Presence of *Nitrosospira* cluster 2 bacteria corresponds to N transformation rates in nine acid Scots pine forest soils

R.A. Nugroho a,*, W.F.M. Röling b, A.M. Laverman c, H.R. Zoomer a, H.A. Verhoef a

a Institute of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

b Department of Molecular Cell Physiology, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

c Department of Geochemistry, Faculty of Earth Sciences, Universiteit Utrecht, Budapestlaan 4, 3584 CD Utrecht, The Netherlands

Received 7 October 2004; received in revised form 29 November 2004; accepted 7 February 2005

First published online 2 March 2005

Abstract

The relation between environmental factors and the presence of ammonia-oxidising bacteria (AOB), and its consequences for the N transformation rates were investigated in nine Scots pine (*Pinus sylvestris* L.) forest soils. In general, the diversity in AOB appears to be strikingly low compared to other ecosystems. *Nitrosospira* cluster 2, as determined by temporal temperature gradient electrophoresis and sequencing, was the only sequence cluster detected in the five soils with high nitrification rates. In the four soils with low nitrification rates, AOB-like sequences could not be detected. Differences in nitrification rates between the forest soils correlated to soil C/N ratio (or total N) and atmospheric N deposition.

© 2005 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Nitrification; Ammonia-oxidising bacteria; Acid Scots pine forests; Environmental factors

1. Introduction

Nitrification is carried out in most ecosystems by autotrophic bacteria and this process is often considered rate limited by the activities of ammonia-oxidising bacteria (AOB). These bacteria are responsible for the oxidation of ammonia to nitrite by the enzymes ammonia monooxygenase and hydroxylamine oxidoreductase [1,2].

All known terrestrial AOB to date belong to a monophyletic assemblage of *Nitrosospira* and *Nitrosomonas* in the β-subdivision Proteobacteria [2,3]. This assemblage originally consists of seven 16S rRNA gene sequence clusters [3]. Koops et al. [4] have expanded the subdivision of AOB by including environmentally retrieved 16S rRNA gene sequences. Molecular analysis revealed that *Nitrosospira* cluster 2 dominated in acid soils [3,5,6]. *Nitrosospira* cluster 3 and *Nitrosomonas* cluster 7 were major AOB in tilled and fertilised soils [7–9], while *Nitrosospira* cluster 0 dominated under undisturbed and unfertilised grassland [9,10]. Generalisation regarding the relationship between clusters and environmental variables cannot be made as other 16S rRNA gene (rDNA)-based studies showed that *Nitrosomonas europaea* cluster 7 can also be present in acid soils [11] and in unfertilised grasslands [9], and *Nitrosospira* cluster 3 in both fertilised and unfertilised grassland [9]. Therefore, it remains unclear how the composition of AOB communities varies due to different environmental factors.

Our aim is to improve the understanding on the relation between the presence of AOB and environmental
factors in acid Scots pine forest soils and the consequences for the in situ N transformation process. We, therefore, (i) quantified the N transformation rates, i.e. net mineralisation, net nitrification and net ammonification rates in nine Scots pine forest soils with different NH$_4^+$--N concentrations; (ii) established correlations between soil properties, i.e. total C, total N, C/N ratio, Ca content, pH, initial NO$_3^-$--N and NH$_4^+$--N concentrations and N transformation rates and (iii) tested the hypothesis that soils with different NH$_4^+$--N concentrations contain different autotrophic AOB and that this difference correlates to N transformation rates.

A culture-independent method was used to examine the presence of AOB in nine acid Scots pine forest soils that have been subjected to different levels of N deposition for many years. Molecular biological approaches have significant advantages for analysis of in situ AOB communities [12], as analysis can be carried out without enrichment and isolation of pure cultures.

