

Microbial activity and bacterial composition of H₂-treated soils with net CO₂ fixation

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Abstract

The effects of H₂ gas treatment of an agricultural soil cultivated previously with a mixture of clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) on CO₂ dynamics and microbial activity and composition were analyzed. The H₂ emission rate of 250 nmol H₂ g⁻¹ soil h⁻¹ was similar to the upper limit of estimated H₂ amounts emitted from N₂ fixing nodules into the surrounding soil ([Dong, Z., Layzell, D.B., 2001. H₂ oxidation, O₂ uptake and CO₂ fixation in hydrogen treated soil. *Plant and Soil* 229, 1–12.]). After 1 week of H₂ supply to soil samples simultaneously with H₂ uptake net CO₂ production declined continuously and this finally led to a net CO₂ fixation rate in the H₂-treated soil of 8 nmol CO₂ g⁻¹ soil h⁻¹. The time course of H₂ uptake and CO₂ fixation in the soils corresponded with an increase in microbial activity and biomass of the H₂-treated soil determined by microcalorimetric measurements, fluorescence in situ hybridization analysis (FISH) and DNA staining (DAPI). Shifts in the bacterial community structure caused by the supply of H₂ were recorded. While the H₂ treatment stimulated β- and γ-subclasses of Proteobacteria, it had no significant effect on α-Proteobacteria. In addition, FISH-detectable bacteria of the Cytophaga–Flavobacterium–Bacteroides phylum increased in numbers.

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1. Introduction

As an obligate byproduct of biological N₂ fixation large amounts of H₂ are produced (Simpson and Burris, 1984). In the rhizosphere of legume crops, H₂ is released when the rhizobia lack uptake hydrogenase enzyme activity (Uratsu et al., 1982). Simulations of H₂ flow by Dong and Layzell (2001) resulted in an average rate of 30–254 nmol H₂ g⁻¹ soil h⁻¹ at a distance of 1–4 cm from active legume nodules. Accordingly, about 240,000 l H₂ ha⁻¹ per season would be produced by a legume crop fixing 200 kg N ha⁻¹ (Dong et al., 2003). Conrad and Seiler (1979) have shown

that H₂ emitted into the rhizosphere is efficiently consumed and does not leave the soil. Soils oxidize H₂ (La Favre and Focht, 1983) and this process is linked with an increase in microbial biomass (Popelier et al., 1985). Dong and Layzell (2001) studied a H₂-treated soil using simultaneous measurements of H₂, CO₂ and O₂ and found an increase in H₂ uptake capacity accompanied by an increase of CO₂ fixation. At concentrations above 680 ml H₂ l⁻¹ even net CO₂ uptake could be attained. McLearn and Dong (2002) further showed a causal connection between H₂ uptake and CO₂ fixation due to microbial activity. Termination of the H₂ treatment resulted in a net CO₂ production, which could be switched back into a net CO₂ fixation through resupply of H₂. Furthermore, H₂ uptake ability as well as CO₂ fixation or evolution by the soil were eliminated by autoclaving or addition of antibiotics. Also, after addition of glucose, the H₂ uptake ability decreased while the CO₂ evolution rate increased considerably. McLearn and Dong (2002) suggest that the microbes responsible for H₂ uptake switched from

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using H_2 as the source of energy and reductant for CO_2 fixation to using glucose. After an initial lag period Dong and Layzell (2001) observed an effective H_2 and net CO_2 uptake in H_2 -treated soil using $147 \text{ nmol } H_2 \text{ g}^{-1} \text{ soil h}^{-1}$. In our H_2 treatment, we chose an average rate of $250 \text{ nmol } H_2 \text{ g}^{-1} \text{ soil h}^{-1}$. This represented the upper limit of estimated H_2 amounts emitted from N_2 fixing nodules into surrounding soil (Dong and Layzell, 2001).

