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# Finding the missing link between diversity and activity using denitrifying bacteria as a model functional community

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The recent development and application of numerous methods mainly based on 16S rDNA analyses have brought insights into the questions of which and how many bacterial populations can be found in a given ecosystem. A new and challenging question for microbial ecologists has emerged from the exploration of this diversity: what is its significance for ecosystem functioning? We propose the denitrifying bacteria as a model microbial community for understanding the relationship between community structure and activity, and have summarized the recent progress in studies of this functional community.

## Addresses

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## Introduction

The advent of 16S rRNA gene-based techniques in the late 1980s paved the way for a flood of publications on bacterial diversity. The application of these methods provided new insights into the composition and structure of microbial communities in various environments, but the time has now come to address the significance of this microbial diversity. Basic issues such as the consequences of the loss or introduction of microbial populations for ecosystem functioning, or whether some of the microorganisms involved in the same process are redundant, remain unresolved. Analysis of the functional communities may be the key to a better understanding of these issues.

Denitrification, the anaerobic reduction of nitrate, nitrite and nitric oxide to nitrous oxide or nitrogen gas, is the major biological mechanism by which fixed nitrogen returns to the atmosphere from soil or water, thereby completing the N-cycle (Figure 1). This stepwise reduc-

tion is an alternative respiration pathway that is used in the absence of oxygen by phylogenetically heterogeneous microorganisms capable of many different functions of importance in ecosystems [1]. Most belong to various subclasses of Bacteria, although the ability to denitrify has also been found in some Archaea and in fungi. Owing to the high degree of taxonomic diversity among denitrifiers and to its role in the N-cycle, the denitrifying community might serve as a good model for investigating the value of microbial biodiversity in ecosystem functioning. This choice of the denitrifying community as a model for microbial ecology is strengthened by the facts that: most of the genes involved in the denitrifying process have already been described [2,3]; antibodies for the corresponding enzymes are available; and the activity of this community can easily be monitored.

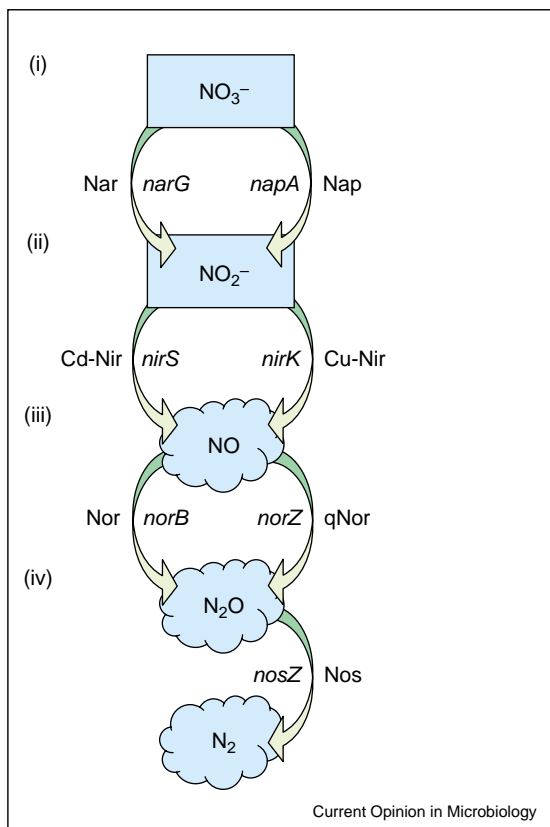
In this review, we highlight studies that have provided new insights into the composition, density or activity of the denitrifying community and might therefore contribute to our understanding of the relationship between denitrifying community diversity and activity (Figure 2).

## Denitrifier diversity – new approaches and new problems

With the development of direct DNA extraction, the focus has been on using denitrification genes as molecular markers to describe the diversity of the denitrifying community [4]. Although, as outlined below, this approach has led to new knowledge, primer and probe development remains an ongoing process. Accumulation of complete sequences from microbial genome projects or from newly isolated denitrifying bacteria is the best way to continually refine primers and probes [5].

