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REGULAR ARTICLE

A cost-effective high-throughput microcosm system for studying nitrogen dynamics at the plant-microbe-soil interface

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Abstract In the present study a new microcosm system was evaluated for its suitability to investigate nitrogen dynamics between soils, plants and microbes. Five different agricultural soils were homogenized and transferred in the test tubes, and kept under controlled conditions in a climate chamber for 4weeks. Soils differed clearly in nitrogen pools and microbial population structures but less in their activities. Bacterial and fungal community compositions and soil properties, except gross N transformation rates, remained stable and reproducible during the test period in all soils. ^{15}N tracer studies showed that N uptake patterns of barley as well as plant growth were linear in the initial growth period. Overall, the presented microcosm system proved to be a powerful tool to elucidate N pathways in soil-plant-microbe systems. In future studies the microcosm system may greatly help generating new insights in the complex processes and controls of nitrogen biogeochemical cycle in agricultural systems.

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Introduction

Intensive agricultural plant production depends on the input of mineral fertilizers, which is mainly provided in the form of urea, NH_4^+ or NO_3^-. A major drawback is that only 30–50% of the applied N fertilizer is taken up by the crop plants (Cassman et al. 2002; Smil 1999). A significant amount of the applied N is lost from the agricultural fields, leading to a variety of adverse environmental effects, namely the pollution of ground water by NO_3^- and the emission of the potential greenhouse gases NO and N_2O (reviewed by Tilman et al. 2002). In the last decade a wealth of studies addressing the consequences of intensive N fertilization have been conducted, ranging from field studies (e.g. Chu et al. 2007) to laboratory experiments (e.g. Well et al. 2006).

A major problem of studying N dynamics in soils is that the N cycle in soils is highly complex as it includes several transformation processes of one N form to another (Hayatsu et al. 2008). The results of soil studies are dependent on the characteristics of the specific soil tested, such as pH, soil texture, initial organic matter content and nutrient content (Grigatti et al. 2007). Elucidating N pathways in soils requires not only studying N pools and transfer rates of N among these pools, but also the explicit consideration of N cycling activities by soil microorganisms. The latter mediate a large range of biochemical N transformations in soils and are therefore critical to soil C and N cycling. The importance and complexity of soil microbiology has often been stressed, and many studies have focused on understanding and predicting soil-microbe dynamics in soils, which proved to be challenging due to the immense heterogeneity of soils (reviewed by O'Donnell et al. 2007). Within microbial communities, soil bacteria have received most attention so far but there is increasing evidence for an important role of fungi in these N transformation processes (Hayatsu et al. 2008). Nutrient cycling dynamics are often scale-dependent (Ettema and Wardle 2002), but also the fact that different soil microorganisms may occupy different ecological niches (microsites) and therefore play different roles in the nutrient cycling, increases the difficulty of understanding soil-microbe interactions (Myrold and Posavatz 2007).

Nevertheless, it remains important to elucidate the role and controls of individual processes involved in N turnover, and its response to N fertilization. To accomplish this, approaches that integrate biogeochemical, physiological, microbiological and agronomic studies are essential (Hirel et al. 2007). As it proved difficult to achieve this goal in a highly reproducible and controlled manner in a natural system, laboratory format microcosm systems may provide a solution to this challenge (Copley 2000). Until now a variety of laboratory format studies have been conducted, focusing on greenhouse gas emissions (e.g. Sanchez-Martin et al. 2008), the characterization of microbial community compositions (e.g. Gordon et al. 2008) or plant versus soil microbe N uptake (Harrison et al. 2008). However, we are not aware of any study using a microcosm design which allows the parallel investigation of the major key factors in the N cycle of soil-plant-microbe systems. To turn a laboratory format test system into powerful and reliable tool for soil studies it is necessary to consider and overcome some major obstacles. For example, microcosm studies may generate poorly reproducible results due to soil heterogeneity and because some factors, such as soil bulk density or soil moisture content, cannot be kept constant over a longer period of time (Grigatti et al. 2007; Jensen et al. 1996; McDowell et al. 2006). Additionally, when using microcosm systems the validity of measurements conducted on soils removed from their original field setting is uncertain, due to various manipulations for the experimental setup (Madsen 2005). Therefore, a proper evaluation of microcosm systems regarding their suitability for soil-plant-microbe studies is crucial.

In this study we present a new, simple and cost-effective laboratory format microcosm system, for standardized high-throughput analysis of nutrient dynamics in soil-plant-microbe-atmosphere systems. The main objectives were to evaluate the microcosm system for its reproducibility and reliability to simultaneously study key processes of the N cycle. Therefore a series of experiments was conducted using five different agricultural soils, which were treated in the same, standardized way and were kept in the test tubes under controlled conditions for 1month. During this test period, samples were taken

for analyses of soil N pools, N transformation processes, N losses, as well as bacterial and fungal populations. The main aim of the experimental setup was to evaluate the suitability of the test system for different types of soils, studying the variance of chemical and microbiological soil properties during the incubation period and investigating plant growth and plant N uptake using the model plant barley.

Material and methods

Test tube design

The design of the microcosm system reflects the idea to study several important parameters at the same time. The system is based on 50ml polypropylene centrifuge tubes (Greiner Bio-one, #227261) complemented with two stainless steel sieves above the tube cones. One sieve was placed at the bottom (27mm in diameter, 1mm thick, mesh size 1mm) for stability and a second sieve above the first one (27mm in diameter, 0.2mm thick, mesh size 5μm) to keep the tube cones free from soil particles or from roots when plants are grown in the microcosms. Eight holes (1mm in diameter) were drilled into the tube cones to provide sufficient aeration of the soil from the bottom and to allow the removal of leachates when conducting leaching experiments. Additionally, we designed a retrofit kit for further elongation to meet individual requirements of different experimental setups. This retrofit kit consists of a polypropylene tube (20cm length, 30mm in diameter) which fits exactly onto the centrifuge tubes. To ensure an airtight closure between the two tubes, a 5mm strong rubber seal was used as connecting strap. The whole system can be closed airproof with rubber seals (commercial bottle caps) at the bottom and butyl rubber septa (Suba-Seal 57, Sigma-Aldrich, #Z124680) at the top, allowing sampling of headspace gas with gas tight syringes. A detailed scheme of the microcosm setup is given in Fig. 1.

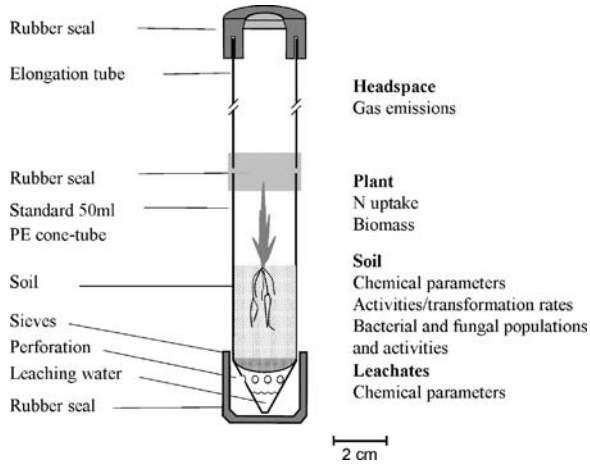

Fig. 1 Setup of the microcosm system to study interactions between soil, plant, atmosphere and microbial communities

Site description, soil packing effects and experimental setup

Soil samples were collected in April 2006 from five sites in the vicinity of Vienna, Lower Austria, Austria, representing different bedrocks, soil textures, pH values, water, and humus contents (Table 1). All sites were used as agricultural fields with the exception of Riederberg, which was a grassland site. All soil types are widely distributed and are frequently used for barley cultivation in this area. Detailed site characteristics and soil properties are given in Table 1.

