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Nitrification along a grassland gradient: Inhibition found in matgrass swards

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ABSTRACT

Measurements along a hill-slope vegetation gradient in nutrient-poor grasslands from acid grasslands via matgrass swards to calcareous grasslands showed increased ammonium to nitrate ratios in the matgrass swards. These results generated the research question whether there might be a difference in nitrification activity or nitrifying community composition between the different zones in this hill-slope gradient. In each of the vegetation types along the gradient, soil samples were taken in five grassland nature reserves. Potential nitrification rates have been determined as an indication of the size of the active ammonia-oxidising microbial communities. Additionally, the dominant ammonia-oxidising sequences related to the β-Proteobacteria have been determined by a Polymerase Chain Reaction (PCR) based on the 16S rRNA gene in combination with Denaturing Gradient Gel Electrophoresis (DGGE) at one of the nature reserves.

Compared to the top and lower zones of the vegetation gradient (i.e. acid grasslands and calcareous grasslands, respectively), potential nitrification rates were clearly repressed in the middle, matgrass swards zone. In contrast to the differences in potential nitrification activities observed in one of the nature reserves (Bemelerberg), no differences in dominant ammonia-oxidising sequences were observed at this location. One sequence belonging to cluster 3 of the *Nitrosospira* lineage appeared to be dominant among the sequences belonging to the ammonia-oxidising species of the β-Proteobacteria in all vegetation zones. Nitrification was apparently inhibited by the vegetation, whereas no shift in nitrifier populations could be shown. The possible role of repressed nitrification in the decline of this vegetation type is discussed.

1. Introduction

The nutrient-poor grasslands on hill slopes in South Limburg are potentially one of the most species-rich ecosystems in the Netherlands (Willems, 2001; WallisDeVries et al., 2002). Up to the beginning of the twentieth century, they were abundant in the form of common land, grazed by sheep led by shepherds. From the beginning of the previous century management practice changed and a strong decline in number of patches and area of these species-rich grasslands occurred. In the 1980s sheep grazing was reintroduced in most of them. Over the past 25 years, the floristic biodiversity of the calcareous grasslands increased in response to these restoration measures, although full restoration has not been accomplished yet. However, the botanical quality of the matgrass sward vegetation decreased even further (Bobbink and Willems, 2001).

One factor that is thought to be important in causing biodiversity loss in European matgrass swards is nitrogen availability (e.g. Roelofs et al., 1996; Bobbink et al., 1998; Roem et al., 2002). Preliminary measurements of soil inorganic nitrogen concentrations in the above-mentioned grasslands showed increased ammonium to nitrate ratios in the matgrass sward vegetation, compared to the other vegetation types in this gradient. It is hypothesized that in this part of the vegetation gradient the process of nitrification is inhibited.

The process of nitrification, i.e. the oxidation of ammonium to nitrate, is performed by two physiologically different groups of bacteria. The group of ammonia-oxidising bacteria (AOB) and ammonia-oxidising archaea (AOA) converts ammonium to nitrite, which is then transformed by the group of nitrite-oxidising bacteria (NOB) to nitrate. For the onset of nitrification the characteristics of

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the group of AOB/AOA is of most importance. Potential nitrification activities had been measured before in calcareous grasslands from the same area and turned out to be significantly ($P < 0.01$) correlated with the Most Probable Numbers of ammonia-oxidising bacteria (Stienstra, 2000). Both parameters were negatively correlated with the time period that passed since the fertilization of the grasslands had been stopped. In addition, analysis of the AOB-related 16S rRNA genes from DNA isolated from the soils of these grasslands, showed a change in the community composition of β-proteobacterial ammonia oxidisers: A predominance of cluster 3 of the *Nitrosospira* lineage in early stages of succession after finishing fertilization was replaced by cluster 4 of the same linage of β-AOB in later stages (Kowalchuk et al., 2000). As mentioned above, the success of the restoration measures taken in the nutrient-poor grasslands on hill slopes in South Limburg aimed at improving floristic biodiversity, varied between the vegetation types in the gradient. In line with the observations of Stienstra (2000), it is hypothesized that the restoration measures produced different communities of ammonia-oxidising betaproteobacteria giving rise to different ammonium to nitrate ratios along the vegetation gradient.

