Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of \textit{nir} gene fragments

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Abstract

Temporal and spatial variation of communities of soil denitrifying bacteria at sites receiving mineral fertilizer (60 and 120 kg N ha$^{-1}$ year$^{-1}$) and cattle manure (75 and 150 kg N ha$^{-1}$ year$^{-1}$) were explored using terminal restriction fragment length polymorphism (T-RFLP) analyses of PCR amplified nitrite reductase (\textit{nirK} and \textit{nirS}) gene fragments. The analyses were done three times during the year: in March, July and October. \textit{nirK} gene fragments could be amplified in all three months, whereas \textit{nirS} gene fragments could be amplified only in March. Analysis of similarities in T-RFLP patterns revealed a significant seasonal shift in the community structure of \textit{nirK}-containing bacteria. Also, sites treated with mineral fertilizer or cattle manure showed different communities of \textit{nirK}-containing denitrifying bacteria, since the T-RFLP patterns of soils treated with these fertilizers were significantly different. Also, these sites significantly differed from the control plot (no fertilizer treatment), whereas the patterns for low and high N-additions were barely separable from each other. Sequencing and phylogenetic analysis of 54 \textit{nirK} clones revealed that the major part of the \textit{nirK}-containing bacteria investigated belonged to a yet uncultivated cluster of denitrifying bacteria.

Keywords: Denitrification; Soil; Fertilizer; Diversity; T-RFLP; \textit{nir} genes

1. Introduction

Biological denitrification is a dissimilative microbiological redox process, where nitrogen oxide functions as an electron acceptor in the energy production. It involves a stepwise reduction of nitrate via nitrite to the gaseous end products nitric oxide (NO), nitrous oxide (N$_2$O) and, in most cases, molecular nitrogen (N$_2$), which all are released into the atmosphere. Denitrification is of importance for several reasons: it plays a major role in the nitrogen cycling in soils [1], N$_2$O is a significant contributor to stratospheric ozone depletion and global warming [1,2], and it results in major nitrogen losses from fertilized agricultural soils [3].

The activity of denitrifying bacteria in soil shows pronounced seasonal variations [4]. However, virtually nothing is known about seasonal variations of the composition of denitrifying communities in soil. Published N$_2$O emission rates from a number of agricultural soils indicate that there may be differences in N$_2$O emission caused by different fertilizer types [5]. Denitrification as well as nitrification may contribute to N$_2$O emission, but under moist and oxygen-depleted conditions, denitrification is generally the major source of N$_2$O [5] and therefore, N$_2$O emission may reflect the denitrification rate. In agreement with this, studies of the effect of fertilizer source showed that both N$_2$O emission and denitrification vary between soils treated with different types of N-containing fertilizers, e.g. non-fertilized sites, sites treated with mineral fertilizer or sites treated with urea [6–8].

It is often assumed that the denitrifying communities in soils are identical in terms of the enzymatic processes.
given equal physical and chemical conditions. This implicit assumption, combined with the lack of methods to study the composition of the denitrifying community in detail, has led to an emphasis on soil physics and substrate supplies rather than on the composition and physiological properties of the active organisms [9].

To challenge this assumption, Munch [10] showed that the N²O:N² ratio during soil denitrification primarily depends on the composition of the denitrifying microflora. Other studies [9,11] have demonstrated that microbial factors and phylogeny of denitrifiers are of great importance for regulating the N₂O flux and should be taken into account when creating models of N₂O emission from soil.

Thus, the composition of the denitrifying community may be a crucial factor in regulating the emission of N₂O to the atmosphere and in regulating denitrifying activity in competition with aerobic heterotrophic activity.

Determination of microbial communities formerly was restricted to cultivation-based techniques, which are insufficient for studying naturally occurring bacteria, since less than 1% of these bacteria are cultivable by the current techniques [12]. Recently, molecular techniques able to overcome this problem have been developed and are used more and more frequently. Since denitrifying bacteria form a functional group with members widely distributed over the phylogenetic tree [13], the 16S rDNA is not suitable for community analysis of denitrifying bacteria. Instead, functional genes involved in the denitrifying process, encoding the key enzyme nitrite reductase (nir) [14,15] or nitrous oxide (nos) [16,17], have been used in community analyses. Two structurally different nitrite reductases are found mutually exclusive among denitrifiers; nirK, encoding a copper-containing nitrite reductase, and nirS, encoding a Cd₁-containing nitrite reductase [18]. In this study, we investigated the composition of soil microbial populations containing nirK or nirS.

