

An unknown respiration pathwaysubstantially contributes to soil CO2 emissions.

Vincent V. Maire, Gaël Alvarez, J J. Colombet, Aurélie A. Comby, Romain R. Despinasse, E E. Dubreucq, M M. Joly, Ac A. Lehours, Véronique Perrier, Tanvir T. Shahzad, et al.

▶ **To cite this version:**

Vincent V. Maire, Gaël Alvarez, J J. Colombet, Aurélie A. Comby, Romain R. Despinasse, et al.. An unknown respiration pathwaysubstantially contributes to soil CO2 emissions.. Biogeosciences Discussions, 2012, 9, pp.8663-8691. hal-00964427

HAL Id: hal-00964427
https://hal.science/hal-00964427

Submitted on 28 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Biogeosciences Discuss., 9, 8663–8691, 2012
www.biogeosciences-discuss.net/9/8663/2012/
doi:10.5194/bgd-9-8663-2012
© Author(s) 2012. CC Attribution 3.0 License.

Biogeosciences Discussions

This discussion paper is/has been under review for the journal Biogeosciences (BG).
Please refer to the corresponding final paper in BG if available.

An unknown respiration pathway substantially contributes to soil CO_2 emissions

V. Maire[1], G. Alvarez[1,2], J. Colombet[3], A. Comby[1], R. Despinasse[4], E. Dubreucq[5], M. Joly[1], A.-C. Lehours[3], V. Perrier[5], T. Shahzad[1], and S. Fontaine[1]

[1]INRA, UR 874 UREP, 63100 Clermont-Ferrand, France
[2]Clermont Université, VetAgro Sup, 63000, Clermont-Ferrand, France
[3]University of Clermont Ferrand, UMR 6023 LMGE, 63177 Aubière, France
[4]INRA, UMR 1095 UBP, 63100 Clermont-Ferrand, France
[5]Montpellier SupAgro, UMR 1208 IATE, 34060 Montpellier, France

Received: 31 May 2012 – Accepted: 23 June 2012 – Published: 18 July 2012

Correspondence to: S. Fontaine (sebastien.fontaine@clermont.inra.fr)

Published by Copernicus Publications on behalf of the European Geosciences Union.



Abstract

The respiratory release of CO_2 from soils is a major determinant of the global carbon cycle. It is traditionally considered that this respiration is an intracellular metabolism consisting of complex biochemical reactions carried out by numerous enzymes and co-factors. Here we show that the intracellular enzymes released from dead organisms are stabilized in soils and have access to suitable substrates and co-factors to permit function. These enzymes reconstitute an extracellular oxidative metabolism (Exomet) that may substantially contribute to soil respiration (16 to 48 % of CO_2 released from soils in the present study). Exomet and respiration from living organisms should be considered separately when studying effects of environmental factors on the C cycle because Exomet shows specific properties such as resistance to high temperature and toxics.

1 Introduction

Knowledge of the metabolic pathways through which organic carbon is oxidized into CO_2 is fundamental to understand the global carbon cycle and its interactions with climate (Fontaine et al., 2007; Heimann and Reichstein, 2008). Outside the metabolic environment of a cell, organic molecules are generally highly stable. For instance, the physical oxidation of one molecule of glucose requires a temperature greater than 500 °C. In contrast, a complex cascade of biochemical reactions (i.e. glycolysis and the Krebs cycle) mediated by numerous intracellular enzymes makes the oxidization of organic C possible at low temperature within living cells. The function of these respiration-carrying enzymes depends on various co-factors (e.g. NAD^+), on being located adjacent to other enzymes, and on physiological properties of the cell (e.g. redox potential) (Krebs, 1981; Burns, 1982; Rich, 2003). Given this complexity and the fragility of respiratory enzymes, it is traditionally considered that respiration is strictly an intracellular metabolism. However, some studies have shown persistence of substantial production

BGD
9, 8663–8691, 2012



of CO_2 in soils where microbial life has been strongly reduced by exposition to toxics ($ClCH_3$, orange acridine) or irradiation (Peterson, 1962; Ramsay and Bawden, 1983; Lensi et al., 1991; Trevors, 1996; Kemmitt et al., 2008). This production of CO_2 cannot be explained by the activity of extracellular enzymes previously secreted by soil microorganisms since these enzymes only hydrolyze organic matter and do not release CO_2. To date, the cause of CO_2 production in soils where microbial life has been minimized is unknown and questions our basic knowledge of biology.

Our findings challenge the idea that respiration is strictly an intracellular metabolism, demonstrating the existence of an extracellular oxidative metabolism (Exomet) reconstituted by respiratory enzymes released from dead organisms in soils. The manuscript is organized in four key parts. We first demonstrate the possibility of an Exomet by incubating a cell-free yeast-extract containing respiratory enzymes with glucose in sterilized water and soil. The Exomet induced by the yeast-extract was quantified by measuring CO_2 and O_2 fluxes in water and soil microcosms. Second, the protective role of soil particles (minerals and humus) for respiratory enzymes is demonstrated by incubating three enzymes involved in glycolysis and the Krebs cycle in five top soils sampled from different regions of the world (Table 1). The third part of the manuscript is devoted to the quantification of Exomet contribution to the CO_2 emissions from the living (non-sterilized) studied soils. This contribution is quantified with a method combining modeling and incubations of sterilized and non-sterilized soils. Finally, we study some Exomet properties including resistance to high temperature and toxics.

2 Materials and methods

2.1 Soil sampling and sterilization

For each of the five studied sites (Table 1), twenty independent soil samples were collected from the 0–20 cm soil layer. Soil samples from each site were pooled to make a composite sample per site. Soil was sieved at 2 mm and was then used to determine

BGD
9, 8663–8691, 2012



pH, texture and organic matter content and to conduct incubation experiments. The soils of Bugac (Hungary), Laqueuille (France), Ponta Grossa (Brasil), Soro (Denmark) and Theix (France) presented textures from sandy-silted to silty-clay soils, pH from 4.3 to 8.6 and three types of land use (grassland, forest and crops). The soil of Theix was used for all investigations whereas the other soils were used to generalize the key findings of this study.

Some incubation experiments involved the use of sterilized soils. We tested different methods of soil sterilization (γ-irradiation, autoclaving and dry heating) during preliminary investigations. Irradiation was chosen among other sterilizing methods for its efficiency to kill soil micro-organisms and for its moderate effect on soil enzymes (see S1 for details of investigations on soil sterilization). The preservation of soil enzymes was important for quantifying the Exomet contribution to soil CO_2 emissions (see the Sect. 2.4). Soils were sterilized by γ-irradiation at 45 kGy (^{60}Co, Ionisos, ISO14001, France).

