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Validation of the traditional calorespirometric procedure using external respirometry to quantify the calorespirometric ratio of soil microbial metabolism

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ABSTRACT

The calorespirometric ratio is a metabolic indicator that can be useful in soil science for understanding thermodynamics and the carbon use efficiency of soil microbial metabolism. Calculating calorespirometric ratios for soil microbial metabolism involves the development of calorespirometric procedures using calorimeters of the heat conduction type. The calorespirometric measurements for soil microbial metabolism are recent and their calculation requires sensitive calorimeters and accurate measurements. Their interpretation for soils has been developing during the last years, but accuracy of the methods involved in their calculation are still under question. This paper analyses the precision of the traditional calorespirometric method to yield these calorespirometric ratios by comparison with analysis of respiration measured in parallel using chromatography. Results indicated that the CO_2 data obtained by chromatography and calorespirometry are significantly correlated with each other and with the heat rate of the soil microbial metabolism. The comparison of the CO_2 data from both methods by the paired sample Wilcoxon signed rank test yielded no statistically significant differences. Nevertheless, small changes in the CO_2 determinations, due to the method of measurement or to the reproducibility of these indices in the soil replicates, affected the experimental quantitative values of the calorespirometric ratios. Differences in the quantitative values were large enough in some of the samples to yield distinct interpretations of the results.

1. Introduction

Calorimetry is a discipline that emerged on the basis of Thermodynamics in the 19th century. The concept of heat and energy sparked an important scientific revolution that impacted all areas of knowledge including biology and medicine. L. Pasteur was the first scientist to introduce the role of the heat on microbial fermentations in 1864 (Battley, 1987) without measuring that heat which involved the beginning of the scientific interest to measure the heat from microbial metabolism.

The first calorimeters that were sensitive enough to detect the heat rate of cells and microbial metabolism appeared at the beginning of the 20th century. These calorimeters were defined as isothermal calorimeters and were specifically designed for measuring the heat production of bacteria for the first time in 1913 (Von Körösy, 1913). Since then, the design of calorimeters has been continuously evolving to study the role of energy in life sciences (Tamiya and Yamamoto, 1933; Calvet, 1948). Their development and evolution was inspired by the fact that microorganisms usually live in water and soil, both systems with a high heat capacity that contributes to keep constant the temperature of their environment, that is at isothermal conditions (Battley, 1987). This concept is implemented for the sensitive calorimeters nowadays and it has always been closely linked to the concern of how efficiently living systems manage the energy that they need to stay alive. The possibility to measure the energy involved in living systems was soon applied to soils after the introduction of heat conduction calorimeters (Ljungholm et al., 1979) and it has not stopped since then (Sparling, 1981; Kimura and Takahashi, 1985; Tissot, 1999; Wadsö, 2009).

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Soil is one of the living systems that remains largely unknown from a thermodynamic perspective (Hansen et al., 2018) due to their biological and chemical complexity. The number of scientists developing this knowledge is relatively small compared to other scientific fields which has resulted in the slow evolution of bioenergetics applied to soil science.

The application of calorimetry to soil microbial metabolism primarily focuses on understanding the metabolic efficiency of the soil microbial population (Barros and Feijóo, 2003; Harris et al., 2012; Bölscher et al., 2020). These experiments are based on the biothermodynamics of microbial growth reactions developed over the last century to measure the energy use efficiency of different strains of bacteria and yeasts responsible for synthesizing products of interest for the industry (Battley, 1987; Heijnen and Van Dijken, 1992; von Stockar et al., 2011). Understanding the energy use efficiency in soil is not related to industrial purposes yet, but can be relevant for diagnosing the soil health, studying the impact of different managements on soil, and providing information about the role of the soil microbial metabolism in maintaining the soil carbon (C).

Equations determining the energy use efficiency of soil microbial growth reactions rely on thermodynamic functions such as the enthalpy change and Gibbs energy change of the microbial growth reaction. These reactions require an energy and C source. In soil, the natural source is the soil organic matter (SOM). Until recently, SOM had not been thermodynamically characterized for the necessary combustion and formation reactions involved in the equations defining C and energy use efficiency (Barros et al., 2024). As a result, most of the experiments studying the metabolic energy use efficiency of microorganisms in soils needed to use external C sources that were thermodynamically characterized such as glucose and cellulose (Barros and Feijóo, 2003; Harris et al., 2012; Bölscher et al., 2016). This scarcity of studies connecting the microbial metabolism with the original energy and C source in soils, SOM, is a significant gap in current research.

The possibility of linking the soil microbial metabolism to SOM is being developed through calorimetry by calculating a metabolic indicator called the calorespirometric ratio (CR) of microbial metabolism. The CR is the quotient between the heat rate (R_q) and the CO_2 rate (R_{CO2}) released by a cell and/or microbial metabolic reaction. It is linked to the nature of the substrate being metabolized and the C use efficiency of a metabolic reaction by the following equation (Hansen et al., 2004):

$$R_q/R_{CO2} = -\ (1 - \gamma_s\ /\ 4)\ \Delta H_{O2}\ -\ \Delta H_B\ (\varepsilon\ /\ 1-\ \varepsilon) \tag{1}$$

Where γ_s is the chemical oxidation state of the substrate being metabolized, ΔH_{O2} the oxycaloric equivalent, ΔH_B the heat of reduction of organic compounds by removal of oxygen, and ε the substrate C conversion efficiency (Hansen et al., 2004). The calorespirometric ratio is experimentally determined through a calorespirometric procedure that is widely used to study the metabolism of resting cells (Criddle et al., 1990). This procedure was first adapted to soil microbial reactions in 2010 (Barros et al., 2010) and applied to microbial growth reactions stimulated in soil by the addition of glucose (Barros et al., 2010) and to soil basal metabolism (Barros et al., 2011) which does not involve microbial growth. The calorespirometric measurement under basal metabolic conditions directly connects the calorespirometric ratio to the oxidation state of the substrate being metabolized because under those conditions the second term of equation (1) is cancelled (Hansen et al., 2004). This information is currently valuable for unravelling the thermodynamics of soil microbial reactions of SOM biodecomposition and predicting the expected C use efficiency if SOM is used as a C and energy source for microbial growth, something still unexplored.

The beginning of the experiments to obtain calorespirometric data from soil microbial metabolism showed that the CR is highly variable in soil and a sensitive indicator that requires accurate measurements to obtain trustworthy information (Herrmann and Bölscher, 2015; Wadsö, 2015). The stability of the base line of the microcalorimeter used for these measurements, high sensitivity, and delivering of data by clean plots (some devices generate noisy signals that needs suitable signal processing for smoothing the plots to calculate the CR) are essential conditions to obtain realistic values.

