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1 **Copper phytoavailability in vineyard topsoils as affected by pyoverdine supply**

2

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26 **Abstract**

27 Pyoverdine (Pvd) is a bacterial siderophore produced by some *Pseudomonads* species
28 that can bind copper in addition to iron in soil. Pvd is expected to alter the dynamics
29 and the ecotoxicity of Cu in vineyard soils. This study investigated the extent to
30 which the mobility and the phytoavailability of Cu varied among vineyard soils with
31 different pH and how they were affected by a supply of Pvd. Pvd was supplied (or
32 not) to ten vineyard topsoils with pH ranging from 5.9 to 8.6 before metal was
33 extracted with 0.005 M CaCl₂. Cu mobility was assessed through its total
34 concentration and Cu phytoavailability through its free ionic concentration measured
35 in the CaCl₂ extract. Cu mobility varied by a factor of six and Cu phytoavailability by
36 a factor of 5000 among the soil samples. In the CaCl₂ extract, the concentration of
37 Cu²⁺ was not correlated with the concentration of total Cu but was correlated with pH.
38 This revealed that Cu phytoavailability depends to a great extent on Cu complexation
39 in soil pore water, the latter being highly sensitive to pH. Adding Pvd enhanced the
40 mobility of Cu in the soils including in carbonate soils. The Pvd-mobilization factor
41 for Cu varied from 1.4 to 8 among soils, linked to the availability of Fe and Al in the
42 solid phase and to Pvd partitioning between the solid and the liquid phase. Adding
43 Pvd reduced the concentration of Cu²⁺ in CaCl₂ extract, which challenges the idea of
44 using Pvd-producing bacteria to promote Cu phytoextraction.

45

46 **Keywords**

47 Metals, siderophores, CaCl₂ extract, complexation, ion-selective electrode,
48 bioaugmentation.

49

50

51 **1. Introduction**

52 Copper-based fungicides such as the Bordeaux mixture are applied to the surface of
53 vine leaves before each rainy episode to control downy mildew caused by
54 *Plasmopara viticola*. After each rainfall event, part of the Cu applied is washed off
55 and accumulates in the soil, especially in the topsoil where its concentration can reach
56 several hundred mg Cu kg⁻¹ soil dry weight in old vineyards (Komarek et al., 2010).
57 Although the amount of Cu applied as Cu-based fungicide has markedly decreased
58 over the past century, completely replacing Cu in viticulture is difficult to envisage in
59 the medium term (Andrison et al., 2018), especially in organic viticulture where the
60 use of synthetic fungicides is prohibited. In 2018, the European Union renewed the
61 authorization to use active substance copper compounds in agriculture for seven years
62 (EU 2018/1981). Cu contamination of vineyard soils will therefore continue.

63 Copper is an essential micronutrient for all living organisms. In plants, Cu is an
64 essential cofactor of numerous proteins involved in the control of the cellular redox
65 state, the transport of electrons in chloroplasts and mitochondria, and remodeling of
66 the cell wall (Printz et al., 2016). However, excess Cu in planta has a detrimental
67 effect on plant growth and may even jeopardize survival. Photosynthetic reactions are
68 among the most important sites of inhibition by Cu (see Kupper et al., 2009 for a
69 description of the mechanisms of “shade” and “sun” reactions caused by excess Cu).
70 Because Cu is redox active, its excess can also lead to the generation of reactive
71 oxygen species (ROS) thereby damaging biomolecules (DNA, proteins). Whether Cu
72 is phytotoxic at the concentrations and in the forms in which it is present in vineyard
73 topsoils remains unclear. While Cu contamination is often evoked as a reason for the
74 reduced growth of grapevine plants after planting and/or the slow establishment of
75 cover crops in interrows observed in some vineyards, direct evidence for Cu

76 phytotoxicity has very rarely been reported *in situ*. In our opinion, this is because (i)
77 plant phenotypes associated with Cu toxicity are not specific, (ii) Cu phytotoxicity is
78 *a priori* related to the concentration of Cu²⁺ in soil pore water (Sauve et al., 1996) and
79 not to the indicators of Cu bioavailability proposed by soil analysis laboratories that
80 are based on chemical extraction methods, and (iii) Cu phytotoxicity is not necessarily
81 direct but may result from induced nutritional deficiency, for example iron deficiency,
82 as observed by Michaud et al. (2007) in durum wheat cultivated in former vineyard
83 soils. Consequently, we need to better understand the determinism of Cu phytotoxicity
84 in vineyard soils.

85 Siderophores are organic chelators (150-2000 Da) released by some soil bacteria (e.g.
86 *Pseudomonads*, *Streptomyces*) to meet their Fe requirements under Fe starvation
87 (Visca et al., 2007). Although siderophores are generally considered as biological
88 agents of Fe uptake, they may also play significant roles in the biogeochemical
89 cycling and biological uptake of other metals, notably Cu. Indeed, Cu is the divalent
90 transition metal for which siderophores exhibit the highest affinity (Cornu et al.,
91 2017b). In addition, the presence of Cu in the surrounding medium has been shown to
92 enhance bacterial production of siderophores (Braud et al., 2010). Promoting the
93 colonization of siderophore-producing bacteria in vineyard topsoils could therefore
94 help manage Cu phytoavailability and phytotoxicity. Recent studies on siderophores
95 by our team (Cornu et al., 2014; 2017b) revealed that pyoverdine (Pvd) was the most
96 likely siderophore to affect the mobility of Cu in the soil. To gain insight into how
97 Pvd-producing bacteria alter the phytoavailability and the phytotoxicity of Cu in
98 vineyard topsoils, we need to investigate how Pvd affects the speciation of Cu in soil
99 pore water and how excess Cu impairs the Pvd-induced mobilization of Fe, notably in
100 carbonate soils.

101 The first objective of this work was thus to shed light on the phytotoxicity of Cu in
102 vineyard soils by measuring the Cu^{2+} concentration in the pore water of a series of
103 vineyard topsoils, in order to better understand its variability and determinism and to
104 compare it with Cu^{2+} phytotoxicity thresholds. The second objective was to evaluate
105 the potential role of Pvd-producing bacteria in the remediation of Cu-contaminated
106 soils by disentangling the effect of a supply of Pvd on the dynamics (mobility,
107 phytoavailability) of Cu and Fe in the same series of soils.

108

109 **2. Materials and Methods**

110 2.1 Soil samples

111 Ten Cu-contaminated vineyard topsoils (0-20 cm) were used in this study. The
112 samples were collected in 2012 in the AOC (i.e. registered designation of origin) zone
113 of Pessac-Léognan (immediately south of Bordeaux, France). Soil samples were air
114 dried and sieved to 2 mm before analysis. Table 1 summarizes the results of the soil
115 analyses conducted by a soil-testing laboratory (LAS, INRA Arras, France), using
116 standard methods. All the soil samples had total Cu contents of $117 \pm 22 \text{ mg kg}^{-1}$ and
117 EDTA-extractable Cu contents (Cu_{EDTA}) ranging from 53 to 132 mg kg^{-1} . The soil
118 samples had a coarse texture due to their high sand fraction and rather low organic
119 matter (OM) content ($< 3\%$) as well as a low cation exchange capacity ($< 10 \text{ cmol}_{\text{c}}+$
120 kg^{-1}), except soil H. The soil samples differed mainly in their pH, which ranged from
121 5.9 in soil A to 8.6 in soil J. Five of the ten samples were carbonate soils (soils F to J),
122 the five others (soils A to E) were not.

