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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 10 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 15

not likely obey to the same laws and respond differently to abiotic factors.

1 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.

²⁵ It is traditionally considered that SOM mineralization results from the activity of soil microbial communities through biological catalyzed processes including both extracel-



lular 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, as-

- similated 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 U⁺ expected in the ADP surface to an electron of the cascade.
- ¹⁰ 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 lev-
- els (Peterson, 1962; 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. (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 biochemical organization 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 CO_2 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 (Peterson, 1962; 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

¹⁵ electron microscopy on tangential ultration sections of soil, DNA and RNA soil content and flow cytometry. The production and the isotope composition (δ^{13} C) of CO₂ were monitored in sterilized and non-sterilized soils during 91 days. We also tested whether the EXOMET in sterilized soils can carry out complex cascade of biochemical reactions by incorporating ¹³C-labelled glucose and by quantifying emissions of ¹³C-CO₂ (Fig. 1).

2 Material and methods

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2.1 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 = $23.9 \pm 1 \text{ g C kg}^{-1}$). 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 ensure that any cultivable cells were present in soil after irradiation, we inoculated culture medium for bacteria (LB agar) and fungi (Yeast Malt agar) with irradiated soil. After irradiation, some sets of soil samples were exposed to autoclaving at 121 °C during variable periods (0.5, 1, 1.5, 2, 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 CO_2 gas (80 % N₂, 20 % O₂) and incubated in the dark at 20 °C for 91 days. Non-irradiated living soil was also incubated
- ¹⁰ 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
- soils" and IAS-t referred to irradiated and autoclaved soils with 't' referring to the time of autoclaving.

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

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The amount and isotope composition (δ^{13} C) of CO₂ accumulated in flasks during the incubation period were quantified using a cavity ring down spectrometer analyser coupled to a small injection system (Picarro 2131-i analyser, Picarro Inc., Santa Clara, CA, USA). 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 C-CO₂ emission rate.

2.3 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).

2.4 Isotope systematic

⁵ 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 δ^{13} C) from the Vienna Pee Dee Belemnite (VPDB) international standard material. The carbon isotope composition is expressed in parts per thousand (‰) according to the expression: $\delta^{13}C = (R_{sample}/R_{VPDB}) - (1) \times 1000$. The car-¹⁰ bon isotope fractionation was calculated as follows: $\Delta\delta^{13}C$ (‰) = $(\delta^{13}C-DOC - \delta^{13}C-CO_2)/(1 + \delta^{13}C-CO_2)$.

2.5 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
¹⁵ buffer (PBS 1X, 0.01 MNa₄P₂O₇) 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
²⁰ analysis. Total cells counts were performed using a FACSCalibur flow cytometrer (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 cellu-

²⁵ lar abundance was determined on plots of side scatter vs. green fluorescence (530 nm wave-length, fluorescence channel 1 of the instrument. Each sample was analyzed for



1 min at a rate of $20 \,\mu L \,\text{min}^{-1}$. FCM list modes were analyzed using CellQuest Pro software (BD Biosciences, version 4.0). Cell density was expressed as cells $\times \,g^{-1}$ of soil (dry mass).

2.6 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 (12 000 × *g*, 2 min) to pellet soil samples. Aliquot of soil sample (0.05 g) was fixed for 1 h 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 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 × 5 min), 70 % ethanol (3 × 15 min), 100 % ethanol (3 × 20 min) solutions. To improve the resin
- permeation, the sample was incubated in a propylene oxide bath (3 × 20 min). To allow the sample to soak resin, soil sample was incubated overnight in a bath containing propylene oxid and Epon 812 resin (ration 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 ra-
- ²⁰ zor lane (Gilette) 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 (Pelanne Instruments, Toulouse, France). Each grid was negatively stained for 30 s with uranyl acetate (2%),
- ²⁵ rinsed twice with 0.02 µm 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 × mm⁻² of soil.



2.7 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 Labora-

tories, 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.

2.8 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 ${}^{13}C_6$ -glucose (${}^{13}C$ abundance = 99 %). This amendment corresponds to 2.6 mg glucose g⁻¹ soil. Incubation and gas measurements were performed as previously described.

