Biology and Fertility of Soils (2023) 59:609–618
https://doi.org/10.1007/s00374-023-01725-5

ORIGINAL PAPER

Lysis of soil microbial cells by CO_2 or N_2 high pressurization compared with chloroform fumigation

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Received: 9 December 2022 / Revised: 31 March 2023 / Accepted: 4 April 2023 / Published online: 18 April 2023
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Abstract

The classical chloroform fumigation-incubation (CFI) and fumigation-extraction (CFE) methods are nowadays among the most used for determining soil microbial biomass, although the chloroform lysing of microbial cells is not always complete. Here, we have tested a physical method, used for sterilizing foods but never in soil, based on N_2 or CO_2 high pressurization (N2HP or CO2HP, respectively) to cause microbial cell lysis. The N_2HP and CO_2HP were tested on two soils differing for their organic matter content, one agricultural (AGR) and one forest (FOR), and firstly were compared with the CFI. The CO_2 extra-flush from both soils during 10-d incubation by N_2HP was lower than that by CFI method, whereas that by CO2HP was greater. Then, the lysis by CO2HP was compared with that by the CFE method by varying CO_2 pressure and duration. The CO2HP, at proper conditions, was more efficient than CFE method to cause the lysis of soil microbial cells. Moreover, both CO_2 pressure value and duration were important in increasing the extractable organic C compared to the CFE. The most successful combination of high CO_2 pressure and duration was 4.13 MPa and 32 h. However, we cannot exclude that CO2HP might have caused the release of soil organic C not ascribable to living organic matter. Further studies using ^{13}C and/or ^{15}N-labeled microbial cells should assess the release of abiotic organic C.

Keywords Microbial biomass · Cell lysis · Chloroform fumigation-incubation · Chloroform fumigation-extraction · CO_2 high pressurization

Introduction

Soil microbiome, a small but highly dynamic living organic matter pool, plays a pivotal role in nutrient cycling and thus it is important in affecting soil fertility (Schloter et al. 2018). Due to its nature, soil microbiome quickly responds to biotic and abiotic factors, thus becoming a sensitive indicator of most disturbances and changes occurring in the soil ecosystem (Laudicina et al. 2012).

Accurate and rapid methods, such as the chloroform ($CHCl_3$) fumigation-incubation (CFI) and the $CHCl_3$ fumigation-extraction (CFE) have been used to measure the size of soil microbiome, i.e. the soil microbial biomass C (SMBC) (Jenkinson and Powlson 1976; Vance et al. 1987). Both methods involve the fumigation of soil for 24 h by $CHCl_3$ vapours, but $CHCl_3$, an alkyl halide, is toxic to both humans and the environment (Lionte 2010). Furthermore, the 24 h $CHCl_3$ fumigation does not lyse all soil microbial cells. Badalucco et al. (1990, 1992) demonstrated that amounts of phenol-reactive C and anthrone-reactive C, i.e. ascribable to sugars, represented higher proportions of total extractable C after than before $CHCl_3$ fumigation, suggesting that some non-biomass sugars were solubilized during the 24 h fumigation. In addition, since the CO_2 produced during the 24 h $CHCl_3$ fumigation in 8 different soils ranged from 20 to 85% of that biotically produced (respired) in the absence of $CHCl_3$, Badalucco et al. (1997) questioned that the percentage of microbial biomass surviving chloroform exposure, or partially lysed, was not negligible. The efficiency of $CHCl_3$ in lysing microbial cells depends on soil texture, due to its low diffusion in clayey soils (Badalucco et al. 1997). However, even in sandy loam soils, not all soil microbial cells were lysed, as shown by Toyota et al. (1996) reporting that approximately 10% of bacterial colony forming units survived a 5-day chloroform fumigation. Likely, the exopolysaccharides secreted by bacteria might

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have protected bacterial cells from the action of chloroform vapours. Later, Haubensak et al. (2002), investigating the reliability of the CFE method in organic and mineral soil horizons of 11 mature forests, demonstrated that different fumigation conditions ($CHCl_3$ exposure from 1 to 10 days at field moist or field capacity water content) resulted in different C/N ratios of the organic compounds made extractable by $CHCl_3$ fumigation from soil. Moreover, Alessi et al. (2011) demonstrated that a significant amount of chloroform was adsorbed by the clay fraction of soil and not removed by increasing the chloroform evacuation time or subjecting the fumigated soil to high N_2 pressures. It was suggested that the CFE method should be corrected for the soil clay content. Finally, Rotbart et al. (2020) showed that the residual (i.e. non-evacuated) chloroform concentrations in the fumigated soils correlated positively with soil organic matter content. Therefore, the assessment of soil microbial biomass through $CHCl_3$ fumigation methods, based on prior disruption of microbial cells for the subsequent extraction (CFE) or mineralization (CFI) of intracellular matter still shows some problems.

