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## **Fulvic acids from Amazonian anthropogenic soils: Insight into the molecular composition and copper binding properties using fluorescence techniques**

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1 **Fulvic acids from Amazonian anthropogenic soils: Insight into the molecular**  
2 **composition and copper binding properties using fluorescence techniques**

3

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16

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25

## Abstract

26

27 Fulvic acids (FA) are one of the components of humic substances and play an important  
28 role in the interaction with metallic species and, consequently, the bioavailability,  
29 distribution and toxicity of metals. However, only a few studies have investigated these  
30 FA properties in specific environment, such as anthropogenic soils. Therefore,  
31 knowledge about FA molecular composition as well as the FA-metal interaction is  
32 essential to predict their behavior in the soil. For this reason, the aim of this study was  
33 to investigate the molecular composition of FA extracted from two sites in an  
34 anthropogenic soil (*Terra Mulata*), from the Amazon region, as well as their  
35 interactions with Cu(II) ions as a model. Results from  $^{13}\text{C}$  NMR, infrared and elemental  
36 analysis showed that these FA are composed mostly by alkyl structures and oxygen-  
37 functional groups, e.g., hydroxyl, carbonyl and carboxyl. The interaction with Cu(II)  
38 ions was evaluated by fluorescence quenching, in which the FA showed both high  
39 quantity of complexing sites per gram of carbon and good affinity to interact with the  
40 metal when compared with other soil FA. The results showed that the complexation  
41 capacity was highly correlated by the content of functional groups, while the binding  
42 affinity was largely influenced by structural factors. In addition, through the lifetime  
43 decay given by time-resolved fluorescence, it was concluded that static quenching took  
44 place in FA and Cu(II) interaction with the formation of a non-fluorescent ground-state  
45 complex. Therefore, this fraction of soil organic matter will fully participate in  
46 complexation reactions, thereby influencing the mobility and bioavailability of metal in  
47 soils. Hence, the importance of the study, and the role of FA in the environment, can be  
48 seen especially in the Amazon, which is one of the most important biomes in the world.

49 **Keywords:** *Terra Mulata* soils, humic substances, metal complexation, PARAFAC,  
50 lifetime

## 51 **1. Introduction**

52       The Amazon biome has worldwide importance (Coe et al., 2013) due to its extent,  
53 biodiversity, and acting as a large carbon reservoir (Batjes and Dijkshoorn, 1999).  
54 Amazonian anthropogenic soils, also called *Terra Mulata* (TM) soils are found in some  
55 Amazon regions. They are dark colored soils, with a high nutrient content and high  
56 fertility (dos Santos et al., 2020; Oliveira et al., 2018; Lucheta et al., 2017; Fraser et al.,  
57 2011; Cunha et al., 2009; Glaser et al., 2000), contrasting with other Amazonian soils  
58 which are highly acidic, with low fertility and cation exchange capacity (Soares et al.,  
59 2005). Their formation is still unclear, but researches have pointed out that, human  
60 activity in the pre-Columbian past has resulted in a large organic matter and nutrient  
61 accumulation over the years (Clement et al., 2015; Novotny et al., 2009). Therefore,  
62 such soils have been considered as a model for sustainable agriculture (dos Santos et al.,  
63 2020; Novotny et al., 2009).

64       The organic matter characteristics vary among soils (Baird and Cann, 2012),  
65 making the characterization and documentation of each TM site very important once the  
66 area of occurrence is restricted and limited, in order to know as much as possible about  
67 their characteristics and particularities (Novotny et al., 2009). Studies have shown that  
68 the organic matter from Amazonian anthropogenic soils is recalcitrant, due to the  
69 presence of carbon nanoparticles with graphite-like cores, in which their structures are  
70 very stable (Archanjo et al., 2014; Oliveira et al., 2018). This graphite-like structure  
71 prolongs the carbon residence time in the soil.

72       Humic substances (HS) are the main constituents of soil organic matter, and are  
73 formed from the remains of plant and animal degradation (Stevenson, 1994). According  
74 to its solubility in aqueous medium, HS is usually grouped in three fractions: humic  
75 acids (HA), fulvic acids (FA) and humin (HU). In the literature several studies are  
76 reported, with the most diverse purposes, involving HA from soils, due to its great

77 agronomic importance (dos Santos et al., 2020; Bento et al., 2020; Canellas and  
78 Façanha, 2004; da Silva et al., 2020; Nebbioso and Piccolo, 2011; Novotny et al., 2009;  
79 Senesi et al., 2003; Tadini et al., 2020; Vergnoux et al., 2011). FA also have a great  
80 influence on soil functionality, however, the amount of studies regarding FA from soils  
81 in the literature is less compared to HA.

82 FA plays an important role in the mobility of metal ions, affecting their  
83 distribution, toxicity, and bioavailability in soils (Tadini et al., 2020; Zhu and Ryan,  
84 2016; Zhu et al., 2014). Thus, the study of its role in the environment is also of great  
85 value. This fraction of HS is the most reactive, and the most mobile, due to its solubility  
86 with an abundance of oxygen-functional groups, that are responsible for the high  
87 complexation capacity with metals, especially copper ions (Tadini et al., 2020; Esteves  
88 da Silva et al., 1998). Copper is an essential micronutrient for plant growth and  
89 development, but both excess and lack can be harmful (Yruela, 2005). Therefore,  
90 maintaining adequate concentrations of this element is necessary.

