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Short communication

Nitrosomonas europaea-like bacteria detected as the dominant β-subclass *Proteobacteria* ammonia oxidisers in reference and limed acid forest soils

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Abstract

Net nitrification in intact soil cores and the community of ammonia-oxidising bacteria were studied in acid Norway spruce (*Picea abies* (L.) Karst) and sessile oak (*Quercus petraea* (Matt. Lieb.)) soils (Haute Ardenne, east Belgium) 18 months after treatment with 5 t ha^{-1} dolomite lime. Liming caused a significant increase in nitrification in the upper soil layers (0.15 m) of both stands. DGGE (denaturing gradient gel electrophoresis) profiling after β-subclass ammonia oxidiser-specific polymerase chain reaction (PCR), combined with hybridisation and sequencing of excised DGGE bands revealed a dominance of *Nitrosomonas europaea*-like sequences, independent of soil horizon, tree species and lime treatment. A minority *Nitrosospira*-like population was detected, which showed affinity to nitrosospiras previously detected in acid soil. These results contrast with several reports suggesting a dominance of *Nitrosospira*-like organisms among ammonia oxidiser communities in acid soils. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: 16S rDNA; DGGE; *Picea abies*; *Quercus petraea*; Nitrification; Dolomite lime

Chemolithoautotrophic nitrification plays an important ecological role in terrestrial ecosystems and is frequently associated with soil acidification, nitrate and cation leaching, aluminium mobilisation and N_2O emissions (Carnol, 1999; Carnol et al., 1997a,b). Despite 100 years of research, it is not clear whether known nitrifying strains are active in acid forest soils, as pure culture isolates typically cease activity below pH 5.5–6.0. Ammonia oxidation is the first, and usually rate-determining, step of the nitrification process (Belser, 1979), and all known terrestrial autotrophic ammonia oxidisers belong to a monophyletic group within the β-subclass *Proteobacteria* (Koops and Möller, 1992) comprised of two genera, *Nitrosomonas* and *Nitrosospira* (Head et al., 1993). Each of these genera contain at least four sub-groupings (Stephen et al., 1996; Purkhold et al., 2000), and recently developed molecular strategies focussing on 16S rRNA genes have shown that the presence of certain sequence clusters can be correlated with specific environmental conditions (Stephen et al., 1996; Kowalchuk et al., 2000a; Kowalchuk and Stephen, 2001). Conventional and molecular analyses have indicated a dominance of nitrosospiras among the ammonia-oxidising bacteria inhabiting most terrestrial environments (Hankinson and Schmidt, 1984), and a grouping dubbed *Nitrosospira* cluster 2 has been associated specifically with acid soils (Stephen et al., 1996, 1998; De Boer and Kowalchuk, 2001). In contrast, *Nitrosomonas* species have been detected in swine-manure fertilised plots (Ceccherini et al., 1998), enrichment cultures (Stephen et al., 1996), and wastewater treatment systems (Logemann et al., 1998), supporting the common view of their preference for high ammonia and high pH environments.

Knowledge on the interactions between variations in environmental conditions and the community of ammonia-oxidisers is currently limited and information on acid forest soils is particularly scarce. This is the first study aiming at identifying ammonia oxidisers present in soils under Norway spruce (*Picea abies* (L.) Karst) and a sessile oak (*Quercus petraea* (Matt. Lieb.)) stands and relating responses to a dolomite lime treatment (Dulière et al., 1999) to a possible shift the ammonia oxidiser community. Our hypothesis was that the ammonia-oxidising communities of these acid forest soils would be dominated by *Nitrosospira* cluster 2, and that this dominance would decrease in response to lime-induced pH increases.

Six replicate plots (225 m^2) of adjacent oak and spruce stands (50°34′N, 6°02′E) were limed around a central tree in

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Table 1
Characteristics of control and limed soil samples from spruce and oak stands, 18 months after liming (*significant difference between control and limed (*T*-test, $p < 0.05$, $n = 6$); NNO_3 net: net nitrate production during 60 days laboratory incubation (mg 100 g [dry weight]$^{-1}$))

Species	Horizon	Treatment	Organic matter content (%)	pH_{H_2O}	NNO_3net
Picea	Organic	Control	81	3.8	3.0
		Limed	75	4.9*	8.6*
	Mineral	Control	43	3.8	1.9
		Limed	46	3.9	5.0*
Quercus	Organic	Control	54	4.1	1.7
		Limed	62	4.8*	9.8*
	Mineral	Control	21	4.1	1.0
		Limed	31	4.2	5.6*

