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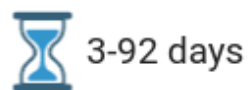
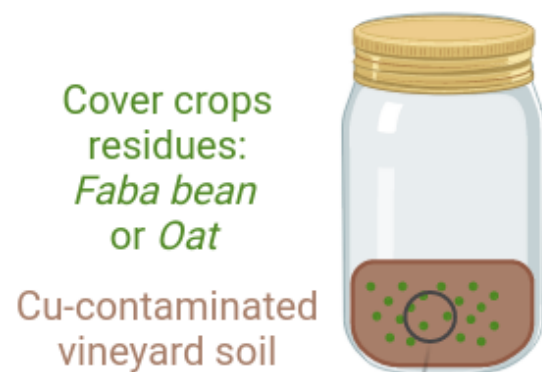
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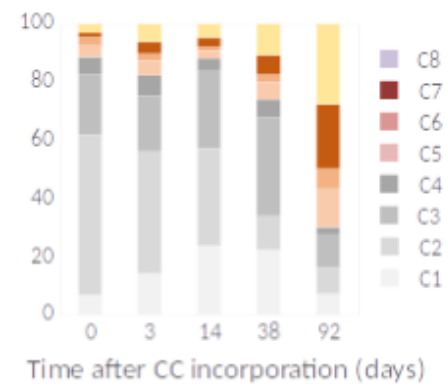
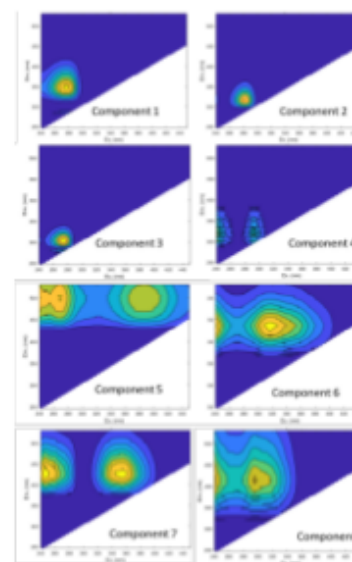
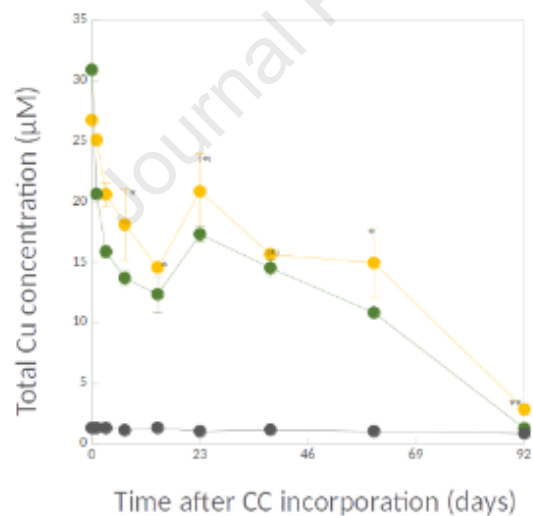
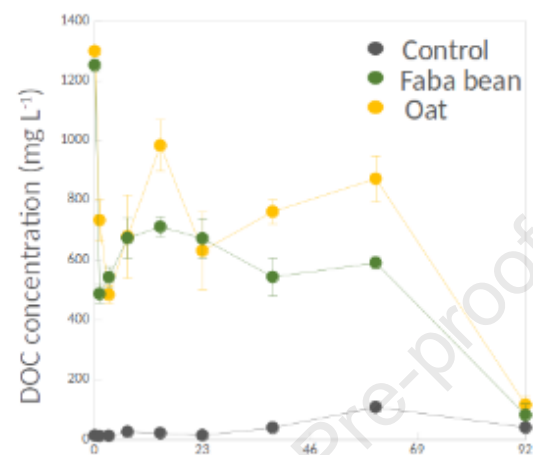
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3-92 days

0.01 M KCl extraction

- Concentration and fluorescent properties of dissolved organic matter
- Total and free Cu ion concentration



1 **Changes over time in organic matter dynamics and copper solubility in a vineyard**
2 **soil after incorporation of cover crop residues: insights from a batch experiment**

3

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12 Abstract

13 Cover crops (CCs) are increasingly used in viticulture because they benefit the soil and
14 the environment in many ways. This study investigated the extent to which the
15 incorporation of CC residues altered organic matter (OM) and Cu dynamics in a Cu-
16 contaminated vineyard topsoil. A 92-day incubation period was used to monitor
17 changes over time in carbon mineralization, carbon hydrolytic enzyme activity,
18 concentration and optical properties of dissolved organic matter (DOM), and Cu
19 solubility after the addition (or not) of two CC residues, oat or faba bean. The results
20 revealed that adding CCs transitorily increased the concentration of DOM in soil
21 solution, as well as the activity of C hydrolytic enzymes and C mineralization rates.
22 DOM content was approximately two orders of magnitude higher in CC-amended soils
23 than in the control soil on day 0, after which it gradually decreased to reach
24 concentrations similar to those measured in the control soil on day 92. Analyses of
25 DOM optical properties showed that its molecular weight and degree of humification
26 increased over time with a decrease in its concentration. The close relationship between
27 DOM and Cu concentrations in the soil solution suggests that degradation of CCs
28 releases soluble forms of C capable of complexing and solubilizing Cu, and hence that
29 incorporating CC residues can transitorily increase the solubility of Cu in vineyard
30 topsoils. Despite their different C:N ratios, oat and faba bean had almost the same effect
31 on Cu dynamics, implying that C inputs played a prominent role in explaining the
32 interactions between OM and Cu within the timeframe of our experiment. In conclusion,
33 this study enabled recommendations on how to mitigate the risk of Cu ecotoxicity
34 associated with incorporating CCs in Cu-contaminated vineyard soils.

35

36

37 Keywords

38 Dissolved organic matter; enzyme assay; ion-selective electrode; metals; PARAFAC;
39 viticulture

40

41 1. Introduction

42 The long-term use of copper-based fungicides in vineyards to prevent the downy
43 mildew of vine led to the accumulation of Cu in vineyard soils. Concentrations as high
44 as several hundred mg Cu kg⁻¹ soil have been measured in the topsoil of old vineyards
45 around the world (see Komarek et al., 2010 and Mackie et al., 2012, and references
46 therein). Although the average application rate of Cu-based fungicides is now limited,
47 for instance to 4 kg Cu ha⁻¹ year⁻¹ in Europe (EU 2018/1981), Cu continues to
48 accumulate in vineyard soils, especially in organic viticulture where the use of synthetic
49 fungicides is prohibited. A recent meta-analysis by Karimi et al. (2021) of Cu
50 ecotoxicity suggests that Cu can reduce the biomass, the activity and/or the diversity of
51 soil microorganisms at a dose of 200 kg Cu ha⁻¹, which corresponds to a concentration
52 in the topsoil (0-20 cm) of about 70 mg Cu kg⁻¹ soil, compared with 91 mg Cu kg⁻¹,
53 which is the mean concentration of Cu in French vineyard soils as assessed based on a
54 Land Use/Cover Area frame Survey (LUCAS) survey (Ballabio et al., 2018). Soil Cu
55 contamination is thus suspected to be detrimental to the biological quality of at least
56 some vineyard soils, as suggested by Dequiedt et al. (2011), who showed that the
57 average soil microbial biomass was lower in vineyards (and orchards) than in
58 grasslands, forests and in other cropping systems.

59 Copper bioavailability, i.e. the degree to which Cu is absorbed or metabolized by
60 ecological receptors (ISO 17402), depends to a great extent on the pH and organic
61 matter content of the soil as both affect the concentration of free Cu ion (Cu²⁺) in the

62 soil solution (Sauvé et al., 2000; Bravin et al., 2009; Ouédraogo et al., 2022), which is
63 the main (and usually the only) form in which Cu is taken up by plant roots (FIAM;
64 Campbell, 1995). Therefore, to manage the risk of Cu ecotoxicity in vineyard plots
65 contaminated by Cu, particular attention must be paid to agronomic practices that affect
66 the pH and organic matter dynamics in soil.