91 The chelating properties of FA can either reduce toxicity when there is an excess  
92 of copper, or prevent it from precipitating as insoluble hydroxide, which renders it  
93 inaccessible to the plant (Boguta and Sokołowska, 2020; Bertoli et al., 2016; Stevenson,  
94 1994). Tadini et al. (2020) studied the interaction of copper with FA from Amazonian  
95 Spodosols. The FA showed greater interaction and quantity of complexation sites over  
96 the soil profile than the HA obtained from the same local. Moreover, the effect of the  
97 addition of pig slurry on the complexation properties of FA from a Calcic Luvisol was  
98 evaluated by the FA interaction with copper (Plaza et al., 2005). It was observed that the  
99 amendment with pig slurry decreased the binding affinity and complexation capacity of  
100 soil FA compared to the control, and this observation was explained by the decrease in  
101 oxygenated functional groups in FA. In addition, different soils have different

102 proportion of functional groups, which leads to changes in affinity for metals.  
103 Therefore, since soil functionality depends on this, it is important to understand the  
104 molecular composition of FA, and how it interacts with metallic ions.

105 Fluorescence excitation-emission matrix (EEM) coupled to parallel factor analysis  
106 (PARAFAC) has proved to be a powerful technique to investigate the binding properties  
107 between organic matter with metallic species, and this due to the high sensitivity, low  
108 cost and low sample volume (dos Santos et al., 2020; Tadini et al., 2020; Yuan et al.,  
109 2015; Mounier et al., 2011; Stedmon and Bro, 2008). Further, because different  
110 chemical species have different fluorescence lifetimes, it is also possible to study the  
111 fluorescence quenching mechanism through time-resolved fluorescence (TRF).  
112 Thereby, the possible mechanisms includes dynamic or static quenching (dos Santos et  
113 al., 2020; Nouhi et al., 2018).

114 Thus, the aim of this study was the molecular characterization of fulvic acids  
115 extracted from *Terra Mulata* soils. In addition, the interaction of fulvic acids with  
116 Cu(II) ions were assessed by fluorescence quenching and time-resolved fluorescence in  
117 order to provide quantitative data on the complexation capacity, conditional stability  
118 constant and complexation mechanism. And, in the end, provide subsidies for those  
119 seeking to understand the intrinsic characteristics of these soils to later try to mimic  
120 experimentally the chemical nature of its organic matter.

121

## 122 **2. Materials and Methods**

### 123 **2.1 *Terra Mulata* sampling**

124 Soil sampling was performed following the 2000 United States Environmental  
125 Protection Agency method (US EPA, 2000). Two different sampling areas of TM were  
126 selected: the first one is from an open area, covered with native and low vegetation  
127 (called TM I; 3°04'05.45" S and 58°33'51.11" W) and the second one is from a closed

128 area with native vegetation (called TM II; 3°04'05.17" S and 58°34'11.68" W). All areas  
129 are located near Itacoatiara city, in Amazonas State (Brazil) (SISBio collection  
130 authorization by the Chico Mendes Institute for Biodiversity Conservation, n° 50042 -2  
131 and registration in the National System for the Management of Genetic Heritage and  
132 Associated Traditional Knowledge (SisGen), No. A0018C2). Soil samples were  
133 collected from layers, 0 to 30 cm deep, air-dried, and sieved through a 2 mm mesh to  
134 remove roots and fragments. Soil characterization can be found in Oliveira et al. (2018)  
135 and Santana et al. (2019).

136

## 137 **2.2 Extraction of fulvic acids**

138 The FA extractions were performed following the International Humic  
139 Substances Society (IHSS) recommendations (Swift, 1996). HS were extracted from  
140 TMs in a proportion of 1:10 (soil:extractor), using 0.1 mol L<sup>-1</sup> NaOH aqueous solution  
141 under N<sub>2</sub> in a mechanical shaking for 4 h. The alkaline extracts were separated by  
142 centrifugation. Therefore, the solutions were acidified with 6 mol L<sup>-1</sup> HCl aqueous  
143 solutions until pH~1 was achieved. This procedure was carried out to separate the HA  
144 and FA fractions: HA fraction was precipitated while FA remained in the supernatant.  
145 Subsequently, this solid-liquid mixture was centrifuged. For FA purification an  
146 adsorption resin (XAD-8) was used, and the alkaline elution passed through a cation  
147 exchange resin (Amberlite IR 120, H<sup>+</sup> form). Subsequently, the extracts of FA were  
148 freeze-dried for further analysis.

149

## 150 **2.3 Characterization of fulvic acids**

### 151 **2.3.1 Elemental analysis**

152 Elemental analyses of FA were performed on solids using an Elemental  
153 Analyzer instrument (2400 Series II CHNS/O, Perkin Elmer, Walther, Massachusetts,

154 USA). Two replicates were performed for each sample. The ash content was determined  
155 in a muffle furnace at 750 °C for 4 h (three replicates). The O content was obtained as  
156 shown in Equation 1. After, C, H, N, S, and O contents were recalculated on an ash-free  
157 basis. The H/C and O/C atomic ratios were determined from elemental analysis (dos  
158 Santos et al., 2020; Giovanela et al., 2010; Stevenson, 1994).

