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1 **Functional traits of a broad-niched metallophyte along a toxicity gradient: disentangling**
2 **intra and inter-population variation**

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24 **Declarations of interest: none**
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26

27 **Abstract**

28 Studies on the relative contribution of inter- and intra-population trait variations in broad-
29 niched species along edaphic gradients are still scarce. Here, we explore the variation of eight
30 traits in five populations of *Cyanotis longifolia*, a broad-niched metallophyte thriving on very
31 broad gradients of soil copper concentration in Central Africa. Variation at species level was
32 decomposed into covariation with copper, variation among sites, and residual variation. The
33 proportion of the variance explained by the site and by the local copper gradient in each site
34 ranged from 10 to 32% and between 5 and 51% respectively, depending on trait. At the
35 species level, specific leaf area decreased and leaf Cu and Co concentrations increased with
36 increasing soil Cu concentration. Surprisingly, traits related to plant size show a quadratic
37 response with higher values on both high and low soil Cu concentrations. This could be
38 accounted for by population-specific patterns of covariation with soil copper. Population
39 specific traits responses were found, with some populations exhibiting no significant response
40 while the others sometimes show opposing variation patterns. The reasons for such
41 idiosyncratic patterns are discussed in terms of interactions with other soil factors influencing
42 Cu availability and toxicity. In further studies on trait variation along toxicity gradients, we
43 recommend to investigate the small scale edaphic variability and to measure traits at the
44 population level to capture the diversity of functional responses.

45

46

47 **Keywords:** metal gradient, facultative metallophyte, intraspecific variation, copper
48 availability, phenotypic plasticity.

49

50 **1. Introduction**

51 Within species variation in functional traits is now recognised as an important component of
52 community trait variation and community assembly processes (Siefert et al 2015, Hart et al
53 2016). It is composed of both within population and between population variations. Most
54 studies focus on between population trait variation along altitudinal and latitudinal gradients
55 (Souto et al 2009, Fajardo & Piper 2011) but studies comparing within species variation along
56 several similar environmental gradients are scarce. However, growing evidence indicates that
57 intra-population variation can be as large as inter-population variation along soil depth, light,
58 temperature, nutrients or altitude gradients (Bresson et al 2011, Wellstein et al 2013, Lemke et
59 al 2015, Harzé et al 2016, Pfennigwerth et al 2017). This can result from two, mutually non
60 exclusive mechanisms i.e. phenotypic plasticity (e.g. Valladares et al 2014) and local genetic
61 variation (e.g. Byars et al 2007, Robson et al 2012). Both genetic and phenotypic differences
62 of adaptive traits have been documented at very small spatial scales (Bradshaw 1959, Linhart
63 & Grant 1996), particularly in relation to metallic trace elements (MTE) enriched soils
64 (Antonovics et al 1971, Al-Hiyaly et al 1993).

65 Soil metal contamination represents stringent selective pressure that can promote the
66 evolution of locally adapted ecotypes (Słomka et al 2011, Wójcik et al 2015). Plant
67 evolutionary ecology studies often compare metallicolous and non metallicolous populations,
68 based on the assumption that the differences in ecological conditions between MTE-enriched
69 site and the non-contaminated neighbourhood produce a clear-cut difference in plant
70 phenotype. However, soil conditions are often extremely variable both among and within
71 metalliferous sites which can lead to intraspecific differences in tolerance strategies, ion
72 contents or functional traits values (Lefebvre 1974, Lange et al 2017b, Stein et al 2017, Frérot
73 et al 2018). Although metallophytes tolerance have been extensively studied from a
74 physiological point of view, most studies on metallophytes have focused on sites that have

75 been recently contaminated by human activities (Baker et al 2010) and functional traits
76 variation in metallophytes across large gradients of metal concentration in the soil remains
77 largely understudied. Functional traits are expected to vary along toxicity gradients because of
78 selection for optimal trait values in local environments (Garnier et al 2004) and because of
79 trade-offs between growth or reproduction and metal tolerance (Wilson 1988, Ernst et al 1990
80 but see Harper et al 1997). Facultative metallophytes along natural MTE gradients offer
81 excellent opportunities to study mechanisms and patterns of trait variation due to soil toxicity,
82 which can be due to phenotypic plasticity or local genetic variation.

83 In South East of the DR Congo (Upper Katanga), natural outcrops of Cu-rich bedrocks occur
84 in the form of several hundreds of “copper hills”. These hills typically appear as islands of
85 herbaceous vegetation emerging from the surrounding woodland matrix (Duvigneaud &
86 Denaeyer-De Smet 1963). Copper concentration in the soil often increases by three orders of
87 magnitude from the base to the top of the hill (Séleck et al 2013). Many other chemical and
88 physical factors vary as well, such as phosphorus and organic matter content, and pH (Séleck
89 et al 2013, Pourret et al 2016). Each copper hill comprises several habitats, depending mainly
90 on soil Cu and Co concentrations, as well as other soil chemical or physical properties, slope
91 and microtopography (Duvigneaud & Denaeyer-De Smet 1963, Saad et al 2012, Séleck et al
92 2013). This variation in MTE concentration in the soil, coupled with the extensive diversity of
93 microhabitats can have a strong influence on the vegetation structure, allowing a large number
94 of species to coexist on a single hill (Saad et al 2012, Ilunga wa Ilunga et al 2013, Faucon et
95 al 2016). Delhaye et al (2016) found clear patterns of variation in community weighted mean
96 values of traits i.e. decreasing leaf area, increasing specific leaf area (SLA, the area of a leaf
97 divided by its dry mass) and foliar MTE content along a gradient of increasing soil Cu. Most
98 of this variation is due to species turn-over along the Cu gradient. In addition, a number of
99 species occupy a very broad niche along the copper gradient and might exhibit significant

100 intraspecific traits variation. However, Cu and Co availability to plants is probably highly
101 variable between sites, depending on the local soil properties and the rhizosphere
102 biogeochemical processes (Lange et al 2014). Species' functional response to metal
103 concentration in the soil could therefore be highly site-specific. Recent works have found
104 within species variation in Cu tolerance in this flora, correlated to soil copper content of
105 native sites, suggesting local adaptation (Faucon et al 2012, Peng et al 2012, Lange et al
106 2018). Therefore, copper hills represent an interesting model to explore trait variation within
107 species along natural MTE gradients.