Nitrite reductase, which catalyzes the reduction of soluble nitrite into gaseous nitric oxide, is the key enzyme in the denitrification process, so a primer system was first developed to amplify the *nirS* and *nirK* genes that encode the cytochrome *cd<sub>1</sub>* and copper nitrite reductases, respectively [6,7]. Different populations of denitrifying bacteria were successfully detected when these primers were applied to DNA extracted from aquatic habitats. Further work on marine sediments and soils showed a very high diversity of *nir* sequences with novel clusters, considerably different to the sequences obtained from isolated bacteria (see, for example, [5,8–10]). Parallel efforts were made to target the genes encoding the other denitrifying reductase [11–14,15]. These studies also revealed major gene clusters with little overlap with the clusters harbouring genes from isolated strains, thus confirming that most

Figure 1



Denitrification pathway. (i) Reduction of soluble nitrate to nitrite is catalyzed either by a membrane-bound (Nar) or a periplasmic nitrate reductase (Nap). (ii) Reduction of soluble nitrite to nitric oxide gas is catalyzed by either a copper (Cu-Nir) or a cytochrome  $cd_1$  nitrite reductase (Cd-Nir). (iii) Reduction of NO to  $\text{N}_2\text{O}$  is catalyzed by either a two-component type (Nor) or single-component type (qNor) of nitric oxide reductase. Lastly, (iv) reduction of nitrous oxide to dinitrogen is catalyzed by the nitrous oxide reductase (Nos).

of the denitrifiers present in the environment remain uncharacterized. A more systematic exploration of the denitrifying community was attempted by PCR amplification of the nitrite and nitrous oxide reductase genes with subsequent terminal restriction fragment length polymorphism (T-RFLP) analysis [16,17] and denaturing gradient gel electrophoresis (DGGE) [5<sup>\*</sup>]. Recent studies have simultaneously targeted genes encoding different reductases of the denitrification pathway [18,19]. To date, most effort has been put into studies of denitrifier diversity in soil and marine ecosystems.

Another approach used to characterize microbial communities and their function in the environment is DNA microarray technology, which is based on the DNA-DNA hybridization principle [20]. A gene array for assessing *nirS* and *nirK* diversity and distribution was first developed by Wu *et al.* [10]. To minimize the inconsistency among probe-target  $T_m$ s and to allow for similar

