Title:

SOIL CARBON DIOXIDE EMISSIONS CONTROLLED BY AN EXTRACELLULAR OXIDATIVE METABOLISM IDENTIFIABLE BY ITS ISOTOPE SIGNATURE.

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19   **ABSTRACT**

20   Soil heterotrophic respiration is a major determinant of carbon (C) cycle and its interactions with climate.
21   Given the complexity of the respiratory machinery it is traditionally considered that oxidation of organic C
22   into carbon dioxide (CO_2) strictly results from intracellular metabolic processes. Here we show that C
23   mineralization can operate in soils deprived of all observable cellular forms. Moreover, the process
24   responsible of CO_2 emissions in sterilized soils induced a strong C isotope fractionation (up to 50 ‰)
25   incompatible with a respiration of cellular origin. The supply of ^{13}C-glucose in sterilized soil led to the
26   release of $^{13}CO_2$ suggesting the presence of respiratory-like metabolism (glycolysis, decarboxylation
27   reaction, chain of electron transfer) carried out by soil-stabilized enzymes and by soil mineral and metal
28   catalysts. These findings indicate that CO_2 emissions from soils can have two origins: 1) the well-known
29   respiration of soil heterotrophic microorganisms and 2) an extracellular oxidative metabolism (EXOMET)
30   or, at least, catabolism. These two metabolisms should be considered separately when studying effects of
31   environmental factors on the C cycle because they do not likely obey the same laws and respond differently
32   to abiotic factors.

33

34   **INTRODUCTION**

35   Mineralization of soil organic matter (SOM) into CO_2 and mineral nutrients is central to the functioning of
36   eco- and agro-systems in sustaining nutrient supply and plant primary production. Soil carbon (C)
37   mineralization is also a major determinant of the global C cycle and climate by releasing from land surfaces
38   an equivalent of ten times the anthropogenic emissions of CO_2 (IPCC, 2007; Paterson and Sim, 2013).
39   Therefore, knowledge of the metabolic pathways by which SOM is oxidized is crucial to predicting both
40   the food production and the climate under a changing environment.

41   It is traditionally considered that SOM mineralization result from the activity of soil microbial communities
42   through biological catalyzed processes including both extracellular depolymerization and cellular
43   metabolisms. Extracellular depolymerization converts high-molecular weight polymers like cellulose into
44   soluble substrates assimilable by microbial cells. This depolymerization is performed by extracellular
45   enzymes released in soil through microbial cell excretion and lysis (Burns et al., 2013). In cells, assimilated
46   substrates are carried out by a cascade of endoenzymes (Sinsabaugh et al., 2009; Sinsabaugh and Follstad
47   Shah, 2012), along which protons and electrons are transferred from a substrate to intermediate acceptors
48   (*e.g.* NADP) and small C compounds are decarboxylated into CO_2. At the end of the cascade, the final
49   acceptor (*e.g.* O_2 under aerobic conditions) receives the protons and electrons while the gradient of H^+
50   generated is used by ATP-synthase to produce ATP (Junge et al., 1997).
51   Given the complexity of its machinery it is often believed that respiration is strictly an intracellular
52   metabolic process. However, this paradigm is challenged by recurrent observations of persistent substantial
53   CO_2 emissions in soil microcosms where sterilization treatments (*e.g.* γ-irradiations) reduced microbial
54   activities to undetectable levels (Blankinship et al., 2014; Kemmitt et al., 2008; Lensi et al., 1991; Maire et
55   al., 2013; Ramsay and Bawden, 1983; Trevors, 1996). Maire *et al.* (2013) addressed this issue and proposed
56   that extracellular oxidative metabolisms (EXOMET) contribute to soil respiration. According to these
57   authors, intracellular enzymes involved in cell oxidative metabolism are released during cell lysis and retain
58   their activities in soil thanks to the protective role of soil particles. These enzymes are able to oxidize ^{13}C-
59   glucose to $^{13}CO_2$ using O_2 as the final electron acceptor suggesting that all or part of the cascade of
60   biochemical reactions involved in cell oxidative metabolism are reconstructed outside the cell (Maire et al.,
61   2013). As an alternative explanation Blankinship *et al.* (2014) proposed that some decarboxylases, retaining
62   activities outside the cell in sterilized soils, catalyze CO_2 emissions through decarboxylation of intermediary
63   metabolites of the Krebs cycle. Whereas differing in the complexity of the proposed mechanisms, these

64 results (i) suggest that CO_2 emissions from soils are not only dependent to the bio-physicochemical
65 environment provided by the cells, (ii) indicate that the soil micro-environment heterogeneity offers a range
66 of physicochemical conditions allowing endoenzymes to be functional.

67 Despite these recent advances, the paradigm that only a cell can organize the complex machinery achieving
68 the complete oxidation of organic matter, at ambient temperature, remains established in the scientific
69 community (see published discussions generated by Maire *et al.,* 2012). In this vein, some authors suggested
70 that CO_2 emissions from γ-irradiated soils can result from "ghost cells" (non-proliferating but
71 morphologically intact cells) which conserve some cellular metabolic activities during prolonged periods of
72 time (Lensi et al., 1991; Ramsay and Bawden, 1983).

73 The objective of the present study was to determine whether a purely extracellular oxidative metabolism
74 (EXOMET) can occur in a soil deprived of active and "ghost" cells. To this aim, high doses of γ-irradiations
75 and different time of soil autoclaving were combined to suppress both biomass and necromass ("ghost"
76 cells). The presence/absence of active and non-active cells in soil was checked by observations with
77 transmission electron microscopy on tangential ultrathin sections of soil, DNA and RNA soil content and
78 flow cytometry. The production and the isotope composition ($\delta^{13}C$) of CO_2 were monitored in sterilized and
79 non-sterilized soils over 4 periods through 91 days of incubation. We also tested whether the EXOMET in
80 sterilized soils can carry out complex cascade of biochemical reactions (e.g. an equivalent of glycolysis and
81 Krebs cycle) by incorporating ^{13}C- labelled glucose and by quantifying emissions of ^{13}C-CO_2 **(Fig 1)**.

