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1 **Linking molecular size, composition and carbon turnover of extractable soil**
2 **microbial compounds**

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20

21 **Abstract**

22 Microbial contribution to the maintenance and turnover of soil organic matter is significant.
23 Yet, we do not have a thorough understanding of how biochemical composition of soil
24 microbial biomass is related to carbon turnover and persistence of different microbial
25 components. Using a suite of state-of-the-art analytical techniques, we investigated the
26 molecular characteristics of extractable microbial biomass and linked it to its carbon turnover
27 time. A ^{13}C plant pulse labelling experiment was used to trace plant carbon into
28 rhizosphere soil microbial biomass, which was obtained by chloroform fumigation extraction
29 (CFE). ^{13}C content in molecular size classes of extracted microbial compounds was analysed
30 using size exclusion chromatography (SEC) coupled online to high performance liquid
31 chromatography–isotope ratio mass spectrometry (SEC-HPLC-IRMS). Molecular
32 characterization of microbial compounds was performed using complementary approaches,
33 namely SEC-HPLC coupled to Fourier transform infrared spectroscopy (SEC-HPLC-FTIR)
34 and electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry
35 (ESI-FT-ICR-MS). SEC-HPLC-FTIR suggests that mid to high molecular weight (MW)
36 microbial compounds were richer in aliphatic CH bonds, carbohydrate-like compounds and
37 possibly P=O derivatives from phospholipids. On the contrary, the lower size range was
38 characterised by more oxidised compounds with hydroxyl, carbonyl, ether and/or carboxyl
39 groups. ESI-FT-ICR-MS suggests that microbial compounds were largely aliphatic and richer
40 in N than the background detrital material. Both molecular characterization tools suggest that
41 CFE derived microbial biomass was largely lipid, carbohydrate and protein derived. SEC-
42 HPLC-IRMS analysis revealed that ^{13}C enrichment decreased with increasing MW of
43 microbial compounds and the turnover time was deduced as 12.8 ± 0.6 , 18.5 ± 0.6 and 22.9
44 ± 0.6 days for low, mid and high MW size classes, respectively. We conclude that low MW
45 compounds represent the rapidly turned-over metabolite fraction of extractable soil microbial
46 biomass consisting of organic acids, alcohols, amino acids and sugars; whereas, larger
47 structural compounds are part of the cell envelope (likely membrane lipids, proteins or
48 polysaccharides) with a much lower renewal rate. This relation of microbial carbon turnover
49 to its molecular size, structure and composition thus highlights the significance of cellular
50 biochemistry in determining the microbial contribution to soil carbon cycling and specifically
51 soil organic matter formation.

52

53 Keywords: Soil carbon, Microbial biomass, Chloroform fumigation extraction, HPLC-FTIR,
54 ESI-FT-ICR-MS, HPLC-IRMS

55

56 **1 Introduction**

57 Microbial growth and activity largely control soil carbon cycling (Liang and Balser, 2011;
58 Schimel and Schaeffer, 2012). It is readily accepted that the majority of plant organic carbon
59 passes through the soil microorganisms, a fraction of which is used for cellular energy needs
60 and the rest for biomass build-up; and that microbial biomass forms soil organic matter
61 (SOM) mostly from cell fragments (Gleixner, 2013; Kögel-Knabner, 2002; Miltner et al.,
62 2012). The contribution of microbial biomass to maintenance and accumulation of SOM is
63 significant, some estimates suggest it could be as high as 80% of organic carbon in soil
64 (Kindler et al., 2009; Liang et al., 2011; Simpson et al., 2007; Six et al., 2006). The residence
65 time of microbial compounds in soil has been attributed to its molecular structure and
66 biochemical composition as well as ecosystem specific effects (Simpson et al., 2007;
67 Throckmorton et al., 2012; Tremblay and Benner, 2006). However, we do not have a
68 thorough understanding of the dependencies of C turnover and persistence of microbial
69 compounds on their molecular size and composition.

70 A widely used method to obtain microbial biomass from soil is biocidal fumigation using
71 chloroform that lyses microbial cells and releases their contents which is followed by its
72 extraction using K_2SO_4 solution (Tate et al., 1988; Vance et al., 1987). It is likely that
73 chloroform fumigation does not lyse certain microbial groups with tougher cell envelopes and
74 that the K_2SO_4 extraction selectively extracts only specific molecular compounds out of the
75 lysed cellular products (Malik et al., 2013). Notwithstanding these shortcomings, chloroform
76 fumigation extraction (CFE) has been extensively used to estimate soil microbial biomass
77 carbon (Franzluebbers, 1999; Philippot et al., 2012) and its source and turnover time when
78 coupled with stable isotope analysis (Dijkstra et al., 2006; Ryan and Aravena, 1994).
79 However, in spite of its popularity in soil biology there is still no knowledge of the molecular
80 structure and composition of the CFE microbial fraction. One of the aims of this report is to
81 provide this understanding that is essential in making reliable ecological interpretations from
82 CFE results. We applied two state-of-the-art molecular profiling tools to characterize the
83 extracted microbial compounds: size exclusion chromatography (SEC) high performance
84 liquid chromatography coupled with Fourier transform infrared spectroscopy (SEC-HPLC-

85 FTIR) and electrospray ionization Fourier transform ion cyclotron resonance mass
86 spectrometry (ESI-FT-ICR-MS). While SEC-HPLC-FTIR quantifies different functional
87 groups in size classes of the dissolved natural organic matter (Landry and Tremblay, 2012);
88 ESI-FT-ICR-MS is able to identify the elemental formulae of thousands of molecular ions
89 over a wide mass range (Reemtsma, 2009; Sleighter and Hatcher, 2007). A combination of
90 these complementary tools allows detailed molecular profiling investigations by revealing
91 different information. Relating this information to stable carbon isotope ratios ($^{13}\text{C}/^{12}\text{C}$) of
92 size separated compounds, when a ^{13}C tracer is applied in an experimental system, allows one
93 to associate the molecular fingerprints to inherent carbon turnover rates. SEC-HPLC-IRMS
94 involves size exclusion chromatography coupled online with an SEC-HPLC-IRMS interface
95 that enables molecular size-dependent separation of organic compounds followed by direct
96 online stable carbon isotope analysis of the eluted size fractions (Malik et al., 2012).