In this study, we tested a new technique for lysing soil microbial cells by the high gas pressurization of soil. The gas can penetrate and fill soil microbial cells, and when the applied gas pressure is suddenly released, the absorbed gas can rapidly expand inside the cells, causing the mechanical rupture of the cell, like a popped balloon (Fig. S1). Likely, the enhanced concentration of gas might cause an increase in fluidity of the lipid double-layer of the microbial membranes, thus increasing their permeability to gas, which can rapidly diffuse into microbial cells (Spilimbergo and Bertucco 2003). Moreover, an intensive localized cooling due to the Joule–Thomson effect, when pressurized gas is expanded, might also play a role in cell lysis during the decompression (Garcia-Gonzalez et al. 2007). This technique, which was first reported by Fraser (1951), and then implemented, involves the sterilization/inactivation of microbial cultures in liquid medium or heat-sensitive foods, such as milk or jams (Nakamura et al. 1994; Garcia-Gonzalez et al. 2007), but it has never applied in soil.

The aim of this study is to test an alternative approach to $CHCl_3$ fumigation for lysing soil microbial cells by exposing the soil to gases at high pressure, followed by rapid depressurization via the gas release. Different combinations of gas pressure, type of gas used, and time of pressurization were tested because these factors affect the penetration of the gas inside the cells. The lysis of soil microbial cells by high pressurization with CO_2 or N_2, here called CO2HP or N2HP, respectively, was determined by measuring either the amount of CO_2 released during ten days of soil incubation, or the organic C extracted by a KCl solution after soil pressurization, and these values were compared with those obtained by CFI and CFE methods, respectively.

Materials and methods

Soils and experimental setup

Two soils, one agricultural (AGR) and one forest (FOR), with different physico-chemical properties, mainly with contrasting organic matter amounts, were collected to assess the response of CO2HP and N2HP approaches with variable soil types. Both soils were collected in Sicily (Italy) by a spade: the AGR soil was surface (0–20 cm) collected at Camporeale, PA province, (37°54′53.6" N 13°04′47.9" E, *Typic Haploxerert*, vineyard) after discarding plant residues and stones from the surface, in February 2020; the FOR soil was collected at San Fratello, ME province, (38°02′12.6" N 14°36′27.1" E, *Typic Haploxerept*, oak forest) at 5–20 cm in order to exclude L/O horizons, in July 2020. About 30 kg were sampled for each soil. Immediately after their collection, soils were sieved (Ø < 2 mm), stored at room temperature and their chemical and physical properties determined (Table 1). A soil aliquot was pre-incubated at 25 °C and at 50% of the water holding capacity (WHC) for 7 days, in order to stabilize the biological activity, before the lysis of soil microbial cells by $CHCl_3$ fumigation, CO2HP or N2HP methods. Three distinct and independent experiments were carried out: Experiment I in November 2020; Experiment II in January 2021; Experiment III in February 2021.

Table 1 Main soil properties

Name	Site	Sand[1] (%)	Silt[1] (%)	Clay[1] (%)	pH[2]	EC[3] (mS/cm)	Limestone[4] (%)	TOC[5] (%)	TN[6] (%)
AGR	Camporeale (PA)	62	11	27	6.8	0.18	0.2	2.1	0.11
FOR	San Fratello (ME)	57	26	17	6.7	0.30	n.d	10.3	0.35

[1] Texture, pipette method; [2] pH, soil:water ratio 1:2.5 (w/v); [3] Electrical Conductivity, soil:water ratio 1:2.5 (w/v); [4] Total limestone (calcimeter); [5] Total Organic C (TOC, Walkley–Black); [6] Total N (TN, Kjeldahl)

n.d., not detectable

CO_2 and N_2 high pressurization (CO2HP and N2HP)

The pressurization experiments were conducted using the Cells Disruption Bomb equipment (Parr Instrument Company, USA), which is a non-stirred steel pressure vessel with an internal volume of 1850 mL and a manometer, located in the upper part, giving internal pressure values up to 20.7 Mpa (Figure S2).

Four aliquots of 15 g for each pre-incubated soil and for each pressurization treatment (see later) were put into 50 mL plastic Falcon tubes, which were placed without caps in the steel vessel (it can contain up to 12 tubes per pressurization cycle). The vessel was tightly closed and incubated at around 40 °C, as recommended by the manufacturer to allow the spatially homogeneous gas diffusion. In order to evaluate the best cell lysis by the high pressurization method, the gas pressure ranged from 2.76 to 5.51 MPa and the duration from 2 to 32 h (Table 2). At the end of each pressurization treatment, the outlet valve of the vessel was immediately opened. The complete depressurization took from 20 to 40 s, depending on the pressure x duration combination, as verified by the manometer connected to the vessel.

$CHCl_3$ fumigation

After AGR and FOR soils pre-incubation, $CHCl_3$ fumigation was performed according to Jenkinson and Powlson (1976). Four soil aliquots (each 15 g) were put inside glass beakers and fumigated with $CHCl_3$ (stabilized with amylene), in a sealed desiccator (40 cm diameter) for 24 h at 22 °C in the dark. Then, the fumigant was removed by a vacuum pump by ten cycles of suction (2 min each) and air re-introduction (1 min each).

Determination of organic C released before (control) and after $CHCl_3$ fumigation, CO2HP or N2HP

In the Experiment I, soil organic C released following $CHCl_3$ fumigation, CO2HP or N2HP was determined similarly to the CFI method (Jenkinson and Powlson 1976), i.e. by measuring the evolved CO_2 during the 10 days incubation. Evolved CO_2 was also determined from the untreated soil (control). Then, in the Experiments II and III, which only differed for the combination of CO_2 high pressures and durations, the released organic C, which was extracted with 2 M KCl, was determined similarly to the CFE (Vance et al. 1987). This salt was preferred to K_2SO_4 since the extracted organic C (see later) was determined by elemental analysis (Murage and Voroney 2007).