To verify both hypotheses, which relates to negatively affected nitrification and a different community composition of the β-AOB in the matgrass sward vegetation as compared to both other vegetation types, the PAA was determined in nutrient-poor grasslands on slopes in five nature reserves. In addition, the dominant type of β-AOB was determined by molecular analysis for the different zones in the gradient of one of the nature reserves.

2. Materials and methods

2.1. Study site description

The studied grassland gradient only occurs in the most southern part of the Netherlands, in the province of Limburg. Between 100 and 150 m above sea level, we find loess and acid gravel deposits from the river Meuse on top of the calcareous substrate, resulting in a unique 30–60 m long vegetation gradient with from the top downwards acid grasslands (*Thero-Airion*, Schaminée et al., 1996) on typic dystrudept soils, via matgrass sward vegetation (*Nardo-Galion*, Schaminée et al., 1996) on dystric eutrudept soils to calcareous grassland (*Mesobromion*, Schaminée et al., 1996) on typic to rendollic eutrudept soils (Soil Survey Staff, 1999). Along the whole gradient (para)lithic contacts occurred. The acid grasslands are characterized by many annuals, e.g. *Rumex acetosella* and *Aira praecox*, next to different heathland moss and lichen species. The matgrass sward vegetation includes diagnostic species like *Danthonia decumbens*, *Stachys officinalis* and *Hieracium umbellatum*, and the calcareous grasslands contain typical species like *Sanguisorba minor*, *Briza media* and *Linum catharticum*. In the present study, data from five nature reserves are included: Bemelerberg (50°51′03″N, 5°46′05″E), Hoefijzer (50°50′58″N, 5°46′29″E), Zure dries (50°48′11″N, 5°44′46″E), Tiendeberg (50°48′34″N, 5°40′03″E) and Schiepersberg (50°49′55″N, 5°46′42″E). All of these five reserves contain a well developed grassland gradient or parts of it.

2.2. Soil sampling

For the chemical characteristics of the soil, mixed soil samples of the upper 10 cm (auger diameter 2 cm) were collected after removing the vegetation along the vegetation gradient at the Bemelerberg reserve in spring 2005, and at the Zure dries, Tiendeberg and Schiepersberg in spring 2007. The soil samples were stored at 4 °C and processed within 3 days. To determine pH and and NO_3-concentrations, we extracted soil (15 g) on a rotary shaker for 1 h (100 rpm) with 100 ml demineralised water, and for the measurement of NH_4-concentrations, 100 ml of a 0.2 M KCl-solution was used. The soil suspensions were centrifuged for 5 min at 4000 rpm. Supernatants were filtered through a Whatman GF/C-filter and stored at −20 °C until further analysis. Soil moisture was determined after drying at 105 °C for 48 h NH_4^+ and NO_3^--concentrations were determined colorimetrically using a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). Total inorganic nitrogen is defined as the sum of NH_4^+ and NO_3-concentrations.

The size of the active ammonia-oxidising microbial community was estimated by measuring the potential ammonia-oxidising activities in soil samples. Therefore, in each of the vegetation types along the gradient six soil samples were taken at the Bemelerberg and Hoefijzer (spring 2005). In spring 2007 an extra set of data was added, by sampling ten soil samples along the vegetation gradients at the Zure dries, Tiendeberg and Schiepersberg. The soil samples of the five nature reserves ($n = 66$) were collected and processed according to the procedure described above for the determination of pH at the start of the potential measurements. To determine the total N, a destructive soil analysis was carried out. A dried, ground and sieved (sieve size 0.2 mm) soil sample is weighed into tin foil containers (around 3 mg). The containers are burnt with oxygen (dry combustion) in a stream of Helium gas, with Cr_2O_3 as a catalyst (1050 °C). Components are measured by a thermal conductivity detector TCD (Pella and Colombo, 1973).