159

$$160 \quad O\% = 100 \% - (C\%, H\%, N\%, S\% + ashes\%) \quad (1)$$

161

### 162 **2.3.2 Infrared spectroscopy**

163 Infrared spectra were obtained using a spectrophotometer coupled with an  
164 attenuated total reflectance (ATR) accessory equipped with a single reflection diamond  
165 crystal (ATR-FTIR) (Perkin Elmer, Spectrum Two UATR, USA). The solid material  
166 was placed directly on the ATR crystal and analyzed using the 4000-400  $\text{cm}^{-1}$  spectral  
167 range with 20 scans and a resolution of 4  $\text{cm}^{-1}$ .

168

### 169 **2.3.3 Solid-state $^{13}\text{C}$ -CPMAS-NMR spectroscopy**

170 The  $^{13}\text{C}$ -CPMAS-NMR of FA was performed employing a Bruker AVANCE  
171 400 MHz spectrometer (Bruker, Billerica, MA, USA) equipped with a magic angle spin  
172 (MAS) probe. A zircon cylindrical rotor with a diameter of 4.0 mm was packed with 80  
173 mg of sample. The spectra were acquired using a rotor rotation frequency of 10 kHz, 1 s  
174 of recycle time, a contact time of 1 ms and 5000 scans. The spectral areas were  
175 integrated and divided into six chemical shifts: alkyl C (0-44 ppm),  $\text{OCH}_3/\text{NCH}$  (44-64  
176 ppm), O-alkyl C (64-93 ppm), anomeric C (93-113 ppm), aromatic C (113-160 ppm)  
177 and carbonyl C (160-200 ppm) (Hu et al., 2019; Xu et al., 2017).

178

179



## 180 2.4 Interaction of fulvic acids with Cu(II)

### 181 2.4.1 Fluorescence quenching

182 The interaction of Cu(II) with FA was studied by fluorescence quenching. The  
183 concentration of FA in all experiments was kept constant at 10 mg L<sup>-1</sup>. Twelve solutions  
184 with different concentrations of Cu(II) (Cu(ClO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O; Sigma-Aldrich, 98%), ranging  
185 from 0 to 11.2 mg L<sup>-1</sup> were added to FA solution in a buffer medium with 0.1 mol L<sup>-1</sup>  
186 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at pH 7.0. The  
187 equilibrium time was determined based on the changes in the fluorescence signal and  
188 was kept at 30 min. EEM spectra were obtained on a spectrofluorometer (F4500,  
189 Hitachi, USA) equipped with a xenon lamp (450 W). The spectra were acquired in the  
190 scanning ranges 220-700 nm for emission and 220-500 nm for excitation. The excitation  
191 and emission slits were fixed at 5 nm, the scan speed was set at 2400 nm min<sup>-1</sup>, and the  
192 detector voltage was 700 V. The EEM spectra obtained were treated using the  
193 chemometric tool known as parallel factor analysis (PARAFAC). The number of  
194 components responsible for fluorescence was assessed by the core consistency  
195 diagnostic (CORCONDIA) (dos Santos et al., 2020; Mounier et al., 2011). In addition,  
196 the complexation parameters were determined by the optimization proposed by Ryan  
197 and Weber (1982), as simplified by Equation 2.

198

$$I = \left( \frac{I_{res} - 100}{2KC_L} \right) \left[ (KC_L + KC_M + 1) - \sqrt{(KC_L + KC_M + 1)^2 - 4K^2C_LC_M} \right] + 100 \quad (2)$$

199

200 where I is the fluorescence intensity during the titration process, I<sub>res</sub> is the residual  
201 fluorescence intensity, K is the conditional stability constant, C<sub>M</sub> and C<sub>L</sub> represents the  
202 total concentration of metal and ligand in mol L<sup>-1</sup> in the solution system, respectively.

203 Both  $C_L$ ,  $K$  and  $I_{res}$  were optimized to give the smallest residue between theoretical and  
204 experimental values.

205

## 206 **2.4.2 Time-resolved fluorescence**

207 Through the results of fluorescence quenching, three samples out of twelve were  
208 selected for lifetime measurements, i.e., three samples containing different copper  
209 concentrations for each FA interaction. The first one contained no copper ( $C_0$ ) and two  
210 others contained  $0.7 \text{ mg L}^{-1}$  ( $C_{0.7}$ ) and  $5.6 \text{ mg L}^{-1}$  ( $C_{5.6}$ ), respectively. Before each  
211 measurement, the sample was bubbled with nitrogen for at least 10 min to avoid  
212 photodegradation by dissolved  $O_2$  (dos Santos et al., 2020). The excitation wavelength  
213 was at 266 nm corresponding to the fourth harmonic of a nanosecond laser Nd:YAG  
214 (Quanta-Ray INDI, Spectra Physic, Germany), for which excitation (1024 nm) occurred  
215 with a pulse width of 5 ns and a repetition of 20 Hz. The fluorescence signal was  
216 detected using an intensified charge-coupled device (CCD) camera with a 35 ns gate  
217 width and six hundred spectra were accumulated at each time step of 0.2 ns. The  
218 spectral deconvolution and associated lifetime values were obtained by using a  
219 homemade MATLAB<sup>®</sup> program.

