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Microbial community changes during humification of ^{14}C-labelled maize straw in heat-treated and native Orthic Luvisol

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Summary

The microbial communities in agricultural soils are responsible for nutrient cycling and thus for maintaining soil fertility. However, there is still a considerable lack of knowledge on anthropogenic impacts on soils, their microflora, and the associated nutrient cycles. In this microcosm study, microorganisms involved in the conversion of crop residues were investigated by means of classical microbiological and molecular methods such as denaturing gradient gel electrophoresis (DGGE) of PCR (polymerase chain reaction) amplified 16S rRNA genes. ^{14}C-labelled maize straw was humified by the naturally occurring microflora in native and in ashed soils, from which organic carbon was removed by heating at 600°C. The humic acids synthesized in the microcosms served as indicators of the humification process and were analysed by ^{13}C-NMR spectroscopy. Ashed, autoclaved and native soil exhibited similar microbial and physicochemical dynamics after inoculation with a soil suspension. Bacterial counts and DGGE analyses showed that in the first few weeks a small number of rapidly growing *r*-strategists were principally responsible for the conversion of maize straw. As the incubation continued, the bacterial diversity increased as well as the fungal biomass. ^{13}C-NMR spectroscopy of 26-week old soil extracts revealed that structures typical of humic substances also evolved from the plant material.

Introduction

The positive impact of soil organic matter (SOM) on the chemical, physical and biological properties of soils is crucial for soil fertility. Humification of SOM is governed by biological processes that play a central role in carbon and nutrient cycles. Hence, the turnover of organic matter with its seasonal input and output dynamics, which induces biotic and abiotic heterogeneity, is of special interest. In good agricultural practice a loss of organic carbon due to mineralization or leaching can be balanced by the incorporation of crop residues, which are subsequently humified by abiotic and biotic processes (Kumar & Goh, 2000). The agricultural practice of crop residue incorporation, which helps to improve soil quality by restoring plant nutrients, also imposes at least a temporary sink for atmospheric carbon dioxide (Sauerbeck, 2001). However, there is still a great lack of understanding on how microbial activity, as well as soil physical and chemical characteristics, determines carbon dynamics. Detailed knowledge about these microbial processes and interactions with the abiotic environment is required, if anthropogenic impacts in the form of land management, fertilization, crop protection or others on the soil and its microflora are to be evaluated (Buckley & Schmidt, 2003; Liebich *et al*., 2003).

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Previous investigations of microbial processes were limited to culturable species, with the drawbacks that they disregard possibly 99% of the native microbial population (Torsvik *et al*., 1990) and that those organisms do not necessarily represent the most dominant species *in situ*. While there is still a potential to increase the number of culturable microorganisms, when addressing soil ecological questions, the application of cultivation-independent biochemical and molecular techniques is of special importance, as today's cultivation-dependent techniques favour organisms with high growth rates under optimal conditions (so called *r*-strategists). However, organisms with the ability to degrade complex organic matter in soil ecosystems are often adapted to (comparably slow) growth at limited substrate availability (*K*-strategists) and thus are often underestimated when assessed with culture-dependent techniques (Torsvik *et al*., 1990). While the molecular techniques are aiming to open the door to the black box of soil microbiology, NMR spectroscopy is nowadays one of the most often employed methods to elucidate the chemical structure of soil organic matter, with the result that the understanding of SOM has changed dramatically in recent years (Preston, 1996; Schnitzer, 2000; Conte *et al*., 2004).

However, these developments in molecular biology and soil chemistry have so far not been combined to characterize the structure of a soil microbial community and its impact on the humification of organic plant material. By combining these molecular and spectroscopic approaches with classical microbiological techniques, we were aiming to see whether expected changes in soil microbial community composition during the degradation of dead plant material are accompanied by changes in the functional properties with respect to growth and substrate use strategies as well as to their culturability under standard laboratory conditions. Combined microbiological and physicochemical results should also be used to validate the suitability of our microcosms for research on humification processes and possible anthropogenic impact thereon (Liebich *et al.*, 2003).

Materials and methods

Soil microcosms

The microcosm experiments were carried out in 250-ml Erlenmeyer flasks equipped with carbon dioxide traps according to Anderson (1975). Soils were taken from the plough layer (0–20 cm) of an Orthic Luvisol (FAO soil classification, 3.3% sand, 80.6% silt, 16.1% clay, 0.92% C_{org}, 0.081% N_{total}, pH 7.0) from Merzenhausen, Germany (6°17′46′E, 50°55′47′′N, 93 m altitude) and sieved at 2 mm. The microcosms differed by the pre-treatment of the soil matrix and were characterized as: ashed soils, which enabled the spectroscopic characterization solely of newly produced humic substances by NMR; autoclaved soils, which served as a linkage between the other two treatments; and native soils containing the native SOM content. The ashed soils were produced by heating at 600°C for 48 h. The autoclaved soils were sterilized three times with wet heat at 121°C for 60 minutes and native soils were air-dried at room temperature.

As a source for new humus formation, 2 g maize straw (air-dried, pieces < 5 mm, 1% of the mass ^{14}C-radiolabelled, with a total ^{14}C-activity of 5.6 kBq; Wais, 1997) was mixed into the microcosms containing 100 g of soil, either ashed (A), autoclaved (B) or native (C). While the native soil already contained a viable native microbial community, ashed and autoclaved soils were inoculated with 1 ml of a soil suspension, which was gained by vigorous shaking for 5 minutes of 1 g fresh soil with 9 ml of a Tween buffer (5.0 g litre^{-1} Tween 80, 0.1 g litre^{-1} NaCl, 0.02 g litre^{-1} $CaCl_2{\cdot}H_2O$, 0.2 g litre^{-1} $Mg_2SO_4{\cdot}7\ H_2O$, pH 7.0). By using a soil suspension including smaller soil particles, we were able to omit some of the disadvantages related to the extraction of viable microorganisms.

The water content in the soils was adjusted to 40% water-holding capacity, corresponding to 22 ml H_2O in native soils and 24 ml H_2O in the heat-treated soils. Additionally 4 g H_2O were applied to compensate for the water uptake by the maize straw due to swelling. The microcosms were fertilized with a calcium ammonium nitrate fertilizer for agricultural use, which was applied at 0.2 g N kg^{-1} soil. This amount is in the range of agricultural practice and corresponds to 200–300 kg N ha^{-1} depending on soil density under the presumption that the total amount stays in a plough layer of 40 cm depth. The microcosms were incubated at 20°C in the dark for 26 weeks.