108 One of the species with the broadest ecological range on copper hills is *Cyanotis longifolia*
109 Benth. (Commelinaceae). It is a very polymorphic, self compatible, hemicryptophytic species
110 common in tropical Africa (Owens 1981, Faden 2012). *C. longifolia* is widespread on copper
111 hills and was previously reported as a Cu and Co hyperaccumulator (Malaisse et al 1979). In
112 this paper, we explore the within species variation of functional traits in *C. longifolia* in
113 relation to gradients of soil Cu concentration. Using populations growing on 5 copper hills,
114 we examine the patterns of traits variation at the species level. Then, we examine the relative
115 contribution of inter- and intra-population functional trait variation along MTE gradients. We
116 test the hypothesis that such variation can be explained by within- and between sites
117 differences in soil available copper content.

118 More precisely, we ask:

- 119 - How do *C. longifolia* functional traits vary along increasing copper concentration in
120 the soil? We expect a change to traits related to a more stress tolerant strategy with
121 increasing the copper content in the soil.

- 122 - What is the relative contribution of intra- versus inter-population variation to the
123 species level variation? We expect the local copper gradient on each site to be the
124 major source of variation in functional traits.
- 125 - Do all populations show similar patterns of trait variation along metal gradients? We
126 expect that the soil copper gradient produces a strong filtering effect on functional
127 traits resulting in similar patterns of traits variation between sites.

128

129 **2. Material and methods**

130 *2.1. Study sites*

131 Five sites were selected in the Copperbelt region in the south-east of D.R. Congo, between
132 Tenke-Fungurume and Lubumbashi (Table 1). Each site was divided in 3 distinct habitats,
133 based on topography and vegetation physiognomy, following Duvigneaud & Denaeyer-De
134 Smet (1963), i.e. from base to top of hill: the dambo (high savannah on horizontal soil around
135 the hill), mid-slope (grassland rich in geofrutex), and the upper slope, (open grassland rich in
136 annual species). From the dambo to the upper part of the slope, soil MTE concentrations
137 increase by two orders of magnitude (Duvigneaud & Denaeyer-De Smet 1963, Séleck et al
138 2013). On each site, a population of *C. longifolia* is present and individuals grow from the
139 bottom to the top of the hills, encompassing a large range of edaphic conditions.

140 *2.2. Plant sampling and traits measurement*

141 In January 2013, in each site, individuals of *C. longifolia* were collected along a transect
142 ranging from the bottom to the top of the hill in order to capture the whole range of soil metal
143 concentrations within each site. Population size of *C. longifolia* was small in several sites and
144 habitats. Therefore, sample size did not exceed 10 percent of the individuals in each habitat,

145 i.e. 22 to 65 replicates depending on site (Table 1). Sampled plants were at least 2 m from
146 each other to avoid multiples samples of the same individual due to vegetative reproduction.

147 On each individual, 6 functional traits related to size (vegetative height: VH, leaf area: LA,
148 rooting depth: RD), resource use and conservation (specific leaf area: SLA, leaf water
149 content: LWC) and vegetative reproduction (clonal distance: CD) were measured. Leaves
150 were collected for determination of concentrations of copper (Cu_{pl}) and cobalt (Co_{pl}). All
151 morphological traits were measured according to Pérez-Harguindeguy et al (2013) on healthy
152 adult plants with leaves exposed to full sunlight. VH was measured from the base of the plant
153 to the top of the upper leaf. LA was measured on the two, fully developed, youngest
154 undamaged leaves using ImageJ software on photographs of fresh leaves. LWC and SLA
155 were measured on the same leaves after drying at 105°C for 48 hours. Leaf traits were
156 averaged at the individual level. Recently, Vojtkó et al (2017) emphasized the importance of
157 clonal traits in response to habitat filtering. Therefore, the maximum CD was measured as the
158 distance between the two farthest ramets of a same individual and the RD was measured after
159 digging up carefully each individual.

160 Leaves used for SLA measurement were also used to determine MTE concentrations. Fresh
161 leaves were washed in a 1% Alconox[®] solution, and rinsed (Faucon et al 2007). The dry
162 samples were ashed at 500°C for 12h. The ashes were diluted in 1 ml of H₂O and 2 ml of
163 HNO₃ 65% (Hoenig & de Kersabiec 1996) and heated at 100°C for 5 minutes. Each solution
164 was filtered and diluted in H₂O in order to obtain a 25 ml solution. Cu_{pl} and Co_{pl} were
165 determined by inductively coupled plasma mass spectrometry (ICP- MS) (Thermo Scientific
166 XSERIES2).

167

168

169 *2.3. Soil analyses*

170 A soil sample (approximately 50g dry weight) from the rhizosphere of each sampled plant
171 was collected. Each sample was air dried for one week and sieved at 2 mm before analysis.
172 Rock (gravel) content was assessed as the (1 - mass proportion) of the 2 mm sieved dried soil.
173 Soil pH was measured with a glass electrode on a 5:1 demineralised water:soil sample.
174 Organic matter content was measured by calcination for 12 hours at 550°C on 2 g soil
175 samples that were previously heated at 105°C for 48 hours in order to remove any interstitial
176 water (Pansu & Gautheyrou 2006). Mineral elements were extracted with 1M ammonium
177 acetate-EDTA (pH 4.65) for 60 min using 5 g of dry soil in 25 ml of solution (Cottenie et al
178 1982). The supernatant was filtered and analysed by inductively coupled plasma-optical
179 emission spectrometers (ICP-OES; Vista MPX, Varian Inc., Palo Alto, CA, USA) for the
180 following elements: Cu, Co, Mn, Mg, P, K and Fe.