97 The objective of this study was to link the molecular size and structure of extractable
98 microbial compounds to their carbon turnover time. CFE derived soil microbial biomass from
99 a plant $^{13}\text{CO}_2$ pulse-labelling experiment over a time series was analysed to estimate the
100 turnover time of its molecular size classes and to gain the molecular profiles of both bulk
101 microbial fraction as well as its size classes. The combination of analytical tools used here
102 allowed us to profile the CFE-derived microbial compounds and ascertain if different
103 compound size classes have variable turnover time. The methods also provide valuable
104 information on the molecular characteristics of compounds in soil organic matter, which has
105 been discussed in relation to the microbial contribution to SOM formation.

106

107 **2 Materials and methods**

108 2.1 Soil sampling and experimental setup

109 Soil from an arable field at the Jena Biodiversity Experiment located in Jena, Germany was
110 used in a greenhouse experiment. *Dysphania ambrosioides* (formerly *Chenopodium*
111 *ambrosioides*), a temperate herb, was grown in soil mesocosms and after 3 months of plant
112 growth; a $^{13}\text{CO}_2$ pulse labelling was performed for 10 h at a CO_2 concentration of 350-400
113 ppm in an airtight glass chamber. The plants were returned to the greenhouse at the end of the
114 labelling period and destructive soil sampling was performed at 1, 3, 12 and 24 h, then at 2, 4,
115 7, 14, 21 and 28 days after the pulse labelling. Rhizosphere soil from 3 mesocosms was
116 sampled at each time point. Soil was then sieved to <2mm, fine roots were extensively

117 removed (this excludes mesofauna and plant residues) and stored at -20 °C. Details about the
118 experimental design and the sampling strategy are given elsewhere (Malik et al., 2015).

119 2.2 Microbial biomass extraction

120 Microbial biomass from soil was obtained using a slightly modified version of the CFE
121 method (Vance et al., 1987; Malik et al., 2013). Soil (7 g wet weight) was fumigated in a
122 desiccator with chloroform gas for 24 h followed by repeated (8 times) evacuation. Organic
123 matter was extracted from fumigated and non-fumigated control soils with 0.05 M K₂SO₄
124 solution in a ratio of 1:4 (w/v). The mixture was homogenized on an orbital shaker (250 rev
125 per min, 30 min), centrifuged for 5 min at 12,000 g and then filtered using prewashed
126 Whatman filter paper. The resultant dissolved organic matter (DOM) was acidified and
127 purged with nitrogen gas in order to remove the dissolved inorganic carbon (Scheibe et al.,
128 2012). Fumigated and non-fumigated K₂SO₄ extracts from all time points and replicates (n
129 =30) were measured for stable carbon isotope ratios using SEC-HPLC-IRMS, whereas only
130 two composite samples from ten randomly pooled K₂SO₄ extracts were used for the other
131 detailed molecular analyses because as expected the microbial biomass content and
132 composition did not change in our steady state experimental system (Malik et al., 2015). The
133 K₂SO₄ extracts were directly measured on SEC-HPLC-IRMS without any further treatment
134 but for the other analyses a solid phase extraction (SPE) was performed in order to
135 concentrate and desalt the DOM. Fumigated and non-fumigated DOM was acidified to pH 2
136 and applied to activated SPE cartridges (Bond Elut PPL cartridge; 1g, Agilent Technologies;
137 Dittmar et al., 2008). CFE protocol blank (K₂SO₄ extract without any soil sample) and SPE
138 cartridge blank were also maintained throughout the molecular fingerprinting analyses. CFE
139 microbial biomass and background non-fumigated DOM represented approximately 1.2 and
140 0.3 % of total organic carbon, respectively, in the studied soil system.

141 2.3 SEC-HPLC-FTIR analysis

142 The SPE extracts were dissolved in methanol in order to obtain a DOC concentration of ~ 3
143 mg mL⁻¹. 40 µL of this solution was injected into an Agilent 1200 HPLC system equipped
144 with the Polymer standards service (PSS) SUPREMA analytical Linear S (8 x 300 mm; 5 µm)
145 SEC column. Otherwise, the SEC-HPLC-FTIR technique and calibration used were the same
146 as previously described (Landry and Tremblay, 2012). Before deposition and FTIR analysis,
147 UV detection was carried out at a wavelength of 254 nm. SEC separated DOM was deposited
148 as tracks onto a rotating germanium disk where a background spectrum was taken on a

149 portion of the track where no DOM was eluting. Each band of every FTIR spectrum was
150 integrated, valley-to-valley. The absolute absorbance area of each band present in the protocol
151 blank was subtracted from the absolute area of the same band in the samples. Concentration
152 factors were considered for these subtractions. The corrected absorbance area of the band was
153 normalized by the corrected total absorbance area of the spectrum to obtain the relative
154 absorbance (%) for the band. These relative values enabled us to follow variations in the
155 proportion of each peak present in different spectra (i.e., in different samples and for different
156 MW).

157 2.4 ESI-FT-ICR-MS analysis

158 Mass spectral analyses were performed on a Bruker Solarix 15 Tesla FT-ICR-MS (Bruker
159 Daltonics, USA) in negative ionization mode. Prior to analysis the SPE DOM extracts were
160 diluted to achieve a dissolved organic carbon concentration of 20 mg/L in a 1:1 mixture of
161 methanol and water (v/v). The samples and blanks were injected into the ESI source with a
162 flow rate of 120 $\mu\text{L/h}$ and an ESI needle voltage of -4 kV. The recorded m/z range was
163 between m/z 150 and 2000. 500 transients with an ion accumulation time of 0.2 s were added
164 up to one spectrum. A list of 67 compounds that covered the relevant m/z range was used for
165 linear internal mass calibration with a maximum mass error of 0.1 ppm. Molecular formula
166 assignment considered C, H, O, N, S and P using a self-written Matlab routine (Koch et al.,
167 2007; Stenson et al., 2003).