Soil incubation was carried out in 125 mL glass jars sealed with rubber stoppers holding silicone septa and placed in the dark at 22° C for 10 days. The CO_2 accumulated after 1, 3, 7 and 10 days of incubation in the headspace of the glass jars was determined by injecting 1 mL of air from each jar into a gas chromatograph (TraceGC, Thermo Fisher Scientific, Milan, Italy) equipped with a thermal conductivity detector and a Poropak Q column, using He as the carrier (oven 50 °C, injector at 225 °C, column flow 40 mL/min, split mode 10:1). At each CO_2 sampling, jars were ventilated with fresh air for 30 min and then sealed again, after adding distilled water to adjust soil moisture, if needed. Soil extraction was carried out with a sample weight:extractant volume (g/mL) ratio of 1:4. Soil suspensions were horizontally shaken for 45 min at 70 rpm. At the end of shaking, the suspensions were filtered with Whatman No. 42 paper and then organic C was determined by a TOC-L elemental analyzer (Shimadzu Italia, Milano).

Statistical analysis

The results reported are the means with standard deviations of 4 pseudo-replicates ($n=4$) and are expressed on dry soil weight basis at 105 °C. For each of the three described experiments, data were subjected to one-way ANOVA (with replication), with the only factor being the specific treatments applied time by time, i.e. control, fumigation (CFI or CFE), CO2HP at various durations, N2HP at various durations. Before performing the ANOVA, the normal distribution and homogeneity of variance of the data were checked by Kolmogorov-Smirnoff and Levene's goodness-of-fit tests, respectively. Significant differences at $p<0.05$ among various treatments and within each of the three independent experiments were assessed by the Least Significant Difference test (LSD). Statistical analysis was performed

Table 2 Framework of the three experiments performed to compare $CHCl_3$-fumigation based methods (CFI and CFE) with the high pressurization method using N_2 or CO_2 gases (N2HP and CO2HP)

EXP	Comparison	Constant parameter	Variable parameter
I	N2HP—CFI	4.13 MPa	16, 24, 32 h
	CO2HP—CFI	4.13 MPa	16, 24, 32 h
II	CO2HP—CFE	4 h	2.76, 3.44, 4.13, 4.82 and 5.51 MPa
		24 h	2.76, 3.44, 4.13, 4.82 and 5.51 MPa
III	CO2HP—CFE	4.13 MPa	2, 4, 6, 8 h
		4.82 Mpa	16, 24, 32 h

Values of both pressures and durations are reported

using RStudio 2022.02.3 + 492 "Prairie Trillium" (libraries *tidyverse* and *emmeans*).

Results and discussion

Comparison between high pressurization (4.13 MPa) and CFI method in lysing soil microbial cells (Experiment I)

The cumulative CO_2-C (C_{flux}) evolved during 10 days of incubation from AGR soil was lower than from FOR soil for the same treatment (Fig. 1), likely due the lower content in KCl-extractable C (Fig. 2), which may be considered as a proxy of available C (Laudicina et al. 2013). Indeed, the increase in CO_2 cumulated between 7 and 10 days of incubation of the AGR soil, regardless of treatment, was smaller than the correspondent increase present in the FOR soil, likely because the microbial available C was nearly exhausted in the AGR soil, while it was still present even after 10 days in FOR soil (Fig. 1). In addition, the C_{flux} cumulated throughout the whole 10-d incubation for all three AGR soil N2HP treatments did not significantly differ from the control treatment, while in N2HP treated FOR soils the C_{flux} was higher than in control soil by 23.4%, 41.8% and 62.4% after 16, 24 and 32 h of N_2 pressurization, respectively (Tables 3, 4, and 5; Fig. 1). These results could depend on: 1) N_2 gas pressurization at 4.13 MPa for the duration time from 16 to 32 h was ineffective in the AGR soil or scarcely efficient in the FOR soil in lysing microbial cells because none or little extra-flush of organic C was respired in comparison with control; 2) the N_2 gas partly remained inside soil pores even after depressurization, thus avoiding the oxygen penetration into soil pores with the result of slowing down microbial respiration. The nitrogen gas, being a not polar molecule, is poorly soluble in water (its hydrosolubility is 19 mg/L at 20 °C and 0.1 MPa pressure) (Garcia-Gonzalez et al. 2007) and might not have reached living microbial cells inhabiting water films surrounding soil particles. In addition, the lower MBC of AGR than FOR soil (Table 1) may also explain the different effect of N2HP in lysing microbial cells of the two soils.