Mineralization of maize straw

The $^{14}CO_2$ produced in the microcosms from the radiolabelled maize straw was captured in tubes containing 10 g soda lime. Before opening, the flasks were flushed with N_2 to transfer the remaining $^{14}CO_2$ into the soda lime traps. After disassembling the traps, fixed CO_2 was released from the soda lime with 50 ml of 6 M HCl and collected in wash bottles containing 75 ml of an ethanolamine-methanol (3:7) solution. Duplicate aliquots of 1 ml were mixed with 10 ml of a scintillation cocktail (Instant Scint-Gel Plus, Canberra Packard, Meriden, CT, USA) and analysed by liquid scintillation counting (Tri-Carb 25600 TR, Canberra Packard).

Microbial activity

Microbial activity was measured by reduction of dimethylsulfoxide (DMSO) to dimethylsulfide (DMS) according to Alef & Kleiner (1989). Briefly, 10-g aliquots of soil were weighed into airtight glass bottles. After 10 minutes of incubation at 40°C, 2 ml of a 10% (W/V) DMSO solution were added to the bottles and incubated at 40°C for 3 h; 250 µl-aliquots of the headspace were sampled and quantified by gas chromatography (HP 8590 Series II, Hewlett-Packard, Avondale, PA, equipped with a GS-Q Megabore column, Fisons Instruments, Beverly, MA, and a flame-ionization detector).

Culturable bacteria

One gram of homogenized soil was extracted with 9 ml sterile Tween buffer (see above), and 0.1 ml from a dilution series (10^3–10^{-7}) in sterile 0.9% NaCl was plated in duplicates on Difco R2A (BD, Franklin Lakes, NJ, USA) plates, which were incubated for 5 days at 28°C. Colony-forming units (cfu) were calculated as mean values from three independent microcosms.

Soil ergosterol content

Soil ergosterol was extracted according to Eash *et al.* (1996) with a few minor changes as described by Liebich *et al.* (2003). Briefly, 3 g of soil were homogenized in a mixture of 18 ml methanol + 6 ml potassium hydroxide-solution (40 g litre^{-1} KOH in 96% ethanol), followed by 30 minutes of incubation at 85°C. The samples were diluted with 5 ml H_2O and vacuum-filtered. The methanol solutions were extracted with *n*-hexane and concentrated under nitrogen flow. Ergosterol contents of

10-µl aliquots were quantified by HPLC (column: LiChrospher Select-B 60 RP, Merck) with a mobile phase of 80% acetonitrile in water (v/v) at 25°C and a flow rate of 0.8 ml minute^{-1}. Ergosterol peaks were detected with a diode array detector (UV/Vis-Detektor UVD 340, Gynkotek, Germering, Germany) at 282 nm. The UV-spectra of ergosterol standards were used for definitive identification.

Bacterial diversity

Three grams of soil were incubated in 5 ml lysis buffer I (pH 8.0) containing 50 mM Tris, 100 mM NaCl, 25% (w/v) saccharose, 0.6 g polyvinypolypyrrolidone (acid-washed), 4 mg lysozyme, and 2 g glass beads (Ø 0.17–0.18 mm) at 37°C for 30 minutes After this pre-incubation 2 ml Lysis buffer II (50 mM Tris, 50 mM EDTA (ethylenediaminetetraacetic acid), 1% (W/V) sodium dodecyl sulfate, 10% (V/V) Tris-saturated phenol/chloroform/isoamylalcohol 25:24:1, ICN Biochemicals, Aurora, OH, USA) were added, and the suspension was homogenized for 2 minutes (VI 4, Edmund Bühler, Tübingen, Germany). The mixture was immediately transferred into centrifuge tubes and incubated on ice for 60 minutes. Nucleic acids were separated from proteins and cell debris using 5 ml of a phenol/chloroform/isoamylalcohol solution (25:24:1, by volume) and washed twice with 5 ml of a chloroform/isoamylalcohol solution (24:1, by volume). DNA in the aqueous phase was precipitated using 2 volumes of cold ethanol (−20°C) and 1/10 volume of potassium acetate (3 M, pH 4.8) at −20°C. The precipitated DNA was washed twice with cold ethanol (−20°C) and cleaned by agarose gel electrophoresis (1% W/V). DNA was extracted from excised gel pieces with QIAex II (Qiagen, Hilden, Germany).

Aliquots of 5 µl volume were used for amplification in a total of 100 µl buffer containing 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (by volume) Tween, 1.5 mM $MgCl_2$, 200 µM of each dNTP (all Advanced Biotechnologies, Epsom, UK), 420 mM DMSO (Sigma-Aldrich, Steinheim, Germany), 0.1 µM of each primer (MWG Biotech, Ebersberg, Germany) in sterile Milli-Q water (Millipore, Eschborn, Germany). The primers L1401 (5′-CGG TGT GTA CAA GAC CC-3′) and U968GC (5′- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC -3′) are specific for the eubacterial 16S rRNA genes (Nübel *et al.*, 1996) with U968GC containing a GC clamp (Muyzer *et al.*, 1993). After performing a hot start for 10 minutes at 94°C, 0.5 µl Red Hot Taq Polymerase (5 U µl^{-1}, Advanced Biotechnologies) was added. Polymerase chain reaction (PCR) was carried out in 34 cycles, each consisting of 1 minute at 94°C, 1 minute at 54°C, and 1 minute at 72°C, followed by a final extension period of 10 minutes at 72°C. Amplification products were purified by spin columns (QIAquick, Qiagen, Hilden, Germany) and analysed by electrophoresis in 1% (w/v) agarose gels.

DGGE was performed with a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA) by using polyacrylamide gels (6% (W/V) acrylamide, 0.1% (W/V) ammonium persulfate, 0.1% (by volume) TEMED (N,N,N′,N′- tetramethylethylenediamine) in TAE (Tris-acetate-EDTA) buffer (Bio-Rad)) with gradients from 40 to 60% of denaturant (100% denaturant corresponds to 7 M urea and 40% formamide (V/V) in TAE buffer) at 60°C at a constant voltage of 50 V for 16 h. DNA-bands were visualized by silver staining as described by Meyer *et al.* (1991).