67 Cover crops (CCs) are increasingly used in agriculture because of the benefits they
68 provide to the soil and the environment. Incorporating CCs in agricultural rotations is
69 one way of increasing soil organic carbon in agroecosystems (Poepflau and Don, 2015)
70 thereby also improving soil structure, biological activity and mineral fertility (Blanco-
71 Canqui et al., 2015). In addition, CCs can limit the growth of weeds, provide habitats
72 for beneficial insects and predators, and protect the soil against erosion (Blanco-Canqui
73 et al., 2015). Mixtures of CCs of different functional types, legume, grass and brassica,
74 are sown in the vineyard inter-rows in the fall (Abad et al., 2021), and incorporated in
75 the soil in spring, soon after the vine bud break. The use of CCs in Cu-contaminated
76 vineyards raises the question of how incorporating their residues affects the solubility
77 and bioavailability of Cu in the topsoil. The few studies carried out on this topic (Merritt
78 and Erich, 2003; Cui et al., 2008) showed that CC incorporation increased the solubility
79 of Cu in soil, likely due to the Cu-mobilization potential of dissolved organic matter
80 (DOM) released during aerobic degradation of their residues (Wershaw et al., 1996).
81 As described in the context of the “Birch effect” (Birch, 1958), soil microbial activity
82 is likely to alter the characteristics of DOM over time, thereby also altering the effect
83 of CCs on Cu dynamics. However, knowledge is still lacking on temporal changes in
84 the quantity and the quality of DOM after CC incorporation, as well as on the
85 relationship between DOM quality, Cu complexation and Cu solubility in this context.
86 Another pending question is the effect of CCs of different functional types on DOM

87 release and Cu dynamics. Incorporating CCs with a low C:N ratio, such as legumes, is
88 suspected to release more DOM more rapidly, and consequently to have a distinct effect
89 on the timing and magnitude of Cu solubilisation than incorporating CCs with a high
90 C:N ratio such as grasses (Cotrufo et al., 2013). However, this hypothesis remains to be
91 tested, notably for the CC species used in viticulture.

92 In this study, we investigated the effect of incorporating CC residues on OM and Cu
93 dynamics in a vineyard topsoil using a batch experiment. One original feature of our
94 study is our detailed characterization of the quality of DOM based on its optical
95 properties to better understand how the changes it undergoes over time induced by the
96 incorporation of contrasted CCs affects the solubility of Cu. Our specific objectives
97 were (i) to monitor over time any changes in the quantity and quality of DOM as well
98 as in the solubility and the complexation of Cu in soil solution after incorporation of
99 the cover crop residues, (ii) to compare the effect of two CC residues with distinct C:N
100 ratios: oat and faba bean, and (iii) to investigate the relationship between the quality
101 and the Cu-mobilization potential of DOM in this context.

102

103 **2. Materials and Methods**

104 **2.1 Soil**

105 The soil used in the present study was collected in 2021 from the uppermost 15 cm of
106 a Podzol (WRB, 2006) in an organic vineyard plot in the “Graves” AOC (i.e. registered
107 designation of origin) zone immediately south of Bordeaux, France. The soil sample is
108 a non-calcareous soil characterized by 89% sand, 8% silt and 3% clay (ISO 11277), 2.9%
109 organic matter (OM) (NF ISO 10694), a pH (H₂O) of 6.5 (NF ISO 10390), a C:N ratio
110 of 15.3, and a cation exchange capacity (CEC) of 5.6 cmol⁺ kg⁻¹ (NF ISO 23470). The
111 total concentration of Cu was 114 mg kg⁻¹ soil DW (NF X 31-147) and the EDTA-

112 extractable concentration of Cu was 75 mg kg⁻¹ DW (NF X 31-120). After sampling,
113 the soil was sieved to 4 mm and stored at its original humidity, i.e. 8% (w/w) at 4 °C
114 until the start of the experiment.

115

116 2.2 Cover crop residues

117 The two cover crop (CC) species used in this study, oat (*Avena sativa* L.) and faba bean
118 (*Vicia faba* L.), are often grown in the inter-rows of French vineyards (source French
119 Wine and Vine Institute). The CCs were sampled in 2021 in a vineyard plot close to
120 Bordeaux and treated as follows: since only the shoots were harvested, the plants were
121 cut at ground level, washed with deionized water to remove soil particles, oven-dried
122 at 40 °C for 3 days, then ground to a fine powder (< 0.5 mm, Retsch MM 400) and
123 stored in a dry place. The concentrations of C and N in the CC residues were measured
124 using an elemental analyser (Flash EA 1112, Thermo Fisher), and their concentration
125 of Cu by ICP-OES (ICAP 6300, Thermo Fischer) after digestion in a HNO₃/H₂O₂ mix
126 (Linero et al., 2018). As expected, the concentration of N was higher and the C:N ratio
127 was lower in faba bean residues (N: 4.3%, C/N: 10.5) than in oat residues (N: 2.8%,
128 C/N: 14.9). The concentration of Cu was 14.7 mg kg⁻¹ in faba bean and 6.3 mg kg⁻¹ in
129 oat, meaning the amount of Cu added to the soil through the incorporation of CC
130 residues (see section 2.3) was negligible (< 0.2%) compared to the amount of Cu
131 initially present in the soil.

132

133 2.3 Microcosm incubation

134 One hundred grams of dry soil equivalent were placed in a 1 L glass jar and CC residues
135 were added (or not) at a rate of 1 g C_{residue} per 100 g soil DW (Fanin and Bertrand, 2006).
136 The cover crops were incorporated in the soil by mixing, the mixture was then rewetted

137 to 15% humidity (w/w) using deionised water, after which the jar was hermetically
 138 sealed and left for 48 hours at 4 °C. Sampling on day 0 corresponds to the soil extraction
 139 performed at the end of this pre-incubation at 4 °C. Microcosms were then incubated at
 140 20 °C in the dark for 1, 3, 7, 14, 23, 38, 60 or 92 days. A temperature of 20 °C is suitable
 141 to study microbial activity during OM decomposition (McDowell et al., 2006; De
 142 Troyer et al., 2011), and corresponds to the mean temperature of vineyard topsoils, as
 143 measured from May to September 2019 in the “Graves” AOC zone (Pierdet, 2020).
 144 Four repetitions were performed on each sampling date (n= 9) and per CC treatment
 145 (n= 3) giving a total of 108 microcosms. Four additional jars with no soil or CC residues
 146 were used as controls for the measurement of C mineralization (see section 2.4 below).

147

148 2.4 Carbon mineralization

149 To trap the CO₂ produced by C mineralization, a scintillation vial containing 2 M NaOH
 150 was placed in the microcosms sampled on day 92 and renewed after each measurement
 151 (n= 14). Carbon mineralization was measured on days 2, 4, 8, 11, 15, 18, 22, 30, 37, 44,
 152 51, 65, 79 and 92 by back-titration of the unreacted NaOH. For each measurement *i*,
 153 the cumulative amount of C mineralized ($Q_{CO_2,i}$, in g) was estimated using eq. 1:

$$154 \quad Q_{CO_2,i} = \sum_{i=1}^{14} (V_0 - V_i) / 2 \times C \times V_t / V_s \times M_c \quad (1)$$

155 where V_i is the volume of HCl used for NaOH titration for measurement *i*, V_0 the mean
 156 volume of HCl used to titrate NaOH in the control jars (0.004 L), *C* the molar
 157 concentration of HCl used for NaOH titration (0.5 M), V_t the total volume of NaOH in
 158 the scintillation vial (0.015 L), V_s the volume of NaOH sampled in each vial for titration
 159 (0.001 L), and M_c the molecular weight of carbon (12 g mol⁻¹).

160

161 2.5 Enzyme assays

162 We measured the activity of three enzymes that degrade cellulose (β -1,4-glucosidase,
163 1,4- β -D-cellobiohydrolase and α -1,4-glucosidase) and one enzyme that degrades
164 hemicellulose (β -xylosidase) on days 1, 3, 38 and 92, following the procedure described
165 in Bell et al. (2013). Briefly, 2.5 g dry soil equivalent were mixed with 91 mL of 50
166 mM sodium acetate buffer in a blender for 1 min. The resulting slurry was added to a
167 96-deepwell microplate using an eight-channel electronic pipette. Additional quench
168 control replicates of soil slurry with 4-methylumbelliferone (MUB) or 7-amino-4-
169 methylcoumarin (AMC) standard curves (0 - 100 μ M concentrations) and controls
170 without substrate addition (soil slurry + water) were included with each sample. Soil
171 slurries were then incubated at 25 °C for 3 h with fluorescent substrates corresponding
172 to each enzyme measured. After the incubation step, the plates were centrifuged for 3
173 min at 3000 rpm and 250 μ L of supernatant was transferred from each well into a black
174 flat-bottomed 96-well plate. Next the samples were scanned on a Synergy H1
175 microplate reader (Biotek, Winooski, USA) using fluorescence with excitation at 365
176 nm and emission at 450 nm. The enzyme activities were integrated over time to derive
177 the cumulative enzyme activities expressed in nmoles kg^{-1} soil (Fanin and Bertrand,
178 2016).