Simultaneously, with the increasing demand for accuracy in experimental CR determinations, there is emerging literature that demonstrates how CR varies in soil beyond methodological accuracy, and how it is also influenced by soil metabolic functional diversity (Barros et al., 2017) as well as other factors, such as soil water content, degree of soil mineralization (Barros et al., 2016), soil ecosystems (Herrmann and Bölscher, 2015; Barros et al., 2016), metabolic pathway and availability of electron acceptors (Chakrawal et al. 2020, 2021).

Despite the efforts to increase understanding about CR of soil microbial metabolism, and despite the existing interest in this metabolic indicator (Chakrawal et al. 2020, 2021; Kästner et al., 2024) the best method to measure it has not been robustly consolidated yet. Traditionally, calorespirometry involves the use of calorimeters to measure the heat rate of microbial and cell metabolism using a vial containing NaOH as a CO_2 trap (Criddle et al., 1990; Wadsö and Hansen, 2015). This procedure was adapted to soil microbial activity (Barros et al. 2010, 2011) and attempts have been made to improve it with two additional methods: colorimetry (Herrmann and Bölscher, 2015) and redesigning the calorimetric vessels (Wadsö, 2015). However, all of them have their own advantages and disadvantages introducing new challenges that have yet to be resolved. Also, none of them have been contrasted with each other. This current state of research on the use of the CR in soil science inspired us to undertake this work with the objective to test the accuracy of the traditional calorespirometric procedure to measure CO_2 rates and to contribute to optimized approaches for CR determinations. The method adapting the traditional calorespirometry to soil (Barros et al. 2010, 2011) using a NaOH trap inside the calorimetric ampoule to monitor the CR, both continuously or averaged under different intervals of time, is tested here by comparing the heat and CO_2 data obtained by calorespirometry with those determined through parallel measurements by gas chromatography.

2. Materials and methods

2.1. Soil sampling sites

Soil samples were collected in Sierra de Aralar (581712, 4757137 - UTM Zone 30 N ETRS89 datum) Navarra, Spain. It occupies a surface of 2177.57 Ha. The altitude above sea level of all the plots selected for sampling ranged between 958 and 1141 m. Sierra de Aralar has a temperate oceanic climate, classified as *Cfb* according to the Köppen-Geiger climate classification. The average annual rainfall is 1317 mm with the highest concentration of rain in the autumn and winter seasons, and snowfall on a greater number of days from January to March. The average temperature is 11.4 °C. Vegetation is mainly constituted by beech forest (*Fagus sylvatica* L.), a few codominant species of *Pinus silvestris* and small contribution of mix of broadleaved and coniferous trees. The entire area is under forest management. The soil in Sierra de Aralar is classified as Umbric Dystrochrept and it is a clay soil (Hoyos et al., 1978). The average soil depth in this area is lower than 50 cm.

Within this area and no more than 5 km apart from each other, eight different plots were selected for the soil samples collection under the following criteria: six of the plots support beech forest under a traditional uniform shelterwood forestry method since 1904, with a rotation period of 120 years according to the forest management plan (Official Technical Report of Navarra govern, 2008). These six plots are symbolized as SF plots; the remaining two plots have beech forest under a new trend close to nature (CTN) forest management since 2007, with a rotation period of 8–10 year according to the official forestry plan too. These two plots are symbolized as CTN. SF and CTN represent the forest management supported by the plots selected for soil sampling. The two CTN plots are paired plots with recent (R samples) and nonrecent interventions (N samples) with an almost uneven-aged structure.

The six SF plots were classified based on their approximate tree age selecting two plots representing the youngest ones (0–40 years) named as state 1 (01 samples), two plots of intermediate tree age (40–80 years) named as state 2 (02 samples) and two plots as the oldest ones (80–120 years) named as state 3 (03 samples) all of them following the natural forest age classes that involves plots with trees of different ages into the above reported ranges. All these plots are paired plots with recent forestry intervention (R samples) and without recent forest intervention (N samples). This means that each plot under recent intervention has the respective replicate without that recent intervention classified through natural forest age classes. Intervention consists in differing thinning procedures depending on the natural forest age class involving canopy opening. Plots under recent intervention present greater openness favouring understorey growth.

The overall sampling design involves 4 plots without recent intervention with their respective replicates under recent intervention. 3 plots represent the SF forestry, each of those with trees at different ages and 1 plot represents the CTN method. All these plots and their locations are summarized in Table 1.

2.2. Soil sampling

In each plot, six sampling points were chosen approximately 5 m away from a randomly selected initial point used as reference. These sampling points are evenly spaced at 60° intervals (0°, 60°, 120°, 180°, 240°, and 300°). It's worth noting that the positions selected for sampling may undergo minor adjustments to avoid excessive proximity to trees or other elements that could potentially distort the nature of the sample (Lestido-Cardama et al., 2024). At each sampling point, samples were taken following a depth gradient, distinguishing, on the one hand, the organic horizons (L/F) from the mineral soil layers, and within the mineral soil layers, two depths, from 0 to 5 cm and from 5 to 10 cm from the soil mineral surface.

Table 1
Name and description of each plot with their corresponding coordinates (X and Y) and altitude (Z) in meters above sea level. Coordinates are provided in UTM Zone 30 N with the ETRS89 datum (EPSG:25830).

Plot name	Description	X	Y	Z
SF01R	Uniform Shelterwood Method (0–40 years) Recent intervention	579,685.96409	4,757,382.30500	1141.40179
SF01N	Uniform Shelterwood Method (0–40 years) Without Recent intervention	580,288.83637	4,756,948.31215	1096.52727
SF02R	Uniform Shelterwood Method (40–80) Recent intervention	580,872.59324	4,756,596.46344	1060.52932
SF02N	Uniform Shelterwood Method (40–80 years) Without Recent intervention	579,759.68242	4,757,290.44848	1134.01790
SF03R	Uniform Shelterwood Method (80–120 years) Recent intervention	583,260.70815	4,757,469.87453	975,94796
SF03N	Uniform Shelterwood Method (80–120 years) Without Recent intervention	583,945,97884	4,758,757,01341	958.20092
CTNR	Close to nature Recent intervention	582,794.00436	4,756,281.61089	1091.40739
CTNN	Close to nature Without Recent intervention	583,322.59377	4757256.40638	998.44290

Samples from the six sampling points were merged into a single sample, differentiating each soil layer, and placed in polyethylene bags for preservation. Approximately 125 cm^3 (in the form of a 5x5x5cm cube) was taken as the minimum volume from each sampling point, to ensure that the combined sample from each plot and depth reached an approximate volume of 750 cm^3.