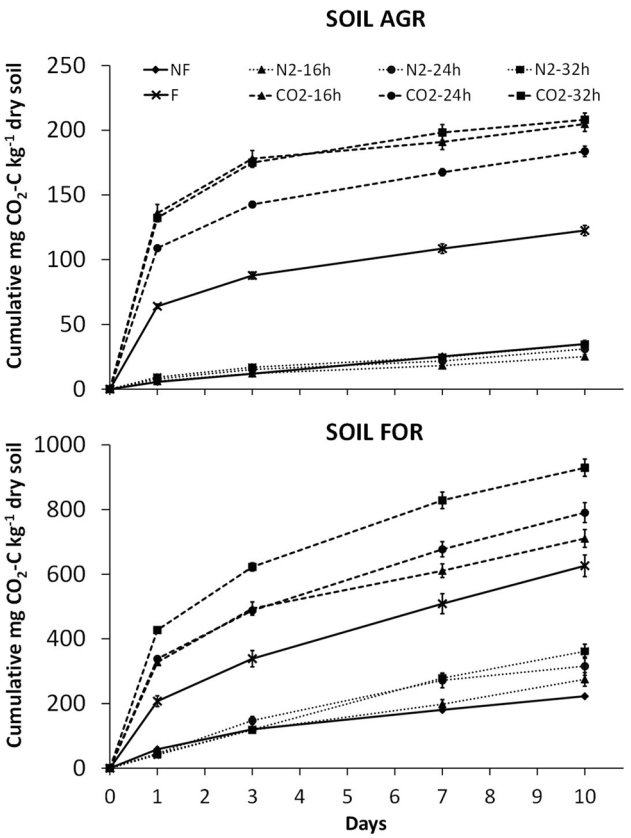

Fig. 1 Cumulative CO_2 emitted from AGR and FOR soils during a 10-days incubation at 25 °C and 50% of WHC, subjected to constant high pressure (4.13 MPa) for 16, 24, and 32 h with molecular nitrogen (N_2, N2HP) or carbon dioxide (CO_2, CO2HP). NF, not fumigated (control); F, $CHCl_3$ fumigated. Values represent means ± SD ($n = 4$)

The 10-d C_{flux} of the AGR and FOR $CHCl_3$ fumigated soils was higher than values of the respective unfumigated controls as it increased by 87.7 mg kg^{-1} and 403.4 mg CO_2-C kg^{-1} (Tables 3, 4, and 5; Fig. 1), respectively. These increases in C_{flux} were likely due to the microorganisms surviving the chloroform fumigation, which respired the cytoplasmic materials released from lysed microbial cells (Jenkinson and Powlson 1976).

The cumulative 10-d C_{flux} from both AGR and FOR soils under CO2HP at 4.13 MPa and any pressurization duration was higher than the values of $CHCl_3$ fumigated soils ($p < 0.05$). Likely, the CO2HP was more effective than $CHCl_3$ fumigation in lysing microbial cells, as shown by the higher mineralization of cytoplasmatic material by the surviving microorganisms. Moreover, the C_{flux} did not increase with the duration of CO_2 pressurization in AGR soil, whereas the C_{flux} of the FOR soil was significantly ($p < 0.05$) linearly related to the duration (Tables 3, 4, and 5; Fig. 1). The CO2HP method, compared to CFI method, increased the C_{flux} of the AGR soil by 62.4%, as an average, whereas the C_{flux} of the FOR soil increased by 13.5%, 26.3% and 48.5% with CO2HP durations of 16 h, 24 h and 32 h, respectively. Therefore, these results suggest that pressurized CO_2 was likely effective in lysing soil microbial cells. The CO_2 can cross microbial membranes because it is a polar molecule (Garcia-Gonzalez et al. 2007). The CO_2 solubility in water at 20 °C and 0.1 MPa pressure is 1690 mg/L, i.e. nearly ninety times higher than that of N_2.

By subtracting the C_{flux} of the untreated control from either 10-d C flux of the $CHCl_3$ fumigated soil or CO2HP treated soil, and applying the Jenkinson's relationship (1988) for estimating MBC by the CFI method $[MBC = (CO_2 - C_{flux\ Fumigated/CO2HP}\ \text{minus}\ CO_2 - C_{flux\ Control})/0.45]$, MBC value of the CO2HP treated AGR soil at 4.13 MPa and for 16 h was

