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Pierre P. Renault, Meriem M. Ben Sassi, Annette A. Berard

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Improving the MicroResp™ substrate-induced respiration method by a more complete description of CO_2 behavior in closed incubation wells

P. Renault [a,*], M. Ben-Sassi [a,b,1], A. Bérard [a,1]

[a] INRA, UMR 1114 EMMAH, Domaine St-Paul, Site Agroparc, 84914 Avignon Cedex 9, France
[b] ADEME, 20 avenue du Grésillé, BP 90406, F-49004 Angers Cedex 01, France

A B S T R A C T

The MicroResp™ method allows soil respiration and microbial community physiological profiles to be determined colorimetrically in microplates. This method, however, neglects CO_2 storage in the agar gel carrying the colorimetric indicator, and calcite dissolution associated with CO_2-induced change in soil solution pH. Our objective was to improve the method by taking into account CO_2 in the gel in the calculation of microbial respiration, describing the effect of microbial CO_2 on the pH of the soil solution and calcite dissolution, and checking whether CO_2 distribution among calcite, soil solution, air and gel is near equilibrium after incubation. We propose a thermodynamic equilibrium model describing (a) distribution of CO_2 among calcite, soil solution, gel and air, (b) dissociations of water, carbonic acid, cresol red, and substrates in the gel and soil solution, (c) exchange of adsorbed cations with H_3O^+ in the gel, and (d) calcite dissolution in soil. In-gel experiments were designed to calibrate the model, quantify the rate of CO_2 exchange with air, and compare conservation procedures. On-soil experiments were designed to check whether calcite dissolution is near equilibrium and whether the model predicts the effect of CO_2 on the pH of the solution. In-microplate experiments were designed to assess the effects of incubation period and soil quantity on estimated microbial respiration. The model can describe the distribution and speciation of CO_2 in the gel, the soil solution and the air space of each microplate well. Initial properties of the gel vary with storage: soda lime partly extracts CO_2 supplied as $NaHCO_3$, and dries out the gel, which can skew the calibration. When incubation is over, the proportion of microbial CO_2 in the gel is higher at lower microbial respiration. Incubations shorter than 4 h underestimate microbial respiration due to the slow diffusion of CO_2 in the gel. CO_2 in the soil solution cannot be overlooked; it decreases the soil pH and may promote calcite dissolution in calcareous soil. It is important to precisely estimate initial CO_2 air fraction and to control temperature, which affects both thermodynamic constants and microorganisms.

Keywords:
Soil
Substrate-induced respiration
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1. Introduction

Soil microbial respiration that consumes O_2 may regulate CH_4 and N_2O greenhouse gas emissions and reduction of metals (Lahlah et al., 2009; Parry et al., 2000), organic matter turnover (Schlesinger and Andrews, 2000), and acid–base, complexation and precipitation/dissolution reactions (Dassonville et al., 2004; Lahlah et al., 2009). Profiling microbial respiration on various substrates provides an insight into microbial functional diversity (Chapman et al., 2007; Degens and Harris, 1997).

Microbial respiration has been assessed by O_2 consumption (Garland et al., 2003), by CO_2 production (Cheng and Coleman, 1989), and by coupling the two (Sierra and Renault, 1995). Respiration is more often characterized by CO_2 measurements, which are easier and sensitive (Dilly, 2001). However, many characterizations suffer from common limitations (long gas analysis, large volumes required, complex set-up).

The MicroResp™ method is a miniaturized substrate-induced respiration method that overcomes these limitations (Campbell et al., 2003), and offers a wide range of applications (Ben Sassi et al., 2012; Tlili et al., 2011): it couples the microplate format of the Biolog™ test restricted to cultivable micro-organisms (Garland and Mills, 1991; Stefanowicz, 2006) with the measurement of CO_2 air fraction according to the work of Rowell (1995) on indicator dyes in agar gel. In each closed well of a 96-well microplate, moist soil with or without C substrates is incubated for 6 h in the presence of an agar gel carrying cresol red as indicator dye (Campbell et al.,

* Corresponding author at: INRA, UMR 1114 EMMAH (Environnement Méditerranéen et Modélisation des Agro-Hydrosystèmes), Domaine Saint-Paul, Site Agroparc, 84914 Avignon Cedex 9, France. Tel.: +33 4 32 72 22 23; fax: +33 4 32 72 22 12.
E-mail addresses: pierre.renault@avignon.inra.fr (P. Renault), meriem.bensassi@avignon.inra.fr (M. Ben-Sassi), annette.berard@avignon.inra.fr (A. Bérard).
[1] Fax: +33 4 32 72 22 12.

2003). The method only takes into account CO_2 in the well air space, which is assumed to be of microbial origin (Campbell et al., 2003). The CO_2 air fraction is estimated from gel absorbance at 570 nm (Rowell, 1995). The MicroResp™ method has been widely used on soils with pH < 7 (Burton et al., 2010; Campbell et al., 2003; Gonzalez-Quiñones et al., 2009; Lalor et al., 2007; Macdonald et al., 2009; Wakelin et al., 2008; Yao et al., 2011; Zhou et al., 2011) and occasionally used on soils with higher pH and calcite (Bérard et al., 2011, 2012; Saul-Tcherkas and Steinberger, 2009; Wakelin et al., 2008), although the technical manual recommends restricting it to soil with pH < 7 (Cameron, 2007). The method was modified by Oren and Steinberger (2008a) to include CO_2 in the soil solution and the effects of substrates on the soil solution pH and calcite dissolution, but without considering the effects of CO_2 on calcite dissolution and on the pH of the solution (Stumm and Morgan, 1996). Their improvements, which require measurements on sterilized soil and evaluation of substrate impact on soil pH, have not been widely adopted to date (Garcia-Palacios et al., 2011; Oren and Steinberger, 2008b). To our knowledge, CO_2 in the agar gel has never been taken into account.

Thus the MicroResp™ method still suffers from limitations. First, the volume of in-well gel (0.15 mL) cannot be neglected given the volumes of soil solution (about 0.12 mL) and air space (about 1 mL). The pH of the gel (from 7 to 9 or more) magnifies the problem with more HCO_3^- and sometimes CO_3^{2-} than $H_2CO_3^*$ (i.e., aqueous CO_2 and actual H_2CO_3) in the gel solution (Stumm and Morgan, 1996) and in solutions of calcareous soils (Ström et al., 2001). Second, failing to allow for the fact that increasing the CO_2 air fraction decreases the pH of the soil solution (Stumm and Morgan, 1996) ultimately overestimates the amount of CO_2 in the solution. For calcareous soils, increasing the CO_2 air fraction may also induce calcite dissolution (Stevenson and Verburg, 2006; Stumm and Morgan, 1996; Tamir et al., 2011), leading to an abiotic production of total CO_2 in the soil solution as $H_2CO_3^*$, HCO_3^- and CO_3^{2-}, even without acidic substrates. Third, no check has been made to determine whether the transfers between calcite, soil solution, air and gel can be considered to be in equilibrium. Failure to approach equilibrium would make calibration impossible. In addition, although tackled by Oren and Steinberger (2008a), it remains important to know whether most of the calcite dissolution caused by acidic substrate occurs before or after microplate clamping.

Accordingly, the objective of this study was to improve the MicroResp™ method by (i) including CO_2 in the gel carrying the colorimetric indicator, (ii) refining microbial CO_2 in soil by describing its effects along with those of substrates and calcite on the pH of the solution, and quantifying calcite dissolution, and (iii) checking whether CO_2 distribution among calcite, soil solution, air space and the gel is near-balanced after 6 h incubation.

2. Materials and methods

The study combines modeling of CO_2 distribution during MicroResp™ incubations based on geochemical equilibrium with measurements performed on the gel, on a calcareous soil, and on both combined in the MicroResp™ experimental design. For certain incubations, the soil was sterilized and/or supplied with substrate (e.g., glucose, glucosamine-HCl and Na_2-malate as neutral, acid and alkaline substrates, respectively).