A recent method for studying the structure of prokaryotic communities in environmental samples is terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is a method that relies on variation in the position of restriction sites among sequences, and the determination of the lengths of the terminal fragments.

T-RFLP analysis has been used to characterize a diversity of 16S rDNA genes (see 14,19,20) as well as functional genes such as mercury resistance (mer) [21], nitrite reductase (nir) [14,15] and nitrous oxide reductase (nosZ) [16].

Analysis of T-RFLP data is still in a developing stage. Often, information on the peak heights (i.e. the abundances) of the terminal fragments is excluded in the statistical comparisons, and only the absence or presence of TRFs is included (e.g. [19,20,22,23]). As a consequence, rare species are given the same weight as the common ones, leading to loss of potentially valuable information about the approximate prevalence of a terminal restriction fragment (T-RF). A number of similarity measures exist, able to deal with this type of data (e.g. [24]). One similarity measure has become particularly common in ecology: the Bray–Curtis coefficient, originally introduced in ecological work by Bray and Curtis [25]. In marine ecology studies of community changes [26], the ANOSIM (analysis of similarities) technique has been developed [27], which analyses the pairwise Bray–Curtis similarities between samples. To our knowledge, ANOSIM has never been used in combination with T-RFLP analysis, prior to what we present here, although it is an obvious candidate for comparing similarities.

In this study, we used the ANOSIM technique to compare T-RFLP patterns of nirK and nirS gene fragments from environmental soil samples. Combined with subsequent cloning and sequencing, we address the hypothesis that the amount (60–75 or 120–150 kg N ha⁻¹ year⁻¹) and type (cattle manure or mineral fertilizer) of fertilizer treatment have an impact on the composition of the community of denitrifying bacteria.

2. Materials and methods

2.1. Field site and soil sampling

The experimental field site was located at Rørrendegård, near Taastrup, Denmark (55°40′N, 12°18′E). Average annual temperature since 1955 is 7.7 °C. Average annual precipitation since 1955 is 613 mm. From 1965 to 1995, the field has been treated with mineral fertilizer (60 kg N ha⁻¹ year⁻¹, 0 kg P ha⁻¹ year⁻¹, 0 kg K ha⁻¹ year⁻¹). Since 1996, the crop has been spring barley (Hordeum vulgare) in 1996, 1997, 1999, 2002 and 2003, red clover (Trifolium pratense L.) in 2000 and 2001, and sugar beet (Beta vulgaris L.) in 1998. The atmospheric N deposition in 1994 was 2.77 kg NH₃⁻N ha⁻¹ and 3.25 kg NO₂⁻N ha⁻¹ [28].

In 1996, the field was divided into 200 m × 18 m plots, treated with different amounts and types of mineral fertilizer or cattle manure. The plots were separated by ten meter wide spaces and were characterized by the following treatments: con (control plot, no fertilizer treatment), low-fer (60 kg N ha⁻¹ year⁻¹, 10 kg P ha⁻¹ year⁻¹, 60 kg K ha⁻¹ year⁻¹ fertilizer), high-fer (120 kg N ha⁻¹ year⁻¹, 20 kg P ha⁻¹ year⁻¹, 120 kg K ha⁻¹ year⁻¹ fertilizer), low-man (75 kg N ha⁻¹ year⁻¹, 10 kg P ha⁻¹ year⁻¹, 75 kg K ha⁻¹ year⁻¹ cattle manure) and high-man (150 kg N ha⁻¹ year⁻¹, 20 kg P ha⁻¹ year⁻¹, 150 kg K ha⁻¹ year⁻¹ cattle manure). Mineral fertilizer was applied as 1:1 N–NO₃ and N–NH₄. The cattle manure had a C/N ration of 9.2. The soil pH (in 1:2.5 soil:water
Table 1

Soil characteristics of experimental plots

<table>
<thead>
<tr>
<th>Weight loss by ignition (%)</th>
<th>Dissolved organic carbon (mg g⁻¹ dwt⁻¹)</th>
<th>Total N (%)</th>
<th>Potential denitrification (ng N₂O–N g dwt⁻¹ h⁻¹)</th>
<th>NO₃⁻</th>
<th>NO₂⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>con</td>
<td>18.51 ± 0.01</td>
<td>650 ± 10.6</td>
<td>0.12 ± 0.01</td>
<td>2.58 ± 0.15</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>low-fer</td>
<td>18.24 ± 0.01</td>
<td>573 ± 4.5</td>
<td>0.14 ± 0.01</td>
<td>2.55 ± 0.21</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>high-fer</td>
<td>20.06 ± 0.01</td>
<td>485 ± 4.3</td>
<td>0.12 ± 0.01</td>
<td>2.55 ± 0.21</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>low-man</td>
<td>15.37 ± 0.01</td>
<td>489 ± 3.7</td>
<td>0.13 ± 0.01</td>
<td>2.55 ± 0.21</td>
<td>0.35 ± 0.06</td>
</tr>
<tr>
<td>high-man</td>
<td>18.38 ± 0.01</td>
<td>561 ± 7.7</td>
<td>0.13 ± 0.01</td>
<td>2.55 ± 0.21</td>
<td>0.35 ± 0.06</td>
</tr>
</tbody>
</table>