82

MATERIAL AND METHODS

83

Soil sampling, sterilization and incubation

84

85 Samples were collected in November 2012 from the 40-60 cm soil layer at the site of Theix (Massif Central,
86 France). The soil is sandy loam Cambisol developed on granitic rock (pH=6.5, carbon content = 23,9±1 g
87 C kg^{-1}). For detailed information on the site see Fontaine *et al*. (Fontaine et al., 2007)**.** Fresh soil samples
88 were mixed, sieved at 2 mm, dried to 10 % and irradiated with gamma ray at 45 kGy (^{60}Co, IONISOS,
89 ISO14001, France). To demonstrate the absence of viable cells in soil after irradiation, we inoculated culture
90 medium for bacteria (LB agar) and fungi (Yeast Malt agar) with irradiated soil and we applied CARD-FISH
91 to irradiated soil extracts. Results showed the absence of any microbial proliferation and RNA-producing
92 cells (Maire et al., 2013). After irradiation, some sets of soil samples were exposed to autoclaving at 121°C
93 during variable periods (0.5 h, 1 h, 1.5 h, 2 h, 4 h). Incubated microcosms consisted of 9 g (oven dried basis)
94 samples of sieved soils placed in 120 mL sterile glass flasks capped with butyl rubber stoppers and sealed
95 with aluminum crimps. Microcosms were flushed with a sterilized free CO_2 gas (80 % N_2, 20 % O_2) and
96 incubated in the dark at 20°C for 91 days. Non-irradiated living soil was also incubated as a control. Three
97 microcosm replicates per treatment were prepared. Flasks were sampled at 15, 31, 51 and 91 days of
98 incubation to measure CO_2 fluxes and ^{13}C abundance of CO_2. After each measurement, flasks containing
99 soil samples were flushed with a sterilized free CO_2 gas (80 % N_2, 20 % O_2). All manipulations were done
100 under sterile conditions. In the text and the figures LS mean "living soils", IS mean "irradiated soils" and
101 IAS-t referred to irradiated and autoclaved soils with 't' referring to the time of autoclaving.

102

Carbon dioxide emissions and their isotope composition ($^{13}C/^{12}C$)

103

104 The amount and isotope composition ($\delta^{13}C$) of CO_2 accumulated in flasks during the incubation period
105 were quantified using a cavity ring down spectrometer analyser coupled to a small sample injection module

106 (Picarro 2101-i analyser coupled to the SSIM, Picarro Inc., Santa Clara, CA, USA). A volume of 20 ml of
107 gas was sampled by the analyser. The CO_2 concentration in gas samples ranged from 300 to 2000 ppm of
108 CO_2 in accordance with the operating range of the analyser. The CO_2 concentrations and delta ^{13}C of gas
109 samples were measured at a frequency of 0.5 Hz during 10 min. Value provided by the analyser is the
110 integrated value during these 10 min of measurement. A reference gas with a known concentration of CO_2
111 and delta ^{13}C was injected between samples. For each period of incubation, the cumulated amount of CO_2
112 was divided by the duration of the period (in days) to estimate the mean daily CO_2 emission rate.

113

114 **Content and isotope composition of dissolved organic carbon (DOC)**

115 At the beginning and at the end of the incubation (t = 15 and t= 91 days), DOC was extracted from 5 g of
116 soil with a 30 mM K_2SO_4 solution. After filtration through 1.6 µm (GE Healthcare, Life Sciences,
117 Whatman[TM], Glass microfiber filters), extracts were lyophilized. The lyophilized samples were analyzed
118 with an elementary analyzer (EA Carlo ERBA NC 1500) coupled to an Isotope Ratio Mass Spectrometer
119 (Thermo Finnigan DELTA S) to determine their carbon content and isotope composition (delta ^{13}C).

120

121 **Isotope systematic**

122 We use standard δ notation for quantifying the isotopic composition of CO_2 and of DOC: the ratio R of
123 $^{13}C/^{12}C$ in the measured sample is expressed as a relative difference (denoted $\delta^{13}C$) from the Vienna Pee
124 Dee Belemnite (VPDB) international standard material. The carbon isotope composition is expressed in
125 parts per thousand (‰) according to the expression: $\delta^{13}C = (R_{sample}/ R_{VPDB}) - 1) \times 1000$. The carbon isotope
126 fractionation was calculated as follows: $\Delta\delta^{13}C (‰) = (\delta^{13}C\text{-DOC} - \delta^{13}C\text{-}CO_2)/(1 + \delta^{13}C\text{-}CO_2)$.

127

128 **Soil cell density**

129 At the end of the incubation setting (t = 91 days), cells were separated from soil particles and enumerated
130 by flow cytometry (FC). One gram of soil was mixed with 10 mL of pyrophosphate buffer (PBS 1X, 0.01
131 M $Na_4P_2O_7$) and shaken for 30 min in ice at 70 rpm on a rotary shaker. After shaking, the solution was
132 sonicated 3 times (1 min each) in a water bath sonicator (Fisher Bioblock Scientific 88156, 320W, Illkirch,
133 France). Larger particles were removed by centrifugation (800 × g, 1 min); the supernatant was fixed with
134 paraformaldehyde (4 % final concentration) and stored at 4°C prior to quantification analysis. Total cells
135 counts were performed using a FACSCalibur flow cytometrer (BD Sciences, San Jose, CA, USA) equipped
136 with an air-cooled laser, providing 15 mW at 488 nm with the standard filter set-up. Samples were diluted
137 into 0.02 µm filtered TE buffer, stained with SYBR Green 1 (10,000 fold dilution of commercial stock,
138 Molecular Probes, Oregon, USA) and the mixture was incubated for 15 min in the dark. The cellular
139 abundance was determined on plots of side scatter versus green fluorescence (530 nm wave-length,
140 fluorescence channel 1 of the instrument. Each sample was analyzed for 1 min at a rate of 20µL.min^{-1}. FCM
141 list modes were analyzed using CellQuest Pro software (BD Biosciences, version 4.0). Cell density was
142 expressed as cells × g[-1] of soil (dry mass).

