Title:

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

Authors:

B. Kéraval [1,2,3], A .-C. Lehours[1,2], J. Colombet[1,2], C. Amblard [1,2], G. Alvarez [3,4], S. Fontaine [3]

Authors affiliations

[1] Clermont Université, Université Blaise Pascal, Laboratoire Microorganismes : Génome et Environnement, BP 10448, 63000, Clermont-Ferrand, France

[2] CNRS, UMR 6023, Laboratoire Microorganismes : Génome et Environnement, 63178 Aubière, France

[3] INRA, UR874 (Unité de Recherche sur l'Ecosystème Prairial), 5 Chemin de Beaulieu, 63039 Clermont-Ferrand, France.

[4] Clermont Université, VetAgro Sup, BP 10448, F-6300 Clermont-Ferrand, France

Correspondence to: B. Kéraval (benoit.keraval@gmail.com)

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 (CO_2) 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 CO_2 emissions in sterilized soils induced a strong C isotope fractionation (up to 50 ‰) incompatible with a respiration of cellular origin. The supply of ^{13}C-glucose in sterilized soil led to the release of $^{13}CO_2$ 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 CO_2 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 CO_2 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 CO_2 (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 CO_2. At the end of the cascade, the final acceptor (*e.g.* O_2 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 CO_2 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 ^{13}C-glucose in $^{13}CO_2$ using O_2 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 CO_2 emissions through decarboxylation of intermediary metabolites of the Krebs cycle. Whereas differing in the complexity of the proposed mechanisms, these

results (i) suggest that CO_2 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 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 (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 ($\delta^{13}C$) of CO_2 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 ^{13}C- labelled glucose and by quantifying emissions of ^{13}C-CO_2 **(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 (^{60}Co, 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 CO_2 gas (80 % N_2, 20 % O_2) 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 CO_2 fluxes and ^{13}C abundance of CO_2. After each measurement, flasks containing soil samples were flushed with a sterilized free CO_2 gas (80 % N_2, 20 % O_2). 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 ($^{13}C/^{12}C$)

The amount and isotope composition ($\delta\ ^{13}C$) 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}C 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 CO_2 and delta ^{13}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.

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 K_2SO_4 solution. After filtration through 1.6 µm (GE Healthcare, Life Sciences, Whatman™, 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 ^{13}C).

Isotope systematic

We use standard δ notation for quantifying the isotopic composition of CO_2 and of DOC: the ratio R of $^{13}C/^{12}C$ in the measured sample is expressed as a relative difference (denoted $\delta^{13}C$) 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 carbon isotope fractionation was calculated as follows: $\Delta\delta^{13}C$ (‰) = $(\delta^{13}C\text{-DOC} - \delta^{13}C\text{-}CO_2)/(1 + \delta^{13}C\text{-}CO_2)$.

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 $Na_4P_2O_7$) 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 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 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 x 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 cacohydrate 0.1 M buffer during 10 min. Post fixation was conducted with the 0.1 M cacohydrate 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 x 5 min), 70 % ethanol (3 x 15 min), 100 % ethanol (3 X 20 min) solutions. To improve the resin permeation, the sample was incubated in a propylene oxide bath (3 x 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 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 (Pelanne Instruments, Toulouse, France). Each grid was negatively stained for 30 s with uranyl acetate (2 %), 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 x 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 $^{13}C_6$-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.

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 CO_2 emissions, $\delta^{13}C$-CO_2, DOC, and $\delta^{13}C$-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 (Anon, n.d.).

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 ($1.4\ 10^4 \pm 4.3\ 10^3$ in LS *versus* $9.5\ 10^3 \pm 0.7\ 10^2$ cell.g^{-1} in IS, **Figs. 2b and 2c**). However, two proxies of cellular functionality and activity (DNA and RNA) were substantially decreased by irradiations (-93.5 % ± 1 % for DNA and -74 % ± 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±3 µg C.g^{-1} to 303±17 µg 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±11 µg C.g^{-1} with 0.5 h of autoclaving to 1060± 28.4 µg C.g^{-1} after 4 h of autoclaving (**Fig. 3a**). 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).