181 *2.4. Statistical analysis*

182 Soil parameters were log-transformed if necessary before analysis to meet normality
183 assumptions. Variation of soil properties was explored with a principal component analysis.
184 Soil copper was highly correlated with the first principal component (see supplementary
185 material 1, fig. S1). On copper hills, copper is known to have a strong filtering effect on
186 vegetation structure (Séleck et al 2013) and functional traits variation (Delhayé et al 2016).
187 For these reasons, soil copper was selected as the main gradient of interest in further analyses.
188 The association between soil Cu content and other soil parameters at each site were quantified
189 using Pearson correlation coefficient (see also supplementary material 1).
190 Traits were log-transformed if necessary to meet the normality assumption. Within each site,
191 the amplitude of variation of traits was evaluated using the coefficient of variation (CV) and
192 trait min-max range. To test if large variability in soil conditions induces large variability in

193 functional traits, Spearman correlations between traits CV or range and the range of soil Cu
194 content at each site were used. Species level patterns of traits variation were evaluated by
195 comparing the fit of a linear and a quadratic regression on the data, pooling the five
196 populations. The best model was selected based on AICc criterion. When the difference in
197 $AICc < 2$ (Burnham & Anderson 2004), the linear model was selected. To identify other
198 potentially important soil factors, a redundancy analysis (RDA) was performed to quantify the
199 multivariate variation of traits explained by all soil factors (see methods and results in
200 supplementary material 1). Using this methodology, soil Cu was the factor explaining the
201 most variation and was therefore further used as main gradient of interest.

202 To assess the relative contributions of intra- and inter-site variance to the total trait variance, a
203 set of linear models were built. For each of the 8 traits, three different models were produced
204 to extract the proportion of variance (R^2) explained by the site effect and the local soil copper
205 gradient at each site. The first model uses a simple linear regression where soil copper is the
206 only explanatory variable (R^2_1). This produces a single model forcing the same slope and
207 intercept for all sites. The second model is a linear mixed model in which soil copper is the
208 fixed effect and the site is a random effect (R^2_2). This produces a regression line for each site
209 with different intercepts but the same slope. Finally, the third is also a linear mixed model,
210 with the soil copper content as a fixed variable and the interaction between the site and the
211 soil copper concentration as a random variable (R^2_3). The latter provides a random slope and
212 random intercept model, allowing each site to have different slopes for trait variation along
213 the copper gradient. The proportion of variance explained by the site is obtained by $VarSite =$
214 $R^2_2 - R^2_1$ and the proportion of variance explained by the copper gradient within each site is
215 obtained by: $VarCu*Site = R^2_3 - R^2_2$. The residual variance is calculated by $VarResidual = 1 -$
216 $(VarSite + VarCu*Site)$.

217 Linear regressions by permutation (n = 9999) were conducted for all traits along the copper
218 gradients at each site separately in order to assess the functional response of each population
219 to within-site environmental variation. To test if the different populations show similar
220 patterns of trait variation along the copper gradients, the slopes of regression lines were
221 compared between sites using a covariance analysis (ANCOVA) with a designed contrast
222 matrix to compare slopes but not the intercepts in order to reduce the number of simultaneous
223 tests. The same methodology was also applied with all other measured soil factors as a control
224 and none showed more significant relationships than with soil copper content, suggesting that
225 this is the main factor driving functional traits variation (results not shown). Analyses were
226 conducted in R software 3.0.2 (www.R-project.org) using packages lme4, ade4, ape and
227 multcomp, vegan and proxy.

228

229 **3. Results**

230 *3.1. Soil*

231 Soil Ac-EDTA extractible elements, stone and organic matter contents as well as pH, vary
232 extensively both within and among sites (Table 1 and supplementary material 1, fig. S1a). The
233 range of soil Cu concentration is shortest on Goma and longest on Fungurume. In addition to
234 Cu, each site also shows extensive variation for other Ac-EDTA extractible elements,
235 especially Co, Mn and Ca. Fungurume has the highest values in Cu, Co, Mn, Ca, Mg, P and
236 pH. Goma is characterized by lower concentrations in Cu, Mn and organic matter. Kinsevere
237 contrasts with all the other sites by its lower Co level ($< 17 \text{ mg kg}^{-1}$).

238

239

240 Table 1: Locations, number of plant and soil samples and variation range of soil parameters
 241 for each site. Rock is the soil gravel content (> 2 mm) and MO is the soil organic matter
 242 content, both expressed in percentage of soil mass. Ca, Co, Cu, Fe, K, Mg, Mn and P are
 243 expressed in mg kg⁻¹ (Ac-EDTA extractable).

	<i>Goma</i>	<i>Orthodoxe</i>	<i>Zikule</i>	<i>Kinsevere</i>	<i>Fungurume</i>
Coordinates	S10°35'56" E26°08'20"	S10°36'26" E26°07'37"	S10°38'48" E26°12'48"	S11°21'58" E27°34'53"	S10°36'47" E26°17'10"
Individuals	65	43	22	45	43
Soil samples	55	34	20	12	40
Cu (mg kg ⁻¹)	30 - 992	36 - 1409	30 - 2129	149 - 6387	218 - 7758
Co (mg kg ⁻¹)	2 - 111	6 - 232	8 - 128	1.5 - 17	64 - 850
Mn (mg kg ⁻¹)	37 - 189	65 - 548	29 - 502	74 - 319	37 - 543
Ca (mg kg ⁻¹)	49 - 593	171 - 1080	104 - 458	131 - 568	72 - 2359
Fe (mg kg ⁻¹)	56 - 322	158 - 379	89 - 367	81 - 306	26 - 223
K (mg kg ⁻¹)	36 - 322	79 - 263	99 - 281	109 - 261	32 - 255
Mg (mg kg ⁻¹)	41 - 300	75 - 374	71 - 280	107 - 364	40 - 643
P (mg kg ⁻¹)	0.9 - 12.3	1.2 - 19.1	2.7 - 17	0.9 - 36.6	1.9 - 63.8
Rock (g kg ⁻¹)	2 - 730	25 - 960	100 - 760	32 - 670	67 - 690
MO (g kg ⁻¹)	33 - 75	30 - 118	57 - 200	39 - 166	39 - 172
pH	5.4 - 6.4	5.4 - 6.5	4.6 - 6.3	5.6 - 6.1	5.5 - 7.1

244

245 In all sites except Kinsevere, soil Cu content is strongly positively correlated to Co content
 246 (Table 2). Cu is positively correlated with P and organic matter, significantly so in 4 of 5 sites
 247 (P) and 3 of 5 sites (organic matter). Other correlation patterns between Cu and other
 248 elements are highly site-specific (Table 2). On Fungurume, pH and Ca concentration increase
 249 with Cu concentration in the soil.