143

144 **Density and integrity of cells**

145 At the end of the incubation setting (t= 91 days), abundance of unicellular organisms (prokaryotic and
146 eukaryotic) with a preserved morphology was quantified on soil ultrathin sections (90 nm thick) by TEM.
147 Each step of the soil inclusion protocol was followed by centrifugation (12000 x g, 2 min) to pellet soil
148 samples. Aliquot of soil sample (0.05 g) was fixed for 1 hour in 1.5 mL of a Cacodylate buffer pH 7.4 (0.2
149 M cacodylate, 6 % glutaraldehyde and 0.15 % ruthenium red). Soil was washed three times with cacohydrate
150 0.1 M buffer during 10 min. Post fixation was conducted with the 0.1 M cacohydrate buffer containing 1 %
151 of osmic acid. To facilitate the further penetration of propylene oxide, soil dehydration was made through a
152 gradient of ethanol: 50 % ethanol (3 x 5 min), 70 % ethanol (3 x 15 min), 100 % ethanol (3 X 20 min)
153 solutions. To improve the resin permeation, the sample was incubated in a propylene oxide bath (3 x 20
154 min). To allow the sample to soak resin, soil sample was incubated overnight in a bath containing propylene
155 oxid and Epon 812 resin (ration 1:1), and secondary eliminated by flipping. After polymerization of cast
156 resin on soil preparations (48 h, 50°C), the narrower parts of the molded and impregnated aggregates were
157 pyramidally shaped with a Reichert TM60 ultramill and finally ultra-thin sections (90 nm) were performed
158 with a diamond knife (Ultra 45°, MF1845, DIATOME, Biel-Bienne, Switzerland; Ultramicrotome Ultracut
159 S, Reichert Jung Laica, Austria). Soil cuts were collected onto 400-mesh Cu electron microscopy grid
160 supported with carbon-coated Formvar film (Pelanne Instruments, Toulouse, France). Each grid was
161 negatively stained for 30 s with uranyl acetate (2 %), rinsed twice with 0.02 µm distilled water and dried on
162 a filter paper. Soil ultrathin sections were analyzed using a JEM 1200EX TEM (JEOL, Akishima, Japan).
163 Abundance of morphologically intact cells were expressed as cells x mm^{-2} of soil.

164

Soil DNA and RNA content

166 Two grams of soil were collected at the end of the incubation setting (t= 91 days). Genomic DNA and total
167 RNA were extracted from soil samples and purified using the PowerSoil DNA isolation kit and the
168 PowerSoil total-RNA isolation kit (Mo Bio Laboratories, Inc.), respectively. DNA and RNA content of soil
169 communities were visualized by electrophoresis on a 1 % agarose gel containing ethidium bromide (0.5
170 $g.mL^{-1}$) normalized with a 1 kbp size marker (Invitrogen). Negative control was performed as well.
171 Following electrophoresis, agarose gels were analyzed using ImageJ software (available at
172 http://imagej.nih.gov/ij/). The band intensities were used to quantify the relative content of soil DNA and
173 RNA in sterilized soils related to living soil.

174

Soil incubations with $^{13}C_6$-labelled-glucose

176 Samples (9 g, dry mass basis) of irradiated (45 kGy) and autoclaved (121 °C, 4 h) soil were incubated after
177 addition of sterile solutions (1.53 mL of a 0.086 M glucose solution) of unlabelled- or of $^{13}C_6$- glucose (^{13}C
178 Abundance = 99 %). This amendment corresponds to 2.6 mg glucose g^{-1} soil. Incubation and gas
179 measurements were performed as previously described.

180

Statistical analyses

182 Each treatment was prepared in triplicate (n=3). One-Way ANOVA analysis was used to test the
183 involvement significance of sterilization treatments on CO_2 emissions, $\delta^{13}C$-CO_2, DOC, and $\delta^{13}C$-DOC.
184 Normality was tested using the Shapiro-Wilk test (p>0.05). Equality of variances were tested with a Leven's
185 Test (p<0.05). Student test analyses were used to test the significance of the difference (p<0.05) obtained

186 between each conditions. Those statistical analyses were performed using the PAST software V3.04
187 (Hammer, 2001).

188

189 **RESULTS**

190 **Effect of sterilization treatments**

191 **Microbial cell density and soil DNA and RNA content**

192 Gamma-irradiations did not significantly reduce cellular density as revealed by flow cytometry ($3.1 \times 10^8 \pm$
193 1.3×10^7 cell.g^{-1} in living soil, LS, *versus* $3.2 \times 10^8 \pm 1.1 \times 10^8$ cell.g^{-1} in irradiated soil, IS, **Fig. 2a**) and
194 transmission electron microscopy ($1.4 \ 10^4 \pm 4.3 \ 10^3$ in LS *versus* $9.5 \ 10^3 \pm 0.7 \ 10^2$ cell.g^{-1} in IS, **Figs. 2b**
195 **and 2c**). However, two proxies of cellular functionality and activity (DNA and RNA) were substantially
196 decreased by irradiations (-93.5 % $\pm$ 1 % for DNA and -74 % $\pm$ 6 % for RNA, **Figs. 2d and 2e**). Moreover,
197 RNA and DNA streaks observed on electrophoresis gels indicated that the nucleic acid content of irradiated
198 soils was largely degraded (data not shown).

199 The combination of γ-irradiations and autoclaving decreased cell densities by two orders of magnitude in
200 irradiated and autoclaved soil, IAS (**Fig. 2a**). Results from flow cytometry and transmission electron
201 microscopy showed that the cell density was reduced to < 2% compared to LS. After autoclaving,
202 transmission electron microscopy revealed that the cell density was reduced to undetectable values (**Figs.**
203 **2b**). According to transmission electron microscopy and nucleic acid extract results (**Figs. 2b, 2d and 2e**),
204 the remaining flow cytometry signal in IAS is attributed to auto fluorescent particles and unspecific binding
205 of the fluorescent dyes on debris.