2.3. Potential ammonia-oxidising activities

Potential ammonia-oxidising activities (PAA) as estimation of numbers of actively ammonia-oxidising cells were determined in 250 ml Erlenmeyer flasks containing slurries of 15 g fresh, sieved (4 mm) soil in 100 ml buffered medium with 2 mM $(NH_4)_2SO_4$. The buffer was composed of 2 mM phosphate buffer (an equimolar mixture of KH_2PO_4 and K_2HPO_4, adapted to the prevailing soil pH), because pilot experiments had shown that the potential ammonia-oxidising activities in samples from the calcareous grassland and from the acid grassland were affected by the pH value of the slurries in which the potential activities were measured. For the calcareous grassland relatively high values were obtained at neutral pH, whereas for the acid grassland potential activities were repressed by pH 7 compared to pH 4 (data not shown). Hence, potential ammonia-oxidising activities were determined at pH values comparable with the original soil pH. During the activity measurements, the slurries were permanently shaken on a rotary shaker (RO 20, Gerhardt, Bonn, Germany; 100 rpm) in the dark at a temperature of 27 °C. Subsamples of 3 ml were taken at $t = 0$, 2, 4, 6, 21, 27, 51, 74, 98, 122 and 146 h, and centrifuged for 5 min at 13,000 rpm (Biofuge pico, Heraeus Instruments, South Plainfield, USA), decanted, and frozen (−18 °C) till analysed. At each sampling time, the pH of the incubation medium was checked, and restored to its original value with 0.1 N NaOH or 0.1 N HCl, if necessary. Concentrations of nitrate plus nitrite were measured on a continuous flow analyser (Skalar 40, Skalar Analytical BV, Breda, the Netherlands). Potential ammonia-oxidising activities were calculated from the changes in NO_2- plus NO_3-concentrations in time, using linear regressions. The slope of the regression lines was used as a measure for the potential ammonia-oxidising activity.

2.4. Data analysis

The measurements of the chemical characteristics of the soil samples were analysed with ANOVA (SPSS 15.01) for soil pH, and with non-parametric tests (Kruskal–Wallis) for soil moisture

content, nitrate, ammonium, ammonium to nitrate ratio, total inorganic N, and total N. Post-hoc Tukey-tests were applied to distinguish between the three different vegetation types.

In the samples that were used to measure potential ammonia-oxidising activities, the interaction between the different vegetation types and the pH was tested with a one-way ANOVA procedure and a post-hoc Tukey test (SPSS 15.01). The interaction between the different vegetation types and the potential ammonia-oxidising activity was tested with a non-parametric test (Kruskal–Wallis, SPSS 15.01) and a post-hoc Tukey test.

2.5. DNA extraction and purification

To obtain an indication of the possible role of the community composition of the ammonia-oxidising bacteria in the accumulation of ammonium in some of the vegetation zones and for comparison with former analyses (Kowalchuk et al., 2000), DNA was extracted from 0.5 g dried soil with the mechanical disruption protocol described by Henckel et al. (1999) from 18 samples of the Bemelerberg nature reserve. Subsequently, the extracted DNA was purified with a commercial purification kit (Wizard® DNA Clean-up system, Promega Corporation, USA) according to the manufacturer's recommendations. Purified DNA was resuspended in deionised water. Extraction and purification were verified by electrophoresis of 5 μl of the DNA solution in 1.2% agarose gel and 0.5× TBE buffer (5× Tris–Borate: 54 g Tris base, 27.5 boric acid, 20 ml 0.5 M EDTA) and then visualised by ethidium bromide fluorescence.

2.6. PCR amplification of β-proteobacterial 16S rRNA genes

PCR fragments of the 16S rRNA gene of the β-subclass of the ammonia-oxidising Proteobacteria were generated with the βAMO161f–βAMO1301r primer set (McCaig et al., 1994). These PCR fragments were used as a template for a nested PCR using the CTO primer set as described by Kowalchuk et al. (1997). This second primer set amplified an approximately 465 bp fragment. In this paper we refer to this fragment as the CTO fragment. The primers are degenerated with specificity and sensitivity clearly defined (Kowalchuk et al., 1997, 1999; Koops et al., 2006). PCR amplification was performed in a total volume of 25 μl containing 1× PCR Mg-free buffer, 200 μM of each deoxynucleotide, 1.75 mM $MgCl_2$, one units of *Taq* DNA Polymerase (Invitrogen, Tech-Line USA), 400 ng μl^{-1} BSA (purified bovine serum albumin, New England BioLabs, Beverly, MA, USA), 0.5 μM of each primer, and 2.5 μl of 1:100 diluted βAMO161f-βAMO1301r PCR fragment as template. Reactions were performed in a Multiblock Thermocycler System (Thermo Electron, USA) according to the following program: 3 min denaturation at 94 °C; 35 cycles of: 30 s denaturation at 92 °C, 60 s annealing at 57 °C and 45 s elongation at 72 °C; the last step was 5 min elongation at 72 °C. All the amplification reactions were verified loading 5 μl amplification products in 1.2% agarose gel, separated by electrophoresis in 0.5× TBE, stained in ethidium bromide solution and visualised with UV.

