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Importance of denitrifying microorganisms in terrestrial ecosystems – focus on soil micromycetes

Bachelor thesis

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Anotation

This thesis summarizes the aspects of ecological importance regarding the denitrification of soil and discusses its origin in fungal denitrifiers. Also it describes the biochemical mechanism of fungal denitrification together with the options for denitrification measuring and micromycelial denitrifiers cultivation.

Bibliographic description

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Abstract

Denitrification, or the respiratory reduction of nitrate/nitrite up to nitrous oxide or molecular nitrogen, is an important biogeochemical process of the global nitrogen cycle. This process is mediated by various communities of microorganisms, including all three domains of life, since the bacteria, archaea, as well as eukaryotic fungi were proven to exhibit denitrifying activity. However, the bacterial denitrification is a thoroughly described process, unlike the fungal denitrification, which is about to be described in more detail yet.

Fungal denitrifiers might play an important role in the decrease of nitrate/nitrite level in terrestrial and aquatic ecosystems, since many of successfully isolated fungal genera were proven to possess functional denitrifying reductases. However, the majority of fungal denitrifiers do not possess the N_2O reducing enzyme (nitrous oxide reductase), so the main end product of their denitrifying activity is the nitrous oxide (N_2O) instead of N_2.

The N_2O is a potent greenhouse gas causing also severe damage to ozone layer. As the denitrification is a major source of global N_2O emissions, it is very important to understand the mechanisms and regulations of fungal denitrification processes.

Nevertheless many of the methods used for investigation of microbial denitrification are limited by several deficiencies, since they are usually based on direct cultivation or amplification/quantification in PCR cycles. Although, the metagenomic approach combined with active gene screening and NextGen sequencing seems to be a promising tool for the investigation of the new denitrifying microbial communities, as the metagenomics are cultivation-independent approach.

Aims of the thesis:

1. To gather knowledge concerning the process of denitrification with focus on denitrification of soil fungi.
2. To review experimental methods, which can be used in order to study the microbial denitrifying activity and to highlight the deficiencies of presently used approaches and methods.

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1 Introduction

The process of denitrification is an important biochemical process involved in the global nitrogen cycle (Fig 1). The denitrification was first reported by Kluyver and Donker (1926),

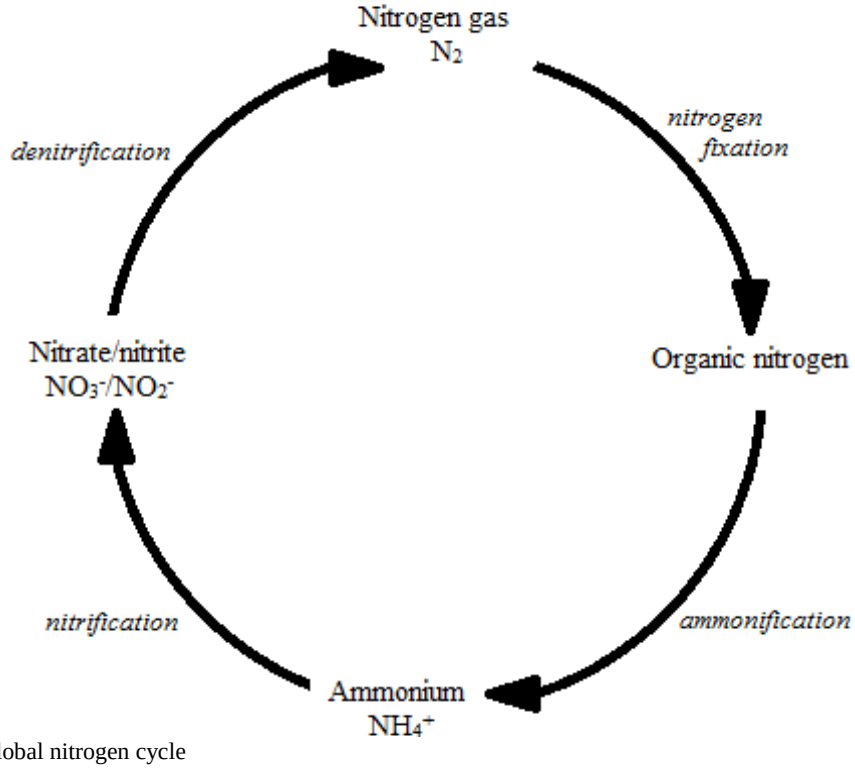

Figure 1: The global nitrogen cycle

who identified the denitrification as a possible way to maintain cellular respiration in bacteria during anaerobic conditions. However, it has been discovered quite recently that the denitrification can be performed not only by the bacteria, but also by fungi and archaea (Zumft, 1997; Shoun *et al.*, 1991, 1992; Tsuruta *et al.*, 1998; Cabello, 2004).

During the process of denitrification, the nitrate and/or nitrite is transformed into gaseous N_2O or N_2 via a series of reductive reactions catalysed by four denitrifying reductases (nitrate, nitrite, nitric oxide and nitrous oxide reductases). These enzymes use the electrons gained by the degradation of various organic compounds and pollutants representing a source of carbon in order to respire the oxidized nitrogen species, and therefore, to produce energy in the form of ATP (Beauchamp *et al.*, 1989; Zumft, 1997). This thesis describes the individual denitrifying enzymes, their catalytic mechanism, and compares the denitrifying reductases of the fungal and bacterial denitrifiers.

The denitrification was described to occur in various aquatic and terrestrial ecosystems. Spatially distributed models of the total global denitrification rates suggest that the aquatic ecosystems are the locations of highest denitrifying activity (88 %), followed by terrestrial

soils (22%; Seitzinger *et al.,* 2006). The terrestrial denitrification proceeds mainly at the aerobic and anaerobic interphase, where the fluctuating levels of accessible oxygen do not allow permanent aerobic respiration (Reddy *et al;* 1989). However, a strong denitrifying activity is often found in areas with high amounts of introduced nitrogen compounds such as fertilized fields and pastures. The denitrification of terrestrial microorganisms is also enhanced in often flooded places, like riparian and riverside soils, wetlands and rice fields (Vorhoeven *et al.*, 2006; Brůček, 2009; Jirout *et al.*, 2011). The denitrification of aquatic ecosystems proceeds particularly within heavily nitrate contaminated areas such as urban locations (Slater & Capone; 1987). The denitrification is also enhanced in the biochemically active stream sediments, poorly ventilated corners of the ocean, and in seafloor sediments. Also, there are some specific habitats of denitrification, such as digestive tracts of higher animals, earthworms, and insects (Karsten & Drake; 1997).

