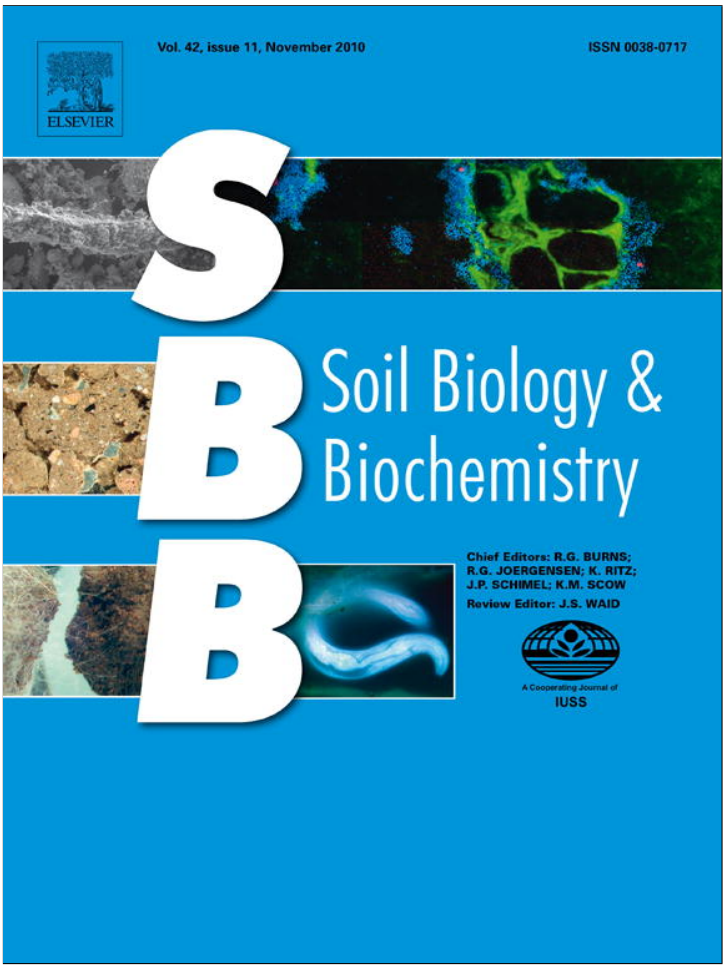

Soil Biology & Biochemistry 42 (2010) 1944–1951

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Do climate warming and plant species richness affect potential nitrification, basal respiration and ammonia-oxidizing bacteria in experimental grasslands?

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ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form
6 July 2010
Accepted 9 July 2010
Available online 23 July 2010

Keywords:
Climate warming
Plant species richness
Above and belowground link
Diversity-ecosystem functioning
Ammonia-oxidizing bacteria
Nitrification
Basal respiration

ABSTRACT

Ammonia-oxidizing bacteria (AOB) are key organisms in the N cycle, as they control the first, rate-limiting step of the nitrification process. The question whether current environmental disturbances, such as climate warming and plant diversity losses, select for a particular community structure of AOB and/or influence their activity remains open. The purpose of this research was to study the impact of a 3 °C warming and of plant species richness (S) on microbial activity and diversity in synthesized grasslands, with emphasis on the nitrification process and on the diversity (community structure and richness) of ammonia-oxidizing bacteria (AOB). We measured soil chemical characteristics, basal respiration, potential nitrification and AOB diversity in soils under increasing plant species richness ($S = 1$, $S = 3$, $S = 9$) at ambient and (ambient +3 °C) temperature. Species were drawn from a 9-species pool, belonging to three functional groups: forbs, legumes and grasses. Mixtures comprised species from each of the three functional groups. Warming did not affect AOB diversity and increased potential nitrification at $S = 3$ only. Under warmed conditions, higher plant species richness resulted in increased potential nitrification rates. AOB richness increased with plant species richness. AOB community structure of monocultures under legumes differed from those under forbs and grasses. Clustering analysis revealed that AOB community structure under legume monocultures and mixtures of three and nine species grouped together. These results indicate that functional group identity rather than plant species richness influenced AOB community structure, especially through the presence of legumes. No clear relationship emerged between AOB richness and potential nitrification whatever plant species richness and temperature treatment. Our findings show a link between aboveground and belowground diversity, namely plant species richness, AOB richness and community structure. AOB richness was not related to soil processes, supporting the idea that increased diversity does not necessarily lead to increased rates of ecosystem processes.

1. Introduction

Owing to increased human activities over the past centuries, ecosystems are currently faced with severe environmental changes such as global warming and plant diversity losses. Numerous studies have explored the link between plant diversity and ecosystem functioning (Hooper and Vitousek, 1997; Hector et al., 1999; Tilman et al., 2001) or ecosystem stability (Wardle et al., 2000; Tilman et al., 2006). While a positive link between plant productivity and plant diversity has been shown in most studies (Hooper et al., 2005), various responses have been found in studies addressing the diversity–stability relationship (Tilman et al., 2006; De Boeck et al., 2008). Furthermore, through the quality and the quantity of root exudates and litter (Wardle, 2002), and plant nutrient-foraging strategies (Campbell et al., 1991), above and belowground components of terrestrial ecosystem are connected. Increasing plant species richness may therefore affect soil microorganisms through the promotion of a greater diversity, quality and quantity of available resources (Wardle, 2005; Orwin et al., 2006; Meier and Bowman, 2008). Some evidence on the impact of plant species richness and community composition on belowground diversity in grasslands indicates that plant species richness

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doi:10.1016/j.soilbio.2010.07.006

regulates soil microbial community composition (Chung et al., 2007) and that plant community composition controls bacterial community composition in the rhizoplane (Nunan et al., 2005) or rhizosphere only (Kowalchuk et al., 2002). It has been also demonstrated that the composition of plant communities affects individual functional groups of soil microorganisms like methanotrophs (Knief et al., 2003), ammonia-oxidizing bacteria (Kowalchuk et al., 2000) and denitrifying bacteria (Cavigelli and Robertson, 2000). Plant species richness and community composition may therefore influence both ecosystem productivity and belowground diversity. Specific information on the effect of plant species richness on AOB is currently lacking.