Values followed by different letters are significantly different, Tukey’s test (p = 0.05).

Non-standard abbreviations: con = control plot (no fertilizer treatments), low-fer = low fertilizer (60 kg N ha⁻¹ year⁻¹, 10 kg K ha⁻¹ year⁻¹; cattle manure), high-fer = high fertilizer (120 kg N ha⁻¹ year⁻¹, 120 kg K ha⁻¹ year⁻¹; cattle manure), low-man = low manure (150 kg manure ha⁻¹ year⁻¹; cattle manure), high-man = high manure (300 kg manure ha⁻¹ year⁻¹; cattle manure). Nitrous oxide reductase activity was measured at room temperature as accumulation of N₂O in 1/16 ml sealed incubation bottles flushed with N₂ and added 0.5 mM potassium nitrate, 0.5 mM glucose, 0.5 mM sodium acetate, 0.5 mM sodium succinate, and 10% acetylene. Nitrous oxide reductase activity was measured after three times extracting nitrate from the soil and incubated as above (without nitrate and acetylene addition) after adding 100 ppm N₂O (final concentration).


2.2. Extraction of total DNA and PCR of nir gene fragments

Total DNA was isolated from three 0.5 g replicates for each field sample, using Fast DNA SPIN Kit for Soil (BIO 101, Carlsbad, California) as specified by the manufacturer. Isolated DNA was visualized under UV light after electrophoresis on agarose gels followed by staining in ethidium bromide. Extracted DNA was predominantly of high molecular weight (>10 kb). The extracted DNA was stored at −20 °C.

The three DNA isolations from each field sample comprise the biological replicates and were kept separate throughout the downstream analysis.

Fragments of the nirK and nirS genes were amplified using primer sets nirK1F and nirK5R for nirK, which give a 512–515-bp product, and nirS1F and nirS6R for nirS, which give an approximately 890-bp product [29]. For T-RFLP, the forward primers were fluorescently labelled with FAM (nirS1F) or TET (nirK1F).

PCR on DNA samples was done using puRe Taq Ready TO GO PCR Beads (Amersham Biosciences, Uppsala, Sweden) and 35 pmol of each primer in a total volume of 25 μl in a DNA Engine Dyad Peltier Thermal Cycler (MJ Research, Waltham, Massachusetts). Samples were amplified using the following PCR conditions: an initial denaturation step at 93 °C for 30 s, followed by 35 (nirK) or 39 (nirS) cycles of denaturation at 93 °C for 25 s, annealing at 54 °C (nirK) or 58 °C (nirS) for 30 s, and extension at 72 °C for 30 s (nirK) or 40 s (nirS). Cycling was completed at 72 °C for 5 min.

PCR on cloned nirK sequences (see below) was done using PCR Master (Amersham Biosciences, Uppsala, Sweden) and 30 pmol of each primer in total volume of 40 μl. The samples were amplified using the specifications described above.
PCR products for T-RFLP were purified by cutting out the band of the expected size from low melt agarose gels. The PCR products were purified from the gel using QIAEX II Gel Extraction Kit (Qiagen, Chatsworth, California).

2.3. Restriction endonuclease digestion and T-RFLP profiles

Each PCR product was divided in three equal portions and digested for 4 h with 10 U of the three endonucleases: MspI, TaqI and HhaI (New England BioLabs, Beverly, Massachusetts). For all three endonucleases NEBuffer II was used. After digestion, the endonucleases were inactivated by heat as specified by the manufacturer.

After DNA digestion, size standards labelled with Texas Red (MegaBACE ET900-R, Amersham Biosciences, Uppsala, Sweden) were added to the samples, which were desalinated using Sephadex G-50 (Amersham Biosciences, Uppsala, Sweden), denatured at 94 °C for 1 min, and kept on ice before injection in the MegaBace 1000 DNA Sequencing system sequencer (Amersham Biosciences, Uppsala, Sweden), where the T-RFLP run was done. Samples were injected at 3 kV for 3 min, and separation was performed at 7 kV for 180 min in MegaBACE Long Read Matrix (Amersham Biosciences, Uppsala, Sweden).

