Metagenomics of the Microbial Nitrogen Cycle

Theory, Methods and Applications

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The nitrogen (N) cycle is one of the most important nutrient cycles in the earth and many of its steps are performed by microbial organisms. During the cycling process greenhouse gases are formed including nitrous oxide and methane. In addition, the use of nitrogen fertilizers increases freshwater nitrate levels, causing pollution and human health problems. A greater knowledge of the microbial communities involved in nitrogen transformations is necessary to understand and counteract nitrogen pollution.

Written by renowned researchers specialised in the most relevant and emerging topics in the field, this book provides comprehensive information on the new theoretical, methodological and applied aspects of metagenomics and other 'omics' approaches used to study the microbial N cycle.

Recommended for microbiologists, environmental scientists and anyone interested in microbial communities, metagenomics, metatranscriptomics and metaproteomics of the microbial N cycle. This volume provides a thorough account of the contributions of metagenomics to microbial N cycle background theory, reviews state-of-the-art investigative methods and explores new applications in water treatment, agricultural practices and climate change, among others.

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Quantification of Functional Microbial Nitrogen Cycle Genes in Environmental Samples

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Abstract

The nitrogen (N) cycle comprises a large number of oxidative and reductive reactions that are catalysed by wide variety of enzymes. Genes coding for most of the N-cycle enzymes have been shown to be present in a diverse polyphyletic group of microorganisms, including bacteria, archaea and fungi. Therefore, a 16S rRNA phylogeny-based approach to study those microbial populations is not possible. Because cultivation-dependent methods are selective for certain microorganisms, molecular methods have been developed to study the ecology and to assess abundance and diversity composition of nitrogen cycling microorganisms in environmental samples. DNA extraction followed by PCR amplification of genes that encode key functional enzymes of the N-cycle are used to study which genes and/or phylotypes are functionally important in the environment. Methods for DNA isolation and purification from environmental samples will be addressed whilst considering the main functional gene targets used to study the nitrogen fixation, nitrification and denitrification processes within the nitrogen cycle. The fluorescence-based quantitative real-time polymerase chain reaction (qPCR) has proven useful for quantification of nucleic acids in samples obtained from numerous diverse sources. Here, we describe relevant experimental conditions for utilization of qPCR to quantify the 16S rRNA, *amoA* and *nar*/*nap*, *nirK*/*nirS*, c-*nor*/q-*nor* and *nos* genes that encode synthesis of key enzymes involved in redox reactions of the N-cycle.

Introduction

Nitrogen (N) is a key element for all forms of life as it makes part of essential compounds such as proteins, nucleic acids, hormones, etc. Despite its abundance in the atmosphere (~ 80%), availability of N in a form suitable for plant and animal consumption is a major constraint to life on our planet. Most of the N in the atmosphere is found in the form of dinitrogen gas (N_2), which is inaccessible to eukaryotes and many bacteria. In the biogeochemical N cycle, N utilization begins with the conversion of bio-unavailable N_2 gas to bio-available ammonia (NH_4^+) accomplished by the so called diazotrophs, either in free-living or in symbiotic associations (with plants or other organisms). This process is called biological N_2-fixation and has a central role in nitrogen availability, and thus in supporting life on earth.

N cycle

Biological nitrogen fixation

The conversion of dinitrogen into ammonia is catalysed in all diazotrophs by the nitrogenase enzyme complex in an ATP-dependent manner. Nitrogenase is composed by two components that are named according to their main functional subunits, dinitrogenase reductase (Fe protein) and dinitrogenase (Mo–Fe protein). The enzyme is encoded by the *nif* (H, D, K, Y, B, Q, E, N, X, U, S, V, W, Z) genes, of which the *nifDK* genes are structural genes that encode the NifD/K (α and β subunits of the dinitrogenase) and the *nifH* gene codes for the γ2 homodimeric azoferredoxin subunit NifH of the nitrogenase complex (Dixon and

Kahn, 2004). The nitrogenase enzyme complex is highly sensitive to oxygen, due to the fact that oxygen reacts with the iron component of the proteins. Nevertheless, the free-living aerobic bacteria have developed several mechanisms to overcome such limitation in soils, for example by maintaining a very low level of oxygen in their cells or by producing extracellular polysaccharides (Dalton and Postgate, 1969; Yates, 1970). In the symbiotic N_2-fixing organisms, such as *Rhizobium*, the plant leghaemoglobin regulate the supply of oxygen to the nodule tissues in order to maintain the low oxygen level within the nodules (Fischer, 1994; Downie, 2005; Oldroyd and Downie, 2008). Ammonia produced by biological N_2-fixation is subsequently incorporated into cellular biomass mainly via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway. Alternatively, glutamate dehydrogenase (GDH) may also be involved in aerobic ammonium assimilation.

Nitrification

In addition to its incorporation into organic nitrogen compounds, ammonia can be oxidized to nitrate (NO_3–) by nitrifying (ammonia oxidizing, AO) Archaea (AOA) and Bacteria (AOB) in a two-step process called nitrification. First, the transmembrane Cu-containing enzyme ammonia monooxygenase catalyses the oxidation of ammonium to hydroxylamine taking two electrons directly from the quinone pool. In the second step, a trimeric multihaem c-type hydroxylamine oxidoreductase (HAO) converts hydroxylamine into nitrite in the periplasm. Then, nitrite is oxidized to nitrate by the membrane-associated iron–sulfur molybdoprotein nitrite oxidoreductase (NXR). The *amoA*, *amoB* and *amoC* genes in AOB comprise the functional *amo* operon (Sayavedra-Soto *et al.*, 1998; Hommes *et al.*, 1998; Norton *et al.*, 2002) and homologue genes have been found in in Archaea (Könneke *et al.*, 2005; Treusch *et al.*, 2005), but these genes have low similarity to their bacterial counterparts.

A major recent discovery in relation to the nitrification process has been the role of Archaea in ammonia oxidation (Könneke *et al.*, 2005; Treusch *et al.*, 2005; Wuchter *et al.*, 2006), which has led to study the presence of ammonia oxidizing Archaea and Bacteria in many ecosystems (Leininger *et al.*, 2006; Nicol *et al.*, 2008; Di *et al.*, 2009; Vissers *et al.*, 2013; Peng *et al.*, 2013). Because of the essential role of Amo in ammonia oxidation, Amo-encoding genes are excellent molecular markers to study the occurrence of Archaea and Bacteria in different environmental conditions, the *amoA* gene being most often used for this purpose (Rotthauwe *et al.*, 1997; Purkhold *et al.*, 2000; Francis *et al.*, 2005).