Moreover, a modification of the soil microbiota may in turn affect soil processes, providing a positive or negative feedback on plant productivity. Despite the crucial role of soil microorganisms in mediating belowground processes, the issue of how the diversity of soil microbiota influences processes such as decomposition or nutrient mineralization remains poorly studied (Balser et al., 2001). Some research revealed that microbial community composition can influence or constrain ecosystem functioning. For example, Cavigelli and Robertson (2000) and Bremer et al. (2007) showed that the composition of denitrifier communities affects the N_2O emission from soil.

Balser and Firestone (2005) have shown that microbial soil processes, principally those strongly linked to microbial phylogeny, i.e. nitrification or denitrification, were related to microbial community composition.

Current theory suggests that the influence of microbial community composition on ecosystem functioning may be more important for 'narrow processes' regulated by a restricted groups of microorganisms than for 'broad processes' carried out by many redundant species (Schimel, 1995).

Furthermore, information on the impact of environmental changes (e.g. warming) on the diversity of microorganisms and the processes they control is scarce. When microorganisms are faced with environmental constraints altering resource availability or abiotic factors (temperature, moisture), they may employ adaptive strategies and trade-offs to survive. This in turn could lead to a shift in community composition, as microorganisms better suited to the new environmental conditions have a competitive advantage (Balser et al., 2001). Warming may affect microbial community structure and richness directly by modifying abiotic factors like temperature and moisture, or indirectly through changes in resource availability (Schimel et al., 1994). Ammonia-oxidizing bacteria (AOB) and the nitrification process have been reported sensitive to moisture and temperature conditions (e.g. Gleeson et al., 2008; Szukics et al., 2010). Furthermore, cultured AOB have revealed different Km values for substrate (NH_4) (Koops and Pommerening-Röser, 2001).

We investigated the effect of warming and plant species richness on the nitrification process, which can be considered as a physiologically and phylogenetically narrow process (Schimel, 1995) and on basal respiration, which can be considered as a physiologically and phylogenetically broad process. The first, rate-limiting step of nitrification is carried out by ammonia-oxidizing bacteria (AOB). All AOB enriched or isolated from soils to date share their overall physiology (aerobic autotrophs), are closely related and belong to the β-*Proteobacteria* (Stephen et al., 1996). At least ten clusters can reproducibly be recognized within the phylogenetic tree based on 16s rDNA sequences, five of which belong to the genus *Nitrosospira* and five to the genus *Nitrosomonas* (Purkhold et al., 2000; Koops et al., 2003). Recently, some studies (e.g. Nicol et al., 2008) suggested a role of non-thermophilic *Crenarchaea* in soil ammonia oxidation. However, measured nitrification rates were more closely related to the ammonia oxidizer transcriptional levels than to those of Crenarchaea (Nicol et al., 2008).

The objectives of this research were to study the influence of a 3 °C atmospheric warming and of plant species richness in synthesized grassland on (i) AOB functioning (potential nitrification) and microbial activity (basal respiration) (ii) AOB richness and community structure and (iii) to investigate the relationship between AOB richness and function. We hypothesized that increased plant species richness may lead to increased AOB richness and functioning.

2. Materials and methods

2.1. Experimental set-up and soil sampling

An experimental platform containing 288 artificially assembled grassland model ecosystems was established in July 2003 at the Drie Eiken Campus of the University of Antwerp (Belgium, 51°09′N, 4°24′E). The platform consisted of 12 sunlit, climate controlled-chambers facing south (2.25 m^2 ground area). Half of the chambers were at ambient temperature (unheated chambers) while the other half were continuously warmed 3 °C above the fluctuating ambient values (heated chambers). Annual mean temperature was 9.6 °C and annual precipitation averaged 776 mm. Each chamber contained 24 grassland communities of species richness (S): $S = 1$ ($n = 9$), $S = 3$ ($n = 9$) and $S = 9$ ($n = 6$). These communities, each containing 30 individuals, were created using nine species from three functional groups: forbs (*Bellis perennis* L., *Rumex acetosa* L., *Plantago lanceolata*), grasses (*Dactylis glomerata* L., *Festuca arundinacea* Schreb., *Lolium perenne* L.) and legumes (*Trifolim repens* L., *Medicago sativa* L., *Lotus corniculatus* L.). These species were selected according to several criteria: presence in temperate grasslands, perennial life cycle, preference for loam or clay soil and known differences in productivity, temperature and drought resistance. Plant communities at $S = 3$ and $S = 9$ contained an equal number of species of each of these three functional groups, and at $S = 3$ each species was represented by the same number of individuals. For $S = 9$, 1 species of each functional group was randomly selected to be represented by 4 individuals, the other species were represented by 3 individuals. All six $S = 9$ communities contained all species, but had different internal arrangements. Each plant community was grown in containers (24 cm inner diameter × 60 cm height) filled with sieved, thoroughly mixed soil collected from a 25 year-old corn field. The soil consisted of 76.3% silt, 14.8% clay and 8.7% sand. Initial soil characteristics were (means ± SE): pH 6.44 ± 0.08, total C 1.60% ± 0.18, total N 0.15% ± 0.01; the exchange complex was largely dominated by Ca^{2+} (85%), followed by Mg^{2+} (8%) and K^{+} (4%). The quantity of water supplied to the chambers was calculated weekly from the difference between the soil moisture outside and inside the unheated chambers, in order to generate the same soil water content. From June 2004 onwards, irrigation was based on precipitation data from a nearby weather station (Lint, Belgium) rather than on soil water content, and the precipitation data were corrected for the artificial enhancement of evapotranspiration in the chambers. As the heated chambers received the same amount of water than unheated ones, any enhanced consumption resulted in (increased) soil drought. Further details on the experimental platform, growth and climate conditions can be found in Lemmens et al. (2006) and De Boeck et al. (2008).