The terrestrial denitrification and particularly its rates are enhanced/inhibited by many external biotic and/or abiotic factors. This thesis summarizes the main abiotic factors having a significant influence to the terrestrial denitrification like soil moisture, temperature, or nitrate/nitrite concentration. However, the biotic factors, which are not mentioned in this work, such as the composition of microbial population or microbial competition also play an important role in the enhancement/inhibition of denitrifying activity. Although, the functioning of these factors, as well as the whole concept of denitrification regulation at the level of whole microbial populations, is still not fully understood and requires further elucidation.

The denitrification has important ecological implications, since this process ends in many cases by release of nitrous oxide, particularly in the case of fungal denitrification. One of the reasons is the N_2O depletes the stratospheric ozone. According to Intergovernmental Panel on Climate Change (IPCC; 2013), the estimated amount of N – N_2O released globally into the atmosphere from terrestrial ecosystems alone represents approximately 30–45 % of the total N_2O emissions. Thus the denitrification is considered as a very important process in the ecological sense, particularly for limiting the N_2O emissions and maintaining the climatic homeostasis, respectively.

Several approaches can be used for the measurements of denitrifying activity and discovery of new denitrifying species in various ecosystems. This thesis summarizes some of the methods based on direct cultivation, PCR, and ^{15}N isotopes, used in order to investigate the microbial denitrification together with their positive and negative aspects. Although the terrestrial denitrification is challenging process to measure precisely, primarily due to the

high microbial diversity (spatial and temporal) and high biotic and abiotic (temperature, oxygen, nutrients) gradients in soil environment.

Another particular challenge in denitrification measurements is that small areas (hot-spots) and brief periods (hot-moments) frequently account for increased nitrogen gas flux activity (Parkin; 1987). The hot-spot nature of denitrification process is understood as an important factor influencing the denitrification rates. However, the lack of understanding of these phenomena represents a huge obstacle in the *in situ* denitrification measurements and the modelling of biogeochemical cycles across varying spatiotemporal scales.

The improved preservation of viable denitrifying microbial communities under laboratory conditions by growth media may also reveal new valuable information about the principles and regulations of the process itself. However, the cultivation techniques have been used in various scientific branches of microbiology for decades, and they are still not able to permit the cultivation of the vast majority of living microorganisms (Doty *et al*; 2013). The growth media used in the studies of denitrifying micromycetes are also inside the scope of this work.

2 Microorganisms with denitrification ability

2.1 Bacteria

The denitrifying bacterial species were found within all major bacterial groups except for the *Enterobacteriaceae*, obligate anaerobes, and Gram-positive bacteria other than *Bacillus* spp. In fact, most of the denitrifying bacteria are heterotrophic facultative anaerobes taxonomically belonging into the various subclasses of the phylum *Proteobacteria*. However, there is no perceptible pattern of distribution of denitrifying genes among prokaryotic organisms (Zumft; 1997).

Denitrifying bacteria may prosper in many diverse habitats, although the biggest part of global bacterial denitrifying activity was found in aquatic ecosystems (particularly in continental shelf sediments; Seitzinger *et al.,* 2006). The largest denitrifying bacterial communities in terrestrial denitrification of soil are usually found in the rhizosphere (Starkey, 1958; Woldendorp, 1963).

The bacterial denitrification proceeds on the cytoplasmic membrane and in the periplasmic space. There the respiratory nitrate reduction generates proton-motive force by a redox loop mechanism in order to generate energy for the cell (Zumft, 1997; Cabello, 2004).

The bacterial denitrifying systems involve the complete reduction of nitrate up to molecular nitrogen. The following bacterial genera are just few examples, which were proven to exhibit an observable denitrifying activity: *Thiobacillus, Pseudomonas, Paracoccus, Bacillus, and Alcaligenes.* Moreover, the *Pseudomonas fluorescens* and *Alcaligenes* spp. have been reported as the numerically dominant bacterial denitrifiers worldwide.

However, not all of the denitrifying bacteria are able to convert the nitrous oxide into nitrogen gas. The truncated denitrification pathway, which ends with the release of N_2O, was described for *Corynebacterium nephridii*, *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, and most carboxidotrophic bacteria (Gamble *et al.*, 1977; Zumft, 1997).

2.2 Archaea

The major archaeal phyla crenarchaeota and euryarchaeota both contain species possessing genes encoding the nitrate and nitrite reductases. This statement was proven by the discovery of putative nitrate transporters and nitrate/nitrite reductases within various archaeal genomes (Cabello; 2004).

The crenarchaeota are mostly hyperthermophiles and anaerobic respirers, and euryarchaeota include methanogens and extreme halophiles. Thus, these organisms usually inhabit locations with extreme conditions, like hydrothermal vents, oil deposits, and hot springs (Woese *et al*., 1990; Brown & Doolittle, 1997).

The putative denitrifying mechanism of archaea (based on the sole model organimsm – *Pyrobaculum aerophilum*) involves reduction of nitrate to nitrogen gas similarly as bacteria. Although, the subcellular localization of archaeal denitrification might be slightly different, since the active site of nitrate reductase in *P. aerophilum* is assumed to be located at the exterior surface of the cytoplasmic membrane. However this mechanism would imply the coupling of this process to a yet unidentified proton-translocating complex (de Vries & Schröder, 2002; Cabello, 2004).

Respiratory nitrate reductases have been isolated from several denitrifying halophilic euryarchaeota, including the *Haloferax* species, but also within other members of the archaeal domain, like in the *Pyrobaculum* and *Haloarculum* genera. According to the study of Cabello (2004), some of archaeal denitrifiers are capable of conducting the complete denitrification pathway (from NO_3^- up to N_2), like *Haloferax denitrificans* and *Pyrobaculum aerophilum*. Although, some archaea such as *Haloarcula marismortui* and *Ferroglobus*

placidus are not able to reduce N_2O (Werber & Mevarech, 1978; Tomlinson *et al*., 1986; Völkl *et al*., 1993; Vorholt *et al*., 1997; de Vries & Schröder, 2002).