2.4. Analysis of T-RFLP profiles

After the T-RFLP run, the raw data were analyzed by the software program MegaBACE Genetic Profiler Version 1.5 (Amersham Biosciences, Uppsala, Sweden). Even though the program made a peak table with all peaks in the electrophogram, we examined all electropherograms manually to check that only true peaks (and all of them) were included in the peak table and that every peak was included only once. Peaks with a height below 75 absorbance units were excluded from the analysis.

To avoid detection of primers and uncertainties of size standards, terminal fragments smaller than 37 bases were excluded from the analysis. Because we could not exclude the possibility that uncut fragments were not cut due to inefficient digestion rather than lack of restriction sites, these fragments were excluded from the analysis. Uuncut fragments comprised on average 2.3% of the summed peak height.

To standardize the results, the heights of every peak were divided with the summarized height for all the peaks of the sample in concern, i.e. the peak heights were standardized to percentage of the total peak height.

We ended up with a frequency table (i.e. a T-RFLP profile), showing the relative peak height for each detected T-RF for every sample, and these profiles were our basis for further statistical analysis.

2.5. Statistical analysis of T-RFLP profiles

All the statistical work with T-RFLP profiles was done with the Primer 5 software (Primer E, Plymouth, UK).

2.5.1. Similarity matrices and MDS plots

Similarities were calculated between every pair of samples as the Bray–Curtis similarity coefficient [25], using the standardized T-RFLP profiles and the similarity function in Primer 5. The similarity matrices were used for multidimensional scaling (MDS) plots and analysis of similarity (see below).

Non-metric MDS were created by the rank order of the Bray–Curtis similarity matrices. MDS constructs a configuration of the samples, in a specified number of dimensions, which attempts to satisfy all the conditions imposed by the Bray–Curtis similarity matrix [26], i.e. the MDS algorithm tries to construct a sample map whose inter-point distances have the same rank order as the corresponding Bray–Curtis similarity matrix. Generally, MDS gives a better representation of data compared to, e.g. PCA analysis. Also, MDS does not assume linearity in correlations between parameters, a phenomenon rarely observed among biological relationships [26]. For more information on MDS, see Schiffman [30].

2.5.2. Analysis of similarity

In order to test for the impact of different factors on the variation in the similarity data, we used the ANOSIM function in Primer 5, which is an analysis of similarity based on the rank ordering of the Bray–Curtis similarities. With suitable replicate samples, this technique tests for differences in community structure by combining permutation tests with the general Monte Carlo randomization approach [31]. The null hypothesis (H0) states that there are no differences in community composition between sample intervals. To test the null hypothesis, a test statistic, Global R, that contrasts the variation between pre-defined clusters with variation within clusters, is computed. The R value is compared to a predicted permutation distribution, given H0 is true. This distribution is calculated by a chosen number of random permutations of the samples; in this study we used 10,000. If H0 is true, the observed R value will fall within the range of the computed permuted distribution [26].

2.5.3. Similarity percentages of T-RFs

The Similarity percentages (SIMPER) routine in Primer 5 can be used to identify the species (i.e. in our case T-RF peaks) responsible for particular aspects of the multivariate structure found in the Bray–Curtis similarity matrix, i.e. determining the peaks responsible for the sample grouping found in the MDS plots [26]. The routine computes the average dissimilarity between all pairs of inter-group samples and then breaks this
2.6. Cloning and sequencing of nirK gene fragments and phylogenetic analysis

Purified PCR products from the five different plots from March and October were cloned using the TOPO TA Cloning kit for Sequencing (Invitrogen, Carlsbad, California). A number of clones from each cloning experiment was randomly selected for further analysis. A small amount of cell material from a clone was picked up with an inoculating loop and the inserts were PCR amplified as described above. Only clones which gave a PCR product of the expected size when run on an agarose gel were analyzed further. PCR products from clones were screened by restriction fragment length polymorphism (RFLP) after digestion as described above. The digested products were separated by gel electrophoresis in a 3.5% agarose gel. The RFLP patterns were manually compared (by eye). A selection of clones representing both identical and distinct RFLP patters was selected for sequencing. Clones were grown at 37 °C overnight and plasmids were isolated using QIAprep Spin Miniprep (Qiagen, Chatsworth, California).