In November 2004 and 2005, respectively 16 and 28 months after the beginning of the experiment, all containers from two chambers (one unheated and one heated; a total of 48 containers) were destructively sampled. Grassland communities were harvested for biomass assessment and results are reported elsewhere (De Boeck et al., 2008). In November 2004, we performed extensive analyses, including soil analysis, AOB diversity and activity at two soil depths (upper: 1–5 cm; lower: 6–10 cm). In November 2005,

due to material constraints, analyses were limited to microbial activities and soil characteristics in the lower layer, selected on the basis of the results of 2004. We collected bulk soil, which we defined as soil not adhering to roots. However, in pot experiments, most soil can be considered to be influence by roots, as rooting density is generally high. The soil was sieved aseptically (4 mm mesh) and homogenised. For biomass estimation, roots were collected manually, dried (2 days at 70 °C) and weighed. In 2004, sub-samples of the soil were freeze-dried and stored at −20 °C until molecular analysis. The remaining soil collected in 2004 and the soil collected in 2005 was stored at 4 °C until analyses (within one week).

2.2. Soil characteristics and microbial activities

Soil moisture, $pH_{(H_2O)}$ and loss on ignition were determined according to Allen (1986). NH_4–N was extracted with 1 M KCl (1:5, w:v) and analysed colorimetrically using a continuous flow analyser (Autoanalyser 3, BranLuebbe, Germany).

Potential nitrification was determined using the 'shaken soil-slurry method'(Hart et al., 1994). This method involved shaking 10 g of field moist, sieved soil in 100 ml of a solution (pH 7.2) containing 1 mM PO_4^{3-} and 15 mM NH_4^+, at 25 °C in the dark. These experimental conditions (moisture, pH, oxygen, ammonium and phosphate availability) assure a maximum nitrate production rate, in the absence of N immobilization and denitrification. We took homogenised sub-samples (15 ml of the slurry) at 2, 6, 22, 26 and 30 h after the start of the incubation. These sub-samples were filtered and stored at −20 °C until analysis. NO_3–N concentrations were analysed colorimetrically with a continuous flow analyser (Auto-Analyzer3, BranLuebbe, Germany). Nitrification rates were calculated by linear regression of nitrate concentrations over time ($\mu g\ N\ g^{-1}\ d^{-1}$).

Basal respiration (BR, CO_2 evolution without added substrate) was estimated as CO_2 accumulation in the headspace of an amber bottle (250 ml, Supelco, USA) from 20 g field moist soil, at 15 °C in the dark (2005 sampling only). Gas samples (4 ml) were taken at 0, 20, 24 and 28 h and were analysed by gas chromatography (GC8000, Interscience, Belgium). Before measurements, soil samples were kept for 12 h at room temperature for equilibration (Lovell and Jarvis, 1998). The CO_2–C production rates were estimated by linear regression of CO_2–C against time ($\mu g\ CO_2\text{–}C\ g^{-1}\ h^{-1}$).

2.3. Analysis of the ammonia-oxidizing bacteria diversity

AOB community structure was assessed by 16s rDNA denaturing gradient gel electrophoresis (2004 sampling only). Genomic DNA was extracted from 0.2 g of freeze-dried soil using the UltraClean Soil DNA kit (MoBio, Solana Beach, CA) according to manufacturer's instructions with a minor modification: 200 µl of $AlNH_4(SO_4)_2$ 100 mM were added before the first step of lyses to remove soil-based inhibitors through chemical flocculation (Braid et al., 2003).