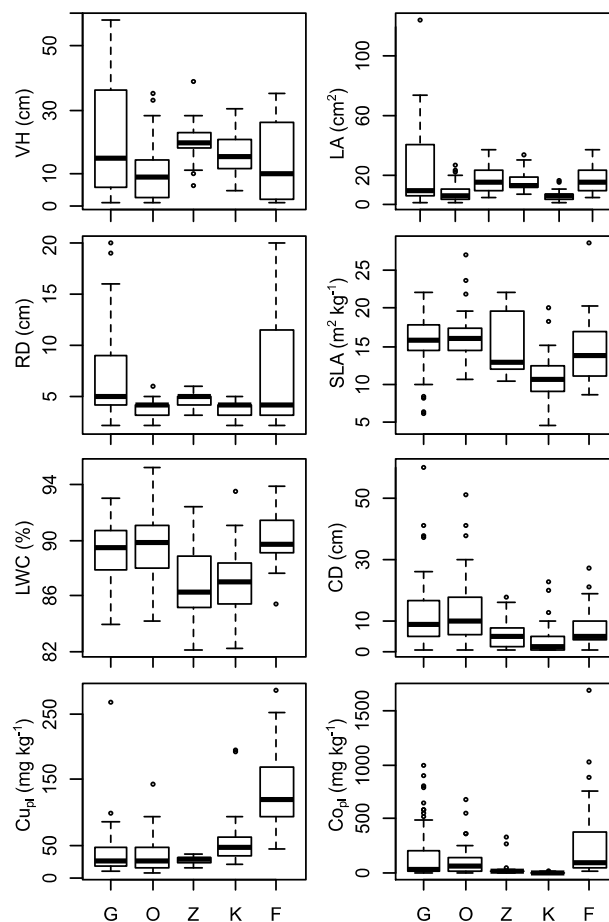
250

251 Table 2: Correlation coefficients between soil Cu concentration (Ac-EDTA extractable) and
 252 other soil factors at each site. Significant correlation coefficients (p<0.001) in bold.

	<i>Co</i>	<i>Mn</i>	<i>Ca</i>	<i>Fe</i>	<i>K</i>	<i>Mg</i>	<i>P</i>	<i>Rock</i>	<i>MO</i>	<i>pH</i>
<i>Goma</i>	0.95	0.07	0.09	-0.19	-0.28	-0.32	0.74	0.61	0.05	0.01
<i>Orthodoxe</i>	0.89	0.84	0.08	0.11	0.43	0.05	0.96	0.50	0.81	-0.38
<i>Zikule</i>	0.83	0.86	0.00	-0.46	-0.07	0.18	0.60	-0.24	0.30	0.39
<i>Kinsevere</i>	-0.80	-0.42	0.67	0.11	0.25	0.33	0.97	0.67	0.80	-0.18
<i>Fungurume</i>	0.96	-0.60	0.80	-0.71	0.29	0.24	0.99	0.57	0.63	0.70

253

255 There is extensive variation both within and among populations for all traits (Fig. 1 and
 256 supplementary material 1, fig S1b). The population Goma has the largest range of size related
 257 traits and clonal distance (VH: 57 cm, LA: 122 mm², CD = 60 cm) and covers the whole
 258 range of trait values (Fig. 1). Fungurume has the highest foliar Cu and Co, up to 286 and 1700
 259 mg kg⁻¹ respectively. Using both traits CV and range (Supplementary material 2), there is no
 260 significant correlation between traits and soil Cu variability- e.g. the largest variability (CV
 261 and range) in size traits are found along the shortest soil Cu gradient (Goma).

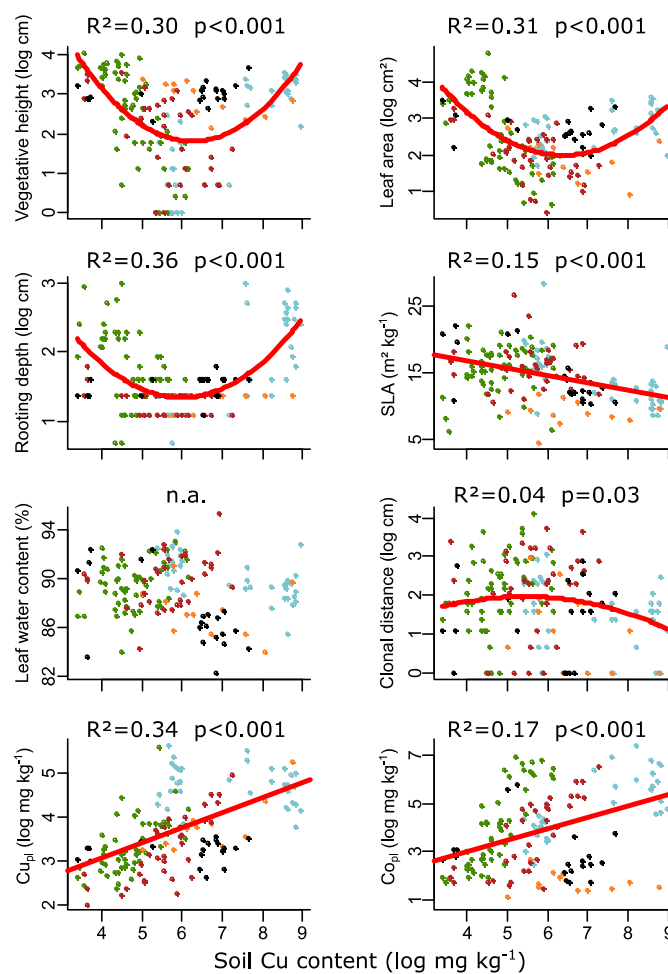


262

263 **Fig. 1:** Variation of eight traits for the five populations (G= Goma, O = Orthodoxe, Z =
 264 Zikule, K = Kinsevere, F = Fungurume). VH: vegetative height, LA: leaf area, RD:
 265 rooting depth, SLA: specific leaf area, LWC: leaf water content, CD: clonal distance,
 266 Cu_{pl} and Co_{pl} leaf Cu and Co content. The thick bar represents the median; the box
 267 contains the inter-quartile range (IQR) and the whiskers are the maximum 1.5 IQR.