These samples were transported to the laboratory in polyethylene bags to preserve their humidity. They were sieved (2 × 2 mm) and kept at 4 °C in polyethylene bags for two months before calorespirometric measurements and chromatography. Another part of the sieved mineral soil was dried for elemental analysis and water content measurements. WHC was measured by a percolation method. The LF samples were ground to homogenize for TOC and pH analysis. TOC was analysed with a Thermo Flashsmart analyser (Thermoscientific flashsmart) after removing the inorganic carbon with HCl 1: 1. pH was measured with a pH-meter in a solution of deionized water and soil 1:1. H and N percentages were determined in a LECO CHNS analyser.

2.3. Calorespirometric measurements

10 g of soil stored at 4 °C were adjusted to 50 % of their water holding capacity and incubated at the temperature of the calorimeter (25 °C) inside polyethylene bags with a small water container inside. Stabilization after the adjustment to 50 % of the water holding capacity was monitored by calorimetry. It took about 3–5 days for the samples used in this study. After the stabilization period, 1 g of the mineral soil samples and about 0.4–0.8 g of the LF samples were placed into a TAM III microcalorimeter (TA Instruments) in 4 ml stainless steel ampoules. Each of these samples had their respective replicate that is introduced inside the TAM III with a small vial with NaOH 0.4 M (Barros, 2018). A total of six samples were run simultaneously at 25 °C and each experiment took 48 h. The ampoules without the NaOH yield the heat flow rate of the soil microbial metabolism (ϕ_0). The sample with NaOH has a more exothermic signal than that from the ampoule without NaOH. This signal involves the heat flow rate from soil microbial metabolism and that from the reaction between the NaOH and the CO_2 from soil microbial respiration (ϕ_1). The R_{CO2} is determined as follows (Wadsö and Hansen, 2015):

$$R_{CO2} = (\phi_1 - \phi_0) \ / \ \Delta_{abs}H \tag{2}$$

where $\Delta_{abs}H$ is the enthalpy of absorption of carbon dioxide by the NaOH, −108,5 kJ mol^{-1} at the NaOH concentration 0.4 M.

The measurement with the NaOH takes 24 h. After that time the two soil replicates with and without the NaOH are taken out the calorimeter and open to remove the NaOH. Once the NaOH is removed, the two replicates are taken into the calorimeter again for 24 h h more. During this time, reproducibility of the heat flow rate is checked to assess the validity of ϕ_0 as the reference line to determine the R_{CO2} and to approach the uncertainty of the CO_2 measurements.

The quotient between the heat flow rate without the NaOH (ϕ_0) and the rate of the CO_2 release (R_{CO2}) gives the calorespirometric ratio (CR) of the soil microbial meatabolism. By this procedure the R_{CO2} and the CR are measured continuously and can be displayed as plots as the heat flow rate (Barros et al., 2011).

Both, ϕ_0 and R_{CO2} plots, can be integrated versus time to yield de heat rate (R_q) and the accumulated CO_2 at different intervals of time. This procedure was applied to the incubation times of the soil replicates used for the chromatographic measurements of CO_2. The quotient between the R_q and the R_{CO2} integrated for different intervals of time (2, 24 and 48 h) give the calorespirometric ratios (CR) averaged for those time intervals.

2.4. Chromatography measurements

In parallel to the calorespirometric measurements, 1.0 g of the same mineral soil samples and about 0.4–0.8 g of the same LF samples inside the TAM III, are introduced in 4 ml glass ampoules which are sealed as the ones in the TAM III. These replicates are incubated at 25 °C for 2, 24 and 48 h in three separate vials. At each of those times a volume of 2 ml of CO_2 is extracted from the glass ampoule with a syringe and injected into a gas chromatograph with a FID detector (Agilent Technologies 7890 A) to quantify the CO_2 concentration produced by the soil samples. The 2 ml sampling is taken just once from each vial at their respective incubation times. A background measurement of the CO_2 concentration in the room air was conducted using an empty ampoule incubated under the same conditions as the soil samples.

2.4.1. Statistical analysis

Comparison of data was done by a pair sample Wilcoxon signed rank test. CO_2 data from chromatography were converted from ppm to micromole CO_2 per gram of soil to perform the comparison. The raw data from chromatography and calorespirometry before and after conversion of units from ppm to micromole CO_2 per gram of soil were analysed by Pearson's correlation. The statistical analysis was applied to a total of 66 data per analysis ($N = 66$, $p < 0.05$) after removing the samples where the measurements failed (6 samples from 72 analysis).

3. Results

Calorespirometric measurements yielded plots like the ones shown in Fig. 1. The experimental period with NaOH inside the ampoule with soil (Fig. 1(a)) yielded the R_{CO2}, as summarized in Fig. 1(b) (step 1). This data can be shown as a plot of R_{CO2} versus time in mol CO_2 per second (Fig. 1(b)). By this way, the evolution of R_{CO2} during the measurement is continuously monitored as done with the heat flow rate in microwatts (ϕ) in Fig. 1(a). The quotient between the heat flow rate of the sample used as a reference (ϕ_0) and R_{CO2} gives the calorespirometric ratio (CR) through step 2, which can also be shown as a plot (Fig. 1(c)). This allows for the continuous monitoring of the CR along with the measurement. The plot of R_{CO2} versus time can be integrated over time in seconds and represented versus time in hours after integration through step 3 as indicates Fig. 1(d)). This plot shows the cumulative pattern of CO_2 with time and can be fitted to the function that best represents the cumulative trend. We found two different trends in the accumulation of CO_2 in the samples used in this study: linear and hyperbolic. These functions were determined individually for each sample using Pearson's correlation for linear trends and allow us to calculate the expected CO_2 levels at 48 h. This method can be applied to the heat flow rate (ϕ) but the heat rate at 48 h could be experimentally determined in this case.

The calculation of the CO_2 and heat accumulated within a specific time interval (days or hours) provides an averaged CR that can be useful for comparisons among different soils and for studies unravelling the thermodynamics of soil ecosystems. That involves integration of ϕ to be converted into joules.

Table S1 (supplementary material) displays the raw data of the total heat, given in mili joules per gram of soil sample, obtained by integrating the ϕ versus time plots at the intervals of time when CO_2 was measured by chromatography. It also reports the CO_2 values obtained through calorespirometry and chromatography in their respective units.