123

124 2.2 Pyoverdine

125 2.2.1 Production, purification and characterization

126 *Pseudomonas fluorescens* (ATCC 13525) was used to produce Pvd. First, bacteria
127 were grown overnight at 25 °C in half-strength Tryptic Soy Broth (TSB 1/2) under
128 shaking at 200 rpm. The bacterial cells were then centrifuged, washed twice with
129 sterilized KCl (9 g L⁻¹) and inoculated in modified Dworkin & Foster (DF) medium
130 with no iron (see composition in suppl. Table T1). To increase the yield of Pvd, DF
131 cultures were grown for seven days at 25 °C under shaking (200 rpm), in a fed-batch
132 mode. Culture was stopped after seven days by adding 2% trichloroacetic acid to
133 precipitate cells that were then eliminated by centrifugation for 4 min at 7650 g. Next,
134 the Pvd-containing supernatant was filtered successively through 1.6-, 1.2- and 0.7-
135 µm fiberglass filters and adjusted to pH 6 before being purified in a two-step
136 procedure. First, the Pvd-containing supernatant was put in contact with a
137 hydrophobic polyaromatic resin (Amberlite® XAD-4) at a ratio of 100 mL of resin per
138 liter of supernatant for 24 h at 4 °C. After each contact (n=4), the resin was recovered
139 by filtration through a sintered glass and the Pvd was eluted from the resin with 100%
140 methanol and concentrated by evaporation. The Pvd concentrate was then loaded onto
141 a C₁₈ column (Lichroprep® RP-18) with a capacity of 20 mL (Ø 1.8 cm, height 8 cm),
142 eluted with methanol/H₂O 70/30 (v/v) and concentrated by evaporation prior to
143 storage at -20 °C.

144 Purified Pvd was analyzed by HILIC-ESI-MS following the protocol of Flis et al.
145 (2016), (for details, see supplementary data). Three major Pvd isoforms were
146 identified with the molecular formula C₄₉H₇₃N₁₅O₁₈, C₄₉H₇₂N₁₄O₁₉ and C₅₀H₇₄N₁₄O₂₀
147 and a molecular weight of 1159.52, 1160.50 and 1189.52 Da, respectively. This agrees
148 with the mean mass proposed by Budzikiewicz (2004) for the Pvd isoforms produced
149 by the strain ATCC 13525 of *P. fluorescens*. The semi-structural formulae of the three
150 isoforms are shown in suppl. Fig. F1.

151

152 2.2.2 Preparation and quantification

153 Pvd stock solution (1 mM) was prepared 24 h prior to use by diluting lyophilized Pvd
154 in ultra-pure water. The concentration of Pvd in the stock solution was assessed by
155 measuring absorbance at 400 nm ($\epsilon = 19\,000\text{ M}^{-1}\text{ cm}^{-1}$ for Pvd at pH 7) (Albrecht-
156 Gary et al., 1994) in an aliquot adjusted to pH 7 by adding phosphate buffer. The
157 concentration of Pvd in the soil extraction solution (see section 2.3.) was assessed by
158 measuring the difference in absorbance at 380 nm between the Pvd+ and the Pvd-
159 treatment in each soil. The measurement was made at 380 nm (Helios Epsilon,
160 Thermo Spectronic) because Pvd absorbance is not sensitive to pH changes at this
161 wavelength (Moll et al., 2008). Beforehand, standard additions of Pvd (0, 33, 66 μM)
162 were made and absorbance was recorded at 380 nm to determine the molar extinction
163 coefficient (ϵ) of Pvd in the extraction solution for each soil. In eight out of the ten
164 soils, a ϵ value of $14\,000\text{ M}^{-1}\text{ cm}^{-1}$ led to a recovery rate of more than 95% between
165 the theoretical and measured Pvd concentrations. For soils E and H, whose organic
166 matter content was higher than the eight others, the ϵ value was set at $12\,500\text{ M}^{-1}\text{ cm}^{-1}$.
167 ¹.

168

169 2.3 Soil extraction procedure

170 Soil samples were rewetted to 90% WHC and equilibrated for two weeks at 20 °C
171 before being used for the assay. This delay avoids working during the full microbial
172 flush, which usually follows soil rewetting and affects the dynamics of metals in soils
173 (Cornu et al., 2007). Small disks of wet soil (5 g DW soil, 3 mm thick, 32 mm in
174 diameter) placed in plastic lids were supplied with Pvd (Pvd+) or not (Pvd-) and
175 equilibrated for 24 h at 20 °C. Pvd was supplied at a concentration of $200\text{ }\mu\text{mol kg}^{-1}$

176 soil DW by adding 1 mL from a 1 mM Pvd stock solution (replaced by ultra-pure
177 water in the controls) to the surface of soil disks. In each treatment, metal extraction
178 was performed as follows: 5 g of wet soil were shaken with 9 mL of 0.005 M CaCl₂.
179 The concentration in the CaCl₂ extract was set at 0.005 M (instead of 0.01 M) to
180 better mimic the extraction power of the soil pore water. As described in Cornu et al.
181 (2014), all the suspensions were agitated for 2 h at 20 °C, centrifuged at 5,000 *g* for
182 10 min and the supernatant was filtered through 0.2 µm cellulose acetate filters. After
183 the pH and the concentration of Pvd were measured (see below), the 10 mL extraction
184 solution was divided into two subsamples: one stabilized with 2% HNO₃ (v/v) and
185 used to measure the total metal concentrations and one stabilized with 1mM NaN₃ to
186 measure free Cu activity. The two subsamples were stored at 4 °C until analysis. Each
187 treatment was conducted in triplicate.

188

189 2.4 Analyses of extraction solutions

190 pH and the concentration of Pvd were measured directly after soil extraction. pH was
191 measured using a combined microelectrode (E16M331, Radiometer Analytical) and
192 the concentration of Pvd using the protocol described in section 2.2.2. Total
193 concentrations of Al, As, Cd, Cu, Fe, Mo, Ni, Pb, Si and Zn were determined by ICP-
194 MS (7700x, Agilent Technologies) at the central analytical department of the
195 University of the Basque Country (UPV/EHU, Bilbao, Spain) from a subsample
196 acidified with 2% HNO₃. Free ionic Cu activity was measured using a cupric ion-
197 selective electrode (ISE, 9629BNWP, Thermo Scientific Orion) according to the
198 procedure described in Djae et al. (2017). Briefly, the ISE was calibrated daily within
199 the 6.6 to 11.2 range of pCu (= -log₁₀ a(Cu²⁺)) in Cu solutions buffered with
200 iminodiacetic acid and potassium phthalate. The calibration curve ($r^2= 0.992$, $n= 50$)

201 showed 85% slope efficiency compared with the theoretical Nernstian slope (suppl.
202 Fig. F2). The concentration of free ionic Cu^{2+} was calculated from pCu and the Cu^{2+}
203 activity coefficient. The latter was given by the extended Debye-Hückel equation
204 found in Ritsema (1993), assuming the ionic strength of all the extraction solutions
205 was fixed by the 0.005 M CaCl_2 background electrolyte.

206

207 2.5 Statistical analyses

208 Data were processed using SYSTAT 11 Edition 2004 (SPSS Inc., 233S. Wacker Drive,
209 Chicago, USA). Principal component analysis (PCA) was used to study groups of
210 correlations among the variables monitored in the CaCl_2 extract. Two-way ANOVA
211 was performed on parameters monitored in the CaCl_2 extract to assess the significance
212 of treatment effects (soil, Pvd supply) and their interaction. Tukey's HSD tests were
213 performed at 5% to identify the parameters monitored in the CaCl_2 extract that were
214 significantly affected by the supply of Pvd in each soil.

