



Non-phototrophic CO₂ fixation by soil microorganisms

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Abstract

Although soils are generally known to be a net source of CO₂ due to microbial respiration, CO₂ fixation may also be an important process. The non-phototrophic fixation of CO₂ was investigated in a tracer experiment with ¹⁴CO₂ in order to obtain information about the extent and the mechanisms of this process. Soils were incubated for up to 91 days in the dark. In three independent incubation experiments, a significant transfer of radioactivity from ¹⁴CO₂ to soil organic matter was observed. The process was related to microbial activity and could be enhanced by the addition of readily available substrates such as acetate. CO₂ fixation exhibited biphasic kinetics and was linearly related to respiration during the first phase of incubation (about 20–40 days). The fixation amounted to 3–5% of the net respiration. After this phase, the CO₂ fixation decreased to 1–2% of the respiration. The amount of carbon fixed by an agricultural soil corresponded to 0.05% of the organic carbon present in the soil at the beginning of the experiment, and virtually all of the fixed CO₂ was converted to organic compounds. Many autotrophic and heterotrophic biochemical processes result in the fixation of CO₂. However, the enhancement of the fixation by addition of readily available substrates and the linear correlation with respiration suggested that the process is mainly driven by aerobic heterotrophic microorganisms. We conclude that heterotrophic CO₂ fixation represents a significant factor of microbial activity in soils.

Abbreviations: FYM – farmyard manure; LSC – liquid scintillation counting; PCR – polymerase chain reaction; RuBisCo – ribulosebiphosphate carboxylase/oxygenase.

Introduction

Commonly, soil without vegetation growing on it is regarded as a source of CO₂. In fact, the net flux of CO₂ is directed from the soil towards the atmosphere (Raich and Potter, 1995; Raich and Schlesinger, 1992). On a yearly time scale, the C input by plant litter to soils is nearly balanced by soil respiration due to the mineralizing activity of microorganisms (Chad-

wick et al., 1994). CO₂ fixation therefore does not seem to be a quantitatively significant process in bulk soil. However, in certain soil microenvironments, the uptake of CO₂ may be larger than mineralization of carbon, e.g. in the vicinity of legume nodules where H₂ concentrations are elevated in the soil air because H₂ is formed as a byproduct of the N₂ fixation, i.e. by *Rhizobium* species. This results in a net uptake of CO₂ by autotrophic hydrogen-oxidizing bacteria (Dong and Layzell, 2001). At least on a small spatial scale, CO₂ fixation may thus outweigh respiration.

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CO₂ may be assimilated on a low level by soil microorganisms and will contribute to the formation of microbial biomass. The process involved could be chemolithoautotrophic growth, e.g. of nitrifying, Fe or sulphide-oxidizing bacteria. Most of these organisms use the Calvin cycle for CO₂ fixation (Schlegel 1985). As the large subunit of the key enzyme of this cycle, the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) is encoded by the *cbbL* gene, the presence of this gene in soil can be used to investigate the presence of these organisms.

Heterotrophic organisms are also known to require CO₂ for growth, although CO₂ provides only a certain percentage of the biomass C of these organisms (Krebs, 1941). Furthermore, it was recently found that, during growth on certain oxo-compounds, not only anaerobic bacteria, but also aerobic *Rhodococcus* and *Xanthobacter* species perform carboxylation reactions of the substrate, which contribute substantially to biomass formation from CO₂ (Clark and Ensign, 1999; Ensign et al., 1998). *Thiobacillus* sp. were also shown to incorporate more than 10% of the cell C from CO₂ during both mixotrophic and heterotrophic growth (Perez and Matin, 1982).

For the bulk soil C budget, CO₂ fixation is of minor importance. However, the process may considerably change the isotopic signature of the microbial biomass because of the distinct isotopic compositions of different CO₂ pools, soil organic matter and the microbial biomass. Atmospheric CO₂ is relatively enriched in ¹³C compared to plant residues, soil organic matter and microbial biomass. In contrast, CO₂ in the soil air is relatively depleted compared to atmospheric CO₂ (Flessa et al. 2000), because it originates from mineralization of plant residues and soil organic matter. If isotopically heavy atmospheric CO₂ enters the soil by gas volume flux, e.g. during or after water infiltration or soil compaction, and is assimilated by soil microorganisms, the isotopic composition of their biomass will be correspondingly enriched. It is difficult, however, at this state of knowledge, to estimate the size of the isotopic shifts in microbial biomass and soil organic matter due to CO₂ fixation, because they depend on (1) the isotopic composition of the soil air, (2) the intensity of gas exchange between soil air and the atmosphere, (3) the percentage of biomass carbon originating from CO₂, and (4) the isotopic fractionation related to the fixation processes involved. Knowledge about the process of CO₂ fixation would be of high interest, because the determination of the shift in natural ¹³C abundance in soil organic matter after a change

from C₃ to C₄ vegetation or vice versa is considered to be a reliable and convenient method to determine carbon turnover times and fluxes between carbon pools (for a review see Balesdent and Mariotti, 1996).