Characterization of humic acids

Before incubation and after 26 weeks, humic acids were extracted from combined soil samples of parallel batches and measured by ^{13}C liquid NMR spectrometry. ^{13}C-NMR spectra were compared with the spectrum of humic acids extracts from native soil that had not been amended with maize straw. Soil samples were fractionated with basic and acidic agents as formerly described for the extraction of soil-bound residues (Khan & Ivarson, 1982; Liebich *et al.*, 1999), without a prior treatment with organic solvents. Briefly, 20 g of soil were extracted three times with 40 ml 0.5 M NaOH for 16 h under N_2. The combined supernatants were acidified with HCl until a pH of 1–1.5 was established. The precipitate contained the humic acids fraction. Prior to NMR analyses, humic acids were washed three times with water to reduce the salt content and were subsequently dissolved in 3.5 ml 0.14 M deuterated NaOH (2–12 mg C ml^{-1}). ^{13}C-NMR spectroscopy was carried out in 10-mm tubes by inverse-gated decoupling as described by Wais *et al.* (1996) with an AMX 400 NMR spectrometer (100.6 MHz, Bruker, Rheinstetten, Germany). Acquisition time was 0.172 s, pulse width 45°, relaxation delay 1.0 s, and the decoupler off during relaxation. Line broadening was carried out at 50 Hz.

C/N ratios

For determining C/N ratios, the soils were coarsely ground and triplicate samples (2 mg) of each soil were oxidized at a temperature of 1000°C in flowing oxygen in the CHNS analyser (CHNS-932, LECO, St Joseph, MI, USA). CO_2 and NO_x combustion gases were measured by selective IR detectors. The nitrogen content was determined by thermal conductivity detection.

Statistical analyses

For each treatment and each sampling time (day 0 and after 1, 2, 3, 4, 6, 10, 20 and 26 weeks), triplicate microcosms were set up, incubated at 20°C in the dark, and analysed in total upon sampling. Data for mineralization of ^{14}C-labelled maize straw, microbial activity, soil ergosterol content, and colony forming units were analysed by a one-way ANOVA using Origin 7G (OriginLab Corporation, Northampton, MA, USA).

Results

Microbial activity (DMSO reductase rates)

The progression of the microbial activity assessed by DMSO reductase rates (Figure 1a) in all cases followed a sigmoidal curve with large initial values followed by a reduction of the microbial activity. At the start of the incubation (day 0), DMSO reductase rates could only be detected in native soil (C, 320 ng DMS g^{-1} (dry soil) h^{-1} (Figure 1a), which subsequently increased to a maximum of 500 ng DMS g^{-1} h^{-1} in the fourth week.

In the reinoculated ashed and autoclaved soils, microbial activity never reached the values initially observed in the native soils. At the peak of the straw degradation (cf. Figure 1d), DMSO reductase rates rose to 220 and 290 ng DMS g^{-1} h^{-1}, respectively. However, this increase was not steady, but interrupted by an intermediate decrease after 4 weeks of incubation, which was also observed after 2 weeks in the native soil. Maximum DMSO reductase rates were measured in the reinoculated autoclaved soils after 10 weeks and in the ashed soils after 20 weeks of incubation.

In comparing the different treatments, it becomes apparent that up to the 10th week when the microbial activity was similar in all treatments, there was a significant ranking of increasing DMSO reductase rates, from the microcosms with ashed soil (A) < autoclaved soil (B) < native soil (C) ($P < 0.05$), with the single exception that no significant difference was measured between ashed and autoclaved soils in the 3-week-old microcosms.

Culturable bacteria

In contrast to the microbial activity data, the number of colony-forming units (cfu, Figure 1b) decreased after 1 week of incubation, when the maximum counts occurred. Only directly after the reinoculation of the ashed and the autoclaved soils was the number of colony-forming units less ($< 10^6$ cfu g^{-1} soil) than the counts in the native soil (37×10^6 cfu g^{-1}, data not shown). After 1 week, maximum bacterial counts were observed in all microcosms with values between 1.3×10^9 and 1.6×10^9 cfu g^{-1} in the ashed and the reinoculated autoclaved soil and significantly smaller counts in the native soil (C, 0.54×10^9 cfu g^{-1}, Figure 1b). During the course of the experiment, counts from the ashed and the autoclaved soils were similar (with the exception of the samples from week 10) and decreased almost continuously after the maximum in the