A region of the small subunit (16S) ribosomal gene spanning the V2–V3 variable domains was amplified using polymerase chain reaction (PCR) and the ammonia oxidizer specific primer set: GC-clamped CTO 189f-ABC/CTO 654r (Kowalchuk et al., 1997; Invitrogen, Merelbeke). Each 25 µl PCR mixture contained 0.8 µM of each primer, 150 µM of each dNTP (Bioline Ltd, London, UK), 1% bovine serum albumine (Fluka Analytical, St Gallen, Switzerland), 2.5 µl of Accubuffer 10× and 1.25 U Accuzyme polymerase (Bioline, Luckenwalde, Germany). Amplification was performed by using an initial denaturation step at 94 °C (2 min); 35 cycles of 92 °C (30 s), 59 °C (1 min), 72 °C (45 s + 1 s $cycle^{-1}$) and a final extension of 72 °C (5 min). PCR products differing in melting properties were separated using denaturing gradient gel electrophoresis (DGGE). This technique yields a banding pattern were the number of DNA bands reflects the genotypes of the bacterial populations (Bloem and Breure, 2003). The DGGE gel was performed using 6% acrylamide gel with a 30–60% denaturant gradient, where 100% denaturant was defined as 7 mM urea plus 40% formamide. The gel was run at 60 °C for 10 min at 200 V followed by 16 h at 80 V, using the "DCode universal mutation detection system" (Bio Rad laboratory, Hercules, CA). The gels were stained for 30 min with SybrGold (Molecular Probes, Leiden, The Netherlands) before visualization by a CCD camera under a blue-light transilluminator (Dark Reader, Clare Chemical, Ross on Wye, UK). Samples were run alongside reference marker using cluster controls derived from clones of known sequences (Stephen et al., 1996; Kowalchuk et al., 1997). The reference marker was used as a standard during gel normalization and analysis, to ensure gel-to-gel comparability. The DGGE profiles were analysed and compared using Gel Compar II (Applied Maths, Kortrijk, Belgium). Similarities in AOB community structure of samples (under different treatments and for both soil depths) were assessed using hierarchical clustering (Fromin et al., 2002). Similarity matrices were generated from binary data (based on the position of the bands, and not on the sequence) for each gel lane (each sample) using Dice Coefficient as follows: $C_D = 2j/\mathrm{uo}(a+b)$, where j = number of bands in common between lanes A and B, a = the total number of bands in lane A, and b = the total number of bands in lane B. The clustering algorithm 'unweighted pair group method with arithmetic averages (UPGMA)' was used to construct the corresponding dendrogram. In these dendrograms, samples with a similar DDGE banding pattern group together, indicating a similar AOB community structure. The number of bands observed in each DGGE lane was used as an indicator of richness (AOB richness) since each band in the DGGE gel is likely to be derived from one phylogenetically distinct population (Ampe and Miambi, 2000).

DGGE has been recognized as a powerful, reliable and reproductive tool for the study of the general structure of microbial communities. Some shortcomings, such as the limited sensitivity for the detection of rare communities or the separation of very small fragments have to be considered when using this technique. In this study, the sensitivity has been improved by the application of a group-specific PCR (Muyzer, 1999), by the use of AOB-specific primers.

2.4. Statistical analysis

Statistics for all variables were performed separately for each soil depth and time of sampling, as they were not considered as experimental factors in our design. As 2-way ANOVA showed significant interactions, one-way ANOVA for unbalanced designs (proc GLM) was applied for analysing the effect of plant species richness and warming on soil properties, microbial activities and AOB richness at each depth and at each time of sampling using SAS (SAS, SAS Institute Inc., 1985). The difference in root biomass between soil depths was further investigated by 1-way ANOVA for each time of sampling and chamber separately. Multiple comparisons of means were performed by a Duncan post-hoc test ($\alpha = 0.05$). The relationships between variables were examined using Pearson's correlations.

3. Results

3.1. Influence of plant species richness and climate warming on soil characteristics and on root biomass

All soil samples had a neutral pH_{H_2O} ranging from 7.2 to 8.2 (Table 1). Significant effects of plant species richness (S) on $pH_{(H_2O)}$

Table 1
Chemical characteristics and root biomass of soils sampled from containers, 16 (2004) and 28 months (2005) after planting.

Time of sampling	Temperature	Depth	Plant species richness S	pH (water)	Organic matter content (LOI, %)	Extractable NH_4^+–N (mg kg^{-1})	Root biomass (g)
2004	Unheated	Upper	$S = 1$	8.0 (0.1)aA	3.96 (0.09)aA	6.89 (0.46)aA	17.5 (5.8)aA
			$S = 3$	8.0 (0.1)aA	4.12 (0.07)aA	1.11 (0.24)bA	22.3 (4.3)aA
			$S = 9$	7.4 (0.1)bA	3.98 (0.06)aA	0.12 (0.12)bA	25.3 (6.7)aA
		Lower	$S = 1$	7.5 (0.1)aA	3.47 (0.07)bA	0.85 (0.35)bA	7.7 (3.9)aA
			$S = 3$	7.5 (0.1)aA	4.00 (0.02)aA	1.89 (0.48)aA	12.8 (3.6)aA
			$S = 9$	7.3 (0.1)aA	3.98 (0.06)aA	0.72 (0.49)bA	14.5 (4.4)aA
	Heated	Upper	$S = 1$	7.9 (0.1)aA	4.16 (0.03)aA	6.39 (0.57)aA	12.9 (3.7)aB
			$S = 3$	8.1 (0.1)aA	4.10 (0.13)aA	0.80 (0.35)bA	13.8 (2.4)aB
			$S = 9$	8.2 (0.1)aA	4.10 (0.05)aA	1.31 (0.41)bA	11.2 (2.3)aB
		Lower	$S = 1$	7.5 (0.1)aA	3.51 (0.06)bA	0.08 (0.08)bB	4.7 (1.6)aA
			$S = 3$	7.6 (0.1)aA	3.83 (0.05)aA	0.53 (0.30)bB	6.3 (1.4)aA
			$S = 9$	7.3 (0.1)aA	4.00 (0.06)aA	1.75 (0.51)aA	4.5 (0.7)aA
2005	Unheated	Lower	$S = 1$	7.2 (0.1)bA	4.24 (0.14)bA	0.82 (0.41)bA	27.7 (15.2)aA
			$S = 3$	7.4 (0.1)abA	4.46 (0.09)aA	2.40 (0.48)aA	26.5 (4.6)aA
			$S = 9$	7.5 (0.1)aA	4.33 (0.07)bA	1.78 (0.07)bA	44.8 (12.3)aA
	Heated	Lower	$S = 1$	7.3 (0.1)bA	4.26 (0.04)bA	0.27 (0.11)bB	24.3 (8.7)aA
			$S = 3$	7.4 (0.1)abA	4.51 (0.03)aA	0.34 (0.11)bB	29.1 (3.2)aA
			$S = 9$	7.5 (0.1)aA	4.14 (0.08)bA	1.62 (0.46)aA	28.1 (4.0)aA