168 To remove detected masses or peaks that were not measured significantly, several criteria
169 were applied. First, m/z with a signal-to-noise ratio of the maximum of each m/z ($s/n_{\text{Max},i}$) ≤ 5
170 were discarded (Pohlabein and Dittmar, 2015 and references therein). The noise was defined
171 as the minimum intensity across all detected masses without blanks. $s/n_{\text{Max},i}$ was defined for
172 each m/z_i individually by dividing the maximum intensity of each m/z_i by the noise. Second,
173 only detected masses that occurred more than one time in the set of measured samples were
174 kept. Third, all detected masses with a s/n_{Blanks} ratio ≥ 20 were removed. To determine
175 s/n_{Blanks} the average of signal intensity across all measured blanks was divided by noise. After
176 removing detected masses according to these criteria, the remaining detected masses were
177 normalized to the sum of intensities. For the following data analyses, only detected masses
178 with assigned molecular formulae were considered. For the list of non-fumigated samples
179 only formulae that were detected in both replicates were considered, the same was applied to
180 the list of peaks in the fumigated samples. Identified molecular formulae were assigned to

181 compound groups based on established molar ratios, aromaticity index and heteroatom
182 contents (Seidel et al., 2015a, 2015b; Text S1). Formulae present in both fumigated and non-
183 fumigated DOM extracts were considered as signatures of bulk SOM, whereas those unique
184 to fumigated extracts were identified as microbial. In addition, a differential spectrum
185 approach was used, wherein the relative intensities of non-fumigated samples were subtracted
186 from those of the fumigated samples resulting in m/z with positive and negative values.
187 Formulae that were present in higher abundance above a threshold (10% of the median
188 intensity of the top 1 % of formulae ranked according to intensity) in either fumigated
189 (positive values) or non-fumigated (negative values) were considered and identified as
190 microbial or bulk SOM-related, respectively. More details about the data analysis are given in
191 Supporting Information, Text S1.

192 2.5 SEC-HPLC-IRMS analysis

193 Stable carbon isotope measurements were carried out using an HPLC system coupled to a
194 Delta+ XP IRMS through an LC IsoLink interface (Thermo Fisher Scientific, Germany). SEC
195 was performed on a mixed bed analytical column (TSK-GEL GMPWXL- 7.8 mm × 30 cm;
196 Tosoh Bioscience, Germany). 100 µL aliquot of soil extracts was injected using an
197 autosampler (Surveyor autosampler, Thermo Fisher Scientific) into the mobile phase that
198 consisted of phosphate buffer 20 mM (pH 6.2) maintained at a constant flow rate of 500 µL
199 min⁻¹ using a Surveyor MS pump. Apparent MW was obtained using a calibration curve
200 plotted with standards having known MW (Malik et al., 2012, 2013 for technical details).
201 Empirical C turnover time (synonymously referred to as mean residence time) of microbial
202 size classes was obtained by estimating the pulse ¹³C dilution rate using an exponential
203 function in SigmaPlot (Malik et al., 2015).

204

205 **3 Results**

206 3.1 SEC-HPLC-FTIR

207 The SEC chromatograms obtained from UV detection shows the distribution of fumigated and
208 non-fumigated DOM with molecular weight ranging from 300 to 6500 Da (Supporting
209 Information, Figure S1). The majority of fumigated and non-fumigated DOM was between
210 500 Da and 3500 Da with peak maxima around 1400 Da. Another peak was observed only in
211 fumigated extracts between 300 Da and 480 Da with peak maxima around 400 Da. The same

212 two peaks were seen in SEC chromatograms obtained with FTIR detection (total spectrum
213 absorbance) but their relative intensities differed when compared with UV absorbance peaks
214 (not shown). FTIR absorption bands at differing wavenumbers were assigned to functional
215 groups on the basis of published literature on natural organic matter and related complex
216 molecules (Bellamy, 1975; Landry and Tremblay, 2012 and references therein). Unique
217 presence of functional groups in fumigated extracts or their increased abundance in fumigated
218 relative to non-fumigated DOM was the criteria used to identify microbial molecular
219 structures (Figure 1). In general, fumigated DOM was richer in aliphatic CH bonds (3050-
220 2830 cm^{-1}), carbohydrate-like compounds and possibly P=O derivatives (1080 cm^{-1}) (Figure
221 1-B, G; Table 1; Davis and Mauer, 2010). The distribution with MW suggests that these
222 functional groups or structures were more abundant in mid to high MW CFE-derived
223 microbial compounds. OH (3700-2700 cm^{-1}), C-O/C-O-C or C-OH (1400-1200 cm^{-1}) and C-
224 N or C-O bonds (1250 cm^{-1}) were more abundant in low MW fumigated DOM (Figure 1-A,
225 E, F; Table 1). The highest proportion of C-N or C-O groups in fumigated DOM was
226 observed below 565 Da, which includes masses assigned to the low end of mid-MW and all
227 the low MW.

228 3.2 ESI-FT-ICR-MS

229 Molecular formula assignment revealed that 59 formulae were unique to fumigated DOM and
230 112 formulae were more abundant in fumigated relative to non-fumigated extracts (Figure
231 S2A-B). These 171 formulae were considered as signatures of microbial compounds. 659
232 formulae were present in both fumigated and non-fumigated extracts and therefore linked to
233 the background SOM. Another 47 of them that were present in higher abundance in non-
234 fumigated relative to fumigated DOM were also considered as SOM-derived (Figure S2C).
235 Among the SOM related molecular formulae 80.6 % (569) were characterized with no
236 nitrogen (N), 17 % (120) with 1 N and the rest 2.4 % (17) with 2-4 N. On the contrary,
237 molecular formulae identified as microbial were rich in N; 53.2 % contained at least 1 N
238 (Table 2). 17 % (29) of microbial molecular formulae were linked to peptides, compared to a
239 tiny 2.5 % (18) of SOM-related formulae. Aromaticity index (AI) estimated for each formula
240 identifies the molecules as non-aromatic ($\text{AI} \leq 0.5$), aromatic ($0.5 < \text{AI} < 0.67$) or condensed
241 aromatic ($\text{AI} \geq 0.67$; (Koch and Dittmar, 2006). The percentage of non-aromatic formulae that
242 were identified as microbial were higher (87.1 %) than those identified as SOM related (75.2
243 %; Table 2). Unique insights were obtained when the extracted formulae were plotted in the

244 H/C (hydrogen/carbon) versus O/C (oxygen/carbon) space called van Krevelen diagrams
245 (Figure 2; (Kim et al., 2003; Sleighter and Hatcher, 2007). Comparing the van Krevelen
246 analysis of microbial and SOM related molecular formulae with that of standard compound
247 classes we could roughly infer that most microbial compounds extracted using CFE were
248 lipid, amino sugar or protein derived. Assigned compound groups varied in relative
249 abundance in microbial and SOM-related fraction; polyphenols and highly unsaturated
250 compounds dominated the SOM-related fraction whereas unsaturated aliphatic and peptide
251 like compounds were present in a higher fraction in the microbial extract (Figure 2E-F, Table
252 S1). The mass:charge ratio (m/z) provides an indication of the MW of the microbial
253 compounds because they were singly charged, which allowed us to investigate the molecular
254 size to structure relationship. Most of the microbial formulae with higher m/z ratio were
255 devoid of N and fell in the van Krevelen space for lipids, whereas those with relatively lower
256 m/z ratio contained 1-2 N and fell in the protein and/or amino sugar van Krevelen space
257 (Figure 2A-D). SOM-related formulae with higher molecular weight fell in the lignin and
258 tannin van Krevelen space where those on the lower range were widely distributed across the
259 van Krevelen diagram.