Soil samples (each ~25kg) were collected from 0 to 20cm depth from all sites, and immediately stored at 4°C until further analysis. Prior to the start of the experiments soils were homogenized, sieved (< 2mm) and stored at 4°C for 10days. Different amounts of moist soil were weighed into the test tubes and either centrifuged (1min, 187*g*) or packed by continuously increasing the weight from above up to 17.1 kN m^{-2} to reach a final volume of 30ml. After packing, soils were sampled in 4 equal parts (13mm height increments in the test tubes). Subsequently soil water content, based on oven-dry weight (in % soil DW), and bulk density (in g cm^{-3}) of each increment was observed (Fig. 2). Thus the optimal amount of moist soil for each site was determined (Table 2).

For the following experiments the respective soil aliquots were weighed and centrifuged into the test tubes (1min, 187*g*). The centrifuge (Sigma 3–15K) was supplemented with a swing-out rotor (Sigma 11133) and additional round carriers for the test tubes (Sigma 17049). The microcosms were kept under controlled conditions in a climate chamber with a 15h/9h day/night cycle at 21/18°C temperature and 55% relative air moisture. During 2weeks of equilibration the soil water content of each soil was

Table 1 Site characteristics and general properties of five soils (0–20cm soil layer) collected in the vicinity of Vienna, Lower Austria, Austria

	Purkersdorf	Riederberg	Maissau	Niederschleinz	Tulln
Soil type	Gleyic Cambisol from sandy loamy flysch	Calcaric Cambisol from clay flysch	Cambisol from silicate material	Chernozem from Loess	Pseudogley on Planosol
Geographic site	48°12′25″ N 16°10′37″ E	48°15′0″ N 16°04′0″ E	48°34′0″ N 15°49′0″ E	48°35′59″ N 15°10′24″ E	48°20′0″ N 16°03′0″ E
Altitude (m. a. sl.)	248	384	341	244	180
Management	Winter barley	Grassland	Arable field	Arable field	Arable field
Water condition	Moist	Changing water conditions	Dry	Moderately dry	Moderately moist
Clay (%)	1.7	29.1	43.2	17.7	0
Silt (%)	64.9	53.8	48.3	74.2	70.4
Sand (%)	33.4	17.2	8.6	8	29.6
pH (KCl)	5.67	6.63	6.99	7.15	6.21
$CaCO_3$ (%)	0.06	2.11	0.2	8.5	0.04
Exchange capacity (mval%)	11.2	33.2	8.9	15.4	37.9
Base saturation (%EC)	81.4	82.1	92.3	98.1	70.4
Bulk density (g DW ml^{-1})	1.06	0.52	1.13	0.96	0.70

adjusted gravimetrically to water contents given in Table 2, which represent 70% of field capacity of homogenised soils after the packing procedure in the microcosms. Thereafter, every 2weeks five replicates of each soil were taken at random and analysed for fluxes of CO_2, N_2O and CH_4, for C and N pools, gross N mineralization and gross nitrification rates, microbial biomass C and N, dehydrogenase and nitrate-reductase activities and bacterial and fungal community patterns. During the test period of 4weeks samples were left untreated, except daily adjusting the soil water content.

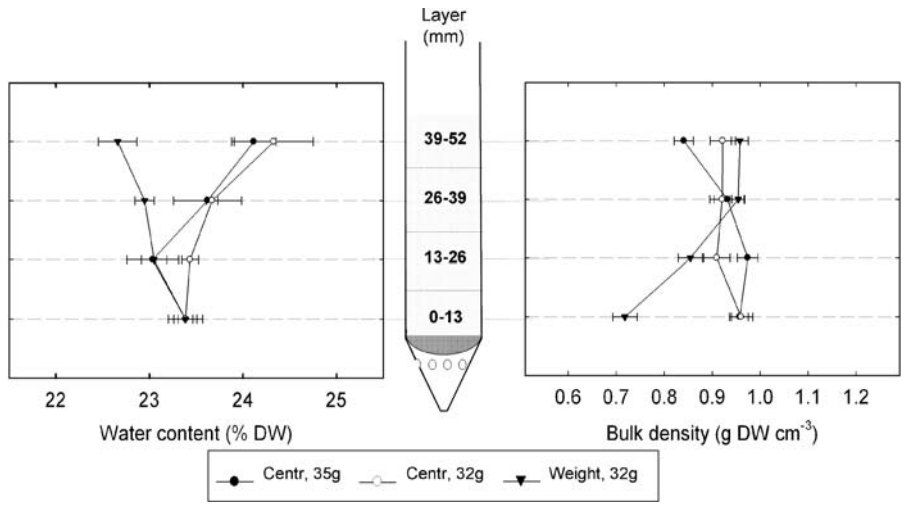

Fig. 2 Vertical gradient of soil water content and soil bulk density of soil Niederschleinz in the microcosm system using three different filling procedures. Soil was centrifuged into the test tubes, using either 35g FW (full circles) or 32g FW (open circles). Triangles represent values where soil (32g FW) was packed by gradually increasing the weight from the top to a final weight of 1kg. Error bars represent standard errors (n = 5)

Table 2 Soil fresh weight, water content, nitrogen and carbon pool sizes and transformation rates of five soils collected in the vicinity of Vienna, Lower Austria, Austria