In addition, knowledge about the process of CO₂ fixation may change the assessment of possible risks related to the formation of bound residues from organic pollutants, pesticides and herbicides applied to soil. The usual way to quantify the formation of these residues in soils is to incubate ¹⁴C-labelled pollutants in soil and to analyze for non-extractable radioactivity (e.g. Burauel and Führ, 2000). These investigations, however, do not provide any information about the structures in which the radioactive carbon is bound. Investigations with ¹³C-labelled phenanthrene showed that the reaction products found in the hydrolysable fraction of bound residues formed during a 50-day incubation in soil included both functionalized derivatives of phenanthrene and labelled amino acids (Richnow et al., 2000). The soil microbial community had thus used part of the pollutant as a C source. The usual assimilation pathway of pollutant-derived C into microbial biomass will be the direct use of the pollutant or its metabolites as C source. However, the C could also be assimilated as labelled CO₂ after partial or complete mineralization of the labelled pollutant (Kästner et al., 1999).

To date, only limited knowledge is available on the rate, extent, and dynamics of CO₂ fixation in soils, particularly in terms of non-phototrophic microbial activity. To elucidate the carbon turnover and fluxes in soil, various soils were incubated in the presence of ¹⁴CO₂. The incorporation of the radiolabel into the soil organic matter fraction was balanced and compared with the overall respiration activity of the native soils and of soils fumigated in order to inhibit microbial activity. In addition, the effect of supplementing the soils with readily degradable carbon sources in order to enhance the activity of heterotrophic microbes in the soil was analysed.

Materials and methods

Incubation experiments with labelled CO₂

To investigate the process of carbon dioxide fixation, three different experiments were performed. The first experiment was designed to check whether ¹⁴C fixation can be observed with the chosen experimental design and for balancing the ¹⁴CO₂ fixation in soils without analyzing the dynamics of the

process. A synthetic soil high in organic matter (Einheitserde type 0 from Einheitserde-Werke, Germany, <http://www.einheitserde.de>) was used, which had the following characteristics: C_{org} , 319 mg g⁻¹; organic matter (determined as loss on ignition), 578 mg g⁻¹; water content (determined as weight loss at 105 °C), 120% (w/w). All data are referred to soil dry weight. The soil was a mixture of peat (70%) and clay (30%) whose pH value had been adjusted to 6 by liming. For incubation with ¹⁴CO₂, 25 g of the moist soil was filled in 250 ml glass bottles sealed with PTFE Mininert[®] valves. A small amount of ¹⁴CO₂ (100 nmol, specific activity 1 TBq mol⁻¹, released from Ba¹⁴CO₃) was then added with a microsyringe. Two of four samples were fumigated with 2 ml CHCl₃ each to inhibit microbial activity. The bottles were incubated in the dark at room temperature for 6 weeks. The gas phase over the soil samples was analyzed periodically for CO₂ formation and O₂ consumption by gas chromatography. The O₂ depletion was compensated for by periodically adding pure oxygen to the vessels in order to keep the O₂ concentration in the gas phase higher than 10% (v/v). At the end of the incubation period, the soil samples were allowed to degas under ambient atmosphere for 24 h.

A second experiment was designed to investigate the kinetics of the processes related to CO₂ fixation in soil. For better comparability to field conditions, an arable soil from an agricultural long-term experiment in Halle, Germany ('Ewiger Roggenbau') was used, which regularly received farmyard manure (FYM) amendments (12 t ha⁻¹ a⁻¹, FYM I plot). The carbon content of the soil was 19 mg g⁻¹, and it had a pH of 6.0 (0.01 M CaCl₂). Sand, silt, and clay contents were 69%, 23%, and 8% (Merbach et al., 1999), respectively, and the water holding capacity was 28.3% (w/w). The incubation was conducted in glass bottles (600 ml volume) sealed by teflon-lined butyl rubber septa. The lids containing the septa were equipped with butyl rubber stoppers to which a canula was mounted in order to minimize leakage. The canula was usually stoppered, but enabled repeated gas sampling without piercing the septum every time. The soil was moistened to 60% of the water holding capacity and was supplied with 5 mg farmyard manure per g of dry soil. Farmyard manure was chosen as a supplement with readily degradable C compounds for further enhancing heterotrophic microbial activity because the farmed soil regularly received farmyard manure as organic fertilizer. We were thus able to increase microbial activity while simulating natural conditions as far

as possible. Three replicates of both sample and control bottles for each sampling time (5, 11, 21, 39, and 81 days after addition of ¹⁴CO₂) were prepared, each containing 25 g of moist soil/farmyard manure mixture (corresponding to 21.4 g dry weight). Prior to the start of the experiment, a similar set of control bottles was fumigated under vacuum with CHCl₃ for 24 h. During the entire incubation period, CHCl₃ remained in these bottles in order to permanently suppress microbial activity. To increase the initial CO₂ content in the bottles, a small tube containing 0.245 mmol NaHCO₃ was placed in each bottle. CO₂ was released from the NaHCO₃ by injecting 6 M HCl into this tube. In addition, 20 kBq of ¹⁴CO₂ (released from NaH¹⁴CO₃ in a vial with a septum) was added by a microlitre syringe and the exact initial activity of every bottle was determined as described below. The bottles were incubated in the dark at 20 °C.

