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1 **Humic fractions from Amazon soils: Lifetime study and humification process by**
2 **fluorescence spectroscopy**

3

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16 **ABSTRACT**

17

18 The Amazon forest is an important and primordial ecosystem, as it controls the
19 dynamics of soil organic matter (SOM) in addition to being considered one of the most
20 important carbon reservoirs in the world. Based on that, this study evaluated the molecu-
21 lar properties of humic (HA) and fulvic (FA) acid from Amazon soil, with and without
22 Cu(II) ions, by Time Resolved Laser Fluorescence Spectroscopy (TRLFS) and Fluores-
23 cence Spectroscopy (FS). Fluorescence excitation-emission matrix (EEM) was obtained
24 by FS, and EEM spectra were treatment by canonical polyadic/parallel factor analysis
25 (EEM-CP/PARAFAC). The results showed that the humification index (A_{465nm}) deter-
26 mined by FS is directly dependent on fluorophores CII/CI ratio in these soils with depth
27 ($r = 0.89$), especially for HA samples, in which demonstrated that the CII consist more
28 complex and humified structure of the SOM. The decay of the lifetime showed the pres-
29 ence of static quenching in the humic fractions. From the decay life data provided by
30 TRLFS and the different correlations with carbon content and A_{465nm} , it can be deduced
31 that fluorescence suppression modifies the molecular properties of the humic fractions
32 with the addition of Cu(II) ions, such as altering the mobility and availability of the
33 metal in the complexation reactions in these Amazonian Spodosols, mainly for fulvic
34 fraction.

35

36 **Keywords:** Humic fractions, Lifetime, Molecular changes, Humification process,
37 Amazon soil.

38 **1. Introduction**

39 Geochemical studies of soil formation in tropical regions, such as the Amazon
40 Forest, are important and primordial, as it controls the dynamics of soil organic matter
41 (SOM), and considered to be one of the most important carbon reservoirs in the world
42 (Cerri et al. 2000; Mafra et al. 2007; Carvalho et al. 2010; Anjos et al. 2021; Huamán et
43 al. 2021; Zaman, 2022). The Amazonian vegetation is quite diversified covering
44 different soil formations, especially the Spodosols systems. These soils are
45 characterized by having an organic litter on a thick sandy horizons overlying more
46 clayey horizons, and the organic matter (OM) produced in the superficial horizon is
47 transferred and accumulated in a deeper profile around 1 to 3 m, forming horizons rich
48 in OM (known as Spodic horizons, Bhs) (Montes et al. 2011; Lucas et al. 2012; Ishida
49 et al. 2014; 2018). Hydromorphic Spodosols from the upper Rio Negro basin can
50 accumulate large amounts of organic carbon in the surface layer around 14 ± 1 Pg of
51 Carbon (Cerri et al. 2000; Montes et al. 2011), demonstrating a high carbon storage
52 capacity in these soils in which can contribute to CO₂ mitigation in this ecosystem and
53 global scale.

54 The main components of SOM are humic substances (HS), i.e. humic acids (HA),
55 fulvic acids (FA) and humin (HU), which can be obtained by acid/alkali extractions
56 (Stevenson, 1994). These naturally occurring by-products of SOM have been
57 systematically studied (Nebbioso et al. 2015; Olk et al 2019a,b; Machado et al. 2020).
58 Fluorescence spectroscopy is frequently used in the literature to rapidly assess the
59 structural and molecular characteristics of OM and their humic fractions (Senesi and
60 D'Orzio, 2005; Tadini et al. 2015, 2018, 2020; Liu et al., 2020; Mohinuzzaman, et al.
61 2020; Soares da Silva et al. 2020; Mielnik et al. 2021; Rinot et al. 2021), recently
62 highlighting the use of Time Resolved Laser Fluorescence Spectroscopy (TRLFS)
63 (Nicolodelli et al. 2017; Nouhi et al., 2018; Dos Santos et al., 2020a,b). This technique
64 can measure the decay of the fluorescence intensity of a sample after excitation,
65 allowing the determination of the fluorescence lifetime in sample fluorophores (Millar,
66 1996). Besides, it can discriminate different chemical species, such as OM, fluorescent
67 metallic ion and their complexes, since they can have different decay lifetime and
68 spectral shapes (Nouhi et al., 2018; Dos Santos et al., 2020a,b).

69 In the literature there are many studies using the TRLFS for proteins and nucleic
70 acids characterization, especially tryptophan, due to its fluorescence being very sensi-
71 tive to environmental factors (Collins et al. 2011). However, there are few studies that

72 use this technique in the characterization of fluorophores in humic fractions, especially
73 HA and FA (Lukman et al. 2012; Nouhi et al., 2018; Dos Santos et al., 2020a,b) and this
74 technique has been growing mainly in the last two years (Figure S1).