	Purkersdorf42	Riederberg	Maissau	Niederschleinz	Tulln
Fresh weight (g microcosm^{-1})	42	31	40	32	32
Initial water content (% DW)	27.9	42.1	24.2	19.9	33.7
Water content(% DW) at equilibrium	25.2 (0.7)	43.4 (1.0)	24.5 (0.8)	18.0 (0.6)	34.5 (1.1)
Total C (mg C g^{-1} DW)	16.2 (0.5)b	47.7 (0.9)e	13.7 (0.3)a	26.4 (0.2)c	29.3 (0.5)d
Total N (mg N g^{-1} DW)	1.63 (0.05)a	4.78 (0.07)d	1.45 (0.05)a	1.86 (0.02)b	3.27 (0.05)c
C/N	9.96 (0.09)c	9.98 (0.05)c	9.43 (0.12)b	14.17 (0.07)d	8.95 (0.03)a
TDC (µg C g^{-1} DW)	40.2 (1.4)b	61.3 (0.8)c	41.0 (1.5)b	31.2 (1.3)a	33.1 (2.4)a
NO_3-N (µg N g^{-1} DW)	18.6 (1.2)a	17.6 (2.4)a	42.3 (1.3)b	19.3 (1.0)a	15.9 (1.4)a
NH_4-N (µg N g^{-1} DW)	1.31 (0.23)a	2.15 (0.17)b	1.18 (0.21)a	1.35 (0.18)a	1.28 (0.15)a
Available PO_4^- (µg P g^{-1} DW)	0.66 (0.03)a	0.07 (0.02)a	3.07 (0.10)c	1.33 (0.07)b	5.83 (0.29)d
$\delta^{15}N$	5.28 (0.12)ab	4.72 (0.06)a	5.50 (0.29)b	6.50 (0.10)c	6.65 (0.12)d
$\delta^{13}C$	−27.25 (0.05)a	−26.35 (0.07)b	−26.42 (0.07)b	−19.13 (0.11)d	−23.92 (0.06)c
N_{mic} (µg N g^{-1} DW)	14.6 (0.8)a	179.7 (12.2)c	10.1 (1.1)a	22.9 (2.5)a	52.9 (2.5)b
C_{mic} (mg C g^{-1} DW)	0.37 (0.03)a	2.27 (0.05)c	0.15 (0.008)a	0.30 (0.03)a	1.29 (0.11)b
C/N $_{mic}$	20.1 (6.9)b	9.9 (2.6)a	7.9 (4.8)a	14.1 (2.2)a	24.7 (0.9)b
CO_2 (µg C g^{-1}DW d^{-1})	101.1 (22.8)c	74.4 (10.2)bc	42.2 (5.3)b	6.2 (6.1)a	56.8 (14.4)b
N_2O (ng N g^{-1}DW d^{-1})	366 (66)b	239 (62)ab	163 (43)a	106 (10)a	152 (56)a
CH_4 (µg C g^{-1}DW d^{-1})	0.57 (0.24)*ns*	−1.37 (1.03)*ns*	0.49 (0.71)*ns*	0.55 (0.30)*ns*	0.03 (0.88)*ns*
Dehydrogenase (µg TPF g^{-1} DW d^{-1})	7.17 (0.87)b	35.78 (1.96)e	16.51 (1.04)c	20.37 (0.38)d	3.61 (0.11)a
Nitrate reductase (µg N g^{-1}DW d^{-1})	74.7 (6.38)ab	52.7 (19.3)a	153.4 (10.2)b	93.8 (5.6)ab	224.9 (9.72)b
Mineralization (µg N g^{-1}DW d^{-1})	2.28 (0.49)b	0.26 (0.05)a	1.76 (0.44)b	0.40 (0.13)a	0.22 (0.05)a
Nitrification (µg N g^{-1}DW d^{-1})	4.67 (0.37)*ns*	6.22 (1.89)*ns*	12.09 (3.51)*ns*	3.40 (0.61)*ns*	2.77 (1.16)*ns*

Values represent means (± standard errors; $n = 5$) at the start of experiment (time 0; after preequilibration in soil microcosms for 10days). Different letters in rows indicate significant differences between sites (Oneway-ANOVA, LSD post-hoc test, $P < 0.05$). TDC Total dissolved carbon, N_{mic} Microbial biomass N, C_{mic} Microbial biomass C, *ns* not significant

Measurement of gas fluxes and chemical analysis

For gas sampling, test tubes were closed at both sides with butyl rubber seals and gas samples were taken immediately after closing and after 1h. With a gas-tight syringe head space air (10ml) was transferred into evacuated headspace vials and kept at 4°C until analysis. After gas sampling, the test tubes were opened again, soils were quantitatively retrieved, homogenized and prepared for further analyses.

Gas samples were analysed within 48h as described by Kitzler et al. (2006b) by automated headspace gas chromatography. Briefly, the GC was equipped with a 63Ni electron capture detector to quantify N_2O concentrations and a flame ionization detector and a methanizer to quantify CO_2 and CH_4 concentrations. Gas emission rates were then assessed by the linear increase of headspace gas concentration over the closure period.

Aliquots (2g) of homogenized soil were extracted in 15ml $CaSO_4$ (10mM) and subsequently anions were determined by ion chromatography (DX 500, Dionex, Vienna, Austria) and conductivity detection. NO_3^- was separated on an anion exchange column (AS11, 250 x 4mm i.d., Dionex, Vienna, Austria) after chemical suppression (ASRS-Ultra, Dionex) and linear NaOH gradient elution (0.5mM to 37.5mM within 10min at a flow rate of 2ml min^{-1}, with a column temperature of 35°C). Ammonium was extracted from aliquots (2g) of homogenized soil with 15ml KCl (1M) and determined by a modified indophenol reaction method (Kandeler and Gerber 1988).

Microbial biomass C and N in soils were analyzed by chloroform fumigation-extraction technique as described by Amato and Ladd (1988) and was calculated as the difference in N concentration between fumigated and non-fumigated soil samples. Briefly, aliquots of fresh soil (2g) were fumigated over chloroform (ethanol-free) for 24h at 22°C. Both, fumigated and non-fumigated soil samples were extracted with 15ml KCl (1M) for 60min before filtering. Total dissolved C

and N in the KCl extracts were determined by an automated C analyzer (Shimadzu, TOC-VCPH, Japan) and a total nitrogen measuring unit (Shimadzu, TNM-1, Japan). A conversion factor for microbial biomass C (K_{EC}) and N (K_{EN}) of 0.45 was applied for incomplete extraction (Jenkinson et al. 2004).

Nitrate reductase activity was measured as described by Kandeler (1996) using 2,4-dinitrophenol as nitrite reductase inhibitor and subsequent colorimetric determination of nitrite at 520nm. Dehydrogenase activity was quantified by the triphenyltetrazolium chloride (TTC) method according to Alef (1995).

An aliquot (4g) of soil was dried at 70°C and weighed to determine soil moisture. Dry soils were ground in a ball mill (Retsch MM2000). Total N and C, as well as natural ^{15}N and ^{13}C abundance of ground soils were then measured by isotope ratio mass spectrometry (IRMS) using an elemental analyser (EA 1110, CE Instruments) connected in continuous flow-mode to a gas isotope ratio mass spectrometer (DELTA^{PLUS}, Finnigan MAT). The natural abundance of ^{15}N and ^{13}C was calculated as follows:

$$\delta^{15}N\,[‰\,\text{vs. at} - \text{air}] = (R_{sample}/R_{standard} - 1) \times 1000$$
$$\delta^{13}C\,[‰\,\text{vs. V} - \text{PDB}] = (R_{sample}/R_{standard} - 1) \times 1000$$

where R is the ratio of ^{15}N / ^{14}N for nitrogen and ^{13}C / ^{12}C for carbon isotope abundance. The standard deviation of repeated measurements of a laboratory standard was 0.15‰ for $\delta^{15}N$, and 0.10‰ for $\delta^{13}C$.

