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Clermont Ferrand, 06th June 2016

Dear Editor,

Please find enclosed a revised version of our manuscript “Soil carbon dioxide emissions controlled by an extracellular oxidative metabolism identifiable by its isotope signature”.

We would like to thank you and the referees for the constructive comments which did help us to improve the quality of our manuscript.

We hope that this new version will meet the expectations of referees and the standards of *Biogeosciences*.

Best regards,

Benoit Kéraval

Anonymous Referee #1 (Received and published: 28 April 2016)

“This MS presents interesting data on CO₂ released from non-cellular origin in soil. The MS follows up on the previous paper by Maire et al., published in this journal in 2013. The primary goal of this MS is to provide further evidence of the extracellular oxidative metabolism by comparing CO₂ released from soil that has undergone different levels of sterilization. An additional goal was to observe whether or not the extracellular metabolic mechanism can break down a relatively complex organic molecule using isotopically labeled glucose.

The MS has improved immensely since the first iteration, especially with the addition of figure 1 and other clarifications made throughout the text. The methods are appropriate for the questions asked and they have been meticulously carried out. The statistical component is easier to understand, but a few details need to be attended to (see below). The discussion addresses the hypotheses and goals described in the introduction and the author’s have pointed out the relevance of their findings to our current understanding of soil carbon metabolism and how their results can guide future research.”

Response: We really appreciate the careful analysis of our findings made by the referee. We also thank the referee for the recommendations formulated with the aim to improve our manuscript during the two stages of the reviewing process.

“I find the study novel and the results to be very interesting. I think, however, there are a few questions remaining within the results that highlight that the extracellular metabolism is still in the hypothesis phase and that the conclusions the authors draw should reflect this.”

Response: We agree that EXOMET remains in the hypothesis phase. Therefore, page 16 - line [20-23], our terms were moderated: “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.”

“My first question concerns the isotope results. From figure 3d, we see CO₂ that is very depleted in the heavy isotope (-40 to -55 ‰ at the beginning of the experiment that becomes even more depleted (-50 to -75 ‰, before returning to the beginning values. The authors suggest that this is related to the DOC concentration associated with each autoclave level; however, what is curious to me is that there were no significant differences between the DOC ¹³C, if the logic is that a low concentration leads to higher fractionation, then we should expect DOC enriched in ¹³C, but we actually see the opposite (the value in the first bar of fig 4b is about 1‰ depleted relative to the other treatments).”

Response: In fact, figure 3b presents the delta ¹³C of DOC at the beginning of experiment, that is, before the EXOMET might have changed the delta ¹³C of DOC due to its isotopic discrimination activities (this is specified in the figure caption). Therefore, it is not surprising to see any important difference between treatments. However, we agree that the causal link between the magnitude of

fractionation and the DOC content is not certain and deserves other studies. We added two sentences (page 14 line 15, page 15 line 17) conveying this message.

“Along this line of reasoning, it seems that a change in the isotopic fractionation should shift linearly only within a treatment, but because there is only a total sample size of 3 and the within treatment DOC concentration variability was small, this cannot be tested. What was done instead, was a comparison across the treatments and I don’t entirely agree with this interpretation, simply because the relationship presented in figure 3E is not simply a matter of DOC concentration but also whatever effects (biotic and abiotic) resulted from the treatments.

Thus, I feel the concentration effect as an explanation to the isotopic fractionation effect to be unsatisfying. The precise mechanism seems to still lie within a black box and this study has provided evidence for the extracellular metabolic breakdown of glucose, but much more research remains to fully clarify the processes behind it.”

Response: As explained above we agree with these ideas and we have added two sentences acknowledging the limits of our study and explaining what can be done to progress.

Lastly, I think the readers would appreciate it if the authors could put their results in context with what we know already about the isotopic signature of soil respiration. For example, we know that the range extends (normally) from -30 to -23‰ in C3 dominated systems. If the non-cellular breakdown of carbon in soil was significant then shouldn’t we expect these values to be much more depleted? Furthermore, how does this theory fit within the diel and seasonal understanding that we have of soil respiration? Perhaps this phenomenon will only be relevant in certain types of soils or climates.”

Response: We have added the following paragraph to discuss this idea:

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

Detailed comments:

“Page 3 line 28: Aren’t most of these enzymes in soils of cellular origin?”

Response: To avoid confusion we changed the sentence by: “(i) suggest that CO_2 emissions from soils are not only dependent to the bio-physicochemical environment provided by the cells”.

“Page 4 Line 17: probably want to clarify that the sampling was not made continuously.”

Response: We changed the sentence Page 4 Line 17 by: “The production and the isotope composition ($\delta^{13}\text{C}$) of CO_2 were monitored in sterilized and non-sterilized soils over 4 periods through 91 days of incubation.”.

“Line 18: maybe reference a biological analog to the “complex cascade of biochemical reactions” to give the reader an idea about what you are describing.”

Response: We changed the sentence Page 4 Line 18 by: “We also tested whether the EXOMET in sterilized soils can carry out complex cascade of biochemical reactions (e.g. an equivalent of glycolysis and Krebs cycle) by incorporating ^{13}C -labelled glucose and by quantifying emissions of ^{13}C - CO_2 (Fig 1).”

“Page 5 Line 2: The beginning of this sentence is confusing – are you trying to make sure that cells were there or were not there.”

Response: We changed the sentence Page 5 Line 2 by: “To demonstrate the absence of viable cells in soil after irradiation, ...”

“Section 2.2 I am not aware that picarro sells an injection system for gas samples. Is this a customized unit? Can you also describe how the data were used from the analyzer? For example, normally an injection will have distinct tails as the sample moves through the system, did you take the peak value, integrate, or average over this pulse? Can you also describe the concentration range of your samples and whether or not calibration was necessary?”