206 **Dissolved organic carbon (DOC) and its isotopic composition**

207 Both γ-irradiations and autoclaving modified the soil chemistry as revealed by the analysis of the aqueous
208 phase at the beginning of the experiment. The aqueous phase contained much more DOC in irradiated soil
209 than in untreated soil (37 ± 3 µg C.g^{-1} to 303 ± 17 µg C.g^{-1} in LS and IS, respectively (**Fig. 3a**). Autoclaving
210 further increased DOC content which gradually accumulated according to the time of autoclaving, from
211 557 ± 11 µg C.g^{-1} with 0.5 h of autoclaving to 1060 ± 28.4 µg C.g^{-1} after 4 h of autoclaving (**Fig. 3a**).
212 Similarly, the δ^{13}C-DOC gradually increased from -27.4 ± 0.4 ‰ in LS to -24.9 ± 0.12 ‰ in IAS-4h (**Fig.**
213 **3b**). In all soil microcosms, DOC content and δ^{13}C of DOC did not significantly change over time (data not
214 shown).

215 All soil microcosms emitted CO_2 throughout the incubation (**Fig. 3c**). The daily CO_2 emission rate increased
216 significantly ($p < 0.05$) with time in LS whereas it gradually declined in IS (**Fig. 3c**). All IAS microcosms
217 exhibited similar dynamics of daily CO_2 emission rate: the high daily CO_2 emission rate recorded during
218 the first period of incubation (0-15 days) strongly decreased during the second period (15-31 days) and
219 stabilized thereafter (**Fig. 3c**).

220 Cumulated CO_2 emissions from LS and IS were not significantly ($p < 0.05$) different throughout the 91 days
221 of incubation (24.4 ± 1.5 and 21.9 ± 1.3 µgC.g^{-1} in LS and IS, respectively) but were significantly ($p < 0.05$)
222 higher than cumulated CO_2 emissions from IAS treatments (16.8 ± 1.5 µgC.g^{-1})(Data not shown). The
223 duration of autoclaving has no effect on cumulated CO_2 emissions. At the end of the incubation, the
224 percentage of initial DOC oxidized to CO_2 was low for all sterilized soils (< 7.2%) and decreased with the
225 duration of autoclaving (from 2.9 to 1.8% for IAS 0.5H and IAS 4H, respectively)(See Supplement S1).

226 The δ^{13}C-CO$_2$ from LS decreased with time, from -22.2 ± 0.1‰ to -28.9 ± 0.3‰. The δ^{13}C-CO$_2$ strongly
227 decreased with the intensity of sterilization treatments, from -29.2 ± 1‰ in IS to -75.4 ± 2.8‰ in IAS with
228 4h of autoclaving (**Fig. 3d**). This pattern of values was maintained throughout the incubation but the
229 difference of δ^{13}C-CO$_2$ between living and sterilized soils was maximal during the two intermediate periods
230 (P2 and P3).

231

232 **Carbon isotope fractionation during DOC mineralization**

233 The δ^{13}C strongly deviated between DOC and CO$_2$ in all sterilized soil microcosms (**Fig. 3e**) indicating
234 substantial C isotope fractionation during DOC mineralization. This isotope fractionation gradually
235 increased with the intensity of the autoclaving treatment, from 13.2 ± 0.7 ‰ in IAS with 0.5h of autoclaving
236 to 31 ± 2.5 ‰ in IAS with 4 h of autoclaving. The isotope fractionation was significantly and positively
237 correlated to the DOC content (r = 0.96, **Fig. 3e**). The δ^{13}C deviation between DOC and CO$_2$ in LS was <
238 4‰ (data not shown).

239

240 **Response of sterilized soil to supply of unlabelled and ^{13}C$_6$ labelled glucose**

241 The supply of unlabelled or labelled glucose in IAS with 4h of autoclaving did not significantly change total
242 CO$_2$ emissions (data not shown). The δ^{13}C values of CO$_2$ released from microcosms with unlabelled glucose
243 ranged from -40.2 ± 0.6 ‰ to -53.8 ± 1.2 ‰ (**Fig. 4**). The CO$_2$ released from microcosms with ^{13}C-glucose
244 showed progressive ^{13}C enrichment with time, from δ^{13}C= 127.8 ± 1.3 ‰ to 657± 1.7 ‰ after 12 and 34
245 days of incubation, respectively (**Fig. 4**). At the end of the incubation, the amount of ^{13}C-glucose released
246 as CO$_2$ corresponded to 0.01% of glucose input.

247

248 **DISCUSSION**

249 **Irradiation & autoclaving: an efficient combination to remove all traces of cell from soils.**

250 Demonstrating that complex soil matrices are truly devoid of intact cell is a challenging task. In previous
251 studies, measures for assessing abundance and activity of cells in γ-irradiated soils ranged from cultivation
252 (Blankinship et al., 2014; Maire et al., 2013), live-dead staining (Blankinship et al., 2014), fluorescent *in*
253 *situ* hybridization (Maire et al., 2013), biomass estimation (Maire et al., 2013), to biomarkers concentrations
254 (Buchan et al., 2012). All gave the same conclusion: a high proportion of dead but intact cells remained
255 after γ-irradiations of soil samples (Blankinship et al., 2014; Lensi et al., 1991; Maire et al., 2013). We found
256 a similar result using flow cytometry, transmission electron microscopy and estimation of DNA and RNA
257 content of soil (**Fig.2**).

258 To remove the remaining cells, we combined γ-irradiations with a time-gradient of autoclaving to analyze
259 the kinetics of microbial cellular lysis. To ensure that none cell with a preserved morphology remained in
260 soil aggregates we performed *in situ* observations with transmission electron microscopy on tangential
261 ultrathin sections of soil. This approach allows avoiding the pitfalls of methods involving dilute suspensions
262 of soil extracts (*i.e.* incomplete elution of microorganisms (Li et al., 2004). The combination of both
263 sterilization treatments allowed suppressing all observable cell structure (**Fig.2**). Our results also indicate
264 that the sterility of soil microcosms was maintained until the end of incubation.

265 By destroying the microbial biomass and releasing its content in soil, the sterilization treatments led to an
266 accumulation of DOC (**Fig.3a**). The increasing DOC accumulation with increasing time of autoclaving
267 likely resulted from desorption of organic carbon from soil particles (Berns et al., 2008) and/or from
268 depolymerization of carbohydrates (Tuominen et al., 1994) since microbial biomass was mostly lysed after
269 0.5h of autoclaving.