75 An important characteristic of humic fractions in soil is their availability to inter-
76 act with metals by complexation reactions in which are responsible for biological avail-
77 ability, accumulation and mobility in the soil environment (Senesi et al. 1991). An es-
78 sential of growth of plants at soil is copper, which acting important metabolic reactions
79 such as photosynthesis and protein synthesis (Yruela, 2005; Gonzaga et al. 2020). Based
80 on this, the hypothesis of this study is that the interaction of divalent metal ion (repre-
81 sented by the copper(II) metal) with SOM, will affect its humification process due to the
82 formation of the metallic complex (Cu(II)-SOM) in which will be verified by TRLFS.
83 To test this hypothesis and obtain a higher level of molecular and complexation knowl-
84 edge of the humic (HA) and fulvic acids (FA) from Amazon Spodosols with and with-
85 out added Cu(II) ions, it will be using the Time Resolved Laser Fluorescence Spec-
86 troscopy (TRLFS), Fluorescence Spectroscopy (emission-excitation matrix, EEM) and
87 mathematical method known as canonical polyadic/parallel factor analysis (CP/
88 PARAFAC).

89

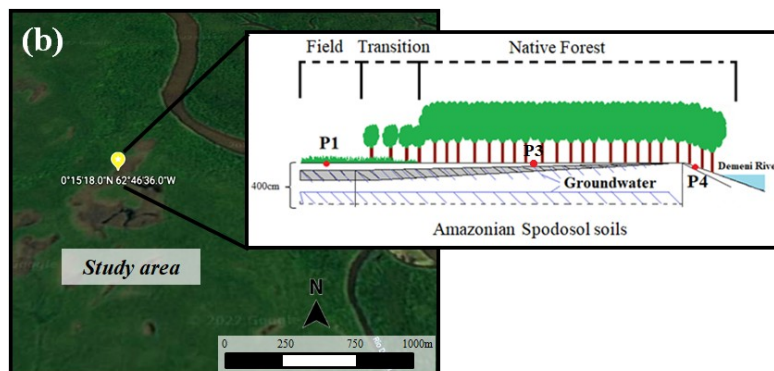
90 **2. Materials and Methods**

91 *2.1. Study area*

92 The study area is located to the north of the city of Barcelos, near the Demeni
93 River (0° 15' 18" N; 62° 46' 36" W) and the soils were classified as Spodosol according
94 Soil Taxonomy (Soil Survey Staff, 2014). The area is tropical climate with average
95 temperature of 26°C and 3,000 mm of mean annual rainfall (Alvares et al. 2013). The
96 samples were from: (a) a permanently waterlogged Spodosol covered by herbaceous
97 meadow of shrubs and grass, locally named "*campina*" (P1); (b) a seasonally
98 waterlogged Spodosol (P3) and (c) a well-drained Spodosol under rainforest vegetation
99 (P4) (Figure 1), more details in Tadini et al. (2018).



100



101

102 **Figure 1:** Soil sample collection sites of the Amazonian Spodosol, Amazon State,
 103 Brazil: (a) Aerial map of the study area (Image obtained from Google Earth in February
 104 2022) and (b) schematic illustration of the Amazonian Spodosol evaluated in this study.

105

106 2.2. Preparation of the samples for analysis

107 Soils samples at different depths (0 to 390 cm) were collected at the different
 108 pedologic horizon types according described in Tadani et al. 2018. To isolate the humic
 109 (HA) and fulvic (FA) acids fractions, the procedure recommended by the International
 110 Society of Humic Substances was used (Swift, 1996). Detailed information regarding
 111 extraction and purification of the humic fractions can be found in Figure S2 in
 112 Supplementary Material.

113

114 2.3. Fluorescence Spectroscopy

115 To allow spectroscopic fluorescence comparison ionic strength and pH were con-
116 trolled. The dilution factor was chosen to have no inner effect at final concentration fol-
117 lowing the description in Tadini et al (2018) in which a concentration test was previ-
118 ously performed to verify the best condition to be used in these studies. The HA and FA
119 samples were dissolved in a 0.05 mol L⁻¹ sodium bicarbonate solution (NaHCO₃) ob-
120 taining a solution with a concentration of 12.5 mg L⁻¹ of AF or AH (pH = 8.0) and left
121 in an ultrasonic homogenizer for 10 minutes to complete dissolution all the samples. A
122 spectrofluorimeter (model LS50B from Perkin Elmer) equipped with a Xenon lamp
123 (240 V) and connected to a computer was used.