^{15}N flux measurements

Gross N mineralization and gross nitrification rates were measured by modified ^{15}N pool dilution assays (Barrett and Burke 2000; Bengtson et al. 2006). The soil cores of each tube were labelled uniformly by adding ^{15}N-enriched (99 atom% excess) solutions of $^{15}NH_4Cl$ for mineralization and $K^{15}NO_3$ for nitrification reaching a total of 12µg ^{15}N per test tube. Homogenous distribution of applied N was ensured by inserting a 7-cm long side-hole needle to the bottom of the soil cores in 4 positions and slowly injecting the labelled solution (400µl each injection) while withdrawing the needle (Pörtl et al. 2007). It has been pointed out previously (Murphy et al. 2003), that uniform labelling of soil is essential for ^{15}N pool dilution essays, therefore a preliminary experiment was conducted, proving that using this technique ^{15}N was indeed homogenously distributed. After the injection of the labelled solution, aliquots of soil were taken from 8 different positions following vertical and horizontal gradients through the total soil core of the microcosm and analysed for their ^{15}N contents, which did not differ significantly ($P < 0{,}01$; data not shown). After incubation at 22°C for 4h, one set of the samples was homogenized and aliquots (2g) of soil were extracted with 15ml KCl (1M) for 60min to stop the assays. After 48h, the second set of samples was stopped in the same way. All extracts were kept at −20°C until further analysis. NH_4^+ and NO_3^- from the KCl extracts were isolated for ^{15}N analysis by a modified micro diffusion technique (Pörtl et al. 2007). Briefly, the KCl extract was transferred to a glass bottle (50ml) and NH_4^+ was converted to NH_3 by addition of ~200mg MgO. NH_3 was captured in acid traps (glass-fibre filter discs containing 10µl of 2.5M $KHSO_4$, enclosed in PTFE tapes) during 5days of incubation on a shaker at 37°C. Acid traps were removed and dried for 3days over concentrated H2SO4 in a desiccator. A new trap was added to the bottle followed by 0.4g of Devarda's alloy and 200mg MgO to convert NO_3^- to NH_3, and the incubation process was repeated. Dried filter discs were removed from the PTFE sealing and folded into tin capsules for subsequent analysis of ^{15}N contents using IRMS. Gross N mineralization and nitrification rates were calculated using the equations developed by Kirkham and Bartholomew (1954) and as modified by Bengtson et al. (2006).

Plant growth and plant ^{15}N uptake

Sieved soil from the five sites was filled into the microcosms as described above (fresh weight and water contents as given in Table 2, centrifugation for 1min at 187*g*). Seeds of winter barley (*Hordeum vulgare* L. cv. Morex) were germinated on moist filter paper for 2days and seedlings were then transferred to the microcosms (one plant per microcosm). During the following 13days plants were continuously harvested from each soil in five replicates for determination of plant dry weight. To determine the kinetics of plant soil N uptake, soil from Purkersdorf was labelled with 1mM $^{15}NH_4Cl$ (10 atom% ^{15}N, 25ml kg^{-1} fresh soil). After applying ^{15}N solution the soil

was sieved again, homogenized and equilibrated for 1week at 4°C before packing. Plant seeding and harvests were performed as above. ^{15}N and N concentration was determined in plants grown in Purkersdorf soil by IRMS.

Seed-derived N was calculated by a two-source mixing model:

$$\%N_{seed} = 100*(\delta^{15}N_{max} - \delta^{15}N_{tx})/(\delta^{15}N_{max} - \delta^{15}N_{seed}),$$

where $\delta^{15}N_{max}$ represents $\delta^{15}N$ of plants after 21days, $\delta^{15}N_{tx}$ of plants at time x, and $\delta^{15}N_{seed}$ of dry seeds.

Bacterial and fungal community patterns

For DNA extraction 0.5g of each soil, taken in three replicas from each sampling time, were processed with the FAST DNA Spin kit for soil (Q-Biogene, Germany) as described by the manufacturer. As a functional marker for nitrifying bacteria, a 491 bp fragment of ammonium monooxygenase catalytic subunit A (amoA) was amplified by PCR using primers amoA1F and amoA2R (Rotthauwe et al. 1997). The forward primer was FAM labelled. PCR reactions were performed in 25µl reaction vials containing 1 x buffer, 3mM $MgCl_2$, 0.2mM dNTPs, 1 U of FIREpol Polymerase (Solis BIODYNE) and 0.24µM of primer. To enhance amplification efficiency 1µl DMSO per reaction and 1µg/µl bovine serum albumin (BSA) were added. As target 25 to 100ng of DNA was applied. Amplifications were performed in a Whatman T1 thermocycler using the following program: an initial denaturing step at 95°C for 5min, followed by 35 cycles of 1min at 95°C, 1min at 60°C, 1min at 72°C and a final extension step at 72°C for 10min. PCR products were checked by standard agarose gel electrophoresis on 1% agarose gels.

Terminal restriction fragment length polymorphism (T-RFLP) analysis was applied to obtain a community profile of ammonium oxidizing bacteria. Two PCR products were pooled for each sample to reduce PCR bias. Pooled amplicons were digested with the restriction enzyme AluI in reaction mixtures (10µl) consisting of 7µl PCR product, 1x buffer, and 0.5µl AluI (10U/µl, Promega). Digestions were performed at 37°C for 4h. Digestion batches were purified by passage through DNA Grade Sephadex G50 (GE Healthcare) columns. Purified product (5µl) was mixed with 15µl HiDi-Formamide (Applied Biosystems) and 0.3µl 500 ROX™ Size Standard (Applied Biosystems) and denatured at 95°C for 2min.

Community profiles of fungi were also analyzed using the T-RFLP method. Fungal internal transcribed spacer (ITS) regions were amplified in 20µl reactions under the following conditions: 20–50ng of template DNA, ReddyMix™PCR Master Mix (ABgene), 3mM $MgCl_2$ (final concentration), 4% DMSO, 1µg/µl BSA, 1µM FAM-labelled ITS1F (Gardes and Bruns 1993) and 1µM ITS4 (White et al. 1990). The cycling conditions were as follows: initial denaturation at 95°C for 2.5min, 30 cycles of 94°C for 30s, 54°C for 30s and 72°C for 45s, and a final extension at 72°C for 5min. All PCR reactions were carried out in triplicate. Pooled PCR products were digested with restriction endonuclease BsuRI (Fermentas, isoschizomer of HaeIII) and purified with the QIAquick PCR Purification Kit (Qiagen).

Detection of FAM-labelled terminal restriction fragments was done by capillary electrophoresis using an ABI 3100 automatic DNA sequencer. T-RFLPs were transformed into numerical data using GenoTyper 3.7 NT software. These raw data were normalized and binned according to Abdo et al. (2006).

Statistical analysis

Significant differences ($P < 0.05$) between soils at the start of the experiment were analysed using one-way ANOVA, followed by a Fisher's LSD post-hoc test with Statgraphics 5.0 (Statistical Graphics Inc., Rockville, MD, USA). Differences between soils and sampling times were analysed by repeated measures ANOVA, followed by a Bonferroni post-hoc test with Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA). Repeated measures ANOVA of microbial community patterns was done by analysing peak areas from the electropherograms of T-RFLPs of ammonium oxidizing bacterial communities and of fungal ITS regions (compare Fig. 3). In detail, peak areas of peaks found in >25% of electropherograms of all harvests and soils were used for statistical analyses. If one or more of the peaks showed a significant difference within one soil, it was considered that there was a significant difference in the microbial community patterns over time. Principal component analyses (PCA) of soil N pools, $\delta^{15}N$ and $\delta^{13}C$ values, N transformation rates and gas emissions were performed with SIMCA-P 11.0 (Umetrics, Umeå, Sweden).