2.2 Demonstration of Exomet by incubating a cell-free yeast-extract in sterilized water and soil

2.2.1 Production of cell-free yeast-extract

Pichia pastoris X33 (Invitrogen, Clare et al., 1991) cells were cultured at 28 °C in a 1.5 l capacity Applikon bioreactor containing synthetic medium described by Boze et al. (2001). This medium contained 80 µg l^{-1} D-biotin, 40 g l^{-1} glycerol and mineral solutions (FM21 and PTM1). The pH of the medium was regulated at 5 using a NH_4OH solution (15 %, v/v) that also served as a nitrogen source. Dissolved oxygen was measured using a polarographic probe and was maintained at over 30 % saturation by stirring up to 1800 rpm and aeration (an injection of 1 l of air per litre of medium per minute). Data acquisition and bioprocess control were carried out using the Applikon biocontroller software. Biomass concentration of culture samples was determined by weighing washed cells after drying at 105 °C to constant weight.

BGD
9, 8663–8691, 2012



The yeast culture was harvested at the end of the growth phase by centrifugation at 25 000 × g for 10 min at 5 °C to yield a hard pellet. To remove the culture media, the sediment was washed and re-decanted three times in potassium phosphate buffer (0.1 M, pH = 6.5 which corresponded to the pH of the Theix soil used for the experiment, V_{yeast}/V_{buffer} = 1/10). The sediment was re-suspended in a small volume of potassium phosphate buffer (V_{yeast}/V_{buffer} = 2/1) before cells were disrupted with a French pressure cell press (100 MPa). Unbroken cells and large cell debris were removed from the yeast extract by fourteen successive centrifugations at 25 000 × g and 5 °C. The yeast extract was filtered under sterile conditions at 0.2 µm to obtain a cell-free extract. Direct microscopic observation confirmed sterility of the yeast extract (S1). The cell-free yeast extract was immediately incorporated into water and soil microcosms under sterile conditions. The cell-free yeast extract contained 28.5 mg ml^{-1} protein (Biuret method) and 0.77 U MDH ml^{-1}.

2.2.2 Yeast extract incubation in sterilized water and soil

The cell-free yeast extract (YE) was incubated in sterilized water (W) or in the irradiated-soil (S) of Theix (Table 1). Experimental microcosms consisted of 5 ml of cell-free yeast extract and 1 ml of ^{13}C labeled glucose with or without 20 g of γ-irradiated-soil (S + G + YE and W + G + YE treatments, respectively) placed in 250 ml flasks. A second dose of glucose was applied after twenty days of incubation in order to determine the persistence of the Exomet. Water (5 ml) with ^{13}C labeled glucose solution (1 ml) and irradiated-soil (20 g) with or without ^{13}C labeled glucose solution (1 ml) were incubated as controls (W + G, S and S + G treatments, respectively). The ^{13}C labeled glucose solution (δ^{13}C = 3712‰) contained 60 mg C – glucose ml^{-1} and was sterilized by filtration at 0.2 µm. Three replicates per treatment were prepared. Soils were incubated at a water potential of –100 kPa. Water and soil microcosms were incubated at 30 °C for 53 days. The Exomet induced by the cell-free yeast extract was quantified by measuring the concentration of CO_2, $^{13}CO_2$ and O_2 in water and soil microcosms throughout the 53 days of incubation. All manipulations were done under

BGD
9, 8663–8691, 2012



sterile conditions and the sterility of microcosms was verified after 13 and 53 days of incubation through direct fluorescence microscopic observations and TSA-FISH method (S1).

2.3 Soil stabilization of respiratory enzymes

2.3.1 Enzyme incubation

In order to quantify the protective role of soil particles (minerals, humus) for respiratory enzymes, three microbial enzymes involved in glycolysis (GHK: glucose hexokinase EC.2.7.1.1, Sigma-Aldrich ref H4502; G6PI: glucose-6-phosphate isomerase EC.5.3.1.9, Sigma-Aldrich ref P5381) and the Krebs cycle (MDH: malate dehydrogenase, EC.1.1.1.37, Sigma-Aldrich ref M7032) were separately incubated in the non-irradiated-soil of Theix for 35 days. A solution with an enzymatic activity of approximately 50 $U\,l^{-1}$ was prepared for each enzyme. A volume of 15 µl of enzyme solution was incorporated in 80 mg soil (dry mass basis) and incubated at 20 °C and –100 kPa. Soils without enzyme amendment were also incubated as controls. Two sets of each treatment (control soil without enzyme and soil with enzyme) were prepared in order to separately quantify the activity of soluble and soil-immobilized enzymes. Immobilized enzymes were assumed to be protected from denaturation and proteolysis (Burns, 1982; Sarkar et al., 1989) and expected to maintain their activity in the long-term (>ten days). Different independent subsets of soil samples were prepared to measure the activity of enzymes at different times between 20 min and 35 days of incubation. The protective role of soil on respiratory enzymes was generalized to the four other soils varying in texture and organic matter content using the G6PI as a model enzyme (S2).

2.3.2 Enzyme activity measurement

The principle of the enzymatic activity measurement was to set up a system of enzymatic reactions where the studied enzyme (red in Fig. 1) was the limiting factor (Nardi

BGD
9, 8663–8691, 2012



et al., 2007). To this end, substrates, cofactors and intermediary enzymes of the enzymatic reaction system were added in excess in soil samples (green in Fig. 1). The activity of the studied enzymes (GHK, G6PI, MDH) was quantified by measuring the formation or the consumption of NADH by spectrometry following Nardi et al. (2007), with some protocol modifications for soil conditions as opposed to leaves. In particular, concentrations of buffer and Mg^{++} solution were increased in order to better control soil pH and precipitate humic acids that could hamper the quantification of NADPH by spectrometry (both humic acids and NADPH absorb at 340 nm). For each of the three enzymes, the enzymatic reaction system and the method of enzyme activity measurement were as follows:

- *GHK (Fig. 1A)*: soil with or without GHK was amended with 300 µl of solution containing buffer (Bicine-NaOH, 100 mM, pH = 8.5), glucose (1.5 mM), $NADP^{+}$ (1.5 mM), Mg^{++} (32 mM) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49, Sigma-Aldrich ref G5885). Production of NADPH following the chemical transformation of glucose in phosphoglucono-δ-lactone was measured by spectrometry at 340 nm.

- *G6PI (Fig. 1B)*: soil with or without G6PI was amended with 300 µl of solution containing buffer (Bicine-NaOH, 100 mM, pH = 8.5), fructose-6-phosphate (1.5 mM), $NADP^{+}$ (1.5 mM), Mg^{++} (32 mM) and glucose-6-phosphate dehydrogenase (2.5 UI^{-1}). Production of NADPH following the chemical transformation of fructose-6-phosphate to phosphoglucono-δ-lactone was measured by spectrometry at 340 nm.

- *MDH (Fig. 1C)*: soil with or without MDH was amended with 300 µl of solution containing buffer (TRIS 100 mM, pH = 6.7), oxaloacetate (1.5 mM), NADH (1.5 mM) and Mg^{++} (32 mM). Consumption of NADH following the chemical transformation of oxaloacetate to L-malate was measured by spectrometry at 340 nm.