April 1996 with 5 t ha^{-1} dolomite lime (55/40) and six replicate control plots in each stand received no treatment (Dulière et al., 1999). Potential nitrification was determined from paired soil cores (0.09 m diameter × 0.15 m length) taken in all 24 plots in October 1997. Cores were divided into organic (Ol, Of, Oh; mean thickness: 0.077 m) and top mineral (Ah; mean thickness of samples: 0.067 m) horizons, and fresh soil was homogenised manually. Nitrification was expressed as the difference between NO_3–N contents in one core per plot analysed immediately and one core incubated for 60 days in the laboratory in the dark at 20 °C and constant humidity. Incubation was performed at field humidity (50%) and water loss minimised by covering cores with a thin polythene film. Furthermore, initial water content was maintained by periodic addition of distilled water. Organic matter was determined as weight loss after ignition at 430 °C for 24 h. pH was determined in 1:10 (organic horizon) and 1:5 (mineral horizon) soil:water suspensions. Exchangeable NH_4^+–N and NO_3^-–N were analysed after extraction (1 h) with 125 ml 6% KCl of 20 g organic soil and 50 g mineral soil (Allen, 1989), followed by steam distillation of 20 ml filtered extract and titration with H_2SO_4 0.005 N. Within the two stands significant differences between limed and control plots were analysed with a *t*-test (SAS Institute Inc., 1989).

Samples from two limed and control plots in each stand (cores taken in January 1998, divided into O and Ah, sieved 4 mm) were selected at random for molecular analyses. Soil sub-samples were washed twice in 120 mM K_2HPO_4. DNA was extracted using a bead-beating technique according to Kowalchuk et al. (1997), with addition of a cell lysis step (shaking with 0.1 volume lysozyme for 15 min at 37 °C) before phenol/chloroform:isoamylalcohol extraction. DNA, suspended in 40 μl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.5), was run on a 1% agarose gel in 0.5 × TAE (1 × TAE is 0.04 M Tris, 0.02 M acetic acid and 1.0 mM EDTA pH 7.5). DNA longer than 10 kb was excised for purification with the QUIA-quick gel extraction kit (Qiagen, Inc., Chatsworth, California). DNA was eluted in 30 μl 10 mM Tris (pH 8.5).

Nested polymerase chain reaction (PCR) amplification was performed with eubacterial-specific 16S rDNA directed primers pA and pH (Edwards et al., 1989), followed by amplification using the CTO primer pair (CTO189f-GC, CTO654r) for the β-subdivision ammonia-oxidising bacteria, as described in Kowalchuk et al. (1997). The resulting amplification products were separated by denaturing gradient gel electrophoresis (DGGE) and run alongside ammonia oxidiser cluster controls derived from clones of known sequence (Stephen et al., 1996, 1998; Kowalchuk et al., 1997). For sequence analysis, DGGE bands were carefully excised and DNA eluded after crushing the gel in liquid nitrogen. Hybridisations were performed using oligonucleotide probes β-AO233 (all β-subgroup ammonia-oxidisers), NspCL2_458 (*Nitrosospira* cluster 2) and NmoCL7_439 (*Nitrosomonas* cluster 7) (Stephen et al., 1998). Hybridisation signals were also quantified as described by Stephen et al. (1998).

After 18 months, dolomite liming had caused a significant pH increase in the organic horizon of the oak and spruce stands (Table 1). Nitrification was significantly increased in the organic and mineral horizons of the limed plots in both stands, probably indicating the presence of acid-sensitive chemolithoautotroph nitrifiers. Total mineral nitrogen production was not influenced by the liming treatment (not shown). It is well known that liming may increase nitrification (De Boer et al., 1993) with unchanged mineralisation when the pH remains below ca. 5.2 (Badalucco et al., 1990). Net mineralisation and nitrification were not significantly different in the two stands.

The DGGE profile revealed one or two similar bands for all samples, irrespective of site, soil horizon and liming treatment (Fig. 1A). The impact of liming on nitrification was therefore not related to a detectable shift in the 16S rDNA DGGE community profile of ammonia-oxidising bacteria. DGGE profiles and hybridisation results were highly reproducible in the comparison of replicate samples, DNA extractions and amplifications. All detectable DGGE bands migrated either in the range typical of *Nitrosomonas* cluster 7 (43.5% denaturant) or *Nitrosospira* clusters 2 and 3 (45% denaturant) (Fig. 1A). The lower signal appeared as a double banding pattern, a phenomenon previously shown to stem from the use of a reverse primer containing an ambiguous base position (Kowalchuk et al., 1997). Hybridisation with sequence cluster-specific probes (Stephen et al., 1998) identified the upper band as *Nitrosomonas europaea* cluster