268 *3.3. Trait variation along the soil Cu concentration*

269 The patterns of variation at the species level are trait specific (Fig. 2). VH, LA and RD show a
 270 highly significant quadratic relationship with soil Cu concentration with higher trait value at
 271 both ends of the gradient. Three traits show significant linear regression with soil Cu: SLA
 272 (negative slope), Cu_{pl} and Co_{pl} (positive slope). Leaf water content shows no significant
 273 relationship and clonal distance shows a negligible ($R^2 = 0.04$) quadratic relationship with soil
 274 Cu concentration.



275

276 **Fig. 2:** Trait variation at the species level, pooling the five studied populations, along the
 277 copper gradient. A linear and a quadratic model were fitted for each trait and the best model
 278 based on the AICc criterion was selected. The red line is the significant regression line. R^2 and
 279 associated p-value are reported. Colour code: Goma (green), Orthodoxe (red), Zikule (black),
 280 Kinsevere (Orange), Fungurume (blue).

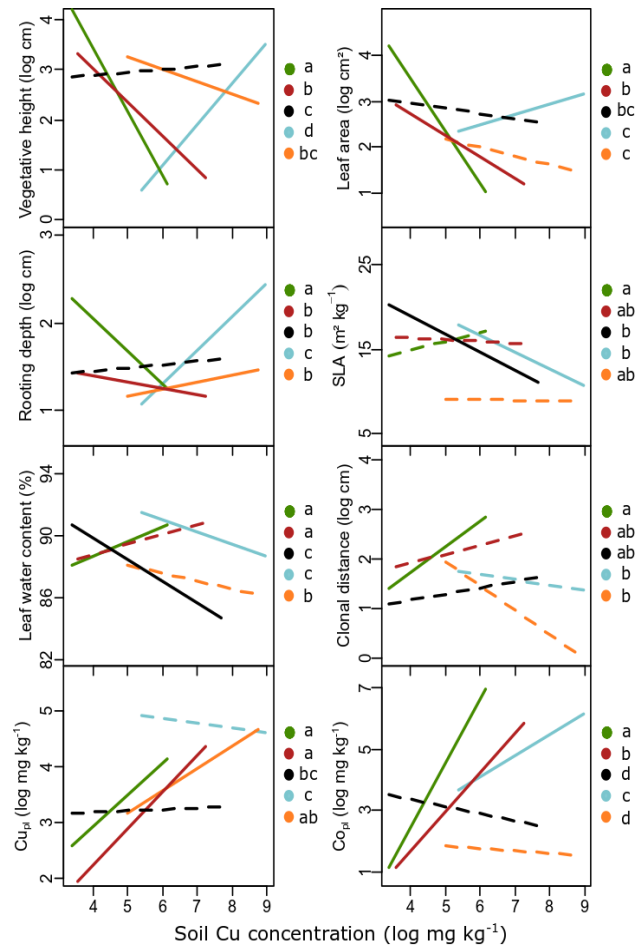
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282 For size related traits (VH, LA and RD) the local soil Cu gradient explains between 28 and 51
 283 % of the traits variation (Table 3). For SLA, LWC and CD, the site effect explains
 284 approximately twice more variance than the gradient effect. Interestingly, Cu_{pl} and Co_{pl} also
 285 show a larger site effect than the soil extractable-Cu effect (1.6 and 1.5 times respectively;
 286 Table 3).

287
 288 Table 3: Percentages of variance explained by the inter-site effect (Among site), the local soil
 289 copper gradient within each site (Cu within site) and the residuals expressed as a percentage
 290 of the total variance.

	<i>Among site</i>	<i>Cu within site</i>	<i>Residuals</i>
<i>Vegetative height</i>	10.6	51.0	38.4
<i>Leaf area</i>	18.1	30.4	51.5
<i>Rooting depth</i>	19.6	28.4	52.0
<i>Specific leaf area</i>	14.3	9.6	76.1
<i>Leaf water content</i>	23.0	13.7	63.3
<i>Clonal distance</i>	10.6	5.2	84.1
Cu_{pl}	24.6	14.6	60.8
Co_{pl}	32.9	22.2	44.9

291
 292 Within each site, a linear regression analysis was conducted to examine the pattern of
 293 variation of each trait as a function of soil Cu. For morphological traits, the regression
 294 analysis and associated covariance analysis reveal highly contrasting, site specific patterns of
 295 trait variation along the Cu gradients (Fig. 3, supplementary material 3). Significant
 296 covariation with soil Cu was found in 1 to 4 sites depending on trait. Significant correlations
 297 in opposite directions were found in 4 of 8 traits (vegetative height, leaf area, rooting depth,
 298 leaf water content).



299

300 **Fig. 3:** Functional traits variation along the copper gradient for the five studied sites. Plain
 301 lines represent significant linear regression, dashed lines are non-significant. Letters in the
 302 legend represent similarities in regression slopes of the different populations for each graph
 303 (see supplementary material 3 for exact values). Colour code: Goma (green), Orthodoxe (red),
 304 Zikule (black), Kinsevere (orange), Fungurume (blue).

305 4. Discussion

306 Intraspecific trait variation is receiving increasing attention in plant community ecology
307 (Siefert et al 2015). However, few studies have focused on traits variation at inter and intra
308 population levels. Here, for the first time, the relative contribution of intra and inter-
309 population trait variation as well as patterns of traits variation in relation to MTE gradients are
310 studied in a broad-niched metallophyte. *Cyanotis longifolia* is able to grow on soil with Ac-
311 EDTA-extractable Cu concentration spanning more than two orders of magnitude (range: 30-
312 7758 mg kg⁻¹). The species occurs in plant communities that show dramatic variations in
313 floristic composition and community averaged trait values, along copper gradients (Séleck et
314 al 2013, Delhaye et al 2016) which are, at least partly, due to the increase in MTE
315 concentrations in the soil.