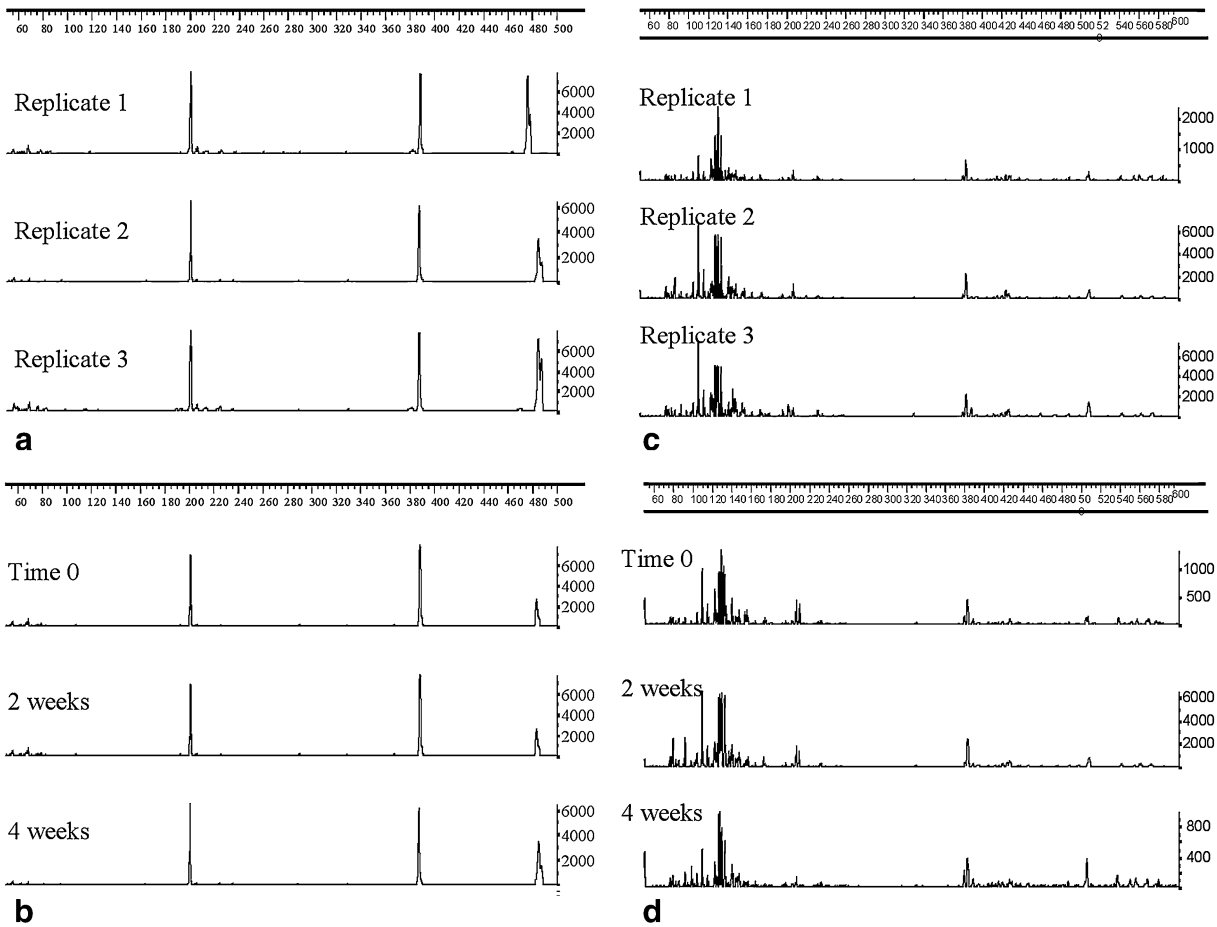

Fig. 3 Microbial community structure of soils collected in the vicinity of Vienna, Lower Austria, Austria and incubated in the microcosm system. Electropherograms of T-RFLPs of (**a**) three replicates of ammonium oxidizing (amoA) bacterial communities at the first sampling event (time 0), (**b**) of amoA bacterial communities at the three sampling events, (**c**) of three replicates of fungal ITS regions at the first sampling event and (**d**) of fungal ITS regions at the three sampling events at site Maissau

Results

Packing effects

Three different packing procedures were used to test for homogeneity of water content and bulk density of the soil along the vertical gradient within the test tubes. Different amounts of soil fresh weight were either centrifuged or packed by increasing the weight from above into the tubes. Clearly, the way of packing the soil into the test tube had a strong influence on water content and bulk density, as with all packing procedures the formation of vertical gradients in the microcosms could be observed (Fig. 2). When 32g moist soil from Niederschleinz was centrifuged (1min, 187*g*) into the tubes, the bulk density increased only in the lowest part of the microcosm by 4.1% ($P < 0.05$). In comparison the bulk density increased by 13.8% ($P < 0.05$) when packing 35g moist soil by centrifugation. When 32g moist soil was packed into the tubes by continuously increasing the weight from the top to 17.1 kN m^{-2}, the observed gradient of bulk density was highest, differing by 25.0% ($P < 0.001$). Vertical gradients in water content due to packing procedures were less pronounced as for bulk density, showing variations of 3–4% in all setups ($P < 0.05$). Similar results in bulk density and water content patterns along a vertical gradient were obtained for the other soil types (data not shown).

Soil N and C pools and activities

Riederberg soil (grassland) differed significantly in most of the measured soil properties from the arable

soils of the other sites (Table 2). The total carbon content of Riederberg soil was nearly double that of the other sites. Additionally, dissolved organic C, total N and NH_4-N contents were significantly higher as well as microbial biomass N and C pools, comprising 3.8% of the total N and 4.8% of the total C pool in the grassland soil. Carbon to nitrogen (C/N) ratios of microbial biomass differed greatly among the five soils, ranging from 7.9 in Maissau soil to 24.7 in Tulln soil. In contrast, soil C/N ratios were similar (between 9.0 and 10.0) with the exception of soil from Niederschleinz having a significantly higher C/N ratio of 14.2 (one-way ANOVA, $P < 0.05$). Further important features of this soil were significantly higher $\delta^{13}C$ and high $\delta^{15}N$ values, being -19.13 ± 0.11 ‰ and 6.50 ± 0.10 ‰ (mean ± SE, $n = 5$), respectively. Niederschleinz was the only Chernozem soil over Loess as bedrock and had much higher carbonate contents (8.5%). At all sites inorganic N pools consisted mainly of NO_3-N while NH_4-N comprised only a small fraction of inorganic N. The proportion of NO_3-N in the inorganic N pool varied between sites, being lowest in Riederberg soil (89.1%) and highest in Maissau soil (97.3%). Maissau soil had the highest NO_3-N contents ($P < 0.05$), comprising 2.91% for the total N pool in the soil, while the total C content was lowest (13.68 ± 0.33mg C g^{-1} DW, $P < 0.05$). Additionally to high NO_3-N contents, gross nitrification rates tended to be highest in Maissau soil (12.1μg N g^{-1} DW d^{-1}). Also gross N mineralization rates were significantly higher in Maissau soil compared to the other sites ($P < 0.05$), but were in the same range in soil Purkersdorf. Generally, gross nitrification rates were higher than gross N mineralization rates in all tested soils. Dehydrogenase activity, as an indicator of total microbial metabolic activity, was highest in Riederberg soil ($P < 0.05$), where also high emissions of CO_2 were found (Table 2). Generally, CO_2 emission rates showed marked differences between the five sites. At site Niederschleinz the lowest CO_2 emission was observed ($P < 0.05$), comprising only 0.02% of the total C pool per day. In contrast, the Purkersdorf soil exhibited the highest CO_2 emission, being 16 times higher than CO_2 emission from soil Niederschleinz and comprising a C loss of 0.62% of the total C pool per day. A similar pattern was found for N_2O emissions, where soil from Niederschleinz showed the lowest and soil from Purkersdorf the highest emission rates, comprising for 0.006% and 0.023% of the total N pools per day, respectively. No significant differences in CH_4 emissions were found between the soils of the different sites ($P > 0.05$).