In order to study the effect of a readily available substrate on CO₂ fixation, a third experiment was conducted without calculating a complete mass balance. The same soil from the previous experiment was used in a similar experimental setup, but without additional farmyard manure amendment. To provide a readily available substrate, 1.75 mmol and 0.32 mmol acetic acid (corresponding to 3.5 and 0.65 mmol C) was added after 36 and 82 days, respectively, to the six bottles scheduled for the following sampling event at 40 and 91 days, respectively. The concentration of CO₂ supplied to the bottles was similar to the concentration in the second experiment, but a lower amount of ¹⁴CO₂ was added (ca. 6 kBq).

Analytical procedures

In the first experiment with the synthetic soil, the CO₂ production and O₂ consumption were determined by gas chromatography of gas samples. CO₂ was separated from the other air components on a Tenax column at 30 °C and detected by mass spectrometry. O₂ was separated on a molecular sieve column at 50 °C and detected by a thermal conductivity detector.

In the second and third experiment, total CO₂ in the gas phase was determined by titration. The vessels from each sampling time were purged with N₂, and the exhaust was bubbled through a series of three CO₂ trapping tubes each containing 10 ml 0.1 M NaOH. The trapped CO₂ was precipitated by addition of 2 ml of 0.5 M BaCl₂ solution. The remaining OH⁻ were titrated to pH 10.5 with 0.05 M HCl and

Table 1. Respiration and $^{14}\text{CO}_2$ fixation during incubation of the highly organic reference soil (Einheitserde type 0)

Incubation time (days)	O_2 consumption rate ($\mu\text{mol O}_2 \text{ day}^{-1} \text{ g}^{-1} \text{ soil}$)	Respiration rate ($\mu\text{mol CO}_2 \text{ day}^{-1} \text{ g}^{-1} \text{ soil}$)	^{14}C bound in soil (% of initial radioactivity)		
			Total	Inorganic	Organic
Einheitserde soil	8	3	18	9	9
Fumigated control	0.8	0.6	<0.5	n.d. ^a	n.d.

^aNot determined.

the CO_2 concentrations were calculated from the acid consumption.

$^{14}\text{CO}_2$ in the gas phase of the vessels was determined using 1 ml gas samples taken with a microliter syringe and injected into a septum-sealed vial containing 0.3–0.8 ml Carbo-Sorb E (Packard Biosciences B.V., Groningen, Netherlands), depending on the expected CO_2 concentration in the gas phase. After absorption of the CO_2 , which was promoted by agitation of the vial, 6 ml of Ultima Gold XR scintillation cocktail (Packard Biosciences) was added and the radioactivity was measured by liquid scintillation counting.

The radioactivity of the solid soil material was determined by combustion of air-dried samples (ca. 0.5 g) in a Biooxidizer (Zinsser Analytic GmbH, Frankfurt/Main, Germany) for 4 min at 900 °C under a flow of O_2 . The CO_2 released by combustion was trapped in Oxysolve C 400 (Zinsser Analytic GmbH), and the radioactivity was measured by liquid scintillation counting.

To check for possible fixation of CO_2 as inorganic carbon (carbonates), selected samples were weighed into the combustion vessels and the carbonates present in these samples were destroyed with 1 M HCl. The samples were dried overnight (60 °C) and were combusted in the Biooxidizer. The radioactivity was determined by liquid scintillation counting. All radioactivity remaining in the acid-treated samples was ascribed to organic compounds.

Liquid scintillation counting was performed with a Wallac 1414 scintillation counter (Perkin Elmer Wallac GmbH, Freiburg, Germany). The counts were corrected for quenching effects by the external standard method and for background activities by including a daily blank. At least 1000 counts were collected from each sample.

For each sampling event of the second experiment, the balance of radioactivity was calculated. Until day 21, the recovery averaged 96% and was always more than 92% of the radioactivity added. At the end of the

experiment (day 81), the recovery decreased to about 75%.

Analysis of the *cbbL* genes encoding for *RuBisCo*

To check for the presence of autotrophic bacteria in the soil, DNA was extracted from different plots of the 'Ewiger Roggenbau' experiment in Halle. The plots investigated were the unfertilized plot, the mineral fertilizer (NPK) plot, and the plot receiving farmyard manure ($12 \text{ t ha}^{-1} \text{ a}^{-1}$, FYM I). The DNA was extracted with the FastDNA spin kit for soil (BIO 101, Carlsbad, CA, USA). Sections of both the 'green-like' and the 'red-like' sequences of the *cbbL* genes, representing different phylogenetic branches of autotrophic bacteria, were amplified using specific primer pairs as described by Selesi et al. (2004). The amplification products were analyzed after electrophoresis on 2.5% agarose gel and staining with ethidium bromide.