260 3.3 SEC-HPLC-IRMS

261 When used in combination with stable carbon isotope analysis the CFE fraction can be used
262 to track the source of microbial carbon. Fumigated and non-fumigated DOM separated using
263 SEC was assigned into three size classes (Figure S3) consistent with those used for SEC-
264 HPLC-FTIR results: less than 408 Da (LMW/low MW), 408-2072 Da (MMW/mid MW) and
265 2072-10510 Da (HMW/high MW). ^{13}C enrichment in microbial compound size classes was
266 estimated from the $\delta^{13}\text{C}$ values of fumigated and non-fumigated DOM size classes using a
267 mass balance. ^{13}C enrichment in all size classes was highest immediately after the pulse
268 labeling of plants and remained so for at least 12 h after the pulse (Figure 3). Among the size
269 classes, highest enrichment was measured in the LMW fraction and it decreased with
270 increasing MW. The mean $\Delta\delta^{13}\text{C}$ of LMW, MMW and HMW microbial compounds 1 h after
271 pulse labeling was 151 ± 48.9 , 107.2 ± 16.5 and 81 ± 29.9 ‰; it decreased to 28.7 ± 8.1 , 29.3
272 ± 4.8 and 17.1 ± 6.7 ‰ by the final sampling point 4 weeks after pulse labelling. The ^{13}C
273 enrichment was fitted into an exponential decay function and the fitted degradation rate
274 constant (k) was used to calculate the turnover time of microbial compound size classes

275 (Figure 3). Empirical C turnover time was estimated at 12.8 ± 0.6 , 18.5 ± 0.6 and 22.9 ± 0.7
276 days for LMW, MMW and HMW size classes, respectively (Table 3).

277

278 **4 Discussion**

279 Chloroform fumigation extraction (CFE) is a widely used technique to quantify soil microbial
280 biomass and although the extraction is incomplete and selective it is the easiest and fastest
281 method of characterising soil microbial biomass. CFE gives lysed cellular components (living
282 microbial biomass) in addition to K_2SO_4 -extractable SOM that includes microbial residues
283 and necromass. Therefore, the non-fumigated extract has to be subtracted from the fumigated
284 one in order to quantify and profile microbial organic matter. The high-resolution analytical
285 tools reported here allowed us to characterise and decipher microbial molecular fingerprints
286 from that of the background SOM. We observed some clear relationships between molecular
287 size and structure of microbial compounds. However, SEC and mass spectrometry measure
288 molecular size using different mechanisms and therefore the size, structure and carbon
289 turnover relationships obtained from these techniques may not be entirely comparable. In
290 addition, the starting material used in the molecular characterization techniques was DOM
291 concentrated through SPE with approximately 60 % recovery (Roth et al., 2015), while that
292 used for isotope analyses was unprocessed fumigated and non-fumigated DOM. This means
293 we are missing a fraction of DOM in the molecular characterization analyses.

294 SEC-HPLC-FTIR analysis revealed that low to mid MW compounds were more oxidised and
295 rich in hydroxyl (OH and C-OH), carbonyl (C=O), amine (C-N), ether or carbohydrates (C-
296 O/C-O-C) and/or carboxyl (COOH) groups suggesting that this is the 'metabolite fraction' of
297 the microbial biomass (Baldock et al., 1990). ESI-FT-ICR-MS suggests that lower MW
298 microbial compounds were enriched in N and fell in the peptide and highly unsaturated
299 compounds' space of van Krevelen analysis and ^{13}C analysis revealed that lower MW
300 compounds have faster turnover. Thus we could conclude that the low MW microbial
301 metabolite fraction consists of fast turnover components often monomers like amino acids,
302 sugars, organic acids and alcohols (Ma et al., 2012). Towards the higher MW range, SEC-
303 HPLC-FTIR observations suggest higher abundance of aliphatic compounds, carbohydrates
304 and phosphate groups indicating the presence of larger cellular biosynthetic components
305 (Simpson et al., 2007). ESI-FT-ICR-MS indicates that mid MW microbial compounds were
306 unsaturated aliphatic compounds, devoid of N and possibly lipid-derived. It is clear from

307 synthesis of data from all three techniques that the majority of microbial compounds were in
308 the mid to high MW range and were polymeric or oligomeric biosynthetic cellular
309 components of lipid, polysaccharide or protein origin (Hart et al., 2013; Spence et al., 2011).
310 SEC-HPLC-IRMS analysis based turnover time estimates suggest that these cellular structural
311 compounds have slower turnover in comparison to the metabolite fraction. These compounds
312 can be degraded into smaller and more oxidised fragments, in agreement with the observation
313 that O-rich functional groups were more abundant in low MW microbial extracts and in bulk
314 SOM. Microorganisms appear to be important contributors of bulk low to mid MW SOM
315 extractable fraction, while bulk high MW SOM seems mostly from plant-derived
316 macromolecules such as lignin and tannin. Through this greenhouse based mesocosm
317 experiment we clearly demonstrate the molecular size and structure relationship to turnover
318 time of microbial compounds. A similar correlation of size and turnover time of microbial
319 compounds has been previously demonstrated in a C3-C4 vegetation change field experiment
320 on two different soil types and this report validates the earlier results (Malik et al., 2013).
321 However, the turnover time of high MW compounds was 22 days that appears to be rather
322 small in the SOM context (Schmidt et al., 2011). This could mean that CFE microbial
323 biomass is largely labile and/or many high turnover time compounds are not extracted (Malik
324 et al., 2015).

325 The complementary molecular characterization tools reported here are neither exhaustive nor
326 specific, but they allowed us for the first time to profile the molecular fingerprints of living
327 microbial biomass as well as the SOM that is largely derived from microbial necromass. CFE
328 is a popular technique in soil biology employed to measure microbial biomass, its source,
329 turnover and fate in soil systems. It is often referred to as “quick and dirty” because there is
330 not much information about the molecular composition of the fumigated and non-fumigated
331 extracts. Assuming a strong dominance of microbial-derived material in the fumigated
332 extracts and by constructing differential spectra, the molecular techniques used in this report
333 suggest that CFE derived microbial biomass is largely lipid, carbohydrate and protein derived.
334 This study sheds light on the hitherto unknown molecular characteristics of the CFE
335 extractable soil microbial biomass.