All soil microcosms emitted CO_2 during all the incubation (**Fig. 3c**). Cumulated CO_2 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 ± 1.3 µgC.g^{-1} in LS and IS, respectively) but were significantly (p <0.05) higher than in IAS (16.8 ± 1.5 µgC.g^{-1}).

The daily CO_2 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-CO_2 from LS decreased through the 4 periods, from -22.2 ± 0.1‰ to -28.9 ± 0.3‰. The δ^{13}C-CO_2 strongly decreased with the intensity of sterilization treatments, from -29.2 ± 1‰ in IS to -75.4 ± 2.8‰ in IAS with 4h of autoclaving (**Fig. 3d**). This pattern of values was maintained throughout the incubation but the difference of δ^{13}C-CO_2 between living and sterilized soils was maximal during the two intermediate periods (P2 and P3).

Carbon isotope fractionation during DOC mineralization

The $\delta^{13}C$ 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 $\delta^{13}C$ deviation between DOC and CO_2 in LS was < 4‰ (data not shown).

Response of sterilized soil to supply of unlabelled and $^{13}C_6$ 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 $\delta^{13}C$ 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 $\delta^{13}C$= 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 ^{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 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 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 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 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 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 CO_2 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 CO_2 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 CO_2 emissions (**Fig. 3c**) and O_2 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, Mn^{3+}/Mn^{2+}) and finally to O_2. 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 ^{13}C-glucose in $^{13}CO_2$ (**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 O_2 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 CO_2 emissions after soil exposure to high temperature and pressure (autoclaving). **Maire et al. (2013)** have already pointed at the exceptional resistance of soil CO_2 emissions to high temperature, pressure and toxics. However, by providing here the evidence of an oxidation of ^{13}C-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 (^{12}C) over heavy (^{13}C) 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 ^{13}C 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 ^{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.

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 EXOMET contributes from 16 to 48 % of soil CO_2 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 ^{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 an example of estimation of $\Delta^{13}C_{EXOMET}$ (**Fig. 3e**), though further studies are needed to verify the genericity of this estimation in other soils. $\Delta^{13}C_{cell}$ for soil microorganisms can be estimated with cell cultures using soil inoculum and different substrate concentrations. This quantification allows determining the isotope composition of CO_2 (‰ delta ^{13}C) released by cell respiration ($\delta^{13}C\text{-}CO_{2cell}$) and EXOMET ($\delta^{13}C\text{-}CO_{2EXOMET}$) in function to DOC content and isotope composition of DOC ($\delta^{13}C\text{-}DOC_{sample}$):

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

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

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

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

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

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

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

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

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

CONCLUSIONS AND IMPLICATIONS

Collectively, our results tend to sustain the hypothesis through which soil C mineralization is driven by the well-known microbial mineralization and an EXOMET carried out by soil-stabilized enzymes and by soil mineral and metal catalysts. These two metabolisms may explain why soil C mineralization is not always connected to size and composition of the microbial biomass (Kemmitt et al., 2008) and why experimental reduction of these microbial components has moderate effects on mineralization rate (Griffiths et al., 2001). Moreover, these two metabolisms should be considered separately when studying effects of environmental factors on the C cycle because they do not likely obey 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.

ACKNOWLEDGEMENTS

This work was supported by the project '*Adaptation and responses of organisms and carbon metabolism to climate change*' of the program CPER (French Ministry of Research, CNRS, INRA, Région Auvergne, FEDER) and by the project EXCEED of the program PICS (CNRS). B. Kéraval was supported by a PhD fellowship from the Région Auvergne and the FEDER.

AUTHOR CONTRIBUTIONS

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

COMPETING FINANCIAL INTERESTS

The authors declare no conflict of interest

REFERENCES

Agren, G. I., Bosatta, E. and Balesdent, J.: Isotope discrimination during decomposition of organic matter: A theoretical analysis, Soil Sci. Soc. Am. J., 60(4), 1121–1126, 1996.

Bernal, J. D.: The Physical Basis of Life, Proc. Phys. Soc. B, 62(10), 597, doi:10.1088/0370-1301/62/10/301, 1949.

Berns, A. E., Philipp, H., Narres, H.-D., Burauel, P., Vereecken, H. and Tappe, W.: Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy, European Journal of Soil Science, 59(3), 540–550, doi:10.1111/j.1365-2389.2008.01016.x, 2008.