2.3 Micromycetes

The abundance of nitrite and nitrous oxide reductases was found to be relatively high among known fungal species, particularly in the centre of the group of *Fusarium* and its teleomorphs. Furthermore, the importance of fungal denitrification is enhanced by several studies proving that fungal denitrification works as a major process in the natural nitrogen cycle (Shoun *et al*; 1992, 2012).

Fungi and mainly saprotrophic basidiomyces inhabit locations of higher concentration of complex organic matter, which can be established within many different ecosystems. Thus, the micromycetes may be isolated from tropical, subtropical and temperate countries. Although they can be also found within unique habitats like cryopegs (lenses of non-freezing hypersaline water in ancient permafrost horizons) and permafrost Arctic sediments of different age (Lodge & Cantrell, 1995; Ozerskaia *et al.*, 2008).

The localization of respiratory reduction of oxidized nitrogen species within the fungal cell is different in comparison with prokaryotic denitrifiers. The denitrification of fungi was proven to proceed on the mitochondrial membrane. This was verified by the analysis of electron donors convenient for fungal denitrifying enzymes performed by Kobayashi *et al.* (1996). They shown that the electrons may be supplied to mitochondrial nitrate reductase by the mitochondrial electron transport system. Thus the reduction of NO_3^- in nitrate reducing fungi such as *F. oxysporum* results in gain of energy in the form of ATP (Kobayashi *et al.*, 1996; Shoun *et al*., 1989, 1992, 1998, and 2012).

However, the vast majority of fungal denitrifiers were proven to be incapable of nitrate reduction (Fujii & Takaya, 2008; Shoun *et al*., 2012). As the BLAST analysis conducted by Shoun *et al*. (2012) shown that none of the investigated fungal genomes contained a *narGHI* homologue, suggesting that the fungal-denitrifying systems containing NaR enzymes represent a minor part of the total fungal denitrifying species only. Thus nitrite becomes the primary substrate for fungal denitrification. However, the fungus *Fusarium oxysporum* and other filamentous fungi, make an exception to this statement, since they are able to synthesise a NADH – NaR enzyme encoded by *niaD* gene (Fujii & Takaya; 2008). Moreover, the most of denitrifying fungi do not possess the nitrous oxide reductase, thus the N_2O becomes the final product of denitrification in fungi (Fujii & Takaya; 2008).

The N_2O release, as the end product of denitrification, was found in the genera *Fusarium*, *Giberella*, *Trichoderma*, *Cylindrocarpon*, *Chaetomium*, *Penicillium*, *Aspergillus*, and

Hansenula and in other members of the Fungi Imperfecti, filamentous fungi, and yeasts. As the most of denitrifying fungi are not capable to form N_2 by reduction of N_2O. However it is presumed, that the nitrogen gas may be formed by a co-denitrification process.

The most efficient denitrifying fungi were found to be *F. oxysporum, F. solani, Chaetomium globosum, Neosartorya fisheri, Penicillium expansum, Trichoderma,* and *Talaromyces* species (Zumft, 1997; Kurakov *et al.,* 1998; Morozkina & Kurakov, 2007; Fujii & Takaya, 2008; Shoun *et al*., 2012).

3 Factors influencing the denitrification process

3.1 Oxygen availability and soil moisture

The oxygen accessibility is a crucial factor influencing denitrification as it has an inhibitory effect on nitrate respiration. The denitrification inhibition by oxygen seems to be specific for nitrate uptake (Hernandez & Rowe; 1987). Although, even if the denitrification is inhibited by the presence of oxygen a low amount of O_2 is required for fungal denitrification induction and cell growth (Zhou *et al*., 2001; Fujii & Takaya, 2008).

However, there are reports proving that some particular species (such as *Thiosphaera pantotropha*) are capable of simultaneous reduction of nitrate and oxygen, what was described as the aerobic denitrification (Robertson & Kuenen, 1984).

The oxygen availability is also tightly connected to the soil water content, since it is known that the increased soil moisture decreases the amount of biologically accessible oxygen, as the oxygen concentration is dependent on the diffusion ability of oxygen in water. Furthermore, the increased water content in soil improves the access of nutrients like nitrates, nitrites or other organic substances (Davidson & Swank; 1986).

According to Ruser *et al.* (2006) the soil moisture also influences the N_2O/N_2 ratio. During their measurements a rapid release of N_2O occurred at humid conditions, when a water-filled pore space (WFPS) reached ≥70%. The production of nitrogen gas was observed in the highest soil moisture level (≥90% WFPS) only but it was substantially smaller than the emission of N_2O.

3.2 Temperature

The temperature of the environment influences growth and the metabolic rates of microorganisms, thus it also influences the denitrification rates (Keeney *et al*., 1979; Maljanen *et al.*, 2009). The denitrification rates increase with the increasing temperature, until the optimum is reached at around 40 °C, then the denitrification rates are reduced as the temperature further increases. However, the optimal temperature for the majority of

denitrifiers highly varies according to individual organisms and their local environment (Powlson *et al*; 1988). The outer temperature limits measured during the terrestrial denitrification were detected below 0 °C at the lower end (Maljanen *et al*., 2009), and about 70 °C in the highs (Keeney *et al*., 1979).

3.3 pH

Soil acidity can also influence the denitrification rates in terrestrial ecosystems, since the denitrification is negatively affected by the increasing soil acidity. The optimal pH for most of microbial denitrifiers is in the neutral range, around pH 7. However the fungal denitrification becomes more relevant in highly acidic soils, where the terrestrial fungi can become dominant, as the soil micromycetes are much more acidity tolerant than bacteria (Robson & Abbott, 1989).

3.4 NO_3^- / NO_2^- concentration

Nitrate availability has been shown to be a stronger control on denitrification than microbial community composition. The reason might be that nitrate is used not only as the electron acceptor in the respiratory chain, but it is also needed for the synthesis of denitrifying reductases (de Vries; 2009).

Laboratory experiments proven that the rate of denitrification depends on the concentration of accessible nitrate. The reaction kinetics of nitrate respiration is influenced by the concentration of NO_3^- present. If the concentration is sufficiently high (about 2 – 5 $mg \cdot kg^{-1}$) the reaction is not dependent on the concentration of NO_3^- and it proceeds via zero order kinetics (Webster and Goulding; 1989). However, when the concentrations of NO_3^- are lower, the reaction rate is limited by the rate of NO_3^- diffusion into denitrification areas, which depends on the soil water content (Ottow *et al*; 1985).