179

180 2.6 Soil extraction and analyses of the extraction solution

181 The soil was extracted on days 0, 1, 3, 7, 14, 23, 38, 60 and 92. Soil extraction was
182 performed as follows: 15 g dry soil equivalent was shaken with 30 mL of 0.01 M KCl
183 at 35 rpm for 2 h at 20 °C, centrifuged at 4600 g for 10 min, and the supernatant was
184 filtered through 0.2 μ m cellulose acetate filters. pH was measured immediately after
185 the solution was extracted using a combined pH microelectrode (E16M331, Radiometer

186 Analytical). The extraction solution was then divided into two subsamples. One
187 subsample was acidified with 2% HNO₃ (v/v) to measure the total concentration of Cu
188 by ICP-OES (ICAP 6300, Thermo Fischer). The other subsample was stored at 4 °C in
189 the dark to measure free Cu ion activity and to characterize dissolved organic matter on
190 the five days following soil extraction. Free Cu ion activity was measured using a cupric
191 ion-selective electrode (ISE, 9629BNWP, Thermo Scientific Orion) according to the
192 procedure described in Cornu et al. (2019), and the concentration of dissolved organic
193 carbon (DOC) was measured using a TOC analyzer (TOC V-CPH, Shimadzu). The
194 concentration of free Cu ion was calculated from free Cu ion activity using the Cu²⁺
195 activity coefficient given by the extended Debye-Hückel equation. The free Cu ion
196 fraction (f_{Cu}) was determined by calculating the ratio of the concentration of free Cu
197 ion to the concentration of total Cu.

198

199 2.7 Optical properties of dissolved organic matter

200 Absorbance and fluorescence of the extraction solutions were measured in a 1-cm
201 quartz cuvette thermostated at 20 °C, using an Aqualog spectrofluorometer (Horiba
202 Scientific). Absorbance spectra were recorded from 239 to 800 nm using a 0.01 M KCl
203 solution as a blank. The spectral slope $S_{275-295}$ used as a proxy of the DOM molecular
204 weight (Helms et al., 2008), was calculated from the slope of the natural logarithm-
205 transformed absorbance spectra in the 275-295 nm wavelength range. To obtain
206 excitation-emission matrix (EEM) fluorescence spectra, excitation scans were
207 performed using a double-grating monochromator (150 Watt Xenon lamp) at
208 wavelengths 240-800 nm at 5 nm intervals. Emission spectra were collected with high
209 CCD detector gain at approximately 0.58 nm (1 pixel) intervals at wavelengths ranging
210 from 245 to 830 nm. When the maximum intensity of the absorbance spectrum was

211 higher than 0.1, the sample was diluted with 0.01 M KCl to avoid internal filter effects.
212 Fluorescence data are expressed in Raman units (RU) after normalization to the area
213 under the Raman peak of an ultrapure water blank at an excitation wavelength of 350
214 nm.
215 EEM spectra were modeled using parallel factor analysis (PARAFAC) in MATLAB
216 R2019a using the DOMFluor toolbox 1.7 (Stedmon and Bro, 2008; Murphy et al., 2014).
217 PARAFAC is a multiway mathematical tool that statistically decomposes the EEM
218 spectra into individual fluorescence components. The PARAFAC model was run for
219 three to eight components with non-negativity constraints. The optimal number of
220 PARAFAC components was determined after split-half analysis, random initialization
221 and visual inspection of the spectral shape of each component and residual from models.
222 For each sample, the relative abundance of each component was expressed through its
223 contribution to the maximum fluorescence intensity (F_{\max}) (Stedmon and Bro, 2008;
224 Murphy et al., 2014). The molecular description of the fluorophore behind each
225 component was made by comparison with the literature and with the "OpenFluor
226 database" (<http://www.openfluor.org/>) (Murphy et al., 2014).

227

228 2.8 Data treatment and statistical analyses

229 Data processing and statistical analyses were performed in R version 4.0.3 (R core
230 Team, 2020). When necessary, the data were log transformed to achieve normal residual
231 distribution. Tukey's HSD tests at 5% were used to identify the parameters that differed
232 significantly among CC treatments. Pearson's correlation tests were used to test the
233 linear relationship between the concentration of Cu and DOC (Fig. 5a), Cu and FI_{C5-C7}
234 (Fig. 5b), free Cu ion and pH (Fig. 5c) and of the free Cu ion fraction and pH (Fig. 5d),
235 in the extraction solution.

236

237 **3. Results**

238 3.1 Soil biological activity

239 Soil biological activity was assessed by analyzing carbon mineralization and the
240 activity of carbon hydrolytic enzymes. As expected, more C was mineralized in the soil
241 supplied with CC residues (i.e. in CC-amended soils) than in the control soil. In CC
242 amended-soils, C mineralization reached maximum within the first three days
243 following CC incorporation, and was slightly higher in the soil amended with oat
244 residues than in the soil amended with faba bean residues (Fig. 1a). The difference in C
245 mineralization between the two CC residues became significant ($p < 0.05$) 22 days after
246 incubation started. At the end of the incubation period, the cumulative amount of C
247 mineralized in CC-amended soils was about 15 times higher than in the control soil,
248 and corresponded to about 20% of the amount of C supplied by the CC residues (24%
249 for oat and 22% for faba bean).

250 On days 3, 38 and 92, the activity of C hydrolytic enzymes was significantly higher (p
251 < 0.05) in CC-amended soils than in the control soil (Fig. 1b). In CC-amended soils, C
252 hydrolytic enzyme activity increased between day 1 and day 3 ($p < 0.05$) and then
253 decreased continuously between day 3 and day 92 ($p < 0.05$) in the soil amended with
254 faba bean residues, whereas it decreased between day 3 and 38 ($p < 0.05$) but then
255 increased between day 38 and day 92 ($p < 0.05$) in the soil amended with oat residues.
256 On day 92, the residual activity of C hydrolytic enzymes was on average 4- to 5-fold
257 higher in CC-amended soils than in the control soil.

258

259 3.2 Characteristics of dissolved organic matter

260 3.2.1 Concentration of DOC and absorbance properties

261 On day 0, i.e. at the end of the pre-incubation period, the concentration of DOC in the
262 KCl extract was almost 100 times higher in CC-amended soils ($> 1000 \text{ mg C L}^{-1}$) than
263 in the control soil ($\sim 15 \text{ mg C L}^{-1}$), and there was no significant difference ($p > 0.05$)
264 between the soil amended with oat residues and the soil amended with faba bean
265 residues (Fig. 2a). The DOC concentration in CC-amended soils decreased by more
266 than 50% during the first three days of incubation, and then increased between day 2
267 and day 14, notably in the soil amended with oat residues. From day 14 to day 92, the
268 concentration of DOC gradually decreased in CC-amended soils, with some variability
269 among sampling dates in the soil amended with oat residues, and on day 92 had reached
270 a value close to that measured in the control soil. On day 0, the absorbance spectral
271 slope $S_{275-295}$ of the KCl extract was higher in CC-amended soils than in the control soil
272 (Fig. 2b), meaning that the incorporation of CC residues initially decreased the average
273 molecular weight of DOM. The $S_{275-295}$ value followed the same trend over time as the
274 DOC concentration: it decreased sharply over the first three days of incubation,
275 increased temporarily between day 3 and day 14, and then decreased from day 14 to
276 day 92 when it reached values close to that measured in the control soil (Fig. 2b).

277

278 3.2.2 PARAFAC components

279 PARAFAC analysis identified eight components whose spectral characteristics and
280 chemical description are detailed in Table 1 and Fig. F1 (suppl. data). In the control soil,
281 DOM was a balanced mixture of components C3, C4, C5, C7 and C8, and its fluorescent
282 properties were relatively well conserved over time (Fig. 3). In CC-amended soils, the
283 fluorescent properties of DOM differed from those of the control, notably during the
284 first 14 days of the incubation period. On day 0, components C2 (55%) and C3 (21%)
285 contributed the most to the DOM fluorescence properties in the soil amended with faba

286 bean residues, while components C1 (36%) and C3 (34%) contributed most to the DOM
287 fluorescence properties in the soil amended with oat residues. DOM fluorescence
288 properties changed over time in both types of CC-amended soils. From day 14 to day
289 92, the contribution of components C5, C7 and C8 increased over time to the detriment
290 of the contribution of components C1 and C3 (and C2 in the soil amended with faba
291 bean residues) in what could be interpreted as a process of DOM humification. As a
292 result, the fluorescence properties of DOM in CC-amended soils differed less from
293 those in the control soil at the end (day 92) than at the beginning (day 0) of incubation.
294 Figure 2c shows changes in the cumulative fluorescent intensity of the PARAFAC
295 components C5, C6 and C7 (FI_{C5-C7}) over time, which we used as a proxy of the
296 concentration of soluble humic substances (see Table 1) in the KCl extract. FI_{C5-C7} was
297 higher in CC-amended soils than in the control soil and followed approximately the
298 same trend over time as the concentration of DOC. The two noteworthy differences
299 between the two variables were that (i) the decrease in FI_{C5-C7} during the first three days
300 was less marked than that of DOC, and (ii) the difference between the two CC residues
301 was larger for FI_{C5-C7} than for DOC.