270

271 **Body of evidence for EXOMET**

272 The irradiated and autoclaved soils showed persistent (>91 days) and substantial soil CO_2 emissions (50-
273 80% of CO_2 emissions compared to LS). Those CO_2 emissions can hardly be ascribed to residual activities
274 of living and "ghost" cells since the sterilizing treatments removed all observable cell structure. Moreover,
275 the substantial C isotope fractionation (from 13 ‰ to 35 ‰, **Fig.3e**) induced by the process responsible of
276 CO_2 emissions is incompatible with a respiration of cellular origin. A substantial contribution of soil
277 carbonates to CO_2 emissions is unlikely because (i) the inorganic carbon pool is very small in the acidic soil
278 used in this study (Fontaine et al., 2007), (ii) the isotopic composition of CO_2 did not reflect the signature
279 of soil carbonates (Bertrand et al., 2007). The decarboxylation of organic compounds by a combustion
280 induced by sterilization treatments is also excluded because (i) CO_2 emissions were persistent throughout
281 the incubation, (ii) the C isotope fractionation during organic C combustion is typically weak (~3‰)
282 (Turney et al., 2006). Finally, irradiation and heating induce a heavy oxidative stress through the formation
283 of hydroperoxides, carboxyls and free radicals. These highly reactive oxidants can lead to organic matter
284 oxidation and decarboxylation. However, this oxidative process can hardly explain the persistent CO_2
285 emissions observed in our experiment since the half-life of highly reactive oxidants is extremely short (i.e.
286 10^{-9} s for free radicals). Moreover, Blankinship *et al.* (2014) have shown that the persistence of soil CO_2
287 emissions after microbial biomass suppression (or at least reduction) is not specific to irradiated soil but
288 also occurs with other methods of sterilization such as chloroform fumigation and autoclaving.

289 The most parsimonious explanation of persistence of CO_2 emissions (**Fig. 3c**) and O_2 consumption (Maire
290 et al., 2013) after soil sterilization is an extracellular oxidative metabolism (EXOMET). By EXOMET we
291 suggest a cascade of chemical reactions where electrons are transferred from organic matter to redox
292 mediators (i.e. $NAD^+/NADH$, Mn^{3+}/Mn^{2+}) and finally to O_2. Those reactions can be catalyzed by respiratory
293 enzymes stabilized on soil particles (Maire et al., 2013) and by minerals and metals present in soil
294 (Blankinship et al., 2014; Majcher et al., 2000). The evidence of a complex oxidative metabolism is
295 supported by the oxidation of ^{13}C-glucose to $^{13}CO_2$ (**Fig. 4**). Indeed, glucose is a stable molecule which must
296 undergo many biochemical transformations before being oxidized to carbon dioxide. The glucose
297 decarboxylation (**Fig. 4**) and concurrent O_2 consumption (Maire et al., 2013) suggest that EXOMET is able
298 to reconstitute an equivalent of glycolysis and Krebs cycle.

299 Mineral catalysts are stable and soil-stabilized enzymes are protected against denaturation (Carter et al.,
300 2007; Gianfreda and Ruggiero, 2006; Nannipieri, 2006; Nannipieri et al., 1996; Stursova and Sinsabaugh,
301 2008)**.** This stability of soil catalysts likely contributes to the maintenance of glucose oxidation and CO_2
302 emissions after soil exposure to high temperature and pressure (autoclaving). **Maire et al. (2013)** have
303 already pointed at the exceptional resistance of soil CO_2 emissions to high temperature, pressure and toxics.
304 However, by providing here the evidence of an oxidation of ^{13}C-labelled glucose in γ-sterilized soil exposed
305 to high temperature and pressure, we show that the complex metabolic pathways of the EXOMET are
306 maintained under these extreme conditions.

307 **Origin of the C isotope fractionation during EXOMET**

308 Our results indicated that the EXOMET preferentially oxidizes organic molecules containing light (^{12}C)
309 over heavy (^{13}C) carbon atoms. Similar strong isotope fractionation has already been described during wet
310 abiotic oxidation of oxalic acid (Grey et al., 2006). The preferential conversion of substrate containing
311 lighter isotopes agrees with classical kinetic and thermodynamic laws. The presence of ^{13}C atoms in a
312 substrate slows its conversion rate because of the higher activation energy request to induce the reaction
313 (Christensen and Nielsen, 2000; Heinzle et al., 2008). Classical works on thermodynamic also indicate that
314 the isotopic fractionation is dependent on substrate concentration (Agren et al., 1996; Goevert and Conrad,
315 2009; Wang et al., 2015). Under limited substrate concentration, the isotope fractionation decreases because
316 the heavy molecules left over during the first stages of reaction are finally carried out by the process.
317 Consistently, the isotopic fractionation induced by the EXOMET was quantified with an excess of substrates
318 (S1). Moreover, the magnitude of isotope fractionation was positively correlated to DOC content (**Fig. 2e**).
319 However, the causal link between the magnitude of fractionation and the DOC content is not certain since
320 the correlation emerges from a compilation of results obtained after different sterilization treatments. Further
321 studies should analyze this causal link in experiments where the DOC content is directly manipulated.

322 Previous studies (Blair et al., 1985; Zyakun et al., 2013) have shown that, contrary to EXOMET, cells
323 induced no or few (< 4‰) C isotope fractionation during respiration. This difference between cell respiration
324 and EXOMET can be explained by two processes. First, substrate absorption by microbial cells is typically
325 limited by substrate diffusion, a process that does not or weakly fractionate isotopes. Second, cells maintain
326 a limited quantity of substrates in the cytoplasm by regulating their substrate absorption and reserves
327 (Button, 1998). This limited substrate availability prevents the preferential use of light C isotope during
328 biochemical reactions of cell respiration.