2.7. Denaturing gradient gel electrophoresis (DGGE) analyses

Approximately 200–300 ng of PCR products were separated by DGGE with a Protean II system (Bio-Rad, USA) according to the protocol of Kowalchuk et al. (1997) for the study of ammonia-oxidising bacteria. The denaturant gradient to separate the nested CTO fragments was 35–55%, with a solution of 8% acrylamide, 7 M urea and 40% formamide defined as 100% denaturing. Gels were run for 17 h at 60 °C in 0.5× TAE buffer (50× Tris–acetate: 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA per litre).

2.8. Sequencing and phylogenetic analyses

From the denaturing gradient gels, selected bands were cut out from the middle parts. In case the DGGE pattern showed only one band, the corresponding band from the original agarose gel that contained a longer fragment with base pairs was cut and used for further analysis. The acrylamide pieces were stored in water overnight at 4 °C for the elution of PCR fragments (Bollmann and Laanbroek, 2002). Bands were used 1:10 diluted as template for a further 25 cycles PCR. Obtained PCR products were separated again with DGGE to confirm recovery of the desired single bands (Zwart et al., 1998). Sequencing reactions were performed with the BigDye Terminator V 3.1. Cycle Sequencing kit (Applied Biosystems, USA) and run on an Applied Biosystems 3130 capillary sequencer. Sequences obtained from DGGE bands were (http://www.ncbi.nml.nih.gov/BLAST) and subsequently aligned with published 16S rRNA gene sequences of cultured ammonia-oxidising bacteria, by means of the fast aligner tool of the ARB software and added to the related neighbour-joining trees by using the parsimony criterion and ad hoc created filters (Ludwig et al., 2004). Alignment of the sequences obtained from the DGGE bands have been done with another fragment of the 16S rRNA gene, i.e. with nucleotides from positions 210 to 506 of the 16S rRNA gene of *Escherichia coli*.

3. Results

3.1. Chemical characteristics of the soil

The soil pH (H_2O) of the different vegetation types was significantly different between all groups and was on average 5.08 for the acid grassland, 5.59 for the matgrass sward vegetation, and 7.69 for the calcareous grasslands (Table 1), whereas the soil moisture content did not show significant differences. The ammonium concentration was decreasing along the research gradient, with lowest values in samples from calcareous grassland, whereas the

Table 1
Summary of the soil characteristics from the different vegetation types collected in spring in the nature reserves Bemelerberg (2005), Hoefijzer (2005), Schiepersberg (2007), Tiendeberg (2007) and Zure dries (2007). Units of measurement for $N–NH_4$, $N–NO_3$, and total inorganic nitrogen are mg kg^{-1} dry soil. Soil moisture content is calculated as (fresh soil-dried soil)/dried soil. Total N is measured as a percentage of the total soil. The standard deviation is between brackets, and significant differences between the vegetation types are indicated by different letters ($P < 0.05$). With asterisks the significance level is indicated: *** = $P < 0.001$; ** = $0.001 > P \leq 0.01$; * = $0.01 > P < 0.05$; n.s. = not significant.