Bertrand, I., Delfosse, O. and Mary, B.: Carbon and nitrogen mineralization in acidic, limed and calcareous agricultural soils: Apparent and actual effects, Soil Biology and Biochemistry, 39(1), 276–288, doi:10.1016/j.soilbio.2006.07.016, 2007.

Blair, N., Leu, A., Muñoz, E., Olsen, J., Kwong, E. and Des Marais, D.: Carbon isotopic fractionation in heterotrophic microbial metabolism , Appl Environ Microbiol, 50(4), 996–1001, 1985.

Blankinship, J. C., Becerra, C. A., Schaeffer, S. M. and Schimel, J. P.: Separating cellular metabolism from exoenzyme activity in soil organic matter decomposition, Soil Biology and Biochemistry, 71, 68–75, doi:10.1016/j.soilbio.2014.01.010, 2014.

Buchan, D., Moeskops, B., Ameloot, N., De Neve, S. and Sleutel, S.: Selective sterilisation of undisturbed soil cores by gamma irradiation: Effects on free-living nematodes, microbial community and nitrogen dynamics, Soil Biology and Biochemistry, 47, 10–13, doi:10.1016/j.soilbio.2011.12.014, 2012.

Burns, R. G., DeForest, J. L., Marxsen, J., Sinsabaugh, R. L., Stromberger, M. E., Wallenstein, M. D., Weintraub, M. N. and Zoppini, A.: Soil enzymes in a changing environment: Current knowledge and future directions, Soil Biology and Biochemistry, 58, 216–234, doi:10.1016/j.soilbio.2012.11.009, 2013.

Button, D. K.: Nutrient Uptake by Microorganisms according to Kinetic Parameters from Theory as Related to Cytoarchitecture, Microbiol. Mol. Biol. Rev., 62(3), 636–645, 1998.

Carter, D. O., Yellowlees, D. and Tibbett, M.: Autoclaving kills soil microbes yet soil enzymes remain active, Pedobiologia, 51(4), 295–299, doi:10.1016/j.pedobi.2007.05.002, 2007.

Christensen, B. and Nielsen, J.: Metabolic network analysis. A powerful tool in metabolic engineering, Adv. Biochem. Eng. Biotechnol., 66, 209–231, 2000.

Fontaine, S., Barot, S., Barré, P., Bdioui, N., Mary, B. and Rumpel, C.: Stability of organic carbon in deep soil layers controlled by fresh carbon supply, Nature, 450(7167), 277–280, doi:10.1038/nature06275, 2007.

Gianfreda, L. and Ruggiero, P.: Enzyme Activities in Soil, in Nucleic Acids and Proteins in Soil, edited by P. P. Nannipieri and D. K. Smalla, pp. 257–311, Springer Berlin Heidelberg, 2006.

Goevert, D. and Conrad, R.: Effect of substrate concentration on carbon isotope fractionation during acetoclastic methanogenesis by Methanosarcina barkeri and M. acetivorans and in rice field soil, Appl. Environ. Microbiol., 75(9), 2605–2612, doi:10.1128/AEM.02680-08, 2009.

Grey, D. C., Damon, P. E., Haynes, C. V. and Long, A.: Carbon-isotope fractionation during wet oxidation of oxalic acid., Radiocarbon, 11(1), 1–2, doi:10.2458/azu_js_rc.11.178, 2006.

Griffiths, B. S., Ritz, K., Wheatley, R., Kuan, H. L., Boag, B., Christensen, S., Ekelund, F., Sørensen, S. J., Muller, S. and Bloem, J.: An examination of the biodiversity–ecosystem function relationship in arable soil microbial communities, Soil Biology and Biochemistry, 33(12–13), 1713–1722, doi:10.1016/S0038-0717(01)00094-3, 2001.

Hammer, Ø., Harper, D. A. T., and Ryan, P. D.: PAST: Palaeontological Statistics Software package for education and data analysis, Palaeontol. Electron., 4, 1–9, 2001.