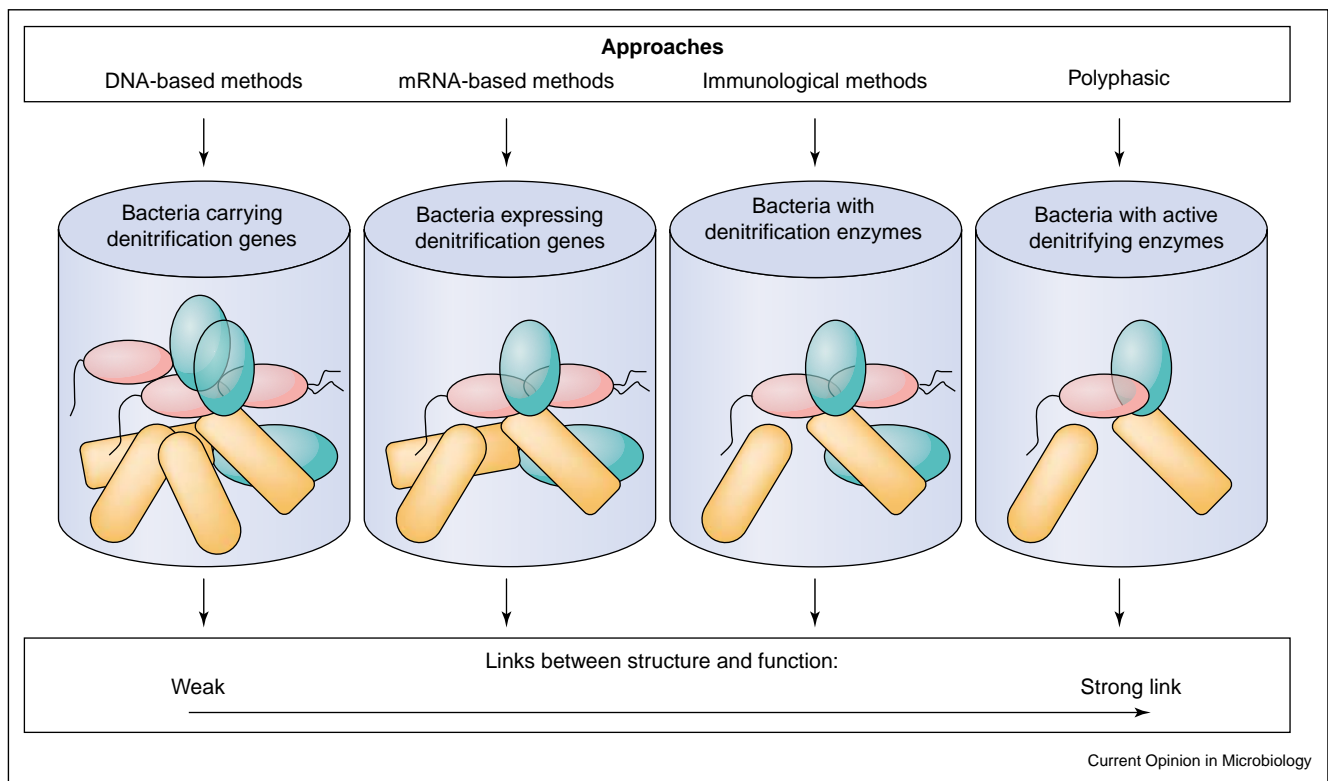
hybridization conditions across the array, Taroncher-Oldenburg *et al.* [21<sup>\*\*</sup>] designed 70-mer oligonucleotide microarrays for *nirK* and *nirS* and evaluated intragenic resolution for the latter. Results from the recent evaluation of a 50-mer oligonucleotide array suggest its possible use for the quantification of denitrification genes [22]. These attempts have demonstrated the potential of microarrays for environmental studies of denitrifiers, but further development is needed to improve sensitivity before they can be put into operation.

Although these new molecular techniques circumvent many of the problems associated with cultivation-dependent approaches, they also bring in other sources of bias [23]. An important ecological limitation with the DNA-PCR-based techniques is that the amplified genes might not be functional, whatever the environmental conditions. In addition, extra-cellular DNA can persist in soil for several months [24], resulting in a possible amplification of DNA from organisms no longer present. Despite these limitations, the molecular approach has proved powerful for evaluating the diversity of bacteria that are genetically capable of performing at least one step in the denitrifying pathway. However, the main ecological limitation of using molecular approaches to investigate a putative link between denitrifier diversity and activity is related to the fact that the denitrification pathway consists of four sequential steps. These steps are catalyzed by at least seven different enzymes encoded by seven different gene clusters. Therefore, even if several genes encoding the different reductases of the denitrifying process are used as molecular markers in the same study, no information can be deduced about the complete denitrifying reaction pathway of the targeted community. Reconstruction of this pathway seems impossible without a cultivation approach. A challenging task would be to integrate the diversity data obtained from the different denitrifying genes to reconstruct the actual denitrifying pathway in the environment. Such reasoned guesswork is necessary to further our understanding of the relationship between diversity and activity. Finally, the simple identification of denitrifying genes in populations is of limited value unless the role, if any, of such populations in the local or global denitrifying activity can also be determined.

### Denitrifier density, forget me not. . .

The next step in understanding the link between diversity and activity in denitrifying communities is to relate the molecular diversity analyses to the density of targeted populations. Mergel *et al.* [25] hybridized soil DNA with probes to detect denitrifying genes. These Southern hybridizations indicated that higher numbers of denitrifying bacteria occurred in the top layer of the soil. DNA-microarray methods have also been tested for the quantification of *nirS* and *nirK* genes [20,21<sup>\*\*</sup>,26], but their sensitivity still needs improvement to ensure better accuracy.