Values are means (±SEM); $S = 1$ ($n = 9$), $S = 3$ ($n = 9$), $S = 9$ ($n = 6$). Different lower case letters indicate significant differences between plant species richness levels for each depth and chamber according to Duncan's test at $P < 0.05$. Different upper case letters indicate significant differences between temperature treatments for each depth and species richness level according to Duncan's test at $P < 0.05$.

were observed in 2004 in the unheated chamber (upper depth, $P = 0.009$) and in 2005 in both chambers (lower depth, $P = 0.046$). In 2004, the $pH_{(H_2O)}$ was higher at $S = 1$ and at $S = 3$ than at $S = 9$. On the contrary, in 2005, pH_{H_2O} was higher at $S = 9$ than at $S = 1$. Despite their significance, these effects were however small (0.2–0.6 unit pH differences).

Organic matter content (LOI) was low (≤4.5%) and differently influenced by plant species richness, according to depth and time of sampling. In 2004, organic matter content was unaffected by plant species richness in the upper depth ($P = 0.651$), whereas it was higher at $S = 3$ and $S = 9$ than at $S = 1$ in the lower depth ($P < 0.0001$). In 2005, the highest ($P < 0.0054$) organic matter content was observed at $S = 3$ (4.5%).

The effect of plant species richness on extractable ammonium depended on sampling time, temperature, and depth (1-way ANOVA; Table 1). In the upper depth for both chambers (2004), extractable ammonium was higher at $S = 1$ than at $S = 3$ and $S = 9$ ($P < 0.0001$). In the lower depth of the unheated chamber (both times of sampling), extractable ammonium was highest at $S = 3$ ($P < 0.0001$), while in the heated chamber (both times of sampling), it was highest at $S = 9$ ($P < 0.0001$). There was no significant effect of plant species richness on root biomass ($P = 0.995$), but in 2004, there were significantly more roots in the upper depth than in the lower depth ($P = 0.0002$).

Warming did not affect pH_{H_2O} (lower depth 2004: $P = 0.8207$; lower depth 2005: $P = 0.7663$; upper depth 2004: $P = 0.148$) nor the organic matter content (lower depth 2004: $P = 0.4576$; lower depth 2005: $P = 0.6323$; upper depth 2004: $P = 0.1574$) whatever the time of sampling, depth and plant species richness level. However, warming decreased extractable ammonium at $S = 1$ (2004: $P = 0.0215$; 2005: $P = 0.185$) and $S = 3$ (2004 and 2005: $P < 0.0001$), in the lower depth (both times of sampling). Warming also significantly decreased root biomass in 2004 ($P = 0.0082$).

3.2. Influence of plant species richness and climate warming on potential nitrification and basal respiration

Mean values for potential nitrification ranged from 0.66 to 1.49 μg N g^{-1} h^{-1} (Fig. 1). In the upper depth (2004 only), neither plant species richness ($P = 0.7135$) nor warming ($P = 0.1980$) influenced potential nitrification. In the lower depth (2004), plant species richness affected potential nitrification only in the heated

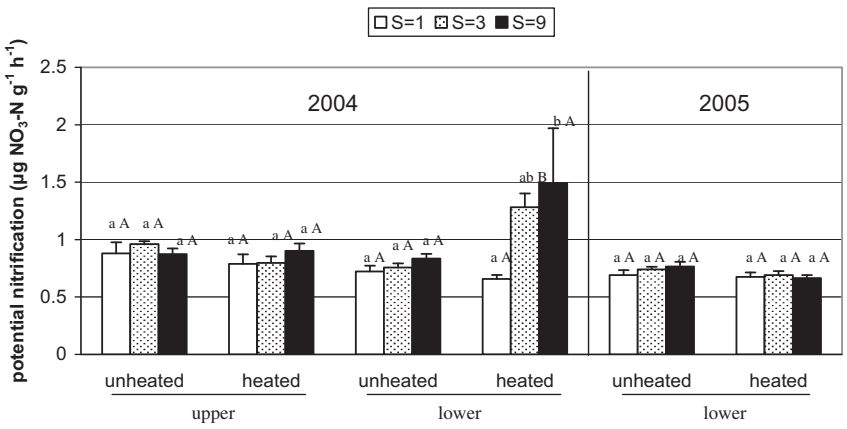

Fig. 1. Effects of plant species richness and warming on potential nitrification in the upper (1–5 cm) and lower (6–10 cm) soil depth. Different lower case letters indicate significant effects of plant species richness at each depth and temperature (one-Way ANOVA; Duncan test; $P < 0.05$). Different upper case letters indicate significant effects of warming at each depth and plant species richness level (one-Way ANOVA; Duncan test; $P < 0.05$). Data are averages (±SEM), $n = 9$ ($S = 3$, $S = 1$), $n = 6$ ($S = 9$).

chamber ($P = 0.0349$), where potential nitrification was higher at $S = 9$ than at $S = 1$ and warming increased potential nitrification only at $S = 3$ ($P = 0.0008$). If we consider plant functional groups for $S = 1$ separately, potential nitrification in the upper layer (2004) at $S = 1$ − legumes (legume monocultures) was significantly higher than under $S = 3$ and $S = 9$, and the lowest potential nitrification was observed at $S = 1$ − forbs/grasses (forbs or grasses monocultures) ($P < 0.0001$, data not shown). In 2005, potential nitrification was not influenced by plant species richness ($P = 0.2694$), or climate warming ($P = 0.7939$).