Hazen, R. M.: Presidential Address to the Mineralogical Society of America, Salt Lake City, October 18, 2005 Mineral surfaces and the prebiotic selection and organization of biomolecules, American Mineralogist, 91(11-12), 1715–1729, doi:10.2138/am.2006.2289, 2006.

Heinzle, E., Yuan, Y., Kumar, S., Wittmann, C., Gehre, M., Richnow, H.-H., Wehrung, P., Adam, P. and Albrecht, P.: Analysis of 13C labeling enrichment in microbial culture applying metabolic tracer experiments using gas chromatography–combustion–isotope ratio mass spectrometry, Analytical Biochemistry, 380(2), 202–210, doi:10.1016/j.ab.2008.05.039, 2008.

IPCC: Changes in atmospheric constituents and in radiative forcing. Climate Change 2007: The Physical Science Basis, Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, edited by: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., and Miller, H. L., Cambridge University Press, Cambridge, UK and New York, NY, USA, 130–234, 2007b.

Junge, W., Lill, H. and Engelbrecht, S.: ATP synthase: an electrochemical ransducer with rotatory mechanics, Trends in Biochemical Sciences, 22(11), 420–423, doi:10.1016/S0968-0004(97)01129-8, 1997.

Kemmitt, S. J., Lanyon, C. V., Waite, I. S., Wen, Q., Addiscott, T. M., Bird, N. R. A., O'Donnell, A. G. and Brookes, P. C.: Mineralization of native soil organic matter is not regulated by the size, activity or composition of the soil microbial biomass—a new perspective, Soil Biology and Biochemistry, 40(1), 61–73, doi:10.1016/j.soilbio.2007.06.021, 2008.

Lensi, R., Lescure, C., Steinberg, C., Savoie, J.-M. and Faurie, G.: Dynamics of residual enzyme activities, denitrification potential, and physico-chemical properties in a γ-sterilized soil, Soil Biology and Biochemistry, 23(4), 367–373, doi:10.1016/0038-0717(91)90193-N, 1991.

Li, D., Velde, B. and Zhang, T.: Observations of pores and aggregates during aggregation in some clay-rich agricultural soils as seen in 2D image analysis, Geoderma, 118(3–4), 191–207, doi:10.1016/S0016-7061(03)00193-9, 2004.

Liu, E., Chen, B., Yan, C., Zhang, Y., Mei, X. and Wang, J.: Seasonal Changes and Vertical Distributions of Soil Organic Carbon Pools under Conventional and No-Till Practices on Loess Plateau in China, Soil Science Society of America Journal, 79(2), 517, doi:10.2136/sssaj2014.02.0069, 2015.

Maire, V., Alvarez, G., Colombet, J., Comby, A., Despinasse, R., Dubreucq, E., Joly, M., Lehours, A.-C., Perrier, V., Shahzad, T. and Fontaine, S.: An unknown oxidative metabolism substantially contributes to soil CO2 emissions, Biogeosciences, 10(2), 1155–1167, doi:10.5194/bg-10-1155-2013, 2013.

Maire, V., Alvarez, G., Colombet, J., Comby, A., Despinasse, R., Dubreucq, E., Joly, M., Lehours, A.-C., Perrier, V., Shahzad, T. and Fontaine, S.: An unknown oxidative metabolism substantially contributes to soil CO2 emissions, Biogeosciences Discuss., 9, 8663, 2012.

Majcher, E. H., Chorover, J., Bollag, J.-M. and Huang, P. M.: Evolution of CO during Birnessite-Induced Oxidation of C-Labeled Catechol, Soil Science Society of America Journal, 64(1), 157, doi:10.2136/sssaj2000.641157x, 2000.

Moyes, A. B., Gaines, S. J., Siegwolf, R. T. W. and Bowling, D. R.: Diffusive fractionation complicates isotopic partitioning of autotrophic and heterotrophic sources of soil respiration, Plant Cell Environ., 33(11), 1804–1819, doi:10.1111/j.1365-3040.2010.02185.x, 2010.

Nannipieri, P.: Role of Stabilised Enzymes in Microbial Ecology and Enzyme Extraction from Soil with Potential Applications in Soil Proteomics, in Nucleic Acids and Proteins in Soil, edited by P. P. Nannipieri and D. K. Smalla, pp. 75–94, Springer Berlin Heidelberg, 2006.