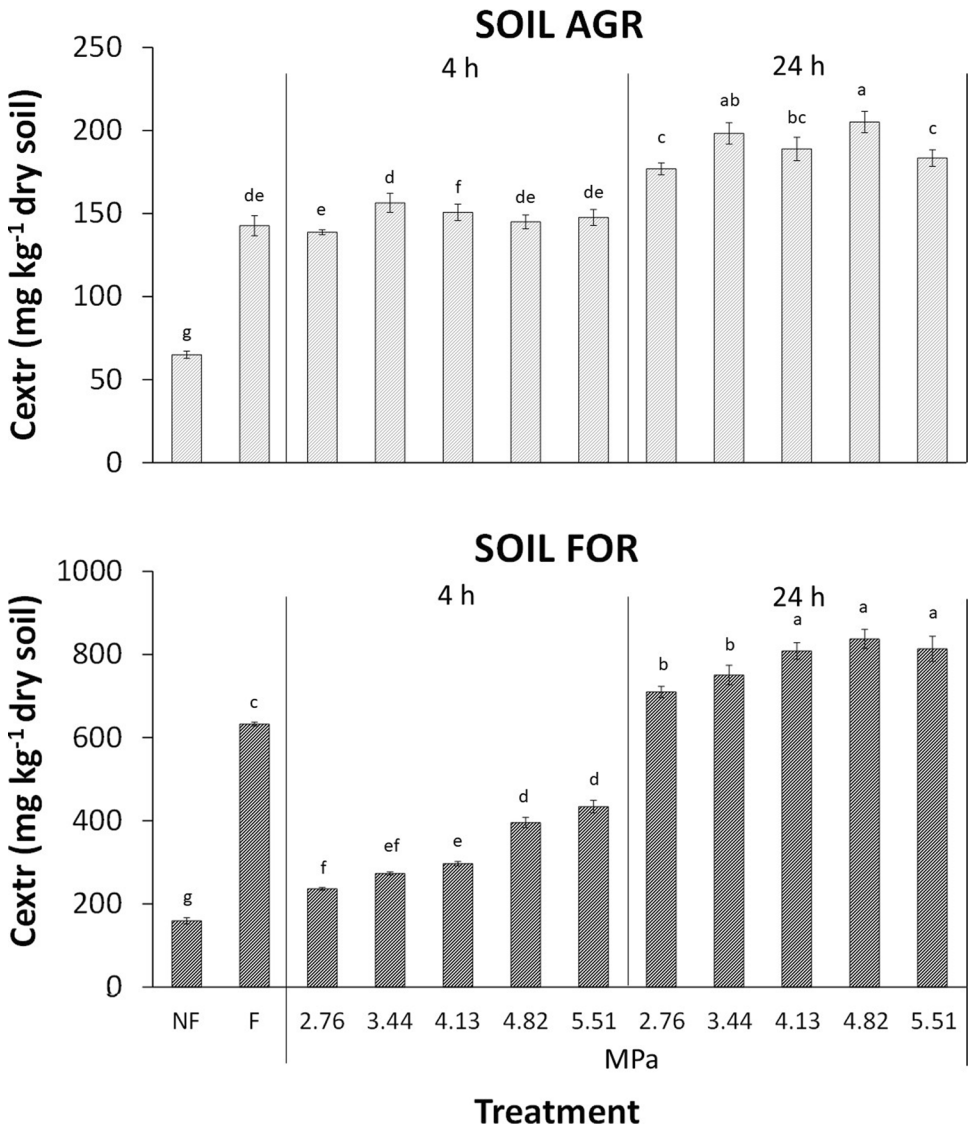

Fig. 2 KCl-extractable C from AGR and FOR soils subjected to various high CO_2 pressures (CO2HP, 2.76, 3.44, 4.13, 4.82 and 5.51 MPa) for 4 or 24 h. NF, not fumigated (control); F, $CHCl_3$ fumigated. Values represent means ± SD ($n = 4$). Different lowercase letters indicate significant differences at $p < 0.05$

Table 3 One-way ANOVA for the three experiments using AGR and FOR soils

		EXP I	EXP II	EXP III
AGR	Fisher value (F)	1616	65.06	351.5
	Pr(> F)	$<2*10^{-16}$	$<2*10^{-16}$	$<2*10^{-16}$
	d.f.* Treatments	7	11	8
	d.f.* Residuals	16	36	27
FOR	Fisher value (F)	290.5	254	213
	Pr(> F)	$1.2*10^{-15}$	$<2*10^{-16}$	$<2*10^{-16}$
	d.f.* Treatments	7	11	8
	d.f.* Residuals	16	36	27

The treatments were: control, $CHCl_3$-fumigated, CO2HP at five pressures (from 2.76 Mpa to 5.51 Mpa) and seven durations (from 2 to 32 h), N2HP at 4.13 Mpa pressure and three durations (16 h, 24 h, 32 h). Tables 4 and 5 show the specific experimental factors of each of the three experiments. Pr (> F) indicates the occurrence probability of the null hypothesis

*Degrees of freedom

almost twice the value of the $CHCl_3$ fumigation-incubation method, whereas the estimated MBC value of FOR soil at 4.13 MPa CO_2 pressure increased with the duration and was up to 75.2% higher than with CFI method (Tables 4 and 5).

It may be speculated that the evolved CO_2 flush of the CO2HP treated soils can also have an abiotic origin, instead of totally deriving from the mineralization of lysed microbial cells. The CO_2 dissolved in water, the quantity of which depends on its partial pressure, reacts with the water molecule to form carbonic acid, which is a weak acid, according to the reaction

$$CO_2(aq) + H_2O \rightleftharpoons H_2CO_3, \text{with } [H_2CO_3]/[CO_2]_{aq} = 1.7 \times 10^{-3} \text{ mol/L(25°C)},$$

i.e. only one molecule of CO_2 dissolved in water every 588 (Garcia-Gonzalez et al. 2007). Moreover, the CO_2 dissolved in water was likely lowered during the high pressurization because the vessel was incubated at 40 °C and the gas hydrosolubility decreases by increasing temperature.