329 It is well known that the delta ^{13}C of CO_2 emitted from soils shows circadian cycle and seasonal fluctuations
330 that reaches up to 5‰ (Moyes et al., 2010). However, it is difficult to link these fluctuations to a modification
331 of metabolic pathways of soil respiration (living respiration versus EXOMET) in response to environmental
332 changes since numerous other processes can contribute to these fluctuations. Moreover, it is likely that the
333 EXOMET does not induce much C isotope fractionation in non-sterilized soils since the DOC content is
334 typically low (**Fig. 3a**) (Liu et al., 2015). Therefore, addition of large amount of DOC is necessary to reveal
335 the C fractionation induced by the EXOMET in non-sterilized soils.

Towards a quantification of EXOMET and cellular respiration in living soils

337 Our findings support the idea that CO_2 emissions from soils are driven by two major oxidative metabolisms:
338 (1) the well-known respiration of soil biota, (2) an EXOMET carried out by soil stabilized enzymes and soil
339 minerals and metals. A first quantification of these metabolisms has been made by Maire *et al*. (2013)
340 suggesting that the EXOMET contributes from 16 to 48 % of soil CO_2 emissions. However, Maire *et al*.
341 (2013) pointed at the need of another method to confirm this substantial contribution of EXOMET. Indeed,
342 their method can lead to some biases. For instance, the soil irradiation used to block cellular activities and
343 estimate the EXOMET induces a flush of respiration due to the release of substrates and enzymes from
344 microbial biomass. This side effect of soil sterilization leads to an overestimation of EXOMET by releasing
345 enzymes and cofactors in soil.

346 The difference in C isotope fractionation between EXOMET and cellular respiration offers another method
347 of quantification of those metabolisms applicable on non-sterilized living soils. The development of this
348 method first requires a quantification of the isotope fractionation (‰ delta ^{13}C) and its dependence to DOC
349 content occurring during cell respiration ($\Delta^{13}C_{cell}$) and EXOMET ($\Delta^{13}C_{EXOMET}$). Our results provide an
350 example of estimation of $\Delta^{13}C_{EXOMET}$ (**Fig. 3e**), though further studies are needed to verify the genericity of
351 this estimation in other soils. $\Delta^{13}C_{cell}$ for soil microorganisms can be estimated with cell cultures using soil

inoculum and different substrate concentrations. This quantification allows determining the isotope composition of CO_2 (‰ delta ^{13}C) released by cell respiration ($\delta^{13}C\text{-}CO_{2cell}$) and EXOMET ($\delta^{13}C\text{-}CO_{2EXOMET}$) in function to DOC content and isotope composition of DOC ($\delta^{13}C\text{-}DOC_{sample}$):

$$\delta^{13}C\text{-}CO_{2cell} = \delta^{13}C\text{-}DOC_{sample} - \Delta^{13}C_{cell} \tag{1}$$

$$\delta^{13}C\text{-}CO_{2EXOMET} = \delta^{13}C\text{-}DOC - \Delta^{13}C_{EXOMET} \tag{2}$$

with $\Delta^{13}C_{cell}$ and $\Delta^{13}C_{EXOMET}$ are functions of DOC content. Based on our results, $\Delta^{13}C_{EXOMET}$ can be determined as

$$\Delta^{13}C_{EXOMET} = 0.037 \times [DOC] - 5.495$$

where [DOC] is dissolved organic C content (μg C g^{-1} soil).

Given that the C isotope fractionation depends on an excess of available substrate, substantial amount of DOC must be added to the living soil before quantifying EXOMET and cell respiration. After substrate addition, cellular respiration (R_{cell}) and EXOMET (R_{EXOMET}) can be separated using the classical isotope mass balance equations:

$$R_{soil} = R_{cell} + R_{EXOMET} \tag{3}$$

$$\delta^{13}C\text{-}CO_{2\,soil} \times R_{soil} = \delta^{13}C\text{-}CO_{2cell} \times R_{cell} + \delta^{13}C\text{-}CO_{2EXOMET} \times R_{EXOMET} \tag{4}$$

where R_{soil} and $\delta^{13}C\text{-}CO_{2\,soil}$ are respectively the total CO_2 emitted by the amended soil (μg C-CO_2 kg^{-1} soil) and its isotopic composition (‰ delta ^{13}C). R_{soil} and $\delta^{13}C\text{-}CO_{2\,soil}$ must be measured in hours following the substrate addition before any substantial growth of soil microorganisms which would lead to an over-estimation of cell respiration. This short-term measurement is also a prerequisite to prevent the microbial uptake of the heavy C isotope left over by the EXOMET. $\delta^{13}C\text{-}CO_{2cell}$ and $\delta^{13}C\text{-}CO_{2EXOMET}$ must be estimated in separate experiments as previously described. Therefore, the two unknowns R_{cell} and R_{EXOMET} can be determined by solving the two equations.

CONCLUSIONS AND IMPLICATIONS

Collectively, our results tend to sustain the hypothesis through which soil C mineralization is driven by the well-known microbial mineralization and an EXOMET carried out by soil-stabilized enzymes and by soil mineral and metal catalysts. These two metabolisms may explain why soil C mineralization is not always connected to size and composition of the microbial biomass (Kemmitt et al., 2008) and why experimental reduction of these microbial components has moderate effects on mineralization rate (Griffiths et al., 2001). Moreover, these two metabolisms should be considered separately when studying effects of environmental factors on the C cycle because they do not likely obey the same laws and respond differently to environmental factors. Soil microorganisms have tight physiological constraints comprising specific environmental conditions (temperature, moisture) and needs in energy and nutrients. The EXOMET is resistant to extreme conditions (e.g. autoclaving) thanks to soil stabilization of enzymes and depends on microbial turnover for the supply of respiratory enzymes. These two metabolisms may interact in many different ways: microbial cells and EXOMET likely compete for available substrates; dying cells are a source of respiratory enzymes and substrate for the EXOMET etc. Further studies are necessary to better understand processes at play and predict the relative importance of EXOMET and cell respiration across ecosystems and climates.

Overall our findings have several implications for biology. They challenge the belief of cell as the minimum structure unit able to organize and achieve cascades of chemical reactions leading to complete oxidation of organic matter. They also suggest that soils have played a key role in the origin of life. Previous studies have shown the role of soil minerals in the concentration and polymerization of amino-acids and nucleic-acids in protein-like molecule during the prebiotic period (Hazen, 2006 ; Bernal, 1949). Our results show that, when all relevant molecules are present, complex biochemical reactions underpinning bioenergetics of life (respiration) can occur spontaneously in the soil. Thus, the first ancestral oxidative metabolisms may have occurred in soil before they were incorporated in the first cell.