Stability of the test tube system during the test period

One major prerequisite for the suitability of the presented microcosm system for future studies is that initial soil properties remain constant after equilibration if no further manipulations are carried out. To verify this, all parameters given in Table 2 were measured again after two and 4weeks and significant changes during this time period were tested by repeated-measures ANOVA (Table 3). None of the measured N and C pools changed significantly ($P > 0.05$). Likewise, $\delta^{13}C$ values of all soils and, with the exception of soil Maissau, $\delta^{15}N$ values also remained stable. With regard to gas emission rates, only N_2O emission from soil Purkersdorf decreased significantly ($P < 0.0001$), while no differences in CO_2 and CH_4 emission rates could be detected in any soil. No significant differences in gross N mineralization and nitrification rates were observed at site Purkersdorf and Riederberg. In soils from Niederschleinz and Tulln, however, gross N mineralization rates increased significantly ($P < 0.0001$), while at site Maissau nitrification rates decreased over time ($P < 0.05$). Nitrate-reductase and dehydrogenase activities did not change significantly during the 4weeks, except in soil Riederberg, where a significant decrease of dehydrogenase activity was observed ($P < 0.05$). The largest influence of incubation time on soil parameters was observed in soil microbial biomass C, which decreased significantly by 29 to 79% at all sites except in soil Niederschleinz. Microbial biomass N, on the other hand, significantly decreased by 63% in soil Riederberg only ($P < 0.0001$). Contrary to microbial biomass, fungal and bacterial (amoA) community structures did not change significantly ($P > 0.05$) in any soil (Fig. 3, showing results for soil Maissau as representative for the others).

To further evaluate the suitability of the microcosm system the variance of the single parameters during the incubation period was determined; even if the absolute value of a parameter under investigation may change during the test period, it is more important that the variance does not increase. This analysis was done by calculating the ratio of the 95% confidence

Table 3 Changes in soil properties and activities and in microbial community composition of five soils collected in the vicinity of Vienna, Lower Austria, Austria and incubated in the microcosm systems over 4 weeks

	P	R	M	N	T	Ratio *CI* t4 / *CI* t0
Total C	ns	ns	ns	ns	ns	0.7
Total N	ns	ns	ns	ns	ns	0.6
TDC	ns	ns	ns	ns	ns	1.0
NO_3^- -N	ns	ns	ns	ns	ns	0.4
NH_4^+ -N	ns	ns	ns	ns	ns	0.3
$\delta^{15}N$	ns	ns	***	ns	ns	0.7
$\delta^{13}C$	ns	ns	ns	ns	ns	0.7
CO_2	ns	ns	ns	ns	ns	0.5
N_2O	***	ns	ns	ns	ns	0.7
CH_4	ns	ns	ns	ns	ns	1.3
Mineralization	ns	ns	ns	***	***	1.4
Nitrification	ns	ns	*	ns	ns	1.6
Dehydrogenase	ns	*	ns	ns	ns	0.7
Nitrate reductase	ns	ns	ns	ns	ns	1.0
N_{mic}	ns	***	ns	ns	ns	0.7
C_{mic}	*	***	*	ns	***	0.5
amoA	ns	ns	ns	ns	ns	na
Fungal ITS	ns	ns	ns	ns	ns	na

TDC Total dissolved carbon, N_{mic} Microbial biomass N, C_{mic} Microbial biomass C, *amo*A Ammonium oxidising bacterial community. *CI* t4, 95% confidence interval of samples after 4weeks; *CI* t0, 95% confidence interval at the first sampling time; na, not applicable; Time effects were calculated by repeated measures ANOVA and Bonferroni post-hoc test (ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$)

intervals (*CI*) of each parameter at the beginning of the test period (t0; after equilibration) and after 4weeks (t4). Only three of all measured parameters, namely gross N mineralization, nitrification and CH_4 emission rates, showed higher confidence intervals at the end of the test period compared to the beginning (Table 3). The variance of all other investigated parameters decreased or remained constant.

Plant growth and ^{15}N uptake

The $\delta^{15}N$ values of two-day old barley seedlings (−0.36 ‰), which were grown in soil from Purkersdorf labelled with $^{15}NH_4Cl$, increased linearly during the subsequent growth period of 11days and reached a $\delta^{15}N$ value of 10.84 ‰ (Fig. 4a). Simultaneously, the proportion of seed-derived N in the plants decreased almost linearly. When plants were 13days old, 60% of total plant N pool consisted of N taken up from the soil. Plant biomass also increased linearly (R^2 = 0.998) during this time, with a gain of 3.7mg dry matter per day (Fig. 4b). When barley was grown in other soils, plant growth rate was similar to Purkersdorf soil; only in Maissau soil plants showed a faster increase of biomass.

Discussion

Handling guidelines and optimizations

The present study describes a new design for a microcosm system that allows investigating the complexity of physiological processes at the soil-plant-microbe-environment interface with high reproducibility at low costs. The outstanding performance that can be achieved with this system is documented by the low variance and the high reproducibility in chemical properties, bacterial and fungal community structures, gas emissions and plant growth. Besides being cost effective and easily available, this microcosm system allows handling of hundreds of replicates requiring only little space. The simple handling allows three to four people to conduct experiments with 400 microcosms or more. Before starting, however, some general considerations using the tube