302

303 3.3 Concentration of total Cu and free Cu ion in the KCl extract

304 On day 0, the concentration of total Cu in the KCl extract was 20-fold higher in CC-
305 amended soils ($\sim 29 \mu\text{M}$) than in the control soil ($\sim 1.3 \mu\text{M}$) and did not differ
306 significantly ($p > 0.05$) between the soil amended with oat residues and the soil
307 amended with faba bean residues (Fig. 4a). Except between day 14 and day 23, the
308 concentration of total Cu in the KCl extract decreased over time in CC-amended soils,
309 whereas it remained fairly constant in the control soil. Except on day 0, the
310 concentration of total Cu in the KCl extract was significantly higher ($p < 0.05$) in the

311 soil amended with oat residues than in the soil amended with faba bean residues (24%
312 on average between day 1 and day 60). As also observed for several variables relative
313 to DOM, on day 92, the concentration of total Cu in the KCl extract was similar in the
314 control soil and in CC-amended soils.

315 In contrast, the concentration of free Cu ion in the KCl extract was significantly lower
316 ($p < 0.05$) in CC-amended soils than in the control soil throughout the incubation period
317 (Fig. 4b). The concentration of free Cu ion in the control soil did not change much over
318 time, i.e. it averaged 5.3 ± 2.9 nM, whereas in CC-amended soils, it varied over time
319 from 0.02 nM to 3 nM, i.e. by more than a factor of 100. On days 3, 7, 14 and 23, the
320 concentration of free Cu ion was significantly higher ($p < 0.05$) in the soil amended
321 with faba bean residues than in the soil amended with oat residues. The free Cu ion
322 fraction (f_{Cu}) was closely ($p < 0.001$) and positively ($r = 0.96$) correlated with the
323 concentration of free Cu ion (data not shown). As observed for the concentration of free
324 Cu ion, f_{Cu} was lower in CC-amended soils than in the control soil, and, in the control
325 soil, did not change much over time, average $0.7 \pm 0.3\%$, whereas in CC-amended soils,
326 it varied over time from 0.0002% to 0.2%, i.e. by a factor of 1000 (suppl. Fig. F2).

327

328 3.4 Relationships between Cu concentration and DOM characteristics in the KCl extract

329 The concentration of total Cu in the KCl extract of CC-amended soils was positively
330 correlated with the concentration of DOC ($r = 0.75$, $p < 0.001$) but was more closely
331 correlated with FI_{C5-C7} ($r = 0.95$, $p < 0.001$) (Fig. 5), notably in the solutions extracted
332 during the period of incubation, i.e. when the quality of the DOM changed most. Figure
333 5b shows that the higher concentration of total Cu in the soil amended with oat residues
334 than in the soil amended with faba bean residues was associated with a higher value of
335 FI_{C5-C7} , i.e., with a higher concentration of humic-like substances (Table 1). In contrast,

336 neither the concentration of free Cu ion nor the fraction of free Cu ion were significantly
337 correlated with DOM characteristics ($p > 0.05$, data not shown) in the KCl extract of
338 CC-amended soils, but were closely ($p < 0.001$) and negatively ($r = -0.94$ and -0.87 ,
339 respectively) correlated with pH (Fig. 5c, Fig. 5d).

340

341 **4. Discussion**

342 4.1 Effect of the incorporation of cover crop (CC) residues on the dynamics of organic
343 matter (OM).

344 On day 0, i.e. before the start of OM degradation, the concentration of dissolved organic
345 carbon (DOC) in the KCl extract increased from 14 mg C L^{-1} in the control to 1300 mg
346 C L^{-1} in CC-amended soils (Fig. 2a). For each CC-amended microcosm, this
347 corresponded to a 250 mg increase in the soluble organic C pool. Assuming that all this
348 DOC originated from CC residues, the soluble fraction of C was around 25% DW in
349 both CC residues, which is in the range of values reported in the literature for different
350 types of aboveground litter (Fanin and Bertrand, 2006) and different cover crops (CCs)
351 (Weiler et al., 2022). The soluble fraction of C in CC residues is usually composed of
352 hydrophilic compounds such as sugars and amino acids (Marstop, 1996; De Troyer et
353 al., 2011). To some extent, this theoretical composition is in agreement with the optical
354 properties of DOM measured on day 0, which showed a lower molecular weight (Fig.
355 2b) and a lower degree of humification (Fig. 3) of DOM in CC-amended soils than in
356 the control soil. The dynamics of organic matter in CC-amended soils occurred in three
357 stages. The first stage, which spanned the first three days, was characterized by high C
358 mineralization rates (Fig. 1a) and a marked decrease in the concentration of DOC in the
359 KCl extract over time (Fig. 2a). This early stage of OM decomposition likely resulted
360 from the rapid mineralization of soluble and readily degradable C forms in CC residues,

361 which stimulated a copiotrophic community due to a marked increase in C availability
362 (Fanin et al., 2014). This hypothesis is supported by the fact that during this stage (i)
363 the average amount of C mineralized (i.e. 150 mg C per microcosm) was the same as
364 the decrease in DOC in the KCl extract, and (ii) the average molecular weight of DOM
365 increased with a decrease in the concentration of DOC.

366 The second stage extended from day 3 to day 60 and was characterized by a shift in the
367 resource-use strategy to target decaying more structural compounds, as indicated by the
368 gradual decrease in C mineralization rates. This second stage is in agreement with that
369 reported in several other studies showing that the rapid increase in CO₂ fluxes after CC
370 incorporation was quickly followed by a slowing down of mineralization rates (Lynch
371 et al., 2016; Ghimire et al., 2017; De Notaris et al., 2020; Li et al., 2021). This is usually
372 due to the fact that microbial communities produce more enzymes to acquire energy
373 and nutrients from the organic residues (Fig. 1b), i.e., they shift from exploitative to
374 acquisitive resource strategies with a high enzyme regeneration cost (Malik et al., 2019).
375 During this stage, the production of CO₂ was not associated with a decrease in the
376 concentration of DOC in the KCl extract. In our opinion, the most likely explanation is
377 that the drop in the concentration of DOM caused by its mineralization was
378 counterbalanced by production of DOM resulting from the degradation of organic
379 carbon originating from the soil and/or CC residues.

380 The last stage extended from day 60 to day 92 and was characterized by a sharp decrease
381 in C mineralization rates due to the depletion of the readily-decomposable compounds
382 coupled with an increase in the proportion of recalcitrant organic compounds. At this
383 stage, the C mineralization rates in CC-amended soils were similar to those in the
384 control soil (Fig. 1a), suggesting that the microbial communities returned to their
385 original status characterized by a low metabolic activity and low respiration rates (Fanin

386 and Bertrand, 2016). The concentration of DOC in the KCl extract decreased
387 considerably during this stage due to a process that excluded DOM mineralization. The
388 two processes we propose are (i) the incorporation of C from DOM decomposition into
389 the soil microbial biomass (Hu et al., 2023), and (ii) the sorption of DOM to the soil
390 solid phase linked to changes in its characteristics, e.g. increased molecular weight and
391 hydrophobicity (Zhou and Wong, 2000) that enhanced its affinity for the soil
392 constituents. The resulting DOM on day 92 was characterized by similar quantity and
393 quality (average molecular weight, humification degree) in CC-amended soils and the
394 control soil. Taken together, these results show that the incorporation of CC residues
395 transiently altered the dynamics of OM and led to significant changes in the quantity
396 and quality of DOM that in turn are capable of altering Cu dynamics.