Denitrification

Finally, denitrification transforms nitrate into N_2 which returns to the atmosphere, thus closing the N cycle in the biosphere. From the biochemical point of view, denitrification is an alternative form of respiration by which nitrate is reduced sequentially to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and finally nitrogen gas, when oxygen becomes limiting (Zumft, 1997; van Spanning *et al.*, 2011; Bueno *et al.*, 2012). The denitrification pathway is performed by more than 60 bacterial genera, and there are evidences that some fungi (Takaya *et al.*, 2002; Prendergast-Miller *et al.*, 2011), Archaea (Treush *et al.*, 2005), and Foraminifera and Gromiida (Risgaard-Petersen *et al.*, 2006; Piña-Ochoa *et al.*, 2010; Koho *et al.*, 2011) are also able to denitrify. During denitrification, reduction of nitrogen oxides is coupled to energy conservation and allows cell to grow under microoxic or anoxic conditions (Zumft 1997; Simon *et al.*, 2008; van Spanning *et al.*, 2011).

Respiratory nitrate reductases

The first reaction of denitrification, this is the conversion of nitrate to nitrite, is catalysed by two biochemically different enzymes, a membrane-bound nitrate reductase (Nar), or a periplasmic nitrate reductase (Nap). Nar enzymes are integral membrane proteins encoded by genes of a well conserved *narGHJI* operon (see reviews by van Spanning *et al.*, 2007; Richardson, 2011; Bueno *et al.*, 2012). Whereas *narGHI* encode the structural subunits, *narJ* codes for a cognate chaperone required for the proper maturation and membrane insertion of Nar. *Escherichia coli* has a functional duplicate of the *narGHJI* operon named *narZYWV*, which physiologically has a function during stress response rather than

anaerobic respiration. In some archaea and bacteria the NarGH subunits are on the outside rather than the inside of the cytoplasmic membrane. This enzyme is supposed to be the evolutionary precursor of the Nar system (Martínez-Espinosa *et al.*, 2007). The Nar enzyme couples quinol oxidation with proton translocation and energy conservation. This respiratory function permits cell growth under oxygen-limiting conditions (Potter *et al.*, 2001; Simon *et al.*, 2008).

Nap is widespread in all classes of denitrifying and non-denitrifying proteobacteria (reviewed in Jepson *et al.*, 2007; Richardson *et al.*, 2007; Richardson, 2011; Sánchez *et al.*, 2011; Bueno *et al.*, 2012). Nap is a two-subunit enzyme, NapAB, with the catalytic subunit NapA located in the periplasm. Eight different genes have been identified as components for operons that encode Naps in different organisms. Most bacteria studied thus far have the *napABC* genes in common. Although Nap is also linked to quinol oxidation, it does not synthesize ATP (Simon *et al.*, 2008). Physiological functions for Nap systems include the disposal of reducing equivalents during aerobic growth on reduced carbon substrates and anaerobic nitrate respiration as a part of bacterial ammonification or denitrification pathways (Potter *et al.*, 2001).

Respiratory nitrite reductases

Two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirS and NirK (for reviews see Rinaldo and Cutruzzola, 2007; van Spanning *et al.*, 2007; Rinaldo *et al.*, 2008; Bueno *et al.*, 2012). They are located in the periplasmic space and catalyse the one-electron reduction of nitrite to gaseous nitric oxide, but neither of the enzymes is electrogenic.

The cd_1 NirS nitrite reductase is encoded by the well-characterized gene clusters from *Pseudomonas aeruginosa* (*nirSMCFDLGHJEN*) and *Paracoccus denitrificans* (*nirXISECFDLGHJN*). In the model denitrifier *Pseudomonas stutzeri* there are two *nir* clusters (*nirSTBMCFDLGH* and *nirJEN*). In contrast to the complex organization of the genes encoding the NirS proteins, the Cu-NirK enzyme is encoded by the *nirK* gene (Rinaldo and Cutrozzola, 2007; Bueno *et al.*, 2012). Although both Nir enzymes are widespread among denitrifiers, there is no evidence that the same species could have both enzymes (Zumft, 1997), and they are unrelated in terms of evolution (Heylen *et al.*, 2006).

Respiratory nitric oxide reductases

The third reaction of denitrification is the reduction of the nitric oxide to the nitrous oxide catalysed by nitric oxide reductase (Nor). Three types of Nor have been characterized, cNor, qNor, and qCuANor (reviewed in Zumft, 2005; de Vries *et al.*, 2007; van Spanning *et al.*, 2005, 2007; van Spanning, 2011; Bueno *et al.*, 2012). The cNor is an integral membrane enzyme encoded by the *norCBQD* operon. The *norC* and *norB* genes encode subunit C and subunit B, respectively, and the *norQ* and *norD* genes encode proteins essential for activation of cNor. Some denitrifiers have additional *norEF* genes, the products of which are involved in maturation and/or stability of Nor activity (Hartsock and Shapleigh, 2010). As a unique case, the Nor of *Roseobacter denitrificans* is similar to cNor, but differs in that it contains copper (Matsuda *et al.*, 2002).

The qNor is a single-subunit enzyme that uses quinol or menaquinol as electron donors and is encoded by the *qnorB* gene. The enzyme has been found not only in denitrifying archaea and soil bacteria (Büsch *et al.*, 2002), but also in pathogenic microorganisms that do not denitrify (Hendriks *et al.*, 2000; de Vries *et al.*, 2003). qNOR is proposed to be the ancestor of all nitric oxide reductases and cytochrome oxidases belonging to the superfamily of haem-copper oxidases (de Vries and Schröder, 2002). The qCuANor has been described in the Gram-positive bacterium *Bacillus azotoformans* (Suharti *et al.*, 2004), but genes encoding qCuANor have not been identified as yet.

Respiratory nitrous oxide reductase

Periplasmic nitrous oxide reductase enzymes (Nos) catalyse the two-electron reduction of nitrous oxide to N_2, the final step in denitrification (reviewed in van Spanning *et al.*, 2005; 2007; Zumft and Kroneck, 2007; van Spanning 2011; Bueno *et al.*, 2012; Pauleta *et al.*, 2013). The enzyme is encoded by the *nos* gene clusters, often composed by the *nosRZDFYLX* genes in members of the Alphaprotebacteria, as *nosX* is missing in Beta-, Gamma- and Epsiloproteobacteria and

Gram-positive species (Pauleta *et al.*, 2013) have shown that. The *nosZ* gene encodes the active subunit of the enzyme, NosZ, and the *nosRX* genes encode proteins with roles in transcription regulation, activation, and Cu assemblage of Nos. The *nosDFYL* genes encode proteins that are apparently required for copper assemblage into Nos, although their specific role still remains unknown (Zumft and Kronek, 2007). A comprehensive phylogenetic analysis of the *nosZ* gene coding nitrous oxide reductase enzymes in genomes retrieved from public databases revealed two distinct clades of *nosZ*, with one unaccounted for in previous studies investigating N_2O-reducing communities. The two clades differ in their signal peptides, indicating differences in the translocation pathway of the enzyme across the membrane. Sequencing of environmental clones of the previously undetected *nosZ* lineage in various environments showed that it is widespread and diverse and at least as abundant as the other (Jones *et al.*, 2013).