Results

The initial experiment with a synthetic reference soil containing high amounts of organic matter was designed to check whether CO_2 fixation in soil could be detected and whether it was related to the general respiration activity of the soil. About $70 \pm 10\%$ of the initially applied radioactivity was recovered in the gas phase after 6 weeks of incubation in the dark. $18 \pm 1\%$ of the radioactivity was bound in the native soil at the end of the experiment (Table 1) and thus the loss of the label amounted to around 12%. After treatment with HCl, $9 \pm 1\%$ of the radioactivity was still detected in the soil (Table 1). The latter fraction can be unambiguously assigned to the organic carbon pool. In the fumigated soil samples without microbial activity, no significant fixation (<0.5%) was found, neither in the organic nor in the inorganic carbon pool.

The respiration of the soil microorganisms resulted in averaged oxygen consumption rates of

Table 2. Respiration and CO₂ fixation during incubation of soil with farmyard manure (FYM) amendment

Incubation time (days)		¹⁴ CO ₂ in gas phase (% of initial)	¹⁴ C bound in soil (% of initial) ^a	Respiration (μmol CO ₂ g ⁻¹ soil) ^b	CO ₂ fixation (% of respiration)
5	Soil + FYM	94.0 ± 7.2	1.1 ± 0.1	12.2 ± 11.9	1.3 ± 0.2
5	Control	95.2 ± 6.6	0.1 ± 0.1	1.0 ± 2.3	0.9 ± 1.1
11	Soil + FYM	95.1 ± 6.0	1.9 ± 0.4	17.7 ± 5.4	2.5 ± 1.2
11	Control	98.5 ± 2.0	0.2 ± 0.1	5.3 ± 2.8	0.3 ± 0.1
21	Soil + FYM	95.2 ± 6.2	2.7 ± 0.4	22.9 ± 1.1	3.0 ± 0.7
21	Control	95.2 ± 4.4	0.3 ± 0.2	n.d. ^c	n.d.
39	Soil + FYM	69.0 ± 21.7	2.9 ± 0.2	22.3 ± 2.3	4.9 ± 2.9
39	Control	84.8 ± 3.3	0.5 ± 0.2	4.8 ± 7.4	1.1 ± 0.6
81	Soil + FYM	70.8 ± 14.0	3.3 ± 0.3	37.9 ± 8.3	2.3 ± 0.7
81	Control	66.3 ± 8.1	0.4 ± 0.1	12.1 ± 8.3	1.0 ± 1.3

^aμmoles CO₂ fixed are presented in Figure 1.

^bInitial amount of CO₂: 11.9 μmol CO₂ g⁻¹ soil.

^cNot determined.

about 8 μmol day⁻¹ g⁻¹ for the native soil and 0.8 μmol day⁻¹ g⁻¹ for the inhibited soil samples. The corresponding CO₂ formation rates were 3 and 0.6 μmol CO₂ day⁻¹ g⁻¹ (Table 1). The respiration gave rise to an increase of the CO₂ concentration in the gas phase of the vessels from 0.04% (v/v; ambient air) up to 12% in the native soil samples and 2.5% in the inhibited samples. As a consequence, the labelled CO₂ was continuously diluted during the course of the experiment. Due to the dilution, it is not really justified to calculate a carbon fixation rate in terms of μmoles of CO₂ fixed per day and g of soil from the data only available for the end of the experiment. Nevertheless, the results give strong evidence for a significant process of biotic carbon fixation from CO₂, particularly in soils containing high amounts of organic matter. Assuming an averaged CO₂ concentration in the gas phase of 6%, we can estimate the amount of CO₂ fixed to be approximately 10 μmol g⁻¹ soil, which corresponds to 8% of the overall respiration and to about 0.04% of the soil C. Half of this amount was bound in organic compounds in this soil.

During incubation of the agricultural soil in the dark with an additional farmyard manure supplement for the second experiment, part of the organic matter present in the soil and in the added farmyard manure was mineralized. After 81 days of incubation, the accumulated respiration amounted to about 38 μmol CO₂ g⁻¹ soil (corresponding to a rate of about 0.5 μmol CO₂ day⁻¹ g⁻¹ soil) in the non-inhibited samples (Table 2). In the fumigated soil, the microbial activity was largely suppressed, resulting in a significantly lower respiration (12 μmol CO₂ g⁻¹

soil, corresponding to a rate of about 0.1 μmol CO₂ day⁻¹ g⁻¹ soil; Table 2).

