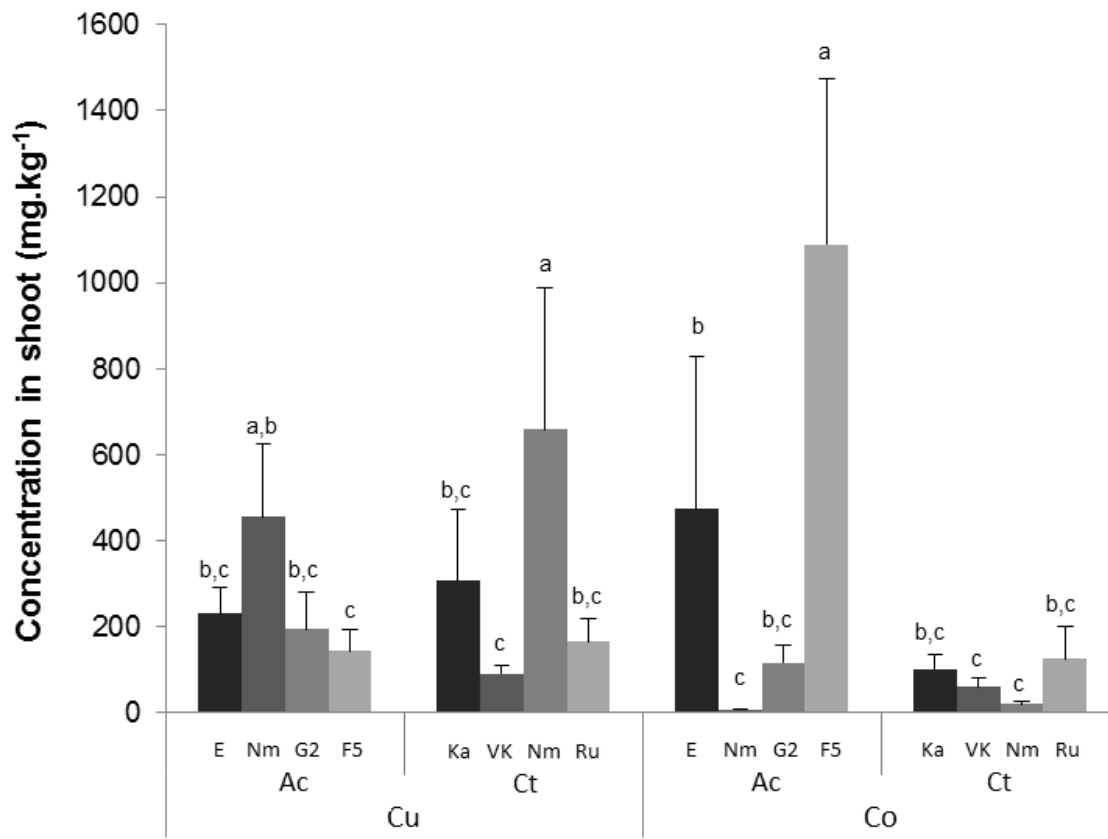
784 Sites are designated as follow: E=Etoile; Nm=Niamumenda; G2=Goma2; F5=Fungurume 5; Ka=Kalabi;  
785 VK=Vallée Karavia; Ru=Ruashi. Ac=*Anisopappus chinensis*; n=79; Ct= *Crepidorhopalon tenuis*; n=67. Error  
786 bars=standard deviation. For each species, there are no significant differences between populations with the  
787 same letter (results of one-way ANOVA followed by post-hoc multiple comparison, Tukey HSD test).  
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789 **Fig.2 Relationships between element concentrations in plants and in soils**