215

216 **3. Results and Discussion**

217 3.1 Variability of Cu mobility and phytoavailability among vineyard topsoils

218 The first objective of this work was to quantify the extent to which the mobility and
219 the phytoavailability of Cu varies among vineyard topsoils with the same total Cu
220 content. To this end, Cu dynamics was analyzed in the control soils (Pvd-). Copper
221 mobility and phytoavailability were assessed, respectively, through the concentrations
222 of total Cu and free ionic Cu measured in the CaCl_2 extract. CaCl_2 extract is usually
223 recommended as a reasonable surrogate for soil pore water (Bravin et al., 2010; Sauve
224 et al., 1997; Kalis et al., 2007), in which plant roots are assumed to take up nutrients
225 (Cu included) mainly in the free ionic form (FIAM; Campbell, 1995). Figure 1a

226 shows that the total Cu concentration in CaCl₂ extract of control soils ranged from
227 1.44 μM (soil J) to 8.75 μM (soil E), i.e. by a factor of six. This variability was
228 already greater than that displayed by Cu_{EDTA} (Table 1). Figure 1b shows that the
229 concentration of Cu²⁺ in the CaCl₂ extract of the control soils varied from 0.06 nM
230 (soil H) to 300 nM (soil A), i.e. by a factor of 5000. Cu phytoavailability thus varied
231 by several orders of magnitude in vineyard topsoils with almost the same EDTA-
232 extractable Cu content (Table 1). No relationship was found between Cu_{EDTA} and the
233 Cu²⁺ concentration in the CaCl₂ extract (p= 0.246, n= 10). Taken together, these
234 results suggest that the current indicators of Cu availability based on Cu extraction
235 with organic complexing agents do not reliably and sensitively detect the risks of Cu
236 phytotoxicity in vineyard soils. In addition, the Cu²⁺ concentration in CaCl₂ extract
237 measured in the soils A and B were of the same order of magnitude as those that led to
238 a 75% reduction (EC₇₅) and a 50% reduction (EC₅₀), respectively, in the relative root
239 growth of durum wheat (Bravin et al., 2010). Assuming that the concentration of Cu²⁺
240 in the CaCl₂ extract is a good proxy of the one in pore water, this result suggests that
241 there is a risk of Cu phytotoxicity in some vineyard soils, at least for graminaceous
242 species, which are particularly sensitive to excess Cu (Michaud et al., 2007; Bravin et
243 al., 2010).

244 The concentration of Cu²⁺ was not correlated with the concentration of total Cu in the
245 CaCl₂ extract (p= 0.883), as underlined by the factor loading plot in Fig. 2. This
246 means that Cu phytoavailability was completely decorrelated from Cu mobility in the
247 ten soils analyzed in this study. This decorrelation shows that the soil properties that
248 determine the mobility of Cu in soil may differ from those that determine its
249 phytoavailability. Therefore, the vineyard soils in which Cu could migrate to the
250 aquifer (if any) may differ from soils in which Cu is phytotoxic. The decorrelation

251 between total Cu and free ionic Cu shows that Cu phytoavailability depends to a large
252 extent on its speciation in pore water. It is thus essential to better identify the drivers
253 of Cu complexation in pore water to improve prediction of Cu phytoavailability in
254 vineyard soils.

255

256 3.2 Soil pH as the main driver of Cu phytoavailability?

257 According to the literature, Cu mobility in soils is primarily related to their pH,
258 organic matter content and, in some cases, to their total Cu content (Sauve et al.,
259 1997). This is why Cu mobility in vineyard soils is usually controlled by liming
260 and/or by the supply of exogenous OM (Brunetto et al., 2016). The present study was
261 carried out on soils with the same total Cu content and with similar texture and OM
262 content (Table 1). Only pH varied among the soils but no correlation was found
263 between Cu mobility and soil pH. Indeed, the concentration of total Cu was not
264 correlated with the concentration of the proton ($p= 0.899$) in the CaCl_2 extract, as
265 underlined by the factor loading plot in Fig. 2. This decorrelation is also evidenced in
266 Fig. 1 where the concentration of total Cu in the controls (Pvd-) did not decrease from
267 the left to the right, i.e. with the rise in pH. By contrast, the concentration of total Cu
268 in the CaCl_2 extract was closely linked to that of Fe and, to a lesser extent, to that of
269 Al, Ni and Pb (Fig. 2, suppl. Table T2). This suggests that the mobility of these four
270 elements depends on the same driving process(es) and hence on the same soil
271 properties in the ten vineyard soils analyzed in this study. We assume that Cu was
272 mobilized mainly by complexolysis while Zn and Cd were mobilized mainly by ion
273 exchange, in agreement with previous works (Al-Wabel et al., 2002; Cornu et al.,
274 2017a). This would explain why the concentrations of Zn and Cd were correlated with
275 each other and with the concentration of protons and were not correlated with the

276 concentration of total Cu in the CaCl₂ extract (Fig. 2, suppl. Table T2).

277 In contrast to Cu mobility, Cu phytoavailability was closely linked to soil pH, as
278 underlined by the strong positive correlation ($r= 0.81$, $p< 0001$) between the
279 concentrations of Cu²⁺ and H⁺ in the CaCl₂ extract (Fig. 2, suppl. Table T2). As
280 expected, the Cu²⁺ concentration decreased with an increase in the pH in the CaCl₂
281 extract (Fig. 3a) because Cu complexation is more pronounced at alkaline pH (Bravin
282 et al., 2009). One originality of the present work is that we quantified the free ionic
283 fraction of Cu in the CaCl₂ extract and show that it varied between 0.002% and 10%
284 among the vineyard soils, mainly depending on their pH (Fig. 3b). The fact that the
285 vast majority of Cu was complexed in pore water at a pH > 6 is in line with the
286 literature (Vulkan et al., 2000; Nolan et al., 2003; Bravin et al., 2009; Ren et al., 2015)
287 and results from the strong affinity exhibited by DOM, notably by humic substances,
288 towards Cu (Milne et al., 2003). The fact that the free ionic fraction of Cu in the
289 CaCl₂ extract was almost zero in carbonate soils is a new finding that suggests that the
290 DOM affinity for Cu is particularly high at alkaline pH and/or that soluble Cu is also
291 held by inorganic ligands (e.g. HCO₃⁻/CO₃²⁻) or inorganic colloids (< 0.2 μm) in these
292 soils. Although perhaps overestimated in this work, in which the soils had contrasting
293 pH but similar OM contents, the link between Cu phytoavailability and pH underlines
294 the need to systematically monitor this parameter during soil analysis. The
295 relationship shown in Fig. 3a suggests that the soil pH should be kept above 6.0 to
296 prevent Cu phytotoxicity. In addition to liming, in Cu-contaminated soils, it is
297 important to reason agricultural practices according to the effect they have on soil pH,
298 especially at the soil-root interface. In this respect, special attention should be paid to
299 nitrogen fertilization, which in some soils, may lead to pH changes of more than one
300 pH unit at the soil-root interface depending on the form of nitrogen supplied (nitric or

301 ammonium) (Chaignon et al., 2002) and exactly where it is applied (Marschner,
302 2012).

303

304 3.3 Effect of Pvd on Cu mobility

305 During complexolysis, complexes are formed at the surface of metal-bearing phases
306 between metals and ligands, which facilitates the release of metals from the solid
307 phase (Brandl and Faramarzi, 2006; Cornu et al., 2017b). This is why the mobility of
308 Cu in soil is assumed to be driven mainly by the concentration of Cu-ligands in the
309 liquid phase. Hence, increasing the concentration of soluble Cu-ligands, for instance,
310 by supplying Pvd is expected to increase the mobility of Cu in soils. This was
311 demonstrated in our previous study (Cornu et al., 2014) in which the mobility of Cu in
312 a calcareous soil increased almost linearly with the dose of Pvd supplied. The present
313 work adds two new insights. First, it shows that the addition of Pvd increases Cu
314 mobility in a wide range of soils. The concentration of total Cu in the CaCl₂ extract
315 was always higher in soils supplied with Pvd (Pvd+) than in the control soils (Pvd-)
316 (Fig. 1a). In this respect, Pvd differs from hydroxamate siderophores such as DFOB,
317 whose specificity for trivalent metals, mainly Fe(III), is too high to increase the
318 mobility of Cu in Fe-rich matrices such as soils (Neubauer et al., 2000; Cornu et al.,
319 2017a). The pH of the CaCl₂ extract was also impacted by the addition of Pvd (suppl.
320 Fig. F3) but not in carbonate soils and not in the sense of acidification, so it does not
321 question the process of “stimulated complexolysis” by which Pvd is assumed to
322 mobilize Cu. The Pvd-induced increase in Cu mobility was observed in both
323 carbonate (F-J) and non-carbonate (A-E) soils. This point is crucial since the bacterial
324 production of siderophore (and consequently of Pvd) is assumed to occur only in
325 carbonate soils, where the availability of Fe is low.