	Acid grassland	Matgrass sward	Calcareous grassland	Statistics
pH_{demi}	5.08 (0.28)a	5.59 (0.49)b	7.69 (0.53)c	$F = 213.50$, $P < 0.000$***
Moisture content	0.26 (0.10)a	0.34 (0.12)a	0.32 (0.09)a	$\chi^2 = 4.00$, $P = 0.135$ n.s.
N in $NO_{3\ demi}$	3.41 (3.84)ab	2.27 (1.95)a	6.26 (3.55)b	$\chi^2 = 7.72$, $P = 0.021$*
N in $NH_{4\ KCl}$	5.27 (4.35)a	4.47 (2.54)a	3.79 (2.82)b	$\chi^2 = 14.30$, $P = 0.001$**
Ammonium:nitrate ratio	2.75 (2.20)ab	4.44 (4.64)a	1.00 (1.41)b	$\chi^2 = 11.58$, $P = 0.003$**
Total inorganic N	8.69 (8.17)a	6.74 (3.01)a	8.02 (3.82)a	$\chi^2 = 1.00$, $P = 0.607$ n.s.
Total N (%)	0.37 (0.06)a	0.40 (0.10)a	0.36 (0.11)a	$\chi^2 = 1.51$, $P = 0.471$ n.s.

lowest amount of nitrate was present in the samples from matgrass sward vegetation. The ammonium:nitrate ratio was 4.44 in the middle (matgrass sward) zone and thus considerably higher than in the neighbouring vegetation types (2.75 & 1.0; Table 1). Total quantities of inorganic nitrogen or total nitrogen percentages did not reveal any differences along the vegetation gradient.

3.2. Potential ammonia-oxidising activities

Within the five reserves, significant lower activities ($P < 0.001$) were encountered in the matgrass sward vegetation (on average 0.1 mg l^{-1} h^{-1}, Fig. 1), when compared to the relatively high potential ammonia-oxidising activities in the acid grassland and in the calcareous grassland (on average 1.3 and 1.5 mg l^{-1} h^{-1}, respectively). The difference in potential ammonia-oxidising activities between the acid and the calcareous grassland was not significant at the 0.05-level. The same significant differences were found when analysing the data from the nature reserve Bemelerberg separately (data not shown). The soil pH (H_2O) of the different vegetation types of the Bemelerberg was again significantly different between all groups and was on average 5.07 for the acid grassland, 5.49 for the matgrass sward vegetation, and 7.61 for the calcareous grassland.

3.3. Diversity of ammonia-oxidising microbial communities

The diversity of ammonia-oxidising microbial communities was established by a nested PCR – DGGE approach for the vegetation zones from the nature reserve Bemelerberg. On the basis of the 16S rRNA gene, no major differences were observed between or within the vegetation types (Fig. 2). All soil samples were dominated by one band, which on the basis of a BLAST analysis of its 553 nucleotides, was most closely related (98% identity) to *Nitrosospira* sp. Nsp2 (Bbband 9; Fig. 3). In all samples, and irrespective of the vegetation type, faint bands accompanied this dominant band. A selection of six faint bands from different locations in the gel was cut from the gel and reamplified. Only two of them turned out to belong to ammonia-oxidising bacteria of the β-subclass of the Proteobacteria. On the basis of a sequence of 402 nucleotides, one of these minor bands was mostly related (97% identity) to *Nitrosospira briensis* (Bbband 2; Fig. 3). This band occurred faintly in most samples and as a subdominant band at one of the matgrass sward samples. The second faint band that belongs to the β-subclass of the ammonia-oxidising bacteria occurred only in a few samples, but apparently again irrespective of the type of vegetation. This latter band was mostly related (99% identity) to *Nitrosospira* sp. Nsp17 (Bbband 17; Fig. 3) on the basis of a sequence of 455 base pairs. All other faint bands were either not reamplifiable after cutting or migrated to the same height of the dominant Bbband 9 in the case of the faint bands in lanes 14 and 16.