Despite the clear demonstrations of pronounced H_2 effects on the biochemical activities of the soil microflora, effects on the microbial community composition are not known. Concerning the H_2 oxidizing bacteria, there is still no common molecular method to detect the diversity in ecosystems (Klüber et al., 1995). The known cultivated H_2 -oxidizing autotrophic bacteria belong mostly to the α - and β -subclasses of Proteobacteria. Therefore, we investigated the effects of H_2 treatment not only on soil microbial biomass and activity, but also on bacterial community structure. Heat production is a general measure of microbial biomass and activity, hence microcalorimetry was used to monitor microbial activity in the soil (Sparling, 1983). DNA-fluorescence staining (DAPI) and fluorescence in situ hybridization (FISH-analyses) were used to determine total counts of bacterial cells and to detect shifts in the bacterial community structure. In the FISH-analyses, fluorescence labelled oligonucleotide probes targeting the 16S rRNA of bacteria are applied to identify bacterial populations without the need of cultivation (Amann et al., 1995). The only prerequisites are the permeability of the bacteria, which is achieved by different fixatives like 4% paraformaldehyde (PFA) or 50% ethanol, and a high ribosome content providing enough 16S rRNA target molecules to stain single cells. Under these conditions bacterial cells can successfully be stained by rRNA directed oligonucleotide probes e.g. by the EUB 338 I, II, III mix specific for all bacteria (Amann et al., 1990; Daims et al., 1999). The community structure of Proteobacteria can be investigated by the application of group-specific probes for α -, β -, and γ -proteobacterial classes (Manz et al., 1992; Neef, 1997). Also other major bacterial groups like Cytophaga-Flavobacteria and the archaeal kingdom can be assessed using the ribosomal probes CF 319a (Manz et al., 1996) and Arch 915 (Stahl and Amann, 1991), respectively. Especially in the α - and β -subclasses of Proteobacteria, some species are known to harbour the 'red-like' RubisCO genes for CO_2 fixation, like several rhizobial species or *Xanthobacter* in the α -group and *Ralstonia* or *Burkholderia* in the β -group (Watson and Tabita, 1997). The chemolithoautotrophic nitrifying bacteria fall into the α -, β -, and γ -proteobacterial classes and can harbour both, the so-called 'green-like' and 'red-like' *cbbL*-genes. The assessment of the above mentioned proteobacterial groups should provide original information about a qualitative shift in the soil bacterial community structure involved in H_2 uptake and CO_2 fixation.

2. Materials and methods

2.1. Soil sampling, characteristics and preparation

The soil under investigation was taken in winter (5°C) from the upper 10 cm of the A_p -horizon of a field cultivated previously with a mixture of clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*) at the research farm Scheuern (Research Network on Agroecosystems Munich). The soil is a loamy cambisol from loess with a loam content of $0.1\text{--}0.15 \text{ kg kg}^{-1}$ soil, organic C content of 1.5%, N content of $0.15\text{--}0.23\%$ and pH 5.5–6.0. After sieving (2 mm) the bulk soil, the water content was adjusted to 50% of the water-holding capacity. The soil was stored in a plastic bag at 4°C for up to a month, before the start of the H_2 treatment.

2.2. H_2 treatment system

The H_2 treatment was applied in a climatic chamber at 14°C over 30 d (Fig. 1). The H_2 treatment employed an H_2 -air mixture of $2000 \text{ ml } H_2 \text{ l}^{-1}$ and $350 \text{ ml } CO_2 \text{ l}^{-1}$ in synthetic air ($80\% N_2$, $20\% O_2$) at a rate of 10 ml min^{-1} . This was equivalent to an exposure rate of about $250 \text{ nmol } H_2 \text{ g}^{-1} \text{ soil h}^{-1}$, which was calculated as the upper limit