220

## 221 **3. Results and discussion**

### 222 **3.1 Characterization of fulvic acids**

223 Table 1 lists the elemental composition, atomic ratios and ashes of fulvic acids  
224 from the two soil areas. In general, both FA samples, from TM I and TM II, showed  
225 oxygen as the main constituent (62.68 and 57.94, respectively), followed by carbon,  
226 hydrogen, nitrogen, and sulfur. This trend is not common for FA (oxygen content higher  
227 than carbon), but can also be seen in Xiaoli et al. (2008). As there are no other studies in

228 the literature on FA from *Terra Mulata* soils, this fact is attributed to the intrinsic  
 229 characteristics of the samples. To further investigate the characteristics of the FA  
 230 samples, the H/C and O/C atomic ratios were calculated from the elemental analysis.  
 231 These ratios are related to the degree of aromatic rings condensation and the content of  
 232 oxygen-functional groups, respectively (Giovanela et al., 2010; Stevenson, 1994).  
 233 Higher values for H/C atomic ratios were found in the FA from TM compared to the FA  
 234 extracted from Cambisols, Podzols and Chernozems, located in China, Italy and  
 235 Canada, respectively (Limura et al., 2012), indicating more contribution of alkyl chains  
 236 in FA from TM. The same behavior (higher values) were found for the O/C atomic ratio  
 237 in the FA from TM, indicating more contribution of oxygen-functional groups (Limura  
 238 et al., 2012).

239

240 **Table 1.** Weight percentage (\*) of carbon (C), hydrogen (H), nitrogen (N), oxygen (O),  
 241 sulfur (S) and ash contents, as well as O/C and H/C atomic ratios for FA from TM.

FA	C (%)	H (%)	N (%)	O (%)	S (%)	O/C	H/C	Ash (%)
TM I	31.32	3.21	1.38	62.68	1.40	1.50	1.23	6.89±0.19
TM II	35.68	3.54	1.71	57.94	1.13	1.22	1.19	6.23±0.24

242 \*Ash-free basis

243

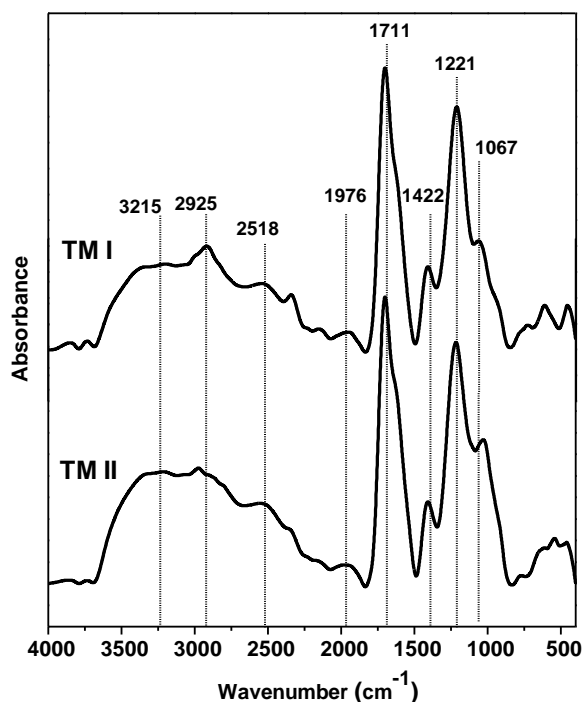
244 The FTIR spectra of FA are shown in Fig. 1. In general, both spectra are slightly  
 245 similar. The broad and intense band in the range of 3500-3000 cm<sup>-1</sup> is related to O-H  
 246 stretching vibration of alcohols, carboxylic acids, phenols and also N-H stretching  
 247 vibration of amide or amine groups (Vergnoux et al., 2011). It can be seen in the region  
 248 of 3000-2800 cm<sup>-1</sup> bands corresponding to alkyl stretching vibration, which was more  
 249 evident for FA from TM 1 (Li and Wu, 2013; Vergnoux et al., 2011). In addition, the

250 absorption near  $2500\text{ cm}^{-1}$  could be attributed to carboxylic acids dimers formed by  
251 hydrogen-bonds (Bertoli et al., 2016).

252 In the region of  $2000$  to  $1000\text{ cm}^{-1}$  it is difficult to precisely interpret due to the  
253 various overlapping bands. Therefore, for a better interpretation of the infrared spectra,  
254 Fig. S1 shows the second derivative curves. This tool has been used to separate  
255 broadband absorption peaks of complex matrices which show new peaks that were  
256 previously imperceptible (Tinti et al., 2015). In the  $1700\text{ cm}^{-1}$  region it is possible to  
257 observe bands in  $1791\text{ cm}^{-1}$  and  $1711\text{ cm}^{-1}$  related to the C=O stretching of carboxylic  
258 acids. This is the most intense and well defined band observed for both FA. In addition,  
259 the aromatic portion of AF can be observed by the appearance of the bands near  $1620$   
260 and  $1530\text{ cm}^{-1}$  in the second derivative curves (Fig. S1), which was not previously  
261 shown in the original spectra (Fig. 1) (Gondar et al., 2005). Additionally, the band near  
262  $1420\text{ cm}^{-1}$  was associated to vibrations of C-H in methylene groups.