BGD
9, 8663–8691, 2012



2.3.3 Relative contribution of soluble and soil-immobilized enzymes to total enzyme activity

After 5 min of incubation with substrates, co-factors and intermediate enzymes, the set of soil samples prepared for quantifying the activity of soluble enzyme was centrifuged at 11 000 × g during 3 min. The supernatant containing the soluble enzymes and substrate was transferred to a micro-plate where the production of NADPH (for GHK and G6PI) and the consumption of NADH (for MDH) were measured by spectrophotometry (340 nm). This measurement corresponded to the activity of soluble enzymes (Soluble Enz). The set of soil samples prepared for quantifying the activity of soil-immobilized enzymes was centrifuged at different times between 5 and 45 min after incubation with substrates, co-factors and intermediate enzymes. The concentration of NADPH and NADH in the supernatant was then measured by spectrophotometry. In the latter case, production of NADPH or consumption of NADH was carried out in the presence of soluble and soil-immobilized enzymes (Total Enz). Activity of enzymes immobilized on soil particles was calculated as the difference (Total Enz – Soluble Enz). Enzyme activity with time (A_j) was expressed as % of initial activity (A_0) of the incorporated enzymatic solution.

2.3.4 Kinetic analysis of total enzyme activity

Different exponential models were tested to fit the decrease in total enzyme activity (Total Enz) with time. The best-fit model considers three pools of enzymes:

$$Y(t) = a \cdot \exp(-b \cdot t) + c \cdot \exp(-d \cdot t) + f \cdot \exp(-g \cdot t)$$

Where t is the time of incubation, a, c and f represent the sizes and b, d and g the decay rates of fast, intermediate and slow pools, respectively. The half life of enzyme pools were calculated as (ln2)/(decay rate).

BGD
9, 8663–8691, 2012



2.4 Contribution of Exomet to soil respiration

A method based on the measurement of CO_2 emissions from irradiated and non-irradiated soils (see the Sect. 2.4b) was developed to quantify Exomet and the respiration of living organisms in the five studied soils. Gamma irradiation, by killing the soil organisms, was considered to stop living respiration whilst preserving soil enzymes (S1) responsible for Exomet. Therefore, CO_2 emissions from irradiated soils were used to estimate the Exomet. However, γ-irradiation also released a large quantity of intracellular enzymes from the killed organisms thereby artificially increasing the Exomet in the irradiated-soils. Taking this effect into account, a model of carbon flux was used to quantify Exomet and living respiration.

2.4.1 Model of carbon flux

In the model, CO_2 emission from non-irradiated soil (R_{ni}) is represented by the sum of living respiration (R_l) and Exomet (R_x) (Eq. 1). After irradiation, a fraction k of R_l is converted to Exomet via the soil stabilization of respiratory enzymes released by the killed organisms. As a result, the sum of Exomet (R_x) and $k.R_l$ determines CO_2 emission from irradiated soil (R_i) (Eq. 2). The model reads as follows

$$R_{ni} = R_l + R_x \quad (1)$$

$$R_l = k \cdot R_l + R_x \quad (2)$$

By fixing the same R_x in the irradiated and non-irradiated soils, the model assumes that irradiation has no effect on the pre-existent Exomet R_x. We discuss here two examples where irradiation could modify R_x. First, the γ-irradiation by denaturing part of soil enzymes could decrease R_x. It is easy to show in this case that the Exomet contribution to soil CO_2 emissions is underestimated by the model. Nevertheless, this underestimation of Exomet is likely to be moderate since the effect of γ-irradiation on soil enzymes is typically low (see review of McNamara et al., 2003). Second, the irradiation by suppressing the microbial uptake of organic substrates could increase the availability of

BGD
9, 8663–8691, 2012



these substrates for Exomet increasing R_x. In this case, the current model would overestimate the Exomet contribution to soil CO_2 emissions. However, Exomet and living respiration are not likely to be in competition for organic substrates. Indeed, Exomet may have preferential access to organic substrate since Exomet-carrying enzymes are adsorbed on soil particles including organic matter. Moreover, most of the soil sites where Exomet can proceed are deprived of microorganisms. Indeed, enzymes responsible for Exomet may diffuse in most soil pores whereas living soil microorganisms due to their size occupy less than 0.5 % of the soil pore space (Paul and Clark, 1989).

Parameter k varies between 0–1 and depends on the fraction of respiratory enzymes released by dead organisms that is stabilized in irradiated-soils. In order to estimate k, we incubated the glucose-6-phosphate isomerase (G6PI) in the irradiated-soils of Theix, Ponta Grossa, Laqueuille, Soro and Bugac for 14 days. This enzyme was selected because it showed the lowest stabilized fraction among the three respiratory enzymes tested in the soil of Theix (Fig. 2). The total G6PI activity was measured throughout the incubation as described in the Sect. 2.3.b. For each soil, we fixed the parameter k equal to the fraction of stabilized G6PI activity assuming that the reaction sustained by the G6PI was the limiting reaction for the Exomet. Other enzymatic reactions could be more limiting for extracellular oxidative metabolism. In this case, k would have a lower value and calculated Exomet would increase. This signifies that the model underestimates the contribution of the Exomet to soil CO_2 emissions. Moreover, parameter k has a limited bearing on the calculation of Exomet. For example, for the soil of Theix, we calculated that a 50 % variation of k caused a 6 % deviation in Exomet.

2.4.2 Soil incubation experiment

Experimental microcosms consisted of 30 g (dry mass basis) samples of fresh sieved soils placed in 250 ml flask. Sets of irradiated and non-irradiated soils were prepared for the five studied soils. Soils were γ-irradiated as previously described. Soils were incubated at 30 °C and a water potential of –100 kPa for 21 days. Sufficient soil microcosms were prepared to permit four destructive harvests and four replicates per

BGD
9, 8663–8691, 2012



treatment. Microcosms were sampled after 2, 6, 13 and 21 days of incubation to determine CO_2 emissions. The sterility of irradiated-soils was verified throughout the incubation by a combination of methods including electron microscopy and molecular tracing of functional RNA-producing microorganisms (S1). The C flux model was constrained with CO_2 emissions from irradiated (R_i) and non-irradiated-soils (R_{ni}) corresponding to the incubation period 13–21 days. The 0–13 day period was excluded because soil stabilization of respiratory enzymes required several days (Fig. 2 and S3).