2.1. Background of the MicroResp™ method

The MicroResp™ experimental design consists of two 96-well microtiter plates placed face-to-face (Campbell et al., 2003). One of the plates, with a capacity of 300 μL · well^{-1}, holds 150 μL · well^{-1} of an agar gel (10 g · L^{-1}) enriched in KCl (0.15 mol · L^{-1}), $NaHCO_3$ (2.5 mmol · L^{-1}) and cresol red dye (32.7 μmol · L^{-1}) to estimate CO_2 air fraction based on gel absorbance at 570 nm. After preparation, this plate is generally conserved for 7 d in a closed environment with soda lime and water, without and with a protective Parafilm during the first day and the 6 following days, respectively. The other plate, with a capacity of 1.2 mL · well^{-1}, holds about 0.45 g · well^{-1} of moist soil with or without substrate. Just before incubation, the plate containing the gel is read with an absorbance microplate reader. The two plates are then sealed together with a silicone rubber gasket with interconnecting holes. After 6 h of incubation, the plates are separated and the plate containing the gel is immediately re-read. We have adapted the method to 24-, 12- and 6-well microplates with silicone rubber gaskets manufactured in our lab to seal identical plates.

2.2. Modeling of in-well CO_2 distribution and its effect on gel absorbance

The modeling study aimed to describe the relationships between gel absorbance, CO_2 air fraction, amounts of total CO_2 in both gel and soil solution ($H_2CO_3^*$ (i.e., aqueous CO_2 and actual H_2CO_3), HCO_3^-, CO_3^{2-}), and calcite dissolution. Thermodynamic equilibrium was considered for transfers between soil, air space and gel, for acid–base reactions in solutions, for exchange of adsorbed cations with H_3O^+ in the gel solution, and for calcite dissolution. For the gel and the soil solution, mass action laws were combined with equations relating the balance of ionic charges and the total quantity of either cresol red or substrate. Mass action laws were written with K' constants combining H_3O^+ activity and concentrations of other species. In soil solution where ionic strength $I \approx 15$ mmol · L^{-1} (Table 3), activities are almost equal to concentrations, so K' constants were assumed to be equal to K constants combining activities only. In gel solution where $I \approx 150$ mmol · L^{-1}, K' constants were estimated from K^* constants combining concentrations only or K constants using species activities only. Deviations between K and K^* are consistent with independent estimates of activity coefficients.

CO_2 distribution among air space, gel and soil solution was described by Henry's law. Henry constants, k_H for water and soil solution and k_H' for saline solution and gel, were estimated from the equations of Harned and Davis (1943) and Weiss (1974), respectively (Table 1). Deviation between k_H and k_H' is consistent with the increase in $H_2CO_3^*$ activity coefficient in the gel calculated by the Pytkowicz's (1975) equation (Table 1). The fugacity coefficient of CO_2 in air was set to 1 (DOE, 1994). H_2O activity in gel and soil solution was set to 1 (Stumm and Morgan, 1996). K_w and K_w^* constants for H_2O ion product were estimated from the equations of Harned and Owen (1958) and Millero (1995), respectively (Table 1). K_{a1} and K_{a2} constants for the first and second dissociation of $H_2CO_3^*$, respectively, were estimated from the equations of Weiss (1974) (Table 1), and K_{a1}^* and K_{a2}^* constants were estimated from the equations of Millero et al. (2007) for NaCl solutions (Table 1), since they differ from those for seawaters (Millero et al., 2006). In the gel, cresol red, symbolized by H_2CR, dissociates into HCR^- and CR^{2-}. The first dissociation is complete, as its pK_{CR1} constant is about 1.1 (El Nahhal et al., 2012; French et al., 2002; Heger et al., 2006; Smith and Matachek, 2002), and the K_{CR2} constant of the second dissociation was estimated by the equation of French et al. (2002) (Table 1). K_{CR2}' was deduced from K_{CR2} and HCR^- and CR^{2-} activity coefficients. An exchange of adsorbed cations with protons in the gel was taken into account empirically (see Eq. (7) below). Activity coefficients of ions in the gel solution were calculated by the Davies equation (Table 1) (Pankow, 1991), except for CR^{2-} where the two negative charges have to be considered independently, as is the case with other diprotic acids used as indicator dyes (Salvatore et al., 1986). We thus extended the relationship that uses specific interaction theory for bromophenol blue (Salvatore et al., 1986) in order to assess realistic variations in the pK_{CR2} of the second dissociation of cresol red at 25 °C (Table 1). In soil, dissociations of acid and alkaline substrates

Table 1
Thermodynamic parameters involved in the calculation of geochemical equilibrium. R is the gas constant (8.31441 J · mol^{-1} · K^{-1}), T the temperature (K), P the air pressure (Pa), and S the salinity of the agar gel solution (expressed as equivalent g NaCl · kg^{-1})).

Constant	Equation	References
k_H: Henry's constant for CO_2 solubilization in pure water	$\frac{1}{k_H} = 10^{\left(-\left(\left(\frac{-2622.38}{T}\right)\right)+(-0.0178471\times T)+15.5873\right)} \times \frac{1000\times R\times T}{P}$	Harned and Davis (1943)
k_H': Henry's constant CO_2 solubilization in saline water	$\frac{1}{k_{H'}} = \exp\left(\begin{array}{l}\frac{9345.17}{T} + 23.3585 \times \ln\left(\frac{T}{100}\right) - 60.2409 + \\ S \times \left(0.023517 + 0.00470356 \times \left(\frac{T}{100}\right)^2 - 0.023656 * \left(\frac{T}{100}\right)\right)\end{array}\right) \times \frac{1000\times R\times T}{P}$	Weiss (1974)
$\gamma_{H_2CO_3*}$: activity of $H_2CO_3^*$ in saline water	$\gamma_{H_2CO_3*} = 10^{0.00035863+(0.00196297\times S)}$	Pytkowicz (1975)
K_w: ion product of water in pure water	$pK_w = -\log_{10}(K_w) = \frac{4470.99}{T} - 6.0875 + 0.01706 \times T$	Harned and Owen (1958)
K_w': ion product of water in saline water	$pK_{w*} = -\log(K_{w*}) = -\log\left(\exp\left(\begin{array}{l}148.9802 + \left(\frac{-13847.26}{T}\right) + (-23.6521 \times \ln(T)) \\ + \left(\left(-5.977 + \frac{118.67}{T} + 1.0495 \times \ln(T)\right) \times \left(S^{0.5}\right)\right) \\ + (-0.01615 \times S)\end{array}\right)\right)$	Millero (1995)
K_{a1}: first dissociation constant of carbonic acid in pure water	$pK_{a1} = -\log(K_{a1}) = -114.3106 + \left(\frac{5773.67}{T}\right) + (17.779524 \times \ln(T))$	Weiss (1974)
K_{a1}^*: first dissociation constant of carbonic acid in saline water	$pK_{a1*} = -\log(K_{a1*}) = pK_{a1} + A_1 + \frac{B_1}{T} + (C_1 \times \ln(T))$ with : $A_1 = 35.2911 \times m^{0.5} + 0.8491 \times m - 0.32 \times m^{1.5} + 0.055 \times m^2$ $B_1 = -1583.09 \times m^{0.5}$ $C_1 = -5.4366 \times m^{0.5}$	Millero et al. (2007)
K_{a2}: second dissociation constant of carbonic acid in pure water	$pK_{a2} = -\log(K_{a2}) = -83.2997 + \left(\frac{4821.38}{T}\right) + (13.5962 \times \ln(T))$	Weiss (1974)
K_{a2}^*: second dissociation constant of carbonic acid in saline water	$pK_{a2*} = -\log(K_{a2*}) = pK_{a2} + A_2 + \frac{B_2}{T} + (C_2 \times \ln(T))$ with : $A_2 = 38.2746 \times m^{0.5} + 1.6057 \times m - 0.647 \times m^{1.5} + 0.113 \times m^2$ $B_2 = -1738.16 \times m^{0.5}$ $C_2 = -6.0346 \times m^{0.5}$	Millero et al. (2007)
K_{CR2}: second dissociation constant of cresol red in pure water	$pK_{CR2} = -\log(pK_{CR2}) = 2.049 + \left(\frac{913.4}{T}\right) + (1.266 \times \log(T))$	French et al. (2002)
$\gamma_{CR^{2-}*}$: activity coefficient for cresol red anion CR^{2-} with 2 e$^-$ charges	$\gamma_{CR^{2-}} = \left(2 \times \left(-0.5107 \times \left(\frac{\sqrt{I}}{1+(1.5\times\sqrt{I})}\right)\right)\right) - (-0.178 * m)$	Empirical equation deduced from
γ_i^*: Davis equation of activity coefficients for ionic compound i, having a charge z_i, except for CR^{2-}	$\log(\gamma_i) = -A \times {z_i}^2 \times \left(\left(\frac{\sqrt{I}}{1+\sqrt{I}}\right) - 0.2 \times I\right)$	Pankow (1991)