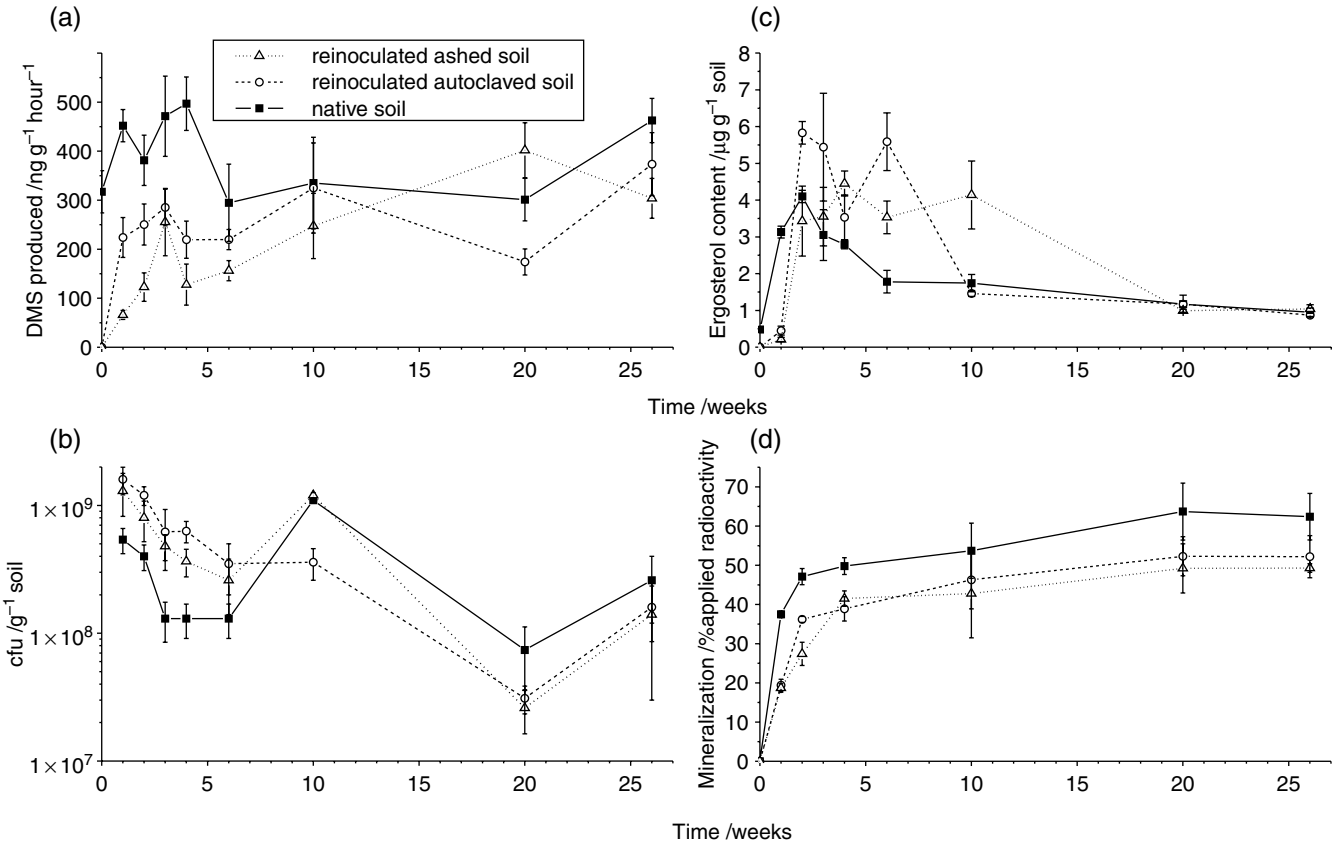

Figure 1 Microbiological data for ashed, autoclaved (reinoculated by a soil suspension) and fresh soil after incorporation of maize straw (± standard deviations). (a) Microbial activity in DMS ng g^{-1} (dry soil) h^{-1}. (b) Colony-forming units as cfu g^{-1} dry soil. (c) Ergosterol content in µg g^{-1} dry soil. (d) Mineralization of the ^{14}C-labelled maize straw (applied radioactivity = 100%).

first week, although microbial activity continued to increase (Figure 1a). In the native soils, the number of colony-forming units dropped rapidly in week three to 0.13×10^9 cfu g^{-1} soil. However, in the same way as in ashed soil, another maximum of 1.1×10^9 cfu g^{-1} was observed after 10 weeks.

Ergosterol contents

The ergosterol content served as an indicator of fungal soil biomass (Figure 1c). At the start of the experiment, ergosterol contents were below the detection limit of 0.2 μg g^{-1} soil in autoclaved and ashed soil, whereas the native soil contained 0.5 μg ergosterol g^{-1} soil. After 1 week of incubation, the values in the native soil (3.1 μg ergosterol g^{-1}) were significantly greater than in the microcosms reinoculated with soil suspensions (each < 0.5 μg ergosterol g^{-1}). In all cases a maximum was reached after 2 weeks (Figure 1c). For the treatments with ashed soil and with native soil this maximum was 3.4 and 4.1 μg ergosterol g^{-1} soil (dry matter), respectively, and for autoclaved soil 5.8 μg ergosterol g^{-1}. After 3 weeks, the ergosterol contents in the microcosms with autoclaved soil and native soil gradually decreased and after the 10th week similar values were measured (1.5 and 1.7 μg ergosterol g^{-1}) whereas the ergosterol content in ashed soil was greater (4.1 μg ergosterol g^{-1}, Figure 1c). From the 20th week all treatments had comparable ergosterol contents between 0.9 and 1.2 μg ergosterol g^{-1} soil.

Degradation of ^{14}C-labelled maize straw

The greatest degradation rates for ^{14}C-labelled maize straw in the soil microcosms was evident within the first weeks of the incubation, but distinct differences between the treatments could be observed (Figure 1d). After 1 week, on average 38% of the radiocarbon applied in the microcosms with native soil was mineralized. In contrast, the $^{14}CO_2$ releases in the autoclaved and the ashed soils (both reinoculated with a soil suspension) were about 19% each. Fast mineralization rates were also observed in the second week of incubation. After 3 weeks, the weekly mineralization rates were $< 3\%$ of the labelled material present at that time. Only in the microcosms with ashed soil, did mineralization rates continue to increase to the end of the fourth week. The 10% greater straw degradation in the microcosms with native soil was maintained until the end of the experimental period and resulted in an overall degradation of 62% of the applied ^{14}C-labelled maize straw. However, in the two reinoculated soils (treatments A and B) only 49% and 52% of the radioactive material applied was converted into $^{14}CO_2$, respectively.

Bacterial diversity

The structure of the soil microbial communities was characterized by denaturing-gradient gel electrophoresis (DGGE). All treatments had similar bacterial community composition after 2 weeks with a small number of bands in the lower half of the gel (Figure 2). In the upper portion of the gel, bands were considerably weaker and indicate a smaller quantity of DNA. After 10 weeks, more bands were detected, each with weaker intensity. However, bands also occurred in the different treatments, which were weaker or not present at all in the other treatments. The overall weaker intensity of bands in the genetic profile of treatment A (ashed soil) was probably caused by a smaller quantity of DNA. This is also supported by the fact that in this treatment the number of colony-forming units (as biomass indicators) was less after 10 weeks of incubation than in the other two treatments. In all treatments, the number of bands is greater in the 10th week than in the earlier sample,

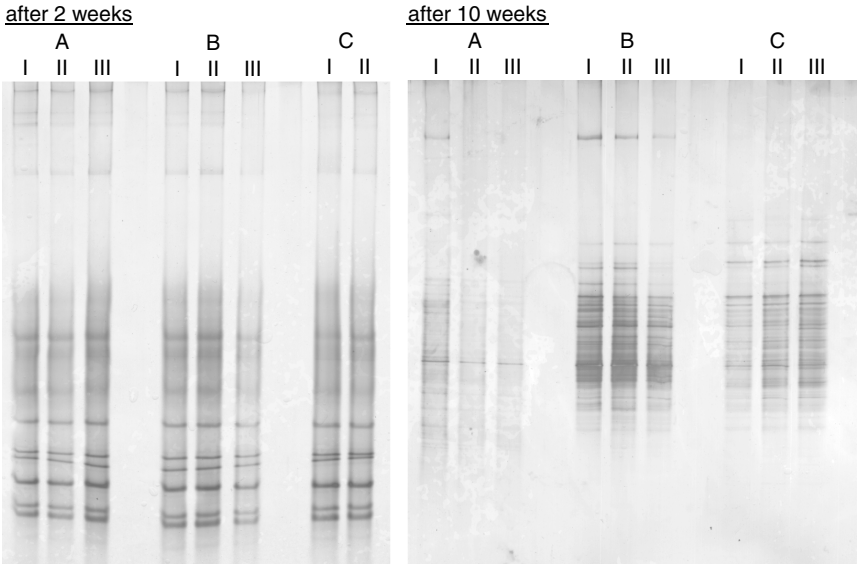

Figure 2 Denaturing-gradient gel-electrophoresis of the PCR products of 16S rRNA genes of the microbial communities in ashed (a), autoclaved (b) and native (c) soil after 2 weeks and after 10 weeks of incubation (I, II and III are genetic profiles from replicate microcosms of each treatment).

suggesting that 2 weeks after straw addition a few bacterial species dominated the bacterial community, which were replaced by a more complex microbial community later on.