Response: We improved this paragraph following your recommendations: “The amount and isotope composition ($\delta^{13}\text{C}$) of CO_2 accumulated in flasks during the incubation period were quantified using a cavity ring down spectrometer analyser coupled to a small sample injection module (Picarro 2101-i analyser coupled to the SSIM, Picarro Inc., Santa Clara, CA, USA). A volume of 20 ml of gas was sampled by the analyser. The CO_2 concentration in gas samples ranged from 300 to 2000 ppm of CO_2 in accordance with the operating range of the analyser. The CO_2 concentrations and $\delta^{13}\text{C}$ of gas samples were measured at a frequency of 30 min^{-1} during 10 mn. Value provided by the analyser is the integrated value during these 10 mn of measurement. A reference gas with a known concentration of CO_2 and $\delta^{13}\text{C}$ was injected between samples. For each period of incubation, the cumulated amount of CO_2 was divided by the duration of the period (in days) to estimate the mean daily CO_2 emission rate.”

Page 8 Section 2.9: It is written that the data were tested for normality, but I couldn't find the test results in the results section- is ANOVA justified or should a non-parametric test be used instead?”

Response: We have indicated the p-values ranges that we used to test the normal distribution of our values and the equality of the variances: Page 8 Line 20 “Normality was tested using the Shapiro-Wilk test ($p > 0.05$). Equality of variances were tested with a Leven's Test ($p < 0.05$).”.

“Page 9 Section 3.12 Were there treatment differences in DOC concentration and the isotopic signature (not simply between dates as indicated in the text).”

Response: There is only one date of measurement, at the beginning of the experiment. We have slightly modified this paragraph in order to clarify the presentation of results: “Both γ -irradiations and autoclaving modified the soil chemistry as revealed by the analysis of the aqueous phase at the beginning

of the experiment. The aqueous phase contained much more DOC in irradiated soil than in untreated soil ($37 \pm 3 \mu\text{g C.g}^{-1}$ to $303 \pm 17 \mu\text{g C.g}^{-1}$ in LS and IS, respectively (**Fig. 3a**)."

"Page 13 line6: I think you mean to say that the "persistence" of emissions or that the emissions were maintained, or something similar."

Response: You are right. We have changed the sentence by: "Moreover, Blankinship *et al.* (Blankinship *et al.*, 2014) have shown that the persistence of soil CO₂ emissions after microbial biomass suppression (or at least reduction) is not specific to irradiated soil but also occurs with other methods of sterilization such as chloroform fumigation and autoclaving."

"Page 15 Section 4.4: This section is a fine theoretical example of how to use isotopic information to calculate the contribution of CO₂ from the extracellular respiration. The only difficulty is the empirical equation derived from figure 3e. This should be removed for the reasons discussed previously and also to avoid others using the equation under the impression that it might be universal (despite any caveat written in the text)."

Response: In fact, we wanted to present this equation as an example of how this fractionation coefficient can be calculated. We agree with you that this coefficient can vary across soils and should not be viewed as a generic coefficient (at least at this step of knowledge). We have modified the paragraph to clarify this point.

"Figure 1: List the sample size in the figure text. Figure 3a-d: show which treatments are significantly different from each other. In the figure heading list the sample size (n)."

Response: Following your recommendations, we have listed the sample size (n=3) in the text of figure 1, 2, 3, 4. We have also showed the differences significance between treatment in figure 3a-b. However, we did not show those last results in figure 3c-d in order to improve the readability of those figures. Standard deviations represent sufficient statistical tools which allow to illustrate the results and the messages described in paragraph 3.1.3.

Anonymous Referee #2 (Received and published: 17 May 2016)

"This excellent study shows the occurrence of extracellular respiration in soils and discusses the involved pathways. Even if addressed in earlier works, the question of extracellular or abiotic production of CO₂ is of broad interest for the conceptual representation of soil organic carbon mineralization. The study is one of the best conducted on this subject. Even if research has to be continued on this question, these are new concepts and ideas in this study, which are worth being published yet. The initial manuscript has been clearly improved in this new version. I therefore consider the manuscript as acceptable for publication."

Response: We thank the referee for his support and help.

168 “Concerning section “4.4. Towards a quantification of EXOMET and cellular respiration in living
169 soils”. Results of figure 3e and corresponding equation page 16 that relates $\delta^{13}\text{C}$ of CO_2 to DOC
170 could be explained through two processes of CO_2 release by exomet: one involving (almost) no
171 fractionation and the other highly fractionating, and probably from carbon derived from
172 extracted/heated organic matter. The linear relationship between $\delta^{13}\text{C}$ and DOC concentration
173 might be as well explained by a proportion of the second process in the CO_2 efflux, which is itself
174 correlated with the extraction level of carbon by treatment, as by a reservoir size dependent kinetic
175 expression of the ^{13}C fractionation factor. The proposed method to quantify exomet through ^{13}C
176 signature thus makes sense, but the equation that relates the isotope fractionation to DOC
177 concentration should not be considered as generic.”

178 **Response:** We completely agree with this point which has also been raised by the first referee. We
179 have modified the text to clarify this limit and suggest studies that could be conducted to overcome
180 these limits (page 14 line 15; page 15 line 17).

181 “According to the data, labelled glucose is a source of exomet CO_2 , but is not the dominant source.
182 The conclusion that exomet can achieve a respiratory-like metabolism doesn’t exclude the
183 occurrence in parallel of more partial mineralization processes, e.g. involving methoxy or
184 carboxyls etc. Complete mineralization of complex molecules such as glucose would furthermore
185 lead to smaller isotope fractionation than observed.”