were taken into account: reactions considered for substrates containing more than one acid or alkaline functional group were chosen based on their pK and the pH of the soil (Table 2). For calcareous soils, the solubility constant K_S for calcite was set to 8.3, mirroring data reported by Stumm and Morgan (1996) at 25 °C. The simulated increase of $[Ca^{2+}]$ during incubations makes it possible to assess the release of abiotic CO_2. Geochemical simulations were performed on soils at various CO_2 air fractions for different substrates and amounts of substrates. pH was adjusted so as to cancel the sum of charges of ions in solution. The variation $\Delta[CO_2]_{tot}$ in total CO_2 concentration in the solution ($H_2CO_3^*$, HCO_3^- and CO_3^{2-}) minus the increase $\Delta[Ca^{2+}]$ in Ca^{2+} amount for calcareous soils between the considered and 0.04% CO_2 air fractions was then fitted by empirical functions:

$$\Delta[CO_2]_{tot} - \Delta\left[Ca^{2+}\right] = a \times \left([CO_2]_a\right)^b - c, \quad (1)$$

Table 2
Retained acid–base reactions for substrates used in MicroResp™ measurements for soils whose pH(water) is about 7.5–8.5.

Substrate supplied	Acid form	Charge of the acid form	Charge of the alkaline form	pKa
Glucosamine-HCl	$C_6H_{13}NO_5$–HCl	+1	0	11.5
Na_2-malate	Na_2–$C_4H_4O_5$	−1	−2	5.1
Alanine	$C_3H_7NO_2$	0	−1	9.71
Glycine	$C_2H_5NO_2$	0	−1	9.58

where the three parameters a, b and c are specific to each combination of soil, substrate type and substrate amount. Considering initial and final CO_2 air fractions eliminates the reference to 0.04% CO_2 that was introduced to facilitate the adjustment of empirical functions. For each soil and each substrate, parameters a, b and c can be obtained by fitting Eq. (1) to geochemical simulations based on equations proposed in this paper. It requires having first at 0.04% CO_2 rough estimates of the sum of charges of ions other than H_3O^+, HO^-, HCO_3^- and CO_3^{2-} in the soil solution and, for calcareous soils, the contribution of Ca^{2+} to this sum. These values can be estimated from chemical analysis and by fitting simulated pH to pH measured on soil slurries at different CO_2 partial pressures.

The measured dimensionless absorbance A_{pH} is the sum of the absorbances of empty microplate A_e, agar gel without cresol red A_g, and cresol red A_{CR}:

$$A_{pH} = A_e + A_g + A_{CR}. \quad (2)$$

A_{CR} may be expressed as a function of HCR^- and CR^{2-} concentrations:

$$A_{CR} = \varepsilon_{HCR^-} \times l_g \times [HCR^-] + \varepsilon_{CR^{2-}} \times l_g \times \left[CR^{2-}\right], \quad (3)$$

where l_g is the path length of light through the gel (m), and ε_{HCR-} and $\varepsilon_{CR^{2-}}$ the molar absorptivities of HCR^- and CR^{2-} (L · mol^{-1} · m^{-1}), respectively. Combining Eqs. (2) and (3) with a mass action law for the second dissociation of cresol red and a related equation for the

stability of total cresol red amount leads to the following estimate of gel solution pH:

$$\mathrm{pH} = \mathrm{p}K_{CR2}' + \log_{10}\left(\frac{A_{\mathrm{pH}} - \left(A_e + A_g\right) - \left(A_{\max} \times \left(\frac{\varepsilon_{HCR^-}}{\varepsilon_{CR^{2-}}}\right)\right)}{A_{\max} + \left(A_e + A_g\right) - A_{\mathrm{pH}}}\right), \quad (4)$$

where $A_{\max}\left(= \varepsilon_{CR^{2-}} \times l_g \times [CR_{tot}]\right)$ is the absorbance of cresol red when it is in CR^{2-} form only. The ratio $\varepsilon_{HCR^-}/\varepsilon_{CR^{2-}}$ was set to 0.0019, in line with Smith and Matachek (2002) at 574 nm. The concentration of $H_2CO_3^*$ in the gel solution may then be estimated as:

$$[H_2CO_3*] = \frac{\Delta q_g + \left(\frac{10^{-\mathrm{pH}}}{\gamma_{H_3O^+}}\right) + 10^{\mathrm{pH}-\mathrm{p}K_w'}}{10^{\mathrm{pH}-\mathrm{p}K_{a1}'} + \left(2 \times 10^{2\mathrm{pH}-(\mathrm{p}K_{a1}'+\mathrm{p}K_{a2}')}\right)}, \quad (5)$$

where Δq_g is the net charge of ions other than H_3O^+, HO^-, HCO_3^- and CO_3^{2-} in the gel solution (mol $c^+ \cdot L^{-1}$). It would be equal to the concentration $\left[Na^+\right]_{NaHCO_3}$ of Na^+ supplied as $NaHCO_3$ (mol $\cdot L^{-1}$) if agar did not affect the solution, which is not the case (Ferreira et al., 2012; Lahaye and Rochas, 1991; Scholten and Pierik, 1998):

$$\Delta q_g = \left[Na^+\right]_{NaHCO_3} + \Delta c^+, \quad (6)$$

where Δc^+ is the net concentration of charges (mol $c^+ \cdot L^{-1}$) of ions released by agar. Δc^+ increases with decreasing pH of the gel, as in other media (Renault et al., 2009), so we assumed the following equation:

$$\Delta c^+ = \Delta c^+_{\min} + \left(\Delta c^+_{\max} \times \left(1 - \frac{\left(\frac{\mathrm{pH}}{\mathrm{pH}_{1/2}}\right)^{\alpha}}{1 + \left(\frac{\mathrm{pH}}{\mathrm{pH}_{1/2}}\right)^{\alpha}}\right)\right), \quad (7)$$

where $\Delta c^+_{\min}$ is the concentration of charges released by agar at a high pH (mol $c^+ \cdot L^{-1}$), $\Delta c^+_{\max}$ the maximum concentration of charges released during acidification (mol $c^+ \cdot L^{-1}$), $pH_{1/2}$ the pH at which one half of the initially-adsorbed charges are released, and α an empirical constant related to the sharp or gentle release of charges with changes in pH around pH ≈ $pH_{1/2}$. $Q_g(CO_2)$, i.e., the amount of CO_2 stored in the gel (mol) as $H_2CO_3^*$, HCO_3^- and CO_3^{2-} is then easily estimated:

$$Q_g(CO_2) = V_g \times [H_2CO_3*] \times \left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{a1}'} + \left(2 \times 10^{2\mathrm{pH}-(\mathrm{p}K_{a1}'+\mathrm{p}K_{a2}')}\right)\right), \quad (8)$$

where V_g is the volume of the gel (L). While the CO_2 air fraction is initially the lab CO_2 air fraction not in equilibrium with the gel, its final value is assumed to be in equilibrium with the gel and estimated from Henry's law (Table 1):

$$[CO_2]_a = k_H' \times [H_2CO_3*]_g, \quad (9)$$

where subscripts a and g stand for air and gel, respectively. The amount of CO_2 stored in the air space is estimated as:

$$Q_a(CO_2) = V_a \times [CO_2]_a, \quad (10)$$

where V_a is volume of the air space (L). Thus variations in CO_2 quantities in both gel and air space are estimated from the amounts of CO_2 in these compartments at the start and the end of incubations, assuming that the gel is not in equilibrium with the air space or the soil solution at the start. By contrast, empirical functions (Eq. (1)) are used to estimate the variations $\Delta Q_s(CO_2)$ of amount of CO_2 in soil solution (mol) minus CO_2 released by calcite dissolution:

$$\Delta Q_s(CO_2) = V_s \times \left(\Delta[CO_2]_{tot,t=6h} - [CO_2]_{tot,t=0}\right), \quad (11)$$

where V_s is the volume of the soil solution (L). A negative value means that abiotic CO_2 production exceeds the total amount of CO_2 stored in the soil solution.