Table 2 shows the elemental soil properties (TOC, H, N), the water content of the samples when they were collected, and their pH. TOC, H, N and water content percentages decreased with increased soil depth.

Table 3 displays the CO_2 data obtained by chromatography and calorespirometry in micromole of CO_2 per gram of soil sample, as well as the heat rate *(R_q),* all of them averaged through 24 h of measurement. The CO_2 rates measured for 24 h of incubation by chromatography and calorespirometry followed the same trend of the TOC, H, and N content of the samples with increasing soil depth, this is decreasing from the LF layer to the mineral soil samples from 0 to 5 and 5–10 cm.

The soil elemental properties (TOC, H, N) were closely and significantly ($p < 0.05$) correlated among them, to the CO_2 rates from

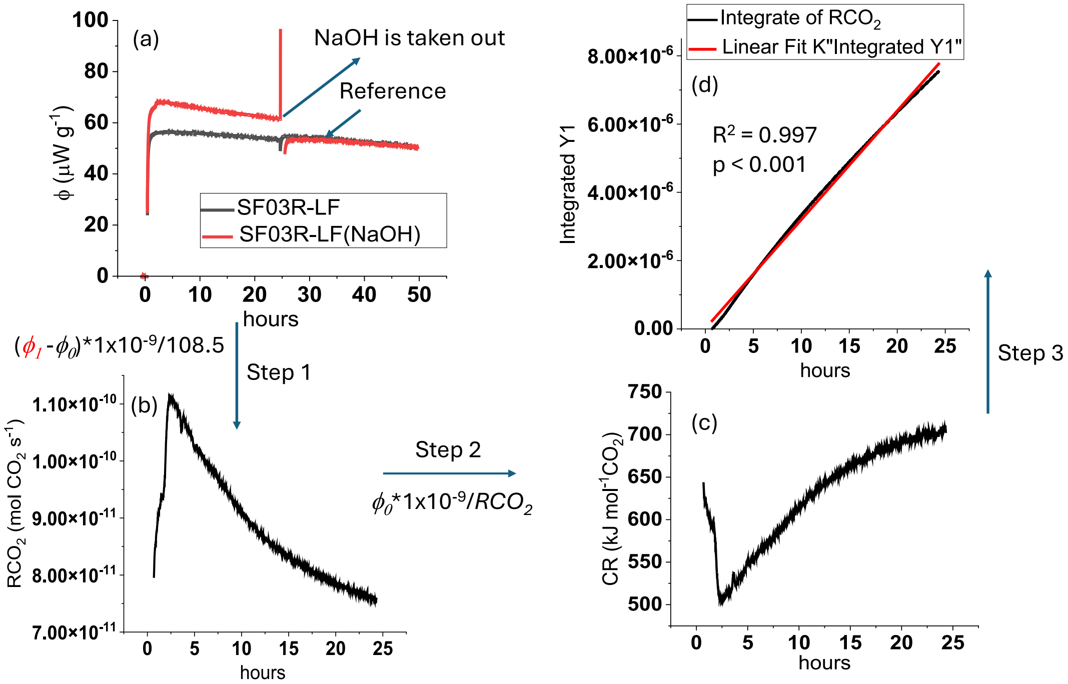

Fig. 1. Analysis of the calorespirometric plots to obtain the CO_2 rate and calorespirometric ratios, CR, of soil microbial metabolism.

Table 2
Percentages of TOC, H, N of the soil samples together with the pH. and the water content of the samples when they were collected.

Samples	Depth	TOC (%)	H (%)	N (%)	pH	Water content (%)
SF01R	LF	27.00	3.89	1.49	5.76	65.09
	0–5	6.54	1.48	0.53	5.17	28.47
	5–10	2.48	1.22	0.35	5.65	21.47
SF01 N	LF	29.87	3.52	1.66	5.36	87.46
	0–5	6.86	1.54	0.47	5.74	29.58
	5–10	5.10	1.31	0.35	6.48	23.86
SF02R	LF	31.16	3.47	1.37	4.80	63.45
	0–5	7.38	1.34	0.44	4.69	32.12
	5–10	4.92	1.10	0.28	4.43	26.25
SF02 N	LF	37.58	4.27	1.83	5.06	67.86
	0–5	9.12	1.54	0.65	5.11	35.91
	5–10	4.17	1.18	0.32	5.26	24.75
SF03R	LF	23.76	3.68	1.47	4.77	60.99
	0–5	7.46	1.78	0.70	4.59	36.27
	5–10	2.31	1.11	0.22	5.01	23.53
SF03 N	LF	34.42	4.10	1.53	4.67	65.89
	0–5	9.13	1.68	0.71	4.53	36.93
	5–10	3.91	1.25	0.32	4.34	30.49
CTNR	LF	32.12	4.37	2.00	5.75	65.41
	0–5	10.66	1.90	0.89	5.55	41.92
	5–10	8.96	1.82	0.84	5.52	36.85
CTNN	LF	28.64	3.37	1.34	5.09	62.22
	0–5	7.04	1.31	0.46	4.83	33.33
	5–10	3.71	1.03	0.25	5.01	25.22

Table 3
Values of the CO_2 produced along 24 h of incubation by chromatography and calorespirometry given in micromole of CO_2 per day and gram of soil (μmol^{-1} d^{-1} g^{-1}), together with the heat rate (R_q) averaged for 24 h of measurement in millijoules per day and gram of soil (mJ $d^{-1}g^{-1}$).

Samples	Depth (cm)	CO_2 ($\mu mol^{-1}d^{-1}$ g^{-1}) Chromatography	CO_2 ($\mu mol^{\text{-}}1d^{-1}g^{-1}$) Calorespirometry	R_q (mJ $d^{-1}g^{-1}$)
SF01R	LF	12.26	7.80	6836
	0–5	1.78	1.03	700
	5–10	0.83	0.31	211
SF01 N	LF	9.82	3.40	3348
	0–5	2.41	0.85	602
	5–10	1.61	1.28	466
SF02R	LF	7.14	4.61	3034
	0–5	1.83	1.51	840
	5–10	0.78	1.66	427
SF02 N	LF	3.22	6.92	3578
	0–5	0.66	2.97	1004
	5–10	0.07	0.07	22
SF03R	LF	3.68	7.46	4675
	0–5	1.03	3.98	1349
	5–10	0.26	0.99	334
SF03 N	LF	7.60	6.24	3533
	0–5	3.82	2.17	1246
	5–10	1.71	0.86	445
CTNR	LF	7.21	ND	2430
	0–5	2.29	2.54	1194
	5–10	1.83	2.14	788
CTNN	LF	7.28	6.93	2430
	0–5	2.11	1.64	1194
	5–10	0.94	0.68	788

chromatography and calorespirometry and to the heat rate of soil microbial metabolism (Table S2. Supplementary material). There was not any correlation involving the pH and the CR determined by both methods.