397

398 4.2 Effect of the incorporation of CC residues on the dynamics of Cu

399 As observed for DOM, the incorporation of CC residues transiently increased the
400 solubility of Cu in the soil. On day 0, the concentration of Cu was higher in the KCl
401 extract of CC-amended soils than in the control soil (Fig. 2a), suggesting that the
402 soluble organic C released by CC residues was able to complex and solubilize Cu.
403 However, the lower Cu:DOC ratio in the KCl extract of CC-amended soils during the
404 initial stage (i.e. from day 0 to day 3, suppl. Fig. F3) may suggest that the affinity for
405 Cu of the ligands released by the CCs was on average lower than that of the soil DOM.
406 This is in line with the low degree of humification of the DOM measured on day 0 in
407 CC-amended soils (Fig. 3) since aromatic substances usually have a higher Cu-
408 mobilizing potential than aliphatic ones (Amery et al., 2008; Borggaard et al., 2019), as
409 well as with the presumed composition of soluble ligands released by CCs based on
410 hydrophilic compounds (e.g. sugars) whose affinity for Cu is rather low (Laurent et al.,

411 2020). The changes over time in Cu solubility in CC-amended soils closely mirror that
412 of DOM, and particularly of humic-like DOM, as evidenced by the close and positive
413 relationship between the concentration of Cu and FI_{C5-C7} in the KCl extract (Fig. 5b).
414 As observed for FI_{C5-C7} , the decrease in the concentration of Cu during the first stage
415 was less pronounced than that of DOC, thus supporting the hypothesis of preferential
416 degradation of aliphatic DOM by copiotrophic microorganisms during the initial stage
417 of decomposition. The subsequent changes in Cu solubility can be interpreted as
418 resulting from the changes in the dynamics of humic-like DOM (Eon et al., 2023),
419 notably, the decrease in Cu solubility during the last stage (i.e. between day 60 and day
420 92) could result from the sorption of Cu-humic DOM complexes onto the soil
421 constituents.

422 The free Cu ion fraction in the KCl extract provides further information regarding the
423 interaction between Cu and DOM. First, the fact that f_{Cu} never exceeded 1.3% in the
424 control soil, and 0.3% in CC-amended soils, confirms that the vast majority of Cu is
425 bound to DOM in the soil solution (Filipovic et al., 2023). Second, the lower f_{Cu} in CC-
426 amended soils compared to in the control soil supports the hypothesis that the soluble
427 organic ligands supplied by CC residues were able to complex Cu. Third, the negative
428 relationship between f_{Cu} and pH (Fig. 5d) is evidence that the complexation of Cu by
429 DOM is highly sensitive to pH, and that the affinity of DOM to bind Cu is higher at
430 high pH, i.e. when more functional groups at the DOM molecules are deprotonated
431 (Nierop et al., 2002), as shown in previous studies by our group (Cornu et al., 2019;
432 Ouédraogo et al., 2021). This last point also explains why the changes in the
433 concentration of free Cu ion over time (Fig. 4b) were not related to the dynamics of
434 DOM but rather to changes in pH in CC-amended soils. During the incubation period,
435 pH varied from 5.5 to 7.0 in the KCl extract of CC-amended soils (suppl. Fig. F4), for

436 reasons linked to CC decomposition, possibly the production / decarboxylation of
437 organic anions (Yan et al., 1996), which in turn made the free Cu ion concentration vary
438 from 0.03 to 3 nM, i.e. by a factor of 100. However, the lower concentration of free Cu
439 ion in CC-amended soils had nothing to do with pH itself, but was rather the result of
440 an increase in the concentration of Cu-complexing DOM, as evidenced by the distinct
441 relationships between the two variables observed in the control soil and in CC-amended
442 soils (Fig. 5c). In this respect, our results contrast with those of a study performed by
443 Cui et al. (2008) in which the decrease in the concentration of free Cu ion in the soil
444 solution after the addition of rice straw was mainly attributed to an increase in pH.
445 Taken together, these results highlight that the incorporation of CC residues transiently
446 altered the dynamics of Cu through its impact on the dynamics of DOM and pH, and
447 led to significant changes in the solubility and the speciation of Cu in soil that may alter
448 its ecotoxicity and leaching, as suggested by Merritt and Erich (2003).

449

450 4.3 Differences between the two CC residues

451 One objective of this study was to assess whether the effect of CC incorporation on the
452 dynamics of OM and Cu in soil differed between oat and faba bean, i.e. between CC
453 residues with contrasted C:N ratios. Our results do not support the hypothesis that the
454 lower C:N ratio of faba bean makes it more degradable by soil microorganisms than oat
455 and, hence, has more impact on the dynamics of OM and Cu. Indeed, the effect of CC
456 incorporation on the dynamics of OM and Cu was similar in magnitude and timing in
457 the two species (Figs. 1, 2, 4). The absence of an effect of the C:N ratio during the first
458 stage could be due to two factors: (i) soil microorganisms primarily relying on
459 accessible energy from the soluble C fraction, and (ii) non-limiting availability of N in
460 the local environment (Fanin et al., 2014). These results are evidence that the

461 composition of C forms in CCs plays a crucial role in explaining microbial activity in
462 the early stages of CC decomposition (Maxwell et al., 2020), while the C:N ratios only
463 play a secondary role, especially when nutrients are readily available in the soil
464 environment (Fanin et al., 2020). Furthermore, the incorporation of dried ground CC
465 residues into the soil likely facilitated the rapid release of significant quantity of soluble
466 forms of C, thereby potentially reducing differences in mineralization rates between the
467 two types of residues in our experiment.

468 During the second stage of decomposition, the degradation of oat residues was even
469 slightly more pronounced than that of faba bean residues, for reasons that could be
470 linked to the fact that the organic carbon in grasses is less protected by association with
471 minerals than organic carbon in legumes (Zhang et al., 2022). This was evidenced by a
472 higher C mineralization rate in the soil amended with oat residues (Fig. 1a), coupled
473 with a higher concentration of DOC (Fig. 2a) and of humic-like DOM (Fig. 2c) from
474 day 3 to day 60. During this stage, the higher concentration of humic-like DOM in the
475 soil amended with oat residues was associated with a higher concentration of Cu (Fig.
476 4a) and a lower fraction of free Cu ion (Fig. 4b) in the KCl extract. Taken together,
477 these results support the hypothesis that the CC residues with the greatest potential to
478 increase the solubility of Cu in soil are those whose degradation most increases the
479 concentration of DOM, notably that of humic-like DOM. Hence, the incorporation of
480 CCs with a high fraction of soluble C such as *Robinia pseudoacacia* (Fanin and
481 Bertrand, 2006) is suspected of having a particularly strong impact on the dynamics of
482 Cu, and likely on those of other trace metals such as Pb (Borggaard et al., 2010), in soil.
483

484 **Conclusion**

485 This work highlights the fact that incorporating cover crop (CC) residues can transiently

486 disrupt the dynamics of Cu in vineyard topsoil. CC residues were shown to increase the
487 solubility of Cu in the soil for 60 to 90 days after their incorporation, mainly due to the
488 dissolved organic matter released by their decomposition. Provided the Cu-DOM
489 complexes behind Cu solubilization are sufficiently labile to contribute to Cu uptake by
490 soil organisms, the incorporation of CC residues could increase the bioavailability of
491 Cu and consequently the risk of Cu ecotoxicity in vineyard topsoil. However, the rates
492 and forms of incorporation of CCs tested in our experiment, which were chosen and set
493 to enable us to disentangle their impact on the dynamics on DOM and Cu, are not the
494 same as the rates and forms of CC incorporation normally used at field scale. The effect
495 of incorporating CCs on the dynamics of DOM and Cu in soil is therefore likely less
496 pronounced and spread out over a longer period of time in real vineyard plots than in
497 the present study, due to presumably lower and slower release of soluble organic C.
498 Taken together, these results underline the need to ensure the stability of the organic
499 matter added to Cu-contaminated vineyard soils, as its stability will determine its effect
500 on Cu dynamics and ecotoxicity. In this respect, composting CC residues before their
501 incorporation could mitigate their detrimental effect on Cu solubility in vineyard topsoil,
502 while simultaneously helping control primary inoculum of downy mildew.

503

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512

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Figure captions

Figure 1. Changes in the cumulative amount of C mineralized (a) and the activity of C hydrolytic enzymes (b) over the 92-day incubation period, in a vineyard soil after incorporation of faba bean (green) or oat (yellow) residues. No cover crop residue was incorporated in the control soil (gray). The data presented are average values \pm one standard deviation (shown as bars in the figure) calculated from four replicates per treatment. For a given sampling day, (*), *, ** and *** indicate that the amount of C mineralized (a) or the activity of C hydrolytic enzymes (b) in the soil amended with oat differed significantly from that measured in the soil amended with faba bean at a probability level of $p < 0.1$, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Figure 2. Changes in the concentration of dissolved organic carbon (DOC) (a), the slope of the absorbance spectra in the 275-295 nm wavelength range ($S_{275-295}$) (b), and the cumulative fluorescent intensity of the PARAFAC components C5, C6 and C7 (FI_{C5-C7}) (c) over the 92-day incubation period in a 0.01 M KCl extract of a vineyard soil after incorporation of faba bean (green) or oat (yellow) residues. No cover crop residue was incorporated in the control soil (gray). The data presented are average values \pm one standard deviation (shown as bars in the figure) calculated from four replicates per treatment. For a given sampling day, (*), *, ** and *** indicate that the concentration of DOC (a), $S_{275-295}$ (b) or FI_{C5-C7} (c) in the soil amended with oat differed significantly from that measured in the soil amended with faba bean, at a probability level of $p < 0.1$, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Figure 3. Changes in the contribution of the eight components identified by PARAFAC

analysis to the maximum fluorescence intensity of dissolved organic matter (DOM) over the 92-day incubation period, in the control soil (a), in soil amended with faba bean (b), and in soil amended with oat (c) residues.