263 The contribution of carbohydrate-like compounds to the composition of FA can  
264 be assessed by the appearance of bands referring to the asymmetric stretch of C-O-C  
265 and C-O (around  $1200$  and  $1100\text{ cm}^{-1}$ ). Further, the bands at  $1055\text{ cm}^{-1}$  and  $1080$   
266  $\text{cm}^{-1}$  may have diverse origin. It may be either from C-O stretching or O-H groups, and  
267 even vibration of minerals, such as Si-O (Drosos et al., 2009; Giovanela et al., 2010;  
268 Stevenson, 1994).

269



270

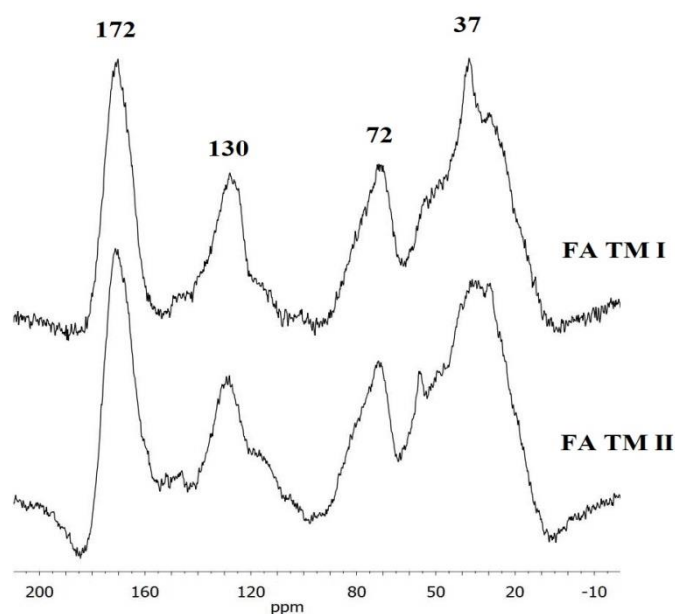
271 **Fig. 1.** FTIR spectra for FA extracted from TM soils.

272

273 As shown in Fig. 2, the  $^{13}\text{C}$ -CPMAS-NMR spectra of both FA exhibited the  
 274 same peaks. The results revealed the presence of alkyl and aromatic carbons, substituted  
 275 or not by heteroatoms and all chemical shifts were consistent with those observed in soil  
 276 FA by other authors (Hu et al., 2019; Xu et al., 2017; Xiaoli et al., 2008). Besides, the  
 277 spectra could be divided into six regions assigned to signals from alkyl C (0-44 ppm),  
 278  $\text{OCH}_3/\text{NCH}$  (44-64 ppm), O-alkyl C (64-93 ppm), anomeric C (93-113 ppm), aromatic  
 279 C (113-160 ppm) and carbonyl C (160-200 ppm) (Hu et al., 2019; Xu et al., 2017).

280 Chemical shifts near 37 ppm were assigned to long chain  $(\text{CH}_2)_n$  similar to those  
 281 present in cutin, suberin, and plant waxes (Lorenz et al., 2000). This peak was evident in  
 282 both FA. Furthermore, the peaks, in the 71-74 ppm range, centered at 72 ppm, were  
 283 attributed to the overlapping of carbons number 2, 3 and 5 in the pyranosidic cellulose  
 284 and hemicellulose structures. In addition, the signal at 130 ppm was assigned to  
 285 unsubstituted aromatic carbons in FA structures. The major peak at 172 ppm

286 represented the carbonyl C of carboxylic acid, ester and amide (Hu et al., 2019; Xiaoli  
287 et al., 2008), suggesting a high content of these functional groups.



288

289 **Fig. 2.** Solid-state <sup>13</sup>C-CPMAS-NMR spectra of FA samples.

290 The total area integration of spectra, made to obtain the relative intensity of each  
291 chemical shift, is shown in Table 2. The results revealed that alkyl and carbonyl  
292 functional groups were the dominant carbon components in FA structures, followed by  
293 aromatic C, OCH<sub>3</sub>/NCH, O-alkyl C, and anomeric C. As for the infrared analysis, both  
294 FA also had a very similar composition according to the <sup>13</sup>C NMR results. A small  
295 difference can be observed between samples, in which the FA from TM I have a slight  
296 increase in oxygenated groups and a slight decrease in hydrophobic functional groups,  
297 such as the aromatic and alkyl portions, and this when compared to the FA from TM II.  
298 This trend could also be seen through the H/C and O/C atomic ratios.

299

300

301

302 **Table 2.** Relative value (% of total area) obtained from  $^{13}\text{C}$ -CPMAS-NMR spectra of  
 303 FA samples.

$^{13}\text{C}$ NMR region (ppm)						
FA	200-160	160-113	113-93	93-64	64-44	44-0
<b>TM I</b>	19.29	16.40	0.32	15.76	16.08	32.15
<b>TM II</b>	17.82	17.82	0.33	14.85	16.17	33.00

304

305 Therefore, as previously reported, stable alkyl structures, rich in oxygen-  
 306 functional groups were found in the FA structures (Jouraiphy et al., 2008). In this study,  
 307 it was possible to observe the same patterns in  $^{13}\text{C}$  NMR, infrared and elemental  
 308 analysis. In addition, greater functionalization was observed in FA from TM compared  
 309 to other FA in the literature (Gondar et al., 2005; Lorenz et al., 2000; Xiaoli et al., 2008;  
 310 Xu et al., 2017). The presence of these functional groups (carbonyl, carboxyl, hydroxyl,  
 311 phenolic and aromatic portions) is extremely important, since they could act as reactive  
 312 sites in metal complexation reactions, and, in addition, reaffirms the particularity of this  
 313 kind of soil and the importance of FA in soil systems.