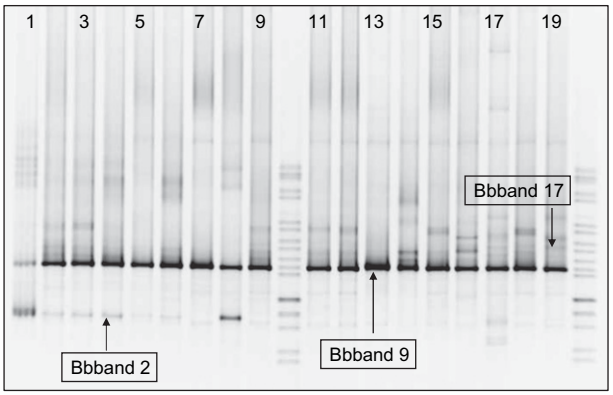

Fig. 2. DGGE-banding pattern of ammonia-oxidising bacteria of the β-subclass of the Proteobacteria obtained by nested PCR from DNA isolated from soil samples from Bemelerberg nature reserve. Lanes 1–6 represent samples from the acid grassland; lanes 7–9 and 11–13 samples from the matgrass sward vegetation; lanes 14–19 samples from the calcareous grassland; lanes 10 and 20 are marker lanes. Indicated are the true bands belonging to the β-subclass of the ammonia-oxidising Proteobacteria. The two bands above Bbband 9 in lanes 14 and 16 migrated at the height of BBband 9 after cutting and reamplification. All other bands were not reamplifiable after cutting.

The dominant sequence of ammonia-oxidising bacteria encountered in all samples belongs to cluster 3 of the genus *Nitrosospira* (Purkhold et al., 2003). The two other subdominant sequences of this functional group (*Nitrosospira* sp. Nsp17 and *N. briensis*) also belong to this cluster. The dominant *Nitrosospira* sp. Nsp2 and the subdominant *Nitrosospira* sp. Nsp17 are even closely related to each other according to Purkhold et al. (2003). Originally these strains have been isolated from German and Icelandic soils, respectively (cf. Purkhold et al., 2003).

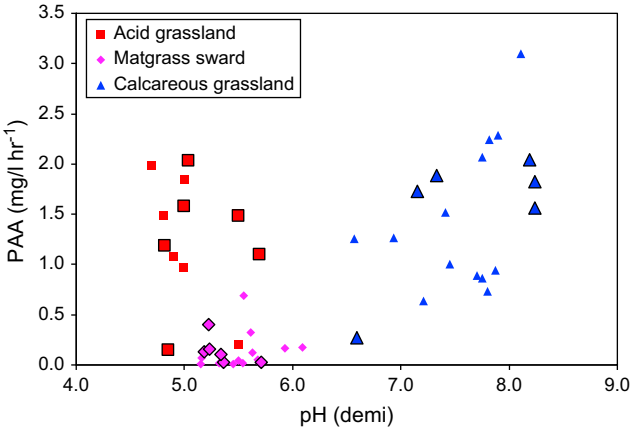

Fig. 1. Potential Ammonia-oxidising Activities (mg l^{-1} h^{-1}) measured in samples from the different vegetation types at the nature reserves of Bemelerberg, Hoefijzer, Schiepersberg, Tiendeberg and Zure dries. The pH (demi) represent the soil pH after extraction with demineralised water for 1 h. The bigger symbols indicate samples from the Bemelerberg that were used in the DNA-analysis. The average R^2 of the linear regressions used for the calculation of the potential activities was 0.98 for the acid grassland samples (s.d. 0.04), 0.91 for the matgrass sward samples (s.d. 0.12), and 0.99 for the calcareous grassland samples (s.d. 0.01).

4. Discussion

We identified clear differences in the potential ammonia-oxidising activities of the soil samples from the different vegetation zones. Hence, the first hypothesis that increased ammonium to nitrate ratios in the matgrass sward vegetation were due to repressed nitrification was supported by the results obtained with the measurements of potential ammonia-oxidising activities. The values measured in calcareous grassland were of the same order as those determined by Stienstra (2000) for earlier stages of succession in calcareous grasslands from the region. The observed repressed activity of the ammonia-oxidising community in matgrass sward vegetation was not due to a lack of ammonium neither in the field nor during the potential rate measurements, as this was provided in excess during the activity measurements. The pH also does not seem to limit the activity of the ammonia-oxidising bacteria, because at the samples from acid grassland with an even lower pH, nitrification occurred. The findings of the present study are in contradiction with general assumptions that nitrification is rapid in soils with a pH $\geq$ 6 and slower in soils with a pH $\leq$ 5 (Subbarao et al., 2006a). This difference in potential activity, which points to differences in numbers of active, ammonia-oxidising cells, was not reflected by differences in the bacterial community