The CO_2 values obtained by chromatography significantly correlated to the CO_2 determined by calorespirometry ($n = 66$, $r = 0.85$, $p < 0.05$) (Pearson's correlation) (Fig. 2). It also correlated to the heat rate ($n = 66$, $r = 0.89$, $p < 0.05$) (Data from Table S1). Correlation between the CO_2 from calorespirometry and the heat rate was close to that obtained with the CO_2 data from chromatography ($n = 66$, $r = 0.92$, $p < 0.05$).

The evolution of these CO_2 data for the three soil layers from each sampling plot can be seen in Fig. 3. There are different quantitative CO_2 values measured by both methods in some samples but their evolution with increasing soil depth is the same in all samples: lower CO_2 respiration with depth.

The comparison of the CO_2 data measured by both methods was made by a paired sample Wilcoxon signed rank test because the CO_2 data given by each of the methods did not pass the normality test. Results yielded non-significant differences between the CO_2 data from calorespirometric measurements and chromatography.

Since the data obtained after 2 h of experiment can be affected by initial distortions (see Fig. 1) and low values of CO_2 and heat rates, and because the CO_2 production by calorimetry at 48 h is extrapolated from the first 24 h, the comparison of the CR values determined by both methods is done with the data obtained after 24 h of incubation. Fig. 4 shows that CO_2 data from both methodologies give different quantitative CR values and distinct evolution of the CR with soil depth.

One of the advantages of the calorespirometric procedure is to track the evolution of the CO_2 rate and the CR continuously along with the entire time of the measurement inside the calorimeter, as shown in Figs. 1 and 5 respectively. Fig. 5 shows different trends in the evolution of the CR with time. There are samples where the CR of the soil basal metabolism keeps stable along with the time of measurement while others present a trend of the CR to increase with time or an unstable evolution during the measurement in the calorimeter.

4. Discussion

This study demonstrates the reliability of the traditional calorespirometric procedure for measuring the rate of CO_2 production and the calorespirometric ratio in soils. The significance of calorespirometric ratios in soil science is growing, as they offer valuable insights into the biochemistry of soil microbial reactions. However, further exploration in this area is limited by the lack of research validating the methods used for their experimental determinations. For this reason, in this paper the CO_2 rates and calorespirometric ratios were measured using two distinct procedures to enhance the traditional calorespirometric method (Hansen et al., 2004; Barros et al., 2011; Yang et al., 2024) since it never

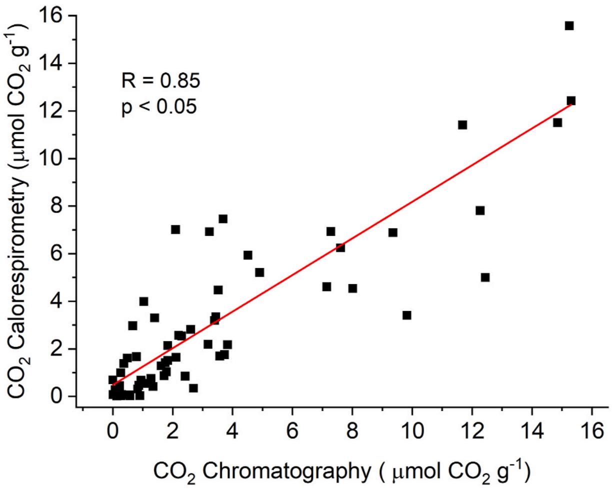

Fig. 2. Results of the correlation between the data of CO_2 obtained by chromatography and calorespirometry ($n = 66$; $p < 0.05$).

