Supporting Online Material for

An unknown respiration pathway substantially contributes to soil CO_2 emissions.

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SUPPLEMENT 1: STERILITY OF SOILS, YEAST EXTRACT AND MICROCOSMS.

Efficiency of γ-irradiation to kill soil organisms and maintenance of microcosm sterility were analyzed in detail during the incubation of soil from Theix for 21 days (see the soil incubation experiment described in section 2.4 of the main text for details). This analysis was based on combination of complementary microbiological and molecular methods, simulation of contamination and determination of method sensitivity. Results of this detailed analysis are presented below in the "Main investigation" section. After efficiency of γ-irradiation and maintenance of microcosm sterility were verified, we used a simplified approach to control the absence of contamination for other incubation experiments (Incubation of the four other studied soils, Yeast-extract incubation described in section 2.2 of the main text). Results of these investigations are presented in the "Complementary investigations" section. The last section of this supplementary information on sterility presents methods and sensibility analysis of TSA FISH.

A. MAIN INVESTIGATION

1. Description of experiments

Several preliminary tests comparing various methods of sterilization (γ-irradiation, heating and autoclaving) were carried out (data not shown) before selecting the method presented here. The γ-irradiation was chosen as sterilizing method for its efficiency to kill soil micro-organisms and for its moderate effect on soil enzymes. Preservation of soil enzymes was particularly important to quantify EXOMET. Dose of γ-irradiation used in our experiments to sterilize soils was 45 kGy. Typically, γ-irradiation at 20 kGy eliminates all cultivable bacteria, actinomycetes and fungi (McNamara et al., 2003).

Here we present results of thorough investigations on efficiency of γ-irradiation to kill soil micro-organisms and maintenance of sterility of microcosms throughout the incubation period. These investigations were carried out on irradiated and non-irradiated soils from Theix incubated for 21 days. Four complementary methods were applied to the soils: i) Microscopic observations and enumeration of morphological intact cells by transmission electron microscopy; ii) quantification of microbial biomass; iii) search for the presence of γ-resistant cultivable microorganisms on three culture media (*e.g. Deinococcus radiodurans*, Warcup, 1950); and iv) count of functional RNA-producing microorganisms in soils by using fluorescence *in-situ* hybridization (TSA FISH). Moreover, we developed two complementary

approaches to quantify the power of our methods to detect a possible contamination of microcosms. First, the minimum density of living microorganisms detectable by TSA-FISH method was determined in soil. To understand significance of undetectable cells for soil CO_2 emission, we calculated the potential contribution of undetectable cells to CO_2 emission from irradiated-soil by using specific respiration of microorganisms. Second, we experimentally simulated a contamination of irradiated-soil by inoculating it with diluted soil inoculum. This inoculum was prepared from the non-irradiated soil of Theix. The microbial biomass resulting due to inoculation represented 1/1900 of the microbial biomass present in the non-irradiated-soil.

2. Results

Irradiation strongly reduced microbial biomass, from 933 in non-irradiated-soil to 247 mg C kg^{-1} in irradiated-soil (Fig. S1-1A). Transmission electron microscopy showed that irradiation also caused many morphological damages to cells such as membrane disruption and loss of cell turgescence (Fig. S1-1E). Morphologically intact cells were 16 times lower (Fig S1-1D) whereas respiration rate was only 1.25 times lower in irradiated-soil as compared to non-irradiated soil (incubation period 0-2 days, Fig S1-2). Our results also showed that remaining cells were not viable or functional. Indeed, microbial biomass continuously decreased during the incubation period reaching undetectable value at day 13 (Fig. S1-1A) indicating that microbial biomass was lyzed. Furthermore, no micro-organism from any culture media was detected, confirming the absence of cultivable micro-organisms in irradiated-soil (see section B "Complementary investigations"). Finally, functional RNA-producing cells were not detected in irradiated-soil irrespective of sampling date whereas $5.0x10^9$ RNA-producing cells g^{-1} soil were found in non-irradiated living soil (Fig. S1-1B). This showed that cells remaining after irradiation did not provide RNA and hence respiratory enzymes anymore. Of course, we cannot exclude the possibility that a few RNA-producing cells remained in irradiated-soil and were undetected by our methods. However, based on the specific respiratory activity of soil micro-organisms calculated in the non-irradiated soil (respiration per unit cell, Fig S1-1B and S1-2 incubation period 0-2 days), respiration measured in irradiated-soil corresponded at least to an activity of 4.10^9 cells g^{-1} soil. Given that TSA-FISH method was able to detect the presence of as low as 10^5 cells g^{-1} soil (see section C "Methods"), we quantified that the potential contribution of undetectable cells to CO_2 emission from irradiated-soil cannot exceed 1/10,000. Consistently, the simulated contamination of irradiated-soil was immediately detected by the TSA-FISH (Fig. S1-1B). This contamination was followed by a

flush of respiration and growth of microorganisms using the soluble C released by the killed biomass (Figs. S1-1A-C, Fig. S1-2). The growth of microbial contaminants was detected by all methods (microbial biomass, culture, TSA-FISH).