Figure 4. Changes in the concentration of total Cu (a) and free Cu ion (b) over the 92-day incubation period in a 0.01 M KCl extract of a vineyard soil after incorporation of faba bean (green) or oat (yellow) residues. No cover crop residue was incorporated in the control soil (gray). The data presented are average values \pm one standard deviation (shown as bars in the figure) calculated from four replicates per treatment. In (b) the Y-axis scale is logarithmic. For a given sampling day, (*), *, ** and *** indicate that the concentration of total Cu (a), or free Cu ion (b) in the soil amended with oat residues differed significantly from that measured in the soil amended with faba bean residues at a probability level of $p < 0.1$, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Figure 5. Total Cu concentration as a function of the concentration of dissolved organic carbon (DOC) (a), total Cu concentration as a function of cumulative fluorescence intensity of the PARAFAC components C5, C6 and C7 (FI_{C5-C7}) (b), free Cu ion concentration as a function of pH (c) and free Cu ion fraction as a function of pH (d), in a 0.01 M KCl extract of a vineyard soil after incorporation of faba bean (green) or oat (yellow) residues. No cover crop residue was incorporated in the control soil (gray). The correlation coefficient (r) is for the two treatments in which cover crops were incorporated. In (c) and (d), the Y-axis scale is logarithmic and the correlation coefficient (r) is for the log-log relationship.

Table 1. Characteristics of the eight components identified by PARAFAC analysis

Component	Ex / Em (nm)	Fluorophores	DOM description	References
C1	275 / 345	Protein-like, Tryptophan-like	Low humified DOM	[1-3]
C2	280 / 315	Free amino acids, Amino acids bound to proteins	Low humified DOM	[4, 5]
C3	275 / 305	Protein-like, Tyrosine-like	Low humified DOM	[6, 7]
C4	240-290 / 330	Protein-like, Tryptophan-like	Low humified DOM	[8]
C5	270-380 / 500	Salicylic acid, Conjugated fluorophores	Recalcitrant DOM associated with microbial reduction reaction	[8-10]
C6	240-315 / 435	Lignin-like	Humified DOM degraded from lignin	[8]
C7	245-355 / 435	Lignin-like	Aromatic DOM with low N content	[11, 12]
C8	240-305 / 410	Oxidized quinone-like	Microbial-based DOM with low molecular weight and low aromaticity	[1, 13, 14]

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Figure 1
Journal Pre-proof

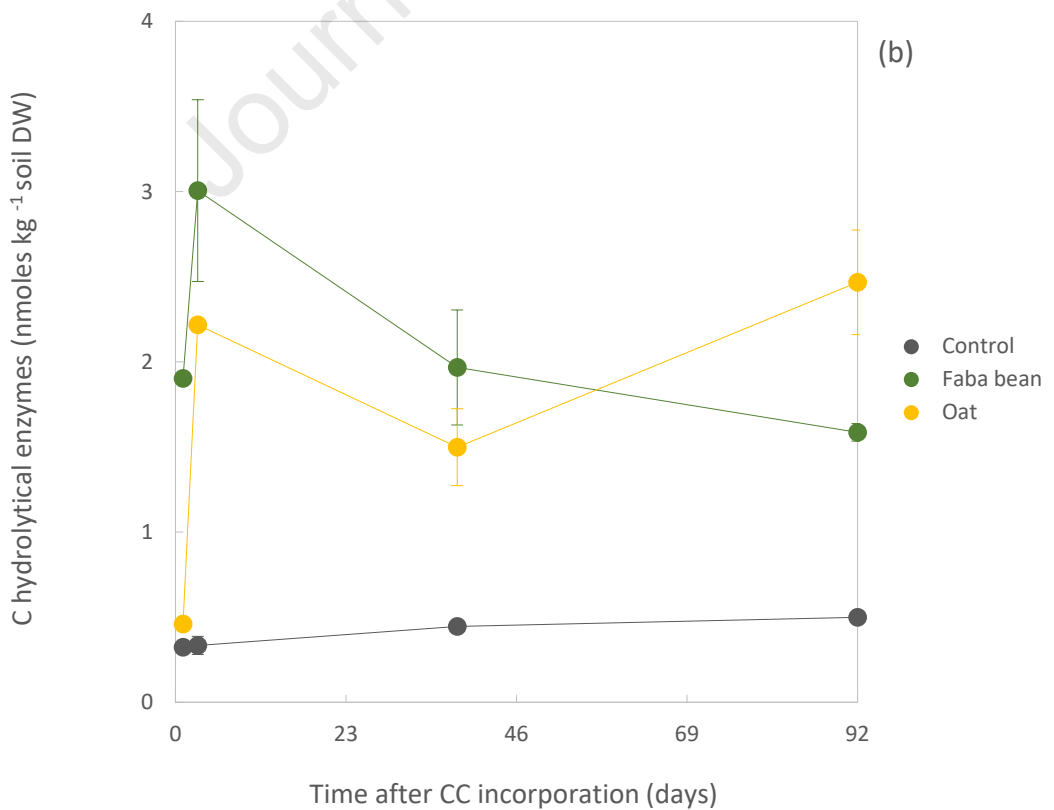
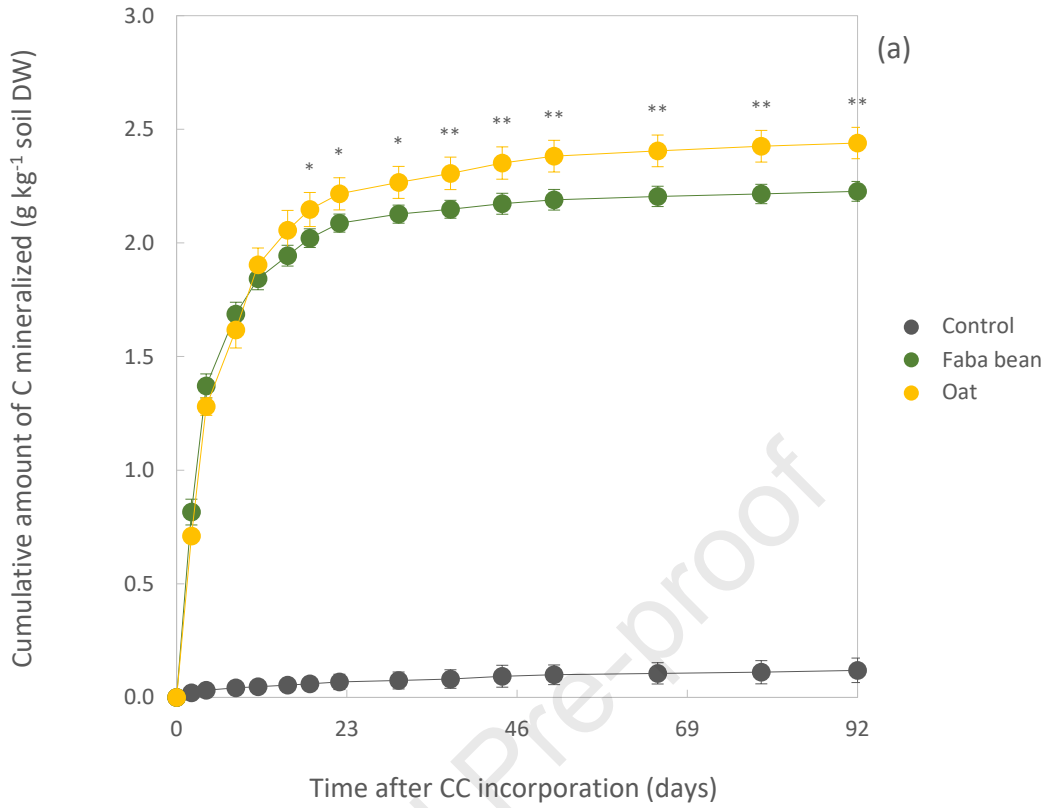