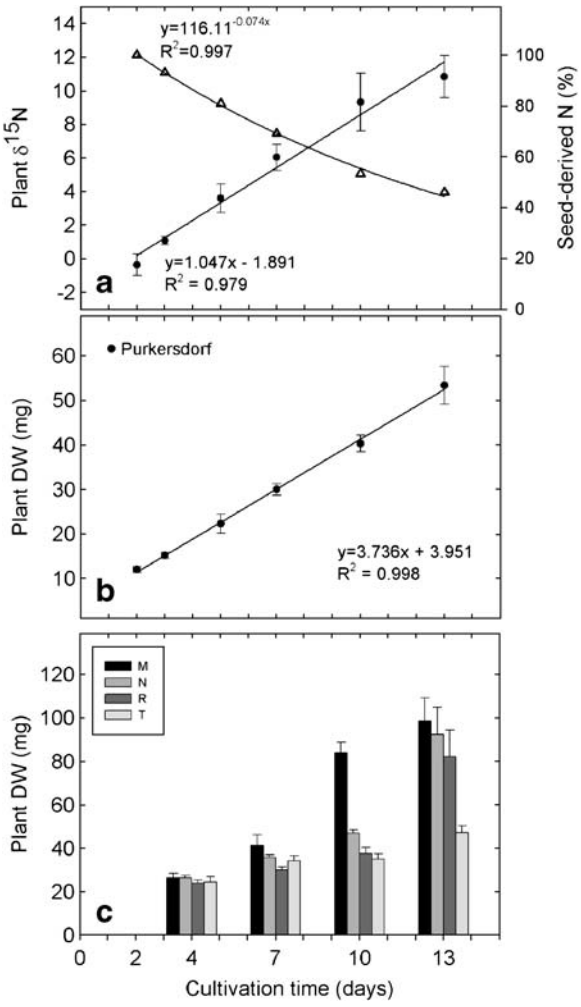

Fig. 4 Time course of barley growth and N uptake in the microcosm system. Increase of plant δ15N (full circles) and simultaneous decrease of seed-derived N (open triangles) in barley plants (a) and increase of plant dry weight (b and c). Seeds of barley were transferred to the microcosms 2 daysafter germination and grown in soil from site Purkersdorf labelled with $^{15}NH4Cl$ (a and b) or (c) in the unlabelled soils from Maissau (M), Niederschleinz (N), Riederberg (R) and Tulln (T). Soils received no fertilizer before or during plant growth. Symbols and bars represent means, error bars represent standard errors (n=5)

design need to be addressed. As in other studies (Grigatti et al. 2007), it was one of the major challenges to establish homogenous conditions of the soils in the microcosms in terms of bulk density and water content. Only slight changes of water content, bulk density and other factors, such as light conditions or temperature, might lead to significant differences in soil processes and plant growth, and the reproducibility of the microcosm system hence would decrease. We found out, that slow centrifugation of the tubes (187g) and using 32g of moist soil, represented the optimal conditions in terms of packing for soil Niederschleinz. With this method it was possible to achieve most homogenous soil bulk density and water content in the tubes (Fig. 2). Moreover, packing density in the tubes (0.9–1.0g cm^{-3} soil volume) closely resembled that of soil in situ (0.96g cm^{-3}, Table 1). Changing the amount of soil used for packing had a great influence on the homogeneity of compaction, e.g. soils were significantly less homogenously packed with only a small increase of fresh weight (3g) for the same centrifugation procedure. As these findings were valid for all five tested soils (data not shown), it is therefore essential to test every soil for homogeneity of soil packing before starting an experiment using the microcosm system. Packing the soil by gradually increasing the pressure from the top, or simply compacting the soil by tipping against the lab bench (data not shown), resulted in significantly higher heterogeneity and should not be applied in such a test system. Even if this may sound trivial, achieving a uniform soil distribution in the tubes was found to be one of the crucial steps for all subsequent analyses using this microcosm approach. This is particularly important for processes that are controlled by soil aeration or redox potentials such as N_2O and CH_4 fluxes or nitrification (data not shown). Prior to further analyses soils should be equilibrated for at least one or 2weeks, to avoid priming effects which are likely to occur during the preparation procedures (Madsen 2005).

Between-soil differences in soil N and C pools and activities

To evaluate the microcosm design for its suitability to study soil-plant-atmosphere interfaces we used five representative soils that are commonly used for cultivation of barley in the vicinity of Vienna, Austria but differ in chemical properties (Table 2). Soil N and C pools and microbial biomass differed significantly between soils (Table 2), and based on these parameters it was possible to clearly distinguish the soils by a principal component analysis (PCA) approach (Fig. 5a). Further, the concentrations of C and N pools within each soil did not change significantly ($P > 0.05$)

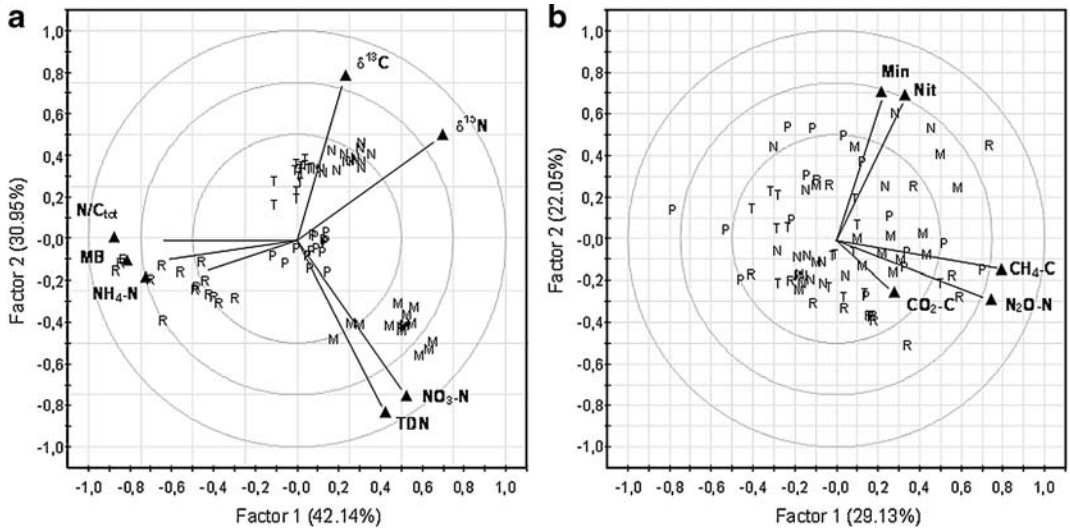

Fig. 5 Principal component analysis of a C and N pools, microbial biomass and $\delta^{15}N$ and δ13C values and b gas emission and gross N transformation rates of five soils collected in the vicinity of Vienna, Lower Austria, Austria. Data points (indicated as letters) represent soils sampled at time 0, after 2 weeks and 4 weeks (P, Purkersdorf; R, Riederberg; M, Maissau; N, Niederschleinz; T, Tulln). Min, gross mineralization rate; Nit, gross nitrification rate; Ntot, total soil nitrogen; Ctot, total soil carbon; Nmic, microbial biomass N; Cmic, microbial biomass C; TDN, total dissolved nitrogen

during the test period (Table 3), showing that without further treatment such as fertilization or plant growth and under constant conditions these parameters remained stable throughout time in the microcosms. The C content of soil microbial biomass, however, decreased significantly in four of the five tested soils, while the N content in soil microbial biomass only decreased at site Riederberg. This change may be a result of initial priming effects after sieving and homogenizing the soils (Kuzyakov et al. 2000), indicating that 10days of equilibration were yet too short. However, these changes appear to be small compared to treatment effects such as fertilization, cutting or plant growth where massive changes in microbial biomass can be expected.