In addition, we assume that the variability in A_{pH} between wells of a given plate results from the variability in light path length l_g. Initial and final absorbances A_{pH} of each well were therefore replaced by absorbances A_{pH}' by taking into account the initial absorbance of the target well $A_{\mathrm{pH}}(t = 0)$ and the mean absorbance $\overline{A_{\mathrm{pH}}}(t = 0)$ across all the wells in a plate:

$$A_{\mathrm{pH}}' = A_e + \left(\left(\frac{\overline{A_{\mathrm{pH}}}(t=0) - A_e}{A_{\mathrm{pH}}(t=0) - A_e}\right) \times \left(A_{\mathrm{pH}} - A_e\right)\right). \quad (12)$$

In this way, A_{pH}, A_g and $A_{\max}$ can be replaced by A_{pH}', $\overline{A_g}$ and $\overline{A_{\max}}$, respectively, in Eq. (4). When $A_e \approx 0$, Eq. (12) approaches the correction of Campbell et al. (2003):

$$A_{\mathrm{pH}}' \approx \left(\frac{\overline{A_{\mathrm{pH}}}(t=0)}{A_{\mathrm{pH}}(t=0)}\right) \times A_{\mathrm{pH}}. \quad (13)$$

At about 80 °C, the gel is distributed in greater amounts than desired, i.e., 0.20, 0.94, 1.74 and 4.09 mL instead of 0.15, 0.664, 1.314 and 3.27 mL for 96-, 24-, 12- and 6-well plates, respectively, and mean gel thickness $\overline{l_g}$ (mm) varies with plates, i.e., 5.51, 4.68, 4.38 and 4.13 mm, for 96-, 24-, 12- and 6-well plates, respectively. Therefore, $\overline{A_g}$ and $\overline{A_{\max}}$ were estimated from measured values on 96-well microplates according to the following equations:

$$\overline{A_g}(n_w = i) = \left(\frac{\overline{l_g}(n_w = i)}{\overline{l_g}(n_w = 96)}\right) \times \overline{A_g}(n_w = 96), \quad (14a)$$

$$\overline{A_{\max}}(n_w = i) = \left(\frac{\overline{l_g}(n_w = i)}{\overline{l_g}(n_w = 96)}\right) \times \overline{A_{\max}}(n_w = 96), \quad (14b)$$

where n_w is the number of wells of the microplate considered.

Thus estimating the amount of microbial CO_2 produced during MicroResp™ incubations hinges on knowing the values of three parameters concerning absorbance (A_e, $\overline{A_g}$ and $\overline{A_{\max}}$) and four parameters concerning the exchange properties of the gel ($\Delta c^+_{\min}$, $\Delta c^+_{\max}$, $pH_{1/2}$, and α). All other parameters may be estimated from the literature.

2.3. Experimental approach

2.3.1. The soil

Measurements were performed on a calcareous cambisol (FAO classification) from the INRA Saint-Paul experimental station (43°91′ N, 4°88′ E) near Avignon. It was cultivated with peas in 2008, but since summer 2008 the soil lay bare. Annual rainfall is about 650 mm. About 20 kg of this soil was sampled in the first 10 cm depth at the edge of a 0.0075 ha experimental field on 8 December 2011. Its moisture was about 21.3 % wt/wt. The soil was air-dried for 4 d in the lab (14 % wt/wt residual moisture), mechanically crushed, sieved at 2 mm for soil analyses and at 2–3 mm for experiments, and stored at 4 °C in hermetically-sealed bags until the beginning of the experiments. Its properties, measured at the Laboratoire d'Analyse des Sols (LAS-INRA) in Arras (France), were as follows: 347 g kg^{-1} $CaCO_3$ and, after decarbonation, 323 g kg^{-1} clay; 259 g kg^{-1} silt; 41 g kg^{-1} sand; 13.2 g kg^{-1} organic C; 1.54 g kg^{-1} total N; 1.4 mg kg^{-1} $N\text{–}NH_4^+$; 101 mg kg^{-1} $N\text{–}NO_3^-$. Soil pH(water) and pH(KCl 1 M) were 8.51 and 7.85, respectively. Chemical properties of solutions for soil-to-solution mass ratios of 1, 2.5 and 5 (Table 3) were used to assess composition of the soil solution in MicroResp™ incubations. For geochemical simulations on this soil, we assumed that the sum of charges of ions other than H_3O^+, HO^-, HCO_3^- and CO_3^{2-} was equal to 4.6 mmol $c^+ \cdot L^{-1}$, including the initial concentration of Ca^{2+} equal to 6 mmol $c^+ \cdot L^{-1}$.

Table 3
Chemical properties of the solutions for mixtures of soil-to-solution mass ratios of 1, 2.5 and 5.

Dilution	pH(water)	Ca^{2+}	Mg^{2+}	K^+	NO_3^-	Cl^-
mmol · L^{-1}						
5	7.56	0.47	0.049	0.212	0.28	0.019
2.5	7.84	0.71	0.078	0.252	0.54	0.025
1	7.88	1.38	0.173	0.360	1.12	0.057
Limit for ω = 31%[a]	7.97	2.96	0.426	0.523	3.06	0.120

[a] Linear regression of Ln values.

2.3.2. Experiments on the gel

The agar product used in this work was the Merk 1614 product. Its properties are presented and compared with those of other market products in Scholten and Pierik (1998).

2.3.2.1. Calibration and assessment of the relationship between A_{pH}' and CO_2 air fraction. We used an EL800 absorbance microplate reader (BioTek Instruments Inc., Winooski, VT) to measure (i) absorbance of empty microplates (A_e), (ii) mean absorbance of microplates filled with gel prepared as in the MicroResp™ method (Campbell et al., 2003), but excluding cresol red ($A_e + \overline{A_g}$), and (iii) mean absorbance of microplates filled with gel with or without added cresol red and in which $NaHCO_3$ 2.5 mmol · L^{-1} was replaced by NaOH 10 mmol · L^{-1} to ensure a pH > 11. The mean maximum absorbance of cresol red as CR^{2-} ($\overline{A_{max}}$) was estimated by the difference in absorbances at pH > 11. Just after reading the absorbances, gel pH was measured in 8 wells of each microplate using a 16-gauge micro-combination needle pH probe (MI-414B, Microelectrodes, Inc., Bedford, NH) gently touching the bottom of the wells and connected to a Bioblock Scientific 93327 pH–EH meter (HANNA Instruments, Smithfield, RI). Additional measurements were performed to assess the relationship between A_{pH}' and the pH of the gel solution: for agar gel with or without added cresol red and buffered at pH ≈ pK_{CR2} by replacing $NaHCO_3$ 2.5 mmol · L^{-1} with H_3BO_3 0.50 mol · L^{-1} and NaOH 0.65 mol · L^{-1}, and for microplates prepared according to the MicroResp™ method after 24 h in sealed bags containing either dry soda lime and water in distinct capsules or air enriched with 1.5% CO_2. To estimate Δc_{min}^+, Δc_{max}^+, $pH_{1/2}$ and α, we set out to obtain the relationship between Δq_g and gel pH after equilibrating the gel with air enriched by 0.5%, 1% and 1.5% CO_2. To better circumvent the effect of the gel, we simultaneously measured the pH of a solution of the same composition, but without agar, equilibrated with CO_2-enriched air. However, the calculations are highly sensitive to gel pH, and 0.5% or more CO_2 bars insight into pHs higher than 7.6. Therefore, parameters Δc_{min}^+, Δc_{max}^+, $pH_{1/2}$ and α were empirically refined to ensure that (i) the calculated CO_2 disappearing from the air space of empty wells over 6 h balanced against the CO_2 stored in the gel of these wells in 96-well microplates, and (ii) the respiration rate estimated for 6 h incubation in wells of 24-well microplates filled with either 0.6, 1.2, 1.8 or 2.4 g of moist soil did not depend on the final CO_2 air fraction in the wells. The relationship between gel absorbance and CO_2 air fraction was checked for microplates filled with gel previously equilibrated 5 d with CO_2 air fractions of 0.5, 1, 1.5 and 2% at about 22.5 °C after being stored uncovered in sealed plastic bags with soda lime and water for 6 d.