C/N ratios in the soil

As primary ashed soil + maize straw, the autoclaved soil + maize straw, and the native soil + maize straw, differed in their C/N ratios, this parameter is suitable for describing the conversion of organic material in the microcosms. The C/N ratio was greatest in the microcosms with ashed soil immediately after starting the experiment. The average value was 20:1. This value was caused mainly by the C/N ratio of the added maize straw (34:1). The C/N ratio in the microcosms with autoclaved or native soil showed average values of 12:1 and 10:1, respectively. However, the data did not differ significantly in a *t*-test ($P < 0.05$). During the incubation, the C/N ratio decreased gradually and after 10 weeks it reached, for all treatments, the level of the native soil with a C/N ratio of 7.2 (Figure 3).

Analysis of humic acids extracts

The effect of humification is particularly clear in the spectrum of humic acids from the ashed soil (Figure 4) as no soil-derived organic carbon was present at the beginning and the spectrum recorded before incubation corresponds to a pure maize straw extract. Thus, the NMR spectrum at the start of the experiment is strongly structured at a chemical shift between 50 and 110 p.p.m. (characteristic for carbohydrates and polyalcohols, Piccolo *et al*., 1990; Hopkins *et al*., 2000). After incubation, peaks within this range of chemical shifts diminished, whereas peaks between $\delta = 25$–45 p.p.m. (methylene groups in aliphatic rings and chains) and between $\delta = 120$–180 p.p.m. (aromatic-C, carbonyl- and carboxyl-groups) increased in number and intensity. Remarkably in this range, there is a new occurrence of peaks around a chemical shift of ~175 p.p.m., which can also be found in the humic acids from the native Orthic Luvisol, but not in the maize straw extract. However, certain signature peaks of the maize straw extract, e.g. at a chemical shift of 153 p.p.m., are still detectable after 26 weeks of incubation.

In the spectra of the humic acids from autoclaved soil + maize straw (Figure 5) and from native soil + maize straw (Figure 6), the signals from the maize straw and the soil matrix overlap. At the beginning, both the characteristic features of the maize straw extract ($\delta = 50$–110 p.p.m.) and the peaks also present in the spectrum of humic acids from the native Orthic Luvisol prior to the addtion of maize straw can be seen (Figure 7). This spectrum can be used as a control for native humic acids spectra from our soil. In the course of incubation, the intensity of the signals typical of maize straw (cf. Figure 4 at the start of the experiment) decrease relative to the signal in the other regions and the spectra become similar to the spectrum of the Orthic Luvisol, with a good concordance between the spectra of treatments B (autoclaved soil) and C (native soil).

Discussion

Mineralization of maize straw in all treatments was characterized by an initial flush of $^{14}CO_2$ production within the first 2 weeks, when at least 50% of the total mineralization activity was observed. This flush is greater than decribed by Recous *et al*. (1995), who worked at lower temperatures.

In the native soil, more ^{14}C-labelled maize straw was mineralized compared with the reinoculated treatments. A more pronounced increase in $^{14}CO_2$ production in the first week of incubation was observed as well as greater absolute values throughout the experiment (Figure 1D). The greater quantity of mineralized straw in the native soil is accompanied by greater microbial activity during the first weeks of incubation compared with the ashed and the autoclaved soils (Figure 1a). This greater total mineralization rate compared with the other treatments may have resulted either from shorter turnover times of newly synthesized microbial biomass or from the

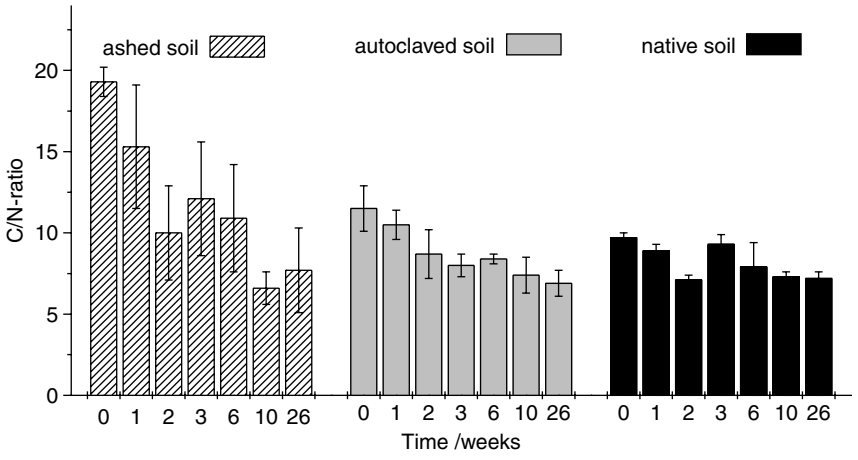

Figure 3 Changes of C/N ratios in soils amended with maize straw within 26 weeks of incubation.

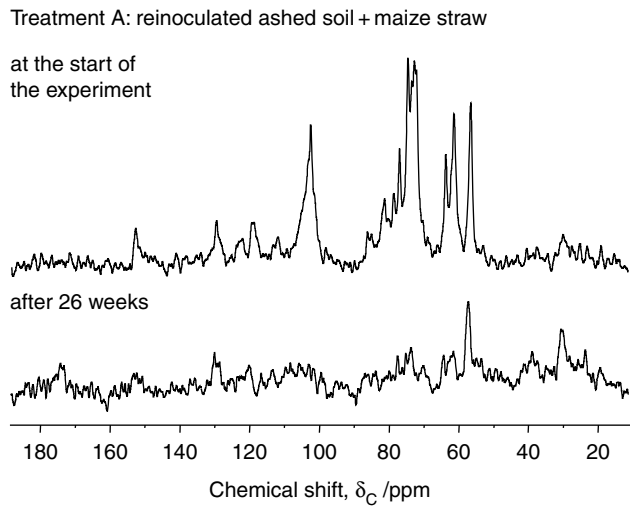

Figure 4 ^{13}C-NMR spectra of humic acids extracts from maize straw decomposing in ashed soil before and 26 weeks after inoculation by a soil suspension (200 000 scans each).

inclusion of more recalcitrant components of the maize straw in the mineralization.