Fixation of the radiolabel from ¹⁴CO₂ proceeded with biphasic kinetics at a relatively fast rate during the initial phase of incubation, and slowed down after 20 days (Figure 1). Even after 81 days of incubation, CO₂ fixation is still detectable. As in the first experiment, we must consider the increasing dilution of ¹⁴CO₂ with CO₂ originating from soil respiration when extrapolating the radioactivity fixed to the amount of CO₂ fixed, because the total amount of CO₂ released by respiration during the course of the experiment was more than 3 times as high as the amount of CO₂ added at the beginning of the experiment. To account for changes of the CO₂ concentration during the sampling intervals, we calculated the amount of CO₂ transferred to the soil using the radioactivity data at a given sampling event and the average of the CO₂ concentrations at the beginning and the end of each particular sampling interval.

Although CO₂ was fixed by the soil, the net flux of CO₂ was dominated by respiration (Table 2, Figure 2). However, CO₂ was fixed during the entire experiment. CO₂ fixation corresponded to an increasing percentage of respiration. The percentage remained in the range of 3 to 5% after an initially lower value of 1.3% which may be due to the disturbance of the soil microbial processes caused by the supplemental farmyard manure and by the moistening of the soil. During the last interval, the percentage decreased to 2.3%. In general, the percentage of respiration accounted for by CO₂ fixation was much lower in the inhibited samples; however, these values carry a high degree of uncer-

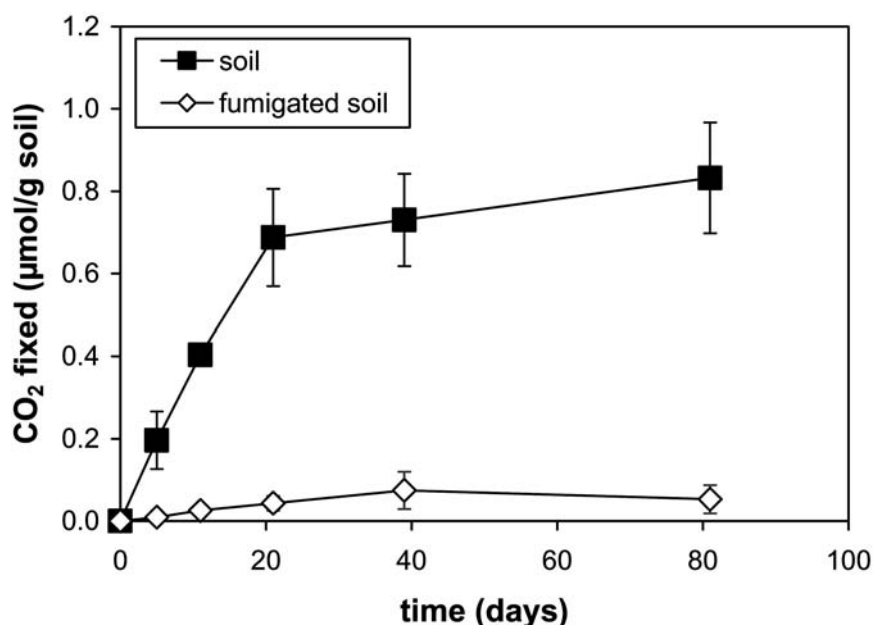


Figure 1. Amounts of CO₂ fixed by the soil microflora during incubation of the farmyard manure-amended soil. Error bars indicate one standard deviation and are shown only where larger than the symbols.

tainty because they are the result of a division of two small numbers with relatively high errors. The plot of CO₂ fixation vs. respiration in the non-inhibited samples reveals a roughly linear relationship between the two processes over the duration of the experiment (Figure 2).

The overall amount of C originating from CO₂ and transferred to the soil was 0.83 µmol g⁻¹ soil after 81 days (Figure 1). This corresponds to about 0.05% of the carbon initially present in this experiment. The transfer was related to biological activity, because it was not significant in the fumigated soils. The analysis of the binding form revealed that a high percentage of the radioactivity was bound as carbonates in the fumigated soil, whereas the majority of the radioactivity in the non-inhibited samples was fixed to organic compounds (Table 3). The absolute amounts of carbonates formed were similar in both the non-inhibited and the fumigated treatments.

When an easily available substrate (acetic acid) was added to the same arable soil incubated initially without any supplement in the third experiment, the percentage of radioactivity transferred to the soil increased significantly in comparison to the initial phase of the experiment (Table 4). The data obtained from this experiment, however, do not allow the amount of additional C fixed in the presence of acetic acid to be calculated.

The analysis of DNA extracted from soil samples originating from different plots of the 'Ewiger Roggenbau' long-term agricultural experiment in Halle, Germany revealed that the 'green-like' (amplicon size 150 bp) as well as the 'red-like' (amplicon size 250 bp) types of the *cbbL* genes were present in the soil from the unfertilized plot and from the plot receiving mineral fertilizer (Figure 3). However, in the farmyard manure-amended plot used for the fixation experiments, only the band of the PCR product of the 'red-like' *cbbL* gene was visible; the band of the 'green-like' *cbbL* gene was not present.