3. Conclusions.

Collectively, these results indicate that the γ-irradiation at 45 kGy is highly efficient to kill soil organisms including the non cultivable micro-organisms. Although some cells can be observed by electron microscopy in the irradiated-soil, they cannot explain the important CO_2 emissions measured in the irradiated-soil. Moreover, these cells are not viable and functional since they cannot grow or produce the RNA required to synthesize proteins and enzymes. We conclude that the γ-irradiation is an efficient method to stop the living respiration and quantify the EXOMET using the model presented in the main text.

The results also indicate that microcosms could be maintained without microbial contamination throughout the incubation period. This signifies that methods developed to maintain microcosms under sterile conditions (manipulation under sterile hood, ventilation of incubated microcosms with filtered air, etc) were efficient. Finally, in case of microcosm contamination (simulated), presence of micro-organisms was immediately detected by the TSA-FISH. Moreover, due to presence of large quantities of labile C in irradiated-soils (released from the killed boil biomass), growth of microbial contaminants was rapid and detected by all methods (microbial biomass, microscopy, TSA-FISH, culture, measurement of flush of CO_2 production).

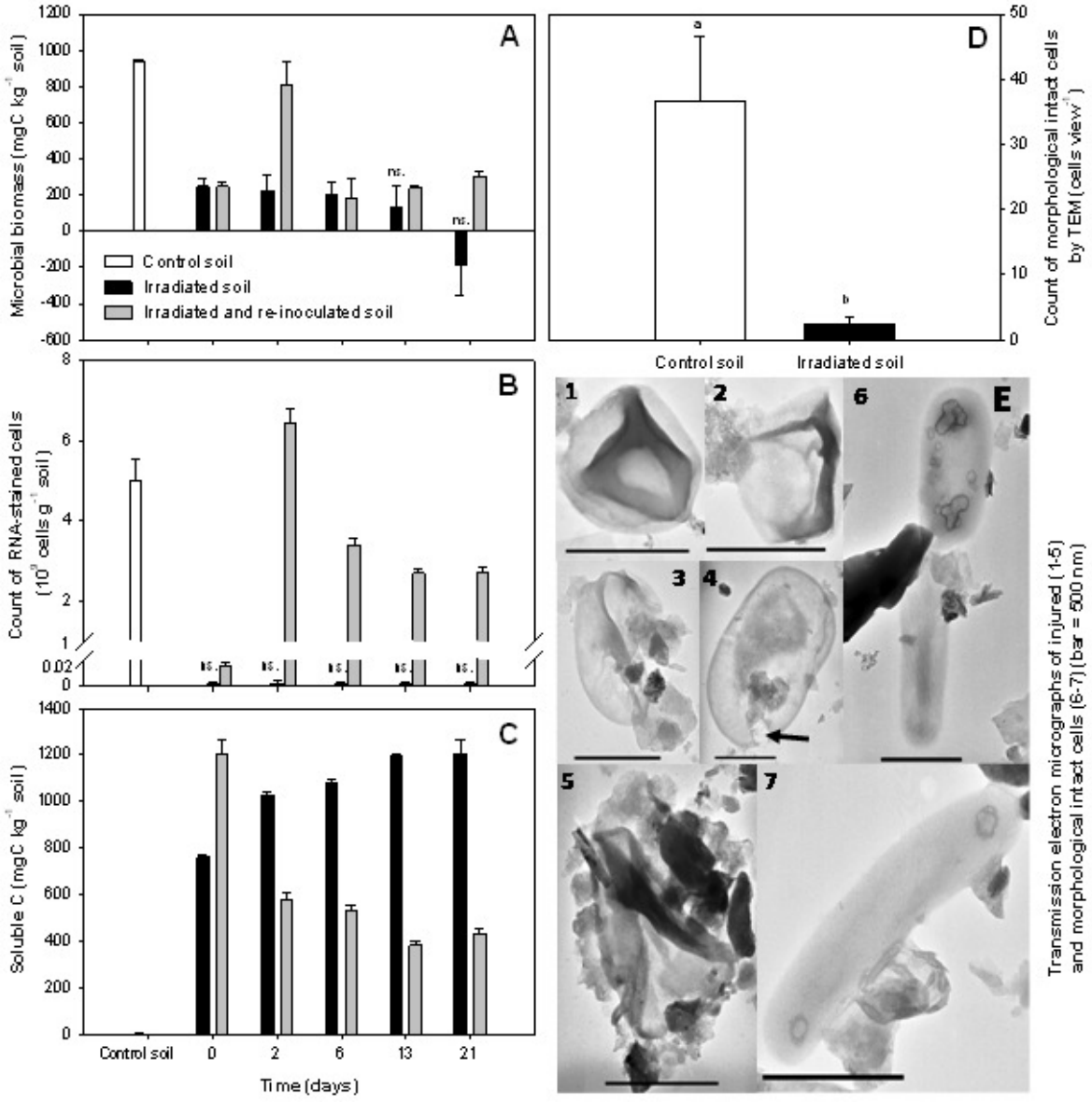


Figure S1-1: Dynamics of (A) microbial biomass, (B) RNA-stained cells and (C) Soluble C in irradiated-soil, irradiated-and-re-inoculated-soil of Theix incubated during 21 days. The non-irradiated soil at day 0 was used as control. Differences from zero of different variables were tested with t-test at 5% *P*-level. ns: non significant. (D) Count of morphological intact cells by transmission electron microscopy in irradiated and control (non-irradiated) soil of Theix. Differences between control and irradiated soil were tested with t-tests at 5% *P*-level. Number of cells in the irradiated-soil was not significantly different from zero (t-test, 5% *P*-level). (E) Transmission electron micrographs of injured (graphs 1 to 5) and morphological intact cells (graphs 6 to 7). The arrow on graph 4 indicates a membrane disruption. The scale black bar corresponds to 500 nm length.

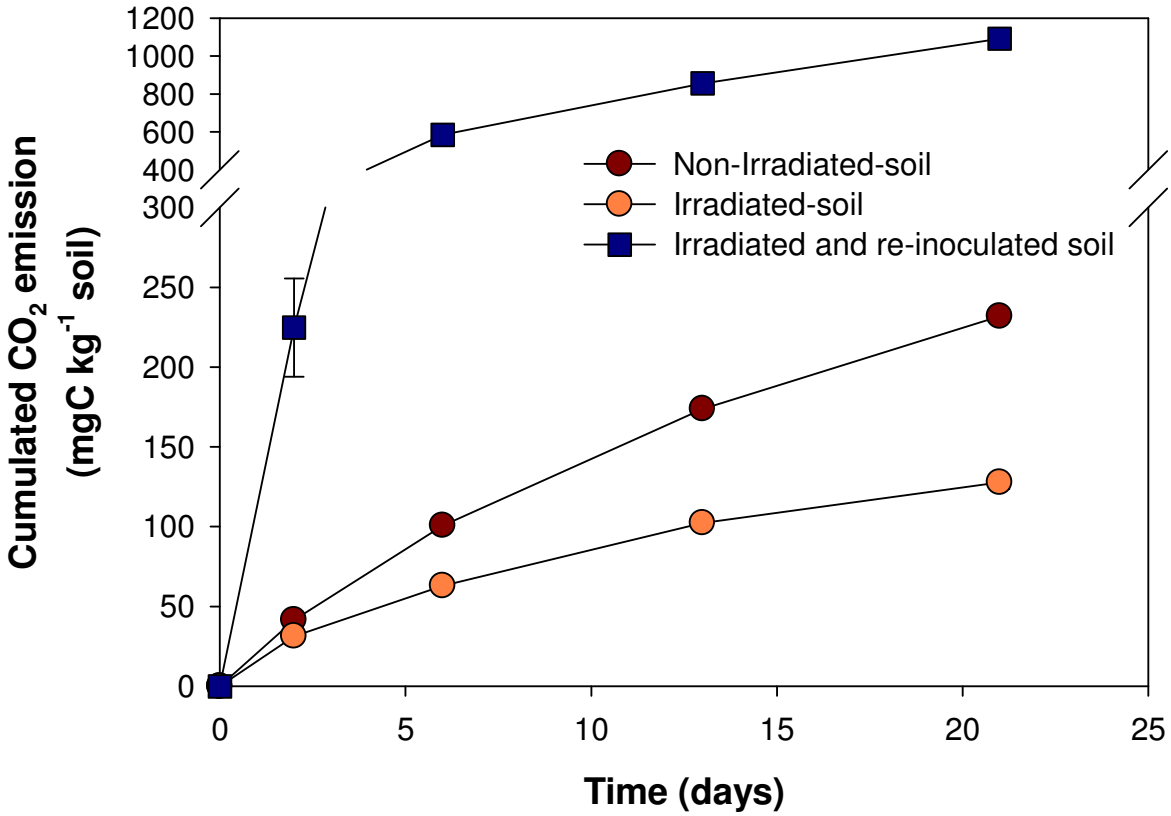


Figure S1-2: Cumulated CO_2 emission from non-irradiated-soil, irradiated-soil, irradiated-and-re-inoculated-soil incubated during 21 days.