Also after 1 week of incubation, microbial activity and fungal biomass are greatest in native soils, suggesting that fungi play an important role in the initial degradation of the straw material. Unlike microbial activity, cfus had already dropped after their maximum value during the first week of incubation. This could be explained by an early end of the logarithmic growth phase. On the other hand, microbial activity and mineralization rates were still large at the same time. A possible change in the composition of the microbial community may have caused a shift from the culturable portion of the population towards non-culturable microorganisms under the conditions applied.

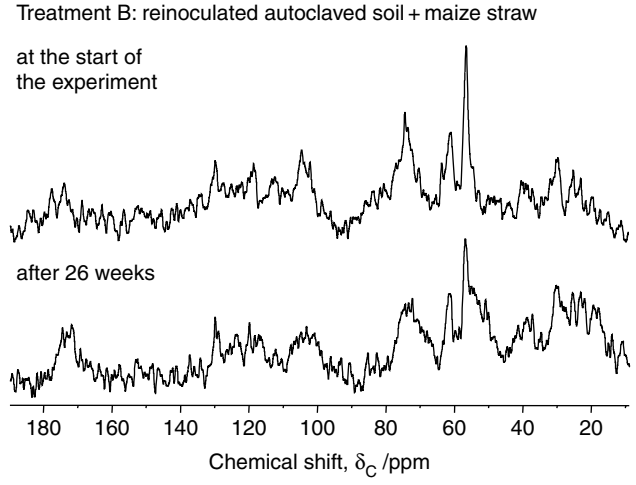

Figure 5 ^{13}C-NMR spectra of humic acids extracts from maize straw decomposing in autoclaved soil before (62 000 scans) and 26 weeks after inoculation with a soil suspension (200 000 scans).

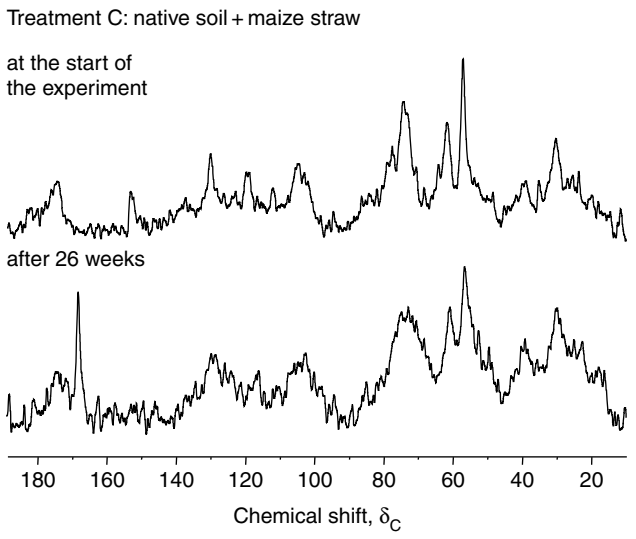

Figure 6 ^{13}C-NMR spectra of humic acids extracts from maize straw decomposing in native soil before and 26 weeks after the start of incubation (200 000 scans).

Right after the addition of organic material, microbes with fast growth rates have a competitive advantage (*r*-strategists, Andrews & Harris, 1986). However, fast growth rates are also characteristic for organisms that accumulate easily under ashed cultivation conditions with single carbon sources (e.g. glucose, starch or amino acids), forming distinct colonies on agar plates. Consequently, the experimental conditions in the reinoculated soils were comparable with 'classical cultivation conditions' used for accumulation, isolation and growth of bacteria at an initial small cell density, which explains the large number of colony-forming units in these treatments in the first weeks of the incubation. However, this does not reflect the total microbial biomass but only the culturable portion of it and total biomass may be even greater in the native soil (which is also suggested by the greater DMSO reductase rates found in the native soil). There, the *r*-strategists are confronted with a greater competition pressure with the autochthonous microflora because of overall greater cell densities at the beginning compared with the reinoculated soils, thus leading to the comparably poor cfu counts. However, the same group of microbes seem to dominate the bacterial community, as shown by the striking agreement of

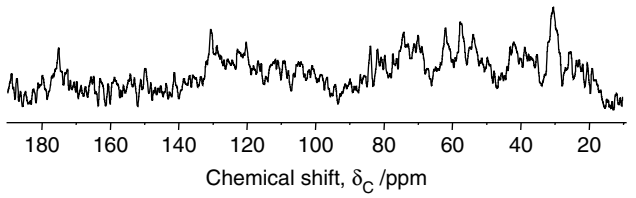

Figure 7 ^{13}C-NMR spectra of humic acids extracts from a typical Orthic Luvisol (70 000 scans).

the DGGE patterns after 2 weeks, each characterized by only a few bands of great intensity.

A shift in the microbial community composition is even more likely when cfus and DMSO reductase rates are compared directly (Figure 1a, b): both measures drop after a first maximum (native soil from week 1 to week 2, reinoculated soils from week 3 to week 4). However, this reduction in cfus and microbial activity is followed by an increase in DMSO reductase rates, whereas cfus continue to drop or remain stagnant. While this could also be a sole function of microbial activity of the existing community, it seems to be more likely that the microbial community composition has changed. Fast-growing bacteria that are adapted to sudden large carbon-input situations may have dominated the initial stage of the straw degradation leading to the poor diversity as detected by the 16S rDNA-patterns. In contrast, the genetic profile of the PCR products of DNA from 10-week-old soils is much richer in bands, with great similarities between the patterns from the different treatments. These similarities are in contrast to the microbiological sum parameters, which did differ between the treatments. This can be explained by the fact that only the most abundant organisms can be detected by the DGGE of PCR products from 16S rRNA genes (Muyzer *et al*., 1993). As in all treatments the *r*-strategists probably represent the largest population due to their rapid growth rates, they also dominated the DGGE patterns at the beginning of the experiment. It is therefore indeed possible that the microbial communities of the different treatments differ from each other in the first 4 weeks but that it was not possible to detect these differences by 16S rDNA-DGGE.