Table 4 Mean, standard deviation ($n=4$) and variation coefficient values of CO_2-C cumulated during 10-d incubation (Exp I-CFI) or values of KCl-extractable C (Exp II-CFE and Exp III-CFE) for AGR soil following the framework reported in Table 2

Pressure value or duration		mg C kg^{-1} dry soil	Standard deviation	CV (Coefficient of variation, %)	MBC* (mg kg^{-1})
EXP I—CFI					
	NF	34.8	0.4	1.2	
	F	122.5	3.9	3.2	194.9
4.13 Mpa – N_2	16 h	25.1	1.8	7.2	n.d
	24 h	31.0	0.5	1.6	n.d
	32 h	34.7	3.1	8.9	n.d
4.13 Mpa – CO_2	16 h	204.8	5.7	2.8	377.8
	24 h	183.8	4.0	2.2	331.1
	32 h	208.2	5.1	2.4	385.2
LSD ($p<0.05$)		6.2			
EXP II—CFE					
	NF	65.0	4.3	6.6	
	F	142.7	12.1	8.5	205.1
4 h – CO_2	2.76 Mpa	138.7	2.9	2.1	194.6
	3.44 Mpa	156.4	11.4	7.3	241.4
	4.13 Mpa	150.7	9.9	6.6	226.3
	4.82 Mpa	145.0	8.3	5.7	211.1
	5.51 Mpa	147.6	9.6	6.5	218.1
24 h – CO_2	2.76 Mpa	176.9	7.2	4.1	295.4
	3.44 Mpa	198.3	12.8	6.5	351.8
	4.13 Mpa	188.9	14.1	7.5	327.1
	4.82 MPa	205.0	12.8	6.2	369.7
	5.51 MPa	183.4	10.1	5.5	312.6
LSD ($p<0.05$)		14.6			
EXP III—CFE					
	NF	54.8	4.1	7.5	
	F	111.3	3.4	3.1	149.3
3.44 MPa – CO_2	2 h	106.8	2.2	2.1	137.4
	4 h	124.3	2.0	1.6	183.6
	6 h	117.4	8.0	6.8	165.3
	8 h	137.6	5.8	4.2	218.6
4.13 MPa – CO_2	16 h	131.8	8.4	6.4	203.3
	24 h	192.5	7.8	4.1	363.5
	32 h	253.1	6.5	2.6	523.7
LSD ($p<0.05$)		8.7			

NF Not fumigated (control); *F* $CHCl_3$-fumigated

*Mean MBC values were calculated for EXP I (CFI method) by the relationship of Jenkinson (1988), while for EXP II and III (CFE method) by the relationship of Vance et al. (1987). See text for details. n.d., not detectable

On the other hand, the CO_2 deriving from the following multiple equilibria $CO_2 \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- \rightleftharpoons CO_3^{2-}$ was negligible because: a) both soils had a pH close to neutrality and thus a significant percentage of the formed carbonic acid immediately dissociated into bicarbonate; b) the CO_2 derived by the above multiple equilibria was likely dispersed during the depressurization phase and before the soil incubation.

Comparison between the CO_2 high pressurization (CO2HP) and the CFE method in lysing soil microbial cells (Experiments II and III)

As mentioned above, the C flux produced during incubation of CO2HP soils could be partially derived from abiotic processes or from the CO_2 trapped in the soil pores even after depressurization, and then slowly released during the

Table 5 Mean, standard deviation ($n=4$) and variation coefficient values of CO_2-C cumulated during 10-d incubation (Exp I-CFI) or values of KCl-extractable C (Exp II-CFE and Exp III-CFE) for FOR soil following the framework reported in Table 2

Pressure value or duration		mg C kg^{-1} dry soil	Standard deviation	CV (Coefficient of variation, %)	MBC* (mg kg^{-1})
EXP I—CFI					
	NF	222.7	3.9	1.8	
	F	626.1	24.1	3.8	896.6
4.13 MPa – N_2	16 h	274.8	18.6	6.8	115.9
	24 h	315.7	25.1	8.0	206.7
	32 h	361.6	7.0	1.9	308.8
4.13 MPa – CO_2	16 h	710.7	32.5	4.6	1084.4
	24 h	790.6	28.0	3.5	1262.1
	32 h	929.5	26.6	2.9	1570.6
LSD ($P<0.05$)		39.5			
EXP II—CFE					
	NF	159.4	15.0	9.4	
	F	632.5	9.2	1.5	1249.1
4 h – CO_2	2.76 MPa	236.2	6.8	2.9	202.7
	3.44 MPa	273.2	7.7	2.8	300.5
	4.13 MPa	296.8	10.1	3.4	362.7
	4.82 MPa	395.7	24.9	6.3	623.9
	5.51 MPa	434.1	29.3	6.7	725.3
24 h – CO_2	2.76 MPa	709.8	27.3	3.8	1453.0
	3.44 MPa	750.6	45.4	6.0	1560.7
	4.13 MPa	808.4	39.9	4.9	1713.3
	4.82 MPa	837.2	46.2	5.5	1789.3
	5.51 MPa	813.6	60.5	7.4	1727.0
LSD ($P<0.05$)		45.9			
EXP III—CFE					
	NF	158.7	12.5	7.9	
	F	689.1	27.9	4.0	1400.3
3.44 MPa – CO_2	2 h	375.0	42.7	11.4	571.2
	4 h	403.2	20.7	5.1	645.6
	6 h	561.1	33.6	6.0	1062.4
	8 h	578.0	30.5	5.3	1107.0
4.13 MPa – CO_2	16 h	811.5	31.4	3.9	1723.4
	24 h	914.7	52.9	5.8	1995.8
	32 h	1129.3	16.2	1.4	2562.4
LSD ($P<0.05$)		59.3			

NF Not fumigated (control); *F* $CHCl_3$-fumigated

*Mean MBC values were calculated for EXP I (CFI method) by the relationship of Jenkinson (1988), while for EXP II and III (CFE method) by the relationship of Vance et al. (1987). See text for details. n.d., not detectable

10 days of soil incubation. In order to verify this potential drawback of the method, we performed a second experiment (Experiment II), comparing the CO2HP with the CFE method, thus determining the KCl-extracted organic C (C_{extr}) produced after $CHCl_3$ fumigation or after CO_2 pressurization/depressurization (Tables 3, 4, and 5).