316 4.1. Species level trait variation

317 Edaphic heterogeneity has significant impact on local intraspecific trait variation, especially
318 in broad-niched species along altitudinal, fertility or aridity gradients (Lenssen et al 2004,
319 Fajardo & Piper 2011, Pakeman 2013, Harzé et al 2016, De Smedt et al 2018). In this study,
320 functional traits of a broad niched species show large variation along short distance MTE
321 gradients (Ac-EDTA extractable). With increasing soil Cu concentration, *C. longifolia* shows
322 a decrease in SLA value concomitantly with an increase in tissues MTE concentrations. This
323 relationship could be attributed to an increasing stress tolerance strategy of the plants with
324 increasing the soil metal toxicity (Potters et al 2007). SLA is related to growth rate and
325 resource use efficiency (Wright et al 2004) and the increase of MTE concentrations in the
326 plant tissues could induce a physiological stress while imposing an investment in metal
327 sequestration mechanisms. The increase in MTE concentration in plant tissues is a well
328 known feature of some species in the copper flora, especially in facultative metallophyte

329 species (Faucon et al 2007, Lange et al 2018). Most surprisingly, at the species level, the
330 covariation of size traits with soil copper was quadratic with lowest values at intermediate soil
331 Cu. The fact that several traits show similar patterns of response is not surprising, especially
332 for traits related to the same functional axis, such as the plant size axis (Díaz et al 2016). Non-
333 linear traits responses along environmental gradients have been previously discussed (e.g.
334 Albert et al 2010) but the origin of these patterns often remains unclear. Here, contrasting site
335 specific responses account for the quadratic response at the species level along the copper
336 concentration gradient. It should be noted however that some traits might vary due to
337 environmental factors independent of the soil MTE toxicity. For instance, size related traits
338 show a negative correlation with the soil rock content (see RDA results in supplementary
339 material 1). The increase in rock content in the soil could imply a lower water retention
340 capacity in the soil, therefore selecting phenotypes adapted to water depletion. However, it
341 was shown that rock content in the soil is not necessarily correlated to a decrease in soil water
342 content in such environments (Delhaye et al 2016). Further studies measuring other soil
343 parameters on a larger number of sites, encompassing many different combinations of
344 parameters, could provide further insight on the actual factors driving traits variation at the
345 species level.

346 *4.2. Within Population trait variation*

347 Along gradients of environmental harshness and within a single species, large phenotypic
348 variations and similar directions of trait variation are generally expected due to the strong
349 restrictive effect of environmental conditions on plants phenotypes (e.g. Kolbas et al 2018).
350 Here, an unsuspected complex pattern of trait variation within and among populations
351 emerged. Intra population trait variations are highly site and trait specific along the studied
352 copper gradients and account for variable proportions of total variance (between 5 and 51%)
353 depending on trait. This is consistent with results found along altitude or resource gradients

354 (Wellstein et al 2013, Lemke et al 2015, Harzé et al 2016, Pfennigwerth et al 2017). Contrary
355 to expectations, covariation with copper was less important compared to the site effect for
356 most traits: only for size related traits was the impact of the local soil copper gradient superior
357 to the site effect. This contradicts the results of a controlled experiment showing that most of
358 the variation in morphological and physiological traits in another pseudometallophyte
359 happened within population (Meyer et al 2010). The large variation of size related traits along
360 local gradients could indicate a trade-off between allocation to size and metal tolerance (Ernst
361 et al 1990). The lower intra-population variation for traits related to resources use tends to
362 show a strong conservatism of these traits along MTE gradients. This is consistent with
363 previously reported results along resources or climatic gradients (Fajardo & Piper 2011,
364 Siefert et al 2015, Harzé et al 2016) but is also consistent with experimental findings on a
365 facultative metallophyte from the copper hills, where SLA vary between populations but not
366 in response to soil metal concentration (Lange et al 2017b). On the contrary, plants MTE
367 contents exhibit large differences both within and between populations as found in other
368 metallophytes (Stein et al 2017, Frérot et al 2018) and show different patterns of covariation
369 with other traits. The large part of the variance explained by the site effect for the plant Cu
370 and Co content (24.6 and 32.9 % respectively) is likely due to the differences in soil metal
371 concentrations as well as other factors that can influence soil MTE availability. On copper
372 hills, Cu availability in the soil is strongly influenced by other edaphic factors, such as pH and
373 organic matter content (Lange et al 2014, Pourret et al 2016). This could play an important
374 role on Fungurume and Kinsevere, where toxicity seems less clear on functional traits and
375 organic matter content co-vary positively with soil Cu content. Soil organic matter content is
376 known to immobilize Cu, leading to less free Cu ions in the soil solution (McGrath et al
377 1988). Further, on Fungurume, the increase in soil pH and Ca content could be due to an
378 increase in carbonates in the soil. Due to the high pH on the most contaminated soils on

379 Fungurume, Cu could be immobilised in the soil (Kabata-Pendias 2010) and therefore
380 represent a less toxic constraint for plants. However, other studies show that organic matter
381 content and pH could not influence the proportion of free Cu^{2+} content relative to the total Cu
382 concentration in the soil (Sauvé et al 1997). On Fungurume, another study shows that a few
383 plant species exhibit a clear decrease in size related traits and an increase in tissues Cu content
384 (Delhaye et al 2016). Population level differences in metal accumulation and tolerance were
385 also demonstrated experimentally in several copper hill metallophytes (Faucon et al 2012,
386 Peng et al 2012, Lange et al 2018) and might be driven by different MTE concentrations in
387 the native soil, therefore suggesting genetic determinism of metal tolerance strategy at the
388 population level. Therefore, the functional traits response to copper toxicity is likely species-
389 specific as well as population-specific within species. Finally, the difference in the behaviour
390 between both (Cu and Co) metals might be related to the greater mobility of Co at the plant-
391 soil interface and the lack of efficient mechanism of Co detoxification (Lange et al 2017a).