2.9 Statistical analyses

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Each treatment were prepared in triplicate (n = 3). One-Way ANOVA analysis was used to test the involvement significance of sterilization treatments on CO₂ emissions, δ^{13} C-

²⁰ CO₂, DOC, and δ^{13} C-DOC. Because of the reduced size of samples used in this study, we tested the normality of our variables using normal probability plots. Student test analyses were used to test the significance of the difference (*p* < 0.05) obtained between each conditions and each sampling dates and for each parameters evaluated during this study. Those statistical analyses were performed using the PAST software ²⁵ V3.04 (Hammer, 2001).



3 Results

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3.1 Effect of sterilization treatments

3.1.1 Microbial cell density and soil DNA and RNA content

Gamma-irradiations did not significantly reduce cellular density as revealed by flow ⁵ cytometry $(3.1 \times 10^8 \pm 1.3 \times 10^7 \text{ cell g}^{-1}$ in living soil, LS, vs. $3.2 \times 10^8 \pm 1.1 \times 10^8 \text{ cell g}^{-1}$ in irradiated soil, IS, Fig. 2a) and transmission electron microscopy $(1.4 \times 10^4 \pm 4.3 \times 10^3 \text{ in LS vs. } 9.5 \times 10^3 \pm 0.7 \times 10^2 \text{ cell g}^{-1}$ in IS, Fig. 2b and c). However, our results showed to absence of any microbial proliferation on inoculated culture mediums for bacteria (LB agar) and fungi (Yeast Malt agar) with irradiated soil. Moreover, 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, Fig. 2d and e) and nucleic acids (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 (Fig. 2b). According to transmission electron microscopy and nucleic acid extract results (Fig. 2b, d and e),
 the remaining flow cytometry signal in IAS is attributed to auto fluorescent particles and
- unspecific binding of the fluorescent dyes on debris.

3.1.2 Dissolved organic carbon (DOC) and its isotopic composition

Both γ -irradiations and autoclaving modified the soil chemistry as revealed by subsequent analyses of the aqueous phase which contained much more DOC in sterilized soils than in untreated soils (37±3 to 303±17µgCg⁻¹ in LS and IS, respectively



(Fig. 3a). Autoclaving further increased DOC content which gradually accumulated according to the time of autoclaving, from $557 \pm 11 \,\mu g \,C \,g^{-1}$ with 0.5 h of autoclaving to $1060 \pm 28.4 \,\mu\text{g}\,\text{C}\,\text{g}^{-1}$ after 4 h of autoclaving (Fig. 3a). Similarly, the δ^{13} C-DOC gradually increased from -27.4 ± 0.4 ‰ in LS to -24.9 ± 0.12 ‰ in IAS-4h (Fig. 3b). In all soil microcosms, DOC content and δ^{13} C of DOC did not significantly change over time (data not shown).

3.1.3 CO₂ emissions and their isotopic composition

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 $(24.4 \pm 1.5 \text{ and } 21.9 \pm 1.3 \mu \text{g C g}^{-1}$ in LS and IS, respectively) but were significantly (p < 0.05) higher than in IAS ($16.8 \pm 1.5 \,\mu g \,C \,g^{-1}$).

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

3.1.4 Carbon isotope fractionation during DOC mineralization

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The δ^{13} C strongly deviated between DOC and CO₂ 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 0.5 h of autoclaving to 31 ± 2.5 % in IAS with 4 h of au-

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toclaving. The isotope fractionation was significantly and positively correlated to the DOC content (r = 0.96, Fig. 3e). The δ^{13} C deviation between DOC and CO₂ in LS was < 4‰ (data not shown).

3.2 Response of sterilized soil to supply of unlabelled and ¹³C₆ labelled glucose

The supply of unlabelled or labelled glucose in IAS with 4 h of autoclaving did not significantly change total CO₂ emissions (data not shown). The δ^{13} C values of CO₂ released from microcosms with unlabelled glucose ranged from -40.2 ± 0.6 to $-53.8 \pm 1.2 \%$ (Fig. 4). The CO₂ released from microcosms with ¹³C-glucose showed progressive ¹³C enrichment with time, from δ^{13} C = 127.8±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 ¹³C-glucose released as CO₂ corresponded to 0.01 % of glucose input.

4 Discussion

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4.1 Irradiation and 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.

- ⁵ 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 0.5 h of autotion claving.