B. COMPLEMENTARY INVESTIGATIONS

Soil incubation experiment. The absence of living micro-organisms in the five studied soils was verified after irradiation (45Gy) and at the end of incubation (21 days). This verification has been carried out through cultural method using three media. No micro-organism from any culture media was detected indicating that all soil microcosms were maintained under sterile conditions (Table S1-1).

Table S1-1: Counting of γ-resistant cultivable microbes in five studied soils after exposition to γ-irradiation (45kGy) and 21 days of incubation.

	Soil names				
Counting after irradiation	**Theix**	**Laqueuille**	**Soro**	**Ponta Grossa**	**Bugac**
Cultivable bacteria	0	0	0	0	0
Cultivable fungi	0	0	0	0	0
D. radiodurans	0	0	0	0	0

Counting after 21 days of incubation					
Cultivable bacteria	0	0	0	0	0
Cultivable fungi	0	0	0	0	0
D. radiodurans	0	0	0	0	0

Yeast-extract incubation. Filtered (0.022 µm) yeast-extract was observed by fluorescent microscopy (x 630, cells were stained by DAPI) to check the absence of cells. The irradiated-soil of Theix used in this experiment was the same that was used for conducting the in-depth investigations on sterility presented in the above section "Main investigation". The absence of contamination during the yeast-extract experiment was verified at days 13 and 53 of the incubation. To this end, aliquots of liquid were sampled in water+glucose+yeast-extract treatment (W+G+YE) and were analyzed by TSA-FISH.

Microscopic observations of filtered yeast-extract confirmed the absence of cells. It was interesting to note the presence of clusters of cellular debris like pieces of membrane. No RNA-producing cells were detected by TSA-FISH confirming that microcosms were maintained under sterile conditions throughout the incubation period (Table S1-2).

Table S1-2: Counting of RNA-stained cells in W+G+YE treatment by TSA-FISH method after 13 and 53 days of incubation.

Incubation day	Count of RNA-stained cells
13	0
53	0

C. METHODS

***Microbial biomass*.** Microbial biomass was determined by the fumigation-extraction technique (Vance et al., 1987).

Transmission electron microscopy. Cells were extracted from soils as recommended by Danovaro *et al*. (2001) with some modifications as described below. An aliquot of 0.1g soil was diluted in 5 ml of sodium pyrophosphate buffer (PPI, 0.01 M). Solution was shaken for 30 min and sonicated three times for 1 min each in a water bath (Bioblock Scientific 88156, 320W). Larger particles were removed by centrifugation at 2000 x g for 1 min and cells contained in the supernatant were fixed with glutaraldehyde (1 % final concentration).

Cells were harvested by centrifuging 200µl of soil extract with 6 ml of sterile water at 35 000 x g for 20 min onto 400 mesh Cu grids (Pelanne Instruments, Toulouse, France). Cells were then stained for 30 s with uranyl acetate (2% wt/wt). Microscopic observations and

enumeration of morphological intact cells were performed at a magnification of 30,000 to 40,000 x using the JEM 1200 EX TEM (JEOL) operated at 80 kV.

Cultivable microorganisms. Gamma-resistant cultivable microorganisms were counted on three different mediums: i) malt medium (10g L^{-1} Malt extract, 14g L^{-1} Agar) for fungi; ii) lysogeny broth medium (25 gLB L^{-1} Broth) for bacteria; iii) TGY medium (10g L^{-1} Tryptone, 1g L^{-1} Glucose, 5g L^{-1} baker Yeast) for *Deinococcus radiodurans*. Isolation plates were prepared by transferring a small amount of soil (±250mg) into a sterilized Petri dish (Warcup, 1950). Ten repetitions per treatment and soil combination were incubated for one month.