336 Unique molecular signatures of the extracted SOM were also obtained by focussing on the
337 non-fumigated DOM fraction. SEC-HPLC-FTIR observations suggest that hydroxyl, carboxyl
338 and ester groups were abundant across the MW range in SOM derived extracts. Interestingly,

339 aliphatic compounds, carbohydrates and phosphate groups were less abundant compared to
340 the fumigated fraction. Complementary ESI-FT-ICR-MS analysis supports the lower
341 abundance of aliphatic compounds and furthermore suggests the lack of nitrogen in most
342 molecules detected. It also identified that SOM-related high MW compounds were likely
343 lignin and tannin compounds, indicators of vascular plants. Those on the lower MW range
344 were also lignin and tannin-related but in addition were also identified as lipid, carbohydrate
345 and protein derived, which are all molecular signatures of microbial biomass (Hart et al.,
346 2013; Kelleher et al., 2006; Malik and Gleixner, 2013; Mann et al., 2015; Spence et al., 2011).
347 This overlap suggests contribution of microbial necromass or microbially processed or altered
348 compounds to SOM formation. It is further substantiated by reports about marine
349 sequestration of carbon in bacterial metabolites where a similar overlap between bacterial and
350 surrounding DOM molecular formulae is observed (Lechtenfeld et al., 2015).

351 The turnover time relationship with molecular size and structure of microbial compounds
352 highlights the importance of cellular biochemistry in determining the microbial contribution
353 to soil C formation. This dependency could be altered in conditions of environmental change
354 as a function of physiological adaptation of soil microorganisms. The latter could lead to
355 change in the balance of microbial anabolic and catabolic processes that is often linked to the
356 capacity of soil microorganisms to regulate the flow of C in soil systems. A more efficient
357 metabolic pathway with higher anabolic fluxes could lead to increased microbial biomass and
358 subsequently higher SOC storage. In this context, the molecular size-turnover time
359 relationship could be useful in understanding the microbial physiological adaptations to
360 climate change and help improving the mechanistic understanding of soil C cycling processes.
361

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378

379 **Figure legends:**

380 Figure 1: FTIR relative absorbance of seven peaks (panels A to G) of size separated DOM in
381 fumigated and non-fumigated samples. A greater proportion of a particular functional group
382 in the microbial fraction is indicated by its higher relative absorbance in the fumigated
383 fraction in comparison to the non-fumigated.

384 Figure 2: van Krevelen plots of ESI-FT-ICR-MS derived formulae over molecular weight
385 (A,B), number of nitrogen (C,D) and compound classes (E,F) that are microbial- (unique to or
386 in higher abundance in fumigated extracts) or soil organic matter (SOM) related (unique to or
387 in higher abundance in non-fumigated extracts). van Krevelen diagrams plot the H/C
388 (hydrogen/carbon) of each assigned molecular formula against its O/C (oxygen/carbon).

389 Figure 3: ^{13}C isotope incorporation in different microbial size classes (n=3) over the
390 experimental period following $^{13}\text{CO}_2$ pulse labeling of plants (LMW: low molecular weight,
391 MMW: mid molecular weight and HMW: high molecular weight).

392

393 **References**

- 394 Baldock, J.A., Oades, J.M., Vassallo, A.M., Wilson, M.A., 1990. Solid-state CP/MAS ¹³C
395 N.M.R. analysis of bacterial and fungal cultures isolated from a soil incubated with
396 glucose. *Australian Journal of Soil Research* 28, 213–25.
- 397 Davis, R., Mauer, L., 2010. Fourier transform infrared (FT-IR) spectroscopy: a rapid tool for
398 detection and analysis of foodborne pathogenic bacteria, in: Méndez-Vilas, A. (Ed.),
399 Current Research, Technology and Education Topics in Applied Microbiology and
400 Microbial Biotechnology. FORMATEX, Spain, pp. 1582–1594.
- 401 Dijkstra, P., Ishizu, A., Doucett, R., Hart, S.C., Schwartz, E., Menyailo, O. V., Hungate, B.A.,
402 2006. ¹³C and ¹⁵N natural abundance of the soil microbial biomass. *Soil Biology and*
403 *Biochemistry* 38, 3257–3266. doi:10.1016/j.soilbio.2006.04.005
- 404 Dittmar, T., Koch, B., Hertkorn, N., Kattner, G., 2008. A simple and efficient method for the
405 solid-phase extraction of dissolved organic matter (SPE-DOM) from seawater. *Limnol*
406 *Oceanogr. Methods* 6, 230–235. doi:10.4319/lom.2008.6.230
- 407 Franzluebbers, A.J., 1999. Introduction to Symposium — Microbial Biomass : Measurement
408 and Role in Soil Quality 30677, 505–506.
- 409 Gleixner, G., 2013. Soil organic matter dynamics: a biological perspective derived from the
410 use of compound-specific isotopes studies. *Ecological Research* 28, 683–695.
411 doi:10.1007/s11284-012-1022-9
- 412 Hart, K.M., Kulakova, A.N., Allen, C.C.R., Simpson, A.J., Oppenheimer, S.F., Masoom, H.,
413 Courtier-Murias, D., Soong, R., Kulakov, L. a, Flanagan, P. V, Murphy, B.T., Kelleher,
414 B.P., 2013. Tracking the fate of microbially sequestered carbon dioxide in soil organic
415 matter. *Environmental Science & Technology* 47, 5128–37. doi:10.1021/es3050696
- 416 Kelleher, B.P., Simpson, M.J., Simpson, A.J., 2006. Assessing the fate and transformation of
417 plant residues in the terrestrial environment using HR-MAS NMR spectroscopy.
418 *Geochimica et Cosmochimica Acta* 70, 4080–4094. doi:10.1016/j.gca.2006.06.012
- 419 Kim, S., Kramer, R.W., Hatcher, P.G., 2003. Graphical method for analysis of ultrahigh-
420 resolution broadband mass spectra of natural organic matter, the van Krevelen diagram.
421 *Analytical Chemistry* 75, 5336–44. doi:10.1021/ac034415p
- 422 Kindler, R., Miltner, A., Thullner, M., Richnow, H.-H., Kästner, M., 2009. Fate of bacterial
423 biomass derived fatty acids in soil and their contribution to soil organic matter. *Organic*
424 *Geochemistry* 40, 29–37. doi:10.1016/j.orggeochem.2008.09.005
- 425 Koch, B.P., Dittmar, T., 2006. From mass to structure: An aromaticity index for high-
426 resolution mass data of natural organic matter. *Rapid Communications in Mass*
427 *Spectrometry* 20, 926–932. doi:10.1002/rcm.2386
- 428 Koch, B.P., Dittmar, T., Witt, M., Kattner, G., 2007. Fundamentals of molecular formula
429 assignment to ultrahigh resolution mass data of natural organic matter. *Analytical*
430 *Chemistry* 79, 1758–1763. doi:10.1021/ac061949s
- 431 Kögel-Knabner, I., 2002. The macromolecular organic composition of Plant and microbial
432 residues as inputs to soil organic matter. *Soil Biology and Biochemistry* 34, 139–162.
433 doi:10.1016/S0038-0717(01)00158-4
- 434 Landry, C., Tremblay, L., 2012. Compositional differences between size classes of dissolved