The C_{extr} of the AGR soil pressurized with CO_2 at five different high pressure values (from 2.76 to 5.51 MPa) for 4 h was not significantly different compared to the C_{extr} of the $CHCl_3$ fumigated soil, thus suggesting that at these conditions the amount of lysed microbial cells was similar to the $CHCl_3$ fumigation. On the contrary, the C_{extr} by the same five high pressurizations but lasting for 24 h, gave an increase of 33.5%, as an average, compared to the fumigated soil (Table 4; Fig. 2). The C_{extr} fluxes of the FOR soil after pressurization for 4 h were always significantly lower than

the values of $CHCl_3$ fumigated soil, although they increased linearly from 2.76 up to 4.82 MPa, whereas the further pressure increases to 5.51 MPa did not enhance the C_{extr} (Table 5; Fig. 2). Nevertheless, the CO_2 pressurization from 2.76 to 5.51 MPa for 24 h gave C_{extr} values always higher than C_{extr} by chloroform fumigation. The highest C_{extr} value occurred again at 4.82 MPa, although it was not significantly different from those observed at 4.13 and 5.51 MPa; the three high pressurizations ranging from 4.13 to 5.51 MPa for 24 h increased the C_{extr} of 29.6%, as an average, compared to the fumigated soil (Table 5; Fig. 2). Likely, the optimal high pressure x duration combination for the CO2HP method to be used for organic C-rich soils with high MBC should be equal or higher than 4.13 MPa for 24 h.

By subtracting the C_{extr} of the untreated control from either the C_{extr} of $CHCl_3$ fumigated soil or the CO2HP treated soil and applying the relationship by Vance et al. (1987) for estimating MBC with CFE method [$MBC = (C_{extr\ Fumigated/CO2HP}$ minus $C_{extr\ Control}) \times 2.64$], the 24 h pressurization at 4.82 MPa of the AGR soil gave an 80% higher value than that of the $CHCl_3$ fumigation (Table 4). The estimate of MBC of the FOR soil subjected to the CO2HP at 4.82 MPa for 24 h was 43% higher than the value of the CFE (Table 5).

In order to find the optimal combination of CO_2 pressure x duration for increasing microbial cell lysis (Tables 3, 4, and 5), we carried out a third experiment (Exp III) with AGR and FOR soils by testing two CO_2 pressures (3.44 and 4.13 MPa) and seven durations (from 2 to 32 h, Fig. 3). The AGR soil, with a low MBC estimated by CFE method, showed that the pressurization at 3.44 MPa for 2 h gave a value of MBC comparable to that of CFE method (Tab. 4; Fig. 3). Prolonging the duration from 2 to 8 h slightly increased the C_{extr}, which was 23.6% higher than that of CFE