Basal respiration ranged from 0.11 to 0.39 μg CO_2–C g^{-1} h^{-1}. Neither plant species richness ($P = 0.6702$), nor warming ($P = 0.0678$) influenced basal respiration significantly (Fig. 2). We observed a positive correlation between basal respiration and organic matter content ($P = 0.02$, $R = 0.32$).

3.3. Influence of plant species richness and climate warming on AOB richness and community composition

Sequencing confirmed that all bands present in DGGE patterns were ammonia-oxidizing bacteria. All AOB sequences were phylogenetically affiliated with the *Nitrosospira* genus (Malchair et al., 2010).

Plant species richness significantly influenced AOB richness (upper depth: $P = 0.0102$; lower depth: $P < 0.0001$) but warming (upper depth: $P = 0.7557$; lower depth: $P < 0.9313$) did not in both depth (Table 2). Plant species richness led to increased AOB richness and the impact was more pronounced in the upper depth where AOB richness was highest under $S = 9$, followed by $S = 3$ and $S = 1$ ($P < 0.0001$). In the lower depth, the highest AOB richness was observed at $S = 9$ and the lowest at $S = 1$ ($P = 0.012$).

Community structure of AOB in the unheated chamber (Fig. 3a), revealed that mixtures ($S = 3$ and $S = 9$) grouped together according to plant species richness level and depth. Under the legume monocultures ($S = 1$), AOB community structure was similar to that of soils sampled under $S = 3$ or $S = 9$ (Fig. 3a). In the heated chamber (Fig. 3b), 2 main nodes appeared: soils from the lower depth under grass and forbs monocultures showed less than 30% similarity with the other DGGE patterns, which formed a joint cluster with two separate sub-clusters. One sub-cluster contained patterns under mixtures and legumes in the upper depth and one sample at $S = 3$ from the lower depth. The second sub-cluster separated patterns under mixtures and legumes in the lower depth from those under forbs and grass monocultures in the upper depth.

To investigate the impact of warming, separate dendrograms for each species richness level were constructed. The dendrogram for soils under $S = 1$ (Fig. 3c) revealed that warming did not influence the AOB community structure, as patterns from heated and unheated chambers did not separate. AOB community structure for soils under legumes (both depths and warming treatments) differed from those under grasses and forbs. At $S = 3$ (Fig. 3d), no effects of depth and temperature on AOB community structure appeared. For soils under $S = 9$ (Fig. 3e), AOB DGGE patterns from the lower depth grouped distinctively from patterns at upper depth. Within these sub-groups, patterns from the same warming treatment clustered together.

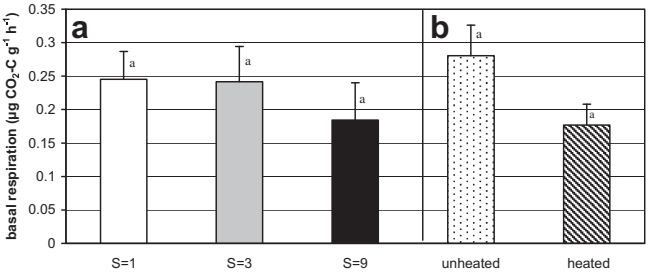

Fig. 2. Effects of plant species richness (a) and warming (b) on basal respiration in 2005 (one-Way ANOVA; Duncan test; $P < 0.05$). Data are averages (±SEM), $n = 18$ ($S = 3$, $S = 1$), $n = 12$ ($S = 9$); $n = 24$ (heated, unheated).

Table 2
Effects of plant species richness and warming on AOB richness (number of DGGE bands), 16 months after planting (2004).

Year of Harvest	Temperature	Depth	Plant specific richness S	AOB richness
2004	Unheated	Upper	$S = 1$	7cA
			$S = 3$	9bA
			$S = 9$	13aA
		Lower	$S = 1$	7bA
			$S = 3$	8abA
			$S = 9$	9aA
	Heated	Upper	$S = 1$	7cA
			$S = 3$	9bA
			$S = 9$	13aA
		Lower	$S = 1$	7bA
			$S = 3$	8abA
			$S = 9$	9aA

Values of AOB richness are means; $S = 1$ ($n = 9$); $S = 3$ ($n = 9$), $S = 9$ ($n = 6$). Different lower case letters indicate significant differences between plant species richness levels at each depth and chamber. Different upper case letters indicate significant differences between temperature treatments for each depth and species richness level according to Duncan's test at $P < 0.05$.

No significant correlations were observed between AOB richness and potential nitrification whatever plant species richness, temperature treatment or depth considered (Table 3).