2.5 Some Exomet properties

Given that we consider Exomet to be carried out by soil-protected slow-cycling enzymes rather than microbe-dependent living respiration with tight physiological constraints, we predicted that Exomet would show specific properties. First, we suggest that Exomet persist in the long-term (>100 days) without microbial production of new enzymes. To test this hypothesis, the irradiated soil of Theix was incubated at 30 °C and a water potential of –100 kPa for 332 days. The CO_2 emission rate was regularly measured during the incubation period in order to calculate the Exomet half-life. Second, we suggest that Exomet resist high temperature, pressure and toxics. To test this hypothesis, we exposed the irradiated-soil of Theix to additional treatments: 150 °C for two hours, autoclaving (137 °C and 2.4×10^5 Pa for 45 min) or chloroform vapors for 24 h. All microcosms were then incubated at 30 °C and a water potential of –100 kPa for 21 days. Four replicates were prepared for each treatment. The CO_2 emission from soils exposed to temperature, pressure or toxics were compared to that of control soil (only irradiated) in order to quantify treatments effects on Exomet.

2.6 Flux measurements

In the incubation experiments where gas fluxes were studied (Sects. 2.2, 2.4 and 2.5), two sets of microcosms were prepared in order to quantify gas exchange and to determine the ^{13}C content of released CO_2. In one set of microcosms, the released CO_2

BGD
9, 8663–8691, 2012



was trapped in NaOH. The ^{13}C abundance of CO_2 was analyzed by IRMS after precipitating carbonates with an excess of $BaCl_2$. CO_2 and O_2 gas concentrations in the other set of microcosms were measured by gas spectrometry.

2.7 Data analysis

All statistical tests were performed with the Statgraphics Plus software (Manugistics, Rockville, MD, USA). General linear model (GLM) procedures, using the LSD method in post ANOVA multiple mean comparison tests, were employed to test effects of soil, treatment (irradiation, substrate and yeast-extract amendment, exposure to high temperature, autoclaving and toxics) and time factors on CO_2 emission and O_2 consumption. When model residuals did not follow a normal distribution, the variables were log-transformed. Non linear regressions were used to analyze kinetics of CO_2, O_2 concentration and enzymatic activity.

3 Results and discussion

3.1 Demonstration of Exomet by incubating a cell-free yeast-extract in sterilized water and soil

The sterility of microcosms was maintained throughout the experiment (S1). The emission of CO_2 was null in water with glucose (W + G, data not shown). Despite sterilization, soils with and without glucose (S and S + G) still emitted CO_2 throughout the incubation (Fig. 3) confirming previous observations (Peterson, 1962; Ramsay and Bawden, 1983; Lensi et al., 1991; Trevors, 1996). The supply of glucose had no effect on soil CO_2 emissions (S versus S + G). In contrast, the combined supply of glucose and cell-free yeast-extract containing respiratory enzymes triggered sudden and enormous respiration fluxes in water and soil (W + G + YE and S + G + YE, respectively). The CO_2 emission from W + G + YE and S + G + YE treatments, respectively

BGD
9, 8663–8691, 2012



represented 725 and 72 times that of control soil (after eighteen hours of incubation, Fig. 3). The released CO_2 originated from added ^{13}C labeled glucose and unlabelled substrates present in yeast-extract and soil (S4). Moreover, CO_2emissions mirrored the consumption of O_2 for all treatments and dates, with the exception of W + G + YE treatment at eighteen hours of incubation (respiratory quotient = 2, Fig. 3a). In this particular case, the availability of O_2 was reduced, metabolic activity was intense and the pressure in microcosms rose to 1.25 atm, all of which indicated the presence of fermentative metabolism.

Our results confirm the idea that the enzymatic cascade leading to the oxidation of organic carbon (i.e. glycolysis and the Krebs cycle) can occur in an extracellular context in soil and water. This indicates that respiratory enzymes can maintain their activity outside the cell and have access to substrate and co-factor flux to function. It is worth noting that the electron transfer to O_2 can also occur in an extracellular context since the emission of CO_2 was coupled to the consumption of O_2 in incubated mesocosms (Fig. 3a). This electron transfer, probably carried out by the cytochromes of cellular debris, permits the regeneration of electron acceptors (i.e. NAD + , Trevors et al., 1982) explaining the persistence of Exomet over 53 days (Fig. 3). Moreover, our results show that fermentative metabolism is another intracellular process that can be reconstituted outside the cell when the availability of O_2 for the Exomet is low.

The Exomet was significantly higher in water than in soil during the first eighteen hours of incbation (W + G + YE versus S + G + YE, Fig. 3b), which can be explained by enzyme inhibitors present in soil (Burns, 1982). However, Exomet continuously decreased with time in water whereas it stabilized in soil at a rate representing 247 % of CO_2 emissions from control soil at day 20. The higher Exomet in soil than in water after 20 days (Fig. 3b) cannot be explained by an exhaustion of C-substrate in water since the second glucose dose had no effect (S5). These results indicate that the soil particles permit Exomet persistence, possibly by preserving the respiratory enzymes from denaturation and proteolysis.

BGD
9, 8663–8691, 2012



3.2 Soil stabilization of respiratory enzymes

Kinetic analysis of total enzyme activities indicated the existence of fast-, intermediate- and slow-cycling pools of enzymes in the soil of Theix (Fig. 2, Table 2). Between 36 and 66 % of the initial enzymatic activity was lost within minutes following enzyme addition to the soil (the difference between initial activity and that at the first measurement 20 min after enzyme addition, Fig. 2). The half-life of this fast pool was too fast to be determined precisely but is <14 min (Table 2). Given the rapidity of their inactivation, enzymes of the fast-cycling pool were likely denatured by physico-chemical processes (Burns, 1982). A second pool of enzymes representing 29–58 % of the initial activity was inactivated more slowly with a half-life of 9–26 h. Soil proteolytic activity may have contributed to the degradation of this pool (Sarkar et al., 1989). Finally, 5–14 % of the initial enzymatic activity was retained in a highly stable form with a half-life of 32–495 days. Figure 2 shows that this long-term persistence of enzymatic activities exclusively relied on enzymes immobilized on soil particles (humus and minerals). These results confirm the protective role of soil particles for respiratory enzymes against physico-chemical denaturation and proteolysis. Our investigations in the other soils gave consistent results with 0.8–3.3 % of the initial enzymatic activity that was stabilized (S2).

3.3 Contribution of Exomet to soil respiration

Despite sterilization by γ-irradiation, the five studied soils released large quantities of CO_2 throughout the incubation period (Fig. 4). After 21 days of incubation, cumulated CO_2 emissions from the irradiated-soil represented 17 to 59 % of that measured in non-irradiated-soil depending on soil type (Fig. 4). The value of model parameter k was determined for the soils of Theix, Ponta Grossa and Laqueuille but not for the soils of Soro and Bugac where large release of humic acids did not permit the measurement of G6PI activity (Table 3 and S3). For the three soils where k could be determined, modeling results indicated that Exomet subtancially contributed to CO_2 emissions from

BGD
9, 8663–8691, 2012



the non-irradiated-soils (Table 3). Exomet was responsible for 16, 21 and 48 % of CO_2 emissions from the soils of Ponta Grossa, Laqueuille and Theix, respectively. These unexpected contributions of Exomet indicate the presence of a large quantity of respiratory enzymes in soils outside cells. This enzymatic pool may result from the long-term accumulation of enzymes released from dead organisms and stabilized on soil particles (Fig. 2).