124

125 2.3.1. Humification Index (A_{465nm})

126 The emission spectra with excitation at 465 nm (slit 10 nm) were obtained
127 with scanning from 480 to 700 nm (slit 10 nm), open filter, 500 nm.min⁻¹ scan speed
128 and the photomultiplier tension was at 700 V. The humification index (A_{465nm}) was de-
129 termined using the total area under the emission fluorescence with excitation at 465 nm
130 with a concentration solution of 12.5 mg L⁻¹ in pH = 8.0 for HA and FA (Milori et al.
131 2002)

132

133 2.3.2. Emission-Excitation matrix (EEM)

134 The EEM fluorescence spectra were acquired for each humic fractions (total-
135 izing 40 EEM samples of the three soils sites) in the scan range between 240-700 nm
136 (slit 10 nm) for emission and 220-510 nm for excitation (slit 10 nm), at 700 nm.min⁻¹
137 scan speed as described in Tadini et al. (2020). Sensitivity was checked every day using
138 ultrapure Raman peak emission. The spectra were treated using mathematical method
139 known as canonical polyadic/parallel factor analysis (CP/PARAFAC) (Bro, 1997; Mur-
140 phy et al. 2013). The CP/PARAFAC analysis decomposes the fluorescence signals from
141 the EEM spectra dataset into simple and independent fluorescent components, in which
142 result at the specifically excitation and emission spectrum (Murphy et al. 2013).

143

144 2.4. Time-Resolved Laser Fluorescence Spectroscopy (TRLFS)

145 A volume of 480 µL of a 8 mg L⁻¹ solution of HA or FA in 1,000 µL of Sodium
146 Acetate Buffer Solution (0.001 mol L⁻¹) were added in a quartz cuvette completed to
147 have a final volume to 3,300 µL with deionized water (pH=8). The prepared sample
148 solution was left for 10 minutes under a nitrogen gas atmosphere to eliminate the O₂ that

149 can induce photodegradation, and then the measurement was performed using a
 150 Quantaaurus (Tau J from Hamatsu model C11367). The time resolved laser fluorescence
 151 spectra were acquired in the scan range between 300-700 nm for emission and with
 152 excitation at 280 nm (LED light source with 5 MHz), while the maximum emission
 153 intensity was 300 counts with counting rate 5.0%, time range 50 ns and filter open. A
 154 known concentration of Cu(II) ions was added to each HA and AF sample and analyzed
 155 by TRLFS. The first HA or FA samples don't have concentration of copper (C_0),
 156 whereas $C_{0.26}$ contained 0.26 mg L^{-1} of Cu(II) (Dos Santos et al., 2020a,b).

157 The fluorescence decay curve was determined by a one-exponential function,
 158 according to equation 1:

$$159 \quad F(t, \lambda) = a_1(\lambda) e^{-\frac{t}{\tau_1}} \quad (1)$$

160 Where τ_1 (s) is value of fluorescence lifetime and $a_1(\lambda)$ is the emission
 161 wavelength coefficient related to component at $t=0$, and t the time in s. The average
 162 lifetime (τ_m) in this study was determined following to equation 2:

$$163 \quad \tau_m = \frac{\tau_{1,A} + \tau_{1,B} + \dots + \tau_{1,N}}{N} \quad (2)$$

164
 165 Where τ_m is average lifetime ($\tau_{1,x}$) for each horizons $x=A, B$ to N and N is
 166 number of horizons were determined in the specific soil or area.

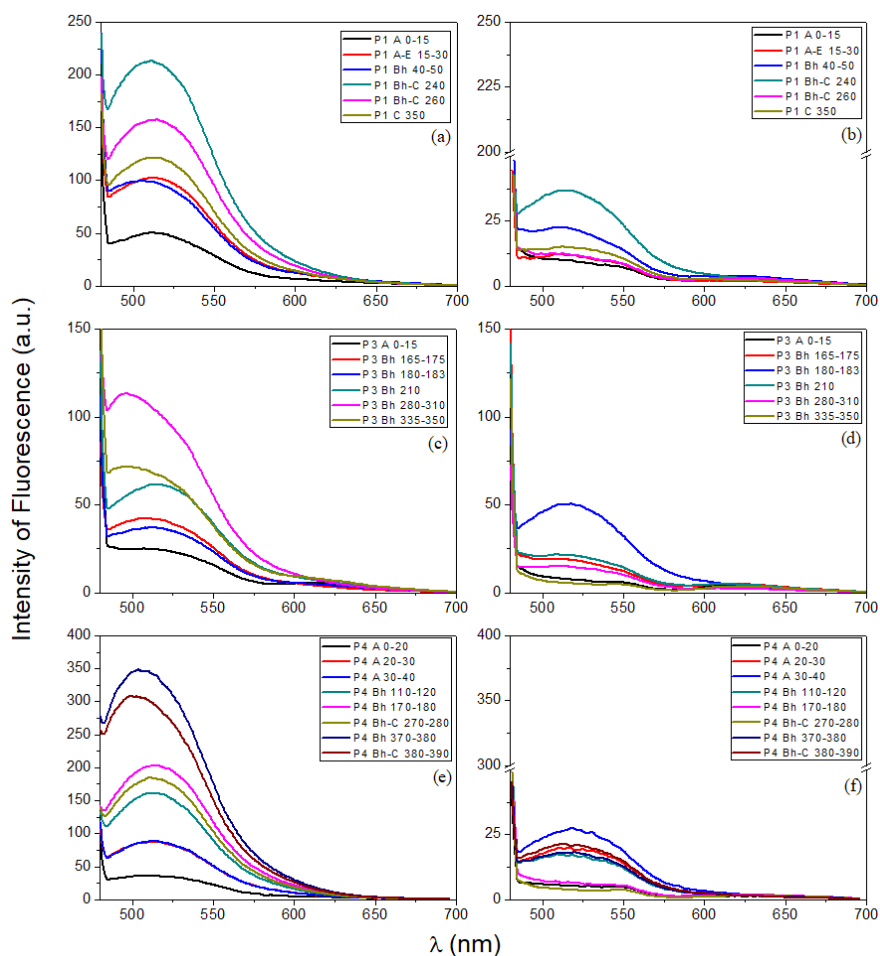
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168