Analysis of N cycle-related functional communities

Up to 10^9 cells/g of bacterial cells have been found in agricultural soils (Sharma *et al.*, 2007; Babic *et al.*, 2008; Dandie *et al.*, 2008; Henry *et al.*, 2008), and cultivation-dependent and -independent methods have shown that functional communities in environmental samples represent up to 5–10% of the total soil bacterial community (Tiedje, 1988; Henry *et al.*, 2006; Herrmann *et al.*, 2008; Jones *et al.*, 2013). Because only a fraction of the bacterial community is cultivable, the culture-dependent isolation techniques are of limited value. Molecular methods that do not require bacterial cultivation have been developed to assess diversity composition of functional communities in environmental samples from soils, waters and sediments. Because the ability to carry out a N cycle-related process (e.g. nitrogen fixation, denitrification, etc.) cannot be associated with any specific taxonomic group, a 16S rRNA phylogeny-based approach to study those populations is not possible. Therefore, existing techniques to study the ecology of microbial communities are based on the use of functional genes, or their transcripts, as molecular markers, and DNA extraction followed by PCR amplification of target gene is currently the most common way to quantify the functional populations in environmental samples (Philippot, 2006; Philippot and Hallin, 2006; Hallin *et al.*, 2007; Smith and Osborn, 2009; Teixeira and Yergeau, 2012).

Restriction fragment length polymorphism (RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) and terminal restriction fragment length polymorphism (T-RFLP) can been used to analyse the predominant populations related with the N cycle in environmental samples. Alternatively, after amplification of a functional gene, cloning and sequencing of the PCR amplicons offer detailed information, though usually more expensive and time-consuming. Comprehensive reviews on molecular methods to assess diversity of N-cycle related communities have been published (Philippot and Hallin, 2006; Hallin *et al.*, 2007; Sharma *et al.*, 2007). Because fingerprinting techniques are based on the number of peaks, or bands, and on their relative intensity, they give estimates of both richness and evenness, but estimation of the total number of functional populations is neglected. To solve this problem, competitive PCR (cPCR) and quantitative real-time PCR (qPCR) can be used. According to MIQUE guidelines (Bustin *et al.*, 2009), the initials RT-qPCR should be used to refer to reverse transcription-qPCR.

cPCR and qPCR

Simultaneous amplification of the target DNA and a control DNA with a known concentration, the so-called competitor, is the theoretical base for competitive cPCR assays. Because target and control DNAs compete for the primers during amplifications, and the method assumes that both DNAs have the same amplification efficiency, the mass ratio between the two amplicons can be used to determine the initial amount of target DNA. This ratio is estimated by agarose gel analysis of PCRs of multiple dilutions of the competitor with the target DNA.

qPCR does not require a gel migration step, is highly reproducible and sensitive, and is less

expensive, laborious and time-consuming than cPCR. Reviews dealing with the advantages and limitations of qPCR have been published (Zhang and Fang, 2006; Smith and Osborn, 2009; Brankatschk *et al.*, 2012; Gadkar and Fillion, 2013). RNA extracted directly from environmental samples can be retro-transcribed to cDNA and used for qPCR, thus providing evidence of gene expression. Using this methodology, N-cycle related genes have been quantified (Nogales *et al.*, 2002; Henderson *et al.*, 2010; Dandie *et al.*, 2011). As for all PCR-based techniques, qPCRs are subjected to well-known biases introduced by, e.g. DNA extraction procedures, primer selection, and PCR conditions. qPCR is currently the main technique used for quantification of housekeeping and functional genes after DNA extraction from environmental samples.

Both cPCR and qPCR technologies rely on the direct proportionality between the intensity of the fluorescent signal measured during the exponential phase of the PCR reaction and the initial amount of target DNA. The copy number of initial target DNA is thereby determined by comparison to a standard curve constructed using target DNA of a known concentration (see 'Calculation of the copy number of standard DNA'). Although the 16S rRNA gene and many functional genes so far studied are present in single copies within bacterial genomes, others such as *nifH*, *amoA*, *narG* and *nosZ* and the housekeeping 16S rRNA gene can be present in more than one copy (McTavish *et al.*, 1993; Flanagan *et al.*, 1999; Klappenbach *et al.*, 2001; Canfield *et al.*, 2005; Jones *et al.*, 2011).

Quantification of functional genes using qPCR

DNA extraction and purification, PCR's inhibition tests, target gene quantification by qPCR, and analysis of the results are conventional steps for quantification of functional bacterial populations in environmental samples. Here, the term environmental sample refers mainly to soil and sediment samples.

DNA extraction

Pioneer methods for soil DNA isolation from environmental samples used mechanical and enzymatic lysis, followed by cleaning of the crude extract and DNA precipitation (Muyzer *et al.*, 1993; Zhou *et al.*, 1996), a methodology that was further improved for simultaneous recovery of DNA and RNA (Hurt *et al.*, 2001; Juniper *et al.*, 2001). It was during evaluation of the effectiveness of nine DNA extraction procedures based on the original data of Miller *et al.* (1999), that a method was developed that provided good quantity and quality DNA (Martin-Laurent *et al.*, 2001). Because this method gave good reproducibility in isolating DNA from different environmental samples, including soils from a range of origins and different physical and chemical characteristics (Philippot *et al.*, 2002; Chèneby *et al.*, 2003; López-Gutiérrez *et al.*, 2004; Martin-Laurent *et al.*, 2004; Čuhel *et al.*, 2010), it was proposed by the Agence Française pour la Normalization (AFNOR) to the International Organization for Standardization (ISO) in 2006.

The ISO standard 11063: soil quality method to directly extract DNA from soil samples

When the need for an international standard for soil DNA extraction was recognized, twelve different soils were used by fifteen independent European laboratories, including France, Finland, Germany, Italy, Spain and Sweden, to evaluate both the reproducibility of the standardized method and the abundance and genetic structure of the total bacterial community. The method was unanimously approved by the ISO as an international standard method (ISO standard 11063) (Petrić *et al.*, 2011a). Later on, the method has also been used to extract DNA from river sediments and agricultural soils (Bru *et al.*, 2011), polychlorinated biphenyls-contaminated sites (Petrić *et al.*, 2011b), technosols (Hafeez *et al.*, 2012) and constructed wetlands (Correa-Galeote *et al.*, 2013). ISO standards give information on the identity and quality of each compound in the protocol, thus providing a complete quality control for users and avoiding the risks associated to commercial kits.