Figure 2



The possible link between microbial community and function can be explored at different resolutions depending on the approach used.

Although numerous studies have been published on the genetic diversity of denitrifying bacteria revealed by direct PCR-based techniques, only five reported the use of such an approach for their quantification. The first was a competitive-PCR (cPCR) for *nirS* developed by Michotey *et al.* [27]. When this method was applied to DNA extracted from marine sediment or water samples, 10–1000 times more cytochrome *cd<sub>1</sub>* denitrifiers were observed than could be estimated with a cultivation approach. The same method was later applied to quantify *nirS* denitrifiers in biofilms [28]. Real-time PCR was also applied to estimate *nirS* gene abundance in marine samples but the primers were too specific and only *nirS* from *Pseudomonas stutzeri* could be quantified [29]. Both competitive and real-time PCR have recently been developed to quantify the *nirK* gene in various environments [30,31].

The major limitation of these molecular quantification techniques is related to the gene copy number by genome, which makes it difficult to transform the obtained gene copy number into number of cells. However, for the denitrification genes, all known genomes are thought to carry only one copy, whereas *narG* varies between one and three. Another limitation is due to the fact that most of the denitrifying steps are catalyzed by different, but func-

tionally equivalent, enzymes. Thus, counting the bacteria that are genetically capable of performing just a single step will require at least two real-time PCRs. In order to understand the ecology of denitrifying bacteria, it is therefore urgent to develop independent methods for enumerating them.

### Denitrification activity – a step further with mRNA?

As stated before, the detection of a functional gene in the environment does not imply that the corresponding activity is present. However, the expression of denitrification genes can be detected by targeting their mRNA. Although researchers have shown that it is possible to extract RNA from various habitats [32], the successful amplification of mRNA from soils is still a challenge. Nevertheless, in 1997, Baumann *et al.* [33] used RNA probes to quantify expressed denitrifying genes in activated sludge, and then in 2002, Nogales *et al.* [34] was the first to report the detection of expressed nitrite and nitrous oxide reductase genes after mRNA extraction directly from environmental samples.

Compared to the eukaryotes, in which the mRNA half-lives are in the hour range, those of the prokaryotes are relatively unstable with half-lives of only a few minutes

[35]. A half-life of 13 minutes was reported in a study monitoring the kinetics of mRNAs for *cd<sub>1</sub>* nitrite reductase [36]. Similarly, the mRNAs for other denitrifying enzymes became apparent almost immediately after a switch to denitrifying conditions and then declined rapidly [37,38]. These data underline the difficulty of monitoring the mRNAs for denitrifying enzymes in the environment. We might also expect the half-life of mRNA to vary between the different bacterial groups within this functional community, suggesting the necessity to analyze a large number of samples over time.

Targeting the mRNAs for the denitrification enzymes will allow a more direct approach to denitrification activity and monitoring of the active denitrifiers, rather than mere indication of their presence. Thus, a comparison of the diversity of the denitrification genes amplified from DNA and mRNA will provide a means of distinguishing potential denitrifiers from those actually expressing their denitrification genes in the environment at the time of sampling. In highly diverse communities, it is easy to consider this diversity as redundancy. mRNA will be useful for determining which populations are active and whether this differs depending on the conditions. This would indicate that such populations are not redundant but complementary.