Nannipieri, P., Sastre, I., Landi, L., Lobo, M. C. and Pietramellara, G.: Determination of extracellular neutral phosphomonoesterase activity in soil, Soil Biology and Biochemistry, 28(1), 107–112, doi:10.1016/0038-0717(95)00108-5, 1996.

Paterson, E. and Sim, A.: Soil-specific response functions of organic matter mineralization to the availability of labile carbon, Glob Change Biol, 19(5), 1562–1571, doi:10.1111/gcb.12140, 2013.

Pignatello, J. J., Oliveros, E. and MacKay, A.: Advanced Oxidation Processes for Organic Contaminant Destruction Based on the Fenton Reaction and Related Chemistry, Critical Reviews in Environmental Science and Technology, 36(1), 1–84, doi:10.1080/10643380500326564, 2006.

Ramsay, A. J. and Bawden, A. D.: Effects of sterilization and storage on respiration, nitrogen status and direct counts of soil bacteria using acridine orange, Soil Biology and Biochemistry, 15(3), 263–268, doi:10.1016/0038-0717(83)90069-X, 1983.

Sinsabaugh, R. L. and Follstad Shah, J. J.: Ecoenzymatic Stoichiometry and Ecological Theory, Annual Review of Ecology, Evolution, and Systematics, 43(1), 313–343, doi:10.1146/annurev-ecolsys-071112-124414, 2012.

Sinsabaugh, R. L., Hill, B. H. and Follstad Shah, J. J.: Ecoenzymatic stoichiometry of microbial organic nutrient acquisition in soil and sediment, Nature, 462(7274), 795–798, doi:10.1038/nature08632, 2009.

Stursova, M. and Sinsabaugh, R. L.: Stabilization of oxidative enzymes in desert soil may limit organic matter accumulation, Soil Biology and Biochemistry, 40(2), 550–553, doi:10.1016/j.soilbio.2007.09.002, 2008.

Trevors, J. T.: Sterilization and inhibition of microbial activity in soil, Journal of Microbiological Methods, 26(1–2), 53–59, doi:10.1016/0167-7012(96)00843-3, 1996.

Tuominen, L., Kairesalo, T. and Hartikainen, H.: Comparison of Methods for Inhibiting Bacterial Activity in Sediment, Appl Environ Microbiol, 60(9), 3454–3457, 1994.

Turney, C. S. M., Wheeler, D. and Chivas, A. R.: Carbon isotope fractionation in wood during carbonization, Geochimica et Cosmochimica Acta, 70(4), 960–964, doi:10.1016/j.gca.2005.10.031, 2006.

Wang, G., Jia, Y. and Li, W.: Effects of environmental and biotic factors on carbon isotopic fractionation during decomposition of soil organic matter, Sci. Rep., 5, doi:10.1038/srep11043, 2015.

Zyakun, A. M., Kochetkov, V. V., Baskunov, B. P., Zakharchenko, V. N., Peshenko, V. P., Laurinavichius, K. S., Anokhina, T. O., Siunova, T. V., Sizova, O. I. and Boronin, A. M.: Use of glucose and carbon isotope fractionation by microbial cells immobilized on solid-phase surface, Microbiology, 82(3), 280–289, doi:10.1134/S0026261713030156, 2013.