4. Discussion

4.1. Linking aboveground diversity to belowground diversity and activity

Our results demonstrate that increased plant species richness in artificial grasslands increased AOB richness. We hypothesize that this positive link could be due to the spatial heterogeneity of resources (NH_4), promoted by plant species richness. Indeed, more diverse plant communities can release more diverse root exudates (Korona et al., 1994; Wardle, 2005), leading to a higher heterogeneity of resources which, after mineralization, may provide a substrate for AOB. Substrate availability for AOB may further be reduced locally by higher plant uptake, which is supported by the increased overall resource uptake observed at higher plant species richness treatments in our experiment (De Boeck et al., 2006). Both root uptake and exudation may therefore have led to the formation of microsites differing in ammonium availability, allowing a higher coexistence of AOB species. This would support the niche complementarity theory (Hector et al., 1999), which can occur through spatial differences in the use of the same resource.

In this study, the community structure of AOB under legume monocultures was more similar to that under higher species richness treatments than under the monocultures of forbs and grasses. This indicates that the effect of increased plant species richness on the AOB community structure was driven by the presence of legumes and not by plant species richness. This is consistent with research of Mokany et al. (2008) who revealed that functional identity is more important than species richness in influencing ecosystem processes in temperate grassland. Temperton et al. (2007) demonstrated that facilitation in mixtures containing legumes was linked to soil N not used by legumes and to a lesser extent to N transfer from legumes. Both mechanisms may lead to locally higher ammonium availability for AOB in soil. Ammonium

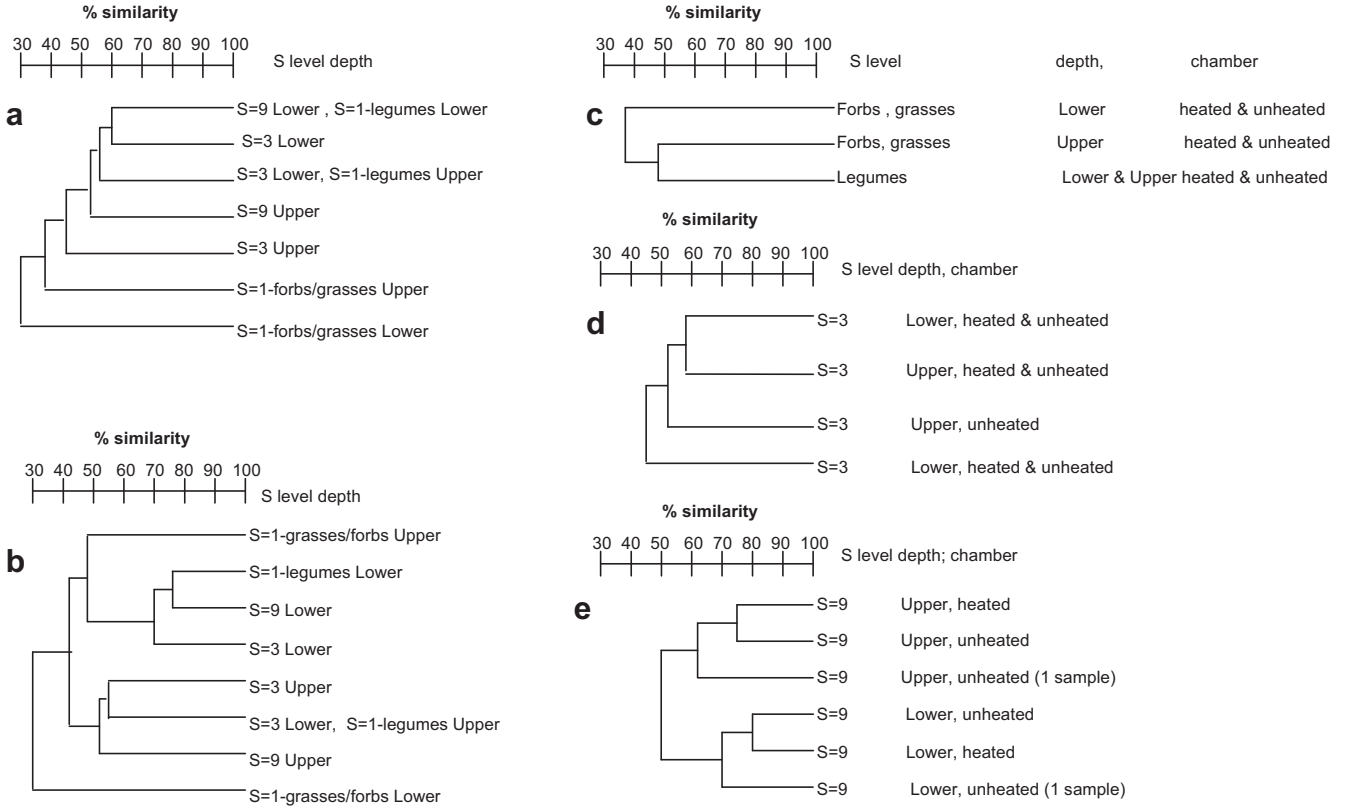

Fig. 3. Similarity dendrograms (UPGMA, Dice coefficient of similarity) of AOB banding patterns calculated from PCR-DGGE patterns obtained in (a) heated and (b) unheated chambers for all species richness levels and at (c) $S = 1$, (d) $S = 3$ or (e) $S = 9$ for both chambers (2004 harvesting).

has previously been recognized as a selective factor able to induce a shift in AOB community composition (Avrahami et al., 2003). This is also in compliance with our findings in monocultures, where we observed that the presence of legumes induced a shift in AOB community structure, presumably due to increased ammonium availability (Malchair et al., 2010). Data on extractable ammonium do not support this hypothesis, but we believe that extractable NH_4 of bulk soil does not reflect NH_4 availability to microorganisms, as demonstrated by Davidson and Hackler (1994).