2.3.2.2. Kinetics of CO_2 exchange between the air space and the gel. Just after being filled with gel, two 96-well microplates were stored for 1 d in sealed plastic bags that contained dry soda lime and water in distinct containers, and two other microplates in sealed plastic bags with air enriched by 1.5% CO_2. Just after opening a bag, one of the two microplates was used for absorbance measurements every 2 to 5 min for 6 h at about 22.5 °C, while the other was used to record the pH of the gel every 1 to 5 min in a single well, using the 16-gauge micro-combination needle pH probe. Beyond 6 h, drying led to cracks in the gel of some wells.

2.3.2.3. Effect of microplate storage on the initial state of the gel. Since soda lime partly dries the gel and extracts CO_2 supplied as $NaHCO_3$, we checked whether absorbance varied between microplates prepared simultaneously and kept for 7 d in a poorly-closed desiccator with dry soda lime and water in separate capsules, in a sealed plastic bag with dry soda lime and water in separate capsules, and in a sealed plastic bag with soda lime and water in the same capsule. In addition, we monitored the kinetics of gel desiccation in microplates stored for 7 d in sealed plastic bags with dry soda lime and water in separate capsules, one of them being covered with a plastic film after 1 d of storage, the other remaining exposed.

2.3.3. Experiment on the soil

2.3.3.1. Ability of the model to simulate the pH of soil solutions for various CO_2 air fractions. Since retained reactions greatly simplify the complex geochemistry of soils, we checked whether they permit the pH of a soil solution to be simulated and whether the calcite dissolution that results from an increase in CO_2 air fraction or the supply of an acid substrate could be described by a thermodynamic equilibrium. The relationship between CO_2 air fraction and pH of the soil solution was assessed on soil slurries with a water-to-soil mass ratio of 1, 2.5 and 5 after equilibrating with either lab air (between 0.04 and 0.12% CO_2) or air enriched by 0.5, 1, 1.5 and 2% CO_2, respectively, by sparging with air and stirring the slurry.

2.3.3.2. Abiotic CO_2 emissions after supplying acid substrate to the soil. Soil was autoclaved at 121 °C and 0.1 MPa for 1 h, incubated for 2 d, then autoclaved a second time for 1 h at the same temperature and pressure to eliminate any microorganisms that were not destroyed in the initial autoclaving (Skipper et al., 1996). Then 24 wells of a 96-well microplate were filled with sterilized soil, while 24 other wells were filled with non-sterilized soil. For each modality, 6 wells each were supplemented with either 0.05 mL of water or with a mixture of 0.025 mL water plus 0.025 mL of a 120 mg · mL^{-1} solution of glucose, glucosamine-HCl or Na_2-malate, respectively. Abiotic emissions due to glucosamine-HCl supply were estimated from 6 h incubation and compared with biotic emissions on unsterilized soil.

2.3.4. MicroResp™ incubations of various times and for various amounts of soil

Three MicroResp™ incubation sets were performed to assess and illustrate the new MicroResp™ data analysis. In a first set of incubations, the wells of four 24-well microplates were filled with 0.6, 1.2, 1.8 or 2.4 g of the same soil at 19 wt.% soil moisture supplemented with 0.033, 0.066, 0.099 or 0.133 mL of water, respectively, and 0.033, 0.066, 0.099 or 0.133 mL of a 120 mg · mL^{-1} solution of glucose. The microplates were then incubated at 22.5 °C for 6 h. Results served to estimate the parameters α and $pH_{1/2}$ and to assess the relative contributions of gel, air space and soil solution to CO_2 storage. In a second set of incubations, the wells of five 96-well microplates were either left empty or filled with about 0.38 g of soil initially at 19 wt.% soil moisture supplemented with 0.05 mL of water or 0.025 mL of water and 0.025 mL of a 120 mg · mL^{-1} solution of glucose, Na_2-malate or glucosamine-HCl. The microplates were then incubated at 22.5 °C for 1, 2, 4, 6 and 8 h. Results served to check whether estimated microbial respiration was incubation time-dependent. A third set of experiments was performed solely to illustrate variations in microbial CO_2 production with changes in substrates. Only one 96-well microplate was used; the wells were filled with about 0.38 g of dry soil initially at 19 wt.% soil moisture and supplemented with 0.05 mL of water or with 0.025 mL of water and 0.025 mL of a 120 mg · mL^{-1} solution of glucose, sucrose, trehalose, mannose, cellobiose, dextrin, glucosamine-HCl, alanine, glycine or Na_2-malate.

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3. Results and discussion

3.1. Calibration of the relationship between A_{pH}' and the CO_2 air fraction

The means and standard deviations of the absorbances of empty microplates (A_e) were 0.030 and 0.001, respectively. The means and standard deviations of the absorbances of the wells filled with gel without cresol red ($A_e + A_g$) were 0.171 and 0.009, respectively. Thus an estimate of $\overline{A_g}$ is 0.141, the variability in A_e can be neglected given its low contribution to A_{pH}, and the standard deviation of A_g is nearly 0.009, leading to a variation coefficient of A_g of about 6.2%. Similar estimates for A_g mean and standard deviation were obtained for agar gel without cresol red and for pH ≈ 11.7 and pH = pK_{CR2}', although slightly lower when borate was used to buffer the gel solution. The means and standard deviations of the absorbances of microplates with gel carrying cresol red and having a pH > 11 were 0.987 and 0.030, respectively, while the means and standard deviations of absorbances of microplates with gel without cresol red and having a pH > 11 were 0.162 and 0.008, respectively. Thus an estimate of $\overline{A_{max}}$ is 0.825. Although an exact estimation of the A_{max} variation coefficient is impossible, it should be lower than that of A_g in our experiment. Using these values and the calculated pK_{CR2} to simulate variations in the absorbance A_{pH} as a function of the pH of the gel solution correctly reflects experimental data (Fig. 1). The pH generally decreases from 9 to 10 at the beginning of incubation (depending on the efficiency of the soda lime to remove CO_2 from the gel) to more than 7.1, a value rarely reached, which corresponds approximately to 2% of CO_2 in the air space.