186 **Response:** We agree with the idea that there are a few questions remaining within the results that
187 highlight that the extracellular metabolism is still in the hypothesis phase. Therefore, page 16 - line
188 [20-23], our terms were moderated: “Collectively, our results tend to sustain the hypothesis through
189 which soil C mineralization is driven by the well-known microbial mineralization and an EXOMET
190 carried out by soil-stabilized enzymes and by soil mineral and metal catalysts.” We have also
191 specified that the causal link between the magnitude of fractionation and the DOC content is not
192 certain since the correlation emerges from a compilation of results obtained after different
193 sterilization treatments. Further studies should analyze this causal link in experiments where the
194 DOC content is directly manipulated and the change over time of the isotopic composition of DOC
195 is quantified (page 14 line 15; page 15 line 17).

Title:

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

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ABSTRACT

Soil heterotrophic respiration is a major determinant of carbon (C) cycle and its interactions with climate. Given the complexity of the respiratory machinery it is traditionally considered that oxidation of organic C into carbon dioxide (CO₂) strictly results from intracellular metabolic processes. Here we show that C mineralization can operate in soils deprived of all observable cellular forms. Moreover, the process responsible of CO₂ emissions in sterilized soils induced a strong C isotope fractionation (up to 50 ‰) incompatible with a respiration of cellular origin. The supply of ¹³C-glucose in sterilized soil led to the release of ¹³CO₂ suggesting the presence of respiratory-like metabolism (glycolysis, decarboxylation reaction, chain of electron transfer) carried out by soil-stabilized enzymes and by soil mineral and metal catalysts. These findings indicate that CO₂ emissions from soils can have two origins: 1) the well-known respiration of soil heterotrophic microorganisms and 2) an extracellular oxidative metabolism (EXOMET) or, at least, catabolism. These two metabolisms should be considered separately when studying effects of environmental factors on the C cycle because they do not likely obey to the same laws and respond differently to abiotic factors.

INTRODUCTION

Mineralization of soil organic matter (SOM) into CO₂ and mineral nutrients is central to the functioning of eco- and agro-systems in sustaining nutrient supply and plant primary production. Soil carbon (C) mineralization is also a major determinant of the global C cycle and climate by releasing from land surfaces an equivalent of ten times the anthropogenic emissions of CO₂ (IPCC, 2007; Paterson and Sim, 2013). Therefore, knowledge of the metabolic pathways by which SOM is oxidized is crucial to predicting both the food production and the climate under a changing environment.

It is traditionally considered that SOM mineralization result from the activity of soil microbial communities through biological catalyzed processes including both extracellular depolymerization and cellular metabolisms. Extracellular depolymerization converts high-molecular weight polymers like cellulose into soluble substrates assimilable by microbial cells. This depolymerization is performed by extracellular enzymes released in soil through microbial cell excretion and lysis (Burns *et al.*, 2013). In cells, assimilated substrates are carried out by a cascade of endoenzymes (Sinsabaugh *et al.*, 2009; Sinsabaugh and Follstad Shah, 2012), along which protons and electrons are transferred from a substrate to intermediate acceptors (*e.g.* NADP) and small C compounds are decarboxylated into CO₂. At the end of the cascade, the final acceptor (*e.g.* O₂ under aerobic conditions) receives the protons and electrons while the gradient of H⁺ generated is used by ATP-synthase to produce ATP (Junge *et al.*, 1997).

Given the complexity of its machinery it is often believed that respiration is strictly an intracellular metabolic process. However, this paradigm is challenged by recurrent observations of persistent substantial CO₂ emissions in soil microcosms where sterilization treatments (*e.g.* γ -irradiations) reduced microbial activities to undetectable levels (Blankinship *et al.*, 2014; Kemmitt *et al.*, 2008; Lensi *et al.*, 1991; Maire *et al.*, 2013; Ramsay and Bawden, 1983; Trevors, 1996). Maire *et al.* (2013) addressed this issue and proposed that extracellular oxidative metabolisms (EXOMET) contribute to soil respiration. According to these authors, intracellular enzymes involved in cell oxidative metabolism are released during cell lysis and retain their activities in soil thanks to the protective role of soil particles. These enzymes are able to oxidize ¹³C-glucose in ¹³CO₂ using O₂ as the final electron acceptor suggesting that all or part of the cascade of biochemical reactions involved in cell oxidative metabolism are reconstructed outside the cell (Maire *et al.*, 2013). As an alternative explanation Blankinship *et al.* (2014) proposed that some decarboxylases, retaining activities outside the cell in sterilized soils, catalyze CO₂ emissions through decarboxylation of intermediary metabolites of the Krebs cycle. Whereas differing in the complexity of the proposed mechanisms, these

results (i) suggest that CO₂ emissions from soils are not only dependent to the bio-physicochemical environment provided by the cells, (ii) indicate that the soil micro-environment heterogeneity offers a range of physicochemical conditions allowing endoenzymes to be functional.

Despite these recent advances, the paradigm that only a cell can organize the complex machinery achieving the complete oxidation of organic matter, at ambient temperature, remains established in the scientific community (see published discussions generated by Maire *et al.*, 2012). In this vein, some authors suggested that CO₂ emissions from γ -irradiated soils can result from “ghost cells” (non-proliferating but morphologically intact cells) which conserve some cellular metabolic activities during prolonged periods of time (Lensi *et al.*, 1991; Ramsay and Bawden, 1983).

The objective of the present study was to determine whether a purely extracellular oxidative metabolism (EXOMET) can occur in a soil deprived of active and “ghost” cells. To this aim, high doses of γ -irradiations and different time of soil autoclaving were combined to suppress both biomass and necromass (“ghost” cells). The presence/absence of active and non-active cells in soil was checked by observations with transmission electron microscopy on tangential ultrathin sections of soil, DNA and RNA soil content and flow cytometry. The production and the isotope composition ($\delta^{13}\text{C}$) of CO₂ were monitored in sterilized and non-sterilized soils over 4 periods through 91 days of incubation. We also tested whether the EXOMET in sterilized soils can carry out complex cascade of biochemical reactions (e.g. an equivalent of glycolysis and Krebs cycle) by incorporating ¹³C- labelled glucose and by quantifying emissions of ¹³C-CO₂ (Fig 1).