AUTHOR CONTRIBUTIONS

This work arose from an idea of S.F. and A.C.L.. B.K, S.F, A.C.L, G.A and C.A designed the experiment. B.K and J.C conducted the experiments. B.K analyzed the data. S.F. identified the C isotope fractionation and conceived the model of quantification. B.K, S.F, A.C.L, G.A and C.A co-wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no conflict of interest

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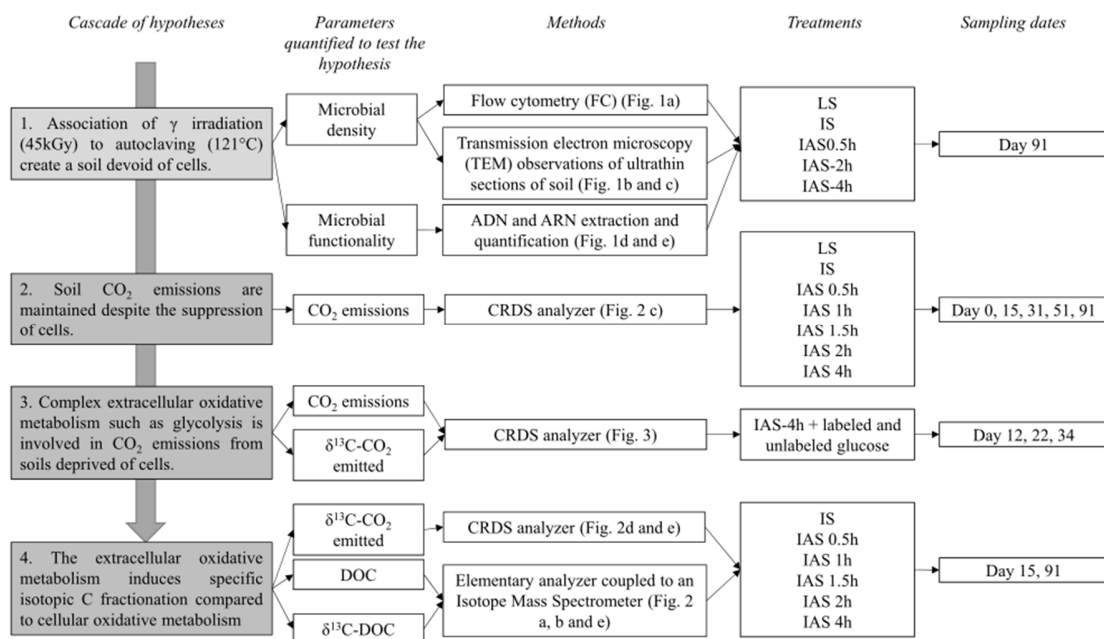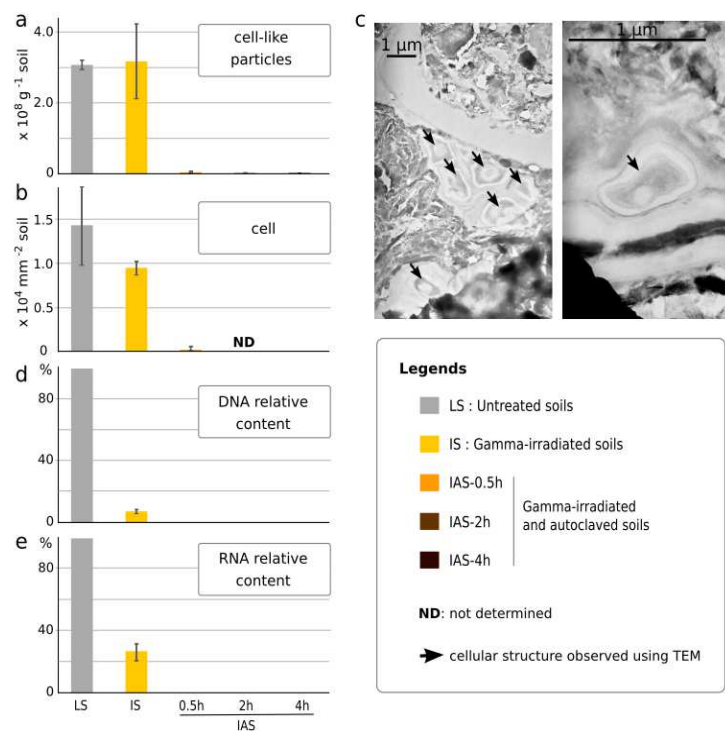531 doi:10.1134/S0026261713030156, 2013.

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Figure 1: General experimental design of the study which include our hypothesis, the parameters, the methods and the samples (n=3 for each date and treatment studied) used to test those hypotheses.

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Figure 2: Impact of sterilization treatments on cellular density, integrity and functionality.