326 Copper was not the only metal whose mobility was affected by the addition of Pvd.
327 As expected, the mobility of Fe and Al was also enhanced in presence of Pvd (Fig. 3),
328 in agreement with the high affinity of Pvd, and more generally of siderophores, for
329 trivalent metals (see above). Surprisingly, Pvd addition significantly altered ($p < 0.05$)
330 the mobility of several other elements, among which Mo, Ni and Si (suppl. Table T3).
331 Adding Pvd enhanced the mobility of Mo and Ni in almost every soil. In the case of
332 Ni, this probably results from a “stimulated complexolysis” in presence of Pvd, given
333 the relatively high affinity of Pvd for Ni(II) ($\log K = 10.9$; Ferret et al., 2014). In the
334 case of Mo, this is most likely the result of the Pvd-induced alkalization (suppl. Fig.
335 F3) since Mo mobility usually increases with an increase in pH (Xu et al., 2006). By
336 contrast, the addition of Pvd reduced Si mobility in all the soils analyzed in this study.
337 Given the short delay observed between Pvd addition and CaCl_2 extraction (24 hours),
338 we suspect that the reduction in Si mobility resulted from enhanced sorption of the
339 forms H_4SiO_4 and polysilicic acid in presence of Pvd, through a process that remains
340 to be determined. It is worth mentioning that the changes in Mo, Ni and Si mobility
341 observed (when observed) in presence of Pvd were of much smaller magnitude than
342 those observed in the mobility of Al, Fe and Cu.

343 This work also highlights the fact that the extent to which Pvd addition affects the
344 mobility of Cu varies widely depending on the type of soil. This hypothesis was
345 reinforced by the highly significant interaction ($p < 0.001$) between the effects of Pvd
346 and soil evidenced by the two-way ANOVA (suppl. Table T3) for the concentration of
347 total Cu in the CaCl_2 extract. The Pvd mobilization factor, calculated as the ratio of
348 the total Cu concentration in the CaCl_2 extract in the presence of Pvd to that in the
349 absence of Pvd, ranged from 1.4 in the soil E to 8 in the soil G. The extent to which
350 Pvd affects the mobility of Cu in soil may rely, on the one hand, on the partitioning of

351 Pvd between the solid and the liquid phase. The more Pvd sorbs onto the soil
352 constituents, the less it affects the mobility of the metals it complexes. The amount of
353 Pvd measured in the CaCl₂ extract was used to calculate the fraction of Pvd sorbed
354 onto the solid phase by difference with the Pvd supplied. As shown in Fig. 5, the
355 fraction of Pvd sorbed onto the solid phase ranged from 73% to 96% among soils
356 (sorption positively correlated with their clay content). Therefore, Pvd is suspected to
357 most affect the mobility of Cu in vineyard soils with a coarse texture like soil A. The
358 strong affinity of Pvd for soil constituents recalls that observed for DFOB in our
359 previous work (Cornu et al., 2017a). Like DFOB, Pvd sorption onto the soil solid
360 phase may partly derive from electrostatic attraction since Pvd, as well as metal-Pvd
361 complexes, is in its zwitterionic form between pH 4 and 8 and is negatively charged
362 above pH 8. By homology with humic substances (Shen, 1999) and considering the
363 specificity of the Pvd molecule, the other mechanisms by which Pvd sorbs onto
364 mineral surfaces involve hydrogen bonding and cation bridging.

365 On the other hand, the extent to which Pvd affects the mobility of Cu in soil may rely
366 on the availability of Fe and Al in the solid phase, i.e. on the size of the kinetically
367 labile Fe and Al pools (Liu et al., 2017). As described above, Fe and Al are in
368 competition with Cu for complexation by Pvd. Both are in large excess in soils
369 compared to Cu, even in the most Cu-contaminated soils. However, the availability of
370 Fe and Al in the solid phase may differ considerably from one soil to another, notably
371 depending on the nature and associated strength of their bonds with the solid phase
372 and on whether they are embedded in the lattice structure of minerals or coated on soil
373 particles. We suspect that the availability of Fe and Al in the solid phase may strongly
374 influence the extent to which Pvd affects their mobility and hence the mobility of Cu.
375 Figure 6a shows the relationship between the amount of Pvd in the CaCl₂ extract and

376 the amount of Fe+Al+Cu mobilized from the solid phase in soils supplied with Pvd.
377 The slope close to one and the low (and almost non-significant) intercept of the
378 regression line suggest that all the Pvd was complexed with Fe, Al or Cu in the CaCl₂
379 extract. This result strengthens the hypothesis that Fe, Al and Cu were mobilized
380 exclusively by complexolysis and as 1:1 M-Pvd complexes in the Pvd+ treatment. It
381 also suggests that Pvd-induced alteration of Fe/Al oxides, if any, within 24 hours, did
382 not affect the mobility of Cu, even though Fe/Al oxides are Cu-bearing phases (Sipos
383 et al., 2008).

384 Pvd speciation in solution was calculated for each soil from the concentration of Pvd
385 measured in the Pvd+ treatment and from the difference in Fe, Al and Cu
386 concentrations measured between the Pvd+ and the Pvd- treatment, in the CaCl₂
387 extract. Figure 6b shows that Pvd was mainly associated with Fe in soils A, B and E,
388 with Al in soil D and with Cu in soils G to J, so that Pvd speciation in solution varied
389 considerably among the soils. We assume that Pvd was most associated with the metal
390 (among Al, Cu and Fe) whose relative availability in the solid phase was the highest.
391 Based on that assumption, Pvd is suspected to most affect the mobility of Cu in
392 vineyard soils in which the availability of Fe and Al in the solid phase is low, which is
393 the case of the carbonate soils G to J. Hence, despite its lower affinity for Pvd than
394 that of Fe and Al, Cu was the metal the most affected by Pvd addition in carbonate
395 soils. This finding is of particular interest because it suggests that Pvd-producing
396 bacteria may substantially alter the dynamics of Cu in these soils. In addition, it
397 supports the hypothesis that in carbonate soils, bacterial siderophores can be diverted
398 from their initial objective of mobilizing Fe to mobilize Cu. This idea was previously
399 suggested by Michaud et al. (2007) for phytosiderophores to explain why symptoms
400 of Fe deficiency were specifically observed in durum wheat grown on calcareous Cu-

401 contaminated soils.

402

403 3.4 Effect of Pvd on Cu phytoavailability

404 Figure 1b shows that adding Pvd significantly ($p < 0.05$) reduced the concentration of
405 Cu^{2+} in the CaCl_2 extract in all soils, by a factor of between 2 and 37 depending on
406 the soil. This reduction is related to the very principle of complexolysis, whose
407 driving force is the reduction in the equilibrium concentration of the free ionic form at
408 the soil-solution interface. In non-carbonate soils (especially in soils B and D), the
409 reduction in the Cu^{2+} concentration may also be linked to the slight alkalization
410 induced by adding Pvd (suppl. Fig. F3).