MATERIAL AND METHODS

Soil sampling, sterilization and incubation

Samples were collected in November 2012 from the 40-60 cm soil layer at the site of Theix (Massif Central, France). The soil is sandy loam Cambisol developed on granitic rock (pH=6.5, carbon content = 23,9±1 g C kg⁻¹). For detailed information on the site see Fontaine *et al.* (Fontaine *et al.*, 2007). Fresh soil samples were mixed, sieved at 2 mm, dried to 10 % and irradiated with gamma ray at 45 kGy (⁶⁰Co, IONISOS, ISO14001, France). To demonstrate the absence of viable cells in soil after irradiation, we inoculated culture medium for bacteria (LB agar) and fungi (Yeast Malt agar) with irradiated soil and we applied CARD-FISH to irradiated soil extracts. Results showed the absence of any microbial proliferation and RNA-producing cells (Maire *et al.*, 2013). After irradiation, some sets of soil samples were exposed to autoclaving at 121°C during variable periods (0.5 h, 1 h, 1.5 h, 2 h, 4 h). Incubated microcosms consisted of 9 g (oven dried basis) samples of sieved soils placed in 120 mL sterile glass flasks capped with butyl rubber stoppers and sealed with aluminum crimps. Microcosms were flushed with a sterilized free CO₂ gas (80 % N₂, 20 % O₂) and incubated in the dark at 20°C for 91 days. Non-irradiated living soil was also incubated as a control. Three microcosm replicates per treatment were prepared. Flasks were sampled at 15, 31, 51 and 91 days of incubation to measure CO₂ fluxes and ¹³C abundance of CO₂. After each measurement, flasks containing soil samples were flushed with a sterilized free CO₂ gas (80 % N₂, 20 % O₂). All manipulations were done under sterile conditions. In the text and the figures LS mean “living soils”, IS mean “irradiated soils” and IAS-t referred to irradiated and autoclaved soils with ‘t’ referring to the time of autoclaving.

Carbon dioxide emissions and their isotope composition (¹³C/¹²C)

The amount and isotope composition ($\delta^{13}\text{C}$) of CO₂ accumulated in flasks during the incubation period were quantified using a cavity ring down spectrometer analyser coupled to a small sample injection module

(Picarro 2101-i analyser coupled to the SSIM, Picarro Inc., Santa Clara, CA, USA). A volume of 20 ml of gas was sampled by the analyser. The CO₂ concentration in gas samples ranged from 300 to 2000 ppm of CO₂ in accordance with the operating range of the analyser. The CO₂ concentrations and delta ¹³C of gas samples were measured at a frequency of 30 mn⁻¹ during 10 mn. Value provided by the analyser is the integrated value during these 10 mn of measurement. A reference gas with a known concentration of CO₂ and delta ¹³C was injected between samples. For each period of incubation, the cumulated amount of CO₂ was divided by the duration of the period (in days) to estimate the mean daily CO₂ emission rate.

Content and isotope composition of dissolved organic carbon (DOC)

At the beginning and at the end of the incubation (t = 15 and t = 91 days), DOC was extracted from 5 g of soil with a 30 mM K₂SO₄ solution. After filtration through 1.6 µm (GE Healthcare, Life Sciences, WhatmanTM, Glass microfiber filters), extracts were lyophilized. The lyophilized samples were analyzed with an elementary analyzer (EA Carlo ERBA NC 1500) coupled to an Isotope Ratio Mass Spectrometer (Thermo Finnigan DELTA S) to determine their carbon content and isotope composition (delta ¹³C).

Isotope systematic

We use standard δ notation for quantifying the isotopic composition of CO₂ and of DOC: the ratio R of ¹³C/¹²C in the measured sample is expressed as a relative difference (denoted δ¹³C) from the Vienna Pee Dee Belemnite (VPDB) international standard material. The carbon isotope composition is expressed in parts per thousand (‰) according to the expression: $\delta^{13}\text{C} = (R_{\text{sample}} / R_{\text{VPDB}} - 1) \times 1000$. The carbon isotope fractionation was calculated as follows: $\Delta\delta^{13}\text{C} (\text{‰}) = (\delta^{13}\text{C-DOC} - \delta^{13}\text{C-CO}_2) / (1 + \delta^{13}\text{C-CO}_2)$.

Soil cell density

At the end of the incubation setting (t = 91 days), cells were separated from soil particles and enumerated by FC. One gram of soil was mixed with 10 mL of pyrophosphate buffer (PBS 1X, 0.01 M Na₄P₂O₇) and shaken for 30 min in ice at 70 rpm on a rotary shaker. After shaking, the solution was sonicated 3 times (1 min each) in a water bath sonicator (Fisher Bioblock Scientific 88156, 320W, Illkirch, France). Larger particles were removed by centrifugation (800 × g, 1 min); the supernatant was fixed with paraformaldehyde (4 % final concentration) and stored at 4°C prior to quantification analysis. Total cells counts were performed using a FACSCalibur flow cytometer (BD Sciences, San Jose, CA, USA) equipped with an air-cooled laser, providing 15 mW at 488 nm with the standard filter set-up. Samples were diluted into 0.02 µm filtered TE buffer, stained with SYBR Green 1 (10,000 fold dilution of commercial stock, Molecular Probes, Oregon, USA) and the mixture was incubated for 15 min in the dark. The cellular abundance was determined on plots of side scatter versus green fluorescence (530 nm wave-length, fluorescence channel 1 of the instrument. Each sample was analyzed for 1 min at a rate of 20µL.min⁻¹. FCM list modes were analyzed using CellQuest Pro software (BD Biosciences, version 4.0). Cell density was expressed as cells × g⁻¹ of soil (dry mass).