The procedure for the ISO standard 11063 is as follows:

1 Sieve samples to < 2 mm.

2 Weight 0.25 g equivalent dry weight aliquots. Samples can be frozen at –80°C until use.
3 To thawed samples, add 0.5 g of 106 µm glass beads, two beads of 2 mm diameter and 1 ml of homogenization buffer (1) extemporaneously prepared.
4 Place the sample into a pre-chilled (–20°C) shaking flask and homogenize it in a mini bead beater system (1.600–1 shaking frequency/min for 30 s).
5 Incubate for 10 min at 70°C. Centrifuge at 14,000 × *g* for 1 min at 4°C.
6 Transfer the supernatant to a new 2 ml microtube. Add 1:10 (v/v) 5 M sodium acetate (pH 5.5) and mix by vortexing. Incubate on ice for 10 min.
7 Centrifuge at 14,000 × *g* for 5 min at 4°C.
8 Transfer the supernatant to a new 1.5 ml microtube. Add 1:1 (v/v) pre-chilled (–20°C) isopropanol. Mix well by manual inversion.
9 Incubate for at least 15 min at –20°C. Centrifuge at 14,000 × *g* for 30 min at 4°C. Remove the supernatant.
10 Wash the pellet with pre-chilled (–20°C) 70% ethanol with precaution to avoid pellet resuspension. Centrifuge for 15 min at 14,000 × *g* at 4°C.
11 Discard the supernatant. Dry the pellet for 15 min at 37°C.
12 Resuspend the pellet in 50 µl ultrapure water.
13 Prepare sample aliquots and store at –20°C until use.

The homogenization buffer is composed of 100 µl of 1 M trishydroxymethylaminomethane HCl (Tris HCl, pH 8.0), 200 µl of 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0), 100 µl of 1 M NaCl, 50 µl of 20% polyvinylpyrrolidone (PVP) 40T, 100 µl of 20% sodium dodecyl sulfate (SDS) and 450 µl ultrapure water.

Other methods for DNA extraction

In addition to ISO standard 11063, other methods are used to extract environmental DNA: (a) cation-exchange (Jacobsen and Rasmussen, 1992) (b) nitrogen-grinding (Volossiouk *et al.*, 1995) (c) microwave-based rupture (Orsini and Romano-Spica, 2001) (d) Nycodenz gradient separation (Bertrand *et al.*, 2005) (e) solvent-based beating (Chen *et al.*, 2006) (f) aluminium-based extraction (Peršoh *et al.*, 2008) and (g) calcium chloride (Li *et al.*, 2011).

In many laboratories, utilization of commercial kits for DNA isolation such as MoBIO PowerSoil DNA kit (MoBIO), ultra clean soil DNA kit (Ozyme, MoBIO), fast DNA spin kit for soil (BIO 101/Q-Biogene) is also frequent.

Purification of DNA

After extraction of DNA, the presence of PCR-inhibitory compounds in the samples is common, and DNA purification is recommended. DNA purification is not part of the ISO standard 11063. DNA purification can be achieved using polyvinylpolypyrrolidone (PVPP) columns and/or Sepharose 4B columns.

PVPP columns can be prepared as indicated by Petrić *et al.* (2011a), as follows:

1 Fill about 1.2 cm of an empty micro-spin chromatography column with PVPP powder.
2 Add 400 µl of ultrapure water to the column and place it in a 1.5 ml microtube.
3 Centrifuge for 2 min at 1000 × *g*. Discard the eluate. Add 400 µl of ultrapure water to the column.
4 Centrifuge for 2 min at 1000 × *g*. At this moment, the column can be kept at 4°C.
5 Add the 50 µl DNA sample to the column and place it in ice for 5 min.
6 Place the column into a new tube and centrifuge at 1000 × *g* for 4 min at 10°C to recover the DNA sample.
7 Quantify the final volume of the sample.

Further DNA purification can be obtained by using Sepharose 4B columns as indicated earlier (Martin-Laurent *et al.*, 2001; Petrić *et al.*, 2011a). Essentially, the protocol is as follows:

1 Fill an empty micro-spin chromatography column with 1 ml of Sepharose 4B solution.
2 Place the column in a 2 ml tube. Centrifuge for 2 min at 1100 × *g* at 10°C. Discard the eluate.
3 Add 500 µl TE buffer (10 mM Tris and 1 mM

EDTA). At this moment, the column can be kept at 4°C.

4 Centrifuge at 1100 × *g* for 2 min at 10°C. Discard the eluate.

5 Place the column in a new 2 ml tube. Add the DNA sample and centrifuge at 1400 × *g* for 5 min at 10°C. Quantify the final volume of the sample.

In addition to PVPP and Sepharose 4B, DNA purification can be achieved using (1) phenol (Tsai and Olson, 1991), (2) elutip-d and Sephadex G-200 columns (Tsai and Olson, 1992), (3) caesium chloride, glassmilk and spearmine (Smalla *et al.*, 1993), (4) PVPP and Microcon-100 columns, microconcentrators (Widmer *et al.*, 1996), (5) agarose gel electrophoresis (Zhou *et al.*, 1996), (6) HR S400 spin columns fast DNA purification kit and elution through Qiagen Mini column (Ranjard *et al.*, 2000), (7) Wizard DNA Clean-Up System (Djigal *et al.*, 2010) and (8) AllPrep DNA/RNA mini kit (García-Lledó *et al.*, 2011) and (8) Geneclean® turbo kit (Glassmilk®-embedded membrane, MP Bio) (Correa-Galeote *et al.*, 2013). A combination of hand-made and kit methodologies have also been used (Leininger *et al.*, 2006; Griffiths *et al.*, 2000; Su *et al.*, 2010).

Although most protocols were originally designed for DNA extraction from soils and sediments, they have also been used to isolate and purify DNA from diverse environmental samples, such as the rizosphere of plants (Ruiz-Rueda *et al.*, 2008), biofilms formed on estuarine rocks colonized by algae (Magalhães *et al.*, 2008), the vermicompost from a fresh olive waste (Vivas *et al.*, 2009), soil-feeding mounds of nematodes or termites (Fall *et al.*, 2004; Djigal *et al.*, 2010) and a bioreactor's biofilm (Calderón *et al.*, 2012). Isolation and purification of DNA from aqueous samples usually requires concentration of the bacterial biomass by filtering the samples through 0.22 µm membranes and further homogenization of the filters (Torrentó *et al.*, 2011). Thermal shocks do not usually increase DNA yield and, in turn, may release humic material. This treatment, however, resulted in extraction of DNA from the Gram-positive actinomycete *Micromonospora* (Frostegård *et al.*, 1999).

Quantity and quality of purified DNA

Quality and quantity of the DNA throughout isolation and purification can be estimated by electrophoresis on agarose gels. Samples can be supplemented with loading buffer (40% sucrose and 0.25% bromophenol blue) before loading the samples on the gel. After electrophoresis, DNA can be visualized by staining with ethidium bromide, GelRed, Sybr Green I, etc. Alternatively, quantification of purified DNA can be determined by spectrometry at 260 nm. Quality of the samples can be checked by measuring absorbance of the sample at 230 nm (shows the presence of organic solvents), 280 nm (indicates the presence of proteins), and 400 nm (suggests the presence of humic acid).