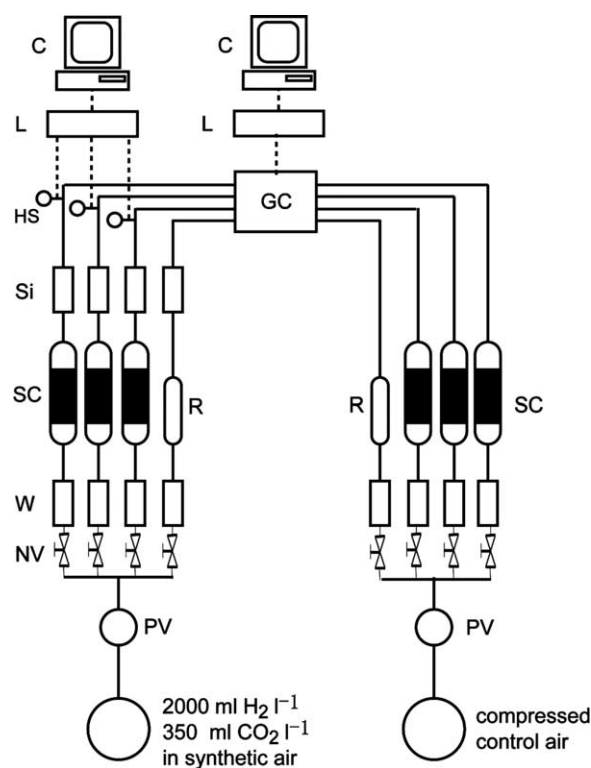


Fig. 1. Gas analysis system for simultaneous measurements of H_2 and CO_2 concentrations in the outlet air of soil columns. (PV: pressure regulating valve, NV: needle valve, W: water flasks, SC: soil columns, Si: silica gel, HS: H_2 sensors, L: data logger, GC: gas chromatograph, R: reference, C: computer).

emitted in the rhizosphere of legumes by Dong and Layzell (2001). As control treatment, soil was exposed to a compressed airflow from outside the laboratory building and provided at the same flow rate as the H₂ treatment. Soil samples (200 g) were filled in plastic pipes (PVC, length 38 cm, bore 6 cm) and exposed to either H₂ or air treatment with three replicates each. For each treatment an empty pipe was used to monitor the CO₂ concentration of the entering gas as a reference. Changes in the CO₂ concentration were measured by gas chromatography (Shimadzu 14B, columns Porapack Q 80–100 mesh). H₂ sensors (Model S121, Qubit Systems Inc., Kingston, Canada) were placed behind the soil samples to continuously measure the H₂ gas concentration in the carrier gas after passage through the soil column. To avoid desiccation of the soil samples, the gas streams were passed through flasks filled with distilled water before entering the soil columns and redesiccated in silica gel before reaching the H₂ sensors, which are sensitive to water vapour.

One soil column per treatment was sampled at five different dates (d 8, 16, 19, 25 and 30) and approximately 15 g of the soil sample were taken for further analyses. While 1 g of the sample was fixed with 4% PFA solution (Amann et al., 1990) and stored at 4 °C for later bacterial counting and FISH-analyses, the remaining sample was used for calorimetric measurements.

2.3. Microcalorimetry

The calorimetric measurements were made using thermal activity microcalorimeters (2277 Bio Activity Monitor, C3-Analysetechnik, Baldham, Germany). Due to differences in temperature (14 °C in the climatic chamber vs. 22 °C used for microcalorimetry) the soil samples were equilibrated 24 h at 22 °C. For the determination of microbial activity (basal heat production in μW) 1 g soil was added to 3.5 ml glass ampoules. Microbial biomass C was determined after substrate-induction (Sparling, 1983) using an amendment of about 0.4% (related to dry weight) yeast extract (1:1 mixed with talcum). As compared to glucose amendment the addition of yeast extract gave a more pronounced stimulation of soil microbial activity with qualitative comparable values (A. Schulz, pers. comm.). Basal microbial activity and biomass were measured with four replicates per treatment. To ensure the metabolic activity of H₂ oxidizing bacteria in the soil columns the ampoules were flushed with a H₂–air mixture (1500 ml H₂ l⁻¹) for 5 min prior to the measurement and sealed thereafter. The control samples were flushed in the same manner to apply comparable experimental conditions. Basal heat production activity values were determined after 6 h and were calculated g⁻¹ soil dry weight (DW). Microbial biomass C was determined before the onset of the second increase in heat production resulting from microbial growth in the substrate amended samples (Heilmann, 1993).