790 **a** Cu in plant and total Cu in soil **b** Co in plant and total Co in soil **c** Cu in plant and free Cu in soil **d** Co in plant  
791 and free Co in soil **e** Cu in plant and bound to MnOx Cu **f** Co in plant and bound to OM Co (*A. chinensis*: n=79;  
792 *C. tenuis*: n=67)  
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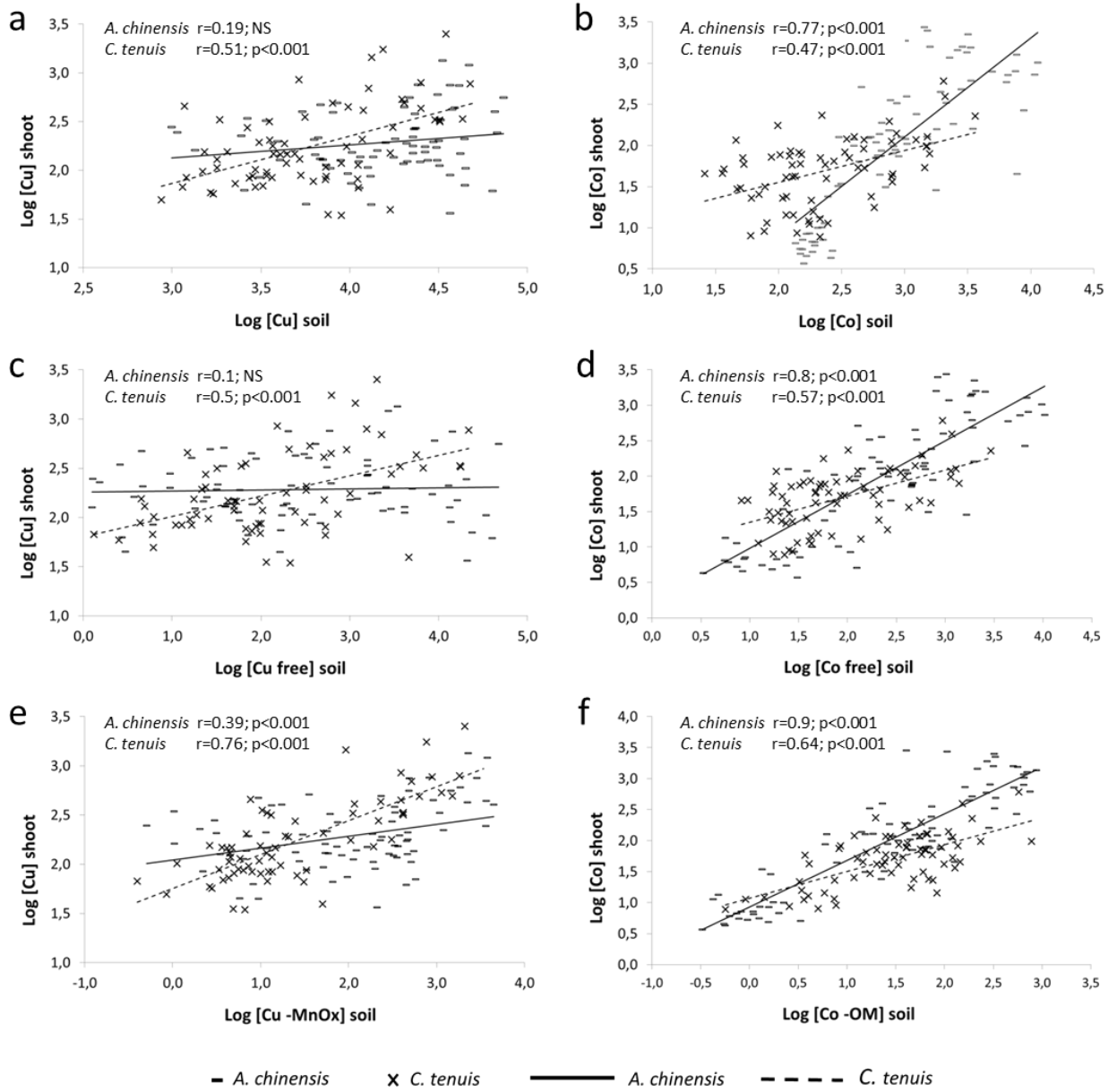
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872 Fig.2



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**Table S1 Total concentrations of elements, pH and organic matter in soils for seven metalliferous sites in Katanga (Dem. Rep. Congo).** F5, Fungurume 5 (n= 20); G2, Goma 2 (n =20); E, Etoile (n=20); Nm, Niamumenda (n=35); Ka, Kalabi (n=17); VK, Vallée Karavia (n=16); Ru, Ruashi (n=18).

Mean	E	Nm	G2	F5	Ka	VK	Ru
Cu (mg.kg <sup>-1</sup> )	12,566	20,833	27,793	18,298	18,624	5,444	3,556
(SD)	(12,502)	(17,899)	(17,117)	(13,285)	(13,392)	(3,315)	(2,407)
Co (mg.kg <sup>-1</sup> )	2,732	168	714	3,451	911	79	657
(SD)	(3,336)	(54)	(247)	(2,614)	(809)	(39)	(677)
pH	6.37	5.70	6.04	6.18	5.60	4.99	5.19
(SD)	(0.64)	(0.57)	(0.17)	(0.58)	(0.31)	(0.23)	(0.25)
OM (%)	6,26	4,57	8,60	11,44	5,28	9,38	12,73
(SD)	(2,75)	(2,05)	(2,68)	(5,30)	(1,93)	(4,25)	(3,41)
Mn (mg.kg <sup>-1</sup> )	2,625	5,710	1,600	1,047	1,070	64	1,023
(SD)	(1,852)	(4,697)	(449)	(223)	(806)	(37)	(926)
Fe (mg.kg <sup>-1</sup> )	42,305	48,534	27,745	21,315	35,250	19,610	51,060
(SD)	(11,460)	(21,237)	(5,753)	(9,387)	(11,309)	(9,654)	(9,128)
Zn (mg.kg <sup>-1</sup> )	123	144	56	27	190	388	152
(SD)	(129)	(91)	(24)	(10)	(121)	(195)	(67)
Mg (mg.kg <sup>-1</sup> )	49,510	48,147	41,595	36,875	22,700	2,084	27,591
(SD)	(44,430)	(20,448)	(7,260)	(22,029)	(25,755)	(470)	(21,195)
Ca (mg.kg <sup>-1</sup> )	642	416	595	1,945	694	578	736
(SD)	(275)	(138)	(160)	(1,710)	(302)	(151)	(295)
K (mg.kg <sup>-1</sup> )	12,940	7,395	6,155	6,085	13,300	5,734	6,766
(SD)	(10,375)	(6,411)	(3,431)	(7,973)	(13,577)	(1,776)	(3,363)