The initial vs. final absorbances of empty wells (i.e., $A_{pH}(t = 0)$ and $A_{pH}(t = 6\ h)$, respectively) strongly suggest that Δc^+_{min} is close to or slightly lower than zero, and that there is no exchange of adsorbed cations with H_3O^+ in the gel solution at pH ≥ 8: the opposite (i.e., $\Delta c^+_{min} \geq 0$ and/or $\Delta c^+ >> 0$) would require an unrealistically high initial CO_2 air fraction in the air space of the wells to explain the change in absorbance measured for these wells (results not shown). The pHs of the solution of the same composition as the gel in the MicroResp™ method but without agar and in equilibrium with 0.5%, 1% and 1.5% CO_2 were 7.49, 7.16 and 6.98, respectively. These values correspond to a net Δq_g of about 3.05, 2.85 and 2.82 mmol $c^+ \cdot L^{-1}$, respectively, which is near the 2.5 mmol $\cdot L^{-1}$ of Na^+ supplied as $NaHCO_3$. By contrast, the pHs of the agar gel in equilibrium with 0.5%, 1% and 1.5% CO_2 were 7.66, 7.41 and 7.22, respectively. These values correspond to a net Δq_g of about 4.50, 5.03 and 4.89 mmol $c^+ \cdot L^{-1}$, respectively, suggesting that Δq_g increases with a decrease in pH in this pH range with a Δc^+_{max} probably higher than 2. We set Δc^+_{max} to 3.0 mmol $c^+ \cdot L^{-1}$ in order to obtain a good fit of the relationship between the air CO_2 fraction and the absorbance at 570 nm on experimental data for 0.5%, 1%, 1.5% and 2% CO_2 (Fig. 2). Parameters $pH_{1/2}$ and α were set to 7.7 and 80, respectively, by minimizing differences in the respiration rates of various amounts of the same soil (see below), making it possible to simulate a majority of cation exchange for pH values between 7.4 and 8.

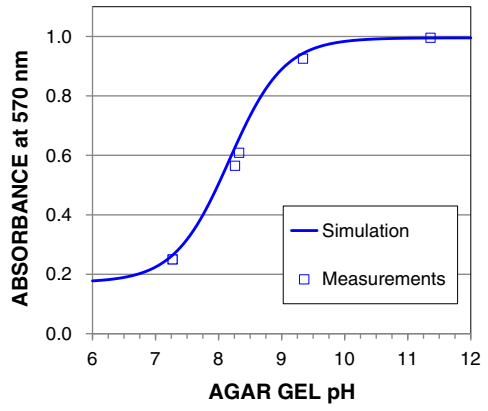

Fig. 1. Relationship between pH of the agar gel and mean of the measured absorbance A_{pH} ($A_{pH} = A_e + A_g + A_{CR}$) over the 96 wells of a microplate. The *X* coordinates of the experimental points are directly-measured pH. The *Y* coordinates of the experimental points are directly-measured absorbances when the gel matched the composition used in the MicroResp™ method, whereas for non-matched gels they were recalculated to replace the absorbance of the modified gel by the actual absorbance of the gel matched to the composition used in the MicroResp™ method.

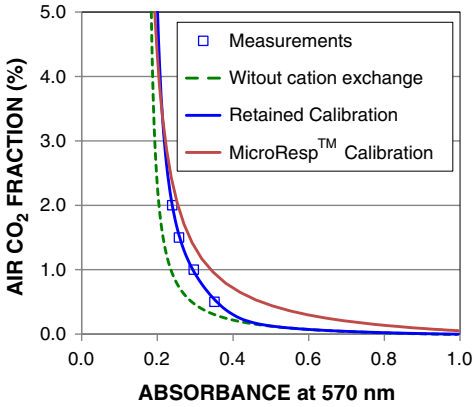

Fig. 2. Relationship between means of measured absorbance A_{pH} ($A_{pH} = A_e + A_g + A_{CR}$) over the 96 wells of a microplate and CO_2 air fraction in equilibrium with the microplates.

3.2. Effects of soil alkalinity, substrate and calcite on soil pH and microbial CO_2 in solution

While solute concentrations were highly dependent on water-to-soil mass ratio (Table 3), pH measured on soil slurries in equilibrium with a range of CO_2 air fractions was only slightly dependent on water-to-soil mass ratio (1, 2.5 and 5 in this work), but highly dependent on CO_2 air fraction (Fig. 3). Simulations performed for soil solutions in equilibrium with calcite and having an initial [Ca^{2+}] near the value extrapolated for soil solutions at the start of MicroResp™ incubations were used to simulate slurry behavior. Although this was not the objective of these measurements, the simulations confirmed that pH(water) (8.51 for our soil) differs from the pH of the soil solution in equilibrium with lab air (pH slightly lower than 8 here), since alkaline soil solution can trap a large amount of gaseous CO_2, but only very slowly in the absence of facilitated transfer (vigorous stirring and bubbling).

Empirical functions were then easily obtained from geochemical modeling to estimate the amount of microbial CO_2 stored in the soil

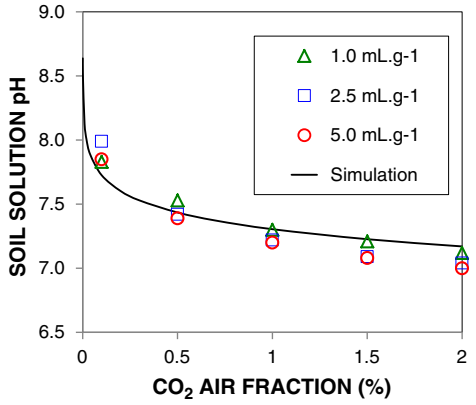

Fig. 3. Measured pH of soil slurry as a function of CO_2 air fraction in equilibrium with it by simultaneous air bubbling and slurry stirring. Simulations were performed for soil at weight moistures as in MicroResp™ incubations.

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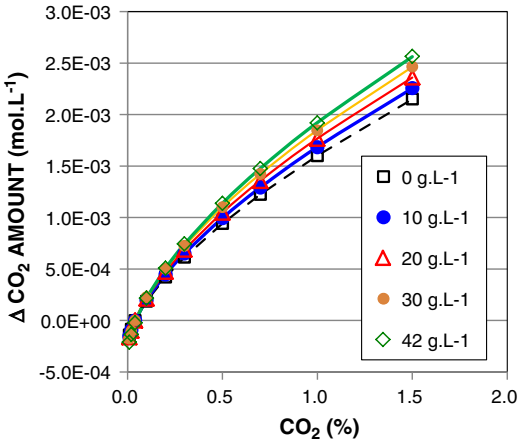

Fig. 4. Simulated amount of total CO_2 (mol · L^{-1}) minus CO_2 derived from calcite dissolution as a function of air space CO_2 fraction for a soil solution in equilibrium with calcite and initially having a sum of charges other than H_3O^+, HO^-, HCO_3^- and CO_3^{2-} equal to $-4.60\ 10^{-3}$ mol c^+ · L^{-1} and $+6.00\ 10^{-3}$ mol c^+ · L^{-1} Ca^{2+}, and for different amounts of Na_2-malate substrate (0, 10, 20, 30 and 42 g · L^{-1}). Zero values indicate that total CO_2 in the soil solution is equal to CO_2 release by calcite dissolution. Negative values mean that calcite dissolution releases more CO_2 than was initially contained in the soil solution, indicating that CO_2 is partly transferred to the air and agar gel in the well.

solution ($H_2CO_3^*$, HCO_3^- and CO_3^{2-}) minus the amount of total abiotic CO_2 emitted by calcite dissolution. The variation of $\Delta[CO_2]_{tot}$ in total CO_2 concentration in the solution ($H_2CO_3^*$, HCO_3^- and CO_3^{2-}) minus the increase $\Delta[Ca^{2+}]$ in Ca^{2+} concentration for calcareous soils between the considered CO_2 air fraction and the 0.04% CO_2 air fraction was then fitted by empirical functions. An example of these functions for several amounts of Na_2-malate is proposed in Fig. 4, and empirical coefficients defined in Eq. (1) for these functions are presented in Table 4 for the relevant soil.

3.3. Kinetics of transfer and calcite dissolution and the effects on MicroResp™ estimation

Microbial respiration rate was assessed in a soil sterilized and subsequently supplied with a slightly acidic substrate (glucosamine-HCl) to indirectly check whether calcite dissolution can be described by a thermodynamic equilibrium system. The results showed that abiotic CO_2 microbial respiration can be neglected, as absolute values were small ($3.50\ 10^{-1}$ µg C–CO_2 · g^{-1} soil · h^{-1}) compared with unsterilized soil enriched with the same substrate (3.23 µg C–CO_2 g^{-1} soil · h^{-1}), and positive or negative sign was dependent on the initial in-lab CO_2 air fraction.