DNA standards for qPCR

Standard DNAs corresponding to the bacterial and archaeal 16S rRNA genes can be obtained after PCR amplification from genomic DNA of *P. aeruginosa* strain PAO1 and the fosmid 29i4 (Quaiser *et al.*, 2002), respectively. Standard DNAs for *nifH* can be obtained from genomic DNA from *Frankia alni* strain ANC14a, the bacterial *amoA* from *Nitrosomonas communis* strain Nm2 and the archaeal *amoA* from the fosmid 54d9 (Treusch *et al.*, 2005). For denitrification genes, *narG* and *napA* DNA are obtained after amplification of genomic DNA from *P. aeruginosa* PAO1, *nirS* from *Pseudomonas fluorescens* C7R12, *nirK* from *Ensifer meliloti* 1021, and *nosZ* from *Bradyrhizobium japonicum* USDA110.

PCR-amplification of standard 16S rRNA and N-cycle related genes

Primers for PCR

Primers used for PCR amplification of the 16S rRNA gene from Bacteria and Archaea, the N_2-fixation related *nifH* gene, the nitrification *amoA* gene from Bacteria and Archaea, and the denitrification *narG, napA, nirK, nirS* and *nosZ* genes are shown in Table 4.1. Quantification of the 16S rRNA gene allows not only calculation of the abundance of the gene, but also calculation of the relative abundance of a functional gene as the ratio between the abundance of the functional gene and the abundance of the 16S rRNA gene.

Reaction mixture

1 1 to 5 ng template DNA (either 16S rRNA, *nifH, amoA, narG, napA nirS, nirK* or *nosZ*).
2 0.6 µmol forward primer.
3 0.6 µmol reverse primer.
4 PCR buffer 1× (2.5 µl).
5 1.5 mmol $MgCl_2$.
6 0.4 mM dNTPs.
7 0.04 units high-fidelity Taq polymerase.
8 Add up to 25 µl ultrapure water.

Thermocycler conditions

The thermocycler conditions for PCR-amplifications of standard Bacteria and Archaea 16S rRNA genes, the *nifH* gene, and Bacteria and Archaea *amoA* genes are shown in Table 4.2, and those for amplifications of the *narG, napA nirS, nirK* or *nosZ* denitrification genes are in Table 4.3.

After amplification, the PCR products are electrophoresed on agarose gels to check size and purity of amplicons, then cloned into a commercial cloning vector and used to transform *Escherichia coli* competent cells. The presence of the insert in the plasmid is verified by PCR using specific primers and further sequencing of the corresponding DNA fragments.

Because sequences corresponding to the polylinker regions of the plasmid are also amplified, they have to be removed. The obtained DNA sequences will 100% match the corresponding sequences of each gene.

Calculation of the copy number of standard DNA

Before linearization of recombinant plasmid containing insert DNA, the existence of a unique cutting site in the DNA sequence can be checked by using the web site http://www.bioinformatics.

Table 4.1 Primers used for PCR amplification of standard 16S rRNA and N cycle-related genes

Primer	Primer sequence (5′–3′)	Target gene	Size of the amplicon (base pair)	Reference
T7	TAATACGCATCACTATAGGG		150	Promega
Sp6	GATTTAGGTGACACTATAG			
341F	CCTACGGGAGGCAGCAG	16S rRNA Bacteria	194	Muyzer *et al*. (1993)
534R	ATTACCGCGGCTGCTGGCA			
771F	ACGGTGAGGGATGAAAGCT	16S rRNA Archaea	226	Ochsenreiter *et al*. (2003)
957R	CGGCGTTGACTCCAATTG			
polR	TGCGATCCSAATGCBGACTC	*nifH*	360	Ducey *et al*. (2013)
polF	ATSGCCATCCTYTCRCCGGA			
AmoA1F	GGGGTTTCTACTGGTGGT	*amoA* Bacteria	490	Rotthauwe et al. (1997)
AmoA2R	CCCCTCKGSAAAGCCTTCTTC			
Crenamo A23F	ATGGTCTGGCTWAGACG	*amoA* Archaea	624	Tourna *et al*. (2008)
Crenamo A616R	GCCATCCATCTGTATGTCCA			
narG-f	TCGCCSATYCCGGCSATGTC	*narG*	174	Bru *et al*. (2007)
narG-r	GAGTTGTACCAGTCRGCSGAYTCSG			
nap3F	TGGACVATGGGYTTYAAYC	*napA*	152	Bru *et al*. (2007)
napA4R	ACYTCRCGHGCVGTRCCRCA			
nirK876F	ATYGGCGGVAYGGCGA	*nirK*	173	Henry *et al*. (2004)
nirK1040R	GCCTCGATCAGRTTRTGGTT			
nirS4QF	AACGYSAAGGARACSGG	*nirS*	425	Throbäck *et al*. (2004)
nirS6QR	GASTTCGGRTGSGTCTTSAYGAA			
nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	*nosZ*	267	Henry *et al*. (2006)
nosZ2090R	CAKRTGCAKSGCRTGGCAGAA			

Table 4.2 Thermocycler conditions for amplification of standard bacterial and archaeal 16S rRNA genes, the *nifH* gene, and bacterial and archaeal *amoA* genes

	Gene			
	Bacterial 16S rRNA	Archaeal 16S rRNA and*nifH*	Bacterial *amoA*	Archaeal*amoA*
Stage1: 1 cycle	10 min, 95°C	10 min, 95°C	10 min, 94°C	10 min, 94°C
Stage 2: 35 cycles	15 s, 95°C	15 s, 95°C	45 s, 94°C	45 s, 94°C
	30 s, 60°C	30 s, 55°C	45 s, 58°C	45 s, 55°C
	30 s, 72°C	30 s, 72°C	45 s, 72°C	45 s, 72°C
Stage 3: 1 cycle	10 min, 72°C	10 min, 72°C	10 min, 72°C	10 min, 72°C

Table 4.3 Thermocycler conditions for amplification of standard *narG*,*nirK*, *nirS*, *napA* and *nosZ* denitrification genes

	Gene		
	narG,*nirK* and *nirS*	*napA*	*nosZ*
Stage 1: 1 cycle	10 min, 95°C	10 min, 95°C	10 min, 95°C
Stage 2: 6 cycles with 1°C decrease by cycle	15 s, 95°C	15 s, 95°C	15 s, 95°C
	30 s, 63°C	30 s, 61°C	30 s, 65°C
	30 s, 72°C	30 s, 72°C	30 s, 72°C
Stage 3: 35 cycles	15 s, 95°C	15 s, 95°C	15 s, 95°C
	30 s, 58°C	30 s, 56°C	30 s, 60°C
	30 s, 72°C	30 s, 72°C	30 s, 72°C
Stage 4: 1 cycle	10 min, 72°C	10 min, 72°C	10 min, 72°C

org/sms2/rest_digest.html in the Sequence Manipulation Suite Program. After digestion with the appropriate restriction enzyme, use any appropriate commercial kit to purify DNA. DNA concentration (ng/μl) can be determined by spectrophotometry as indicated above. To calculate the molecular weight of a DNA fragment, use the formula MW (ng/mol) = bp number × 660 g/mol × 109 ng/g, where MW is the DNA molecular weight, bp is the number of base pairs (nucleotides) in the double stranded DNA, and 660 is the molecular weight of 1 base pair. Then, the molarity (M) of standard DNAs can be calculated as M (mol/μl) = DNA concentration (ng/μl)/MW (ng/mol). Since 1 mol of any DNA contains 6.023×10^{23} molecules (Avogadro's number), then the DNA copy number can be calculated as follow: copy number/μl = M (mol/μl) × 6.023×10^{23} copies/mol. It is recommended to prepare a stock of standard DNAs in 25-μl aliquots containing 0.5×10^{8} copies/μl. Keep them at –20°C until use. Avoid repeated freezing/thawing of the aliquots.