4 Molecular basis of denitrification

4.1 Enzymes of the denitrification cascade

4.1.1 Nitrate reductase (NaR)

Nitrate reductase is the enzyme catalysing respiratory reduction of nitrate into nitrite. The nitrate respiring bacterium *Escherichia coli*, although it is not denitrifying bacterium, has provided the most information about the mechanism of nitrate respiration in bacteria (Zumft, 1997; Bedzyk *et al*., 1999).

The bacterial NaR is a membrane-bound enzyme using NADH as the direct reductant for the purpose of nitrate reduction. The process of electron transfer within the NaR enzyme is performed through all the main parts of the enzyme (flavin and central domains, as depicted

in Fig. 1) to the Molybdopterin domain, where the reduction of NO_3^- to NO_2^- takes place (Zumft, 1997; Bedzyk *et al.*, 1999; Campbell, 1999).

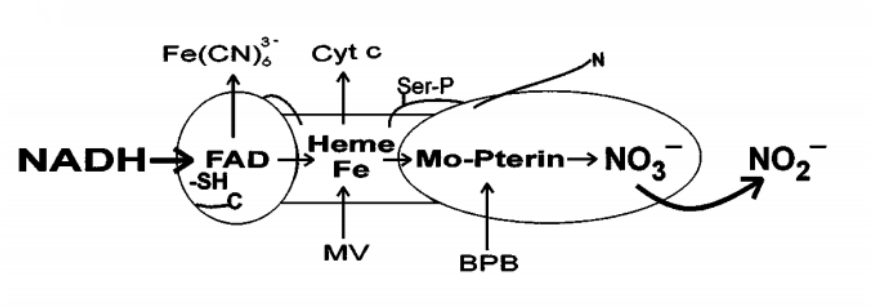

Figure 1: The mechanism of electron transport within bacterial NaR enzyme (taken from Campbell, 1999)

The isolated fungal respiratory nitrate reductases were not found to be orthologous to the bacterial nitrate reductases, suggesting that fungal NaR is evolutionarily distant from bacterial nitrate reductases. However, according to Kobayashi *et al*. (1996) the fungal nitrate reductase might use malate together with pyruvate as the electoron donors for the purpose of respiratory nitrate reduction, since they suggest that NADH alone is not a proper reducing substrate for mitochondrial NaR activity. Furthermore, according to the same study there might be a formate dehydrogenase system that is directly associated with the respiratory chain. This system may support the nitrate reduction, as the formate can serve as an electron donor to the mitochondrial NaR activity. Thus the fungal NaR might resemble NaR activity in *E. coli* and other denitrifying bacteria (Berks *et al.*, 1995; Moreno-Vivian *et al.*, 1999; Zumft, 1997; Kim *et al.*, 2009; Takaya, 2009; Kobayashi *et al.*, 1996).

4.1.2 Nitrite reductase (NiR)

The nitrite reductase is a protein responsible for reduction of nitrite into nitric oxide. It is the key enzyme of the denitrification process, since it catalyses the respiratory step leading to a gaseous intermediate (Zumft; 1997).

Two types of respiratory nitrite reductases can be isolated from the microbial denitrifers. A homotrimeric cytochrome cd_1 encoded by the *nirS* gene, or a copper-based NiR encoded by the *nirK* gene. This variant is better conserved and is used by eukaryotic denitrifiers such as fungi. Moreover, none of the denitrifying eukaryotes was found to harbor the cd_1 – NiR. (Zumft, 1997; Ward, 1995; Hallin & Lindgren, 1999; Kim *et al.*, 2009).

It was found that copper-based NiR enzyme can use azurin or pseudoazurin as the physiological electron donor in bacteria. However, the reaction mechanism of copper-based NiR is still not fully understood yet. The reaction mechanism is assumed to proceed via Cu^{+} - NO_2^{-} and Cu^{+} - NO^{+} complexes upon the connection of this enzyme with nitrite. The question remains whether nitrite is bound in the enzyme initially to Cu^{+} or Cu^{2+} (Zumft, 1997; Kim *et al.*, 2009).

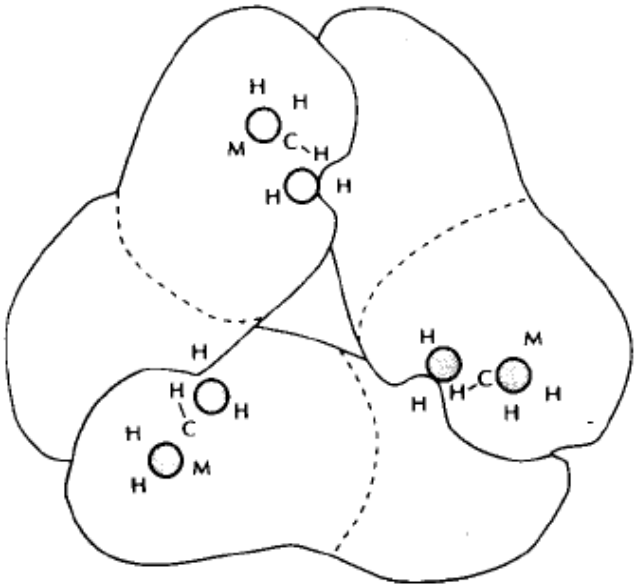

Figure 2: Schematic representation of trimeric Cu-NiR structure of *A. cycloclastes*. The circles represent the location of Cu ions coordinated by cysteine (C), histidine (H), and methionine (M) (taken from Adman *et al*; 1991)

It has been suggested that the fungal nitrite reductases, or at least the NiR protein isolated from bacteria and fungus *F. oxysporum* derived from a common ancestor. One of the reasons is that the fungus *F. oxysporum* synthesizes a small azurin-like protein for the purpose of electron release into the reaction of nitrite (Zumft, 1997; Kim et al., 2009). Thus it seems that the fungal nitrite reductase might resemble the catalytic mechanism of its bacterial homologues.

4.1.3 Nitric oxide reductase (NoR)

Prokaryotic respiratory NO reductases are membrane-bound protein complexes responsible for conversion of nitric oxide into nitrous oxide. The orthologues of NoR were found within various species of bacteria and archaea (Zumft, 1997; Cabello, 2004).