(a) Cell density enumerated by flow cytometry (FC), (b) cell density and integrity determined by transmission electron microscopy (TEM), (c) TEM photographs of ultrathin sections of soil showing cellular structure in LS, (d) DNA and (e) RNA relative contents in soils (dry mass basis). The percentage of DNA and RNA relative contents was estimated using LS as a reference. Standard deviation was estimated using three replicates per conditions (n=3). LS: Untreated soils, IS: irradiated soils, IAS-t: irradiated and autoclaved soils with 't' referring to the time of autoclaving.
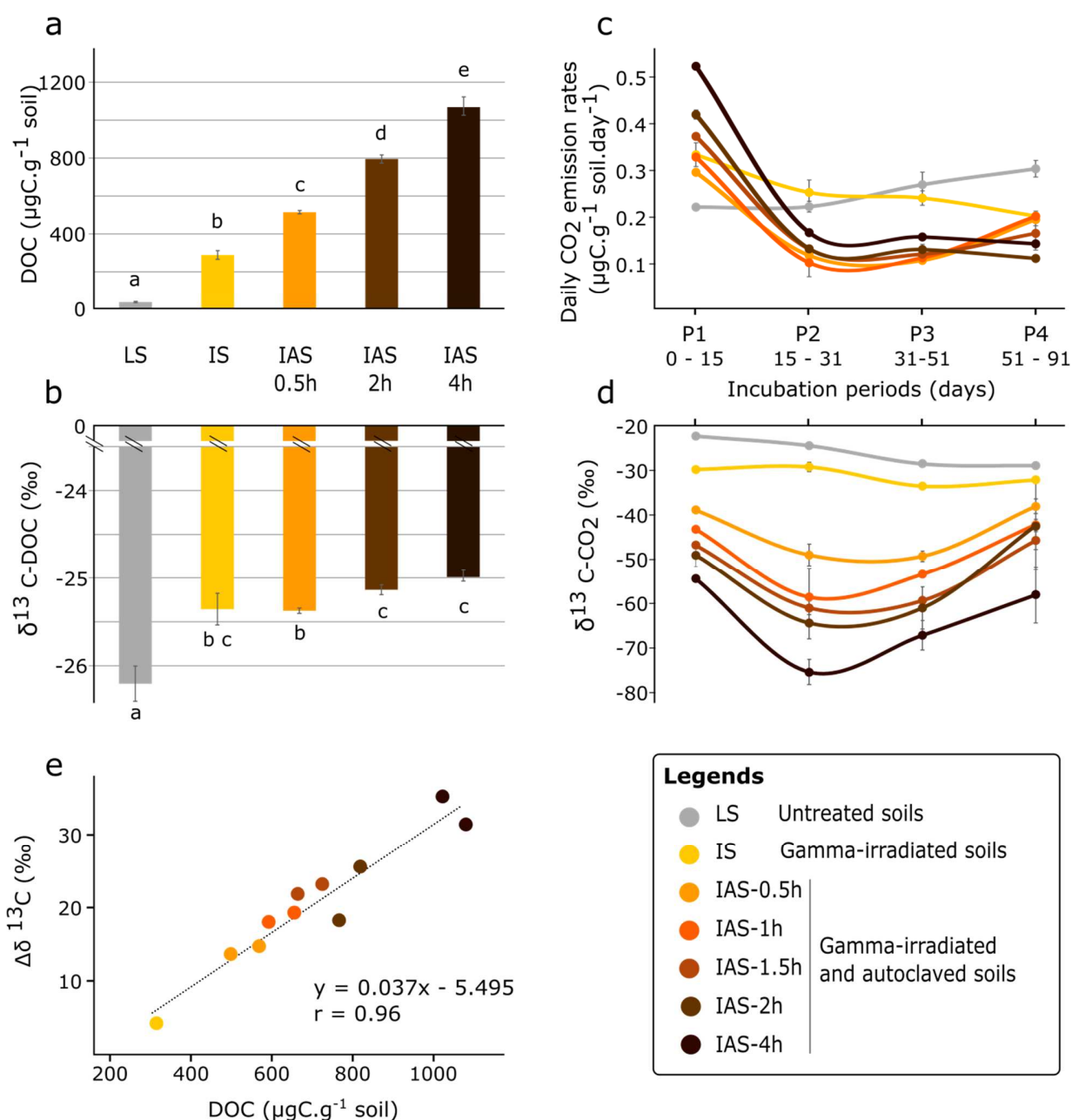
Figure 3: Content and isotopic composition of dissolved organic carbon (DOC) and of CO_2 across time and treatments.

(a) Content and (b) $\delta^{13}C$ of dissolved soil organic carbon content (DOC) at the beginning of incubation, (c) daily $C-CO_2$ emissions rates and (d) $\delta^{13}C$ of CO_2 released during four periods of incubation, (e) correlation between the carbon isotope discrimination ($\Delta\delta^{13}C$ in ‰) induced by the extracellular oxidative metabolism (EXOMET) and the DOC content. The correlation was calculated from data of sterilized soil treatments (IS, IAS-0.5h, IAS-1h, IAS-1.5h, IAS-2h, IAS-4h) analyzed at the beginning and the end of incubation. Standard deviation was estimated using three replicates per conditions (n=3). LS: Untreated soils, IS: irradiated soils, IAS-t: irradiated and autoclaved soils with 't' referring to the time of autoclaving.
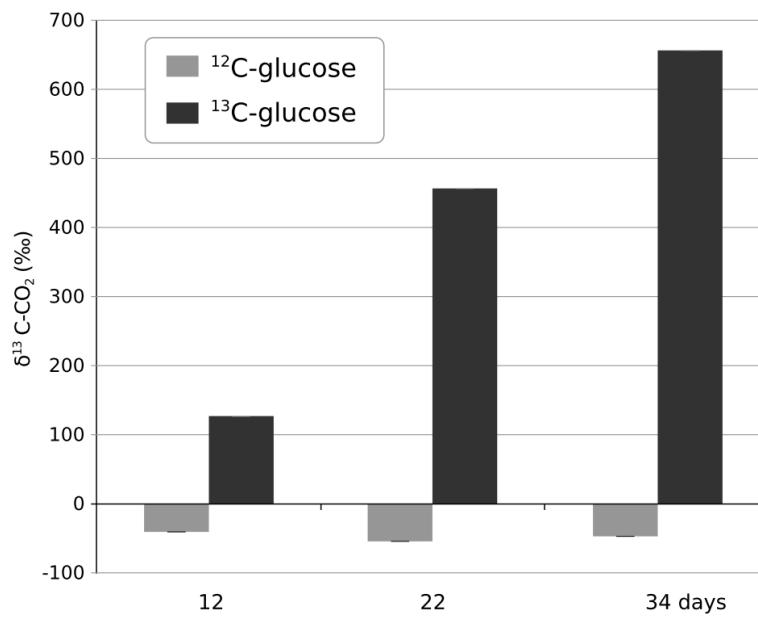
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Figure 4: Kinetic of the δ^{13}C-CO$_2$ released from an irradiated and autoclaved (4h) soil inoculated with ^{13}C-labelled glucose (^{13}C-glucose) or with unlabelled glucose (^{12}C-glucose) through 34 days of incubation. Standard deviation was estimated using three replicates per treatments (n=3).

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