Inhibition test

Sensitivity, accuracy and reliability of any PCR are based on the quality of the template DNA (Nolan *et al.*, 2006). Because organic and phenolic compounds, humic acids, glycogen, fats, Ca^{2+} ions, heavy metals, detergents, antibiotics and constituents of bacterial cells can be co-extracted during environmental DNA extraction (Wilson, 1997) there is the need to avoid the presence of inhibitory compounds in the extracted DNA solution. qPCR is a method of choice to test the presence of inhibitory compounds in DNA from an environmental sample (Opel *et al.*, 2010). During qPCR, inhibition can be detected by changes in (a) the efficiency of the reaction (b) the melting curve due to modifications of the PCR product, c) the relative amounts of the PCR product due to the different inhibition levels.

The absence of PCR inhibitors in the soil DNA extracts can be analysed by mixing it with a known amount of a DNA sample that is supposed not to be targeted in the environmental DNA, the so

Table 4.4 Thermocycler conditions for qPCR inhibition test

Stage1*: 1 cycle	10 min, 95°C
Stage* 2: 35 cycles	15 s, 95°C
	30 s, 55°C
	30 s, 72°C
	30 s, 80°C (data collection step)
Stage 3†: Dissociation stage: melting curve: 30 cycles with 0.5°C increase by cycle	15 s, 95°C
	15 s, 80°C
	15 s, 95°C

*Times and temperatures should be set according to the manufacturer's instructions. Values in the ttable have been employed successfully with different buffers.
†Melting curves can be established by each laboratory. Values in the table are widely used.

called spike DNA (Bustin *et al.*, 2009). Then, this DNA can be amplified by qPCR using appropriate primers. Here, we describe utilization of the pGEM-T Easy vector (Promega) as spike DNA. pGEM-T can be amplified by qPCR using primers SP6 and T7 as described earlier (Henry *et al.*, 2006).

Reaction mixture

1 2 ng template (environmental) DNA.
2 1 × 107 copies of *Sal*I-digested spike pGEM-T Easy vector (without insert).*
3 1 µM T7 primer. †
4 1 µM Sp6 primer.†
5 250 ng T4 Gp32.‡
6 7.5 µl SYBR Green PCR buffer 2× (containing Hot DNA Taq polymerase and dNTPs).
7 Add ultrapure water up to 15 µl.

In separate wells, add (a) template DNA + spike DNA (b) spike DNA without template DNA or (c) no DNA (negative control).

Utilization of SYBR Green PCR buffer is recommended over preparation of a mixture containing each reaction component prepared independently. Keep at 4°C until use (according to manufacturer's instructions). Mix the plate, then centrifuge before qPCR.

Thermocycler conditions

The thermocycler conditions for qPCR to run the inhibition test are shown in Table 4.4.

Gene quantification by qPCR

Standard DNA curves

For qPCR, construction of a standard curve, is required. For that purpose:

1 Prepare serial decimal dilutions ranging from 0.5×10^7 copies/µl to 0.5×10^2 copies/µl from the stock of standard DNAs.
2 Take 2 µl from each of the six DNA dilutions and use them independently as template DNA to run qPCRs. Final DNA copy numbers for each run should go from 1×10^7 copies to 1×10^2 copies. New dilutions for construction of standard DNA curves should be prepared for each qPCR.

External DNA controls

In qPCR, external DNA controls are genomic DNA isolated from *P. aeruginosa* PAO1 to be used for 16S rRNA, *narG*, *napA* and *nirS* qPCR. Similarly, genomic DNA from *F. alni* ACN14a is used as template for *nifH* qPCR. For *nirK* and 16S

*When pGEM-T Easy vector is used, the plasmid containing insert DNA can be linearized using the restriction enzyme *Sal*I.

†The T7 and Sp6 sequences are shown in Table 4.1.

‡Bacteriophage T4 gene 32 protein (T4gp32) can be used to limit the PCR inhibition and enhance the PCR amplification by stabilization of the single-stranded DNA.

rRNA, genomic DNA from *E. meliloti* 1021 can be used. Finally, genomic DNA from *B. japonicum* USDA110 can be used as template DNA for qPCR of the 16S rRNA, *nifH*, *napA* and *nosZ* genes. Since genomes from these bacterial species are completely sequenced, their size and the copy number of the targeted gene per genome are known. After qPCR, the copy number of a target gene per ng of genomic DNA can be calculated. A comparison between the obtained copy number and the expected copy number determined from the initial amount of DNA in the qPCR can be used to assess the reliability of the assay. A usual copy number for external DNA control is 1×10^7.

Bacterial and archaeal species with unsequenced genomes cannot be used to obtain DNA control for qPCR, as the number and sequences of the targeted genes are unknown. This is the case for the archaeal 16S rRNA gene and the AOA and AOB *amoA* genes.

Reaction mixture for qPCR

1 2 ng template (environmental) DNA.
2 1 µM forward primer for 16SrRNA and 2 µM for other N-cycle related genes.*
3 1 µM reverse primer for 16SrRNA and 2 µM for other N-cycle related genes.*
4 250 ng T4 Gp32.
5 7.5 µl SYBR Green PCR buffer 2× (containing Hot DNA Taq polymerase, buffer and dNTPs).
6 Add ultrapure water up to 15 µl.

In separate wells, add (a) template DNA (b) the different dilution for construction of the standard DNA curve (c) external DNA controls or (d) no DNA (negative control).

For q-PCR, utilization of SYBR Green PCR buffer is recommended over preparation of a mixture containing each reaction component prepared independently. Keep at 4°C until use (according to the manufacturer's instructions). Mix the plate, then centrifuge before qPCR.

Thermocycler conditions

Thermocycler conditions for q-PCR of the 16S rRNA gene from Bacteria and Archaea are shown in Table 4.5, those for the *nifH* gene in Table 4.6, those for the *amoA* gene from Bacteria and Archaea in Table 4.7, and those for the *narG*, *napA*, *nirK*, *nirS* and *nosZ* denitrification genes in Table 4.8.