3.4 Some Exomet properties

Significant CO_2 emissions were maintained throughout the 332 day incubation of irradiated-soil from Theix (Fig. 5). We calculated that the half-life of Exomet was 165 days. This result confirms the idea that Exomet can persist in the long-term without microbial production of new enzymes. This persistence reflects soil stabilization of respiratory enzymes released by ancient generations of microbial populations.

Although the treatments applied to the irradiated-soil of Theix (high temperature, pressure and chloroform) are known to denature unprotected enzymes and be lethal for most microorganisms (Koffler et al., 1957; Kashefi and Lovley, 2003; Rainey et al., 2005; Lopez-Garcia, 2007) significant CO_2 emissions persisted during the 21 day incubation period. Based on soil CO_2 emissions (Fig. 6), we estimated that 50, 20 and 10 % of Exomet were resistant to chloroform, 150 °C and autoclaving, respectively. Thus, soil particles not only protect enzymes against denaturation (e.g. Lähdesmäki and Pnspanen, 1992) but also allow the maintenance of complex oxidative metabolism in conditions where life is generally impossible.

4 Conclusion and perspectives

Our findings indicate that CO_2 emissions from soils are driven by two major oxidative metabolisms: (1) the well-established respiration of soil biota, (2) an Exomet carried out by enzymes released from dead organisms and stabilized by soil particles. These two

BGD
9, 8663–8691, 2012



metabolic pathways should be considered separately when studying effects of environmental factors on the C cycle because Exomet shows specific properties including resistance to high temperature. Further experiments are necessary to better understand the molecular mechanisms at play and predict Exomet in different soils and ecosystems. Finally, the reconstitution of intracellular metabolism outside the cell could occur in different environments (water, sediments) and may concern other metabolisms (fermentation, denitrification) since it only requires the presence of dead cells releasing intracellular enzymes.

Our results have several implications. First, the resistance of Exomet to high temperature (150 °C) indicates that the respiratory release of CO_2 from soils is more resistant to extreme temperatures than photosynthetic fixation (photosynthesis in most plants stops at temperature >40 °C). Given that the frequency of extreme temperatures is expected to increase with global warming (IPCC, 2007), these temperatures may shift the photosynthesis-respiration equilibrium towards greater ecosystem CO_2 emissions. Second, the long-term persistence of Exomet-carrying enzymes (Fig. 2 and 5) signifies that current CO_2 emissions from soils depend on past microbial activities. This memory of soils suggests a delay between the modification of microbial activities and its consequence on soil respiration. Such a delay could explain why many short-term experiments manipulating the diversity of soil microorganisms fail to demonstrate links between soil biodiversity and soil function (Griffiths et al., 2001). Thus, the functional redundancy of soil microbial species concluded from these experiments should be re-evaluated. Finally, our results support the idea that soils have played a key role in the origin of life (Bernal, 1949; Hazen, 2006). Previous studies have shown the role of soil minerals in the concentration and polymerization of amino-acids and nucleic-acids provided during the prebiotic period (Bernal, 1949). Our results show that, when all relevant organic molecules are present, complex biochemical reactions underpinning bioenergetics of life (respiration) can occur spontaneously in the soil and without any cellular organization. Thus, the first ancestral oxidative metabolism may have occurred in soil before the presence of the first cells.

BGD
9, 8663–8691, 2012



Supplementary material related to this article is available online at: http://www.biogeosciences-discuss.net/9/8663/2012/ bgd-9-8663-2012-supplement.pdf.

Acknowledgements. We thank D. Tourvieille and the "Plant and Pathogen" team of the UMR-INRA 1095 for their technical support. We thank P. Ambus, L. da Silveira Pontes, and L. Horváth who sampled the soil in Denmark, Brazil and Hungary, respectively; C. Mallet for culture analysis; S. Revaillot, O. Delfosse and J.-L. Ollier for chemical and isotopic analyses; L. Genestoux, C. Chabalier, F.-X. Sauvage, N. Duchatau for respiratory enzyme activity analyses, G. Borrel for gas spectrometry; and V. Allard, P. Barré, S. Barot, R. Pilon and our favorite native English speakerine J. Bloor for their constructive comments on previous versions of the manuscript. The research leading to these results received funding from the French Institutes INRA-INRIA (ARC-VitelBio), the French national agency ANR (DIMIMOS), le Conseil Général d'Auvergne (CPER 2007–2013) and the European Community's 6th and 7th Framework Programs (FP6 and FP7) under grant agreement n° 017841 (Nitro-Europe) and n° 226701 (CARBO-Extreme). All authors have refused to apply for the research bonus scheme set up by the French government in 2010 and have consequently no competing financial interest in this work. This work is dedicated to Waniya Shahzad and Gabin Maire.

This work originated from an idea of SF; VM, GA, ED and SF designed experiments; RD and MJ conducted soil incubations and counting of cultivable microorganisms; JC conducted electron microscopic observations of soils, ACL, RD, VM and SF performed gas spectrometry; ACL and VM performed fluorescence in situ hybridization (TSA-FISH); VM and SF conducted the incubation experiment with intracellular enzymes; GA, VP, ED and SF conducted the incubation experiment with yeast-extract; SF built the model for estimating Exomet in soils; VM, GA, TS and SF wrote the manuscript; and all authors took part in the interpretation of the results.

BGD
9, 8663–8691, 2012



References

Bernal, J. D.: The Physical Basis of Life, London, Routledge and Paul, 1951.

Boze, H., Laborde, C., Chemardin, P., Richard, F., Venturing, C., Combarnous, Y., and Moulin, G.: High-level secretory production of recombinant porcine follicle-stimulating hormone by *Pichia pastoris*, Process Biochem. 36, 907–913, 2001.

Burns, R. G.: Enzyme-activity in soil – location and a possible role in microbial ecology, Soil Biol. Biochem., 14, 423–427, 1982.

Clare, J. J., Rayment, F. B., Ballantine, S. P., Sreekrishna, K., and Romanos, M. A.: High-level expression of tetanus toxin fragment c in *Pichia pastoris* strains containing multiple tandem integrations of the gene. Bio/Tech., 9, 455–460, 1991.

Fontaine, S., Barot, S., Barre, P., Bdioui, N., Mary, B., and Rumpel, C.: Stability of organic carbon in deep soil layers controlled by fresh carbon supply, Nature, 450, 277–280, 2007.

Griffiths, B. S., Ritz, K., Wheatley, R., Kuan, H. L., Boag, B., Christensen, S., Ekelund, F., Sörensen, S. J., Muller, S., and Bloem, J.: An examination of the biodiversity-ecosystem function relationship in arable soil microbial communities, Soil Biol. Biochem., 33, 1713–1722, 2001.