2. Materials and methods

2.1. Field site and forest floor collection

Eight sites located in the Netherlands and one site in Finland (Table 1) were chosen to represent a large range in NH$_4^+$--N concentrations. The forest floor (fragmentation (F) layer) of Scots pine (Pinus sylvestris L.) stand utilized in this study was collected in June 2002. At each site, ten samples (15 × 20 cm) of the F layer were collected randomly from an area of approximately 5 × 5 m, then pooled in a clean plastic bag and returned to the laboratory. Pooling was necessary to make the soil more homogeneous. The field-moist soils were immediately passed through a 4-mm sieve in the laboratory and homogenized by hand. Sieving through 2-mm sieves has hardly any effect on mineralisation [13]. Sub-samples of 30 g of the sieved material were stored at −20°C until molecular analysis (Section 2.4). The remaining sieved material was kept at 5°C, to minimise changes in initial conditions across the soils, for less than a week until further analysis on soil characteristics (Section 2.2) and N transformation rates (Section 2.3). In this study, the F layer was chosen, as in this layer, compared to the litter (L) layer and mineral soil, less spatial variation was observed in mineralisation and nitrification rates and temporal variation in these rates was not significant [14]. In addition to that, denitrifying capacity was lowest in the F layer [6].

2.2. Soil characteristics analysis

Sub-samples of the sieved material were analysed for total N and total C on a Carlo Erba Strumentazione elemental analyser (model 1106) and Ca content on AAS...
(Perkin Elmer, model 1100B). Moisture contents of the soils were determined by drying fresh samples at 50 °C for 3 days. Other sub-samples were extracted with 1 M KCl (15 g field-moist soil:100 ml of 1 M KCl) on a shaker at 200 × g for 1.5 h and filtered through filter paper (Schleicher and Schuell, 595.5). Extractable NH$_4^+$-N and NO$_3^-$-N concentrations were measured on an autoanalyzer (Skalar SA-40), and pH was measured using a Consort P907 pH meter.

2.3. N transformation rate determination

N transformation rates were determined using the method described by Laverman et al. [14]. Before the N transformations experiment, sub-samples for incubation were brought to 65% moisture content by adding demineralised water. Two 250-ml screw-cap bottles were filled with 15 g fresh material each. One bottle was capped with a regular cap and the other with a septum-contained cap. Acetylene 0.1% (vol/vol) was added to a septum-capped bottle and was present throughout the incubation time as acetylene was still detectable in the headspace at the end of the incubation (data not shown). Acetylene was purified by passage through 5 N H$_2$SO$_4$, 5 N NaOH, and water [15]. Both bottles were incubated for three weeks at 25 °C in the dark. This assay was done in triplicate for each soil. At the end of the incubation, 1 M KCl-extractable NH$_4^+$-N and NO$_3^-$-N concentrations were measured in an autoanalyzer. Concentrations of extractable NO$_3^-$-N in soil at time zero and after three weeks were used to calculate net nitrification rate. The net ammonification rate and net N mineralization rate were calculated in the same manner; subtracting initial concentrations of NH$_4^+$-N and (NH$_4^+$ + NO$_3^-$-N), respectively, from those measured at the end of the incubation. All N transformations were expressed on dry weight basis. The percent contribution of net ammonification and nitrification rates to net mineralization rate were calculated as net ammonification and nitrification rates divided by net mineralization rate, respectively.

2.4. DNA extraction from soil and nested PCR

DNA was extracted from approximately 0.3 g sub-samples of soil (at field moisture content) using the FastDNA® SPIN Kit for Soil (FastPrep® FP120 Cell Disrupter). The extracted DNA was cleaned three times with the Wizard DNA clean-up system (Promega, Madison, WI, USA). Independent DNA extractions were done in triplicate. A nested PCR was then carried out for all samples. Universal bacterial-specific 16S rDNA directed primers pAf/pHr [16] were used in the primary amplification. PCR products were amplified from each DNA preparation in 50 μl reactions, 400 nM pAf/pHr, 0.2 mM dNTPs, 10 μg BSA, Taq DNA polymerase (2.5 unit, Promega), the buffer conditions recommended by the manufacturer, and 5 μl of each soil DNA extraction as template source. Reactions were carried out in a T3 thermocycler (Whatman Biometra®). The PCR thermocycling regime was as follows: 3 min at 95 °C, followed by 30 cycles consisting of 1 min at 95 °C, 1 min at 55 °C, and 1 min at 72 °C and a final cycle consisting of 10 min at 72 °C. The PCR products were diluted 100 times and used as template in the second amplification using AOB specific primers, CTO189f-GC/CTO654r [12]. The secondary amplifications used the same reaction volume, reagent concentrations and buffer conditions as described for the primer set of pAf/pHr. The PCR amplifications were performed at 95 °C for 3 min after which 30 cycles were performed at 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and a final cycle consisting of 10 min at 72 °C. PCR products were examined by agarose gel electrophoresis.