Quantification cycle (Cq) and standard curves

SYBR Green is a fluorescent dye that binds non-specifically to double-stranded DNA. In general, after qPCR the software of the thermocycler will retrieve values of fluorescence intensity throughout the amplification cycles. The cycle at which the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase is called the quantitative cycle. Following the MIQE guidelines (Bustin *et al.*, 2009), this cycle should be called the quantification cycle (C_q). This value is inversely proportional to the log value of the initial DNA concentration in the reaction mixture. It is recommended to keep track on the background, exponential amplification, linear amplification and plateau of each curve during qPCR. Finally, a standard curve is drawn by plotting the C_q value of each standard DNA against the common log of the DNA initial copy numbers in each reaction mixture. Because data values involved in the construction of the curve contribute to the final quantification of the environmental sample, the following descriptors of the curve should be reported: the amplification efficiency (E), the linear regression coefficient (r^2) and the y-intercept. Once the standard curve has been obtained, the copy numbers of each DNA sample can be calculated by interpolation of the C_q values in the standard curve. Export the data set to a spreadsheet application and run appropriate statistical analyses. Because the number of a target gene operon per cells is variable (Klappenbach *et al.*, 2001), the gene copy numbers obtained is not related to the number of the cell population. Calculation of the gene copy number per nanogram of DNA instead of gram of soil minimized any bias related to soil DNA extraction efficiency.

Specificity of the qPCR can be validated empirically with a direct experimental evidence, the melting curve and melting temperature (T_m) obtained during the last step of the qPCR being widely used for this purpose (Bustin *et al.*, 2009).

*Primers for qPCR are shown in Table 4.1.

Table 4.5 Thermocycler conditions for qPCR of the 16S rRNA gene from Bacteria and Archaea

	Gene	
	16S rRNA from Bacteria	16S rRNA from Archaea
Stage1*: 1 cycle	10 min, 95°C	10 min, 95°C
Stage 2*: 35 cycles	15 s, 95°C	15 s, 95°C
	30 s, 60°C	30 s, 55°C
	30 s, 72°C	30 s, 72°C
	30 s, 80°C (data acquisition)	30 s, 80°C (data acquisition)
Stage 3†: Dissociation stage (melting curve: 30 cycles with 0.5°C increase by cycle)	15 s, 95°C	15 s, 95°C
	15 s, 80°C	15s, 80°C
	15 s, 95°C	15 s, 95°C

*Times and temperatures should be set according to the manufacturer's instructions. Values in the table have been employed successfully with different buffers
†Melting curves can be established by each laboratory and the values are widely used.

Table 4.6 Thermocycler conditions for qPCR of the *nifH* gene

	nifH gene
Stage1*: 1 cycle	10 min, 95°C
Stage 2*: 35 cycles	15 s, 95°C
	30 s, 55°C
	30 s, 72°C
	30 s, 80°C (data acquisition)
Stage 3†: Dissociation stage (melting curve: 30 cycles with 0.5°C increase by cycle)	15 s, 95°C
	15 s, 80°C
	15 s, 95°C

*Times and temperatures should be set according to the manufacturer's instructions. Values in Table 4.6 have been employed successfully with different buffers.
†Melting curves can be established by each laboratory and the values are widely used.

Table 4.7 Thermocycler conditions for qPCR of the *amoA* gene from Bacteria and Archaea

	Gene	
	Bacterial *amoA*	Archaeal *amoA*
Stage1*: 1 cycle	10 min, 95°C	10 min, 95°C
Stage 2*: 35 cycles	15 s, 95°C	15 s, 95°C
	30 s, 60°C	30 s, 55°C
	30 s, 72°C	30 s, 72°C
	30 s, 80°C (data acquisition)	30 s, 80°C (data acquisition)
Stage 3†: Dissociation stage (melting curve: 30 cycles with 0.5°C increase by cycle)	15 s, 95°C	15 s, 95°C
	15 s, 80°C	15 s, 80°C
	15 s, 95°C	15 s, 95°C

*Times and temperatures should be set according to the manufacturer's instructions. Values in Table 4.5 have been employed successfully with different buffers.
†Melting curves can be established by each laboratory and the values are widely used.

Table 4.8 Thermocycler conditions for qPCR of the *napA*, *narG*, *nirK*, *nirS* and nosZ denitrification genes

	Gene		
	narG, nirK and *nirS*	*napA*	*nosZ*
Stage1*: 1 cycle	10 min, 95°C	10 min, 95°C	10 min, 95°C
Stage 2*: 6 cycles with 1°C decrease by cycle	15 s, 95°C	15 s, 95°C	15 s, 95°C
	30 s, 63°C	30 s, 61°C	30 s, 65°C
	30 s, 72°C	30 s, 72°C	30 s, 72°C
	30 s, 80°C (data adquisition)	30 s, 80°C (data adquisition)	30 s, 80°C (data adquisition)
Stage 3*: 35 cycles	15 s, 95°C	15 s, 95°C	15 s, 95°C
	30 s, 58°C	30 s, 56°C	30 s, 60°C
	30 s, 72°C	30 s, 72°C	30 s, 72°C
	30 s, 80°C (data adquisition)	30 s, 80°C (data adquisition)	30 s, 80°C (data adquisition)
Stage 4**: Dissociation stage (melting curve: 30 cycles with 0.5°C increase by cycle)	15 s, 95°C	15 s, 95°C	15 s at 95°C
	15 s, 80°C	15 s, 80°C	15 s, 80°C
	15 s, 95°C	15 s, 95°°C	15 s, 95°C

*Times and temperatures should be set according to the manufacturer's instructions. Values in Table 4.5 have been employed successfully with different buffers.
†Melting curves can be established by each laboratory and the values are widely used.

When running inhibition tests, absence of inhibition is considered when differences in C_q values are ± 1 cycle. Should inhibition be detected, re-purification of the sample DNA is required. The PCR efficiency of the reaction, as indicated by the slope of the curve as well as the lack of PCR inhibition has to be checked. Dilutions of extracted DNA or addition of a given amount of control DNA to environmental DNA can be used for verification.

Other primers for qPCR

In addition to primers indicated in Table 4.1 for qPCR of N-cycle related genes, other primers can be used for quantification of bacterial and archaeal 16S rRNA genes (Table 4.9), bacterial *nifH* and AOA and AOB *amoA* genes (Table 4.10), and genes involved in denitrification (Table 4.11).

Other N-cycle related genes for qPCR quantification

In addition to bacterial and archaeal 16S rRNA genes, bacterial *nifH* and AOA and AOB *amoA* genes, and *narG, napA, nirS, nirK, norC* and *nosZ* denitrification genes, other genes can be used as a target for quantification of functional genes involved in the N-cycle (Table 4.12).