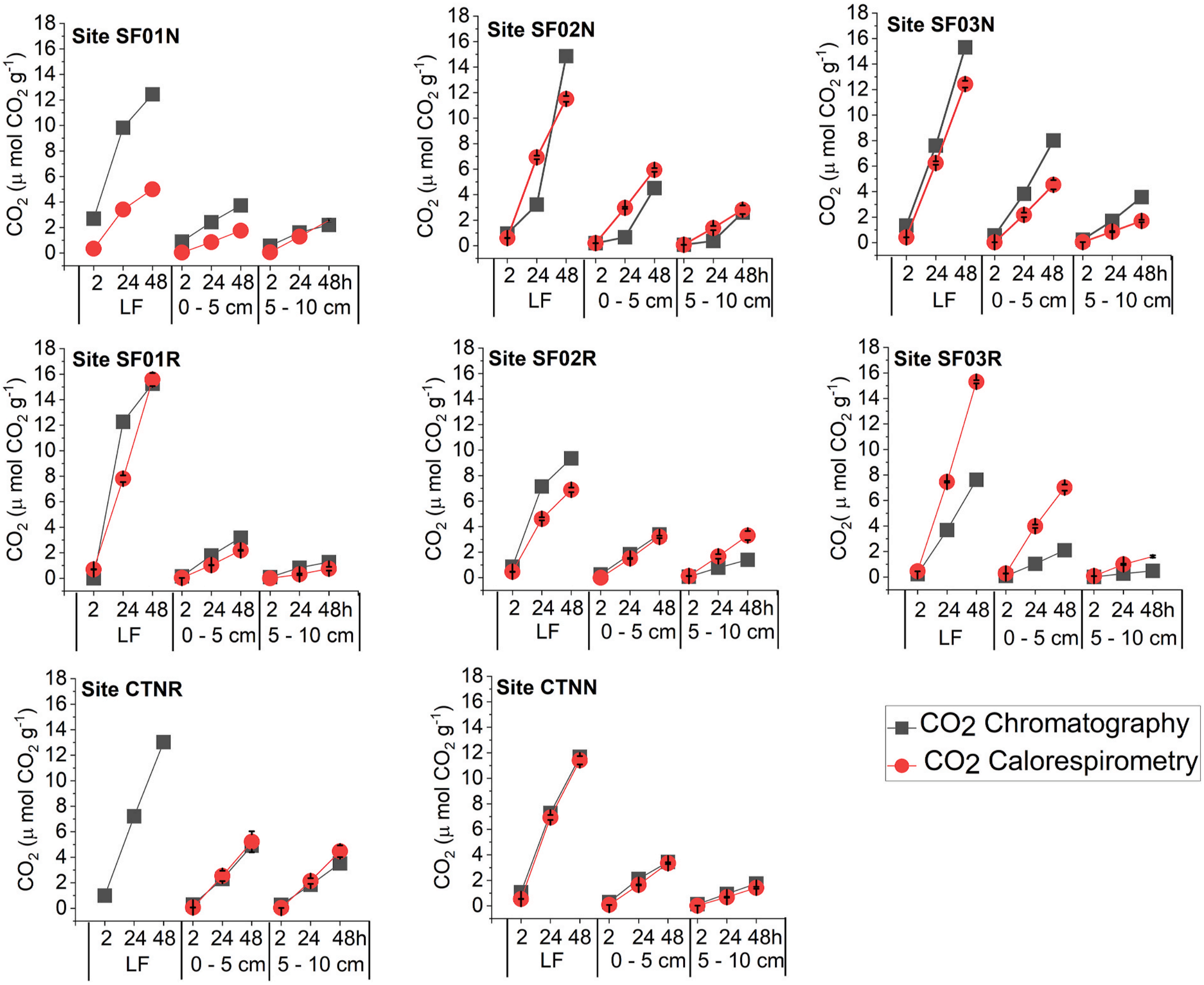

Fig. 3. Comparison of the CO_2 data determined by chromatography and calorespirometry. Each plot represents the results from different sampling sites, showing the averaged CO_2 evolution over 2, 24 and 48 h of measurements from the soil organic layer, LF, and mineral soil samples from 0 to 5 to 5–10 cm depth (x-axis).

had been compared before with other methods to measure the CO_2 rates from soil microbial respiration. The results indicate that the calorespirometric procedure yields CO_2 values that closely match those measured by chromatography in most of the soil samples incubated under identical conditions for the calorimeter and chromatography measurements. The paired sample Wilcoxon signed-rank test showed non-significant differences between the CO_2 data obtained through chromatography and those calculated using calorespirometry. Fig. 3 illustrate the evolution of CO_2 data within the soil samples from different locations and specific depths, validated by a significant correlation between both methods. This correlation could be utilized to adjust the CO_2 values obtained from either of the methods used here, enhancing overall accuracy. Such calibration would contribute to improve the precision of CO_2 determinations under laboratory conditions, particularly for national inventories of CO_2 emissions linked to various soil management practices (Liang et al., 2024).

Furthermore, both CO_2 data showed the same trend to decrease with soil depth. This evolution was directly correlated to the observed drop in the TOC, N and H percentages with increasing soil depth. On the contrary, the pH of the samples did not present any effect on the CO_2 and heat rate of soil microbial metabolism. This is surprising since the effect of pH on soil microbial metabolism is well understood. A possible reason could be that variations of pH among samples are not large enough to cause a noticeable effect on soil microbial metabolism.

These findings strengthen the reliability of calorespirometry as an additional tool for concomitant measurements of the heat and CO_2 rates of soil microbial metabolism. The accuracy of the reported correlation between the chromatographic and calorespirometric measurements can be further improved by increasing the number of soil replicates used to quantify CO_2 derived from soil microbial metabolism.

One of the advantages of calorespirometric measurements in soils is the ability to calculate the calorespirometric ratio (CR) of soil microbial metabolism. This ratio is typically determined by averaging the rates of heat and CO_2 production over specific time intervals, to provide averaged CR values that can be compared among different soil samples (Herrmann and Bölscher, 2015; Barros et al., 2016). However, when calculating this metabolic indicator using CO_2 data obtained through calorespirometry and chromatography, there were samples showing large differences in the averaged CR values. These discrepancies were substantial enough to lead to varying interpretations regarding the nature of the substrates being metabolized. For instance, if we consider the CR values obtained for the samples after 24 h of incubation, all samples from SF03R location exhibited CR values of approximately 1300 kJ mol^{-1} CO_2 when analysed via chromatography while calorespirometry yielded values ranging from about 626 to 336 kJ mol^{-1} CO_2 depending on the specific soil layer (Fig. 4). Other samples, such as those from site SF01R, showed calorespirometric values of CR of 875 kJ mol^{-1} CO_2 in the LF layer and about 678 kJ mol^{-1}CO_2 in the mineral soil samples (Fig. 4) while chromatography returned values ranging from 557 mol^{-1} CO_2 in the LF layer to approximately 391 kJ mol^{-1} CO_2 and 253 kJ mol^{-1} CO_2 in their soil mineral layers, indicating a trend of the CR to decrease with soil depth. All these values derive in differing

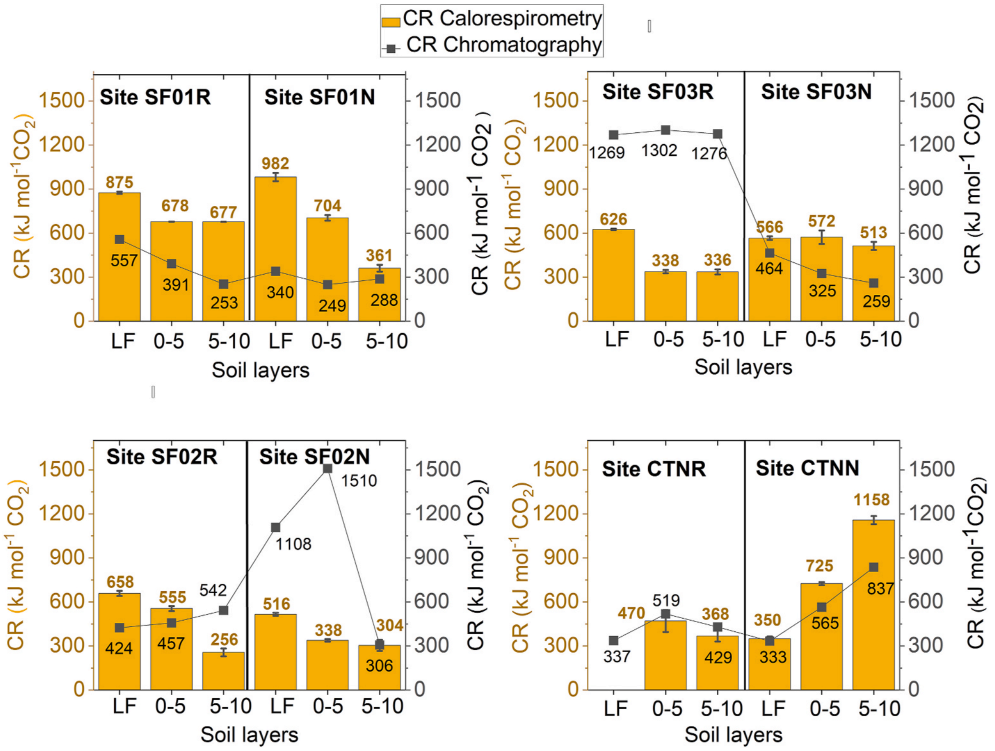

Fig. 4. Comparison of the averaged CR data obtained after 24 h of incubation, given by the CO_2 data measured by chromatography and calorespirometry. Plots display the CR values of the soil samples from the different sites and specific soil layers. Each plot represents a sampling location; x-axis display the different soil layers. LF is the soil organic layer at the soil surface. 0–5 and 5–10 symbolize the mineral soil samples collected at 0–5 cm and 5–10 cm of depth.