The change in AOB community structure under mixtures was related to an increase in AOB richness but unchanged potential nitrification. In contrast, under monocultures of legumes the AOB community shift was associated with decreased species richness and increased nitrification (Malchair et al., 2010). Operating mechanisms under mixtures are, however, different from monocultures of legumes, as patch heterogeneity (explaining higher AOB species richness, see above) and plant N uptake have to be taken into account. Although facilitation may result in patches with higher NH_4 availability, inducing a shift in AOB community structure, this change might not be sufficient for increasing AOB population size and, concurrently, nitrification rates. Indeed, competition for NH_4 between roots and AOB must be considered. Similarly, Hawkes et al. (2005) showed that an increase in gross nitrification rates under exotic grasses was both related to an increase in AOB abundance and a change in AOB community composition. Korsaeth et al. (2001) showed that plant roots can indeed affect N cycling in soil, not by stimulating microbial activities, but by inflicting N starvation on soil microorganisms. This mechanism can also explain the increased potential nitrification rates in the mixtures of the heated soil, where above- and below-ground productivity (De Boeck et al., 2008) and, consequently, competition for ammonium were lower.

Table 3
Pearson's correlation coefficients between potential nitrification and AOB richness, 16 months after planting (2004).

Year of Harvest	Temperature	Plant specific richness S	*P*-value	Coefficient of correlation
2004	Unheated	$S = 1$	0.780	−0.07
		$S = 3$	0.266	0.31
		$S = 9$	0.936	0.03
	Heated	$S = 1$	0.800	−0.06
		$S = 3$	0.203	−0.31
		$S = 9$	0.360	−0.29

The observation that species richness did not influence basal respiration is in agreement with results of other studies (Loranger-Merciris et al., 2006; Habekost et al., 2008) and with those of De Boeck et al. (2007) who found no effect of species richness on soil respiration for *in situ* measurements of our samples. Species richness may lead to qualitative and quantitative differences in substrate supply through litter and root exudates, factors recognized as important factors controlling respiration (Luo and Zhou, 2006). In our experiment, half-yearly mowing and removal of aboveground biomass suppressed the potential influence of aboveground litter on soil respiration. A temperature effect on soil respiration is dependent on soil water status (Luo and Zhou, 2006), and lower soil water content observed at higher species richness (De Boeck et al., 2006) may have limited soil respiration.

4.2. Influence of warming on microbial activity and diversity

Processes governed by microbial activities, such as respiration and nitrification, have generally been reported to be sensitive to

temperature (e.g. Emmer and Tietema, 1990; Davidson et al., 2005). Temperature response curves are, however, derived from data over a large scale of temperatures (0–30 °C). Within the range of the temperature change expected with climate change (1.5–4.5 °C in global mean annual temperature), some authors report increased net nitrification (Hart, 2006) and soil respiration (Rustad et al., 2001; Emmett et al., 2004), while other studies revealed no effect of climate warming on net nitrification (Shaw and Harte, 2001; Emmett et al., 2004) and on soil respiration (De Boeck et al., 2007). In this study, the 3 °C warming did not influence basal respiration and potential nitrification except increased potential nitrification at $S = 3$ (lower depth, 2004). The latter can be explained by decreased root biomass in this treatment which has been ascribed to summer drought (De Boeck et al., 2008). The 3 °C increase in air temperatures in our study, increased soil temperatures by 1.57 °C (De Boeck, unpublished data), which is probably within the range of daily/seasonal fluctuations. Furthermore, temperature optima of heterotrophic soil populations can be considered sufficiently broad to tolerate such limited changes in soil temperature. The decreased above and belowground productivity observed under warmer and drier conditions (De Boeck et al., 2008) could also have limited soil respiration. Indeed, other factors such as substrate availability or soil water availability (Allison and Treseder, 2008) can modulate the impacts of warming on soil processes.

We did not detect any significant effect of warming on AOB community structure and species richness. Although, Avrahami et al. (2003) have reported temperature to be a selective factor for AOB, the shift in AOB community structure was due to a higher increase in temperature (5 °C–15 °C) than in our study. Furthermore, we mainly observed sequences affiliated to cluster 3 (Malchair et al., 2010), which can be observed at most temperatures (Avrahami et al., 2003), so that our findings are not contradictory.

5. Conclusion

Our results demonstrated that there was no consistent effect of warming and plant species richness on microbial activities (basal respiration and potential nitrification). AOB richness (number of DGGE bands) and community structure (DGGE banding pattern) were not influenced by warming, but they were both affected by plant species richness. AOB richness increased with plant species richness. The impact of plant species richness on AOB community structure was driven by functional group identity, i.e. the presence of legumes, possibly through their influence on ammonium availability. In contrast to our hypothesis, no link emerged between AOB richness and potential nitrification. These results show that higher species richness does not necessarily lead to increased rates of ecosystem processes.

Acknowledgements

We thank M.-C. Requier for help with soil sampling and A. Piret for help with soil preparation. This research was funded by the Fund for Scientific Research-Flanders (Belgium), by Liege University (Special Research Fund-FSR) and by Fonds de la Recherche Scientifique (Fonds pour la Recherche Fondamentale Collective-FNRS-FRFC). During the preparation of the manuscript, S. Malchair received financial support from the “Fonds National de la Recherche (FNR-Luxembourg)”. H.J. De Boeck is a post-doctoral research associate of the Fund for Scientific Research – Flanders. We are grateful to Dr. A. Wilmotte for providing access to database and software analytical tools. We thank the two anonymous reviewers for helpful comments on the manuscript.

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