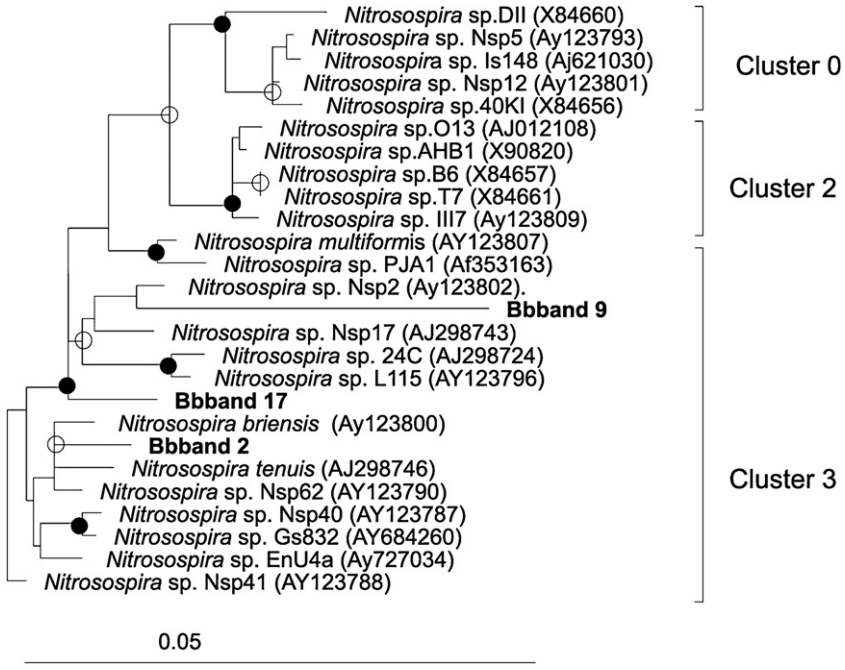

Fig. 3. 16S rRNA-based neighbour-joining phylogenetic tree of the ammonia-oxidising *Nitrosospira* lineage of the β-subclass of the Proteobacteria (Purkhold et al., 2003). Bands retrieved in this study are marked in bold. Scale bar indicate 5% estimated sequence divergence. Boot trap values ≥90% or ≥70% are indicated in the tree by closed and open circles, respectively.

composition. In all three vegetation types, species with 16S rRNA-based sequences that were mostly related to *Nitrosospira* sp. Nsp2 were the dominant ammonia-oxidising species. Hence, we have to reject the hypotheses that different ammonia to nitrate ratios observed in the matgrass sward vegetation were due to different communities of ammonia-oxidising β-Proteobacteria.

In an earlier study in calcareous grasslands in the Province of Limburg, the Netherlands, a shift from cluster 3 of the *Nitrosospira* lineage in early succession fields to cluster 4 of the same lineage of β-AOB in climax vegetation types has been observed (Kowalchuk et al., 2000). These authors proposed that ammonium availability, which was lower in the climax vegetation, may be a steering force affecting the ammonia-oxidising microbial community in grassland soils. As the current research sites have never been fertilised, except for atmospheric N deposition, and the vegetation in these grasslands has been relatively persistent at least since the beginning of the 20th century, our observations seem to correspond more with those of Horz et al. (2004), who found that increased nitrogen deposition altered the structure of the ammonia-oxidising bacterial community towards a community dominated by bacteria most closely related to *Nitrosospira* sp. Nsp2, which is also the dominant β-AOB at our locations and related to cluster 3 of the *Nitrosospira* lineage.