169 **3. Results and Discussion**

170 *3.1. Molecular properties of soil organic matter*

171 Figure 2 shows the fluorescence spectrum ($\lambda_{\text{ex}} = 465 \text{ nm}$) for the sample of HA
 172 and FA extracted from the Hydromorphic Spodosol (a and b), seasonally Hydromorphic
 173 Spodosol (c and d) and Well-drained Spodosol (e and f).



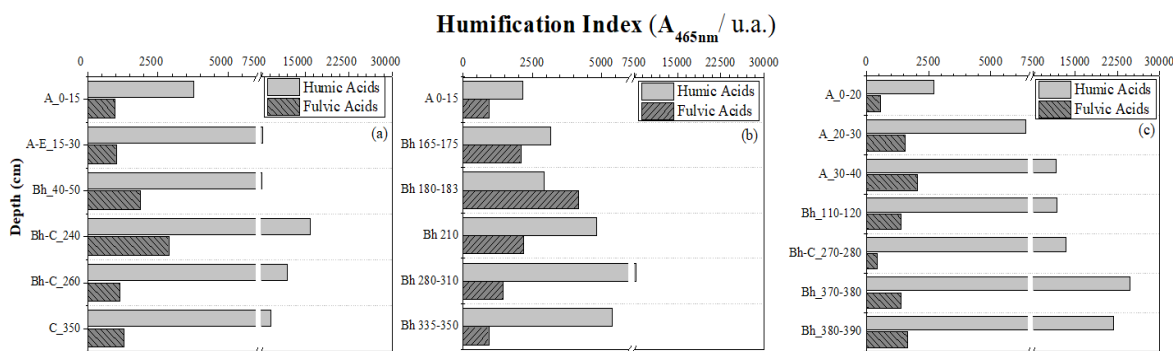
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175 **Figure 2:** Emission spectra with 465 nm excitation for samples of HA (on left) and FA
 176 (on right) ($12,5 \text{ mg.L}^{-1}$, $\text{pH}=8$) extracted from the Hydromorphic Spodosol (P1: a and
 177 b), seasonally Hydromorphic Spodosol (P3: c and d) and Well-drained Spodosol (P4: e
 178 and f).

179

180 The emission spectra for humic fractions (Fig. 2) presented a typical wide band
 181 with a maximum intensity around 500-550 nm (Milori et al. 2002; Fuentes et al. 2006;
 182 Tadini et al. 2018). The excitation in 465 nm is more resonant with aromatic and
 183 resistant groups (Milori et al. 2002) that can associate the SOM transformation stage in
 184 these soils. Since, the maximum intensity of fluorescence was observed with depth for
 185 all Spodosol areas (P1, P3 and P4) in which can be suggests the relevant presence of
 186 molecular components, such as aromatic polycondensation structure (Fuentes et al.
 187 2006; Tadini et al. 2018). The change in fluorescence to longer wavelengths can be
 188 reflecting on a higher concentration of more humified groups especially in these Bh
 189 deepest of these Spodosol. From these two-dimensional spectra, it was possible to
 190 determine the humification index described by Milori et al. (2002), as shown in Figure 3

191 3.