2.5. TTGE analysis, cloning and sequencing

The PCR products were then profiled on TTGE using a Biorad Protein II Electrophoresis Unit (BIO-RAD Dcode™ systems, Hercules, CA, USA) as described by Laverman et al. [6]. Gels (8% polyacrylamide, 1× TAE, 37.5:1 acrylamide: bisacrylamide, 8 M ureum) were run for 16 h at 60 V over temperature range 59.6–64.4 °C, increasing at 0.3 °C h$^{-1}$. Laverman et al. [6] have compared TTGE and DGGE profiling of AOB and found that TTGE banding patterns are similar to those produced by DGGE. TTGE gels were stained with ethidium bromide, washed in distilled water and digitally recorded using the Imager™ system (Appligene, Inc., Illkirch, France). To optimise the sensitivity of DNA detection, we also stained TTGE gel using SYBR gold, one of the most sensitive dyes, and the results were identical to those used ethidium bromide stain (data not shown). Independent DNA extractions and PCRs on the same soil sample revealed similar TTGE patterns (data not shown).

To determine the nucleotide sequence of TTGE bands, the centre of the band was cut out and incubated overnight in 50 μl TE at 4 °C. This solution was used for PCR with primers CTO189f/CTO654r. The products were cleaned using Wizard PCR prep. (Promega, Madison, WI, USA), ligated to the pGEM-T vector, and transformed into Escherichia coli JM109 competent cells (Promega, Madison, WI, USA) as specified by the manufacturer. Transformed colonies were screened for inserts of the correct size by PCR amplification with CTO primer set. The selected clones were amplified with vector primer M13f (Promega, Madison, WI, USA) and analysed on an ALF Express II (Amersham Pharmacia Biotech, Uppsala, Sweden) as described by the manufacturer. The nucleotide sequence of the selected clones was determined using the T7 sequencing kit (Invitrogen).
Biotech.) according to the manufacturer’s instruction. These selected clones were tested for correspondence with the original TTGE bands to make sure that the sequences were the target bands.

2.6. Data analysis

Non-parametric tests were used as homogeneity of variances of the data was not ensured. Mann–Whitney U non-parametric tests were used to analyse differences in environmental factors and N transformation rates between two groups of sites, one group containing all sites that showed the presence of a particular 16S rRNA sequence, the other group containing the sites that did not show this sequence. Correlations between net nitrification rate, net ammonification rate and environmental factors were determined using Spearman’s correlations. All statistical analyses were done using SPSS 11.5 for Windows.

Recovered 16S rDNA sequences were compared to sequences deposited in the GenBank DNA database by using BLAST algorithm [17]. Sequence alignments were performed using ClustalW Multiple sequence alignment program version 1.8 (http://clustalw.genome.jp). Only nucleotide positions that were unambiguously aligned were used in the subsequent phylogenetic analysis. Distance analysis of 427 nucleotide positions of the alignment was performed with Treecon version 1.3b software [18]. Gaps were not taken into account in the analysis. The bootstrap analysis was based on 100 replicates.

2.7. Nucleotide sequence accession numbers

The nucleotide sequences have been deposited in GenBank under accession numbers AY683621–AY683629.