The catalytic mechanism of nitric oxide reductases is still not fully clarified. However, there are three putative mechanisms of NoR – NO complex formation and NO reduction described in the study of Moënne-Loccoz (2007; Fig. 3). Among the three possible catalytic mechanisms the *cis*-Fe_B mechanism was supported as the most probable since it does not proceed via the formation of a heme *b* $\{FeNO\}^7$ species, which is considered to be a potential dead-end product.

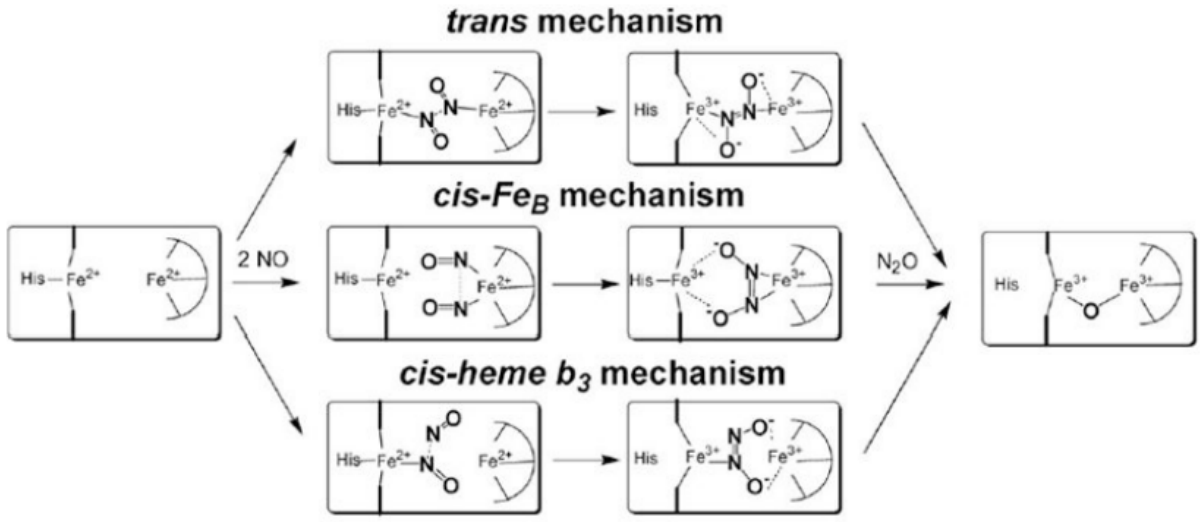

Figure 3: The possible mechanisms of nitric oxide reduction (taken from Moënne-Loccoz; 2007)

Denitrifying fungi use their own homologue of NoR for the purpose of NO reduction, a cytochrome called P450*nor*. The P450*nor* is a soluble monoprotoheme cytochrome containing a protoporphyrin IX with an iron ion in the active center. In addition to NoR activity, P450*nor* was recently proven to exhibit NADH-peroxidase activity, therefore it might serve as a multifunctional detoxifying enzyme (Kizawa *et al.*, 1991; Nakahara *et al.*, 1993; Shiro, 1999; Kobayashi *et al.* 1996; Zumft, 1997; Chao *et al.*, 2008; Shoun *et al.*, 2012).

The characteristically long helix, which is created by the distal part of P450*nor*, enables the direct access of electrons in the form of hydrides from NADH to P450*nor* – NO complex. This feature is not usual among the relatives of P450*nor*, because most enzymes belonging to the P450 superfamily use a flavoprotein reductase in order to transport the electrons into the active site of the enzyme. Furthermore, the P450*nor* of *F. oxysporum* exhibits a closer relationship to bacterial P450s than to eukaryotic P450s. This fact may point to the possibility that fungal NoR activity was acquired by horizontal gene

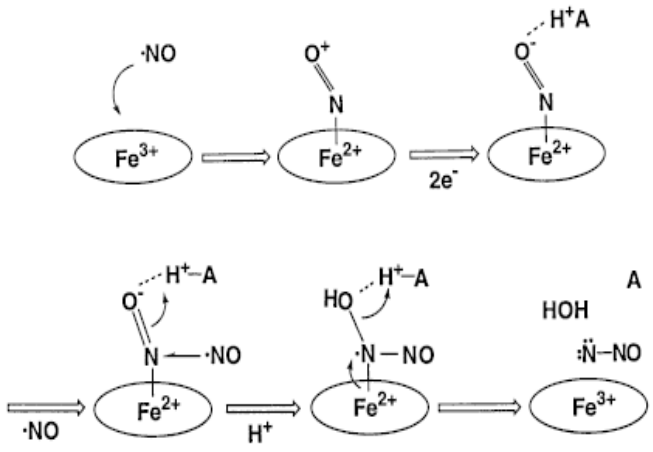

Figure 4: Putative mechanism of N_2O release (taken from Shiro; 1999)

transfer and subsequent modulation of bacterial P450 gene from actinomycetes (Nakahara *et al.*, 1993; Shiro *et al.*, 1995; Takaya & Shoun, 2000; Chao, 2008; Shoun *et al.*, 2012).

4.1.4 Nitrous oxide reductase

The N_2O reductase is the last enzyme of the denitrification cascade, which is responsible for reduction of N_2O into N_2. It was isolated from many Gram-negative denitrifiers, but no Gram-positive bacterium has been found to possess the N_2O reductase yet (Henry *et al*; 2006).

It is supposed that high percentage of denitrifying bacteria lack the *nosZ* gene, which is encoding the of N_2O reductase protein. Thus the end product of denitrification (N_2 or N_2O) is determined, irrespective of soil physicochemical characteristics, not only by the N_2O reductase regulatory mechanisms, but also by the occurrence of this enzyme among the individual denitrifying species (Zumft, 1997; Henry, 2006).

The prokaryotic N_2O reductase is a homodimeric multicopper protein composed of two domains, which both contain copper ions in their structure. The first domain, called Cu_A, is responsible for the uptake of electrons from the oxidized substrate (cytochrome c). The electrons are then transported into the second domain, called the Cu_Z, where the reduction of NO is performed (Zumft, 1997; Haltia *et al.*, 2003; Rasmussen, 2005; Henry *et al.*, 2006).

The denitrifying fungi do not possess functional N_2O reductases, however some of the denitrifying fungi were proven to release N_2. The co-denitrification process was found to be responsible for the fungal N_2O reducing activity, as the co-denitrification results in release of hybrid N_2 molecules (Zumft, 1997; Kumon *et al.*, 2002; Shoun *et al.*, 2012).