192

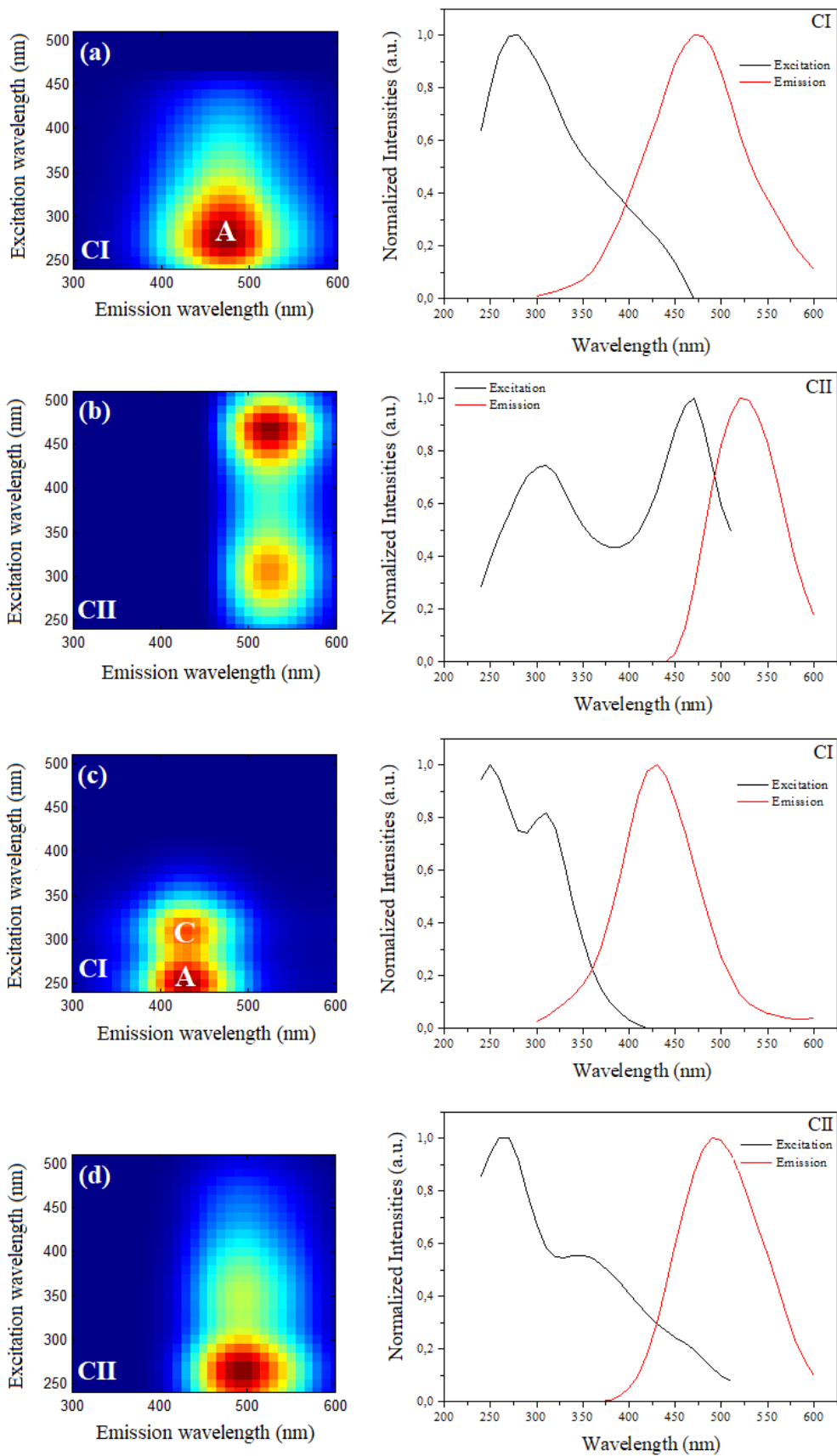
193 **Figure 3:** Humification index (A_{465nm}) values from HA and FA from (a) Hydromorphic
 194 Spodosol (P1), (b) seasonally Hydromorphic Spodosol (P3) and (c) Well-drained
 195 Spodosol (P4).

196

197 Figure 3, showed an increase HA and FA humification indices during the depth
 198 for the Hydromorphic Spodosol (P1) that the highest values found in the deeper Bh-C
 199 horizons (Bh-C 240 and Bh-C 260) and the lowest values were verified in the
 200 superficial horizons. The same behavior was verified to seasonally Hydromorphic
 201 Spodosol (P3) that highlighted the deeper Bh horizons have highest humification index
 202 (Bh 280-310 and Bh 335-350). Particularly, for P3, in the Bh 180-183 horizon showed
 203 an exception behavior comparison other Bh horizons (P1 and P4), in which the higher
 204 value A_{465nm} was from FA fraction that suggest in this horizon FA is more humified than
 205 HA. In Well-drained Spodosol (P4) for HA there was a gradual increase during the
 206 depth, that highest values were obtained for the deepest horizons, Bh 370-380 and Bh-C
 207 380-390. These results suggest that the humic fractions at the surface horizons include
 208 simple, labile and poorly humified compounds, whereas the more complex humified
 209 compounds are leached and accumulated or retained at deeper horizons, especially in
 210 the HA fractions in these areas (Tadini et al. 2018). So, the Amazonian soils
 211 humification indices (A_{465nm}) presented the following descending order: P4 > P3 > P1
 212 for HA samples and P3 > P1 > P4 for FA samples.