### Denitrifying enzymes: the tree hiding the forest

Proteins are better candidates than mRNA to relate community structure and function because they are at the end of the regulatory cascade, so any regulations of transcription, translation or post-translational steps will be taken into account. Only a few studies have reported the application of immunological techniques to target denitrifiers directly in the environment. Metz *et al.* [39<sup>••</sup>] developed new approaches to study the denitrifying populations expressing copper nitrite reductase *in situ*. The phylogenetic affiliation of the populations was determined by immunostaining with an antibody specific for the reductase, combined with 16S rRNA oligonucleotide probes, and flow cytometry to sort out the antibody-labelled cells. Recently, Maron *et al.* [40] proposed an approach to quantify the membrane-bound nitrate reductase in cells extracted from the soil by Nycodenz gradient with a detection limit of 2.65 µg enzyme cell<sup>-1</sup>.

Combining an immunological approach with other techniques could provide the best way to identify active denitrifiers in the environment. However, the detection of a given enzyme only provides a strong hint of the presence of the corresponding activity. A crucial question is how long the enzyme can be detected in the cell after its substrate has disappeared, and information on the stability of the denitrifying enzymes is lacking. Moreover, the specific activity of a given protein can vary depending on the strain. Nitrate reductase activity for *Pseudomonas*

strains, for example, ranged from 0.26 to 1.5 µM nitrite min<sup>-1</sup> mg of protein<sup>-1</sup> [41].

### The missing link between activity and diversity – to be or not to be

Denitrifying bacteria are present in environments such as soil, sediment, wastewater treatment systems, aquatic and marine habitats. However, they are not always denitrifying because their preferred electron acceptor is molecular oxygen. In general, dinitrogen and nitrous oxide emissions by denitrification from ecosystems are positively correlated to the amounts of nitrogen applied and carbon availability [42–44]. Moreover, rainfall, temperature shifts and various other events are often followed by peak nitrogen emissions.

Surprisingly, almost none of the existing models to predict denitrification, nitrogen loss and nitrous oxide emissions in soils take into account the main players in this process — the actual denitrifying bacteria. This implies that either the diversity and density of this functional group are assumed to be the same, whatever the environment, and therefore can be modelled as a constant, or denitrifier diversity and density are unimportant for predicting gaseous nitrogen emission. However, denitrifier populations differ in physiological properties, such as their affinities for electron acceptors and donors or the relative reaction rates of the reductive steps. Thus, the work by Cavigelli and Robertson [45,46], Holtan-Hartwig *et al.* [47,48] and Rich *et al.* [49] suggested that differences in the community composition of soil-denitrifying bacteria would explain differences in denitrification rates and N<sub>2</sub>O production.

### Conclusions

The link between microbial diversity, density and activity in the environment can be investigated by focusing on model functional communities. Possible candidates are the denitrifying bacteria, given that denitrification is a trait found in many taxonomic and physiological groups of bacteria. We are now able to target environmental bacteria with a genetic capacity to denitrify, to quantify them, to access the active bacteria and to measure their activity *in situ* or in the laboratory. The approaches described in this review should now be combined to see how diversity can be related to activity. New approaches, such as the combination of fluorescence *in situ* hybridization (FISH) with microautoradiography or stable isotope probing (SIP) analysis, are next in line for assessment [50<sup>•</sup>]. To link diversity and activity even closer, the future focus should be on the development of new protein-based techniques for detection and quantification of active denitrifying bacteria in the environment. The EU COST action 856 'Ecological Aspects of Denitrification, with emphasis on Agriculture' launched in 2002 has become an important European network that will be useful to fulfill these objectives.

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### Free journals for developing countries

The WHO and six medical journal publishers have launched the Access to Research Initiative, which enables nearly 70 of the world's poorest countries to gain free access to biomedical literature through the Internet.

The science publishers, Blackwell, Elsevier, the Harcourt Worldwide STM group, Wolters Kluwer International Health and Science, Springer-Verlag and John Wiley, were approached by the WHO and the *British Medical Journal* in 2001. Initially, more than 1000 journals will be available for free or at significantly reduced prices to universities, medical schools, research and public institutions in developing countries. The second stage involves extending this initiative to institutions in other countries.

Gro Harlem Brundtland, director-general for the WHO, said that this initiative was 'perhaps the biggest step ever taken towards reducing the health information gap between rich and poor countries'.

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