4.2 Co-denitrification

An unusual phenomenon, called co-denitrification, is often associated with the denitrification of fungi. This process is conducted by combining the nitrogen atoms coming from NO with the nitrogen coming from particular nitrogen donors, and results in formation of hybrid N_2 or N_2O species (Shoun *et al*; 2012).

The nitrogen donor seems to serve also as an internal electron donor for the reduction of NO, since the co-denitrification is independent on the presence of NADH as the reductant. Furthermore, the co-denitrification product was found to vary depending on the redox state of the nitrogen donor. It is presumed that the N_2 is formed from amines, whether the N_2O is formed by the use of imines or azides. However, it is still not known, whether the co-

denitrification product (N_2 or N_2O) is determined by the type of co-substrate or by the composition of denitrifying microbial community (Kumon *et al.*, 2002; Shoun *et al*., 2012).

The denitrifying fungi *Fusarium solani* and *Cylindrocarpon tokinese* were proven to evolve hybrid N_2 species. Nevertheless the fungus *Fusarium oxysporum* evolves just N_2O by co-denitrification. Thus it is suggested that the mechanisms of co-denitrification differ among the individual denitrifying species (Shoun *et al.*, 1992; Tanimoto *et al.*, 1992; Kumon *et al.*, 2002; Shoun *et al.*, 2012).

The co-denitrification was proven to be conducted by the P450*nor* enzyme. However, the denitrifying bacterium *Streptomyces antibioticus* was also shown to evolve small amount of nitrogen gas formed by the co-denitrification. Thus the co-denitrification of this bacterium remains to be clarified, because P450*nor*, which is known to be responsible for co-denitrification in fungi, was not found within any bacterium yet (Kumon *et al.*, 2002; Shoun *et al*., 2012).

5 Measuring the denitrification

5.1 Denitrification enzyme activity (DEA)

The DEA measurement method is a relatively undemanding procedure, which allows large numbers of samples to be run, thus it has been applied in many studies utilizing an acetylene block technique (Revsbech & Sørensen, 1990; von Rheinbaben, 1990; Payne, 1991; Aulakh *et al.*, 1992; Barton *et al.*, 1999).

Box 1: Principle of DEA

The method of DEA measuring is based on the cultivation of denitrifiers under isolated conditions, which are adjusted in order to eliminate the factors having a strong influence on the denitrification process. The sample, which may be enriched in nitrate, is sealed in order to establish anaerobic conditions. Then the sample is incubated under artificial atmosphere. The use of a so called acetylene block (ethyne gas introduced to the incubation inhibits the conversion of N_2O to N_2) enables the determination of N_2O concentration, as the end product of denitrifiaction in real time. The use of DEA approach without the use of acetylene is useful for determination of the ratio of N_2O/N_2. Moreover, in order to stop the expression and function of newly synthesised denitrifying reductases the chloamphenicol may be added to the incubation, so the determined product is the result of catalysis of working enzymes only (Gardner; 2008).

Parsons *et al.* (1991) used the DEA approach in order to investigate the loss of reactive nitrogen for agricultural soils. They found that the loss of nitrogen gas is strongly enhanced/inhibited by particular seasons or seasonal transitions (e.g., snowmelt, early spring) and seasonal events (e.g., litterfall, floods), with soils having the most frequent denitrifying activity in the spring. However, the increased DEA in this study often followed increases in soil moisture.

Myrold (1990) summarizes the previous studies using the acetylene block technique and isotope labelling methods and points out the deviation of results between these two methods,

which reached nearly 30 %. The acetylene inhibition method usually shows about 20 % faster denitrification activity than the method using labelled isotopes. That may be caused by the decrease in the number of electrons needed for the reduction of nitrate only into N_2O (Groffman *et al,* 2006).

Box 2: Determination of N_2O by gas chromatography

The gas chromatography combined with electron capture detector (ECD) is a well known method used for determination of N_2O concentration in gas samples. This method is accurate enough and relatively undemanding, therefore it is often used in rutine analysis. The measuring activity of ECD detector is dependent on the operating temperature and standing current in the detector. The optimal temperature of the detector for determination of N_2O is 250 - 300°C (Rassmussen, Krasnec and Pierotti; 1976).

On the other hand, it was proven that the acetylene block inhibits the process of nitrification, which is closely related to denitrification. That can cause the underestimation of denitrification rates in systems with small and/or dynamic NO_3^- pools, such as sediments. Thus, even if DEA measurements revealed a huge amount of valuable information about the rate and regulation of the denitrification processes in various ecosystems, the results of these analyses may be misrepresentative (Groffman *et al;* 2006).

5.2 ^{15}N isotope labelling

Various ^{15}N tracer methods were used in studies of denitrification in terrestrial and aquatic ecosystems. There are four most common approaches of tracing denitrification by labelled isotopes: isotope fractionation, isotope dilution, ^{15}N mass balances and the direct determination of gasses containing ^{15}N isotopes upon the addition of $^{15}NO_3^-/^{15}NO_2^-$. The latter method has been considered one of the best for soil investigations (Groffman *et al;* 2006).

Box 3: Principle of ^{15}N isotope labelling

The compounds ^{15}N labelled isotopes are utilized by microorganisms in the same way as the non labeled nutrients, which contain mainly ^{14}N isotopes. The ^{15}N isotope concentration can be measured after the addition of $^{15}NO_3^-$ / $^{15}NO_2^-$ prior to the incubation (Groffman *et al,* 2006). The labelled ^{15}N isotopes can be determined by gas chromatography separation followed by detection of atomic emission spectra. The abilities of photodiode array detector allow to determine more elements at the same time, if their specific emission spectra are in a range of 50 nm. Thus the compounds containing ^{14}N and ^{15}N isotopes can be measured at once, since their emission spectra are 420.17 and 421.46 nm respectively (Deruaz *et al.* 1995).

Global mass balance analyses performed by Seitzinger *et al*. (2006) suggest that the terrestrial denitrification must be the major global sink for anthropogenic nitrogen. Mulholland *et al.* (2008) used the data from ^{15}N tracer experiments representing several biomes. This study shown that total biotic uptake and denitrification of nitrate increase with increasing nitrate concentration, but that the efficiency of biotic uptake and denitrification decreases as the nitrate concentration increases. Furthermore Koba *et al.* (1997) used the ^{15}N natural abundance method in order to determine the intermittent occurrence of denitrification

in forested ecosystems. The data from this study showed that variations of NO_3^--N levels and $\delta^{15}N$ value are useful indicators for clarifying the soil nitrate/nitrite dynamics as affected by water mixing, plant uptake, nitrification, and denitrification in forested ecosystems.