Both strands were sequenced directly on the plasmids using dye primer sequencing with the primer M13 reverse or T7 forward (Invitrogen, Carlsbad, California), which binds to sites next to the insert in the plasmid. DNA sequences were determined using a MegaBace 1000 DNA Sequencing system sequencer (Amersham Biosciences, Uppsala, Sweden).

Deduced amino acid sequences from the cloned sequences were aligned with published nirK sequences from the NCBI database. Phylogenetic analysis was performed with a neighbor-joining algorithm and distance calculation according to Jukes and Cantor [32], using Treecon 1.3b [33]. Regions of deletion and insertion were omitted from the analysis because of uncertain alignment. aniA From Neisseria gonorrhoea [M97926] was used as outgroup for the phylogenetic analysis. In order to avoid problems during calculation of the dendrogram, a single sequence was chosen to represent all sequences in each cluster consisting of >98% identical sequences.

2.7. Nucleotide sequence accession numbers

The nirK gene fragments have been deposited in the GenBank nucleotide sequence database under Accession No. AY249326–AY249378.

3. Results

3.1. Amplification of nir gene fragments

nirK Gene fragments were successfully amplified from all 15 field samples (five plots three times during the year) while nirS was successfully amplified only in the five soil samples obtained in March. In July and October, nirS fragments could not be successfully amplified, despite an extensive effort including re-extraction of DNA, change in template concentration and annealing temperature, additional DNA purification procedures and PCR using different DNA polymerases.

3.2. Replication of T-RFLP profiles

Initially, there were three DNA extractions for each field sample, but unfortunately some (12 out of 180) were lost during the experimental process and could not be redone. Therefore, in ten cases there are only two replicates and in one case only one “replicate”. The replicates were used as separate profiles for calculating the Bray–Curtis similarity matrices used in the further analyses and in construction of MDS plots.

3.3. Description of community patterns

Overall, MDS revealed three clearly distinct time groups when nirK was digested with HhaI and MspI (Fig. 1). After digestion with TaqI, the July and October samples grouped together. For nirS, there is a tendency for the low amount replicates to group together in the middle of the plot, distinct from the other replicates (Fig. 2). Stress values for nirK are small, indicating a good ordination with no real prospect of misleading interpretation, while the stress values for nirS (ranging from 0.13 to 0.15) are a bit larger, but still provide a useful two-dimensional picture [26].

3.4. Variation in similarity

In order to test for the impact of the different factors on the variation in the similarity data, the ANOSIM technique was used [27]. This technique calculates a global R-value, which technically lies in the range from −1 to 1. It usually falls between 0 and 1, indicating some degree of discrimination between sites. An outcome of \( R = 1 \) can only be expected if all replicates (of a given factor) are more similar to each other that to any other replicates, whereas \( R = 0 \) only appears if the similarity between and within replicates (of a given factor) will be the same on average.

3.4.1. Time variation

To test for time variation, a two way crossed analysis was used where the two factors were time (March, July,
October) and plot (con, low-f, high-f, low-cm, high-cm). For nirK, the analysis with the three different restriction enzymes showed a clear global time difference (global $R = 1$, $p = 0.0\%$ for HhaI andMspI, global $R = 0.987$, $p = 0.0\%$ for TaqI). $p = 0.0\%$ Indicates that 0 out of 10,000 permutations exceeded the observed value. If the three times are compared pair wise (March–July, March–October, July–October) for the three enzymes, the resulting $R$-values are 1 in eight of the nine cases and 0.94 in the last case (July–October, TaqI), all with a significance level of 0.0%. According to this procedure, the three various time groups were not drawn from the same population; hence, there is significant change in community composition caused by variation in time.

Since the nirS samples were only obtained in March, nothing can be said about the seasonal variation in nirS community structure.
3.4.2. Treatment type variation

Community variations due to mineral fertilizer or manure treatment were tested the same way as variation due to time, but with time and fertilizer as the two factors. The observed test values are shown in Table 2. For nirK, the test values are averaged across all time groups. For nirS, the global tests indicate a difference between the three types of treatment (control, mineral fertilizer, cattle manure), and the pairwise tests indicate that all three treatments significantly differ from each other, with $R$ ranging from 0.465 to 0.915 ($p$ ranging from 0.0% to 0.23%). As with time groups, the various fertilizer type groups were not drawn from the same population. Hence, there is significant change in community composition at the sites treated with different fertilizer types.