411 Hence, adding Pvd increased the concentration of Pvd-Cu but decreased the
412 concentration of Cu^{2+} in the CaCl_2 extract, regardless of the soil. Its effect on Cu
413 phytoavailability therefore depends on the contribution of the Cu-Pvd complex to
414 plant uptake of Cu. According to the FIAM model (Campbell, 1995), its contribution
415 is zero and adding Pvd undoubtedly reduced the phytoavailability of Cu in soils,
416 which calls into question the idea of using Pvd-producing bacteria to promote the soil-
417 to-plant transfer of Cu. However, there have been several reports of deviations from
418 the FIAM model, in which metal complexes also affected metal uptake by plant roots
419 (see Nowack et al., 2006 for a review). These deviations resulted either from the
420 dissociation of metal complexes within the diffusion layer (Degryse et al., 2006)
421 and/or from the passive uptake of non-dissociated metal complexes, such as metal-
422 EDTA (Schaidler et al., 2006), by plant roots. Two studies (Vansuyt et al., 2007;
423 Shirley et al., 2011) showed that plants are able to incorporate iron from Fe-Pvd and,
424 because Pvd was detected in planta, proposed that Fe-Pvd was at least partially
425 incorporated non-dissociated by plant roots. It should be noted that in these studies,

426 Fe-Pvd was supplied at concentrations $\geq 50 \mu\text{M}$ and was the only source of iron. To
427 date, no similar investigations have been conducted on Cu and so the ability of plants
428 to incorporate Cu from Cu-Pvd has never been demonstrated. Our previous study
429 (Cornu et al., 2014) showed that adding Pvd to a calcareous soil significantly
430 increased the uptake of Cu by barley and tomato. The contribution of Cu-Pvd to the
431 root uptake of Cu is therefore not zero. Based on the literature on Cd (Degryse et al.,
432 2006; Panfili et al., 2009), we believe that the contribution of Cu complexes depends
433 above all on their rate of Cu^{2+} release in the diffusion layer, i.e. on their lability.
434 Further investigations are planned to accurately assess the contribution of Cu-Pvd to
435 Cu uptake by plant roots and to estimate the dissociation rate of the Cu-Pvd complex,
436 for instance following the protocols described by Degryse et al. (2006) or Schneider
437 and Nguyen (2011). This will enable a more in depth understanding of the role played
438 by Pvd and Pvd-producing bacteria in the soil-to plant transfer of Cu, notably in
439 vineyard soils.

440

441 **Conclusion**

442 This study assessed differences in the mobility and the phytoavailability of Cu among
443 vineyard topsoils and how they were affected by a supply of pyoverdine. First, our
444 results showed that Cu phytoavailability may differ by a factor of 5000 among soils
445 with almost the same EDTA-extractable Cu content. This work thus highlights the fact
446 that the indicators of Cu availability based on Cu extraction by organic complexing
447 agents such as EDTA do not reliably and sensitively detect the risks of Cu
448 phytotoxicity in vineyard soils. Instead, we propose to use indicators that account for
449 the speciation of Cu in solution, such as the concentration of Cu^{2+} in CaCl_2 extract.
450 Second, our results showed that the complexation of Cu in solution and hence the

451 phytoavailability of Cu are very sensitive to pH. This work thus underlines the need
452 for proper management of pH in vineyard soils, notably in the topsoil, to limit plant
453 exposure (whether by the grapevine itself or by the green manure sown between the
454 rows) to Cu. Third, our results showed that adding Pvd systematically enhances the
455 mobility of Cu in vineyard soils, including in carbonate ones. Hence, Pvd-producing
456 bacteria are expected to affect the mobility of Cu in carbonate soils. In this regard, our
457 results highlight the fact that Pvd can mobilize more Cu than Fe in carbonate soils,
458 thereby strengthening the hypothesis that, in these soils, excess Cu can disrupt the
459 iron acquisition processes set up by organisms (plants, bacteria, fungi) and lead to a
460 Cu/Fe nutritional imbalance. Finally, our results showed that adding Pvd reduced the
461 concentration of Cu^{2+} in the CaCl_2 extract. This raises the question of the contribution
462 of the Cu-Pvd complex to root uptake of Cu as a central issue. We thus now need to
463 quantify the contribution of the Cu-Pvd complex to root uptake to better understand
464 how Pvd-producing bacteria may affect the soil-to-plant transfer of Cu and, thereby,
465 help in the phytomanagement of Cu-contaminated soils.

466

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476

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Figure captions

Figure 1. Concentrations of total Cu (a) and free ionic Cu (b), in 0.005 M CaCl₂ extract from ten Cu-contaminated vineyard topsoils supplied with pyoverdine (Pvd+) or not (Pvd-). For each soil, *, ** and *** indicate that the total Cu concentration or free ionic Cu concentration measured in the presence of Pvd differed significantly from the one measured in the control (absence of Pvd), at the probability level of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. The error bars stand for mean standard deviations and ns stands for non-significant. The red dotted lines in (b) represent the Cu²⁺ phytotoxic thresholds reported for durum wheat by Bravin et al. (2010).

Figure 2. Factor-loading plot for the first two components identified in the principal component analysis of the characteristics of the 0.005 M CaCl₂ extract from ten Cu-contaminated vineyard topsoils. The variables are total concentrations of Al, As, Fe, Cd, Cu (Cu_{tot}), Mo, Ni, Pb, Si and Zn, and concentrations of H⁺ and Cu²⁺.

Figure 3. Free ionic Cu concentration (a) and free ionic Cu fraction (b) as a function of pH, in 0.005 M CaCl₂ extract from ten Cu-contaminated vineyard topsoils. In (a) and (b) the Y-axis scale is logarithmic and the correlation coefficient (r) shown is for the log-log relationship.

Figure 4. Concentrations of total Fe (a) and total Al (b), in 0.005 M CaCl₂ extract of ten Cu-contaminated vineyard topsoils supplied with pyoverdine (Pvd+) or not (Pvd-). For each soil, *, ** and *** indicate that the Fe concentration (a) or Al concentration

(b) measured in the presence of Pvd differed significantly from the one measured in the control (absence of Pvd), at the probability level of $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. The error bars stand for mean standard deviations and ns stands for non-significant.

Figure 5. Pvd sorption onto the soil solid phase as a function of soil clay content (%), in ten Cu-contaminated vineyard topsoils supplied with pyoverdine. Each point corresponds to one soil. Pvd sorption values are means of three replicates.

Figure 6. Relationship between the amount of Pvd in 0.005 M CaCl₂ extract and the amount of Fe+Al+Cu mobilized from the solid phase (a) and Pvd speciation in 0.005 M CaCl₂ extract (b), in the ten Cu-contaminated vineyard topsoils supplied with Pvd. Pvd speciation was calculated from the Pvd concentration and the Pvd-induced increase in Fe, Al and Cu concentrations measured in 0.005 M CaCl₂ extract in the Pvd+ treatment, based on the relationships shown in (a).