213

214 Figure 4 showed the fluorophores obtained by EEM-CP/PARAFAC of the humic
 215 fractions without Cu(II) ions. It can be observed contribution of two components (CI
 216 and CII) for both HA and FA from Amazon Spodosols, whose the core consistency di-
 agnostic (CORCONDIA) were of 93.84 and 97.09% for the HA and FA, respectively.



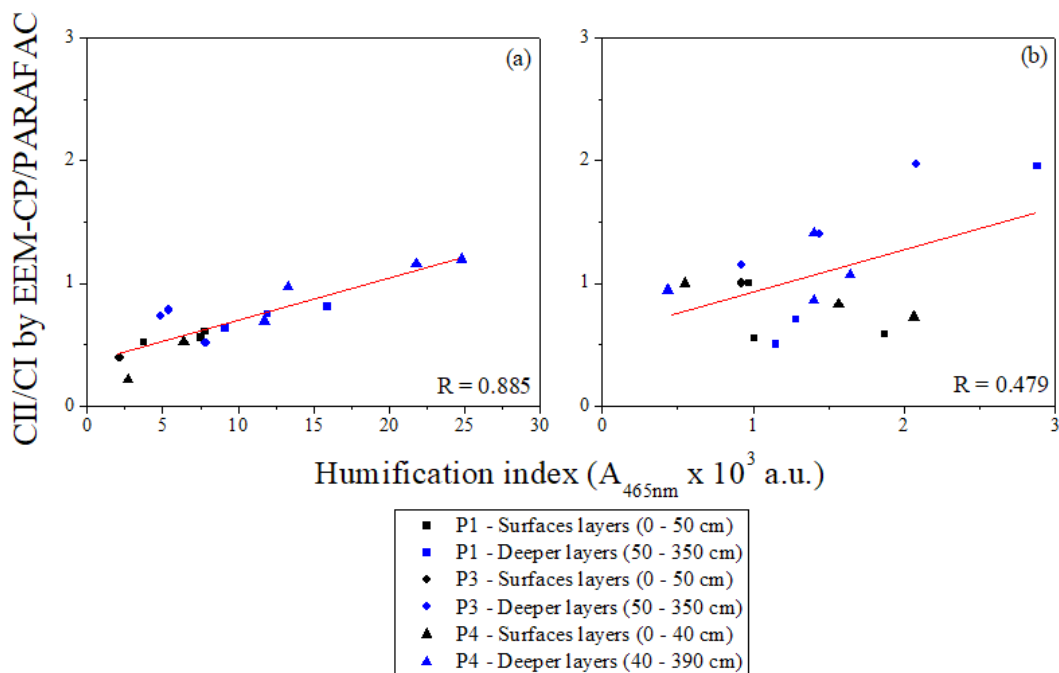
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218 **Figure 4:** Fluorophore obtained by EEM-CP/PARAFAC: CI and CII present in HA (a
 219 and b) and FA (c and d) extracts extracted from Amazonian Spodosols.

220

221 Figure 4a and 4c refers the A peak (CI, $\lambda_{ex}/\lambda_{em}$: 275-320/460nm) and A-C peak
222 (CI, $\lambda_{ex}/\lambda_{em}$: 320-360/400-460 nm) which are characteristic of soil humic and fulvic
223 acids compounds, respectively. The CII for HA (Figure 4b) showed two excitation
224 peaks ($\lambda_{ex}/\lambda_{em}$: 300/525nm e $\lambda_{ex}/\lambda_{em}$: 455/525nm) that refers the complex structure
225 and it is referred as soil humic-like component in literature. Figure 4d showed one peak
226 (CII for FA, $\lambda_{ex}/\lambda_{em}$: 275/500 nm) that can be typically attributed to fulvic-like from
227 soil (Stedmon et al., 2003; Coble et al., 2014; Morais et al. 2021; Tadini et al. 2020;
228 2022). As expected HA present higher emission wavelength compare to FA due to the
229 longer humification process inducing π -conjugation and redshift (Senesi et al. 1991;
230 Tadini et al. 2018).

231 Figure 5 showed the correlation obtained by HA and FA humification index
232 (A_{465nm}) between CII/CI ratios. In Fig. 5a, a good positive correlation was observed ($r =$
233 0.89) for all HA samples, demonstrating that the humification index (A_{465nm}) is directly
234 dependent on CII/CI ratio in these soils. Since, the CII is the most complex fluorophore
235 with aromatic and humified characteristics present in SOM (Fig. 4), in which suggesting
236 the fluorophore CII is acting on the humification index mainly in the deeper horizons of
237 the HA samples, highlighting the Bh horizons from P4 Spodosols that showed higher
238 values of A_{465nm} evidenced in this study. A different behavior was observed for the FA
239 fraction which is not correlated with the humification index (see in Fig. 5b, $r = 0.48$)
240 which suggests the FA composition is less diversified and simpler than the HA
241 structure, and besides, the FA humification index showed a proportion of ten times
242 lower than the HA fractions that corroborating the structure of FA is simpler than HA.