In contrast to nirK, the three enzymes did not show the same pattern for nirS. The observed global $R$-values for HhaI and TaqI indicate a significantly disperse but overlapping fertilizer type groups, while the value for MspI indicates very little dispersion. The pairwise test also showed different patterns for the three enzymes.

<table>
<thead>
<tr>
<th>Fertilizer type</th>
<th>nirK</th>
<th>nirS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global test</td>
<td>0.532</td>
<td>0.449</td>
</tr>
<tr>
<td>con-fer</td>
<td>0.465</td>
<td>0.407</td>
</tr>
<tr>
<td>con-man</td>
<td>0.560</td>
<td>0.241</td>
</tr>
<tr>
<td>fer-man</td>
<td>0.583</td>
<td>0.589</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fertilizer amount</th>
<th>nirK</th>
<th>nirS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Global test</td>
<td>0.269</td>
<td>0.082</td>
</tr>
<tr>
<td>con-low</td>
<td>0.379</td>
<td>0.118</td>
</tr>
<tr>
<td>con-high</td>
<td>0.205</td>
<td>0.080</td>
</tr>
<tr>
<td>low-high</td>
<td>0.241</td>
<td>0.043</td>
</tr>
</tbody>
</table>

3.4.3. Treatment amount variation

Observed ANOSIM test values for the analysis of differences between fertilizer amount groups for nirS and nirK, are shown in Table 2. For nirK, the values are averaged across all time groups. For nirK, the global $R$-values ranged from 0.268–0.282, indicating different but overlapping fertilizer amount groups [26]. A closer look at the pairwise tests showed that the control plots differed from both the high and low fert amount groups (only in one case the $R$-value dropped below 0.25), while the latter groups are barely separable at all ($R$-values ≤0.241 for all three enzymes). For nirS, only the $R$-value for HhaI ($R = 0.370$) indicated a difference between the fertilizer amount groups.

3.5. Sequence analysis of clones

For 57 soil clones, both strands of ca. 515 bp partial nirK fragments were sequenced. In a comparison with National Center for Biotechnology Information (NCBI) database using BLAST search, 54 (95%) of the sequences were confirmed as nirK by showing from 82% to 98% homology to known nirK sequences. The remaining three sequences did not match any sequence in the database. Phylogenetic analysis of the nirK sequences placed them mainly together with other sequences obtained from soil bacteria (soil cluster I and III illustrated on Fig. 3).

3.6. Assignment of known nir sequences to T-RFLP fragments

In a computer simulation, all known nirK and nirS sequences (from NCBI database and own studies) were cleaved with the same three restriction enzymes as were used to cleave the PCR products from soil DNA. The lengths of these theoretical T-RFLP fragments were calculated, and sequences (clones) were assigned to
Fig. 3. Neighbor-joining analysis of nirK gene fragments based on 172 amino acids cloned from agricultural soil, Taastrup, Denmark (MW) (this study), forested upland (U) and marsh (M) soils, Michigan, USA [34], Washington margin sediment [36], forest soil, Germany [39], and Danish forest soil (H). Accession numbers in brackets. Clones obtained from this study are shown in boldface. A single sequence was chosen to represent all sequences in clusters consisting of >98% identical sequences, and the origin and number of the other sequences in each cluster are indicated. The U7 clone also represents 15 M-clones, 13 U-clones and 14 MW-clones, while * includes Ochrobactrum anthropi [AJ224907] and O. sp. [AY078249-51]; ** includes Brucella suis [AE014526]; and *** includes Ensifer sp. [AY078247-8]. Values indicate the percentage of 100 replicate trees supporting the branching order; values below 50 are omitted.
3.7. Similarity percentages

With the SIMPER routine in Primer 5 the number of T-RFs needed to explain 60% of the variation between groups are found (Table 3). For nirK, ANOVA analysis showed a significant seasonal variance for the amount \( \left( p = 0.038 \right) \) and the type \( \left( p = 0.0077 \right) \) of fertilizer. In contrast, no significant variance between the three pair wise comparisons of fertilizer type or amount for nirK \( \left( p = 0.075 \right. \) and \( p = 0.36 \) or nirS \( \left( p = 0.797 \right. \) and \( p = 0.964 \) ) was observed.

4. Discussion

T-RFLP analysis of nirK and nirS gene fragments from natural populations of denitrifying bacteria in agricultural soils was applied to investigate the temporal and spatial variation in the community structure of denitrifying bacteria.