4.2 Body of evidence for EXOMET

The irradiated and autoclaved soils showed persistent (> 91 days) and substantial soil CO_2 emissions (50–80 % of CO_2 emissions compared to LS). Those CO_2 emissions can hardly be ascribed to residual activities of living and "ghost" cells since the ster-²⁰ ilizing treatments removed all observable cell structure. Moreover, the substantial C isotope fractionation (from 13 to 35‰, Fig. 3e) induced by the process responsible of CO_2 emissions is incompatible with a respiration of cellular origin. A substantial contribution of soil carbonates to CO_2 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 CO_2 did not reflect the signature of soil carbonates (Bertrand

²⁵ isotopic composition of CO_2 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) CO_2 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 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 CO_2 emissions observed

- ⁵ 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) showed that the maintenance 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 expressed on a support of the stable stable and expressed on the stable stable of the stable stable stable stable.
- ²⁰ (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 ²⁵ 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,



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we show that the complex metabolic pathways of the EXOMET are maintained under these extreme conditions.

4.3 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. Albeit exceptionally pronounced here, 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, the isotopic fractionation induced by the EXOMET was pos itively correlated to DOC content (Fig. 2e). It is worth noting that similar strong isotope fractionation has already been described during wet abiotic oxidation oxalic acid (Grey et al., 2006).

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 respira-

- tion. 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 reserves of cell respiration.
- ²⁵ use of light C isotope during biochemical reactions of cell respiration.



4.4 Towards a quantification of EXOMET and cellular respiration in living soils

Our findings support the idea that CO_2 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 EX-OMET 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 EX-OMET. 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 10
- 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 (Δ^{13} C) and its dependence to DOC content occurring during cell respiration ($\Delta^{13}C_{cell}$) and EXOMET ($\Delta^{13}C_{EXOMET}$). Our results provide a first estimation of $\Delta^{13}C_{\text{EXOMET}}$ (Fig. 3e). $\Delta^{13}C_{\text{cell}}$ for soil microorganisms can be estimated with cell cultures using soil inoculum and different substrate concentrations. This guantification allows determining the isotope composition of CO₂ ($\infty \delta^{13}$ C) released by cell 20 respiration ($\delta^{13}C-CO_{2cell}$) and EXOMET ($\delta^{13}C-CO_{2EXOMET}$) in function to DOC content and isotope composition of DOC (δ^{13} C-DOC_{sample}):

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

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



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

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

where [DOC] is dissolved organic C content (μ g C g⁻¹ soil).

⁵ 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:

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

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

where R_{soil} and δ^{13} C-CO_{2 soil} are respectively the total CO₂ emitted by the amended soil (µg C-CO₂ kg⁻¹ soil) and its isotopic composition (‰ δ^{13} C). R_{soil} and δ^{13} C-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 respira-

¹⁵ tion. This short-term measurement is also a prerequisite to prevent the microbial uptake of the heavy C isotope left over by the EXOMET. δ^{13} C-CO_{2cell} and δ^{13} C-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.

5 Conclusions and implications

²⁰ Collectively, our results show that 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



(4)

(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 re-

- ⁵ spond 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 re-

actions 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.

Author contributions. This work arose from an idea of S. Fontaine and A.-C. Lehours.
 B. Kéraval, S. Fontaine, A.-C. Lehours, G. Alvarez and C. Amblard designed the experiment. B. Kéraval and J. Colombet conducted the experiments. B. Kéraval analyzed the data.
 S. Fontaine identified the C isotope fractionation and conceived the model of quantification.
 B. Kéraval, S. Fontaine, A.-C. Lehours, G. Alvarez and C. Amblard co-wrote the paper.

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Figure 1. General experimental design of the study. The parameters measured, the methods used and the treatments realized to validate our hypotheses are presented. The sampling dates are also indicated. LS: Untreated soils, IS: irradiated soils, IAS-t: irradiated and autoclaved soils with "*t*" referring to the time of autoclaving.





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 deviations were calculated using three replicates per condition. 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 CO₂ across time and treatments. **(a)** Content and **(b)** δ^{13} C of dissolved soil organic carbon (DOC) at the beginning of incubation, **(c)** daily C-CO₂ emissions rates and **(d)** δ^{13} C of CO₂ 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 deviations were calculated using three replicates per condition. LS: untreated soils, IS: irradiated soils, IAS-t: irradiated and autoclaved soils with 't' referring to the time of autoclaving.