Discussion

The results clearly show that, beside the mineralization of soil organic matter, the fixation of CO₂ is a relevant process in soils; the correlation of fixation rates to the net mineralization is presented here to the best of our knowledge for the first time. Non-phototrophic CO₂ fixation takes place in soils and the majority of the resulting products are organic compounds. The process is mediated by soil microbiota as shown by insignificant CO₂ fixation rates in the fumigated samples. Although respiration is still detectable in these samples, we regard the processes going on in the fumigated soil as abiotic processes or as processes

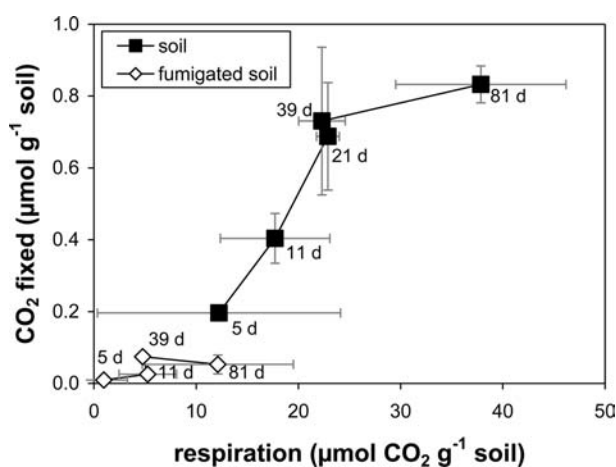


Figure 2. CO₂ fixation vs. respiration during incubation of the farmyard manure-amended soil. Error bars indicate one standard deviation and are shown only where larger than the symbols.

Table 3. Radioactivity bound in the farmyard manure-amended soil and its partitioning into organic and inorganic compounds

	Incubation time (days)	Total radioactivity (Bq g ⁻¹)	Organic compounds (% of total)	Inorganic compounds (% of total)
Soil + farmyard manure	5	11.6 ± 0.4	88	12
Fumigated control	5	0.8 ± 0.3	25	75
Soil + farmyard manure	81	26.9 ± 4.0	96	4
Fumigated control	81	3.4 ± 0.6	9	91

Table 4. Radioactivity bound in soil (% of radiolabel added initially) before and after acetic acid addition to the FYM soil from the 'Ewiger Roggenbau' long-term experiment in Halle, Germany. Acetic acid was supplied in two doses: 1.75 mmol acetic acid after 36 days and 0.32 mmol acetic acid after 82 days. Bold numbers indicate sampling events after acetic acid addition

Incubation time (days)	4	7	18	40	91
Soil	0.6 ± 0.1	0.8 ± 0.0	1.0 ± 0.0	1.8 ± 0.0	3.0 ± 0.1
Fumigated soil	0.3 ± 0.0	0.5 ± 0.2	0.4 ± 0.1	0.3 ± 0.0	n.d. ^a

^aNot determined.

catalyzed by enzymes without the need of cycling redox chains that were inhibited by fumigation with CHCl₃. In addition, the CO₂ fixation can be stimulated by readily biodegradable organic substrates, e.g. acetic acid. Acetic acid was chosen as the substrate because it is a common metabolite in soils which is produced readily when microenvironments of soil such as the inner parts of aggregates become anaerobic i.e. after precipitation events, but it is consumed rapidly by heterotrophic bacteria with increasing availability of O₂ (Küsel and Drake, 1995). This experiment provided additional evidence that the transfer of radioactiv-

ity from ¹⁴CO₂ to soil organic matter was related to presumably heterotrophic microbial activity.

The amount of CO₂ fixed accounted for 2 to 5% of the net respiration and corresponds to up to 0.5 per mille of the organic carbon present in the agricultural soil even after the relatively short incubation period of 81 days. The linear correlation of respiration to CO₂ fixation indicates that the process is essential to microbial respiratory activity and may play an important role in C transformations in soil, even in heterotrophic processes. After an initial lag phase, the fixation showed biphasic kinetics, which is typical