3. Results

3.1. Study sites properties

Table 1 shows the soil characteristics of all study sites. The chosen sites show a large range in initial \( \text{NH}_4^+ \)-N, \( \text{NO}_3^- \)-N and Ca concentrations, total C, total N and pH. \( \text{NH}_4^+ \)-N was the predominant form of inorganic-N, and \( \text{NO}_3^- \)-N concentrations were very low in Schoorl, Nastola, Bentveld and Appelscha, accounting for less than 6% of inorganic N. In the other sites \( \text{NO}_3^- \)-N concentrations were much greater, accounting for 29–38% of inorganic N. Comparisons across all sites revealed that soil from Nastola has the lowest values of the initial \( \text{NH}_4^+ \)-N and \( \text{NO}_3^- \)-N contents, total C and total N compared to other soils, while other sites have shown no consistent trends concerning within site soil properties.

3.2. N transformation rates

The N transformation rates in nine forest soils after 3 weeks of incubation are shown in Fig. 1. At all sites, acetylene treatment resulted in a complete inhibition of net nitrification rate, suggesting that autotrophic nitrification was the dominant process. Concerning the net nitrification rate (Fig. 1(a)), the rates in Schoorl, Nastola, Bentveld and Appelscha were relatively low, less than 0.5 \( \mu \text{g g}^{-1} \text{ dry soil wk}^{-1} \). Error bars are standard deviations. The abbreviations on the x-axis represent the following sites: Sc, Schoorl; Na, Nastola; Be, Bentveld; Ap, Appelscha; Ys, Ysselsteyn; Hu, Huishorst; We, Wekerom; Ro, Roggebotzand; As, Asbak.
prises soils from Roggebotzand and Asbak (29.4–31.6 μg g⁻¹ dry soil wk⁻¹).

Net ammonification rates differed between sites (Fig. 1(b)). The lowest net ammonification rates were in Roggebotzand and Asbak (2.6–3.7 μg g⁻¹ dry soil wk⁻¹). Nastola has a higher rate (8.0 μg g⁻¹ dry soil wk⁻¹) than Roggebotzand and Asbak, while Schoorl, Ysselsteyn, Hulshorst and Wekerom have higher (a factor three) rates than Nastola. The highest rates were found in Bentveld and Appelscha (35.0–37.3 μg g⁻¹ dry soil wk⁻¹).

Furthermore, net mineralization rates in all soils were comparable with the exception of soils from Schoorl and Nastola (Fig. 1(c)). The net mineralization rates in Schoorl and Nastola were lower than other in sites, 22.1 and 8.2 μg g⁻¹ dry soil wk⁻¹, respectively. Moreover, the percent contribution of ammonification and nitrification to net mineralization rates in Schoorl, Nastola, Bentveld and Appelscha are around 99% and 1%, respectively. The percent contribution of these two processes to net mineralization rates in Schoorl, Nastola, Bentveld and Appelscha are around 9% and 91%, respectively.

3.3. Correlation between soil properties and N transformation rates

Table 2 shows Spearman’s correlations between different soil properties. Positive correlations were found between pH and Ca content, initial NO₃⁻N concentration and total N, N deposition rate and total N, and N deposition rate and initial NO₃⁻N concentration. Negative correlations were found between C/N ratio and total N, Ca content and total C, Ca content and total N, pH and total C, initial NO₃⁻N concentration and C/N ratio, and N deposition rate and C/N ratio.

A weak negative correlation was observed between net ammonification rate and net nitrification rate. Net mineralisation was positively correlated to net ammonification rate. Moreover, these rates were also correlated to some soil properties (Table 2). Net nitrification rate was negatively correlated to C/N ratio, and positively correlated to total N, initial NO₃⁻N concentration and N deposition rate. Net ammonification rate was negatively correlated to Ca content and pH. Net mineralisation rate was positively correlated to total N and N deposition rate, and negatively correlated to C/N ratio, Ca content and initial NH₄⁺-N concentration.