TSA-FISH. Functional RNA-producing microorganisms were enumerated using fluorescence *in-situ* hybridization combined to tyramide signal amplification (TSA-FISH). One gram soil sample was extracted in 100 mL of sodium pyrophosphate buffer (PPI, 0.01M) according to protocol described in Battin *et al.* (2001). TSA–FISH was performed as described in Biegala *et al.* (2003) and Mary *et al.* (2006) with some modifications as described below. Soil extract sample was fixed with 1% formaldehyde and harvested on 0.2µm (pore-size) polycarbonate filters. Filters were cut in three parts forming pseudo-replications for each sample. They were then dehydrated in ethanol series (50, 80, 100%, 3 min each) and dried. Before hybridization, prokaryotic cells were partially lysed by placing the filters for 1h at 37°C in 1ml of 100 µg mL^{-1} lysozyme (47000 U mg^{-1}, Sigma-Aldrich). Enzymatic reaction was stopped by rinsing the filter 3 times in 5mL of sterile water for 1min. Filters were then dehydrated in a second ethanol series (50, 80, 100%, 3 min each) and dried. Hybridizations were performed with the probes EUB338 according to hybridization conditions described by Mary *et al.* (2006). Prior to the TSA reaction, cells were equilibrated in 5mL of TNT buffer at room temperature for 15min. For the TSA reaction, 20µL of TSA mix (40% [wt/vol] dextran sulfate stock solution, mixed 1:1 with the amplification diluent of the TSA-Direct kit [NEN Life Science Products Inc., Boston, Mass.], and added [50:1] to fluorescein-tyramide [TSA-Direct kit]) was added, and the mixture was left to incubate for 30min at room temperature in the dark. The unlabeled fluorochrome was then washed out by two subsequent rinses, of 20min in 5mL of TNT buffer prewarmed at 55°C followed by one rinse of 10min in PBS buffer at room temperature.

The filter sections were labeled with 20µL of a mix of 800µL Citifluor, 200µL vectaShield and 1µL of DAPI (1 mg mL^{-1}). The filter sections were inspected and cells were counted under a Leica DMIRB epifluorescence microscope equipped with excitation / emission filters 360/420 nm for DAPI and 490/515 nm for FITC.

Sensitivity of the TSA-FISH. Sensitivity of TSA-FISH method was determined in two steps. After extraction of microbial biomass from non-irradiated-soil of Theix with 100 mL of

sodium pyrophosphate buffer (PPI, 0.01M), soil extract was diluted serially from 10^{-1} to 10^{-8}. Microbial biomass from all soil extracts was stained through TSA-FISH and number of RNA-stained cells was counted. This counting provided first estimation of cell density threshold from which TSA-FISH method was able to detect presence of living cells. However, this estimation was made on diluted living soil extracts where cell/soil-particle ratio was always identical. In contrast, irradiation caused a decrease in cell/soil-particle ratio of irradiated-soil. Therefore, we set up a second experiment in order to determine effect of soil particles on the quantification of cell density. Irradiated-soil of Theix was re-inoculated with diluted soil inoculum prepared in the previous step to obtain 10^5 cell g^{-1} soil. Immediately after soil inoculation, cell density in irradiated-reinoculated soil was quantified by TSA-FISH. If measured density was lower than prepared density (10^5 cell g^{-1}), then soil particles had detrimental effect on the quantification of cell density in the soil. Three replicates per treatment were made.

The first experiment showed that the TSA-FISH method was able to significantly detect presence of living cells from 10^3 cells mL^{-1} (10^{-7} level of dilution, Fig. S1-3A). This number of cells in soil extract corresponded to 10^5 cells g^{-1} soil. In the second experiment, measured cell density matched experimentally prepared cell density (1.6 *vs* 1.7 10^5 cells g^{-1} soil, respectively; Fig S1-3B), indicating that quantification of cell density in soil was not affected by soil particles. We conclude that TSA-FISH applied to our soil was able to detect 10^5 cells g^{-1} soil.