- 435 organic matter from freshwater and seawater revealed by an HPLC-FTIR system.
436 *Environmental Science and Technology* 46, 1700–1707. doi:10.1021/es203711v
- 437 Lechtenfeld, O.J., Hertkorn, N., Shen, Y., Witt, M., Benner, R., 2015. Marine sequestration of
438 carbon in bacterial metabolites. *Nature Communications* 6, 6711.
439 doi:10.1038/ncomms7711
- 440 Liang, C., Balsler, T.C., 2011. Microbial production of recalcitrant organic matter in global
441 soils: implications for productivity and climate policy. *Nature Reviews Microbiology* 9,
442 75–75. doi:10.1038/nrmicro2386-c1
- 443 Liang, C., Cheng, G., Wixon, D.L., Balsler, T.C., 2011. An Absorbing Markov Chain
444 approach to understanding the microbial role in soil carbon stabilization.
445 *Biogeochemistry* 106, 303–309. doi:10.1007/s10533-010-9525-3
- 446 Ma, J., Ibekwe, a. M., Wang, H., Xu, J., Leddy, M., Yang, C.-H., Crowley, D.E., 2012.
447 Assimilable Organic Carbon (AOC) in Soil Water Extracts Using *Vibrio harveyi* BB721
448 and Its Implication for Microbial Biomass. *PLoS ONE* 7, e28519.
449 doi:10.1371/journal.pone.0028519
- 450 Malik, A., Blagodatskaya, E., Gleixner, G., 2013. Soil microbial carbon turnover decreases
451 with increasing molecular size. *Soil Biology and Biochemistry* 62, 115–118.
452 doi:10.1016/j.soilbio.2013.02.022
- 453 Malik, A., Gleixner, G., 2013. Importance of microbial soil organic matter processing in
454 dissolved organic carbon production. *FEMS Microbiology Ecology* 86, 139–148.
455 doi:10.1111/1574-6941.12182
- 456 Malik, A., Scheibe, A., LokaBharathi, P. a, Gleixner, G., 2012. Online stable isotope analysis
457 of dissolved organic carbon size classes using size exclusion chromatography coupled to
458 an isotope ratio mass spectrometer. *Environmental Science & Technology* 46, 10123–9.
459 doi:10.1021/es302467y
- 460 Malik, A.A., Dannert, H., Griffiths, R.I., Thomson, B.C., Gleixner, G., 2015. Rhizosphere
461 bacterial carbon turnover is higher in nucleic acids than membrane lipids: implications
462 for understanding soil carbon cycling. *Frontiers in Microbiology* 6, 268.
463 doi:10.3389/fmicb.2015.00268
- 464 Mann, B.F., Chen, H., Herndon, E.M., Chu, R.K., Tolic, N., Portier, E.F., Roy Chowdhury,
465 T., Robinson, E.W., Callister, S.J., Wullschleger, S.D., Graham, D.E., Liang, L., Gu, B.,
466 2015. Indexing Permafrost Soil Organic Matter Degradation Using High-Resolution
467 Mass Spectrometry. *PloS One* 10, e0130557. doi:10.1371/journal.pone.0130557
- 468 Miltner, A., Bombach, P., Schmidt-Brücken, B., Kästner, M., 2012. SOM genesis: microbial
469 biomass as a significant source. *Biogeochemistry* 111, 41–55. doi:10.1007/s10533-011-
470 9658-z
- 471 Philippot, L., Ritz, K., Pandard, P., Hallin, S., Martin-Laurent, F., 2012. Standardisation of
472 methods in soil microbiology: progress and challenges. *FEMS Microbiology Ecology* 82,
473 1–10. doi:10.1111/j.1574-6941.2012.01436.x
- 474 Pohlabein, A.M., Dittmar, T., 2015. Novel insights into the molecular structure of non-
475 volatile marine dissolved organic sulfur. *Marine Chemistry* 168, 86–94.
476 doi:http://dx.doi.org/10.1016/j.marchem.2014.10.018
- 477 Reemtsma, T., 2009. Determination of molecular formulas of natural organic matter