3.4. β-subdivision AOB-like 16S rDNA

Fig. 2 shows that very similar AOB TTGE banding patterns were recovered for soil samples from Ysselsteyn, Hulshorst, Wekerom, Roggebotzand and Asbak (Fig. 2). These doublet bands co-migrated with products from cloned standards representing Nitrosospira clusters 2 and 3. The TTGE bands apparent for the Schoorl, Nastola, Bentveld and Appelscha samples did not clearly co-migrate with any of the cloned standards (Fig. 2). TTGE patterns of PCR products from independent PCRs on the same sample and from independent DNA extractions showed no variation in the number of bands and migration position (results not shown). These findings confirmed the reproducibility of the PCR-TTGE method. In both reference clone and environmental TTGE patterns, bands often occurred in doublets, which is consistent with previous results which
showed that a single template sequence can give rise to multiple TTGE bands due to an ambiguous position in the CTO reverse primer [12].

Co-migration of bands from Nitrosospira clusters 2 and 3 and the lack of co-migration for bands from four samples with any of the standards prevented unambiguous identification of AOB clusters based on migration pattern alone. It was, therefore, necessary to excise and reamplify TTGE bands for cloning, sequencing and phylogenetic analysis.

Phylogenetic analysis of the recovered sequences derived from Ysselsteyn, Hulshorst, Wekerom, Roggebotzand and Asbak bands placed these sequences (GenBank Accession Nos. AY683625, AY683626, AY683627, AY683628 and AY683629, respectively) in Nitrosospira cluster 2 (Fig. 3), which is in agreement with the TTGE analysis. The similarity between these five sequences ranged from 98% to 100%. The sequences showed the closest affinity to Nitrosospira sp. strains III7 and AHB1. The similarity to these known β-subdivision AOB sequences ranged from 95% to 99%. Phylogenetic analysis of Schoorl, Nastola, Bentveld and Appelscha bands placed their recovered sequences (GenBank Accession Nos. AY683621, AY683622, AY683623, and AY683624, respectively) outside the Nitrosospira Nitrosomonas clade.

Statistical analysis based on non-parametric analysis of variance (Mann–Whitney U test) on soils with and without recovered Nitrosospira cluster 2-like 16S rDNA sequences revealed that soils with recovered Nitrosospira cluster 2-like 16S rDNA sequences were significantly higher in total N, initial NO$_3$$^-$–N concentration and atmospheric N deposition, while C/N ratio was lower in these soils. Net nitrification rate was higher in these soils, while net ammonification rate was lower in these soils.

4. Discussion

In this study, we found that environmental factors, such as C/N ratio of the F layer and atmospheric N deposition affect the presence of the AOB, which may explain the big differences in N transformation rates between the different forest soils. The presence of AOB was determined by cultivation-independent, molecular methods. The different steps (DNA extraction, PCR, and profiling) in such a molecular approach have their pitfalls [19]. However, since all samples were treated similarly, these pitfalls can be considered the same for all samples, allowing between-sample comparisons.

In soils with high net nitrification rates, we detected only Nitrosospira cluster 2-like sequences. In soils with low net nitrification rates, we could not detect any AOB-like sequences even though a nested PCR approach was used. We found only non-AOB sequences in these soils. The detection of non-AOB-like sequences using the CTO primers was not surprising, since these primers have a relatively low specificity [4,20]. Purkhold et al. [20] demonstrated that none of the primers and probes targeting the 16S rRNA or the 16S rRNA-encoding gene of AOB can cover all cultured species along with being 100% specific for these AOB. The predominance of Nitrosospira cluster 2-like sequences detected in this study is not likely to be due to high selectivity of the CTO primers. Kowalchuk et al. [12] tested the selectivity in the CTO primers by performing on DNA templates containing different ratios of clones pH4.2A/6 (Nitrosospira cluster 2) and pH4.2A/23 (Nitrosomonas cluster 6), followed by separation of these two clones in DGGE. The DGGE profiling revealed bands with relative intensities that were in good agreement with ratios between the two clones in the starting DNA template.
We also do not expect that the predominance of *Nitrosospira* cluster 2-like sequences is due to a failure of FastDNA Spin Kit to extract DNA of several types of highly abundant AOB. *Nitrosospira* clusters 3 [21] and 4 [21,22] and *Nitrosospira multiformis* and *Nitrosomonas europaea* [23] could also be detected in soil systems using the FastDNA Spin Kit. Furthermore, Laverman et al. [6] previously used a modified mechanistic cell disruption method to extract DNA from two forest soils, Wekerom and Roggebotzand, that were also addressed in this study and also detected *Nitrosospira* cluster 2, exclusively, based on 16S rRNA gene based analysis. Moreover, Laverman et al. [6] confirmed the dominance by members of *Nitrosospira* cluster 2 by using a set of primers that target the ammonia monooxygenase (amoA) gene. They found that all AOB populations detected in Wekerom and Roggebotzand soils were closest related to a cultured representative of *Nitrosospira* cluster 2, strain *Nitrosospira* sp. AHB1.