interpretations of the substrate being metabolized by soil microorganisms. Generally, CR values are related to the degree of oxidation of the substrate being metabolized by the equation that traditionally defines this metabolic indicator (equation (1)) (Hansen et al., 2004) when there is not microbial biomass gain (because it cancels the second term of equation (1)) and when oxygen serves as the electron acceptor in the biochemical reactions occurring in the soil. Consequently, variations in the quantitative values of CR can be attributed to changes in the nature of the substrates being metabolized. The degree of oxidation of the organic substrates is linked to the energy of that substrate through different thermodynamic models (Patel and Erickson, 1981; Sandler and Orbey, 1991; Gary et al., 1995). Therefore, values of the calorespirometric ratios may approximate the enthalpies of the substrates being metabolized and may be useful for assessing about the oxidation state of those substrates by comparing their CR values to the degree of oxidation and the enthalpy of glucose catabolism ($\gamma_s = 0$; $\Delta_c H = -469$ kJ mol^{-1}C) (Gary et al., 1995; Wadsö and Hansen, 2015) or any other substrates (García et al., 2012).

Considering, for instance, the CR values obtained for the mineral soils in samples from SF01R site after 24 h of incubation (Fig. 4) by both methods mentioned before, chromatography would be indicating metabolism of substrates more oxidized than glucose (391 kJ mol^{-1} CO_2 and 253 kJ mol^{-1} CO_2 in the mineral samples) while the CR values obtained by calorespirometry in the same soil layers denote metabolism of substrates more reduced than glucose and carbohydrates (CR = 678 and 677 kJ mol^{-1}CO_2). Metabolism of substrates more oxidized or more reduced than carbohydrates occur in soil (Gunina and Kuzyakov, 2022). This variation can be attributed to the complex chemical nature of SOM which consists of substrates that can be either more or less oxidized than carbohydrates (Kleber, 2010; Rumpel and Kögel-Knabner, 2011, Willians and Plante, 2018). Soil samples with SOM at a low degree of decomposition, such as the LF samples here, tend to be rich in lignin content, which produces aromatic products during the initial stages of biodecomposition by fungi (Chávez-Vergara, 2014). This characteristic may explain the recorded CR values exceeding 500 kJ mol^{-1} CO_2 by both procedures in samples LF from SF01R site.

The differences observed in the calorespirometric ratios for some samples, obtained through the CO_2 values from both methods, can be attributed to the instability of the calorimetric signal during the first 2 h of measurement (Wadsö, 2015). It could be caused too by the procedure linked to the extraction of CO_2 from the ampoule to be measured by chromatography. When more than 10 % of the headspace inside the ampoules with the soil samples is removed, some air containing CO_2 into the soil mineral matrix could be also pulled out creating a bias in the CO_2 determination by chromatography. This feature would yield CR values lower than those from calorespirometry as happens in the soil samples in Fig. 4. However, in general, the averaged calorespirometric ratios for different time intervals calculated by both methods, varied randomly among some soil samples, while they were similar in others, without any general trend that could be attached to the LF or mineral soil samples. Therefore, justifying these differences is not as straightforward as attributing them solely to the distinct methodologies used. It is important to note that the calorespirometric ratios determined for soils are inherently variable metabolic indices.

Based on existing literature and in alignment with the results presented here, the variability of the calorespirometric ratios can be attributed to the chemical complexity of SOM and to the diversity of the metabolic functions of soils (Herrmann and Bölscher, 2015; Barros et al. 2016, 2017; Chakrawal et al. 2020, 2021). There are previous works demonstrating that some soils have stable and reproducible calorespirometric ratios while others present large variations of these values affecting the replicates of the same soil (Barros et al., 2017). As the experiment was designed here, all the measurements took place with 5 soil replicates incubating at the same time, into the same volume, at the same temperature and into the same kind of calorimetric vessels. It may happen that the activated metabolic functions differ among those replicates if some of the soils from the different sampling plots have higher metabolic diversity than others. This last involves differing enzymatic