314

## 315 **3.2 Interaction of fulvic acids with Cu(II)**

### 316 **3.2.1 Fluorescence quenching**

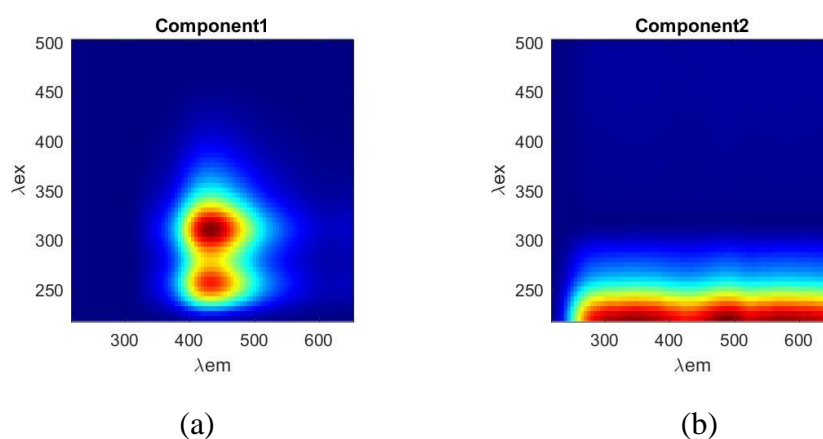
317 The interaction of FA from TM I and TM II with Cu(II) ions was studied by  
 318 fluorescence quenching, in which a two-component model was found for Cu(II)-FA  
 319 with a good CORCONDIA value (98.80%) (Fig. 3a and b). The first component (Fig.  
 320 3a) had a main peak at [ $\lambda_{\text{Ex}}$  260nm/ $\lambda_{\text{Em}}$  430nm] and the second one at [ $\lambda_{\text{Ex}}$  310nm/ $\lambda_{\text{Em}}$   
 321 430nm], which is characteristic of fulvic acids from soil (Tadini et al., 2019). These  
 322 peaks were at shorter emission wavelengths and in the blue region of the visible

323 spectrum, characterizing the presence of simple structures in FA samples (Senesi et al.,  
324 2003), such as alkyl groups. This corroborates with the  $^{13}\text{C}$  NMR measurement, H/C  
325 atomic ratio, and FTIR spectra, showing a high content of this functional group.

326 Fulvic acids has blue shifts for emission wavelengths compared to humic acids  
327 from the same origin (Sierra et al., 2000). Generally, the blue-shift phenomenon is  
328 attributed to the presence of more alkyl structures in samples. Emission fluorescence  
329 intensity in longer wavelengths were shown in humic acids from TM, the same soils  
330 used in this study (dos Santos et al., 2020). The fluorescence intensity in longer  
331 wavelengths is due to the presence of more aromatic structures, which, in the literature,  
332 is called red-shift.

333 The second component (Fig. 3b) presented a continuous emission range,  
334 showing no variation of its contribution during quenching experiments. This component  
335 is probably related to a noise factor in the UV domain (<250nm). Thus, this component  
336 was not included in the discussion.

337



338 **Fig. 3.** Components 1 (a) and 2 (b) obtained by PARAFAC for interaction of FA with  
339 Cu(II).