Density and integrity of cells

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

Soil DNA and RNA content

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

Soil incubations with ¹³C₆-labelled-glucose

Samples (9 g, dry mass basis) of irradiated (45 kGy) and autoclaved (121 °C, 4 h) soil were incubated after addition of sterile solutions (1.53 mL of a 0.086 M glucose solution) of unlabelled- or of ¹³C₆- glucose (¹³C Abundance = 99 %). This amendment corresponds to 2.6 mg glucose g⁻¹ soil. Incubation and gas measurements were performed as previously described.

Statistical analyses

Each treatment was prepared in triplicate (n=3). One-Way ANOVA analysis was used to test the involvement significance of sterilization treatments on CO₂ emissions, $\delta^{13}\text{C}$ -CO₂, DOC, and $\delta^{13}\text{C}$ -DOC. Normality was tested using the Shapiro-Wilk test ($p > 0.05$). Equality of variances were tested with a Leven's Test ($p < 0.05$). Student test analyses were used to test the significance of the difference ($p < 0.05$) obtained

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

RESULTS

Effect of sterilization treatments

Microbial cell density and soil DNA and RNA content

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

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

Dissolved organic carbon (DOC) and its isotopic composition

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

All soil microcosms emitted CO₂ during all the incubation (**Fig. 3c**). Cumulated CO₂ emissions from LS and IS were not significantly ($p < 0.05$) different throughout the 91 days of incubation (24.4 ± 1.5 and $21.9 \pm 1.3 \mu\text{gC.g}^{-1}$ in LS and IS, respectively) but were significantly ($p < 0.05$) higher than in IAS ($16.8 \pm 1.5 \mu\text{gC.g}^{-1}$).

The daily CO₂ emission rate, DER, increased significantly ($p < 0.05$) from P1 to P4 in LS whereas DER gradually declined in IS (**Fig. 3c**). All IAS microcosms exhibited similar dynamics of DER: the high DER recorded during P1 strongly decreased during P2 and stabilized thereafter (**Fig. 3c**).

The $\delta^{13}\text{C}$ -CO₂ from LS decreased through the 4 periods, from $-22.2 \pm 0.1 \text{‰}$ to $-28.9 \pm 0.3 \text{‰}$. The $\delta^{13}\text{C}$ -CO₂ strongly decreased with the intensity of sterilization treatments, from $-29.2 \pm 1 \text{‰}$ in IS to $-75.4 \pm 2.8 \text{‰}$ in IAS with 4h of autoclaving (**Fig. 3d**). This pattern of values was maintained throughout the incubation but the difference of $\delta^{13}\text{C}$ -CO₂ between living and sterilized soils was maximal during the two intermediate periods (P2 and P3).

Carbon isotope fractionation during DOC mineralization

The $\delta^{13}\text{C}$ strongly deviated between DOC and CO_2 in all sterilized soil microcosms (**Fig. 3e**) indicating substantial C isotope fractionation during DOC mineralization. This isotope fractionation gradually increased with the intensity of the autoclaving treatment, from 13.2 ± 0.7 ‰ in IAS with 0.5h of autoclaving to 31 ± 2.5 ‰ in IAS with 4 h of autoclaving. The isotope fractionation was significantly and positively correlated to the DOC content ($r = 0.96$, **Fig. 3e**). The $\delta^{13}\text{C}$ deviation between DOC and CO_2 in LS was < 4 ‰ (data not shown).

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

The supply of unlabelled or labelled glucose in IAS with 4h of autoclaving did not significantly change total CO_2 emissions (data not shown). The $\delta^{13}\text{C}$ values of CO_2 released from microcosms with unlabelled glucose ranged from -40.2 ± 0.6 ‰ to -53.8 ± 1.2 ‰ (**Fig. 4**). The CO_2 released from microcosms with ^{13}C -glucose showed progressive ^{13}C enrichment with time, from $\delta^{13}\text{C} = 127.8 \pm 1.3$ ‰ to 657 ± 1.7 ‰ after 12 and 34 days of incubation, respectively (**Fig. 4**). At the end of the incubation, the amount of ^{13}C -glucose released as CO_2 corresponded to 0.01% of glucose input.

DISCUSSION

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

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

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

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

Body of evidence for EXOMET

The irradiated and autoclaved soils showed persistent (>91 days) and substantial soil CO₂ emissions (50-80% of CO₂ emissions compared to LS). Those CO₂ emissions can hardly be ascribed to residual activities of living and “ghost” cells since the sterilizing treatments removed all observable cell structure. Moreover, the substantial C isotope fractionation (from 13 ‰ to 35 ‰, **Fig.3e**) induced by the process responsible of CO₂ emissions is incompatible with a respiration of cellular origin. A substantial contribution of soil carbonates to CO₂ emissions is unlikely because (i) the inorganic carbon pool is very small in the acidic soil used in this study (Fontaine et al., 2007), (ii) the isotopic composition of CO₂ did not reflect the signature of soil carbonates (Bertrand et al., 2007). The decarboxylation of organic compounds by a combustion induced by sterilization treatments is also excluded because (i) CO₂ emissions were persistent throughout the incubation, (ii) the C isotope fractionation during organic C combustion is typically weak (~3‰) (Turney et al., 2006). Finally, irradiation and heating induce a heavy oxidative stress through the formation of hydroperoxides, carboxyls and free radicals. These highly reactive oxidants can lead to organic matter oxidation and decarboxylation. However, this oxidative process can hardly explain the persistent CO₂ emissions observed in our experiment since the half-life of highly reactive oxidants is extremely short (i.e. 10⁻⁹ s for free radicals). Moreover, Blankinship *et al.* (2014) have shown that the persistence of soil CO₂ emissions after microbial biomass suppression (or at least reduction) is not specific to irradiated soil but also occurs with other methods of sterilization such as chloroform fumigation and autoclaving.