Changes in microbial diversity and community structure might have large-scale consequences for ecosystem functioning and underlying processes (Schimel, 1995; Schimel et al., 2005). In our case, however, environmental conditions, i.e. the presence of specific vegetation and associated habitat conditions, affected the activity of the ammonia-oxidising bacterial community, but not its composition. An explanation for the observed difference in behaviour between the potential ammonia-oxidising activities and the community composition of the ammonia-oxidising Proteobacteria in our soil samples as observed in the Bemelerberg nature reserve may be related to the presence and activity of ammonia-oxidising archaea. Copy numbers of the functional gene for ammonia oxidation in archaea, the so-called archaeal *amoA* gene, might be two orders of magnitude larger in pristine grasslands than the copy numbers of the bacterial *amoA* gene (Leininger et al., 2006). Furthermore, reverse transcription quantitative PCR studies and complementary DNA-analysis using novel cloning-independent pyrosequencing technology applied by these authors demonstrated the activity of the archaea *in situ* and supported the numerical dominance of archaeal over bacterial ammonia oxidisers. In a series of agricultural soils in which pH had been maintained at distinct pH values of 4.5–7.5 for 35 years, Nicol et al. (2008) observed an increased number of copies of the archaeal *amoA* gene with decreasing pH, whereas the copy number of the bacterial *amoA* gene remained more or less the same. At pH 5.3–5.9, the authors observed a two orders of magnitude difference between the *amoA* gene copy numbers of archaea and bacteria, while the *amoA* gene transcript copies were almost three orders of magnitude larger for the archaea. In non-fertilised French grassland soils managed by sheep grazing or mowing, copy numbers of the 16S rRNA gene were of the same order of magnitude for ammonia-oxidising bacteria and archaea (Le Roux et al., 2008). However, based on assumed lower transformation rates for ammonium into nitrite by AOA compared to AOB (Könneke et al., 2005), Le Roux et al. (2008) postulated that the major part of nitrification in the investigated grassland soils was related to AOB activity. Notwithstanding these considerations, we cannot not exclude that active AOA have been responsible for the observed disagreement between potential ammonia-oxidising rates and community compositions as determined for the different parts of our vegetation gradient.

A possible explanation for the observed repression of potential nitrification activity in the matgrass sward vegetation is the mechanism of repression by allelopathic compounds, which has been proposed by Rice and Pancholy (1972). The authors, however, did not provide adequate mineralization data to rule out ammonium shortage as an alternative explanation and also failed to provide *in situ* evidence for plant involvement in nitrification inhibition. Exudation of toxic compounds by plants, produced particularly by plant species from climax vegetation is not

conclusively demonstrated in the field (cf. De Boer et al., 1990). Nevertheless, biological nitrification inhibition has recently been demonstrated by several authors. Exudates from the roots of the grass *Brachiaria humidicola* repressed nitrification (Subbarao et al., 2006b). Furthermore, it was demonstrated in *B. humidicola* that ammonium stimulates the synthesis and release of inhibiting compounds (Subbarao et al., 2007a,b). Grasslands dominated by *Andropogon* contained mostly ammonium, whereas nitrate nearly disappeared as the grassland matured (Lata et al., 1999). Lata et al. (2004) also demonstrated that nitrification can be suppressed or stimulated depending on the ecotype of *Hypparhenia diplandra*. Finally, Zakir et al. (2008) found an increasing effect of plant age and ammonium on the release of nitrification inhibiting compounds by root exudates of *Sorghum bicolor*. As far as we know, such an effect involving natural vegetation has not been published.

Repression of nitrification by plant species of the matgrass sward vegetation could be an adaption to the nutrient-poor circumstances of these grasslands to conserve and use N efficiently, by maintaining inorganic nitrogen in the less mobile form of ammonium and thus preventing it from leaching as nitrate (Vitousek et al., 2002; Herrmann et al., 2005). However, the acid-sensitive species of the matgrass sward vegetation are sensitive to high soil ammonium concentrations, especially in combination with low soil pH (Van den Berg et al., 2005; Dorland et al., 2003; Lucassen et al., 2002; De Graaf et al., 1998). Hence, if plants from the matgrass sward vegetation indeed inhibit ammonium oxidation by the release of allelopathic compounds, then a negative feedback could exist, leading to a decline of this vegetation type. Atmospheric deposition of reduced N (ammonium or ammonia) will increase the rate of this decline as ammonium would not be oxidised to nitrate by the ammonia-oxidising microorganisms and thus not leach to the groundwater. This will lead to a strong dominance of ammonium supply in this vegetation type. Studies are underway to evaluate plant species control of the nitrification process.

5. Conclusions

As hypothesized, the high ammonium to nitrate ratios observed in the matgrass sward part of the vegetation gradient along hillslope grasslands could be ascribed to a repressed nitrification. However, the observed differences in potential ammonia oxidation rates were not related to differences in the composition of the community of ammonia-oxidising β-Proteobacteria. Members of the third cluster of the *Nitrosospira* lineage dominated in all soils independent on the prevailing ammonium to nitrate ratio.

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