Figure 1

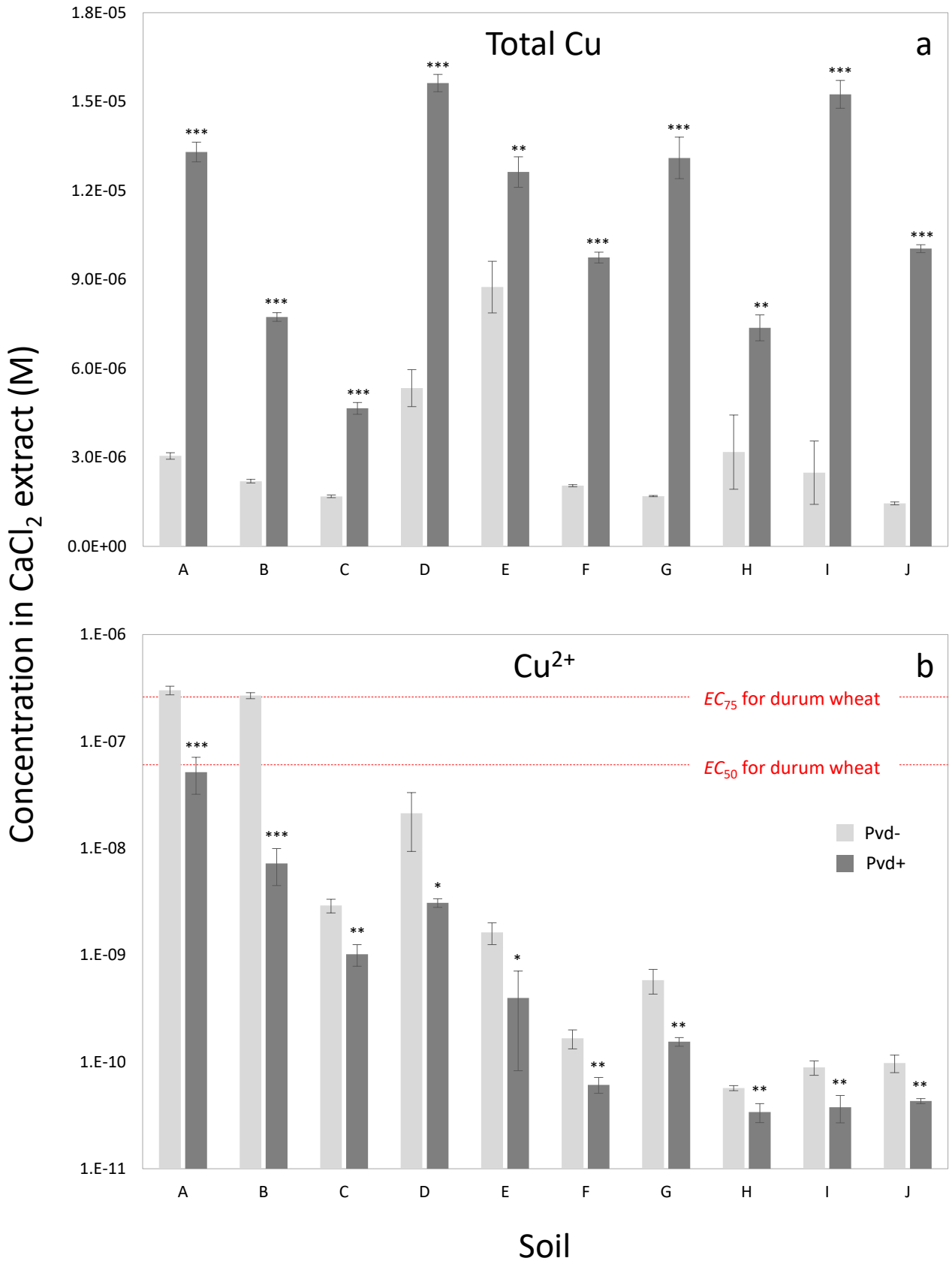


Figure 2

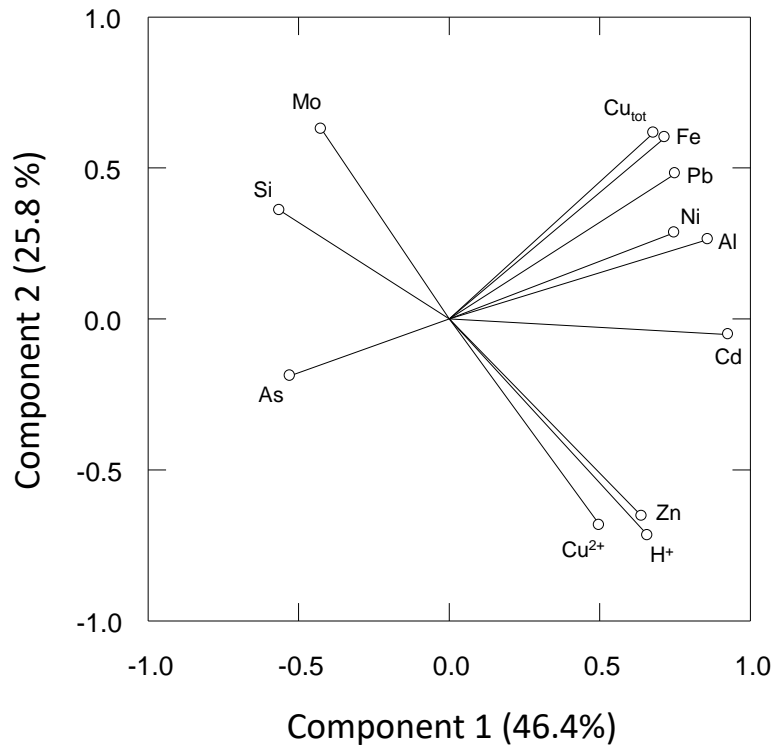


Figure 3

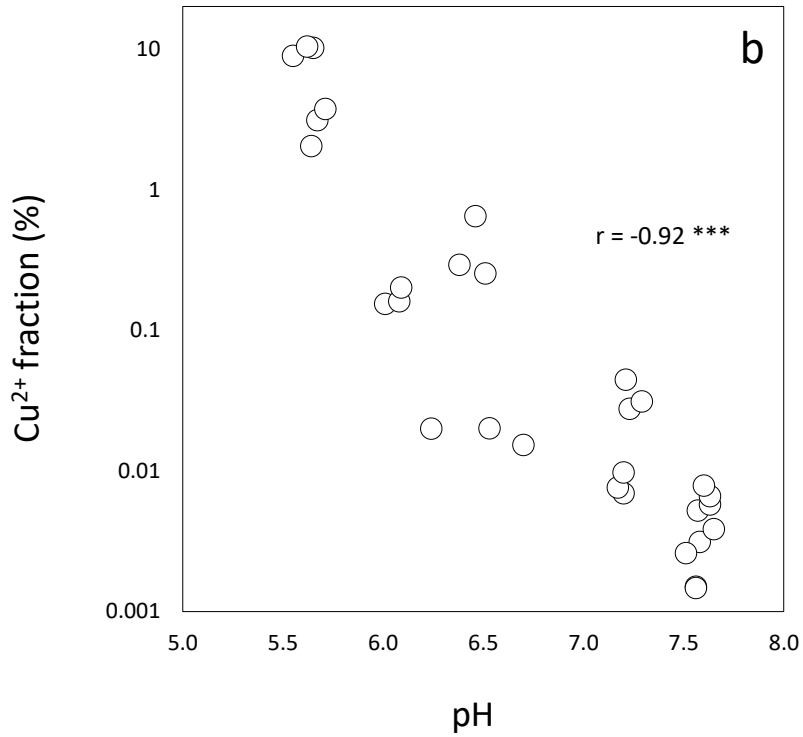
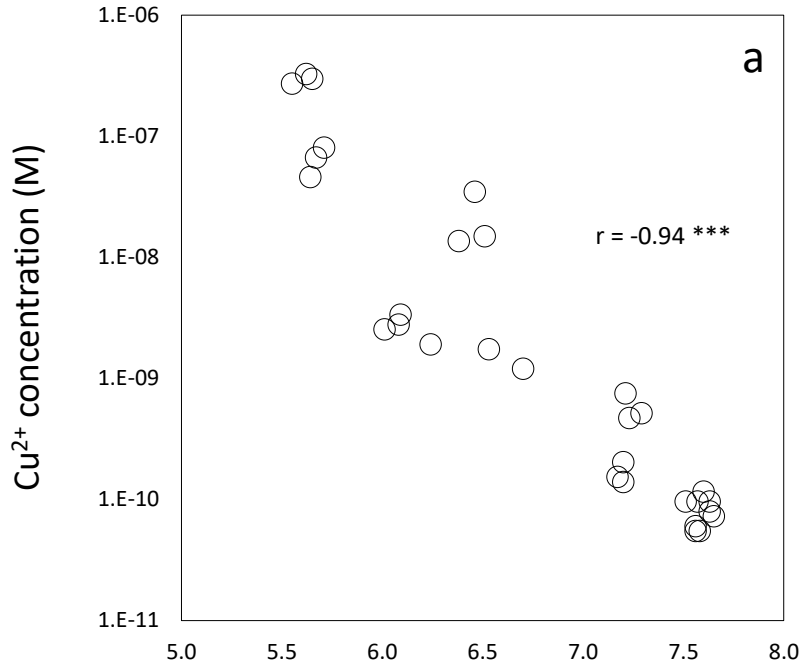