- 478 molecules by (ultra-) high-resolution mass spectrometry. Status and needs. *Journal of*
479 *Chromatography A* 1216, 3687–3701. doi:10.1016/j.chroma.2009.02.033
- 480 Roth, V.-N., Dittmar, T., Gaupp, R., Gleixner, G., 2015. The Molecular Composition of
481 Dissolved Organic Matter in Forest Soils as a Function of pH and Temperature. *PLoS*
482 *ONE* 10, e0119188.
- 483 Ryan, M.C., Aravena, R., 1994. Combining C-13 Natural-Abundance and Fumigation
484 Extraction Methods to Investigate Soil Microbial Biomass Turnover. *Soil Biology &*
485 *Biochemistry* 26, 1583–1585. doi:10.1016/0038-0717(94)90101-5
- 486 Scheibe, A., Krantz, L., Gleixner, G., 2012. Simultaneous determination of the quantity and
487 isotopic signature of dissolved organic matter from soil water using high-performance
488 liquid chromatography/isotope ratio mass spectrometry. *Rapid Communications in Mass*
489 *Spectrometry* 26, 173–180. doi:10.1002/rcm.5311
- 490 Schimel, J.P., Schaeffer, S.M., 2012. Microbial control over carbon cycling in soil. *Frontiers*
491 *in Microbiology* 3, 1–11. doi:10.3389/fmicb.2012.00348
- 492 Schmidt, M.W.I., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I. a.,
493 Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D. a. C., Nannipieri, P., Rasse,
494 D.P., Weiner, S., Trumbore, S.E., 2011. Persistence of soil organic matter as an
495 ecosystem property. *Nature* 478, 49–56. doi:10.1038/nature10386
- 496 Seidel, M., Beck, M., Greskowiak, J., Riedel, T., Waska, H., Suryaputra, I.G.N.A., Schnetger,
497 B., Niggemann, J., Simon, M., Dittmar, T., 2015a. Biogeochemistry of dissolved organic
498 matter in an intertidal sandy beach. *Marine Chemistry* 176, 150–163.
499 doi:10.1016/j.marchem.2015.08.011
- 500 Seidel, M., Yager, P.L., Ward, N.D., Carpenter, E.J., Gomes, H.R., Krusche, A. V., Richey,
501 J.E., Dittmar, T., Medeiros, P.M., 2015b. Molecular-level changes of dissolved organic
502 matter along the Amazon River-to-ocean continuum. *Marine Chemistry* 177, 218–231.
503 doi:10.1016/j.marchem.2015.06.019
- 504 Simpson, A.J., Simpson, M.J., Smith, E., Kelleher, B.P., 2007. Microbially derived inputs to
505 soil organic matter: Are current estimates too low? *Environmental Science and*
506 *Technology* 41, 8070–8076. doi:10.1021/es071217x
- 507 Six, J., Frey, S.D., Thiet, R.K., Batten, K.M., 2006. Bacterial and Fungal Contributions to
508 Carbon Sequestration in Agroecosystems. *Soil Science Society of America Journal* 70,
509 555–569. doi:10.2136/sssaj2004.0347
- 510 Sleighter, R.L., Hatcher, P.G., 2007. The application of electrospray ionization coupled to
511 ultrahigh resolution mass spectrometry for the molecular characterization of natural
512 organic matter. *Journal of Mass Spectrometry* 42, 559–574. doi:10.1002/jms.1221
- 513 Spence, A., Simpson, A.J., McNally, D.J., Moran, B.W., McCaul, M. V., Hart, K., Paull, B.,
514 Kelleher, B.P., 2011. The degradation characteristics of microbial biomass in soil.
515 *Geochimica et Cosmochimica Acta* 75, 2571–2581. doi:10.1016/j.gca.2011.03.012
- 516 Stenson, A.C., Marshall, A.G., Cooper, W.T., 2003. Exact masses and chemical formulas of
517 individual Suwannee River fulvic acids from ultrahigh resolution electrospray ionization
518 Fourier transform ion cyclotron resonance mass spectra. *Analytical Chemistry* 75, 1275–
519 1284. doi:10.1021/ac026106p
- 520 Tate, K.R., Ross, D.J., Feltham, C.W., 1988. A direct extraction method to estimate soil

- 521 microbial c: effects of experimental variables and some different calibration procedures.
522 *Soil Biology and Biochemistry* 20, 329–335. doi:10.1016/0038-0717(88)90013-2
- 523 Throckmorton, H.M., Bird, J.A., Dane, L., Firestone, M.K., Horwath, W.R., 2012. The source
524 of microbial C has little impact on soil organic matter stabilisation in forest ecosystems.
525 *Ecology Letters* 15, 1257–1265. doi:10.1111/j.1461-0248.2012.01848.x
- 526 Tremblay, L., Benner, R., 2006. Microbial contributions to N-immobilization and organic
527 matter preservation in decaying plant detritus. *Geochimica et Cosmochimica Acta* 70,
528 133–146. doi:10.1016/j.gca.2005.08.024
- 529 Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil
530 microbial biomass C. *Soil Biology and Biochemistry* 19, 703–707. doi:10.1016/0038-
531 0717(87)90052-6
- 532

Table 1: Proportion of functional groups in different molecular weight (MW) size classes of microbial compounds as obtained by SEC-LC-FTIR analyses. Number of pluses signifies relative abundance in fumigated soil extracts in comparison to non-fumigated ones.

Functional group	Microbial compounds				
	Low MW	Mid MW		High MW	Bulk
	343-398 Da	453-620 Da	1593-2107 Da	2788-5844 Da	
Aliphatic CH	nd	++	+	++	++
Carbohydrate, P=O	+ [#]	++	+	+	+
OH	++	+			
C-N, C-O	+ [#]	+		#	
C-O-C, C-OH	++				
C=O (COOH)	+ [#]				

= Relative absorbance values between replicates were variable, thus comparison was less reliable

nd = not determined because the signal was too low over the entire FTIR spectra for this part of the track

Table 2: Absolute and relative abundance of ESI-FT-ICR-MS derived formulae identified as microbial and SOM related. Note: AI < 0.5: non-aromatic, AI > 0.5: aromatic and AI > 0.67: condensed aromatic.

	Microbial in %	SOM-related in %	Microbial absolute	SOM-related absolute
Total formulae			171	706
0 N	46.8	80.6	80	569
1 N	41.5	17	71	120
2-4 N	11.7	2.4	20	17
AI ≤ 0.5	87.1	75.2	149	531
0.5 < AI < 0.67	5.8	14.2	10	100
AI ≥ 0.67	7.1	10.6	12	75

Table 3: Estimated turnover time for the different molecular size fractions of soil microbial biomass. Also given is the empirical molecular size range and relative abundance of the size fractions.

Molecular size class	Turnover time (d)	Relative abundance (%)
Low MW (< 408 Da)	12.8 ± 0.6	8
Mid MW (408-2072 Da)	18.5 ± 0.6	80.4
High MW (2072-10510 Da)	22.9 ± 0.7	11.6