Figure 2

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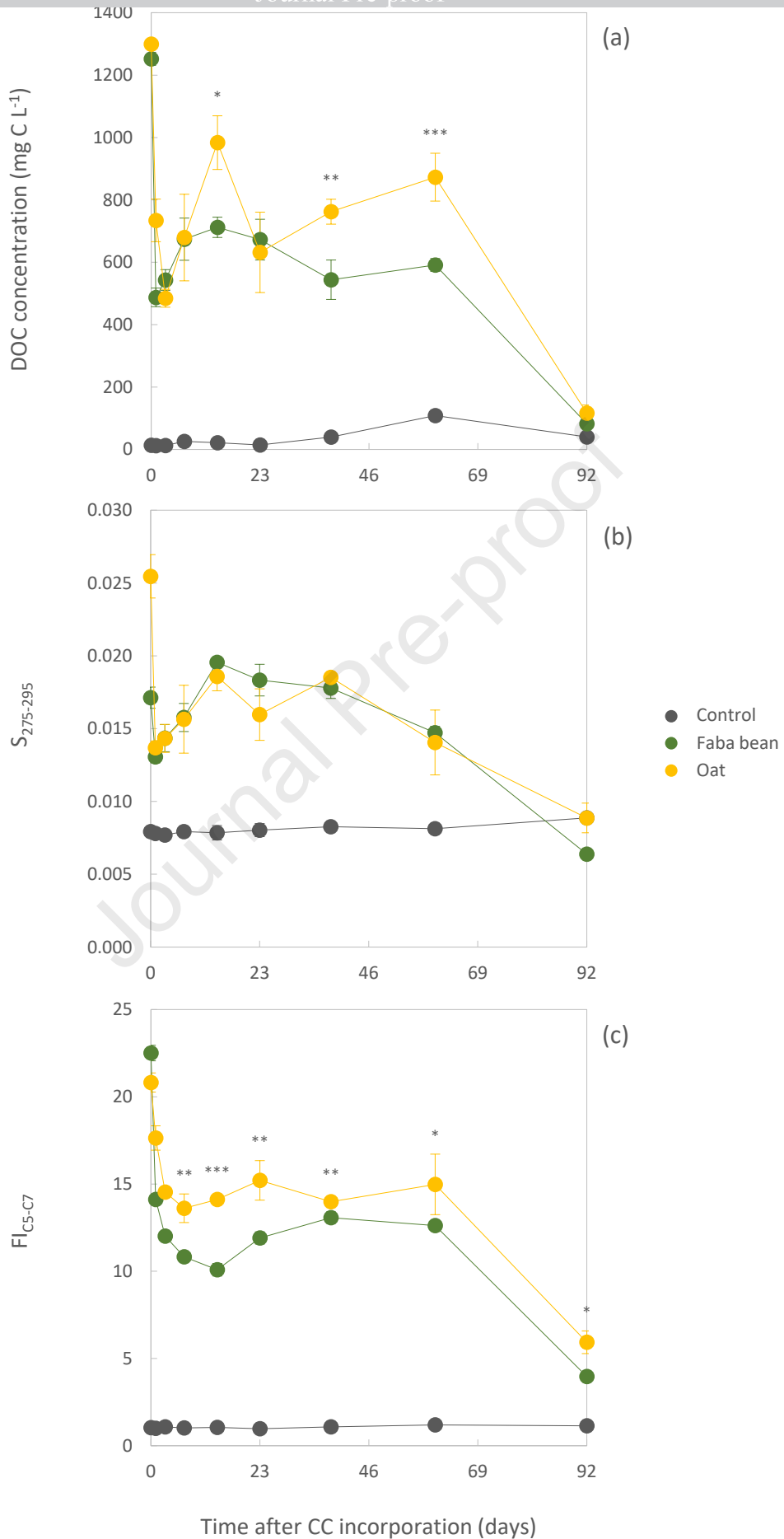
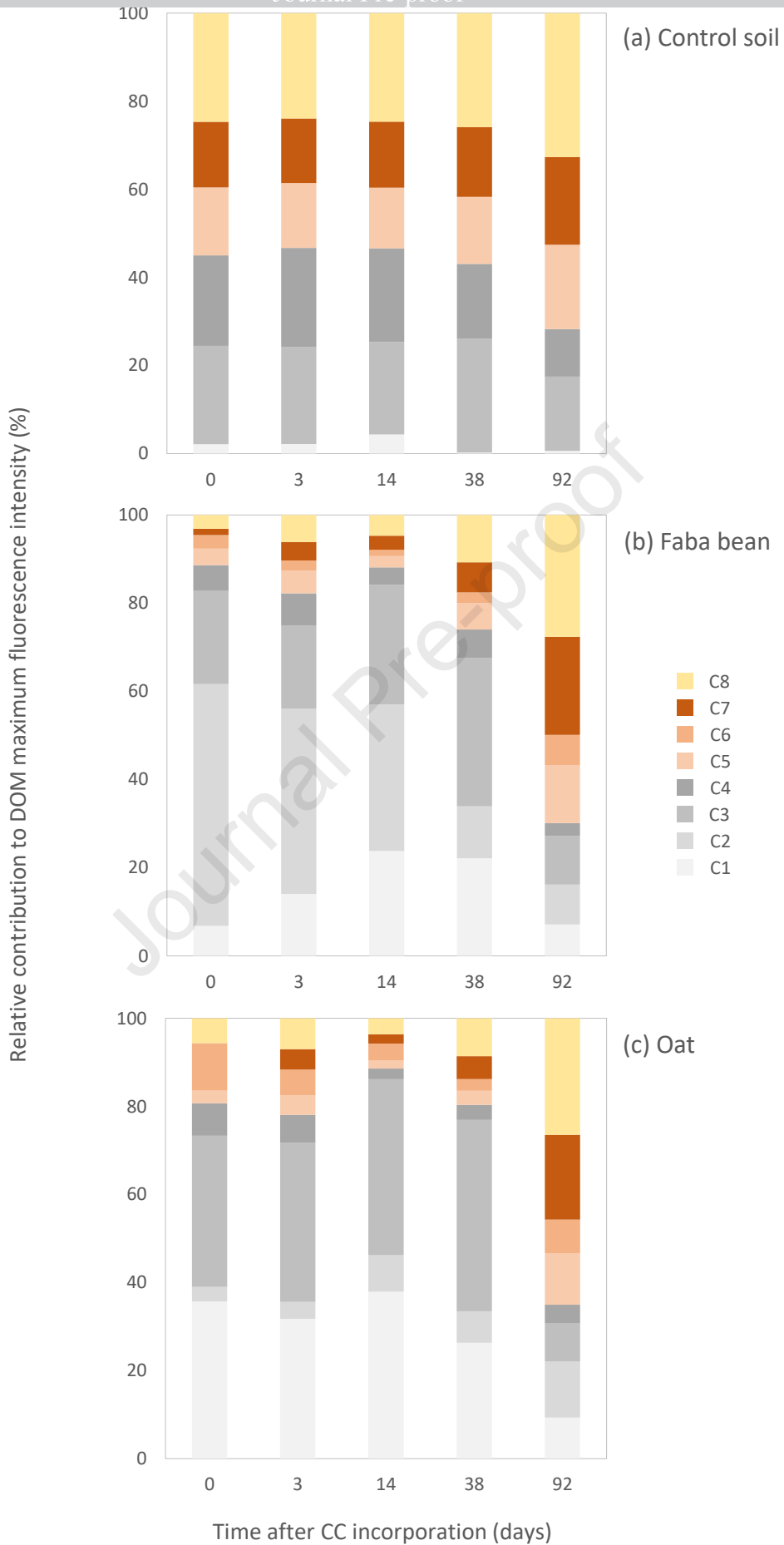
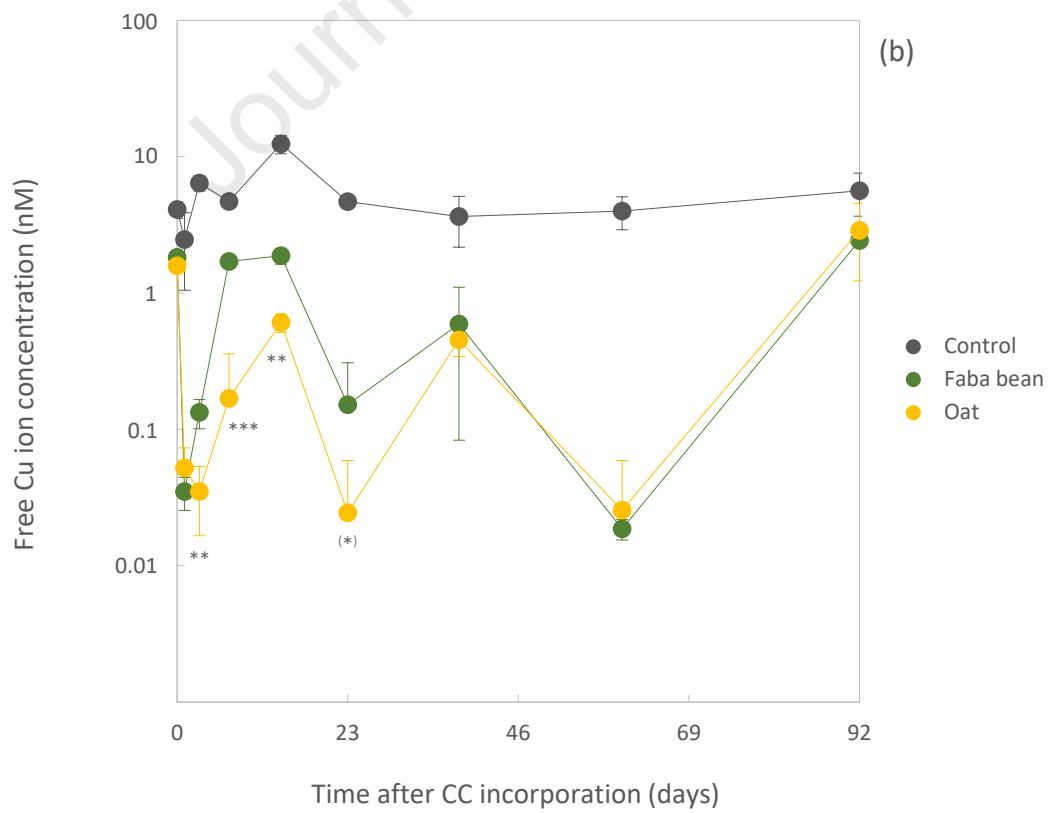
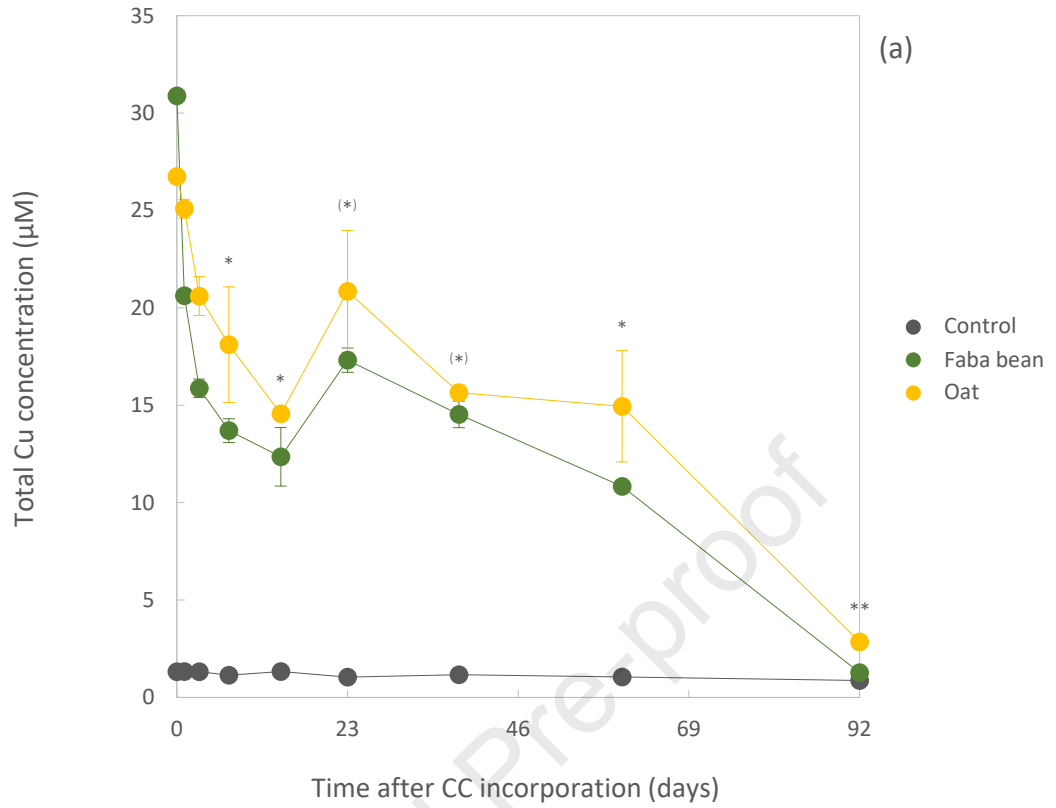
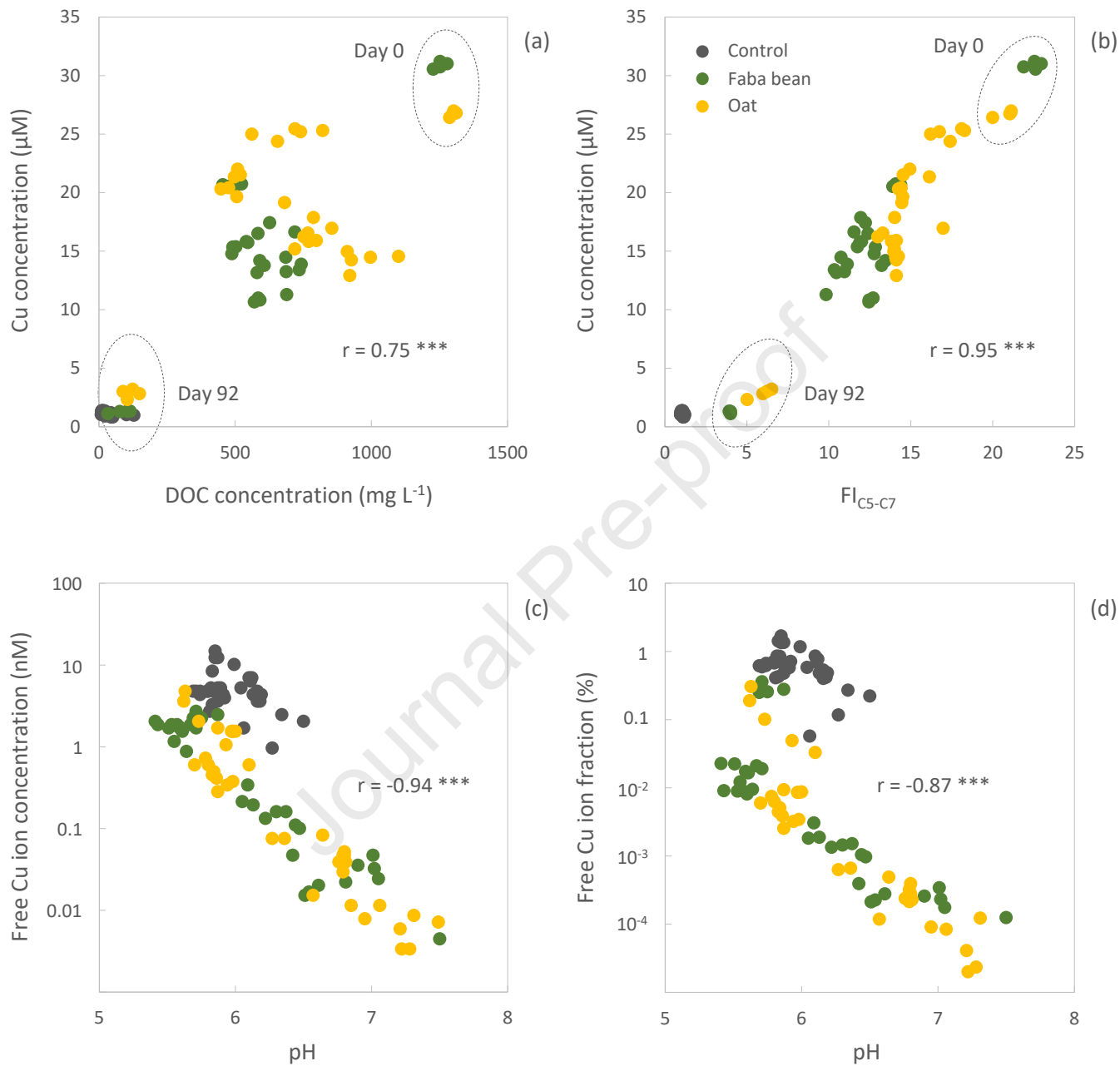


Figure 3

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Highlights

Adding cover crops (CCs) transitorily altered the dynamics of Cu in vineyard soil

Adding CCs transitorily increased the concentration of dissolved organic matter

Optical properties of dissolved organic matter changed over time after adding CCs

Soluble forms of C released by cover crop residues are capable of solubilizing Cu

Oat and faba bean had almost the same effect on the dynamics of DOM and Cu

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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