Hazen, R. M.: Mineral surfaces and the prebiotic selection and organization of biomolecules, Am. Miner., 91, 1715–1729, 2006.

Heimann, M. and Reichstein, M.: Terrestrial ecosystem carbon dynamics and climate feedbacks, Nature, 451, 289–292, 2008.

IPCC: Fourth Assessment Report. Climate Change: 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, 996 pp., 2007.

Kashefi, K. and Lovley, D. R.: Extending the upper temperature limit for Life, Science, 301, 934, 2003.

Kemmitt, S. J., Lanyon, C. V., Waite, I. S., Wen, Q., Addiscott, T. M., Bird, N. R. A., O'Donnell, A. G., 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 Biol. Biochem., 40, 61–73, 2008.

BGD
9, 8663–8691, 2012



Koffler, H., Mallett, G. E., and Adye, J.: Molecular basis of biological stability to high temperatures, Proc. Natl. Acad. Sci. USA, 43, 464–477, 1957.
Krebs, H.: Introductory remarks, philosophical transactions of the Royal Society of London, B, Biological Sciences, 293, 3–4, 1981.
Lähdesmäki, P. and Pnspanen, R.: Soil enzymology: role of protective colloid systems in the preservation of exoenzyme activities in soil, Soil Biol. Biochem., 24, 1173–1177, 1992.
Lensi, R., Lescure, C., Steinberg, C., Savoie, J. M., and Faurie, G.: Dynamics of residual enzyme-activities, denitrification potential, and physicochemical properties in a gamma-sterilized soil, Soil Biol. Biochem., 23, 367–373, 1991.
Lopez-Garcia, P.: Habitability: the point of view of a biologist, in: Lectures in Astrobiology, vol. 2, edited by: Gargaud, M., Martin, H., and Claeys, P., Advances in Astrobiology and Biogeophysics, Springer-Verlag Berlin, Berlin, 221–237, 2007.
McNamara, N. P., Black, H. I. J., Beresford, N. A., and Parekh, N. R.: Effects of acute gamma irradiation on chemical, physical and biological properties of soils, Appl. Soil Ecol., 24, 117–132, 2003.
Nardi, S., Muscolo, A., Vaccaro, S., Baiano, S., Spaccini, R., and Piccolo, A.: Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and krebs cycle in maize seedlings, Soil Biol. Biochem., 39, 3138–3146, 2007.
Paul, E. A. and Clark, F. E.: Soil Microbiology and Biochemistry, Academic Press Inc., San Diego, CA., USA., 273 pp., 1989.
Peterson, G. H.: Respiration of soil sterilized by ionizing radiations, Soil Science, 94, 71–74, 1962.
Rainey, F. A., Ray, K., Ferreira, M., Gatz, B. Z., Nobre, M. F., Bagaley, D., Rash, B. A., Park, M. J., Earl, A. M., Shank, N. C., Small, A. M., Henk, M. C., Battista, J. R., Kampfer, P., and da Costa, M. S.: Extensive diversity of ionizing-radiation-resistant bacteria recovered from Sonoran Desert soil and description of nine new species of the genus *Deinococcus* obtained from a single soil sample, Appl. Environ. Microbiol., 71, 5225–5235, 2005.
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 Biol. Biochem., 15, 263–268, 1983.
Rich, P. R.: The molecular machinery of Keilin's respiratory chain, Biochem. Soc. Trans., 31, 1095–1105, 2003.

BGD
9, 8663–8691, 2012



Sarkar, J. M., Leonowicz, A., and Bollag, J.-M.: Immobilization of enzymes on clays and soils, Soil Biol. Biochem., 21, 223–230, 1989.
Trevors, J. T., Mayfield, C. I., and Inniss, W. E.: Measurement of electron-transport system (Ets) activity in soil, Microb. Ecol., 8, 163–168, 1982.
Trevors, J. T.: Sterilization and inhibition of microbial activity in soil, J. Microbiol. Methods, 26, 53–59, 1996.
Vanderheiden, G. J., Fairchild, A. C., and Jago, G. R.: Construction of a laboratory press for use with the French pressure cell, Appl. Microbiol. 19, 875–877, 1970.

BGD
9, 8663–8691, 2012



Table 1. Site characteristics and soil properties.

	Sites				
	Theix	Laqueuille	Soro	Ponta Grossa	Bugac
Country	France	France	Denmark	Brazil	Hungary
Land use	Grassland	Grassland	Beech forest	Crops	Grassland
Clay/Silt/Sand (%)	26/25/49	19/55/26	10/22/68	14/6/80	10/5/85
pH (–)	6.2	5.3	4.3	7.0	8.6
OM content ($g C kg^{-1}$)	39.0	121.9	33.7	14.8	44.0
CEC ($C_{mol} kg^{-1}$)	21.5	25.1	9.5	3.9	10.2

BGD
9, 8663–8691, 2012



Table 2. Size and half-time of fast, intermediate and slow pools of respiratory enzymes. GHK: glucose hexokinase, G6PI: glucose-6-phosphate isomerase, MDH: malate dehydrogenase. Pool size and half-time have been obtained by fitting the kinetic model presented in Sect. 2.3 to total enzyme activity (Fig. 2).

	Fast pool		Intermediate pool		Slow pool	
Enzyme	Size (%)	Half-life (min)	Size (%)	Half-life (h)	Size (%)	Half-life (days)
GHK	35.8	<14	57.6	8.9	6.6	43.3
G6PI	66.4	<14	28.8	26.4	4.8	31.5
MDH	38.3	<14	47.6	17.1	14.1	495.1

BGD
9, 8663–8691, 2012



Table 3. Activity of glucose-6-phosphate isomerase (G6PI) and model quantification of CO_2 emissions from living organisms (R_l) and extracellular oxidative metabolism (R_x) for the five studied soils. R_{ni} and R_i represent CO_2 emissions from non-irradiated and irradiated soil respectively, for the incubation period 13–21 days. k is the fraction of R_l converted in extracellular oxidative metabolism (Exomet) after irradiation. Contribution (%) of Exomet to soil CO_2 emission was calculated as $R_x/R_{ni} \cdot 100$.