The soil ergosterol content served as an indicator of fungal biomass (Figure 1c). However, measured ergosterol contents were not transformed into total fungal biomass, as direct conversions of ergosterol contents into fungal biomass are still problematic because of variations between different fungal species and their metabolic state (Bermingham *et al*., 1995). Despite these criticisms, ergosterol is considered a reliable method for the relative measure of fungal biomass (Ruzicka *et al*., 2000). While a number of culturable bacteria reached their maxima after 1 week of incubation, ergosterol contents increased until the second week (native soils) or even longer. Because of shorter generation times prokaryotes with rapid growth rates have an advantage over fungi when readily utilizable carbon sources become available (Stamatiadis *et al*., 1990). However, the latter have the ability to utilize more recalcitrant compounds, which could be of advantage to them at a later phase of degradation. This conjecture is supported by the findings of Broder & Wagner (1988), who isolated fungi (e.g. *Penicillium*, *Trichoderma*, *Myrothecium*) in greater numbers at later stages of the crop residue turnover (maize, wheat and soya beans). After 20 and 26 weeks the extractable ergosterol content in all three treatments reached the same level of 0.9–1.2 $\mu g\ g^{-1}$ soil (dry mass). These values are comparable with those found by Jensen *et al*. (2000) in agricultural soils from Danish farms, which have received straw and mineral fertilizers over 5 years, but have not been treated with animal manure. These findings show the significance of our microcosms for agriculture-related research, e.g. to study pesticide side-effects on soil microbial communities (Liebich *et al*., 2003).

The humification of maize straw was followed by using ^{13}C-solution state-NMR of humic acids extracts. Although these humic acids extracts make up only a small portion of the total organic carbon, their characterization via ^{13}C-NMR spectroscopy has a long tradition since the mid-1980s (Schnitzer, 2000). As discussed by Zech *et al*. (1997), humification of crop residues is characterized by increasing ^{13}C-NMR signals related to carboxyl C-, alkyl C- and aromatic C-moieties, while signals related to O–alkyl C are reduced. Thus, from the ^{13}C liquid-NMR spectra of the humic acids extracts in all treatments, the development of functional groups characteristic for humic acids was obvious (cf. Figure 7; Preston & Schnitzer, 1984; Piccolo *et al*., 1990). However, the spectra of autoclaved and native soils were superimposed by the native carbon matrix. Thus, changes in NMR signals as indicators for humification could best be studied in the ashed soils. In particular, the strong decrease in signal intensity at $\delta = 60$–90 p.p.m. and at $\delta = 103$ p.p.m. in the ashed soil (cf. Figure 5) suggests a rapid and preferential consumption of carbohydrates and other polyalcohols. In contrast, the intensity of the peak at $\delta = 55$–60 p.p.m. only decreases later and incompletely. In this range, signals from amino acids (Schnitzer *et al*., 1993) and methoxyl-substituted carbons in lignins (Knicker, 2000) are found. After consumption of the carbohydrates, lignins and proteinaceous material are included to a greater extent into the microbial metabolism although with rather slow degradation rates. Wilson (1985) found half-lives for lignins between 280 and 340 days, and only after consumption of the carbohydrates is their decrease in signal intensity in the NMR spectra detectable. Under limiting conditions, nitrogen immobilized in the soil organic matter can serve as a readily available nitrogen source (Liebich *et al*., 1999). However, nitrogen was applied to the microcosms as an inorganic fertilizer and, at least at the start of incubation, nitrogen deficiency should not have occurred. The decrease of signal intensity in the described ranges was less pronounced in the spectra of the humic acids from autoclaved or native soil due to the background signals of the intact carbon matrix.

In the course of humification, signals indicating the presence of carbohydrates and other polyalcohols ($\delta = 57$–103 p.p.m.) diminish, whereas the number and intensity of the peaks related to newly synthesized alkyl carbon structures ($\delta = 25$–45 p.p.m.) and in the range of aromatic carboxyl and carbonyl groups ($\delta = 120$–180 p.p.m.) increase. In the aromatic range, there is a striking decrease of the signal at $\delta = 153$ p.p.m., which possibly represents oxygen- or nitrogen-substituted aromatic carbon atoms (phenolic OH and/or NH_2 bonded to an aromatic C, Schnitzer, 2000), which, as a function of time, are included in the metabolism of the maize straw (Figure 7).

In all spectra, a perceptible increase in signal intensities was observed in the range of the carboxyl-C, amide-C and ester-C atoms ($\delta = 170$–175 p.p.m., Piccolo *et al.*, 1990; Hopkins *et al.*, 2000; Wais, 1997; Figures 4–6). Whereas native humic acids in the autoclaved and native soils overlap with the added plant material, in the ashed soil no distinct signal with this chemical shift was observed at the start of the experiment, in contrast to the samples taken after 26 weeks. Knicker (2000) found a similar development in ^{13}C-NMR spectra of degraded wheat straw, with clearly more pronounced signal intensity after 4 years compared with the sample taken after 58 days. In our experiment, these signals were not visible in the spectrum of the maize straw extract but they were present in that of the original soil. As these signals are absent in the spectra of the unchanged straw material, they might be regarded as an indicator of humification.

Conclusions

The *r*-strategists were responsible principally for the conversion of maize straw in the first weeks of incubation. This can be seen from the large proportion of culturable organisms that produced identical and very simple genetic profiles in all treatments. The selective conditions were present in all treatments, although greater microbial activity and fungal biomass was detected from the very beginning in microcosms with native soil. At the same time, the culturable organisms were exposed to greater competitive pressure so that in comparison to the reinoculated treatments in the cases of ashed or autoclaved soil, fewer bacterial counts were determined. Two weeks after the start of incubation, the genetic profiles could not be differentiated, which underlines the dominance of fast-growing *r*-strategists for the metabolization of readily available carbon sources in the soil. As the incubation proceeded, the diversity of the genetic profiles increased as well as the fungal biomass, while the bacterial cfu tended to decrease. Together with the ^{13}C-solution state-NMR analyses, this study showed that the microcosms described indeed are suitable for monitoring the humification of crop residues in soil and also possible anthropogenic impacts thereon. During plant residue turnover in agricultural soils, microbial community composition changes from a dominance of fast growing *r*-strategists, which are cultivable under simple laboratory conditions, to a community of greater bacterial diversity.

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References

Alef, K. & Kleiner, D. 1989. Rapid and sensitive determination of microbial activity in soils and in soil aggregates by dimethylsulfoxide reduction. *Biology and Fertility of Soils*, **8,** 349–355.