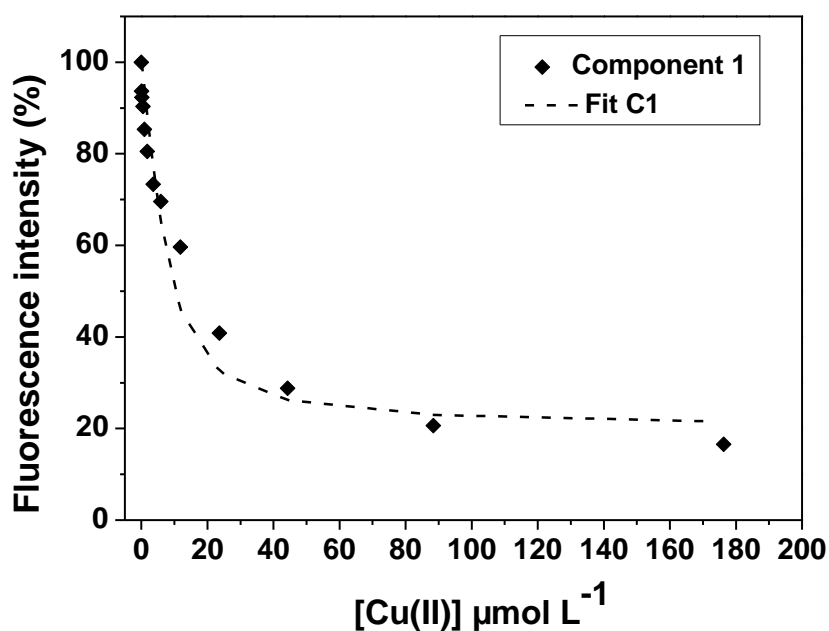
340



341 The data optimization using the 1:1 (ligand:metal) complexation model  
342 proposed by Ryan and Weber (1982) allowed the quantitative evaluation of  
343 fluorescence quenching. Bias was used as an optimization parameter. Mounier et al.  
344 (2011) defined Bias as the sum of the absolute value of the difference between the  
345 experimental, and the calculated fluorescence intensity. In this study, Bias values ranged  
346 from 2.02 to 2.29 (Table 3), which are lower than others found in the literature (Tadini  
347 et al., 2020), indicating that experimental results fit well to theoretical model. Moreover,  
348 the titration curves of component 1 referring to FA from TM I is presented in Fig. 4.  
349 The symbols represent the data obtained experimentally and the solid line show the  
350 theoretical values as a function of Cu(II) addition. FA from TM II showed the same  
351 behavior (Fig. S2).

352 The fluorescence quenching curve for FA from TM I and Cu(II) showed a  
353 fluorescence intensity decay of approximately 84% (Fig. 4). The quenching effect was  
354 strongest at the initial doses of copper (higher slope of the curve), where there were a  
355 large number of active sites that are now coordinating copper, thus forming complexes  
356 (Boguta et al., 2019; Mounier et al., 2011). In addition, the decrease of fluorescence  
357 contribution with increased metal concentration suggested the saturation of accessible  
358 sites, and the fact that the decay did not fall to zero is due to some fluorophores that did  
359 not participate in Cu(II) binding (Boguta et al., 2019; Boguta and Sokołowska, 2020).

360



361

362 **Fig. 4.** Fluorescence quenching curve of FA from TM I determined experimentally  
 363 (symbol) compared with the theoretical (dash line) as a function of Cu(II) addition.

364 The proposed complexation model allowed the calculation of  $C_L$  and  $K$ , where  
 365  $C_L$  represents the concentration of binding sites available for interaction with Cu(II)  
 366 ions and  $K$  is the conditional stability constant of the complex formed. The Log  $K$   
 367 values were 5.57 and 5.38 for the FA components from TM I and II, respectively (Table  
 368 3). These values were higher than those found for FA extracted from Amazonian  
 369 Spodosol (Tadini et al., 2020), Oak Forest soil (Esteves da Silva et al., 1998) and the  
 370 standard FA of Elliot Soil (IHSS sample) (Enev et al., 2016), indicating that the  
 371 complex formed between TM FA and Cu(II) is more stable (Table 3). In addition, high  
 372 stability constant values were reported for the interaction of humic acids also extracted  
 373 from TM with Cu(II), then demonstrating that both humic fractions present a great  
 374 affinity to interact with this metal (dos Santos et al., 2020). The complexation capacity  
 375 ( $C_C$ ) was obtained by dividing the  $C_L$  values, in  $\text{mol L}^{-1}$ , by the carbon concentration  
 376 calculated from the FA carbon content, in  $\text{mg C L}^{-1}$ , and represents the density of  
 377 binding sites (in mol) for a metal (Cu(II)) per gram of carbon in the organic matter

378 (fulvic acids). Therefore, the  $C_C$  values calculated for FA components from TM I and II  
379 were 1.43 and 2.09 mmol of Cu(II)  $g^{-1}$  C, respectively (Table 3). These values were also  
380 higher than the other FA found in the literature (Esteves da Silva et al., 1998; Tadini et  
381 al., 2020), suggesting a high concentration of binding sites available to interact with  
382 Cu(II) ions (Table 3).

383         Gathering the results, both FA from TM showed a high quantity of binding sites,  
384 as well as an affinity for interaction with Cu(II), then, acting in complexation reactions  
385 and consequently the bioavailability of copper. Generally, paramagnetic metal ions,  
386 such as Cu(II), display high stability constants values with FA. It may be explained by  
387 the  $3d^9$  configuration of the divalent copper with incomplete d orbitals. In addition,  
388 studies indicate that the high stability constant for the complex formed is associated  
389 with the increase of acidic functional groups and other oxygen-containing ligand groups  
390 (Guo et al., 2012, 2015). Oxygen is a Lewis base, a small atom and electron donor in  
391 carboxyl, carbonyl and hydroxyl groups. Exhibit high electronegativity, and form strong  
392 bonds with small Lewis acids, such as Cu(II) (Pearson's rule). These oxygenated  
393 functional groups were observed for FA, as shown in  $^{13}C$  NMR, FTIR and Elemental  
394 Analysis. So, FA had good interaction with Cu(II) due to its molecular structure.  
395 Therefore, these results are important for understanding the mobility and fate of copper  
396 in the environment.

397 **Table 3.** Conditional stability constant (Log K) and complexation capacity ( $C_C$ ) for component 1 regarding the interaction of FA from  
 398 *Terra Mulata* with Cu(II) ions and other FA extracted from soils reported in the literature.