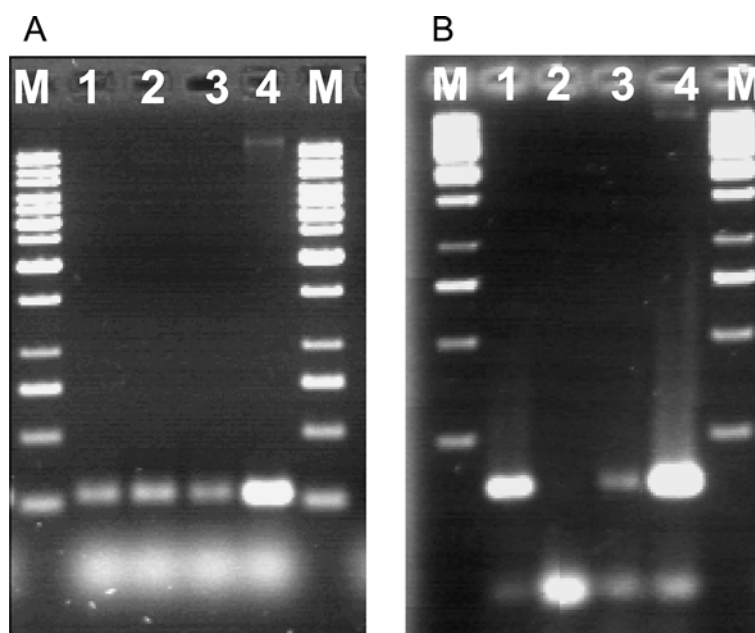


Figure 3. Agarose gels after electrophoresis of the *cbbL* fragments (A 'red-like', B 'green-like') amplified from the soils: 'Ewiger Roggenbau' control (no fertilizer, lane 1), 'Ewiger Roggenbau' FYM I (lane 2), 'Ewiger Roggenbau' NPK (mineral fertilizer, lane 3) and positive control (lane 4, *A. eutrophus* in A, *N. vulgaris* in B). M: 1 kb DNA ladder.

in soil batch experiments to assess microbial activity, with lower rates after 40 days of incubation.

The relative importance of CO₂ fixation may even be underestimated because we can expect the organisms to use internal CO₂, too. For example, in experiments with the fungus *Fusarium oxysporum* the percentage of cell carbon originating from external CO₂ was as low as 0.1 to 1% under oligotrophic conditions (Parkinson et al., 1991). The higher use of external CO₂ by soil microorganisms may be caused by the dominance of small bacteria in the soil microflora. The small size is related to a relatively large surface area and thus increased gas exchange between the cells and their surroundings.

The formation of organic soil compounds from CO₂ strongly depends on microbial activity, but a variable proportion of the CO₂ was also bound as carbonate. The proportion of carbonates differed between the experiments, being higher in the first experiment where a highly organic soil was used than in the second experiment with the farmyard manure-amended agricultural soil. The difference may be explained by the higher CO₂ concentration in the gas phase above the highly organic soil or simply by differences in the local pH values in the two soils. During incubation of the farmyard manure-amended agricultural soil, the CO₂ concentrations in the gas phase remained under

5%, and were thus in the range typically found in soil air. Under these conditions, the amounts of carbonates originating from CO₂ were much lower than with the higher CO₂ concentrations in the experiment with the highly organic 'Einheitserde'. The inhibited controls fixed the largest proportion of CO₂ as carbonates, but the absolute amounts were similar in both treatments. We therefore regard the process of carbonate formation from atmospheric CO₂ as being mainly independent of microbial activity. In the experiment with the synthetic soil, fumigation had not only an effect on incorporation of CO₂ into organic compounds, but also into carbonates. This may be related to a lower pCO₂ in these samples or to changes in the pH due to microbial activity, but it might also indicate biotic fixation of CO₃²⁻ in the 'Einheitserde' soil.

The fixation of CO₂ has also been observed in a previous experiment on the formation of non-extractable residues (Kästner et al., 1999). The authors found that an amount of CO₂ corresponding to that amount which was formed during degradation of anthracene could be fixed to soil organic carbon under similar incubation conditions. Only a minor percentage of the radioactivity was shown to be bound to silicates. The distribution of the radioactivity originating from CO₂ on humic and fulvic acids and humin were similar to those obtained during incubation of

soil with radioactively labelled anthracene, supporting the assumption that, in this case, CO₂ fixation may contribute significantly to the formation of bound residues as determined by incubation of ¹⁴C-labelled substrates. This conclusion was additionally supported by the fact that the 9-¹⁴C label position used in the experiment was not directly converted to microbial biomass during growth on anthracene as a source of carbon and energy (Kästner et al., 1995). In contrast to the present work, Kästner et al. (1999) used a soil/compost mixture with elevated C content. The carbon originating from CO₂ in the non-inhibited samples corresponded to about 0.17% of the soil carbon in the experiment by Kästner et al. (1999). This is higher than found in the current experiment. The higher percentage may be related to a higher CO₂ concentration due to relatively high respiration rates, which in turn resulted from the addition of a high amount of degradable organic compounds supplied with the compost material. Thus, the process may depend on the CO₂ partial pressure and may be faster in soils with elevated CO₂ concentrations.

The processes contributing to CO₂ fixation by soil microorganisms have not yet been characterized. If we assume that the processes are biotic, we have to consider the biochemical reactions likely to be involved in the CO₂ fixation. We can exclude phototrophic processes because the incubations were performed in the absence of light. In natural soils, phototrophic processes are restricted to the top few millimetres of the profile, if they are relevant at all. However, chemolithoautotrophic organisms may be involved in the process. The most abundant pathway of CO₂ fixation used by most aerobic autotrophic bacteria is the Calvin cycle with the key enzyme ribulose-1,5-bisphosphate carboxylase (RuBisCo; Menendez et al., 1999), the large subunit of which is encoded by the *cbbL* gene. The analysis of soils from the 'Ewiger Roggenbau' long-term agricultural experiment in Halle revealed the presence of populations with the 'red-like' and the 'green-like' types of the *cbbL* genes. Interestingly, in contrast to soil from the other plots of the long-term agricultural experiment, the 'green-like' type of the *cbbL* gene could not be detected in the farmyard manure-amended soil used for the present experiment. The amendment with organic matter (in the form of farmyard manure) thus appears to have suppressed the growth of a whole group of autotrophic organisms carrying the 'green-like' *cbbL* gene. This shift in the microbial community may be explained by the higher input of readily available C