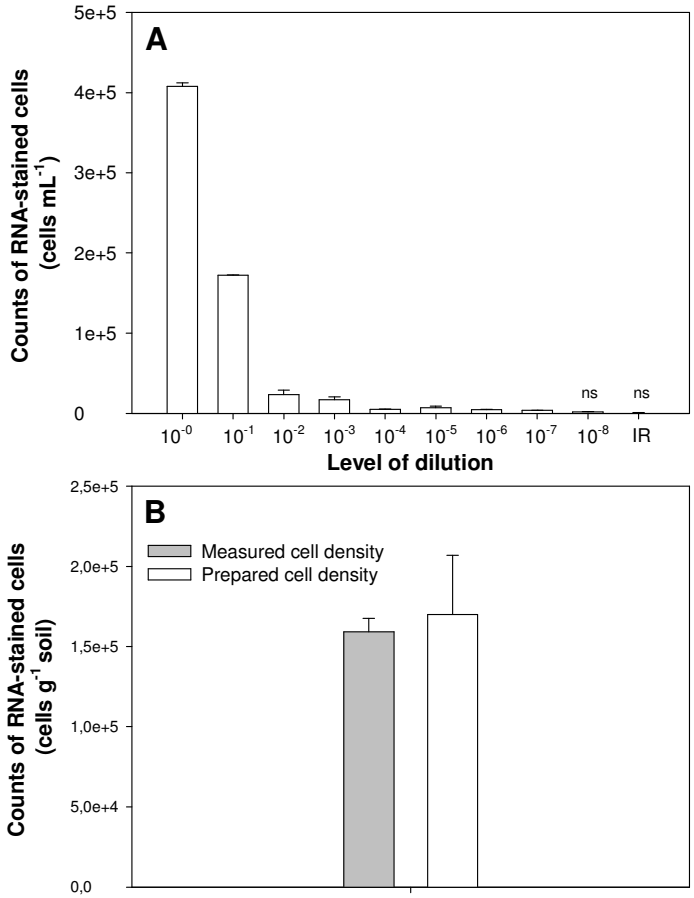


Figure S1-3: A) Count of RNA-stained cells in pure and diluted soil extracts and B) Prepared *vs* measured cell density in the irradiated-re-inoculated-soil. Differences from zero were tested with t-test at 5% *P*-level (ns: non significant).

SUPPLEMENT 2: GENERALIZATION OF RESPIRATORY ENZYME STABILIZATION IN THE FOUR OTHER STUDIED SOILS

The incubation of three enzymes involved in glycolysis (GHK: glucose hexokinase, G6PI: glucose-6-phosphate isomerase) and the Krebs cycle (MDH: malate dehydrogenase) in non-irradiated-soil of Theix showed that 5-14% of initial enzymatic activity was retained in a highly stable form. Here we tested whether the protective role of soil on respiratory enzymes can occur in other soil types. To this end, we incubated G6PI in non-irradiated soil from four other studied sites (Ponta Grossa, Laqueuille, Bugac and Soro) for 13 days. This enzyme was selected because it showed the lowest stabilization rate (Table 2 of the main text). However, spectrometric measurement of G6PI activity at pH=8.5 was not possible in soil from Soro. This soil released huge quantity of humic acids at pH 8.5 hampering measurement of NADPH since humic acids and NADPH both absorbs at 340 nm. To demonstrate the ability of soil from Soro to stabilize respiratory enzymes, G6PI was replaced by MDH. MDH was measured at pH = 6.7, which limited solubilization of humic acids and allowed measurement of NADH concentration.

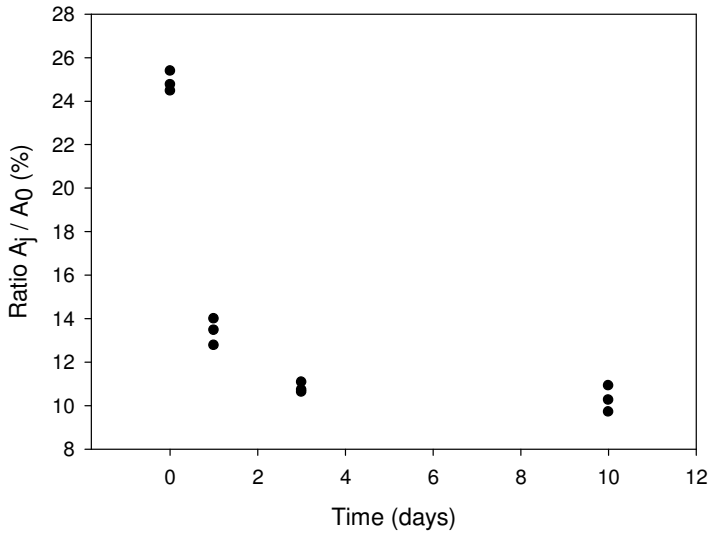
Activity of G6PI in the soils of Ponta Grossa, Laqueuille and Bugac decreased strongly after one hour of incubation and then moderately till day 6 of incubation (Table S2-1). From day 6 to the end of incubation, activity of G6PI was constant indicating that remaining enzymes were retained in a stable pool. These results are consistent with the fast and intermediate cycling pools of enzymes identified in soil of Theix (Table 2 of the main text). Percentage of enzymatic activity stabilized in soil depended on soil type and represented 3.3% for Ponta Grossa, 0.8% for Laqueuille and 2.5% for Bugac (Table S2-1). Activity of MDH in soil from Soro followed the same kinetics as for G6PI but it stabilized at higher percentage (±27%). Collectively, these results indicate that the soil stabilization of respiratory enzymes is common in soils.