243

244 **Figure 5:** Correlation graphs of Humification index ($A_{465\text{nm}}$) vs. CII/CI by
 245 EEM-CP/PARAFAC from: (a) HA and (b) FA samples of the Amazon soils evaluated
 246 in this study (highlighted blue square, point and triangle represent deeper layers from
 247 P1, P3 and P4, respectively).

248

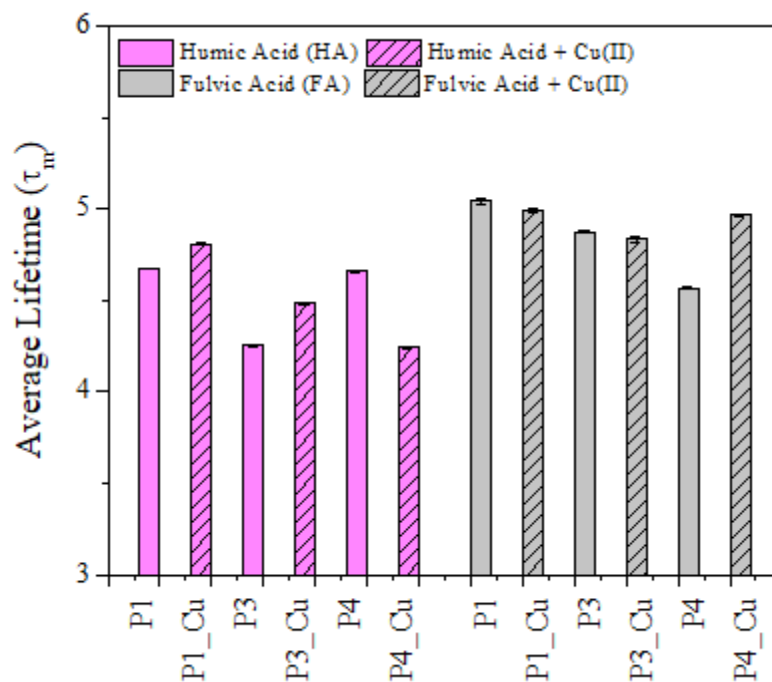
249 3.2. Lifetime properties of humic fractions

250 The humic fractions for this study showed one-exponential decay of lifetime (τ_1)
 251 as described in Table S1 and besides, an interesting factor is that τ_1 decays faster for
 252 FA samples (4,81 ns) than for HA samples (4,53 ns) ($t=0.055$) (Table S1). Then, it can
 253 be suggest the variability is smaller for HA samples (2%) than for FA fractions (10 %),
 254 in which its means the FA samples may be more reactive than HA, and thus, the FA
 255 fractions are easier to bind to metals than HA fractions which can cause a variation of
 256 the lifetime in these fractions (Fig. S4).

257 Figure 6 showed that when Cu(II) ions were added, there was an increase in the
 258 lifetime (τ_m) of the P1 and P3 profile of the HA samples and the P4 profile of the FA
 259 sample, while the P4 profile of the HA sample decreased the τ_m and at the profiles P1
 260 and P3 of the FA samples did not changed of the τ_m . Studies in the literature using
 261 humic (Dos Santos et al. 2020a) and fulvic (Dos Santos et al. 2020b) acids extracted
 262 from Terra Mulata soils in Amazonia and HA extracted from the Saint Lawrence
 263 Estuary in Canada (Nouhi et al. 2018) with Cu(II) showed that for Cu(II) complexation
 264 the lifetime decrease (due to quenching process) that can be observed for HA at the P4

265 area and for FA at the P1 and P3 areas (Figure 6). To regarding the samples showed an
 266 increase of the lifetime, P1 and P3 for HA fraction and the P4 for FA fraction,
 267 distinguished by low SOM transformation evidenced of the low A_{465nm} (Figure 3) and
 268 the presence of simpler structure (Figure 4) in which can be suggesting these fractions
 269 are participating of the complexation reactions and will be influencing the mobility and
 270 metal availability in these soil profiles. Moreover, the dependence of the relative
 271 amplitudes of the fluorescence decay of FA remained almost unchanged in the soil
 272 samples P1 and P3, which showed an invariance of the lifetime values of FA after the
 273 addition of the metal, suggesting that there was no energy transfer between the singlet
 274 states of FA and Cu(II), thus suggest the quenching process in these profiles is static
 275 quenching.