The most parsimonious explanation of persistence of CO₂ emissions (**Fig. 3c**) and O₂ consumption (Maire et al., 2013) after soil sterilization is an extracellular oxidative metabolism (EXOMET). By EXOMET we suggest a cascade of chemical reactions where electrons are transferred from organic matter to redox mediators (i.e. NAD⁺/NADH, Mn³⁺/Mn²⁺) and finally to O₂. Those reactions can be catalyzed by respiratory enzymes stabilized on soil particles (Maire et al., 2013) and by minerals and metals present in soil (Blankinship et al., 2014; Majcher et al., 2000). The evidence of a complex oxidative metabolism is supported by the oxidation of ¹³C-glucose in ¹³CO₂ (**Fig. 4**). Indeed, glucose is a stable molecule which must undergo many biochemical transformations before being oxidized in carbon dioxide. The glucose decarboxylation (**Fig. 4**) and concurrent O₂ consumption (Maire et al., 2013) suggest that EXOMET is able to reconstitute an equivalent of glycolysis and Krebs cycle.

Mineral catalysts are stable and soil-stabilized enzymes are protected against denaturation (Carter et al., 2007; Gianfreda and Ruggiero, 2006; Nannipieri, 2006; Nannipieri et al., 1996; Stursova and Sinsabaugh, 2008). This stability of soil catalysts likely contributes to the maintenance of glucose oxidation and CO₂ emissions after soil exposure to high temperature and pressure (autoclaving). Maire et al. (2013) have already pointed at the exceptional resistance of soil CO₂ emissions to high temperature, pressure and toxics. However, by providing here the evidence of an oxidation of ¹³C-labelled glucose in γ-sterilized soil exposed to high temperature and pressure, we show that the complex metabolic pathways of the EXOMET are maintained under these extreme conditions.

Origin of the C isotope fractionation during EXOMET

Our results indicated that the EXOMET preferentially oxidizes organic molecules containing light (¹²C) over heavy (¹³C) carbon atoms. Similar strong isotope fractionation has already been described during wet abiotic oxidation of oxalic acid (Grey et al., 2006). The preferential conversion of substrate containing lighter isotopes agrees with classical kinetic and thermodynamic laws. The presence of ¹³C atoms in a substrate slows its conversion rate because of the higher activation energy request to induce the reaction (Christensen and Nielsen, 2000; Heinzle et al., 2008). Classical works on thermodynamic also indicate that the isotopic fractionation is dependent on substrate concentration (Agren et al., 1996; Goevert and Conrad,

2009; Wang et al., 2015). Under limited substrate concentration, the isotope fractionation decreases because the heavy molecules left over during the first stages of reaction are finally carried out by the process. Consistently, our results show that the isotopic fractionation induced by the EXOMET was positively correlated to DOC content (**Fig. 2e**). However, the causal link between the magnitude of fractionation and the DOC content is not certain since the correlation emerges from a compilation of results obtained after different sterilization treatments. **Further studies should analyze this causal link in experiments where the DOC content is directly manipulated and the change over time of the isotopic composition of DOC is quantified.**

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

It is well known that the delta ¹³C of CO₂ emitted from soils shows circadian cycle and seasonal fluctuations that reaches up to 5‰ (Moyes et al., 2010). However, it is difficult to link these fluctuations to a modification of metabolic pathways of soil respiration (living respiration versus EXOMET) in response to environmental changes since numerous other processes can contribute to these fluctuations. Moreover, it is likely that the EXOMET does not induce much C isotope fractionation in non-sterilized soils since the DOC content is typically low (Fig. 3a**) (Liu et al., 2015). Therefore, addition of large amount of DOC is necessary to reveal the C fractionation induced by the EXOMET in non-sterilized soils.**

Towards a quantification of EXOMET and cellular respiration in living soils

Our findings support the idea that CO₂ emissions from soils are driven by two major oxidative metabolisms: (1) the well-known respiration of soil biota, (2) an EXOMET carried out by soil stabilized enzymes and soil minerals and metals. A first quantification of these metabolisms has been made by Maire *et al.* (2013) suggesting that the EXOMET contributes from 16 to 48 % of soil CO₂ emissions. However, Maire *et al.* (2013) pointed at the need of another method to confirm this substantial contribution of EXOMET. Indeed, their method can lead to some biases. For instance, the soil irradiation used to block cellular activities and estimate the EXOMET induces a flush of respiration due to the release of substrates and enzymes from microbial biomass. This side effect of soil sterilization leads to an overestimation of EXOMET by releasing enzymes and cofactors in soil.

The difference in C isotope fractionation between EXOMET and cellular respiration offers another method of quantification of those metabolisms applicable on non-sterilized living soils. The development of this method first requires a quantification of the isotope fractionation (‰ delta ¹³C) and its dependence to DOC content occurring during cell respiration ($\Delta^{13}\text{C}_{\text{cell}}$) and EXOMET ($\Delta^{13}\text{C}_{\text{EXOMET}}$). **Our results provide an example of estimation of $\Delta^{13}\text{C}_{\text{EXOMET}}$ (**Fig. 3e**), though further studies are needed to verify the genericity of this estimation in other soils.** $\Delta^{13}\text{C}_{\text{cell}}$ for soil microorganisms can be estimated with cell cultures using soil inoculum and different substrate concentrations. This quantification allows determining the isotope composition of CO₂ (‰ delta ¹³C) released by cell respiration ($\delta^{13}\text{C}\text{-CO}_{2\text{cell}}$) and EXOMET ($\delta^{13}\text{C}\text{-CO}_{2\text{EXOMET}}$) in function to DOC content and isotope composition of DOC ($\delta^{13}\text{C}\text{-DOC}_{\text{sample}}$):

$$\delta^{13}\text{C}\text{-CO}_{2\text{cell}} = \delta^{13}\text{C}\text{-DOC}_{\text{sample}} - \Delta^{13}\text{C}_{\text{cell}} \quad (1)$$

$$\delta^{13}\text{C}\text{-CO}_{2\text{EXOMET}} = \delta^{13}\text{C}\text{-DOC} - \Delta^{13}\text{C}_{\text{EXOMET}} \quad (2)$$