As the chemical composition of the soils studied differed so clearly, we expected to be able to separate the five soils by PCA based on microbial activities and gross transformation rates. Though soil respiration (in terms of CO_2 production) and production of N_2O differed significantly between (some) soils (Table 3), no clustering of sites was evident in this PCA approach (Fig. 5b). This suggests that after equilibration under standardized conditions overall soil activities (e.g. gas fluxes and N transformations) did not allow a clear separation of soils. We attribute this to the generally high variability in trace gas fluxes and gross N transformation rates (e.g. 100–150% coefficients of variation, Parsons et al. 1991), hindering PCA separation of soils according to soil activities. Moreover, it is well known that emissions of trace gases, such as N_2O and CH_4, are controlled by a complex interplay of production and consumption processes, being influenced by many factors, such as water filled pore space as a proxy for soil redox status and aeration (Bateman and Baggs 2005; Kitzler et al. 2006a; Well et al. 2006). In our study we equilibrated the soils at 70% water holding capacity. It is therefore likely that in this experiment the occurrence of aerobic and anaerobic microsites increased the variance in trace gas fluxes reflecting the transient and sensible balance between simultaneous trace gas production and consumption.

Reproducibility of the microcosms and changes of variance of data during time

More important than the quantitative changes of pools or activities during the test period per se, is the reproducibility of each parameter. If soil aliquots are not large enough to be representative or homogenous over all microcosms, or if soil environmental factors, such as temperature or moisture vary between single microcosms during the incubation period, this may

lead to a divergent development of single microcosms and their soil pools, microbial communities and activities with time. In detail this means that the coefficient of variation (measured as 95% confidence interval) of each parameter would increase, due to increasing data variability. This was not the case for most parameters in our study, as standard deviations remained constant or decreased during the 4weeks, even if absolute values changed (Table 3). In the case of gross N mineralization, gross nitrification and CH_4 emissions, there were minor increases in data variance between the first and last sampling event by 30 to 60% (Table 3). However, gross transformation rates generally show high variance (e.g. Parsons et al. 1991) and 4weeks represent a long time period for such fluxes to remain constant. Although not uncommon (e.g. Accoe et al. 2004), it was surprising that gross nitrification rates were higher than gross N mineralization rates in all tested soils. This could be due to over- or underestimation of gross N transformation rates, caused by e.g. high heterotrophic nitrification rates or remineralization of $^{15}NH_4^+$ (Watson et al. 2000). To measure gross N transformation rates using the pool dilution assay further studies are underway to address the effects of homogeneity of ^{15}N labelling and timing of sampling on N fluxes in the microcosm system. However, overall the microcosm system allows a wide range of soil-microbe-atmosphere interactions to be studied, as under controlled conditions each replicate sample developed similarly in the microcosm system.

Bacterial and fungal community patterns

To elucidate N pathways in soils it is not sufficient to study chemical pools and transfers between these pools without investigating soil bacterial and fungal communities. Despite the crucial role they play in the cycling of nutrients in soils, the microorganisms that are actually responsible for the key processes have rarely been described (Madsen 2005). As it is not easily possible to manipulate these communities in a natural system, it may be advisable to conduct additional experiments in a laboratory format microcosm system (Copley 2000). To evaluate our microcosm system for its future suitability to study microbial community patterns and their role and response to fertilizer application, it was crucial that the initial community patterns did not change during the test period in untreated soils and between replicas. T-RFLP analysis of ammonium-oxidising communities (amoA) clearly showed that these community patterns were stable during the 4weeks (Fig. 3b), even though significant changes in microbial biomass carbon could be found (Table 3). Analysis of fungal ITS regions showed that no significant change in fungal communities could be observed as well (Fig. 3d). In the absence of manipulations, e.g. fertilization or plant growth, the bacterial ammonium-oxidizing as well as fungal communities did not change during the test period. Even more importantly, the analysis of replicas from one time point (Fig. 3a and c) showed a consistent community T-RFLP pattern with negligible variations between the replicates. These findings strongly suggest that the microcosm system can also be used to study microbial community structures and functions (e.g. enzyme activities) in soils. Previous studies have shown that bacterial growth rates and activities differ greatly between bulk soil and rhizosphere along barley roots (Soderberg and Baath 1998). These findings were yet not considered in the present study, and will have to be dealt with in follow-up experiments, as here the main aspect was to validate the chemical and microbiological stability of the bulk soils in the microcosms.

Plant growth and N uptake

Since future studies using the test tube system should allow investigations of plant-soil-microbe interactions, plant growth and N uptake were monitored in a time frame of 2–13days after germination (Fig. 4). This time period of 13days proved to be optimal for studying plant N dynamics, as within this time a linear increase of plant biomass as well as ^{15}N uptake was observed. These results demonstrate that, over this time period, model plants like barley can be grown in the test tube system without restriction by soil nutrients and rooting space and the effect of different soil parameter on plant physiology and N uptake can be assessed. When the plants were continuously grown in the microcosms for one more week, no mineral nutrient deficiencies were observed (von Wiren, data not shown) and plants grew vigorously without fertilizer treatment. Nevertheless, experiments using barley should not exceed a time period of 3weeks, as there may occur unexpected and

hardly traceable side effects due to reduced root growth and nutrient uptake in the limited soil volume. Obviously, the increase of plant biomass differed between the different soils (Fig. 4c), most likely because of their different initial physico-chemical conditions.

Already 1day after transferring two-day old barley seedlings to the microcosms the ^{15}N tracer derived from the soil was detected in the plants (Fig. 4a). While the ^{15}N content in the plants increased linearly during the following 11days, the amount of seed-derived N in the plants decreased (Fig. 4a). Plants therefore immediately took up soil inorganic N and N reliance on soil N pools increased continuously over time in the presented microcosms. It therefore appears feasible to investigate plant N uptake patterns and other physiological processes in 3 to 13days-old plants in a highly reproducible way.

Conclusions

This study describes a simple and cost-effective laboratory format microcosm system for high-throughput analyses of N dynamics in soil-plant-microbe-atmosphere systems. Our results show so far unrecorded evidence that several key processes in N dynamics in agricultural soils can be studied in parallel under controlled conditions. The microcosm system is suitable to study soil N cycling or other biogeochemical cycles as well as microbial populations and functions. Further, the microcosm system has the potential to investigate the interplay between plants and microbes in soil N cycle processes. Keeping the microcosms under stable, standardized and reproducible conditions proved to be easily possible, provided a uniform distribution of soil bulk density had been achieved. However, to reliably apply the microcosm system for measurements of gross N transformation rates as well as CH_4 fluxes, further testing is necessary. As pointed out by others, it is important to consider the limitations of such a microcosm system, and that the validity of results has a model character which needs up-scaling at certain points by field studies (Madsen 2005). Nevertheless, this system is a powerful tool to elucidate interactive pathways in soil-plant-microbe-environment systems, and may allow generating new insights and the discovery of new basic mechanisms in the complex processes and controls of the agricultural soil N cycle.

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