sources with the organic fertilizer on the farmyard manure plot. In contrast to the observed lower diversity of autotrophic organisms, we found significant CO₂ fixation in soil from this plot, which was even enhanced by addition of readily degradable carbon substrates. These results suggest that heterotrophic pathways of CO₂ fixation may play a more important role than autotrophic processes, which would tend to be suppressed by an excess of readily available substrates, although both types of processes may occur concomitantly in the soil. However, it must be borne in mind that the mere presence of a gene does not imply that the corresponding process is really proceeding and that a CO₂ fixation activity of chemolithoautotrophic bacteria to the observed extent is not very likely without the addition of inorganic electron donors. In any case, we have to consider alternative pathways of CO₂ fixation.

One pathway used by aerobic Archaea is the 3-hydroxypropionate cycle (Menendez et al., 1999). An alternative pathway is the reductive citric acid cycle which was found in microaerophilic as well as in strictly anaerobic bacteria (Menendez et al., 1999). The strictly anaerobic acetogenic and methanogenic bacteria use the reductive acetyl CoA pathway (Fuchs, 1989). Methanotrophic bacteria can incorporate CO₂ via the serine-pathway (Michal, 1999). However, the major part of the C which these microorganisms use for biosynthesis is derived from methane oxidation to formaldehyde, rather than from CO₂.

In soil, we can expect to find bacteria from all the groups mentioned above. However, all of these groups are confined to appropriate mostly anaerobic microenvironments. In the case of nitrifying bacteria, their relative abundance in the microbial community and their growth efficiency are quite low (Paul and Clark, 1996). In contrast to that, acetogenic bacteria are considered to contribute significantly to C transformations in soil (Drake et al., 1997; Küsel and Drake, 1999). However, their acetogenic activity is also confined to anaerobic microhabitats or transiently anaerobic sites, e.g. aggregate cores. As the soil used in our experiment was not strongly aggregated and the respiration data do not indicate a significant depletion of O₂ in the gas phase, it is not very likely that these anaerobic processes accounted for the CO₂ fixation observed.

Instead, we have to assume that heterotrophic organisms also contribute to CO₂ assimilation. This is strongly indicated by the facts that (1) the addition of readily available C sources enhanced the fixation of CO₂, and that (2) respiration and CO₂ fixation are

coupled. CO₂ fixation during heterotrophic growth can also be inferred from experiments with axenic cultures. For example, during heterotrophic growth of *Thiobacillus novellus*, 13% of the biomass originated from CO₂, not from the organic substrate (Perez and Matin, 1982), indicating that CO₂ is also necessary for heterotrophic growth and that it contributes significantly to cell biomass. Even fungi as heterotrophic organisms depend on CO₂ for normal growth. It has been shown that the growth of *Verticillium albo-atrum* is nearly completely inhibited if grown on glucose or glycerol as C source in the absence of CO₂ (Hartman et al., 1972).

The anaplerotic reactions are important reaction sequences of the central metabolism in heterotrophic organisms including CO₂ fixation (Krebs, 1941). These reactions are needed to regenerate oxaloacetate, the acceptor molecule of the citric acid cycle, when compounds (in particular succinyl-CoA and 2-oxoglutarate) are withdrawn from this cycle and used for biosynthesis of porphyrins and amino acids. These processes are therefore closely related to growth and synthesis of biomass. It was estimated that CO₂ fixation by anaplerotic reactions during heterotrophic growth contributes around 10% of the cell carbon (Perez and Matin, 1982; Sonntag et al., 1995). The contribution of anaplerotic reactions could also explain the lower CO₂ fixation rates relative to respiration towards the end of the experiment: They may be related to the declining nutrient supply resulting in less growth and more maintenance metabolism resulting in lower demand for CO₂.

Another central process involving CO₂ fixation is the synthesis of pyrimidines and purine nucleotides (Michal, 1999) that are essential compounds of DNA and RNA and therefore must be synthesized for cell growth. Incorporation of ¹⁴C into nucleic acids has been shown for some fungi (Hartman et al., 1972; Parkinson et al., 1991). Other perhaps less important reactions including CO₂ fixation steps are the degradation of leucine and the synthesis of biotin (Michal, 1999). However, none of those reactions have previously been considered for the analysis of soil carbon flow in soil. To decide which of these reactions are involved in CO₂ fixation and what their specific contributions are, we need more information about the processes involved. To this end, we are currently studying the partitioning of the label from CO₂ into various different soil fractions and compounds.

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