Figure 4

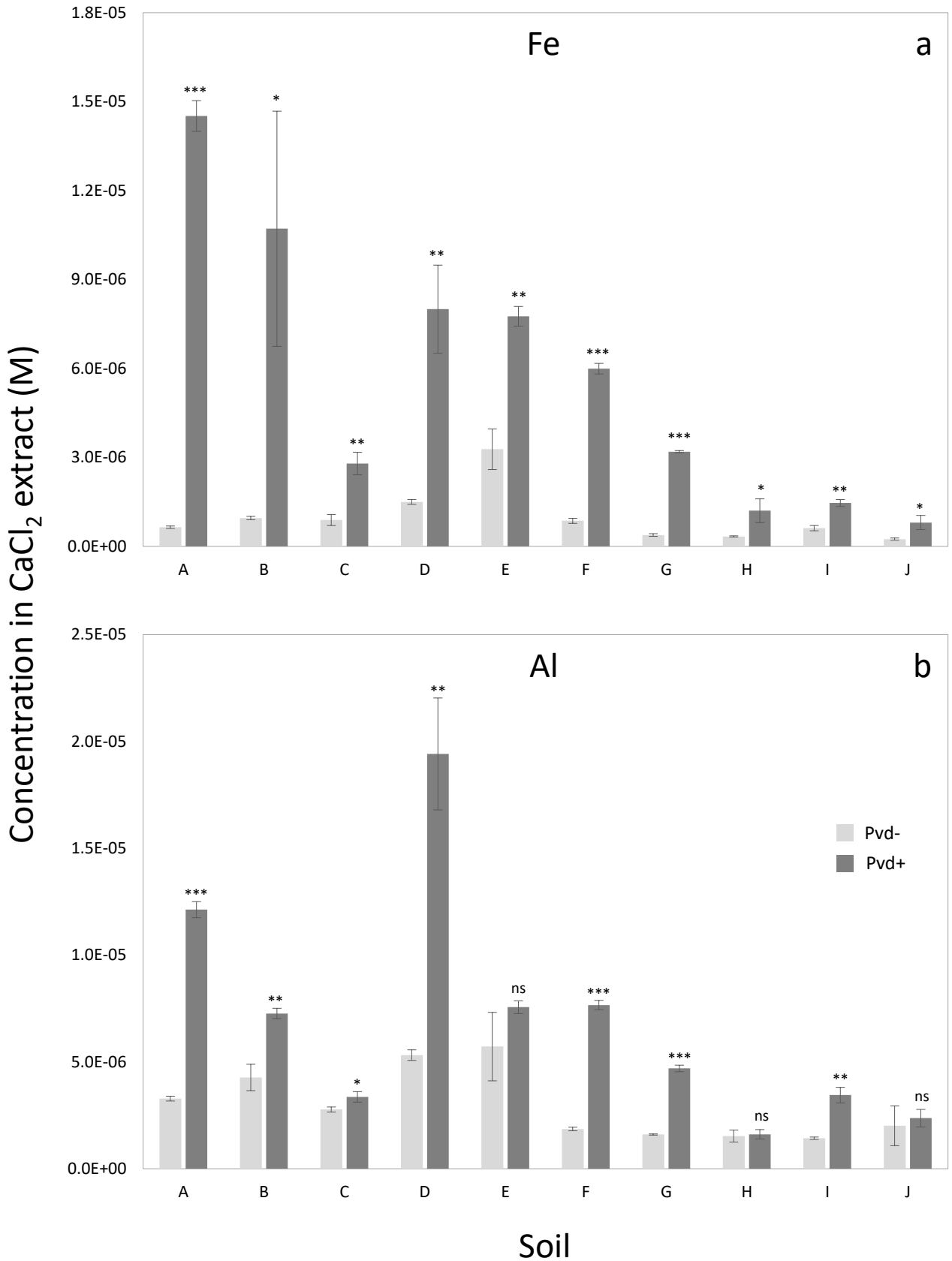


Figure 5

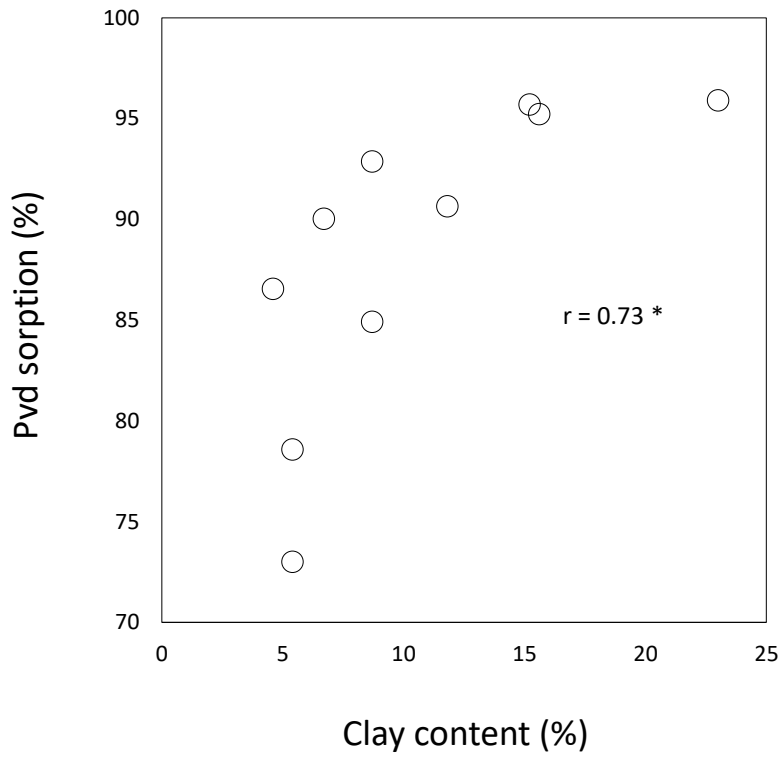


Figure 6

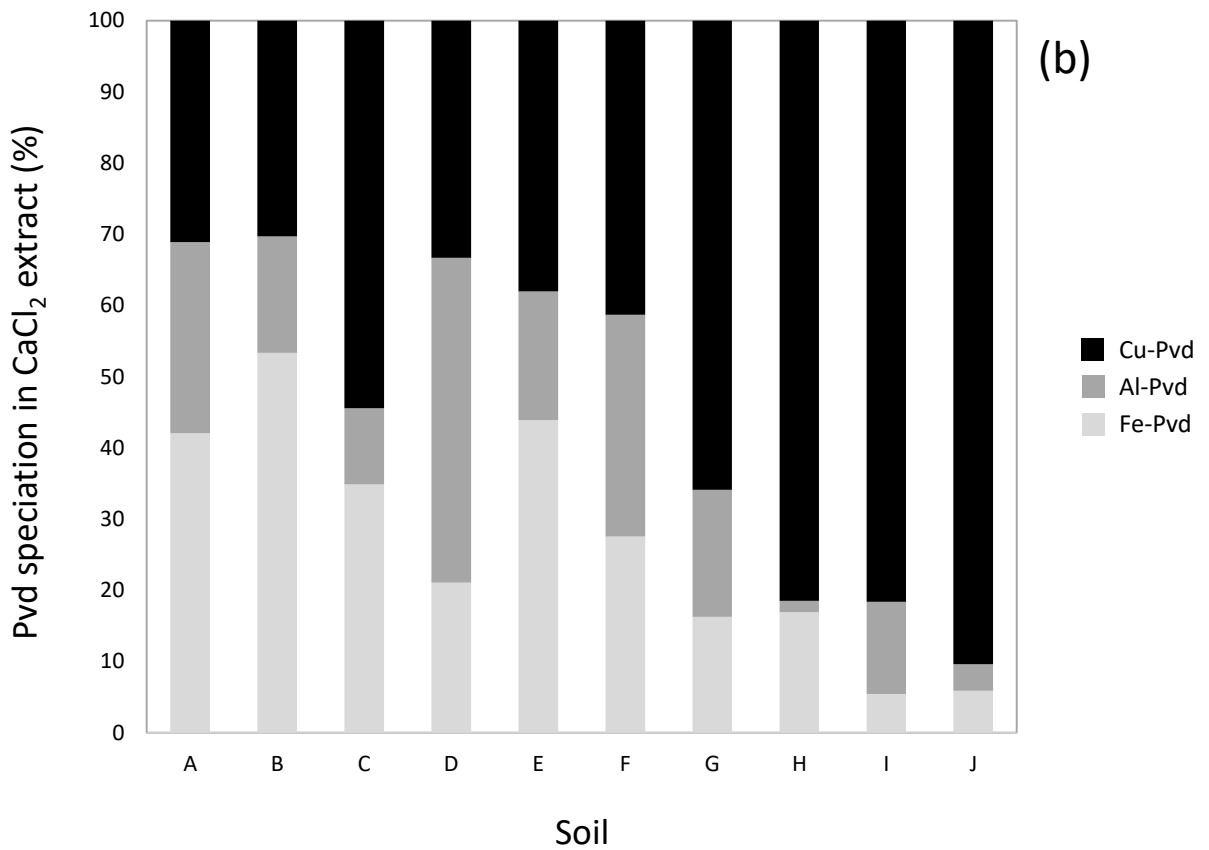
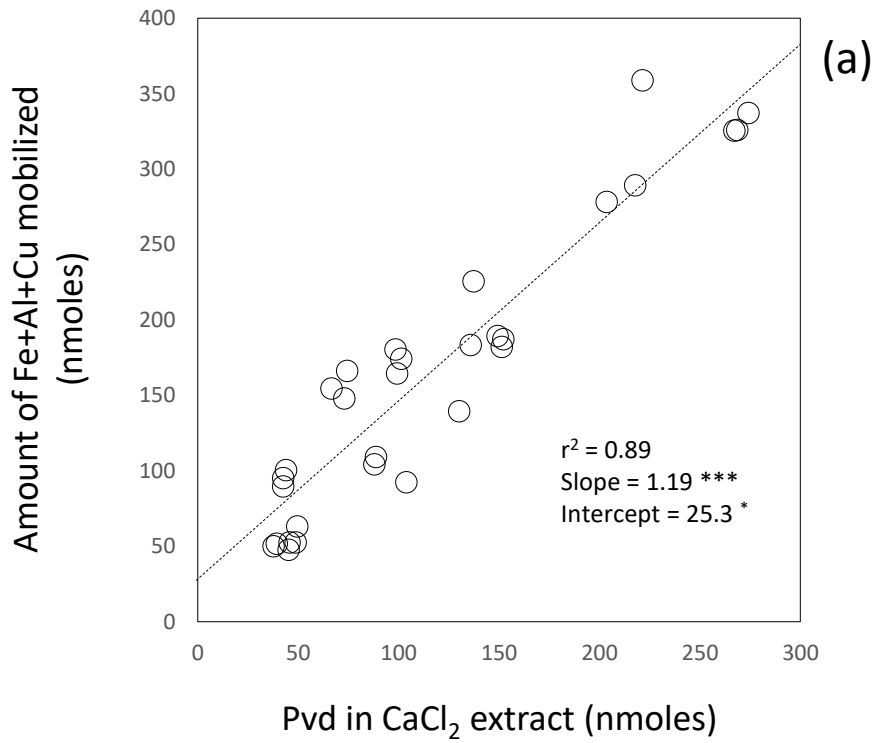


Table 1. Selected physical and chemical properties of the ten Cu-contaminated vineyard topsoils considered in this study. OM stands for organic matter.

	Sand	Silt	Clay ^a	OM ^b	CEC ^c	CaCO ₃ ^d	pH _{water} ^e	Total Cu ^f	Cu _{EDTA} ^g
	g kg ⁻¹			%	cmol _{c+} kg ⁻¹	g kg ⁻¹		mg kg ⁻¹	
A	875	71	54	0.9	1.8	< 1	5.9	104	96
B	835	119	46	1.4	2.2	< 1	6.5	100	88
C	622	222	156	1.3	5.3	< 1	6.8	103	53