2.4. Quantitative determination of the bacterial community size and structure

2.4.1. Cell fixation and immobilization on polycarbonate filters

One gram of soil was added to 9 ml extraction buffer and homogenized in a mixer mill (MM 2, Retsch GmbH and Co. KG, Haan, Germany) for 7 min at 80% intensity. After sedimentation for 15 min 5 ml supernatant were mixed with 15 ml 4% PFA and incubated for 3–4 h at 4 °C according to Amann et al. (1990). The fixed samples were centrifuged (5 min, 5000 rev min⁻¹) and washed three times in phosphate buffered saline (PBS buffer). The pellet was then resuspended in 1 ml 0.01% (w/v) toulidine blue for 1 h to reduce autofluorescence of soil particles (Weber et al., 2001). After washing three times in PBS buffer the sample was stored in an equal volume of PBS-ethanol (1:1) at –20 °C. Then 10 μl of the fixed samples were filtered through wet sterile gauze filters to remove large soil particles. The gauze filters were rinsed four times with 1 ml PBS. The cells were finally filtered onto polycarbonate membrane filters (GTTP 0.2 μm , Millipore, Eschborn, Germany), then dehydrated in 50, 80 and 100% ethanol (3 min each) and dried. Dried filters could be stored in the dark at room temperature for several months.

2.4.2. Fluorescence in situ hybridization (FISH)

The prepared filters were hybridized on glass slides for at least 1.5 h at 46 °C mounted with 200 μl of a hybridization buffer and 20 μl of fluorescently labelled probe (50 ng μl^{-1} Interactiva, Ulm, Germany). The hybridization buffer contained 5 M sodium chloride, 10% sodium dodecyl sulfate (SDS), 1 M Tris/HCl (pH 8.0) and the specific amount of formamide. The oligonucleotide probes used are listed in Table 1. The filters were washed by 15-min immersion in washing buffer at 48 °C containing 1 M Tris/HCl (pH 8.0), 10% SDS, 5 M NaCl and 0.5 M EDTA (pH 8.0). The filters were washed in distilled water to remove salts, air dried and mounted with Citifluor anti-bleaching agent AF1 (Citifluor Ltd., Canterbury, UK).

2.4.3. DAPI staining

For counterstaining with the DNA-binding dye DAPI (4',6-diamidino-2-phenylindole) the hybridized polycarbonate filters were covered with 300 μl DAPI solution (1.75 ng μl^{-1}) and incubated at room temperature for 10 min in the dark. After washing the filters with distilled water they were dried in the dark.

2.4.4. Microscopy

Cells were counted using an Epifluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) equipped with the filter sets 01 (DAPI) and 09 (FLUOS).

Table 1
16S rRNA oligonucleotide probes used for hybridization

Probe	Binding position in <i>E. coli</i> (Brosius et al., 1981)	Specificity	Sequence (5'–3')	Reference
EUB 338-I	16S-rRNA, 338–355	Bacteria (except * and **)	GCTGCCTCCCGTAGGAGT	Amann et al. (1990)
EUB 338-II	16S-rRNA, 338–355	Planctomycetales*	GCAGCCACCCGTAGGTGT	Daims et al. (1999)
EUB 338-III	16S-rRNA, 338–355	Verrucomicrobiales**	GCTGCCACCCGTAGGTGT	Daims et al. (1999)
Alf 968	16S-rRNA, 968–985	α -subclass of Proteobacteria	GGTAAGGTTTCTGC GCGT	A. Neef, unpub Phd thesis, Technical University of Munich (1997)
Beta 42a	23S-rRNA, 1027–1043	β -subclass of Proteobacteria	GCCTTCCCACTTCGTTT	Manz et al. (1992)
Gam 42a	23S-rRNA, 1027–1043	γ -subclass of Proteobacteria	GCCTTCCACATCGTTT	Manz et al. (1992)
CF 319a	16S-rRNA, 319–336	Cytophaga- Flavobacteria- Bacteroides	TGGTCCGTGTCTCAGTAC	Manz et al. (1996)
Arch 915	16S-rRNA, 915–934	Archaea	GTGCTCCCCGCAATTTCT	Stahl and Amann (1991)

3. Results

3.1. H_2 and CO_2 dynamics

The time course of CO_2 production in control and H_2 -treated soils is shown in Fig. 2. The H_2 treatment led to a continuous decrease in CO_2 production after d 8 and finally to a net CO_2 uptake of about $8 \text{ nmol } CO_2 \text{ g}^{-1} \text{ soil h}^{-1}$. The observed CO_2 fixation activity corresponded with the time course of H_2 uptake (data not shown).