	Sites				
G6PI activity (% initial activity)	Theix	Laqueuille	Soro	Ponta Grossa	Bugac
7 days	10.5 ± 0.9	0.5 ± 0.05	nd	5.1 ± 0.4	nd
14 days	10.0 ± 0.4	0.3 ± 0.03	nd	3.1 ± 0.1	nd
Model quantification of the two metabolic pathways of organic C oxidation					
Model inputs					
R_{ni} ($mgC - CO_2 kg^{-1}$)	56.4 ± 0.5	186.4 ± 3.3	200.2 ± 1.5	162.8 ± 0.9	168.8 ± 2.6
R_i ($mgC - CO_2 kg^{-1}$)	30.2 ± 0.9	40.3 ± 1.5	34.6 ± 0.9	30.6 ± 0.8	98.2 ± 3.2
k (%)	10.0 ± 0.4	0.3 ± 0.03	nd	3.1 ± 0.1	nd
Model results					
R_l ($mgC - CO_2 kg^{-1}$)	29.1	146.5	nd	136.4	nd
R_x ($mgC - CO_2 kg^{-1}$)	27.3	39.9	nd	26.4	nd
Contribution of the extracellular oxidative metabolism to CO_2 emission (%)	48.4	21.3	nd	16.2	nd

BGD
9, 8663–8691, 2012



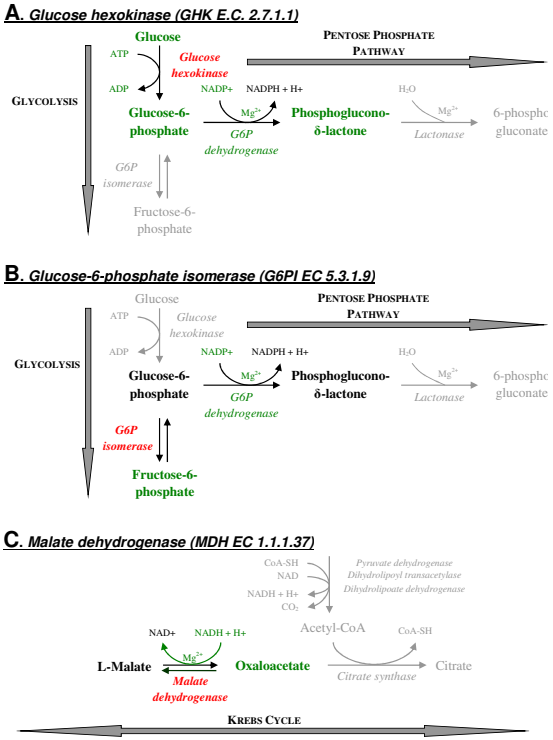


Fig. 1. Enzymatic reactions mediated by the two enzymes involved in glycolysis (A. GHK: glucose hexokinase, B. G6PI: glucose-6-phosphate isomerase) and the enzyme involved in the Krebs cycle (C. MDH: malate dehydrogenase) used to quantify the protective role of soil particles on respiratory enzymes. The principle of the enzymatic activity measurement was to set up a system of enzymatic reactions where the studied enzyme (red) was the limiting factor. For doing this, we added all substrates, cofactors and intermediary enzymes in excess (green). The activity of the studied enzyme (GHK, G6PI, MDH) was quantified by measuring the formation or the consumption of NADH by spectrometry. Reactions surrounding the studied enzymatic (grey) are given for information.

BGD
9, 8663–8691, 2012



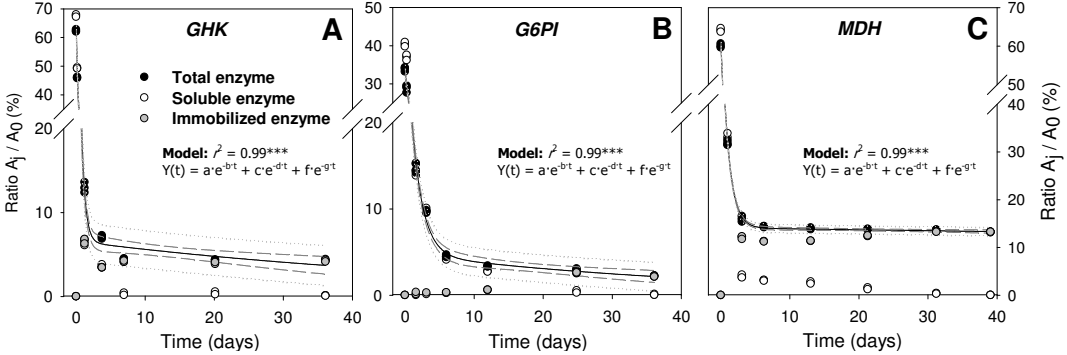


Fig. 2. Activity of three specific enzymes involved in glycolysis and the Krebs cycle following their incorporation in non-irradiated-soil of Theix. Enzyme activity along time (A_j) is expressed as % of the initial activity of enzymatic solution (A_0) applied to the soil. The activities of total (dark circles), soluble (white circles) and immobilized enzymes (grey circles) are distinguished. Full, dashed and dotted lines represent the fit of the kinetic model on total enzyme activity, its confidence and predictive error intervals at 5 % *P*-level, respectively. **(A)** G6PI: glucose-6-phosphate isomerase; **(B)** GHK: glucose hexokinase; **(C)** MDH: malate dehydrogenase.

BGD
9, 8663–8691, 2012



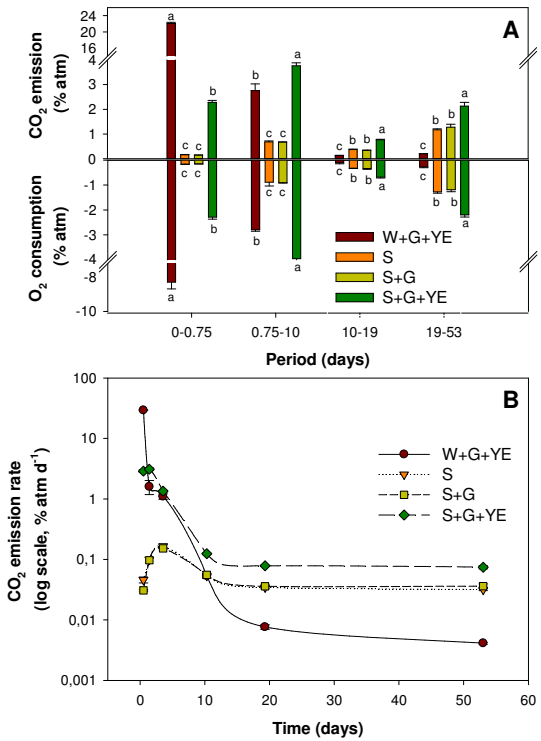


Fig. 3. (A) Total (labeled + unlabelled) CO_2 emission and O_2 consumption from irradiated-soil (S); irradiated-soil + labeled glucose (S + G); irradiated-soil + labeled glucose + yeast-extract (S + G + YE); water + labeled glucose + yeast-extract (W + G + YE) for four periods of incubation. Results are expressed as % of microcosm atmosphere. Water with labeled glucose was also incubated as control, but CO_2 emission from this control was null and not reported. Letters indicate at each sampling date the ANOVA-based differences at 5 % *P*-level among treatments. **(B)** Total (labeled + unlabelled) CO_2 emission rate from water and soil microcosms. Symbols are same to panel A.