To confirm the specificities of the T-RFs, PCR-amplified nirK fragments were cloned and sequenced. This revealed that some, but not all, T-RFs detected in the T-RFLP analysis were found in the clone library. The T-RFs found were not necessarily the most abundant ones, but since cloning may introduce bias, this could not be expected per se. It is more important that the three clones which could not be confirmed as nirK did not correspond to any of the T-RFs found in the T-RFLP analysis, since this would indicate that the nirK primers amplify soil DNA, which is not nirK. Interestingly, all the dominant (with a peak height of more than 10% of total peak height) nirS T-RFs corresponded to in silico digests from sequences from known denitrifying bacteria, e.g. the combination of the observed T-RFs HhaI 109 bp, MspI 141 bp, and TaqI 82 bp corresponded to Pseudomonas stutzeri [X56813]. The in silico digest showed that a combination of T-RFs from five isolates and one clone (Azoarcus tolualcyanus [AY078272], Azospirillum brasilense [AF361787], Azospirillum lipoferum [AF361790], Magnetospirillum magnetotacticum [NZ_AA0464836]), Pseudomonas stutzeri [X56813] and the estuarine sediment clone [AJ404079] theoretically encompass from 56% to 79% of the total peak height in the five soils. This is in contrast to nirK, where no such correspondence between T-RFs and published sequences from known denitrifying isolates were found. Sequencing of nirK clones from our soils confirmed this (see below).

With the nirK primers, PCR amplification was possible from all field samples tested. It was not possible to amplify with the nirS primers from field samples taken in July and October, only from field samples taken in March. There is no certain way to explain the shift from detectable to non-detectable levels of nirS in ecological terms, since very little is known about the environmental preferences of nirS- and nirK-containing denitrifying bacteria. However, in at least two other studies, nirS was undetectable in soil samples using the same primers as we used. Avrahami et al. [15] could not detect nirS fragments from a silt loam soil from Germany, while Priemé et al. [34] were unable to detect nirS from a mixed deciduous upland forest soil in Michigan. In the same study, Priemé et al. successfully amplified nirS from a mixed deciduous marsh in Michigan. In contrast, nirS, was successfully detected in a number of studies of marine habitats [15,29,35,36]. A possible explanation is that the nirS primers selectively amplify nirS genes closely related to those used for the development of the primers [29]. Thus, they may not have been detected if more distantly related nirS fragments were present in the soils investigated here.

Our study showed a distinct seasonal variation in the community structure of nirK-containing denitrifying bacteria represented by T-RFLP and ANOSIM analysis of the nirK gene. This was expected, since a number of cultivation and molecular studies has shown seasonal fluctuations of the dominant members of the bacterial agricultural soil community (e.g. [37,38]. In agreement with this, a cultivation study using DNA hybridisation

Table 3
Numbers of T-RF peaks needed to explain 60% of the variation between groups

<table>
<thead>
<tr>
<th>Fertilizer type</th>
<th>March</th>
<th>July</th>
<th>October</th>
<th>March</th>
</tr>
</thead>
<tbody>
<tr>
<td>con-fer</td>
<td>11 (57)</td>
<td>8 (42)</td>
<td>9 (60)</td>
<td>7 (63)</td>
</tr>
<tr>
<td>con-man</td>
<td>11 (68)</td>
<td>6 (38)</td>
<td>8 (53)</td>
<td>7 (62)</td>
</tr>
<tr>
<td>fer-man</td>
<td>12 (72)</td>
<td>9 (47)</td>
<td>10 (59)</td>
<td>7 (60)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fertilizer amount</th>
<th>March</th>
<th>July</th>
<th>October</th>
<th>March</th>
</tr>
</thead>
<tbody>
<tr>
<td>con-low</td>
<td>11 (63)</td>
<td>6 (40)</td>
<td>9 (58)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>con-high</td>
<td>11 (69)</td>
<td>8 (41)</td>
<td>8 (58)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>high-low</td>
<td>11 (71)</td>
<td>8 (48)</td>
<td>10 (51)</td>
<td>7 (60)</td>
</tr>
</tbody>
</table>