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

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

where [DOC] is dissolved organic C content ($\mu\text{g C g}^{-1}$ 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:

$$R_{\text{soil}} = R_{\text{cell}} + R_{\text{EXOMET}} \quad (3)$$

$$\delta^{13}\text{C-CO}_{2\text{soil}} \times R_{\text{soil}} = \delta^{13}\text{C-CO}_{2\text{cell}} \times R_{\text{cell}} + \delta^{13}\text{C-CO}_{2\text{EXOMET}} \times R_{\text{EXOMET}} \quad (4)$$

where R_{soil} and $\delta^{13}\text{C-CO}_{2\text{soil}}$ are respectively the total CO_2 emitted by the amended soil ($\mu\text{g C-CO}_2 \text{ kg}^{-1} \text{ soil}$) and its isotopic composition ($\text{‰ delta }^{13}\text{C}$). R_{soil} and $\delta^{13}\text{C-CO}_{2\text{soil}}$ 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. $\delta^{13}\text{C-CO}_{2\text{cell}}$ and $\delta^{13}\text{C-CO}_{2\text{EXOMET}}$ 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 to 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 it has been included in the first cell.

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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.

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The authors declare no conflict of interest

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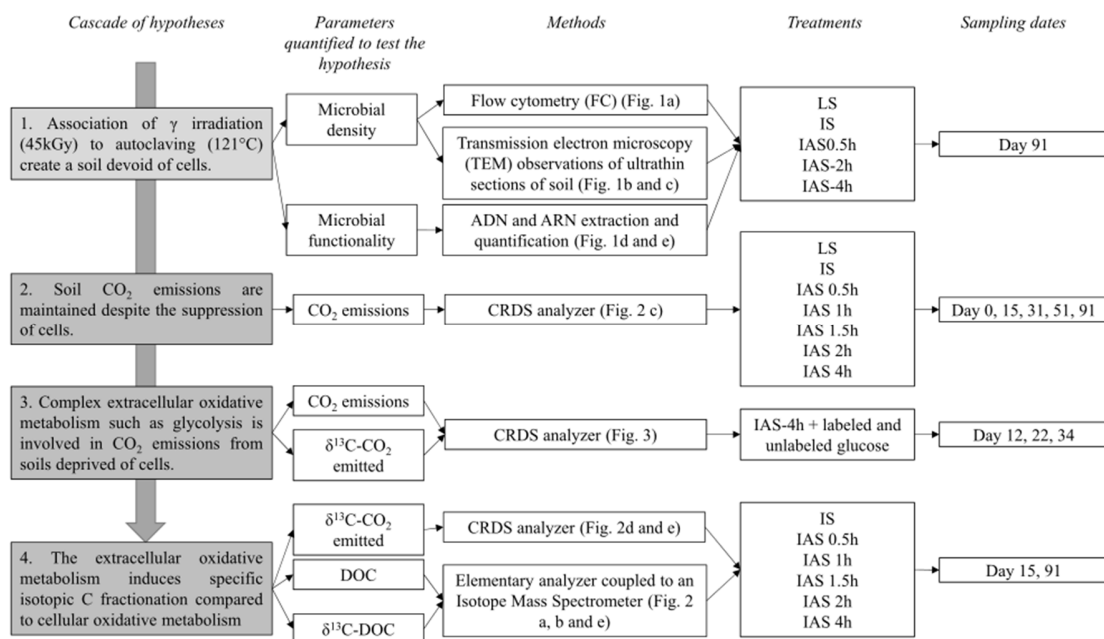
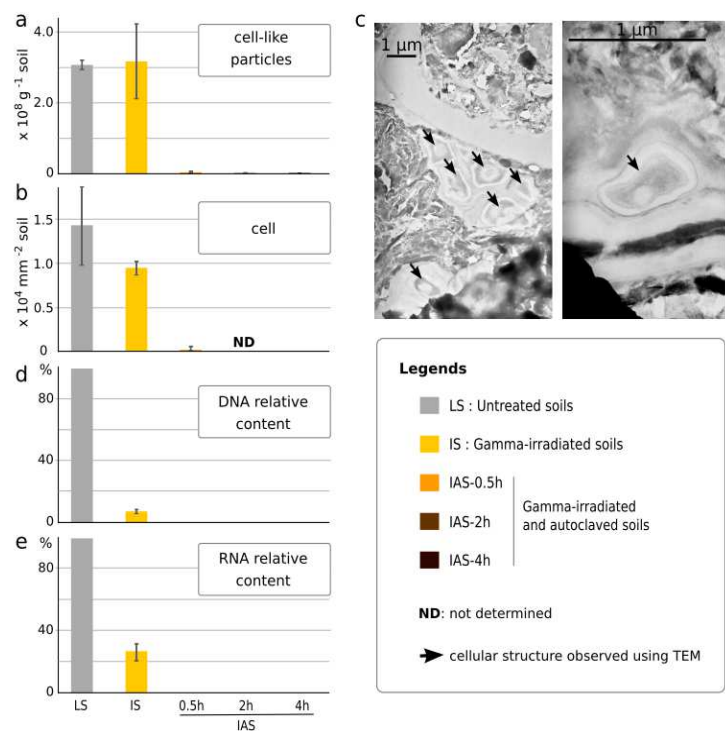


Figure 1: General experimental design of the study which include our hypothesis, the parameters, the methods and the samples (n=3 for each date and treatment studied) used to test those hypotheses.