BGD
9, 8663–8691, 2012



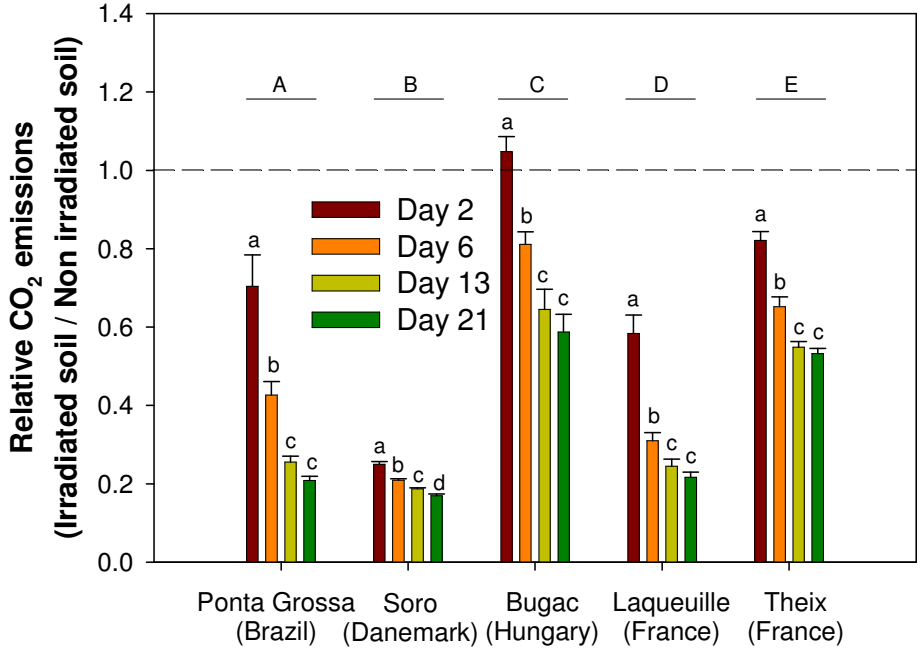


Fig. 4. Relative CO_2 emission between irradiated and non-irradiated soil for the five studied soils. Day 2, Day 6, Day 13 and Day 21 represent days of incubation. Letters indicate the differences at 5 % level between measurements over time (lower case) and between soils (upper case) based on repeated-measures analysis of variance.

BGD
9, 8663–8691, 2012



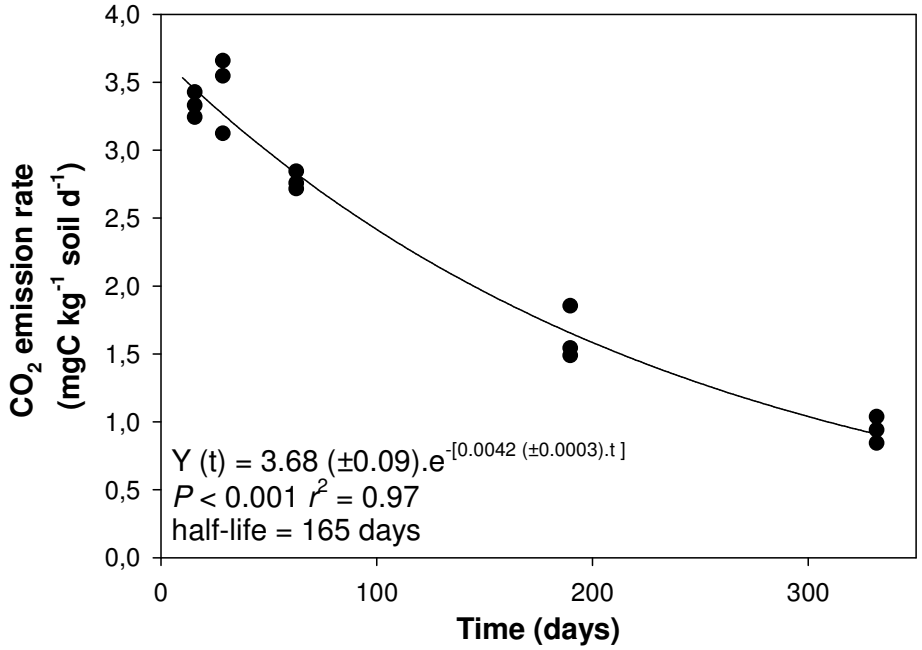


Fig. 5. CO_2 emission rate from irradiated-soil of Theix incubated during 238 days. Half-life ($t_{1/2}$) was calculated from a simple exponential model.

BGD
9, 8663–8691, 2012



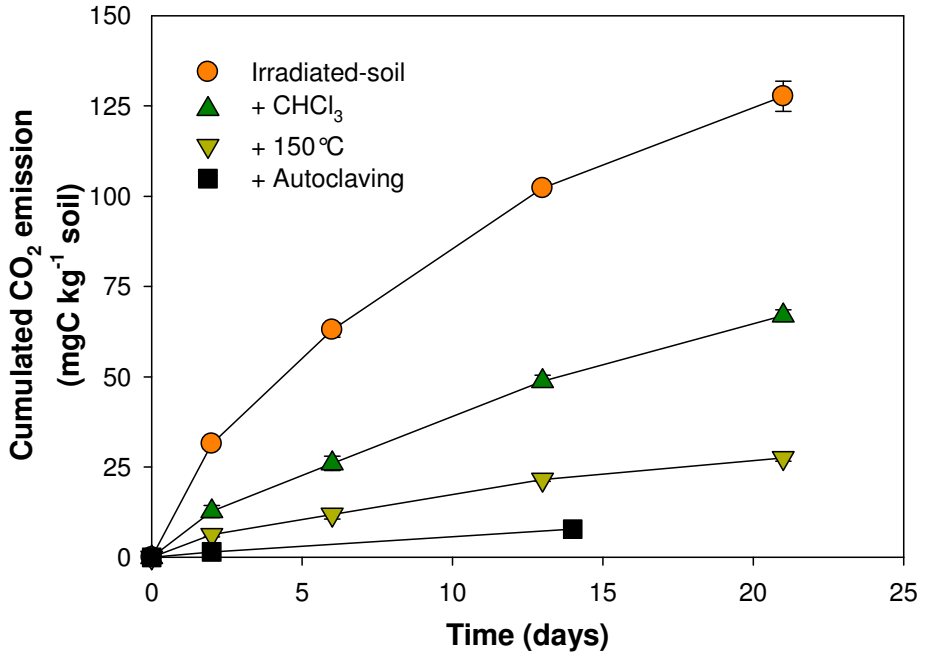


Fig. 6. Cumulated CO_2 emission from soil of Theix after exposure to irradiation (Irradiated-soil), irradiation and 150 °C (+150 °C), irradiation and chloroform fumigation (+$CHCl_3$), irradiation and autoclaving (+Autoclaving).

BGD
9, 8663–8691, 2012