3.2. Microbial activity and biomass determined by microcalorimetry

Until d 16 no significant differences were observed in microbial activity or microbial biomass between the H_2 -treated and control soils. Microbial activity decreased

slightly in the air-flushed control treatment, but rose significantly in the H_2 -treated soil such that values of $14 \mu\text{W g}^{-1} \text{ DW}$ were reached at the end of the experiment, twice as high as in the control soil (Fig. 3). A similar time course of H_2 stimulation was obtained for substrate-induced activity and microbial biomass (data not shown).

3.3. Bacterial cell counts and activity determined by DAPI- and FISH-staining

Total bacterial cell counts determined by DAPI staining increased in the H_2 and the control (air) treatments. While cell counts of the control treatment increased to 9×10^9 cells g^{-1} soil after 19 d, cell counts of the H_2 -treated soil continued to rise and were finally with 16×10^9 cells more than twice as high as those in the control treatment. The portion of bacterial cells stained with the rRNA-directed

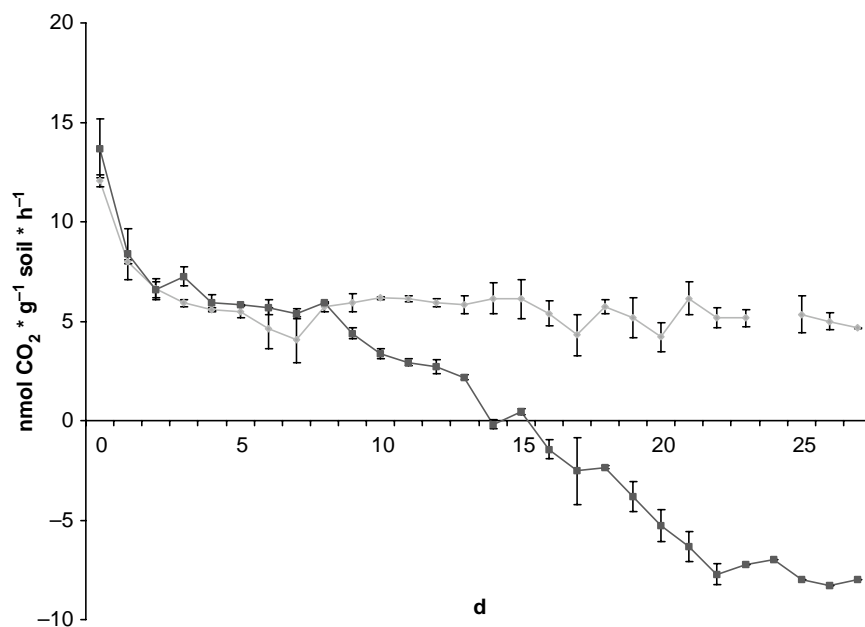


Fig. 2. Time course of CO_2 production in air-treated control soil \square and in H_2 -treated soil \blacksquare . Values are means of two undisturbed microcosms, error bars indicate the standard deviation.