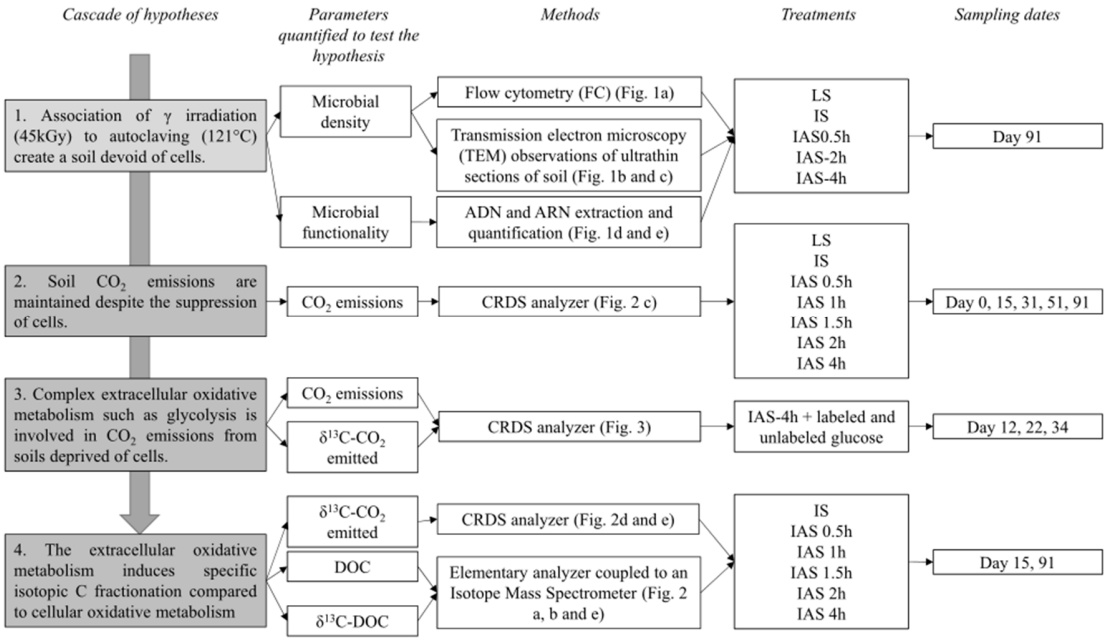


Figure 1: General experimental design of the study which include our hypothesis, the parameters, the methods and the samples (n=3 for each date and treatment studied) used to test those hypotheses.

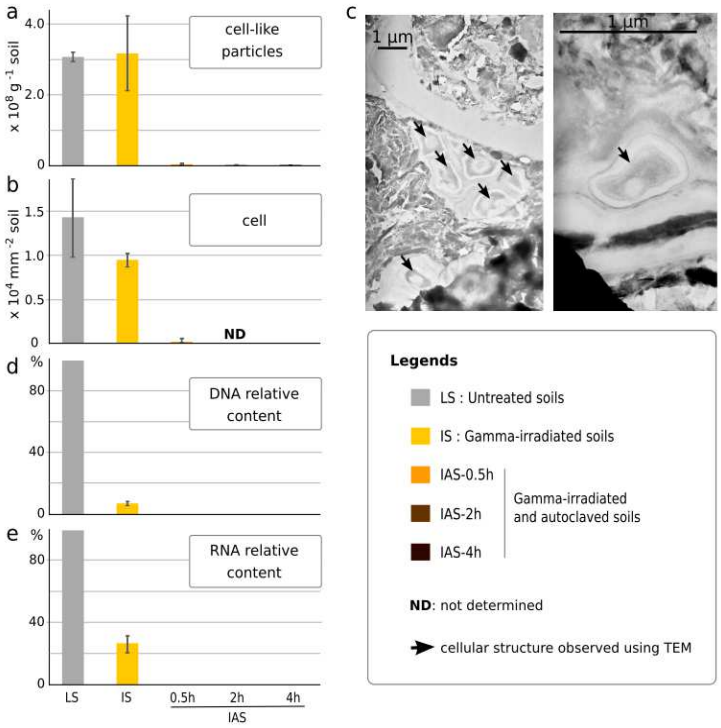
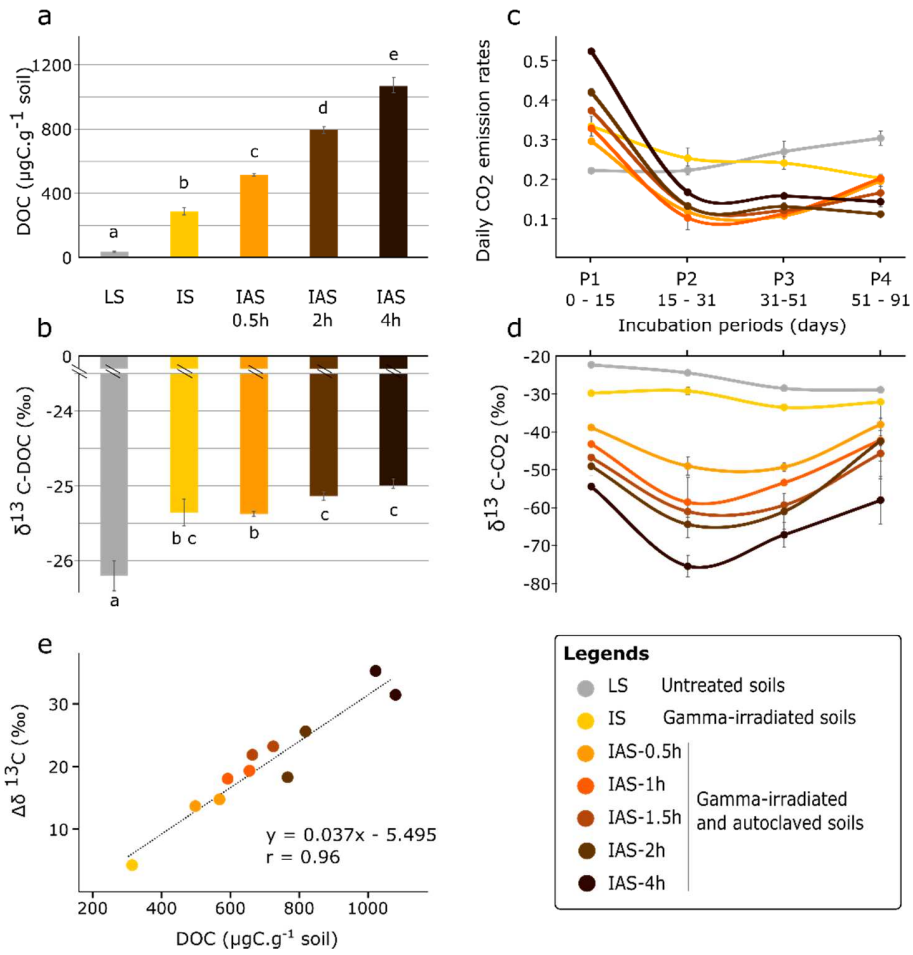