535

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

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

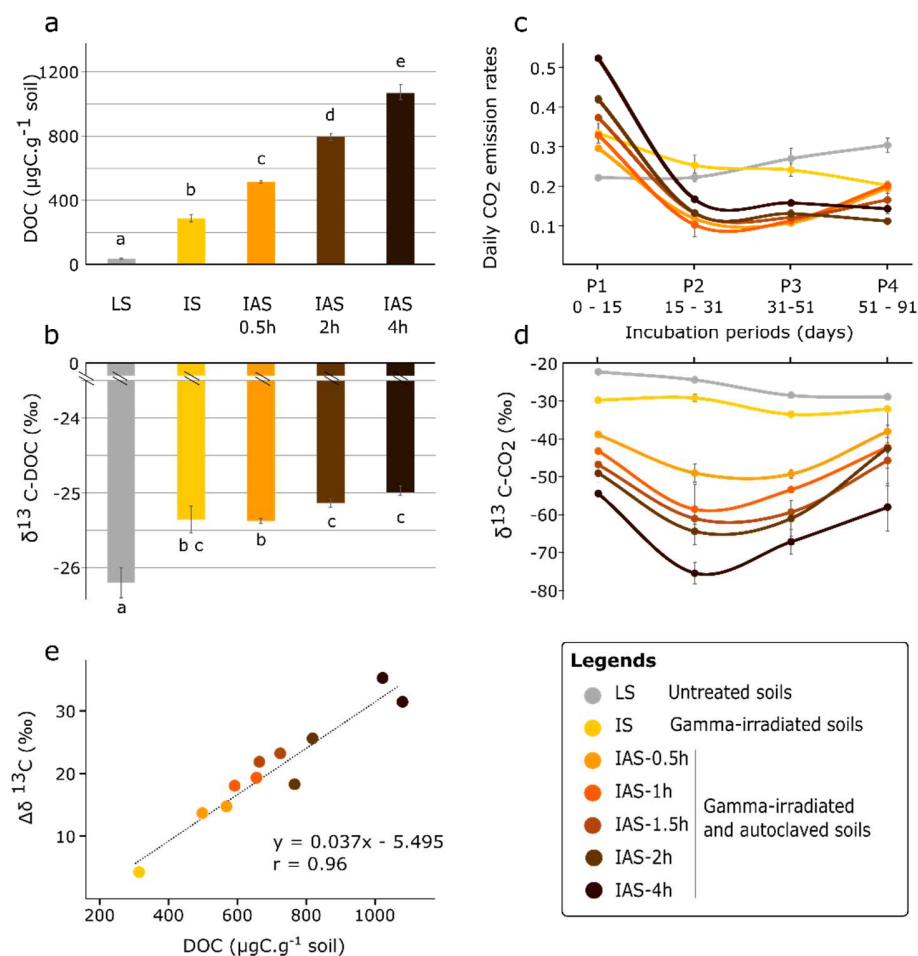


Figure 3: Content and isotopic composition of dissolved organic carbon (DOC) and of CO₂ across time and treatments.

(a) Content and (b) $\delta^{13}\text{C}$ of dissolved soil organic carbon content (DOC) at the beginning of incubation, (c) daily C-CO₂ emissions rates and (d) $\delta^{13}\text{C}$ of CO₂ released during four periods of incubation, (e) correlation between the carbon isotope discrimination ($\Delta\delta^{13}\text{C}$ 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.

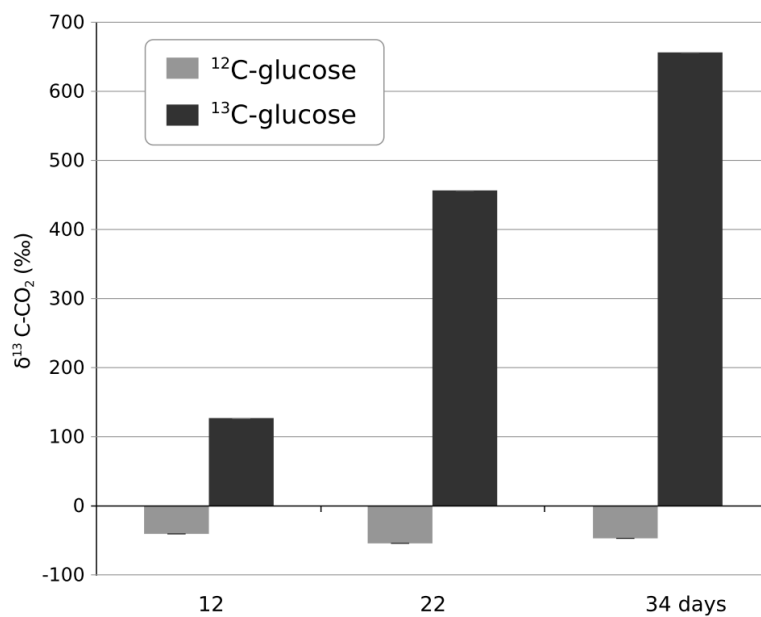


Figure 4: Kinetic of the $\delta^{13}\text{C-CO}_2$ released from an irradiated and autoclaved (4h) soil inoculated with ^{13}C -labelled glucose (^{13}C -glucose) or with unlabelled glucose (^{12}C -glucose) through 32 days of incubation. Standard deviation was estimated using three replicates per treatments (n=3).