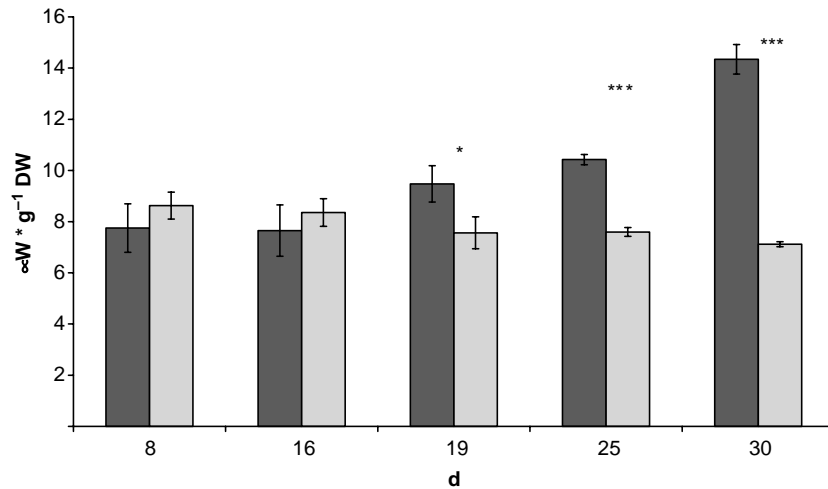


Fig. 3. Microbial activity in $\mu\text{W g}^{-1} \text{DW}$ in H₂-treated and control soil measured by heat production (microcalorimetry). Values are means of four replicates, error bars indicate the standard deviation ($P < 0.05^*$, $P < 0.001^{***}$).

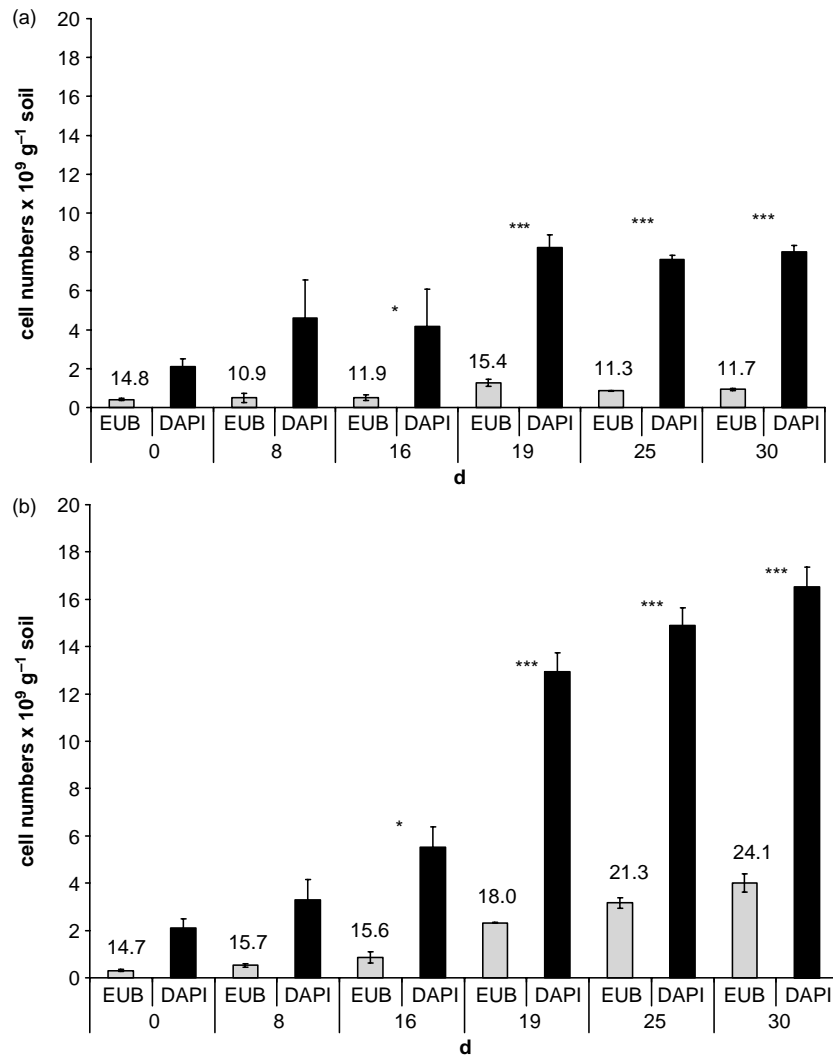


Fig. 4. EUB-positive and total counts (DAPI) of bacterial cells in air-treated control soil (a) and H₂-treated soil (b). Values are means of three counted filters, error bars indicate standard deviation ($P < 0.05^*$, $P < 0.001^{***}$). The percentage of EUB-positive cells within the total cell counts is given above the EUB bars.

oligonucleotide probe set EUB338-I, EUB338-II, EUB338-III using the FISH-method remained at about 12% in the control soil but rose to 24% in the H₂-treated soil by the end of the experiment (Fig. 4a and b). This indicates an increased proportion of bacterial cells with high ribosome content was present in the H₂-treated soil.