Fig. 5a–b illustrates the changes in the pH of the gel solution and in the absorbance A_{pH} of microplates initially stored in sealed plastic

Table 4
Retained coefficients *a*, *b* and *c* used in Eq. (1) to describe variations in amount of microbial CO_2 in the soil solution (i.e., the total amount of $H_2CO_3^* + HCO_3^- + CO_3^{2-}$ reduced by the contribution of calcite dissolution to this pool) with regard to microbial CO_2 at 0.04% CO_2 air fraction:.

Substrate	Concentration	*a*	*b*	*c*
–		$1.83\ 10^{-3}$	$6.45\ 10^{-1}$	$2.28\ 10^{-4}$
Glycine		$5.77\ 10^{-2}$	$2.57\ 10^{-2}$	$5.25\ 10^{-2}$
Alanine		$5.20\ 10^{-3}$	$2.50\ 10^{-1}$	$2.28\ 10^{-3}$
Glucosamine-HCl		$1.85\ 10^{-3}$	$6.40\ 10^{-1}$	$2.33\ 10^{-4}$
Na_2-malate	10	$1.93\ 10^{-3}$	$6.38\ 10^{-1}$	$2.46\ 10^{-4}$
	20	$2.03\ 10^{-3}$	$6.31\ 10^{-1}$	$2.64\ 10^{-4}$
	30	$2.13\ 10^{-3}$	$6.24\ 10^{-1}$	$2.83\ 10^{-4}$
	42	$2.25\ 10^{-3}$	$6.17\ 10^{-1}$	$3.28\ 10^{-4}$

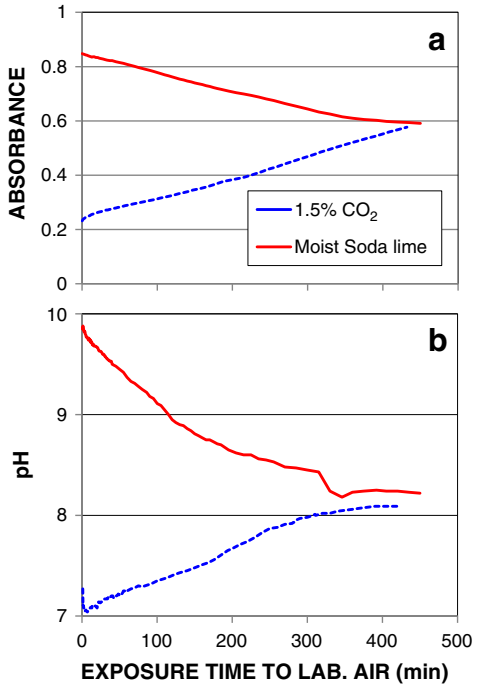

Fig. 5. Changes (a) in absorbance A_{pH} and (b) in the pH of the gel solution of microplates initially stored in sealed plastic bags enriched with either 1.5% CO_2 or with soda lime and water in separate capsules, and exposed to lab air.

bags either enriched with 1.5% CO_2 or containing soda lime and water in distinct capsules and exposed to lab air. Superimposed yellow and pink color layers (corresponding to CO_2-enriched air and CO_2-free air, respectively) in microplate well gel were clearly visible for long periods of these experiments. These observations indicate that CO_2 transfer between the air space and the gel is low and that equilibrium is reached only after about 4 h. This is mainly because low CO_2 transfer in the gel prevents a quick redistribution of CO_2 when it accumulates or disappears in the gel near the interface with air. For MicroResp™ microplate wells where microbial CO_2 production was continuous over the 6 h of incubation, it is reasonable to consider that equilibrium is never reached and that the estimated microbial respiration a priori underestimates actual respiration. However, the relative bias resulting from the equilibrium hypothesis will decrease with increasing incubation times. Staggered incubations (1, 2, 4, 6 and 8 h) of soil enriched with glucose suggest that 4 h is the minimum time requirement for incubations and that the 6 h incubation proposed in the MicroResp™ method is reasonable (Fig. 6).

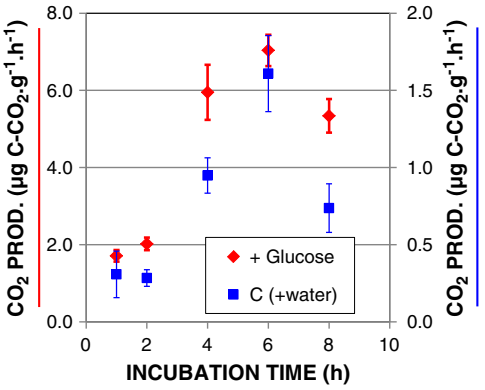

Fig. 6. Microbial respiration rates (µg C–CO_2 · g^{-1} dry soil · h^{-1}) estimated from soil incubation in 96-well microplates for 1, 2, 4, 6 and 8 h, without substrate (*Y* coordinates on the right) and with glucose (*Y* coordinates on the left).

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Initial state of the gel may vary with initial storage time and conditions. For example, the initial absorbance of 96-well microplates varies between simultaneously-prepared microplates stored for 7 d in a poorly-closed desiccator with dry soda lime and water in separate capsules ($\overline{A_{pH}}(t=0)=0.90$), a sealed plastic bag with dry soda lime and water in separate capsules ($\overline{A_{pH}}(t=0)=1.04$), and a sealed plastic bag with soda lime and water in the same capsule ($\overline{A_{pH}}(t=0)=0.97$). For plates stored in sealed plastic bags, differences in $\overline{A_{pH}}(t=0)$ between dry soda lime and water in separate capsules and in the same capsule may have resulted from differences in gel desiccation, which would explain why $\overline{A_{pH}}(t=0)$ exceeds the maximum simulated absorbance $\left(A_e+\overline{A_g}+\overline{A_{max}}=0.996\right)$. The kinetics of gel desiccation in microplates stored for 7 d in sealed plastic bags with dry soda lime and water in separate capsules varies greatly with the presence or absence of a plastic film covering the wells (Fig. 7). The procedure proposed in the MicroResp™ method therefore appears to have been adapted to protect the gel from desiccation. However, the initial absorbance may still vary with the amount of CO_2 extracted by soda lime during plate storage. This amount has to be taken into account in microbial respiration estimates. However, the worst-case scenario would be the gel drying out, which would ultimately modify the value of the sum Δq_g, and skew the calibration. For this reason, it is essential to take all feasible precautions to protect the gel from partial drying. Repeated use of the same microplate gels "regenerated" between consecutive incubations by exposure to soda lime should be ruled out. Finally, we note that microplates with an initial absorbance $\overline{A_{pH}}(t=0)$ of about 0.90 would correspond to a gel of about pH 9.1 and about 0.0073% CO_2 air fraction, indicating that the NaOH partially replaces $NaHCO_3$ within the gel during storage due to CO_2 extraction by the soda lime.

3.4. The effects of soil mass and substrate on respiration estimated from 6 h incubation

The 24-well microplates that were filled with 0.6, 1.2, 1.8 and 2.4 g of the same soil at 19 wt.% soil moisture and supplemented with 0.033, 0.066, 0.099 and 0.133 mL of water and 0.033, 0.066, 0.099 and 0.133 mL of a 120 mg · mL^{-1} solution of glucose, respectively, were then incubated at 22.5 °C for 6 h. Although the results partly contributed to calibration ($pH_{1/2}$ and α were estimated so as to minimize differences in estimated microbial respirations between these four soil weights), they show that estimated soil microbial respiration is not dependent on soil weight and therefore not dependent on CO_2 air fraction at the end of the incubations (Fig. 8). This is all the more interesting as there is a variation in the relative proportions of total CO_2 in the gas phase in the soil solution and in the gel (Fig. 9). The accumulation of CO_2 during the 6 h of incubation is always stronger in the gel than in the air space and the soil solution, especially when final CO_2 content is low. Low final levels of CO_2 air fraction are common for soils incubated without substrates or with inefficient, poorly-used substrates. These results clearly demonstrate that we cannot neglect variations in the amount of CO_2 in the gel. The fraction of CO_2 stored in the soil solution cannot be neglected, but it can be estimated with less precision in view of its low contribution to the total accumulation of microbial CO_2.