392 The absence of correlation between gradient length and functional traits variability in each
393 population contradicts the hypothesis that larger niche implies larger trait variation (Sandquist
394 & Ehleringer 2003, Sides et al 2014) but is consistent with observations along light
395 availability gradients (Lemke et al 2012). This might be due to different, site specific,
396 mechanisms of phenotypic variation on different isolated sites or on soils with different MTE
397 concentrations within each site. It is not known if the covariations with soil Cu are due to
398 phenotypic plasticity or local adaptation. Due to the geographical isolation, site specific
399 responses of traits along local gradients may have evolved due to ecological drift (e.g.
400 Vellend 2010) or local adaptation (Kawecki & Ebert 2004). Short distance genetic
401 differentiation was demonstrated along different types of environmental gradients such as
402 altitude (Byars et al 2007, Leempoel et al 2018) or in response to short distance variation in
403 metal contamination (Jain & Bradshaw 1966, Antonovics et al 1971). Therefore, phenotypic

404 plasticity could be the main mechanism of functional adjustment along short gradients (e.g. at
405 Goma) while local adaptation could appear along large gradients in soil MTE concentrations
406 (e.g. at Fungurume). This might explain the apparent distinct groups of individuals on soil
407 with low and high metal content within the population of Fungurume (see supplementary
408 material 1, Fig. S1). This hypothesis could be tested using genetic analysis and common
409 garden experiments.

410 Traits covariation is also recognised as an important factor explaining the local diversity of
411 phenotypes (Armbruster & Schwaegerle 1996). Trade-offs between traits can produce
412 different local phenotypes selected in particular environments (Laughlin & Messier 2015).
413 Finally, it is likely that other environmental factors such as mycorrhizal fungi or bacteria
414 could improve plants MTE tolerance (Lenoir et al 2016, Sun et al 2010) and are also
415 influenced by local soil conditions (Bauman et al 2016). Therefore, we further advocate for
416 experimental measurement of the variation in population level traits along Cu and/or Co
417 gradients in controlled conditions (Lange et al 2017b) in order to disentangle the relative
418 proportion of phenotypic plasticity and ecotypic variation in *C. longifolia*.

419

420 **5. Conclusion**

421 Our study has revealed a complex pattern in the structure of intraspecific variation in a broad
422 niched species. This suggests that generalization based on one or very few populations may be
423 highly misleading due to the particular combination of local conditions at each site. A species
424 should be sampled from sites encompassing its whole ecological range. Our results also stress
425 that, depending on the spatial scale at which intraspecific variation is explored, very different
426 patterns can emerge. This is likely due to interactions between isolation, various edaphic

427 factors and/or local adaptation. A population specific approach is needed to capture the whole
428 species level traits variation along toxicity gradient.

429

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434

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Supplementary material 1: Multivariate analysis of the variability in plant traits and some soil characteristics and their multivariate correlations.

In order to investigate the extent of variation and differences in soil conditions and functional traits diversity in each sites, we performed 2 principal component analyses using the data presented in the material and methods (Figure S1). Differences between populations are tested using a multivariate analysis of variance by permutation (PERMANOVA).

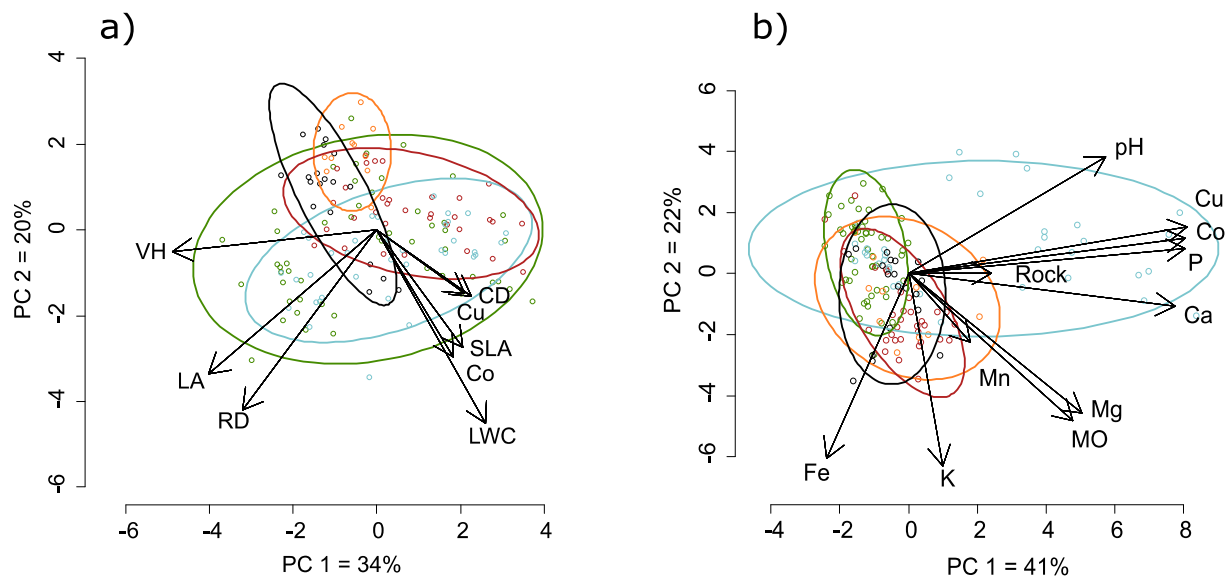


Figure S1: Principal component analysis of traits (a) and soil factors (b) for the 5 studied populations of *C. longifolia*. Colour code: Goma (green), Orthodoxe (red), Zikule (black), Kinsevere (Orange), Fungurume (blue). Ellipses represent the 95% probability of occurrence of each population. Number of points and abbreviations: see main text.

On the principal component analysis of plant traits (Fig S1a), the first three components explain 71% of the total variance. The first PC is correlated to plant vegetative height, PC2 is correlated to leaf area and rooting depth and PC3 is correlated to specific leaf area and leaf copper content. Although intra-population variability is large, the different sites occupy different portions of the multivariate space ($F=9.64$, $R^2=0.21$, $p<0.001$).

On the PCA of soil factors, the first component explains 41% of the variance and is correlated to soil Cu, P, Ca and Co content (Ac-EDTA extractable). The second component explains 22% of the total variance and is correlated to soil Ac-EDTA extractable Fe and Mg content (Fig S1b). The different populations also occupy different portions of the multivariate space ($F=15$, $R^2=0.30$, $p<0.001$).