Nevertheless, we have used only one non-quantitative analysis for the detection of AOB sequences. Since no single technique can be expected to provide a comprehensive view of microbial populations in their environment [2], we might have overlooked other AOB populations possibly present. Therefore, we cannot rule out that additional AOB populations will be found if a combination of different techniques and molecular markers would be applied on all samples.
Soils with high nitrification rates are characterised by high initial $\text{NO}_3^-\text{N}$ concentrations, low C/N ratios (or high total N) and high atmospheric N deposition. According to the atmospheric N deposition map of The Netherlands [24], sites with high nitrification rates can be classified as sites with intermediate to high atmospheric N deposition. Thus, our observations are in agreement with earlier findings [25–29]. As the soils with high nitrification rates were all characterised by a predominance of *Nitrosospira* cluster 2-like sequences, the C/N values and the high atmospheric N deposition of these sites are probably favourable for the presence of *Nitrosospira*. In acid coniferous forest soil profiles with low N deposition ($14.8 \text{ kg N ha}^{-1}\text{ yr}^{-1}$) at a site in South Western Sweden, AOB-like sequences could not be detected either [30].

Net nitrification rates and the presence of *Nitrosospira* cluster 2 were not correlated to soil pH. This may be due to the relatively small range of soil pH values and small number of study sites, although it has been observed in acid forest soil that *Nitrosospira* cluster 2 is present regardless of soil pH [22].

Since we did not detect any other cluster types, this study suggests the dominance of a single 16S rDNA sequence type within natural AOB communities in geographically distant acid Scots pine forest soils. So far, similar little variation of AOB community composition, with the exclusive detection of a single *Nitrosospira* 16S rDNA sequence cluster, was found in a nitrogen-saturated coniferous forest soil [6]. These results are in contrast with other molecular studies of AOB in soil habitats where larger 16S rDNA sequence diversity has been detected [e.g., 3,5,7,12,22]. Environmental factors in our study sites probably affect the activity and presence, but not the cluster type of AOB.

The AOB found in this study at Wekerom is similar to the AOB previously detected at this site by Laverman et al. [6]. They also found little variation between different organic layers and throughout the year in this site. We suggest that this high degree of spatio-temporal stability of AOB in acid Scots pine forest soils is a general phenomenon.

In general, it appears that members of *Nitrosospira* cluster 2 dominate in acidic soils with high nitrification rates [this study, 3,5,6]. However, *Nitrosomonas europaea*-like populations dominating in acidic Belgian forest soils [11] show that this is not always the case. Which and how environmental factors select for certain sequence cluster(s) of AOB remains an enigma. The correlation of N transformation rates to C/N ratio (or total N), atmospheric N deposition and the presence of AOB suggests that N transformation rates are not simply the result of the effects of individual ecosystem properties, but are driven by the interactions between these properties.

Acknowledgements

The authors thank Paul L.E. Bodelier and Manuela Coci for providing reference clones of the AOB. Heikki M. Setälä is acknowledged for providing soil from Nastola, Finland and George A. Kowalchuk for his useful comments on a previous draft.

References