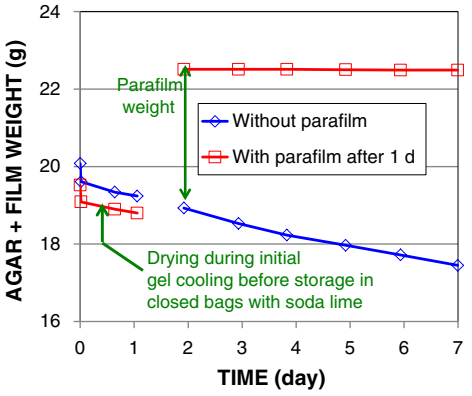

Fig. 7. Change in the weight of microplates filled with agar gel during its initial 7-day storage in sealed bags with or without Parafilm anti-desiccant.

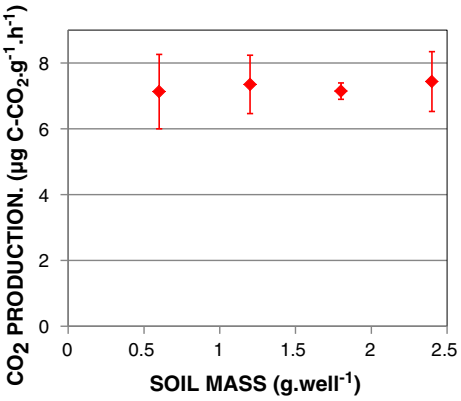

Fig. 8. Microbial respiration rates (µg C–CO_2 · g^{-1} dry soil · h^{-1}) estimated for different amounts of soil (0.6, 1.2, 1.8 and 2.4 g of fresh soil at 19 wt.% soil moisture) supplied with water and glucose.

We then tested this new mathematical procedure for estimating microbial respiration from MicroResp™ incubation on various substrates (Fig. 10). As already observed in Fig. 9, the new method leads to estimates of microbial respiration rates 2 to 4 times higher than estimates considering only CO_2 accumulation in the air space of the wells. Microbial respiration rates decreased in the following order of added substrate: sucrose ≥ glucose ≥ malate ≥ glucosamine-HCl ≥ cellobiose ≥ alanine ≥ mannose ≥ dextrin ≥ trehalose, and microbial respiration rates were between 5 and 15 times stronger in the soil with substrate than in the soil with water only. The coefficients of variation of individual measurements (i.e., in a well) ranged from 6.3% (soil with water) to 53% (soil + trehalose).

4. Conclusion

Here we propose a novel and more complete description of the fate of CO_2 during soil incubation in the wells of microplates used in the MicroResp™ method; for the first time, the storage of CO_2 (as $H_2CO_3^*$, HCO_3^- and CO_3^{2-}) in the gel carrying the colorimetric indicator and the effects of CO_2 on the pH of the soil solution and, in calcareous soils, the dissolution of calcite are now taken into account. The comparison between the experimental data and simulations shows that this improved model makes it possible to simulate the main geochemical processes involved in the fate of CO_2. It yields appropriate estimates of microbial CO_2 production, as shown by 6 h-plus incubations of 0.6 to 2.4 g of soil (in 24-well microplates) An incubation time of 6 h is a good compromise between the need for short incubations to prevent microbial growth/selection and the need for long incubations to reduce the gradient in CO_2 distribution within the gel and between the gel and the air space. The final proportion of CO_2 stored in the indicator gel is far from negligible, especially when the final CO_2 air fraction is low, as is the case for incubation of soil without substrate or with substrates that are difficult to consume. Incubation for less than 4 h may underestimate microbial respiration, since CO_2 dissolution and transfer within the gel are both slow processes. The amount of CO_2 in the gel at the start of incubation depends on the level of CO_2 (supplied as $NaHCO_3$) extraction by soda lime while the gel is in storage. Extracted CO_2 varies with microplate distance to soda lime, duration of the exposure to soda lime, etc. Storage may partially desiccate the gel and modify the

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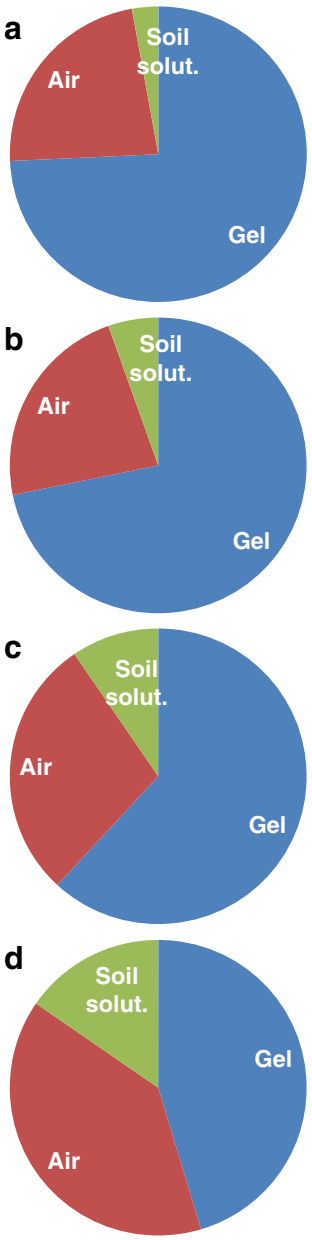

Fig. 9. Distribution of microbial production of CO_2 in microplate well gel, air space and soil solution as a function of (a) 0.6 g, (b) 1.2 g, (c) 1.8 g and (d) 2.4 g of fresh soil (19wt.% soil moisture) supplied with water and glucose, corresponding to estimated final CO_2 air fractions equal to 0.32, 0.57, 0.99 and 1.74% CO_2. For the calculations, abiotic CO_2 originating from calcite CO_2 was assumed to be in the soil solution only. This slightly minimizes the contribution of soil solution to CO_2 storage based on calculations assuming even CO_2 distribution between soil solution, air space and gel.

relationship between CO_2 partial pressure and gel absorbance, and so repeated use of the same microplates "regenerated" between consecutive incubations by exposure to soda lime should be avoided. CO_2 stored in the soil cannot be neglected, but an increase in CO_2 partial pressure decreases the pH of the soil solution and, in the case of calcareous soils, generally promotes calcite dissolution. Neglecting this decrease in pH or the dissolution of calcite would lead to an overestimation of biotic CO_2 production. Since microbial CO_2 production in soils without substrate often leads to a final CO_2 fraction of less than 0.4%, it is important to have a good estimate of the initial CO_2 partial pressure (varying between 0.04 and more than 0.1% in lab air), possibly by having empty wells with gel. It is also vital to keep firm control over

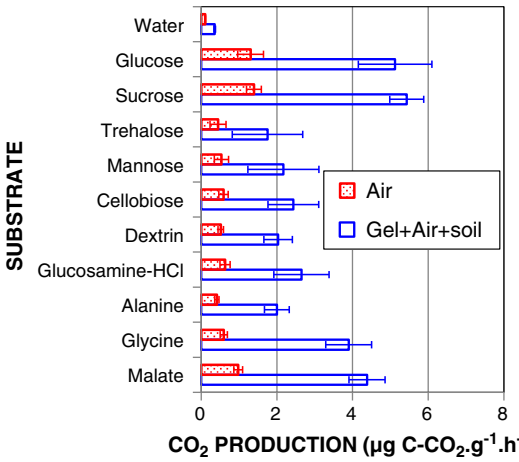

Fig. 10. Example illustrating microbial respiration rate for several added substrates. Microbial respiration rates have been estimated by taking into account either CO_2 accumulation in air only or CO_2 accumulation in the gel, air and soil solution minus the CO_2 issued from calcite dissolution.

temperature, which can affect all the thermodynamic constants as well as microbial activity.

Authors can provide on request an Excel file to perform all calculations.

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