However, the use if this method is limited by the labouring procedures and expensive required instrumentation in comparison with acetylene inhibition method. Furthermore, the addition of $^{15}NO_3^-/^{15}NO_2^-$ to establish high levels of ^{15}N enrichment increases the nitrogen availability and may result in an overestimation of denitrification in systems with N-limitation (Groffman *et al;* 2006).

5.3 Quantitative Polymerase Chain Reaction

Quantitative PCR (qPCR) is suitable for quantification of specific microbial functional guilds including microbial denitrifiers based on functional genes such as *narG* (López-Gutiérrez *et al;* 2004), and *NirK* (Henry *et al;* 2004). In contrast to quatification of 16S rDNA, the *NirK* gene copy number can be directly correlated to cell numbers since only one copy of the *NirK* gene has been identified in denitrifying bacteria (Philippot, 2002).

Box 4: Principle of qPCR

The Polymerase chain reaction is the most commonly used method in molecular biology. It is based on multiple replication of short DNA sequences. The chain reaction itself is performed in cycles, where denaturation (separation of dsDNA) is followed by annealing of primers and synthesis of new DNA. This cycle can proceed thanks to sudden temperature changes. The qPCR method is based on the same principle, the only difference is that the amplification of DNA sequences is recorded in real time. There are two most common methods used for quantification of such results. The first one is based on the use of nonspecific fluorescent dyes, such as SYBR Green, which binds to all DNA strands. The second option is the use of sequence specific DNA chains containing the dye, which can be detected after hybridization with target DNA molecule. By use of a proper detector, the increase of amplified sequences quantity can be measured after each cycle. (Vandesompele; qPCR guide).

The quantitative *nosZ* PCR assay, which was conducted in the study of Henry *et al.* (2006), showed that the N_2O reducing denitrifying bacteria represented less than 5% of the total bacterial communities in the studied soils. However, these results may be misrepresentative, as the PCR-based approaches are limited by demands on the use of specific primers.

Moreover, this method´s biggest disadvantage is the dependence on the recognition of the amplified sequence, which is necessary for the construction of a proper primer. Furthermore, even if this method is capable of the precise arbitrary gene quantification, it is unable to provide any information about the composition of microbial communities (Henry *et al*; 2004).

5.4 Terminal restriction fragment length polymorphism (T-RFLP)

The T-RFLP method can be used for the detection of microbial community diversity and was proven to be sufficient for examining of the new denitrifying genes. The T-RFLP analysis has been used to characterize a diversity of 16S rDNA genes as well as functional genes such as *narG* and *nosZ* (Avrahami *et al.*, 2002; Braker *et al.*, 2001; Scala *et al.*, 2000).

Box 5: Principle of T – RFLP method

The principle of this method is to measure of the degree of polymorphism of terminally restricted DNA fragments (Marsh; 1999). The ends of PCR products are labelled by fluorescent stain on the primers. This modified DNA is then amplified and subsequently the DNA molecule is cut by the restriction enzymes to form the individual terminal restriction fragments. These fragments can be separated either by classical gel electrophoresis or by automated capillary electrophoresis with fluorescent detection system. The profile of microbial community in the tested sample comes as an output from this method, which can be then compared with the database. (Jonátová; 2010).

This method is necessarily not dependent on the cultivability of investigated organisms under laboratory conditions. Moreover, thanks to its reproducibility and the possibility of automation, this method seems to be suitable for the routine analysis of a big number of samples (Groffman *et al*, 2006).

Analysis of similarities in T-RFLP patterns showed a significant seasonal shift in the bacterial community structure of ecosystems containing the *NirK* gene possessing species. On the other hand, the bacterial denitrifiers possessing the *NirS* gene could be isolated only within particular seasonal period (March). Moreover, the investigated sites treated with mineral fertilizers or cattle manure showed different communities of *NirK* containing bacteria, since the T-RFLP patterns of such treated soils were significantly different (Wolsing & Priemé; 2004).

However, Smalla *et al.* (2007) states the major disadvantages of this method are possible imperfect digestion or the need for additional purification steps. Also, the resulting separated peaks in the chromatographic output can represent many Terminally Restricted Fragments (T-RFs), which differ in few base pairs only.

5.5 Denaturation gradient gel electrophoresis (DGGE)

Although the DGGE is a relatively new approach, it was shown to be sufficient for the investigation of *nirK* and *nosZ* genes (Thrönback *et al*; 2004). Furthermore, the combination of DGGE with other laboratory approaches enables a phylogenetic classification of the isolated genotypes by cutting the bands out of the gel, reamplification, cloning and sequencing (Smalla *et al*; 2007).

Box 6: Principle of DGGE

The DGGE method is used for separation of the same size amplified DNA sequences. The separation of these fragments is based on their different composition. Strictly speaking the separation is based on the ratio of base pairs present in the DNA strand (Guanine - Cytosine / Adenine - Tyrosine), because the fragments containing more Adenine – Tyrosine bases are falling apart faster, so they are passing through the gel slower. Theoretically the every single band on the gel represents a single organism. These bands can be visualised by staining with SYBR Gold, which fluoresces under the UV light, or the gels can be stained by silver for example (Thrönback *et al.* 2004).

Enwall, Philipot and Hallin (2005) used DGGE, together with cloning and sequencing, of *nosZ* gene in their approach dealing with long-term fertilization effects on denitrifying communities. They found out that potential denitrification and soil respiration rates increase in the field plots treated with organic fertilizers, similarly as Rochette *et al*. (2000). The study also shown that higher denitrification rates correlate with the total organic carbon concentration in the ecosystem.

However, the study of Thrönback *et al.* (2004) shown that the examination of *nirS* genes by DGGE could not be successfully performed. That was probably caused by either by a high number of melting domains contained in the specific fragment of this gene, or due to the bad resolution of this fragment.

5.6 Metagenomic approach

The metagenomic approach enables the analysis of the whole microbial community composition. This process is based on the direct sequencing of the sample containing a mixture of various nucleic acids coming from diverse organisms. This enables the better understanding of the functions of microbial communities at the level of ecosystems under given conditions, and to better understand the regulatory mechanisms influencing the whole denitrifying ecosystems (Handelsman; 2004).