Numbers are average values from independent treatment with three restriction enzymes. Numbers in parentheses indicate the maximum number of T-RF peaks obtained with a single restriction enzyme. Non-standard abbreviations: con = control plot (no fertilizer), fer = mineral fertilizer, man = cattle manure, low = low amount N (60–75 kg N ha\(^{-1}\) year\(^{-1}\)), high = high amount N (120–150 kg N ha\(^{-1}\) year\(^{-1}\)). Peaks found in the electropherograms, considering that a difference of ±2 bp in the sizes of the T-RFLP fragments is likely to occur due to the nature of the gel separation [14]. For nirK, known clones could be assigned to 11–16% of the T-RFLP peaks, for nirS a little more (17–23%), including all the major peaks (data not shown). Seventy-eight percent of the cloned nirK sequences had theoretical T-RFLP patterns that were also found in one or more electropherograms (data not shown). In silico cleavage of the three clones, which did not match any sequence in the NCBI database, resulted in T-RFLP patterns different from those found in the T-RFLP analyses (data not shown).
showed seasonal fluctuations in the population of denitrifying bacteria in a Norway spruce forest [17]. The seasonal variation was not significantly related to any single of the measured abiotic parameters (data not shown) and may be caused by the combined effect of seasonal changes in a number of measured and non-measured parameters.

It should be noted that replicate field sites were not included in this study. Hence, any difference among sites with different fertilizer treatment may be linked to a range of site-specific properties and not to the fertilizer per se. However, our statistical analysis showed that the populations differed significantly between the sites with the three groups of fertilizer types (control, mineral fertilizer, and cattle manure) investigated. For \textit{nirK}, all the pair wise ANOSIM tests showed a wide and significantly dispersion between the sites with the three types of fertilizer treatment. For \textit{nirS}, two of the three restriction digests showed three overlapping, but significantly disperse groups, while the last digest indicated very little dispersion. These results are in agreement with Bossio et al. [37], who found significant differences in the bacterial community composition between soil treated with mineral or organic fertilizer. For \textit{nirK}, the fertilizer type was a more important determinant of the population structure compared to the fertilizer amount. It seems that there may be a threshold, resulting in a community shift, and that further addition of nitrogen (in the range investigated here) may not be important. Avrahami et al. [15] observed a similar pattern in a laboratory study, showing that medium (58 \( \mu g \) \( NH_4^+ \)-N g \(^{-1} \)) and high (395 \( \mu g \) \( NH_4^+ \)-N g \(^{-1} \)) ammonium addition to a silt soil resulted in the same shift in denitrifying population, based on T-RFLP analysis of \textit{nirK}, compared to no or low (6.5 \( \mu g \) \( NH_4^+ \)-N g \(^{-1} \)) ammonium addition.

The developing view that diversity is likely to be functional significant (e.g. [1,11]) may imply that the observed differences in population structure result in differences in the nitrogen dynamics in the soils and that, for instance, the denitrification rate and N\(_2\)O emission may be altered. We observed a substantial difference in the ratio between nitrous oxide reductase activity and denitrification activity among sites (Table 1), which may influence N\(_2\)O emission rates from the soils. The change in ratio may be linked to the observed changes in community structure (Figs. 1 and 2) or directly to changes in abiotic parameters known to influence the ratio of nitrous oxide:nitrogen emission, like concentrations of soil nitrate and easily degradable organic carbon [3]. However, neither pH, concentrations of inorganic N or dissolved organic C differed between control soil and other soils.

The SIMPER procedure allowed us to identify specific T-RFs with large influence on the shifts in community structure. However, no specific T-RFs could be singled out as having a large influence on the observed shifts in the community structure between sites with different fertilizer type and amount. Instead, several (6–11) T-RFs combined to explain 60\% of the variation between fertilizer type and amount (Table 3). The SIMPER analyses indicated that the number of \textit{nirK} T-RF peaks responsible for the community shifts varied throughout the year, while no such variation in the number of T-RFs responsible for the community shift due to fertilizer type or amount were seen for either \textit{nirK} or \textit{nirS} (Table 3). We cannot explain this seasonal variation.

Phylogenetic analysis placed the majority of \textit{nirK} clones together with \textit{nirK} clones obtained from agricultural soil from Eberstadt, Germany [15], and from forested wetland soil and forested upland soil from Michigan, USA [34] in soil cluster I and III (Fig. 3). These clusters did not contain any known cultivated denitrifying bacteria, indicating that a pool of related yet uncultivated denitrifiers is present in soil from different places. In contrast, no \textit{nirK} clones closely related to marine sediment clones [36] or forest soil clones [39] in soil cluster II were found. One clone clustered together with \textit{Sinorhizobium meliloti}.

In conclusion, we observed a significant and distinct seasonal community shift for \textit{nirK}-containing bacteria, while \textit{nirS}-containing bacteria only were detectable in March. A significant but much smaller spatial variation was observed, which may have been caused by fertilizer type but not by fertilizer amount. The major part of the \textit{nirK}-containing bacteria in the investigated soils belonged to a yet uncultivated cluster of denitrifying bacteria.

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References