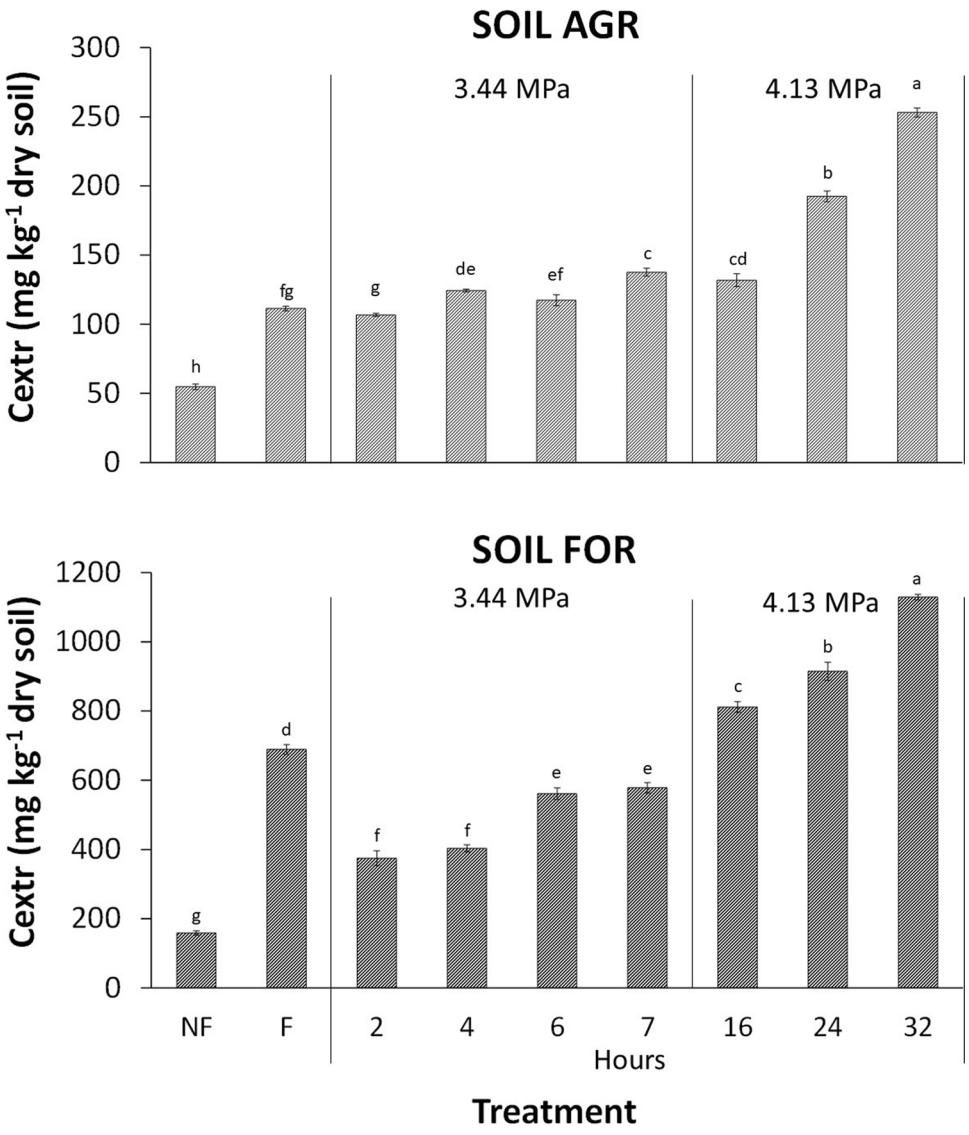

Fig. 3 KCl-extractable C from AGR and FOR soils subjected to two constant high CO_2 pressures (CO2HP, 3.44 or 4.13 MPa) for 2, 4, 6, 8, 16, 24 or 32 h. NF, not fumigated (control); F, $CHCl_3$ fumigated. Values represent means ± SD ($n = 4$). Different lowercase letters indicate significant differences at $p < 0.05$

method. However, the C_{extr} of the CO2HP at 4.13 MPa for 16 h duration was equal to that of 8 h duration, while the 24 and 32 h durations at 4.13 MPa increased the C_{extr} by 46% and 92%, compared to that at 16 h duration, respectively (Tab. 4; Fig. 3). Likely, it seems that with low soil MBC the pressurization duration plays a more important role in lysing microbial cells than the value of the CO_2 pressure.

Both Experiments II and III using FOR soil, i.e. with the highest MBC, showed that the durations up to 8 h and at 3.44 MPa of CO_2 lysed less microbial cells than CFE method; the difference between the two methods decreased by increasing the duration (Table 5; Fig. 3). However, at 4.13 MPa pressure for 16 h duration, the C_{extr} was significantly higher by 17.8% than that of CFE method, while the increase at 24 and 32 h was higher by 32.7% and 63.9%, respectively. Likely, in soils with high MBC, both CO_2 pressure level and its duration affected the microbial cells lysis. It may be speculated that in soils with high MBC there is a broad spectrum of cells differing in their resistance to lysing agents. This may explain the linear progressive increase in C_{extr} of the FOR soil by increasing both pressurization duration and applied CO_2 pressure (Table 5; Fig. 3).

The following two hypotheses are proposed considering our results: 1) the multitude of soil microbial species shows different resistances to lysing agents; likely by increasing the MBC and the biodiversity, the cell resistance also increases; 2) the highest values of CO2HP pressure and duration might have lysed the quiescent or encysted microbial cells and/or made KCl-extractable some additional soil organic C not ascribable to living organic matter.

Conclusions

Our proposed physical approach based on high pressurization by CO_2 (CO2HP) was likely more effective than $CHCl_3$ fumigation in lysing soil microbial cells of two soils differing in organic matter content and MBC values. The soil with higher organic matter content and microbial biomass required higher CO_2 pressures and longer pressurization periods than the soil with lower organic matter. Among the tested combinations, the most successful was CO_2 pressure at 4.13 MPa for 32 h. However, our results do not exclude that the proposed CO2HP method may release some additional soil organic C not ascribable to living organic matter. Therefore, further studies are needed using several soils with different chemical and physical properties. Future research should also analyze the composition of organic C extracted after the CO2HP treatment to ascertain its microbial origin. Finally, the proposed method should be tested with soils added with known abundances of microbial cells labeled with ^{13}C and/or ^{15}N. Microbial diversity of soil before and after the CO2HP treatment should be also tested. These studies can give insights into the C and/or N made extractable by the CO2HP and if it derives from soil microbiome. These studies may also be the basis to set up a new method to determine the soil microbial biomass.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00374-023-01725-5.

Acknowledgements We thank the Editor-in-Chief very much, Prof. Paolo Nannipieri, for the numerous suggested modifications and integrations of the manuscript, which significantly contributed to improving it. This work was partially supported by the University of Palermo's PhD course "Biodiversity in Agriculture and Forestry" [Code D083] funded by the Ministero dell'Università e della Ricerca (MUR, Italy).

Funding Open access funding provided by Università degli Studi di Palermo within the CRUI-CARE Agreement.

Data Availability During the manuscript submission procedure, the corresponding author declared that all the experimental data of this work will be provided upon request.

Declarations

No part of this manuscript has been previously published or submitted elsewhere and no conflict of interest exits in its submission.

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