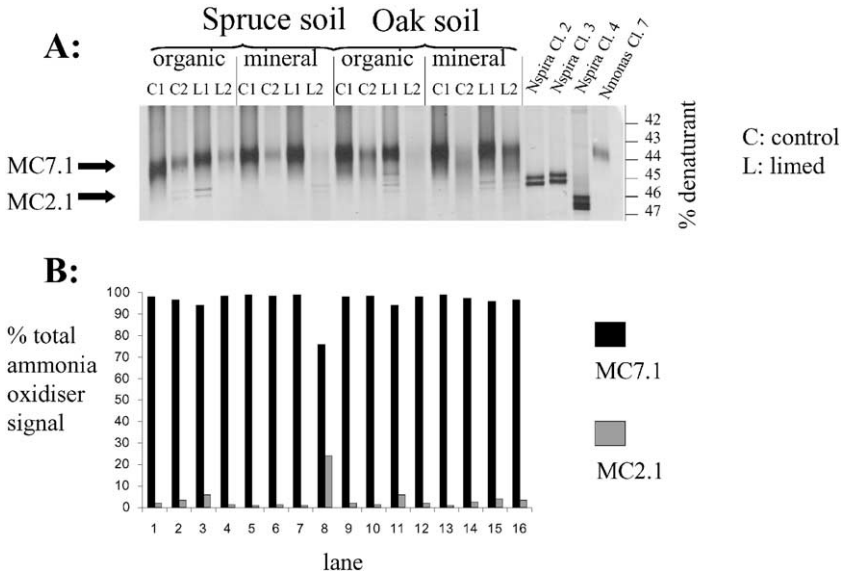

Fig. 1. (A) DGGE gel of acid forest soils. Ammonia oxidiser cluster controls were derived from clones of known sequence. Band designations correspond with those given in the tree figure (Fig. 2). (B) Results of hybridisation analysis. The normalised signal for the two detected clusters equalled nearly 100% of the total as detected using an all β-subgroup ammonia oxidiser probe.

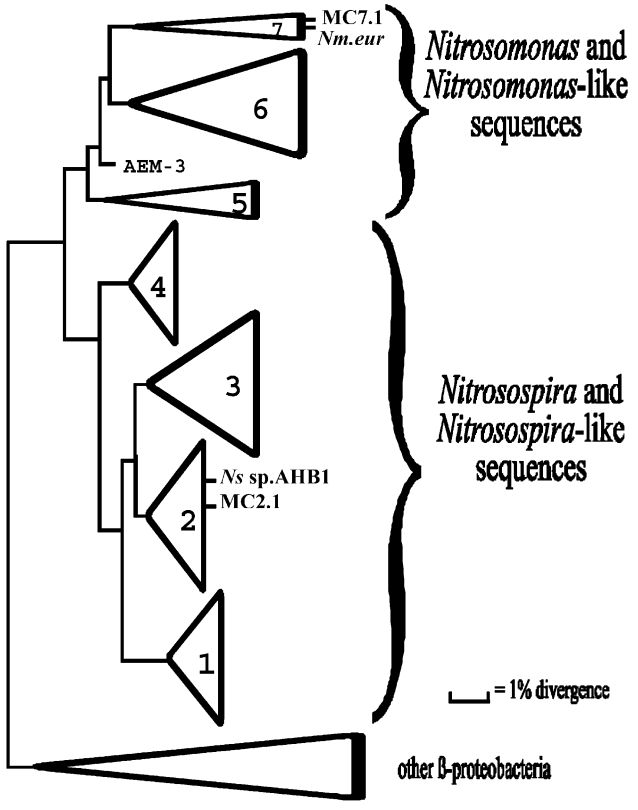

Fig. 2. Schematic phylogenetic tree for the β-subgroup proteobacterial ammonia oxidisers. The construction of the tree was based on a distance matrix and a neighbour-joining analysis, as described by Stephen et al. (1996). The height of each triangle represents the number sequences of the given group used in the analysis; while the length depicts the level of sequence variation within the group. The cluster designations, as proposed by Stephen et al. (1996) are indicated inside the triangles. Brackets give the breadth of the *Nitrosomonas* and *Nitrosospira* genera. 'MC' sequences were derived from excised DGGE bands.

7 and the lower doublet as *Nitrosospira* cluster 2. The *N. europaea* cluster 7 signal dominated all samples, accounting from 78 to 98% of the total ammonia oxidiser hybridisation level (Fig. 1B). Sequences derived from excised DGGE gels (EMBL accession numbers: AJ307984, AJ307985) confirmed hybridisation results (Fig. 2); band MC7.1 was 99.8% identical to *N. europaea* over the sequenced region 500 bp, and MC2.1 99.2% identical to *Nitrosospira* sp. AHB1.

These results suggest a dominance of *N. europaea*-like bacteria among the ammonia oxidisers in these acid soils. This is in sharp contrast with studies on ammonia oxidiser community structure in acid and neutral agricultural soils (Stephen et al., 1996, 1998), and acid and neutral grasslands (Kowalchuk et al., 2000a,b), and other acid forest soils (Kowalchuk and De Boer, unpublished), which all provided evidence for a dominance of *Nitrosospira*-like organisms. Furthermore, previous studies have reported a correlation between the abundance of *Nitrosospira* cluster 2 and soil pH (Kowalchuk et al., 2000a). We found no such correlation. These differences are most probably not the result of procedural artefacts, as the methods used do detect dominance of *Nitrosospira* populations when applied to other samples (Kowalchuk et al., 1997).

In conclusion, we have shown that the nitrification response to a dolomite lime treatment in two stands was not related to a detectable shift in the ammonia-oxidiser community. We report for the first time, the dominance of *N. europaea*-like bacteria among the ammonia oxidisers of highly acid forest soils, thus expanding the niche range of this bacterial lineage. Clearly, greater knowledge of the determinants of niche differentiation is necessary before accurate generalisations and predictions can be made with

respect to ammonia oxidiser distribution and activity in the environment.

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