To assess the multivariate correlations between soil factors and functional traits, we used a redundancy analysis (RDA). The global model computed using all traits and all soil factors explains 34.5% of the total variance (adjusted R^2 , Peres-Neto et al 2006) and is significant while tested with permutation of the residuals of the model (Anderson & Legendre 1999). The global model presents 4 significant axis ($p_{\text{perm}} < 0.001$). After removing the soil factors who showed too much multi-collinearity (Co and P), we selected the factors that contribute significantly to the RDA using a forward selection with two stopping criteria (Blanchet et al 2008). Five edaphic factors were retained, and explain together 27.2 % of the variability. In order of decreasing contribution (adjusted R^2): Cu, gravel content, Mn, Mg and Ca.

On figure S2, SLA is negatively correlated while Cu_{pl} and Co_{pl} are correlated positively with soil Cu content. However, soil Co and P content are also highly correlated with soil Cu content. Plant height, leaf area and rooting depth are negatively correlated with soil gravel content.

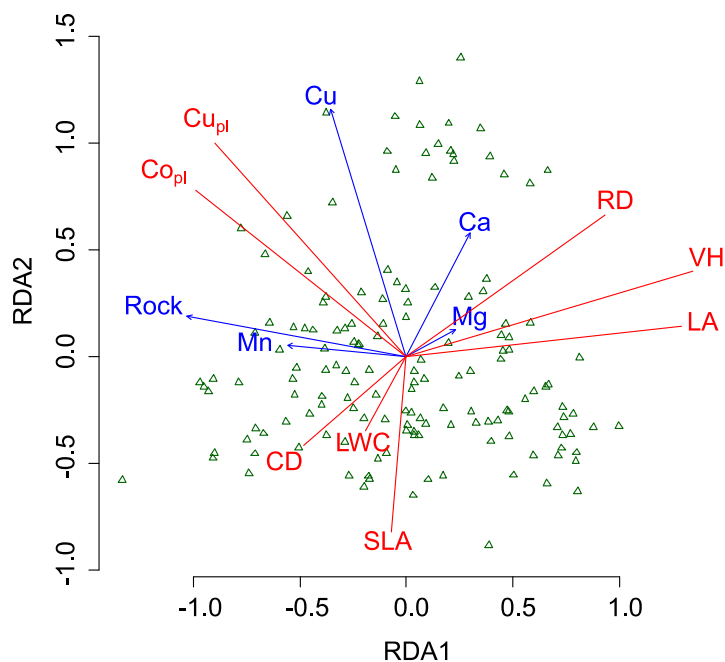


Figure S2: plot of the redundancy analysis on the 8 measured traits and the 5 significant soil parameters explaining 27% of the variation in functional traits. Each point represents an individual. Functional traits are represented by the red vectors and soil parameters by the blue vectors. VH: vegetative height, LA: leaf area, RD: rooting depth, SLA: specific leaf area, LWC: leaf water content, CD: clonal distance, Cu_{pl} : leaf copper content, Co_{pl} : leaf cobalt content, Rock: soil gravel content.

References

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- Blanchet, F. G., Legendre, P., & Borcard, D. (2008). Forward selection of explanatory variables. *Ecology* **89**: 2623-2632.
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Supplementary material S2: Regression tables for the 6 morphological and 6 chemical traits along the soil copper gradient.

	Goma			Orthodoxe			Fungurume			Zikule			Kinsevere		
	Slope	R ²	p	Slope	R ²	p	Slope	R ²	p	Slope	R ²	p	Slope	R ²	p
<i>VH</i>	-18.3	0.53	***	-6.94	0.47	***	7.8	0.75	***	1.34	0.1	ns	-4.66	0.47	*
<i>LA</i>	-1.1	0.48	***	-0.46	0.34	***	0.23	0.36	***	-0.11	0.12	ns	-0.18	0.14	ns
<i>RD</i>	-0.4	0.21	***	-0.07	0.14	*	0.38	0.62	***	0.04	0.12	ns	0.08	0.39	*
<i>SLA</i>	1.1	0.04	ns	-0.19	0	ns	-1.97	0.44	***	-2.14	0.52	***	-0.01	0	ns
<i>LWC</i>	0	0.11	*	0.01	0.07	ns	-0.01	0.38	***	-0.2	0.41	**	-0.01	0.08	ns
<i>CD</i>	0.5	0.14	**	0.18	0.02	ns	-0.1	0.03	ns	0.12	0.03	ns	-0.48	0.25	ns
<i>Cu_{pl}</i>	0.5	0.39	***	0.66	0.61	***	-0.09	0.07	ns	0.03	0.02	ns	0.4	0.62	**
<i>Co_{pl}</i>	2.1	0.72	***	1.27	0.59	***	0.69	0.59	***	-0.23	0.08	ns	-0.08	0.04	ns

Supplementary material S3: Within population coefficients of variation (a) and range (b) of the different traits

a)

	<i>Goma</i>	<i>Orthodoxe</i>	<i>Fungurume</i>	<i>Zikule</i>	<i>Kinsevere</i>
<i>Vegetative height</i>	0.80	0.88	0.86	0.34	0.41
<i>Leaf area</i>	1.06	0.74	0.50	0.47	0.52
<i>Rooting depth</i>	0.56	0.19	0.72	0.16	0.18
<i>Specific leaf area</i>	0.20	0.23	0.28	0.28	0.27
<i>Leaf water content</i>	0.02	0.02	0.02	0.03	0.03
<i>Clonal distance</i>	0.91	0.89	0.83	0.93	1.31
<i>Cu_{pl}</i>	1.01	0.80	0.43	0.23	0.66
<i>Co_{pl}</i>	1.51	1.34	1.27	2.02	0.54

b)

	<i>Goma</i>	<i>Orthodoxe</i>	<i>Fungurume</i>	<i>Zikule</i>	<i>Kinsevere</i>
<i>Vegetative height</i>	57.0	34.0	34.0	32.5	25.5
<i>Leaf area</i>	123	24.9	31.2	26.5	15.4
<i>Rooting depth</i>	18.0	4.0	18.0	3.0	3.0
<i>Specific leaf area</i>	15.9	16.2	19.9	11.5	15.5
<i>Leaf water content</i>	9.1	11.1	8.5	10.2	11.3
<i>Clonal distance</i>	59.0	50.0	26.0	17.0	22.0
<i>Cu_{pl}</i>	257	135	242	21.1	175
<i>Co_{pl}</i>	999	670	1686	322	13.4