D	825	121	54	1.2	2.6	< 1	6.4	111	76
E	794	88	118	2.2	4.9	< 1	6.5	103	73
F	816	97	87	1.9	5.8	4.4	7.9	113	68
G	857	76	67	1.7	4.9	8.4	8.3	154	132
H	661	109	230	3.1	17.5	105	7.6	159	85
I	872	41	87	1.4	7.6	80	7.9	102	73
J	753	95	152	1.7	9.7	35	8.6	121	77

^a ISO 11277

^b NF ISO 10694

^c NF ISO 23470

^d NF ISO 10693

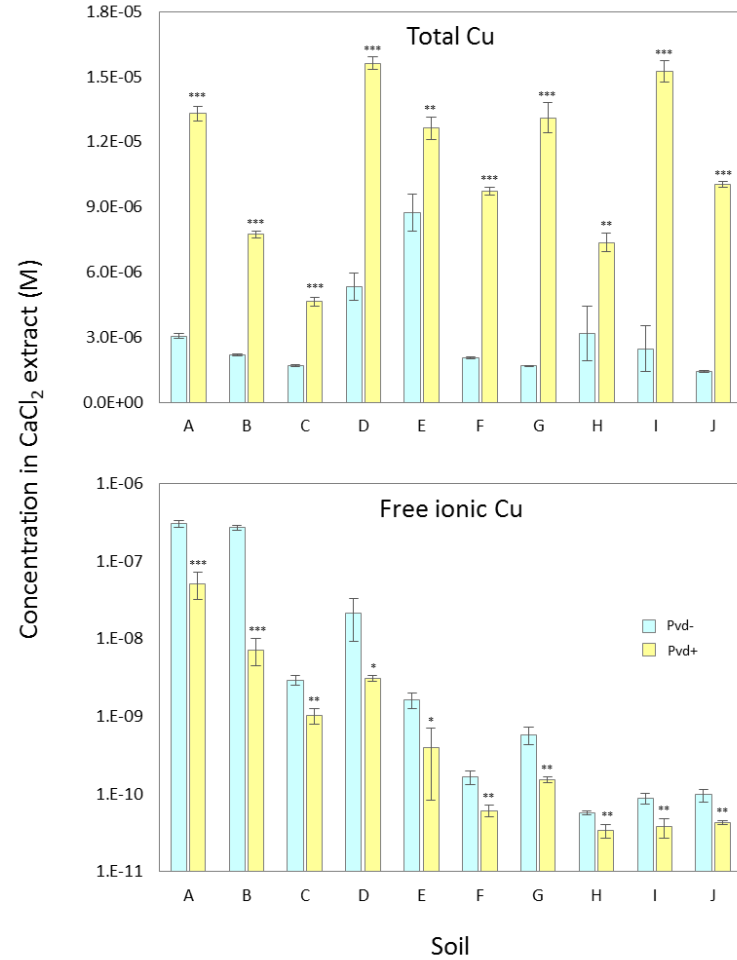
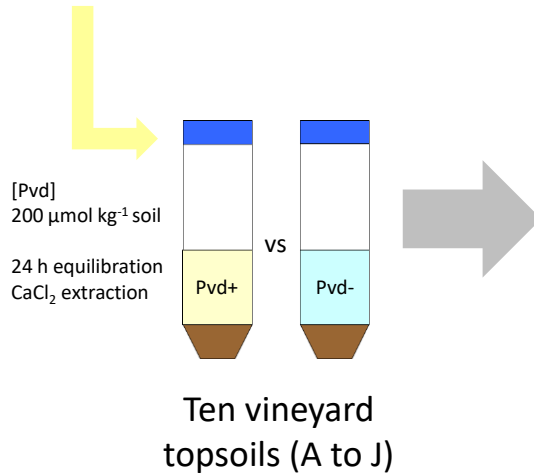
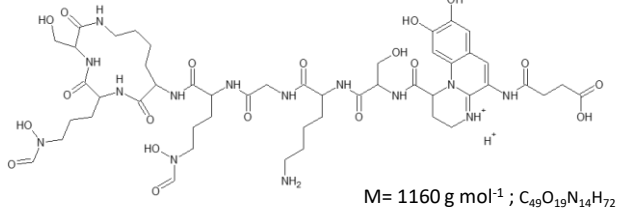
^e NF ISO 10390

^f NF X 31-147/NF ISO 22036

^g NF X 31-120/NF ISO 22036

Graphical abstract

Exogenous Pyoverdine (Pvd)



Increased mobility !



Decreased phytoavailability ?