Anderson, J.P.E. 1975. Einfluß von Temperatur und Feuchte auf Verdampfung, Abbau und Festlegung von Diallat im Boden. *Journal of Plant Diseases and Protection*, **SH 7,** 141–146.

Andrews, J.H. & Harris, R.F. 1986. *r*- and *K*-selection and microbial ecology. *Advances in Microbial Ecology*, **9,** 99–147.

Bermingham, S., Maltby, L. & Cooke, R.C. 1995. A critical assessment of the validity of ergosterol as an indicator of fungal biomass. *Mycological Research*, **99,** 479–484.

Broder, M.W. & Wagner, G.H. 1988. Microbial colonization and decomposition of corn, wheat, and soybean residue. *Soil Science Society of America Journal*, **52,** 112–117.

Buckley, D.H. & Schmidt, T.M. 2003. Diversity and dynamics of microbial communities in soils from agroecosystems. *Environmental Microbiology*, **5,** 441–452.

Conte, P., Spaccini, R. & Piccolo, A. 2004. State of the art of CPMAS C-13-NMR spectroscopy applied to natural organic matter. *Progress in Nuclear Magnetic Resonance Spectroscopy*, **44,** 215–223.

Eash, N.S., Stahl, P.D., Parkin, T.B. & Karlen, D.L. 1996. A simplified method for extraction of ergosterol from soil. *Soil Science Society of America Journal*, **60,** 468–471.

Hopkins, D.W., Chudek, J.A., Haslam, S.F.I. & Webster, E.A. 2000. Application of solid-state NMR to investigate organic biogeochemistry in soils. In: *NMR in Microbiology: Theory and Applications* (eds J.-N. Barbotin & J.-C. Portais), pp. 431–451. Horizon Scientific Press, Wymondham.

Jensen, U.B., Elmholt, S. & Labouriau, R. 2000. Distribution of ergosterol in organically and conventionally cultivated agricultural soils. *Biological Agriculture and Horticulture*, **18,** 113–125.

Khan, S.U. & Ivarson, K.C. 1982. Release of soil bound (nonextractable) residues by various physiological groups of microorganisms. *Journal of Environmental Science and Health Part B*, **17,** 737–749.

Knicker, H. 2000. Biogenic nitrogen in soils as revealed by solid-state carbon-13 and nitrogen-15 nuclear magnetic resonance spectroscopy. *Journal of Environmental Quality*, **29,** 715–723.

Kumar, K. & Goh, K.M. 2000. Crop residues and management practices: effects on soil quantity, soil nitrogen dynamics, crop yield, and nitrogen recovery. *Advances in Agronomy*, **68,** 197–319.

Liebich, J., Burauel, P. & Führ, F. 1999. Microbial release and degradation of nonextractable anilazine residues. *Journal of Agricultural and Food Chemistry*, **47,** 3905–3910.

Liebich, J., Schäffer, A. & Burauel, P. 2003. Structural and functional approach to study pesticide side-effects on specific soil functions. *Environmental Toxicology and Chemistry*, **22,** 784–790.

Meyer, C.G., Tannich, E., Harders, J., Henco, K. & Horstman, R.D. 1991. Direct sequencing of variable HLA gene segments after in vitro amplification and allele separation by temperature gradient gel electrophoresis. *Journal of Immunological Methods*, **142,** 251–256.

Muyzer, G., De Waal, E.C. & Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes

coding for 16S rRNA. *Applied and Environmental Microbiology*, **59,** 695–700.

Nübel, U., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I., Ludwig, W. & Backhaus, H. 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology*, **178,** 5636–5643.

Piccolo, A., Campanella, L. & Petronio, B.M. 1990. Carbon-13 nuclear magnetic resonance spectra of soil humic substances extracted by different mechanisms. *Soil Science Society of America Journal*, **54,** 750–756.

Preston, C.M. 1996. Applications of NMR to soil organic matter analysis: history and prospects. *Soil Science*, **161,** 144–166.

Preston, C.M. & Schnitzer, M. 1984. Effects of chemical modifications and extractants on the carbon-13 NMR spectra of humic materials. *Soil Science Society of America Journal*, **48,** 305–311.

Recous, S., Robin, D., Darwis, D. & Mary, B. 1995. Soil inorganic N availability: effect on maize residue decomposition. *Soil Biology and Biochemistry*, **27,** 1529–1538.

Ruzicka, S., Edgerton, D., Norman, M. & Hill, T. 2000. The utility of ergosterol as a bioindicator of fungi in temperate soils. *Soil Biology and Biochemistry*, **32,** 989–1005.

Sauerbeck, D.R. 2001. CO_2 emissions and C sequestration by agriculture – perspectives and limitations. *Nutrient Cycling in Agroecosystems*, **60,** 253–266.

Schnitzer, M. 2000. A lifetime perspective on the chemistry of soil organic matter. *Advances in Agronomy*, **68,** 1–58.

Schnitzer, M., Dinel, H., Mathur, S.P., Schulten, H.R. & Owen, G. 1993. Determination of compost biomaturity. III. Evaluation of a colorimetric test by ^{13}C-NMR-spectroscopy and pyrolysis-field ionization mass spectroscopy. *Biological Agriculture and Horticulture*, **10,** 109–123.

Stamatiadis, S., Doran, J.W. & Ingham, E.R. 1990. Use of staining and inhibitors to separate fungal and bacterial activity in soil. *Soil Biology and Biochemistry*, **22,** 81–88.

Torsvik, V., Goksyør, J. & Daae, F. 1990. High diversity in DNA of soil bacteria. *Applied and Environmental Microbiology*, **56,** 782–787.

Wais, A. 1997. Non-extractable residues of pesticides in soil: analysis of humic substances and possible bindings of active ingredients and metabolites to humic substances. Doctoral dissertation, University of Bonn, Bonn, Germany.

Wais, A., Burauel, P., de Graaf, A.A., Haider, K. & Führ, F. 1996. Using ^{13}C-NMR spectroscopy to evaluate the binding mechanism of bound pesticide residues in soils. 2. Investigations of model humic acids. *Journal of Environmental Science and Health Part B*, **31,** 1–24.

Wilson, J.O. 1985. Decomposition of [^{14}C]lignocelluloses of *Spartina alterniflora and* a comparison of field experiments. *Applied and Environmental Microbiology*, **49,** 478–484.

Zech, W., Senesi, N., Guggenberger, G., Kaiser, K., Lehmann, J. & Miano, T.M. et al. 1997. Factors controlling humification and mineralization of soil organic matter in the tropics. *Geoderma*, **79,** 117–161.