276



277

278 **Figure 6:** The average lifetime (τ_m) for humic and fulvic acids samples with and
 279 without Cu(II) ions for each study area (P1, P3 and P4).

280

281 Study realized by Tadini et al. (2019) evaluating the same P1 and P4 areas
 282 showed the HA fraction had few variation of C content during the depth whereas FA
 283 fraction had high C variation with depth and an increase value at the deeper horizons. In
 284 this study, P4 area for FA showed differently behavior lifetime (τ_m) compared others
 285 areas P1 and P3, this increase of lifetime added Cu(II) in this area (Figure 6) may be
 286 suggest the decrease amount of C present in the FA structure influencing the mechanism

287 of fluorescence quenching due to the complexation process in this fraction, as
288 confirmed by data correlation observed in Figure S5 (Supplementary Material). In
289 Figure S5a, a good negative correlation was observed ($r = -0.677$) for all FA samples
290 after interaction with Cu(II), demonstrating that the interaction of Cu(II) by influencing
291 of the C amount in SOM structure, especially for FA from P4 soil (see in Fig. S5b, $r = -$
292 0.944) that can be suggesting the functional protection and influence the carbon
293 persistence in these Podzols. Besides, study describe by Tadini et al. (2020) employed
294 the same samples from Amazonian Spodosols observed the HA needed 10 times more
295 carbon in their structures to complex ions metallic than FA fractions using the
296 fluorescence suppression analysis of Cu(II) and Al(III) ions.

297 Figure S6 (Supplementary Material) showed the correlation obtained for HA and
298 FA of the humification index (A_{465nm}) vs. lifetime (τ_1) with Cu(II) ions (a) and without
299 Cu(II) ions (b) for different profiles of soils. It can be observed moderate positive
300 correlation Pearson, $r = 0.614$ and $r = 0.629$ for lifetime (τ_1) to HA and FA (Fig.S6a),
301 respectively. These results suggested lifetime (τ_1) for the HA and FA fractions may be
302 related to the same origin of groups of chemical sites, that it is corroborates to EEM-
303 CP/PARAFAC data (Figure 4b and 4d). These results showed the CII that refers more
304 complex structures (e.g. condensed and aromatic groups) can be conferring a greater
305 aromaticity and recalcitrant carbon in these soils, and consequently, increase the
306 humification index in these humic fractions. Besides, FA fractions showed that when
307 interacting with Cu(II) metal it decreases its humification (Figure S6b), thus
308 demonstrating these fractions have greater mobility and availability of metal
309 complexation than HA these soils. Thus, the employment of the advanced technique, as
310 TRFLS, in this study provided key insights to evaluate the molecular features and
311 carbon sequestration mechanism in the Amazonian Spodosols.

312

313 **4. Conclusions**

314 Conventional Fluorescence and TRFLS reveal differences in the molecular prop-
315 erties of humic fractions samples from Amazon soils with and without Cu(II) ions com-
316 plexation. From of the use of fluorescence spectroscopy technique, a strong positive
317 correlation was observed ($r = 0.89$) between humification index and EEM-CP/
318 PARAFAC components ratio for HA samples, demonstrating that the humification in-
319 dex (A_{465nm}) is directly related on CII/CI ratio in these soils and increasing with depth,
320 and besides, demonstrated the CII consisted by the OM more complex and humified.

321 The decay of the lifetime given by the time-resolved fluorescence, it was concluded that
322 static quenching is mostly probable in these soils. The results suggested lifetime (τ_1) for
323 the HA and FA fractions may be related to the same origin of groups of chemical sites
324 as more humified characteristics determined by the fluorophore CII from EEM-CP/
325 PARAFAC before interaction with Cu(II) ions. Besides, the complexation reaction of
326 Cu(II) metal with the FA fraction is dependent on the carbon amount present in the
327 SOM in these soils in which may be suggesting affect of the carbon persistence in these
328 Amazonian Spodosols. Moreover, this study highlights the possibility of employing
329 TRLFS since it stands out as an excellent spectroscopic indicator for soils' molecular
330 parameter.

331

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340 analysis.

341

342 **Conflict of Interest Statement**

343 There are no conflicts of interest or relationship, financial or otherwise that
344 might be perceived as influencing the objectivity for this research.

345

346 **Authorship**

347 Dr. Amanda M. Tadini developed the original idea, experimental protocol, gener-
348 ated and analyzed data, and wrote the manuscript. Dr. Débora M. B. P. Milori and Dr.
349 Stephane Mounier contributed to the development of the original idea, provided super-
350 vision, and assisted in manuscript preparation. Dr. Houssam Hajjouli contributed to the
351 experimental protocol and data analysis. Dr. Gustavo Nicolodelli contributed to data
352 analysis and revision of the manuscript. All authors contributed to the final version of
353 the manuscript.

354

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