Box 7: Principle of pyrosequencing

The pyrosequencing is the basic principle of many NextGen sequencing technologies. Including the 454sequencer that was the first to achieve commercial introduction (2004). The pyrosequencing method is based on stepwise incorporation of single DNA nucleotides, what leads to release of pyrophosphate. The pyrophosphate initiates a series of reactions leading to production of light by a luciferase enzyme. This feature allows the 454 base recognition software to calibrate the light emmited by the incorporation of every nucleotide. Although, the calibrated base calling is not able to interpret the long stretches of the same nucleotides (>6), thus these sequences are prone to base insertions and deletions during base recognition. However, because the each incorporation step is nucleotide specific, substitution errors are rarely encountered in 454 sequence reads (Mardis, 2008; Přistoupilová, 2008).

High-throughput sequencing methods are not dependent on the cultivability of investigated organisms and it is not necessary to know the requested analyzed gene sequence in advance. Thus, the metagenomic approach combined with active gene screening and NextGen sequencing could reveal new knowledge concerning the yet uncultured denitrifying microbial communities. Furthermore, the NextGen sequencing eliminates the need for DNA fragment cloning into *E. coli*, and it is more than ten times cheaper and less time consuming than the Sanger sequencing method (Mardis, 2008; Přistoupilová, 2008; Demanéche *et al.*, 2009).

On the other hand, Demanéche *et al.* (2009) underlined the difficulty of phylogenetic affiliation of the denitrification genes due to the lack of congruence between the denitrification genes and 16S rRNA trees.

6 Cultivation of denitrifying micromycetes

A large set of denitrifying isolates has been obtained up to date, what supports the research concerning denitrification. Although, this is not fully true for fungal denitrification, since the knowledge concerning fungal denitrification is highly limited in comparison with its bacterial counterpart. So as are the databases of fungal denitrification genes, which are restricted by the limited number of isolated organisms.

The cultivation of denitrifying fungi requires specific cultivation conditions. As the denitrification is an anaerobic process, it is needed to reduce pressure of oxygen during the cultivation. The establishment of the artificial atmosphere during the cultivation can be achieved by flushing with argon, helium or even carbon monoxide (Shoun & Tanimoto, 1991; Lavrent'ev *et al.*, 2008). The temperature is also important for the cultivation of denitrifying microorganisms. The bacterial isolates favor the incubation around 30-37°C, but the fungi require lower incubation temperature (about 26-30°C; Shoun *et al.*, 1989; Shoun *et al.*, 2012). The pH of the growth medium may significantly influence N_2O emissions as well, since the media are acidified by addition of nitrate/nitrite. The reason is that acidic conditions may lead to chemical reactions producing N_2O, thus use of a buffer solution (such as phosphate buffer) is advantageous (Lavrent'ev *et al.* 2008).

Even though the cultivation of microorganisms based on the use of growth media has been used for many decades, and new growth media are able to favor the growth of denitrifiers exhibiting high natural diversity. This technique is still not able to maintain the viability of most organisms. Furthermore, the cultivation of denitrifying micromycetes may be significantly influenced by problems related with soil sampling. The limited cultivation abilities of presently used growth media represent a huge limitation in the research of microbial denitrifiers (Heylen *el al;* 2006).

Box 8: Growth media used for cultivation of denitrifying micromycetes

Various culturing media were used in the past studies of denitrifying fungi. Kurakov *et al.* (1998) successfully used a liquid Czapek medium for precultivation of different fungal strains belonging to classes of Zygomycetes, Ascomycetes and Deuteromycetes. Subsequently, the precultured samples can be examined for purity from bacteria on water agar, meat-peptone agar and glucose-peptone medium.

Hawksworth *et al.* (1995) ascribed the cultivated fungal strains to certain systematic groups by using the malt, Czapek, and corn extract media.

Shoun and Tanimoto (1991) successfully used a liquid glucose-peptone (GP) medium for the cultivation of denitrifying fungus *F. oxysporum*. This medium was also used in the study of Lavrent'ev *et al.* (2008), who used glucose-peptone agar and soil agar as the solid nutrient media.

The so-called mix-culturing (MC) medium used in the study of Liu *et al.* (2006) can be used to cultivate fungal and bacterial denitrifiers together. This is a novel approach, which could reveal valuable information about the cooperation of fungi and bacteria in denitrification.

7 Conclusion

The denitrifying mechanisms are widespread among various microbial prokaryotic and some eukaryotic species such as fungi. Fungal denitrification is considered as an important part of the natural nitrogen cycle, as the denitrificatoin of terrestrial fungi is assumed to be important contributor to global N_2O emissions, and the decrease of global N_2O emissions is crucial for maintaining the climatic homeostasis. Thus, the understanding of fungal denitrifying mechanisms, and particularly its regulatory mechanisms, should be a matter of high interest, since the tendency toward acidification in soil might potentiate the activity of fungi. However, the knowledge concerning the fungal denitrification is still not known to any great extent in comparison with its bacterial counterpart.

The accurate quantification of terrestrial nitrogen cycling is highly important for future studies of denitrification. However, the DEA method, which revealed the most of denitrification rates estimates within various ecosystems, was proven to produce inaccurate results. Although, the increased accuracy might be produced if the results of N_2 and/or N_2O flux measurements could be compared with the outputs of stable isotope approaches, and if such multiple models would be applied at sites with well established mass balances. This approach might allow the comparison of different methods and models with/against each other, thus quantifying the nitrogen cycling more precisely.

Moreover, the metagenomic approach combined with active gene screening and NextGen sequencing might be a useful tool for identification of the various denitrifying communities and for investigation of the denitrification process itself. Since the metagenomic approach enables the analysis not only of the identity, but also functional abilities, of the yet uncultured microorganisms. Thus the metagenomic approach may enable better understanding of the functioning of microbial communities at the ecosystem level under given conditions. Also, this approach may reveal new, yet undiscovered, genome sequences that could be helpful for construction of new primer sets, thus a wider diversity of fungal denitrifiers might be covered. Therefore the reach of the PCR dependent approaches (such as qPCR) would be improved. This approach might be helpful for the investigations of denitrification and its regulation, as the denitrifying reductases harbored by yet undiscovered denitrifying species might be further examined by PCR-based approaches.

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