3.4. Determination of the bacterial community structure

Bacteria belonging to the α -, β - and γ -subclasses of the Proteobacteria were identified by the FISH-technique using the probes Alf968, Bet42a, and Gam42a, respectively. Members of the Cytophaga–Flavobacterium–Bacteroides (CFB) phylum were detected with probe CF319a. α -Proteobacteria increased through enhanced aeration in both treatments to $1\text{--}2 \times 10^8$ cells g⁻¹ soil, but there were no significant differences observable between the H₂-treated

and control soils (Fig. 5a). They constitute a minor component (4–8%) of the FISH-positive population (EUB 338) (Fig. 5b). While the air treatment had no significant effect on cell counts of β -Proteobacteria (3.1×10^8) H₂ treatment caused a highly significant increase up to 1.7×10^9 cells. β -Proteobacteria represented 10% of the EUB-positive bacteria in untreated and control soils, whereas they represent 30% in H₂-treated soils. γ -Proteobacteria were also stimulated through H₂ treatment with cell counts of 5.9×10^8 cells in control soil and 2.1×10^9 cells in H₂-treated soil. The percentage of γ -Proteobacteria did not increase in control soil, but attained 35% of all EUB-positive bacteria in the H₂-treated soil. The CFB community was very small in the untreated and control soils with 1.0×10^7 and 5.4×10^7 cells counted, respectively, but the H₂ treatment resulted in a large increase to 3.1×10^8 cells (Fig. 5a). The percentage of