Figure 2: Impact of sterilization treatments on cellular density, integrity and functionality.

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

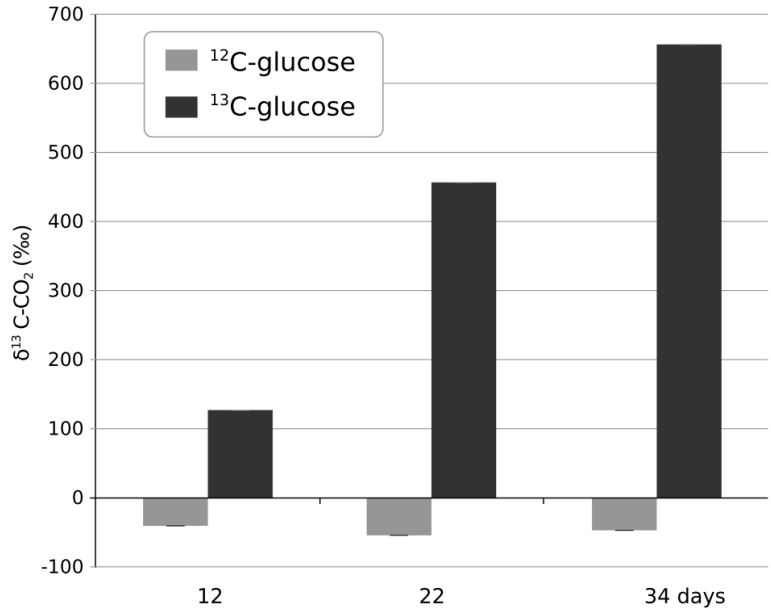


543

544 **Figure 3:** Content and isotopic composition of dissolved organic carbon (DOC) and of CO_2 across time and
545 treatments.

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

553



554

555 **Figure 4:** Kinetic of the $\delta^{13}C\text{-}CO_2$ released from an irradiated and autoclaved (4h) soil inoculated with ^{13}C-
556 labelled glucose (^{13}C-glucose) or with unlabelled glucose (^{12}C-glucose) through 32 days of incubation.
557 Standard deviation was estimated using three replicates per treatments (n=3).

558