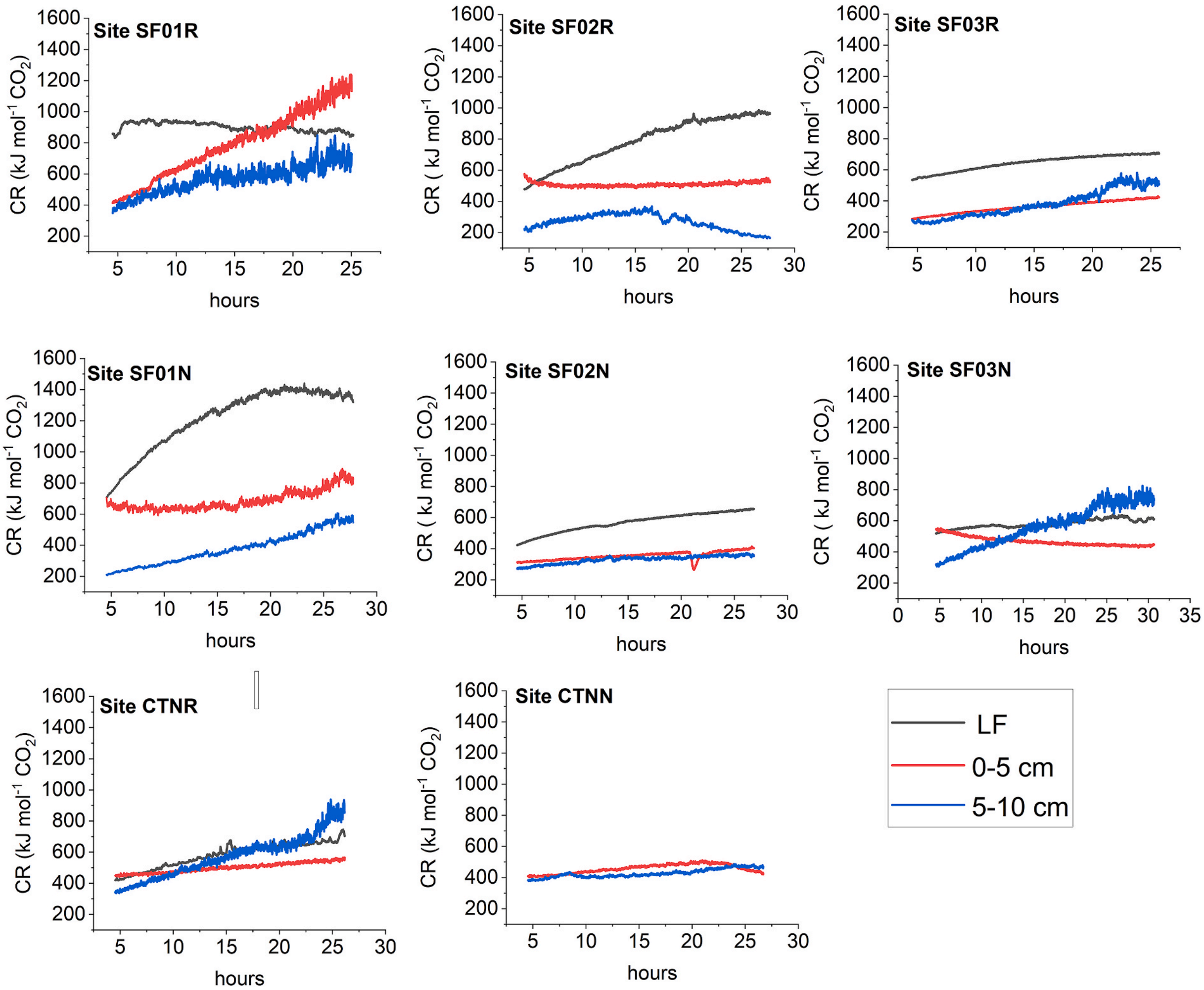

Fig. 5. Evolution of the CR during the calorespirometric measurements of all soil samples used in this work. Each plot represents one sampling site. Each sampling site shows the results obtained for the LF layer and the mineral soils collected at 0–5 cm and 5–10 cm of depth.

structure. Values of the calorespirometric ratios can be also affected if other electron acceptors than oxygen are activated in the soil replicates during the experimental incubation (Chakrawal et al., 2020, Chakrawal et al., 2021). It may occur too that in some of the replicates microbial or seed growth start to take place. They are common incidents in soil incubations that cannot be detected by the CO_2 measurements by chromatography and by averaging the CO_2 and heat rates through intervals of time of 24 h.

One of the advantages of the traditional calorespirometric procedure tested here is to let the continuous monitorization of the CO_2 and heat rates and the CR along with the entire time of measurement in the calorimeter (Barros et al., 2011; Yang et al., 2024). This continuous monitoring of the heat rate enables the detection of microbial growth and seed germination during the measurement. When it takes place, the heat rate profile shifts from a stable signal or a gradual decay—typical of soil basal metabolism—to an exponential or linear increase of the recorded heat flow rate, characteristic of microbial and seed growth (Kimura and Takahashi, 1985, Barja and Nuñez, 1999, Nuñez-Regueira et al., 2006; Schabes and Sigstad, 2006.). Additionally, the ongoing observation of the CR revealed that some soils maintain a stable CR throughout the measurement, while others exhibit a variable CR profile over time, as illustrated in Fig. 5. Therefore, the observed differences in the CO_2 rates and CR values from both methods may result from these features rather than solely from any insensitivity of the procedures or by the fact to be measured by two independent methods.

Another typical trend of the calorespirometric ratio when monitored continuously is to increase with time (Yang et al., 2024) as observed here in Fig. 5 in some of the soil samples. This behaviour could be attached to inhibition of aerobic microbial respiration due to the decay of the O_2 that is available or accumulation of CO_2 inside the calorimetric ampoules with the samples, but it was not the case here. The quantity of O_2 in the calorimetric ampoule can be easily calculated and the duration of the experiments can be adapted to avoid the impact of oxygen depletion (Wadsö and Hansen, 2015; Wadsö, 2015; Yang et al., 2024). Moreover, it is also clearly detected in the profile of the heat flow rate with time (Yang et al., 2024). None of the heat flow rate plots in this work revealed any drastic drop of the heat rate to zero, or close to zero, during the measurement, which is the typical effect when the lack of O_2 limits any biochemical reaction taking place at aerobic conditions. There were no publications about the effect of the depletion of O_2 or accumulation of CO_2 inside the calorimetric ampoules on the soil microbial activity recorded by calorimetry until recently (Yang et al., 2024) and it does not limit the methodology. The soil sample can be replaced by a new replicate if inhibition occurs and the duration of the calorespirometric experiments can be adjusted according to the availability of oxygen into the calorimetric ampoule as part of the routine of the procedure. This work considered all those aspects before designing the experimental phase. Consequently, the observed increase in the calorespirometric ratio monitored by the calorimeter in these experiments may indicate a change in the type of substrate metabolized during the measurement, as well as the activation of different enzymes.

5. Conclusions

Chromatography and calorespirometry are effective methods for tracking and comparing CO_2 respiration rates in different soil layers. However, small variations in the CO_2 data obtained from these methods can result in significant differences in the calorespirometric ratios calculated by averaging the heat and CO_2 rates, causing distinct interpretations regarding the types of substrates metabolized in the soils during the experimental measurement.

Results strengthen the calorespirometric method as a procedure for continuous monitorization of the CO_2 rate and calorespirometric ratio along with time of measurement. This additional information helps to detect incidents during the measurement that could affect the quantitative values of the calorespirometric ratios and their interpretation, such as the existence of microbial growth reactions during the measurement, inhibition of the microbial metabolism due to oxygen depletion or changes in the observed trends and values compatible with the activation of other electron acceptors in the soil samples.

CRediT authorship contribution statement

Verónica Piñeiro: Investigation, Formal analysis, Conceptualization. **Yago Lestido-Cardama:** Resources, Methodology, Investigation. **César Pérez-Cruzado:** Supervision, Resources, Project administration, Investigation, Funding acquisition. **Nieves Barros:** Writing – original draft, Supervision, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Calorespirometric measurements, were done by Verónica Piñeiro at the Laboratory of Elemental Analysis at the RIAIDT services in the Campus of Lugo of the University of Santiago de Compostela.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2025.109812.

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