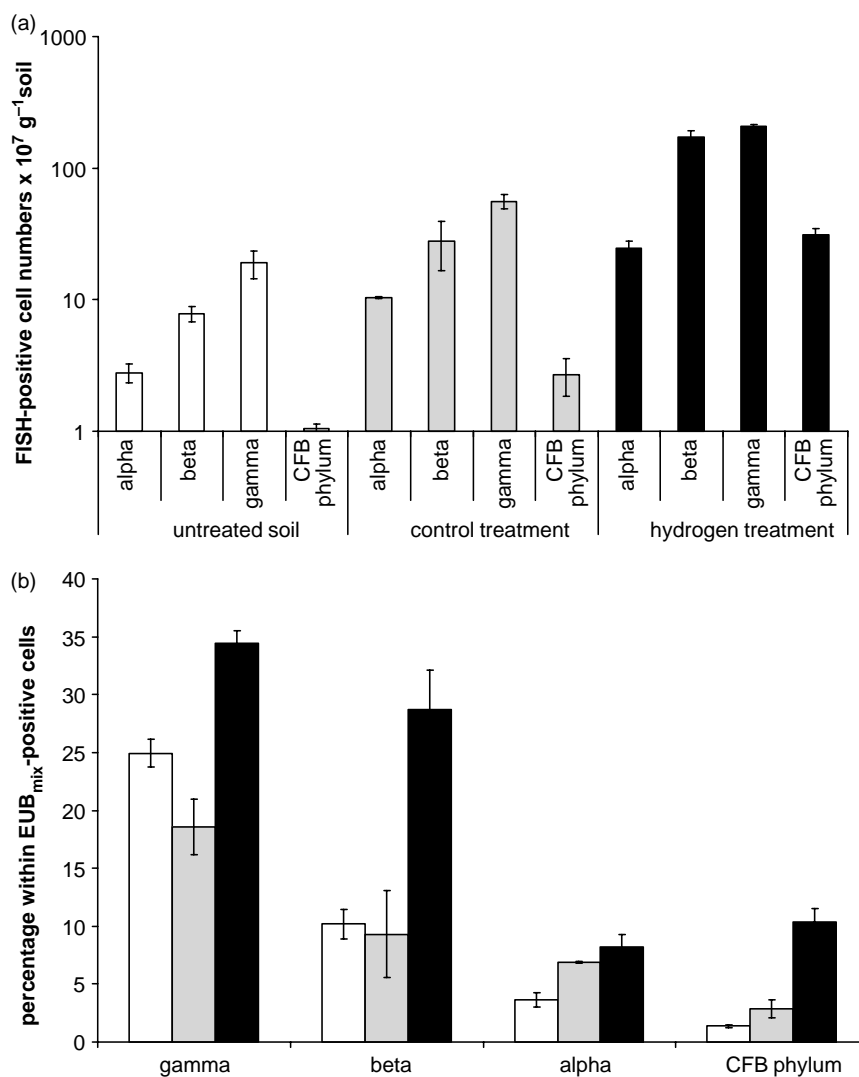


Fig. 5. Bacterial community structure. Cell numbers of FISH-positive bacteria (with high ribosome content) belonging to the α -, β - and γ -subclasses of Proteobacteria and the CFB-phylum (a) and percentage of group-specific counts within the EUB-positive bacteria (b) in untreated soil □, control soil ◻ and H₂-treated soil ■. Values are means of three counted filters, error bars indicate standard deviation.

FISH-positive CFB-cells increased from 2% in the control soil to 10% of all EUB-positive bacteria in H₂-treated soil (Fig. 5b). Oligonucleotides for the domain Archaea (Table 1) were also applied, but FISH-positive Archaea were not detected.

4. Discussion

Our experiments confirmed the effect of H₂ exposure on CO₂ fixation in soils (Dong and Layzell, 2001). Interesting changes in the biomass, activity and structure of the soil bacterial community occurred under these conditions. The concomitant increase in total bacterial cell and EUB-positive cell numbers in H₂-treated soil samples clearly reflects the activation of certain subgroups of bacteria due to this treatment. Applying group-specific phylogenetic probes, bacterial groups taking profit from the H₂ treatment could be identified as β- and γ-Proteobacteria as well as bacteria of the CFB group. The lack of response by the α-Proteobacteria was unexpected, since Rhizobiaceae belong to the α-Proteobacteria including, e.g. *Rhizobium* and *Bradyrhizobium*, which are known to grow autotrophically with H₂ (Watson and Tabita, 1997). Due to the supply of H₂, the proportion of γ-Proteobacteria was about twice as high as in the control soil. The high percentage of γ-Proteobacteria reflects a selective effect towards the γ-Proteobacteria in the rhizosphere of clover with *Pseudomonas* representing the prevailing member (Grayston et al., 1998; Marilley and Aragno, 1999), which can be found in all leguminous rhizospheres (Miethling et al., 2003). The β-Proteobacteria even showed a stronger stimulation and increased their activity 3-fold reaching up to 30% of the EUB-active bacterial population due to the H₂ treatment. Amongst them are some well-known H₂ oxidizing bacteria of which e.g. *Ralstonia eutropha* (previously *Alcaligenes*) is the most familiar one. All Knallgas bacteria isolated by La Favre and Focht (1983) from soil adjacent to nodules lacking the activity of uptake hydrogenases were characterized as *Alcaligenes paradoxus* (Aragno and Schlegel, 1992). However, the most pronounced response was shown of members of the CFB phylum which unexpectedly increased considerably due to the H₂ treatment. Members of CFB phylum are common in soil habitats and play an important role in the degradation of complex biopolymers (Eiler et al., 2003). They possess the ability to decompose cellulose and chitin under aerobic conditions (Reichenbach, 1992). Their stimulation could be interpreted as secondary process initiated by the increased microbial biomass and their products in the H₂-treated soils. Lueders et al. (2004) have shown by the stable isotope labeling technique that the ¹³C-label of C sources is rapidly transferred to bacteria of the CFB group or protozoa in soil food chains. Another explanation could be that CFB populations could possess hydrogenases or autotrophic activity. Most recent molecular evidences showed, that the diversity of autotrophic bacteria harbouring the *cbbL*-gene is much higher as expected in

many phylogenetic groups of soil bacteria in terrestrial habitats (Nanba et al., 2004; Selesi et al., 2005).

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