Table S2-1: Activity of glucose-6-phosphate isomerase (G6PI) and malate dehydrogenase (MDH) after their addition in the non-irradiated-soils of Ponta Grossa, Laqueuille, Bugac and Soro. Enzyme activity with time is expressed as % of the initial activity of enzymatic solution applied to the soil.

	G6PI			**MDH**
Incubation time	Ponta Grossa	Laqueuille	Bugac	Soro
1 hour	7.85 ±0.96	1.49 ±0.18	10.4 ±0.2	42.4 ±6.2
6 days	3.51 ±0.36	0.81 ±0.06	2.31 ±0.36	28.2 ±2.4
13 days	3.32 ±0.07	0.76 ±0.05	2.52 ±0.16	26.9 ±1.8

SUPPLEMENT 3: KINETIC ACTIVITY OF G6PI IN THE IRRADIATED-SOIL OF THEIX.

Figure S3-1: Activity of glucose-6-phosphate isomerase (G6PI) following its addition in the irradiated-soil of Theix. Enzyme activity with time is expressed as % of the initial activity of enzymatic solution applied to the soil.



SUPPLEMENT 4: UNLABELED AND LABELED CARBON RELEASED AS CO_2 IN WATER AND SOIL MICROCOSMS.

The ^{13}C labeling of glucose allowed separating mineralization of added glucose from that of unlabelled substrates present in yeast extract and soil. Fig S4-1 shows that labeled glucose (G) was oxidized in CO_2 in water (W) and soil (S) microcosms with yeast extract (YE) (W+G+YE and S+G+YE treatments, respectively). Moreover, labeled CO_2 emission was greater in S+G+YE than in S+G treatment. These results indicate that an equivalent of glycolysis and the Krebs cycle was reconstituted by enzymes released from dead yeast cells in water and soil. It is interesting to note that emitted CO_2 was equally composed of labeled and unlabelled carbon in water microcosms whereas it was dominated by unlabelled carbon in soil microcosms. This dominance of unlabelled CO_2 in soil microcosms probably reflected dominance of unlabelled substrates over labeled glucose since irradiation released huge quantities of soluble C from killed biomass (Fig. S1-1C). Moreover, these unlabelled substrates released from killed biomass contained many intermediary compounds that could be decarboxylated faster than glucose. These intermediary compounds may also inhibit glucose oxidation since many enzymatic reactions involved in glycolysis and the Krebs cycle are inhibited by presence of reaction products.

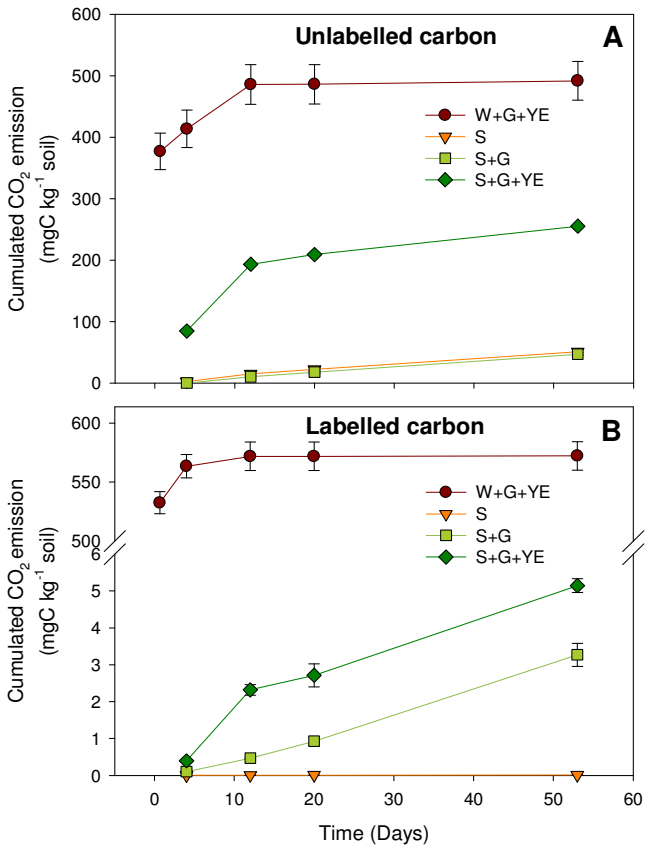


Figure S4-1: Cumulated emissions of (A) unlabelled CO_2 and (B) labelled CO_2 from water and soil microcosms. Symbols are labeled as follows: S, irradiated-soil; S+G, irradiated-soil + labeled glucose; S+G+YE, irradiated-soil + labeled glucose + yeast extract; W+G+YE, water + labeled glucose + yeast extract.