Soil	Metal	Method	Conditional stability constant (Log K)	Complexation capacity ( $C_C$ ) (mmol Cu(II) g <sup>-1</sup> C)	Reference
<i>Terra Mulata I</i>	Cu(II)	Ryan and Weber	5.57	1.43	This study
<i>Terra Mulata II</i>	Cu(II)	Ryan and Weber	5.38	2.09	This study
Amazonian Spodosol	Cu(II)	Ryan and Weber	2.26–5.31	0.11–2.44	(Tadini et al., 2020)
Oak Forest	Cu(II)	Ryan and Weber	4.33–4.60	0.03–0.04	(Esteves da Silva et al., 1998)
Elliot soil (IHSS sample)	Cu(II)	Stern-Volmer	4.45	-	(Enev et al., 2016)

399 - not realized

400

401

402

### 403 3.2.2 Time-resolved fluorescence

404 The quenching process can be induced by two mechanisms. The first one is  
405 static quenching, with the formation of a non-fluorescent ground-state complex, where  
406 the lifetime of the complex is not affected. The second one is dynamic quenching,  
407 which is caused by collision with a quencher and fluorophore during the lifetime of the  
408 excited state, thus, affecting the fluorescence lifetime (Lakowics, 1999). Therefore, as a  
409 complementary technique to fluorescence quenching, TRF was used to assess the  
410 interaction mechanism between FA from TM and Cu(II) and distinguish whether a static  
411 or dynamic quenching process occurred. Furthermore, to the best of our knowledge, this  
412 kind of analysis for any FA from TM is not in the literature, thus, highlighting the  
413 importance of this analysis.

414 Fig. S4 shows the fluorescence lifetime distribution of FA complexes with  
415 copper. Through the deconvolution results, the decay was found to be mono-exponential  
416 ( $\tau_1$ ), i.e., containing only one lifetime. For both FA the average lifetime values observed  
417 were approximately  $5.0 \pm 0.2$  ns. These results fit well with the PARAFAC, in which  
418 the lower lifetime can be associated with component 1 (Fig. 3a), which has a simpler  
419 fluorophore structure.

420 Therefore, the average lifetime values appeared to be independent of metal  
421 addition (Fig. S4). This suggests that the mechanism of fluorescence quenching between  
422 Cu(II) and FA occurred by static quenching, with a non-fluorescent ground-state  
423 complex. Nouhi (2018) reported different lifetime values, but also static quenching  
424 between Cu(II) with FA extracted from the Saint Lawrence Estuary in Canada. This is  
425 because different samples have different fluorescence decay, showing the high  
426 sensitivity and selectivity of TRF. Further, dos Santos (2020) determined the  
427 fluorescence lifetime for the interaction of humic acids from TM and Cu(II). Static

428 quenching was also the predominant complexation mechanism and, when compared to  
429 this study, similar values were found for  $\tau_1$ .

430 In addition, the study of soils in terms of their complexation properties is of  
431 great value and provides important information regarding the fate of metals in the  
432 environment. TM is considered as a model of fertile soil, and researchers have been  
433 focusing on trying to reproduce this kind of soil in the laboratory, or at least,  
434 reproducing its characteristics (Cernansky, 2015). Techniques such as hydrothermal  
435 carbonization has been explored as an alternative to reproduce this anthropogenic soil  
436 particularities (dos Santos et al., 2020; Fregolente et al., 2020; Bisinoti et al., 2019;  
437 Bento et al., 2019; Santana et al., 2019; Melo et al., 2017; Silva et al., 2017; Kambo and  
438 Dutta, 2015). Therefore, it is essential to know the intrinsic characteristics of TM and  
439 this study can be a guide for those who seek to mimic these properties. Furthermore,  
440 there are no previous studies regarding the molecular characterization of fulvic acids  
441 from *Terra Mulata* soils, as well as its interaction with metallic species. This shows the  
442 novelty of the present work, as it brings brand new information about TM, from one of  
443 the sites in the Amazon basin. In addition, by showing their great capacity to interact  
444 with metallic species, the capacity of the technique to measure this and, by providing  
445 new information about the mechanism of complexation, the importance of focusing on  
446 the study of FA is demonstrated, as no information is available in the current literature  
447 about this.

448

#### 449 **4. Conclusion**

450 The fulvic acids extracted from *Terra Mulata* soils were mainly composed of  
451 alkyl and oxygen-functional groups. The excitation-emission fluorescence spectroscopy  
452 coupled to PARAFAC showed that simple structures and less aromatic, which emit

453 fluorescence at low emission wavelengths, were responsible for the fluorescence  
454 phenomena, corroborating with the information presented in the structural analysis.  
455 High complexation capacity and strong binding ability were obtained between Cu(II)  
456 and fulvic acids from *Terra Mulata* soils when compared to other fulvic acids in the  
457 literature. This was due to the fact that the organic matter is well functionalized, with  
458 abundance in carboxyl, carbonyl and hydroxyl groups. Thus, the results showed that the  
459 complexation reactions are strongly correlated to the nature of the organic matter  
460 present in the soil. Furthermore, the interaction mechanism of fluorescence quenching  
461 that occurred between fulvic acids and Cu(II) was static quenching, with a non-  
462 fluorescent ground state complex formation.

463         This study sought to advance the knowledge of the chemical composition and  
464 binding properties of fulvic acids from *Terra Mulata* soils with metals. Therefore, these  
465 findings represent a breakthrough for those who seek to reproduce the intrinsic  
466 characteristics of Amazonian anthropogenic soils.

467

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482

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