Figure 1

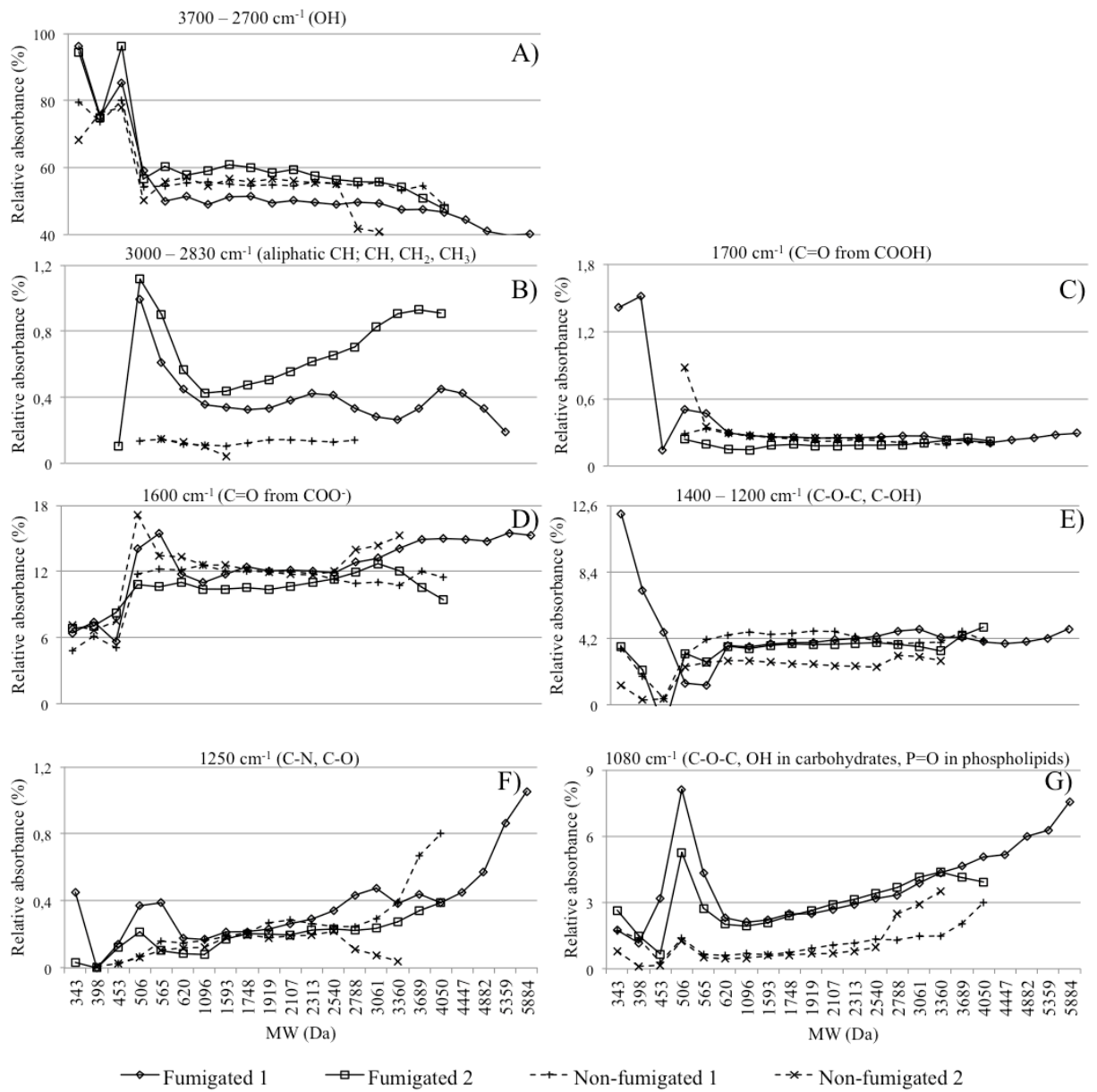


Figure 2

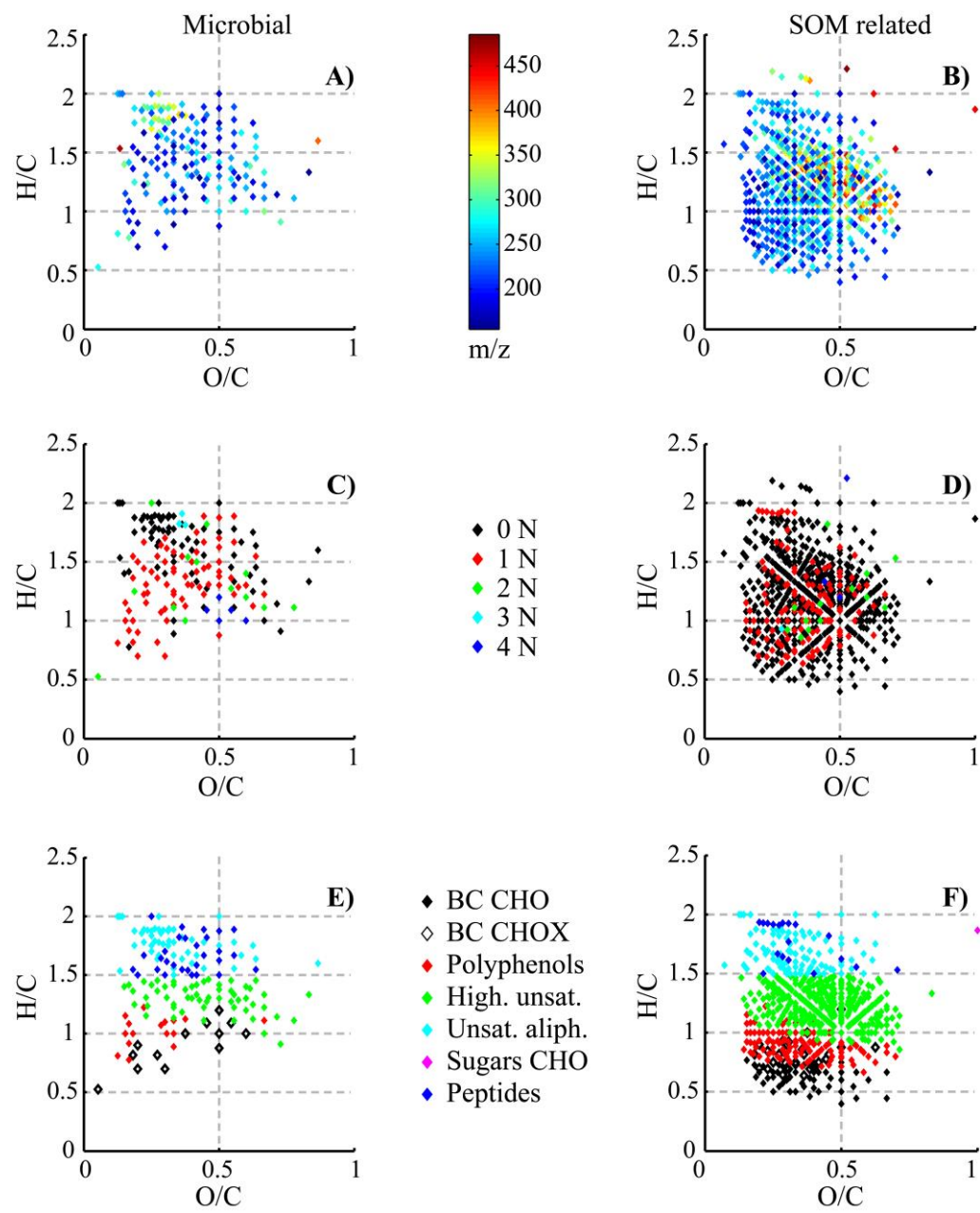


Figure 3