SUPPLEMENT 5: EFFECT OF THE SECOND GLUCOSE DOSE ON MICROCOSM RESPIRATION.

To determine the long-term (>20 days) metabolic capabilities of enzymes from yeast-extract in water and soil (W+G+YE and S+G+YE treatments), two sets of microcosms were prepared. First set received glucose application at the beginning of experiment (results from this set are presented in the main text of manuscript) whereas second set received glucose after 20 days of incubation. Emission of CO_2 from the two sets of microcosms were similar (Fig S5-1) indicating that respiratory activity in microcosms after 20 days was limited by activity of yeast-extract enzymes and not by availability of C-substrate. The higher respiratory activity in S+G+YE than in W+G+YE treatment illustrated the role of soil particles in the stabilization of enzymes and the EXOMET.

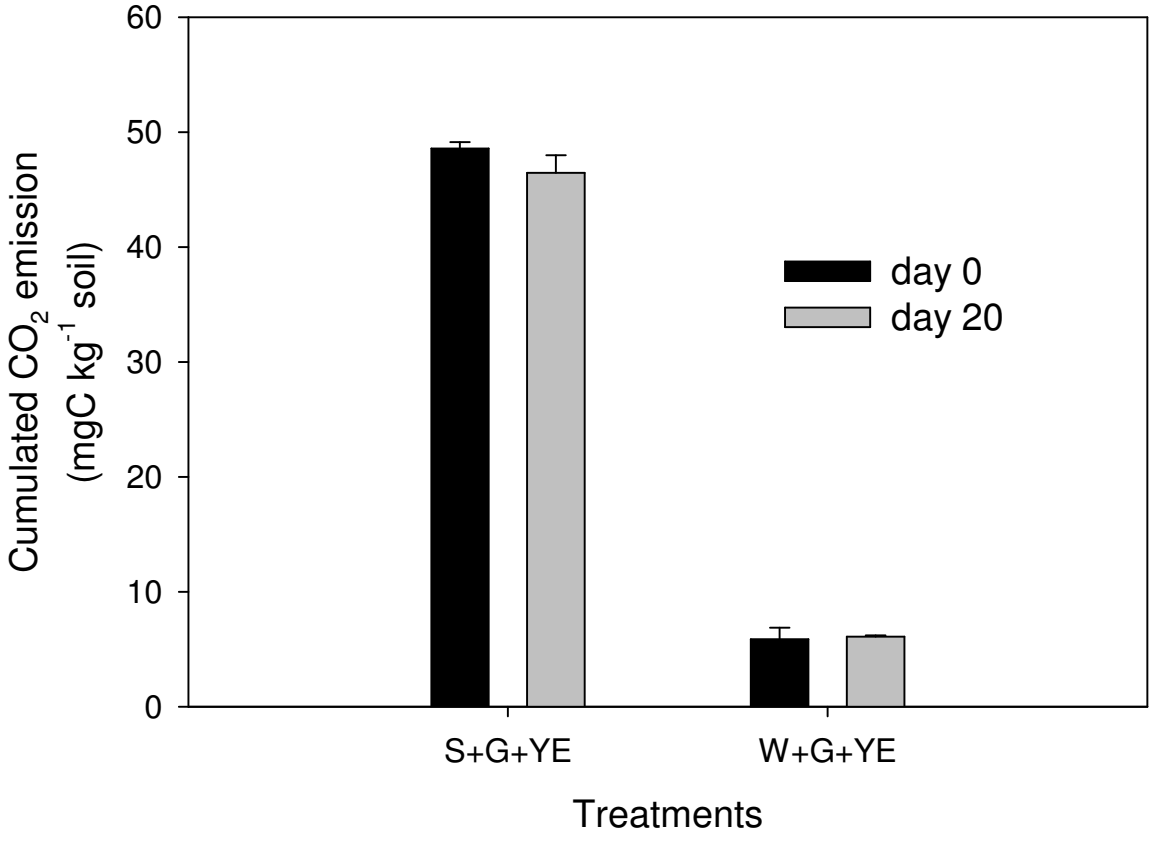


Figure S5-1: Cumulated CO_2 emissions from water and soil microcosms that received the glucose supply at the beginning or after 20 days of incubation. The cumulated emissions corresponded to the incubation period 20-58 days.

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