Future trends and directions

In addition to biological nitrogen fixation, nitrification, dissimilatory nitrate reduction and denitrification, other processes are involved in the redox reactions that constitute the N-cycle. Anaerobic ammonia oxidation (anammox) converts nitrite and ammonium directly into N_2, thus largely contributing to production of N_2 (Kartal *et al.*, 2013). The nitrite-dependent anaerobic methane oxidation (Raghoebarsing *et al.*, 2006) and hyperthermophilic N_2-fixing methane-producing archaea (Mehta and Baross, 2006) are also examples of the metabolic capacity of new nitrogen conversions within the N cycle. Genome sequencing of several N-cycle organisms and accumulation in the databank of either partial or complete sequences from bacterial genome projects and newly isolated Bacteria and Archaea will help to design new primers for the study of microbial communities inside the N

Table 4.9 Other primers for qPCR amplification of 16S rRNA from Bacteria and Archaea

Primer	Primer sequence (5´–3´)	Target gene	Reference
519F	GWATTACCGCGGCKGCTG	16S rRNA *Bacteria*	Torrentó *et al*. (2011)
907R	CCGTCAATTCMTTTRAGTTT		
1055f	ATGGCTGTCGTCAGCT	16S rRNA *Bacteria*	Chon *et al*. (2011)
1392r	ACGGGCGGTGTGTAC		
Parch519f	CAGCCGCCGCGGTAA	16S rRNA *Archaea*	Wuchter *et al*. (2006)
ARC915r	GTGCTCCCCCGCCAATTCCT		
669F	CGACGGTGAGGGATGAAAG	16S rRNA *Archaea*	Xu *et al*. (2012)
88611GR	CCAGGCGGCAGACTTAAC		

Table 4.10 Other primers for qPCR amplification of *nifH* and *amoA* genes

Primer	Primer sequence (5´–3´)	Target gene	Reference
nifHF	AAAGGYGGWATCGGYAARTCCACCAC	*nifH* Bacteria	Morales *et al*. (2010)
nifHRb	TGSGCYTTGTCYTCRCGGATBGGCAT		
ArchamoA-for	CTGAYTGGGCYTGGACATC	*amoA* Archaea	Wuchter *et al*. (2006)
ArchamoA-rev	TTCTTCTTTGTTGCCCAGTA		
ARAMORT1F	GCATCAGTGCTGCGATATTG	*amoA Archaea*	Lopez-Legentil *et al*. (2010)
ARAMORT1R	TGGCTTAGACGATGTACCCAC		
amoAF	STAATGGTCTGGCTTAGACG	*amoA Archaea*	Xu *et al*. (2012)
Arch-amoAR	GCGGCCATCCATCTGTATGT		
Mixture of three primers	CTO189fA-GC, CTO189fBGC and CTO189fC-GC	16S $rRNA_{AOB\ populations}$[1]	Wang *et al*. (2012a)
CTO654r	CTAGCYTTGTAGTTTCAAACGC		

[1]16S rRNA AOB populations.

Table 4.11 Other primers for qPCR amplification of bacterial denitrification genes

Primer	Primer sequence (5´–3´)	Target gene	Reference
1960m2f	TAYGTSGGGCAGGARAAACTG	*narG*	López-Gutiérrez *et al*. (2004)
2050m2r	CGTAGAAGAAGCTGGTGCTGT		
narG328f	GACAAACTTCGCAGCGG	*narG*	Reyna *et al*. (2010)
narG497r	TCACCCAGGACGCTGTTC		
V16	GCNCCNTGYMGNTTYTGYGG	*napA*	Wakelin *et al*. (2007)
V17	RTGYTGRTTRAANCCCATNGTCCA		
F1aCu	ATCATGGTSCTGCCGCG	*nirK*	Enwall *et al*. (2010)
R3Cu	TTGGTGTTRGACTAGCTCCG		
nirK517F	TTYGTSTAYCACTGCGCVCC	*nirK*	Chen *et al*. (2010)
nirK1055R	GCYTCGATCAGRTTRTGGTT		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	Chen *et al*. (2010)
nirS950R	GCBACRCGSGGYTCSGGATG		
nirS2F	TACCACCCSGARCCGCGCGT	*nirS*	Chon *et al*. (2009)
nirS3R	GCCGCCGTCRTGVAGGAA		
nirSsh2F	ACCGCCGCCAACAACTCCAACA	$nirS_{Pm}$[1]	Henderson *et al*. (2010)
nirSsh4R	CCGCCCTGGCCCTGGAGC		

Table 4.11 continued

Primer	Primer sequence (5′–3′)	Target gene	Reference
forward	ACAAGGAGCACAACTGGAAGG T	$nirS_{Ps}$[2]	Grüntzig *et al*. (2001)
reverse	CGCGTCGGCCCAGA		
$cnorB_P$F	CATGGCGCTGATAACGGG	$cnorB_P$[3]	Dandie *et al*. (2007)
$cnorB_P$R	CTTIACCATGCTGAAGGCG)		
$cnorB_B$F	AIGTGGTCGAGAAGTGGCTCT	$cnorB_B$[4]	Dandie *et al*. (2007)
$cnorB_B$R	TCTGIACGGTGAAGATCACC		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	Chen *et al*. (2010)
nirS950R	GCBACRCGSGGYTCSGGATG		
nosZ1F	WCSYTGTTCMTCGACAGCCAG	*nosZ*	Henderson *et al*. (2010)
nosZ1R	ATGTCGATCARCTGVKCRTTYTC		
Forward	AGAACGACCAGCTGATCGACA	*nosZ*	Chon *et al*. (2009)
Reverse	TCCATGGTGACGCCGTGGTTG		
nosZ-F-1181	CGCTGTTCITCGACAGYCAG	*nosZ*	Ma *et al*. (2008)
nosZ-R-1880	ATGTGCAKIGCRTGGCAGAA		
nosZ-II-F	CTIGGICCIYTKCAYAC	nosZ type II[5]	Jones *et al*. (2012)
nosZ-II-R	GCIGARCARAAITCBGTRC		

[1]*nirS* gene from populations of *P. mandelii* and related species ($nirS_{Pm}$-bearing communities).
[2]*nirS* gene from populations of *P. stutzeri* and related species ($nirS_{Ps}$-bearing communities).
[3]*norB* gene from populations of *P. mandelii* and closely related strains ($cnorB_P$-bearing communities).
[4]*norB* gene from populations of *Bosea*, *Bradyrhizobium*, and *Ensifer* spp. ($cnorB_B$-bearing communities).
[5]*nosZ* type II gene from denitrifying bacterial populations.

Table 4.12 Primers for qPCR amplification of others N-cycle related genes

Primer	Primer sequence (5′–3′)	Target gene	Reference
nrfA-2F	CACGACAGCAAGACTGCCG	*nrfA* (encoding formate-dependent nitrite reductase)	Mohan *et al*. (2004)
nrfA-2R	CCGGCACTTTCGAGCCC		
Nitro-1198f	ACCCCTAGCAAATCTCAAAAAACCG	16s $rRNA_{Nitrobacter\ sp}$[1]	Knapp and Graham (2007)
Nitro-1423r	CTTCACCCCAGTCGCTGACC		
675f	GCGGTGAAATGCGTAGAKATCG	16s $rRNA_{Nitrosospira\ sp}$[2]	Knapp and Graham (2007)
Nspra-746r	TCAGCGTCAGRWAYGTTCCAGAG		
NxrB 1F	ACGGGAGACCAAGCCGGG	$nxrB_{Nitrobacter\ sp}$[3] (encoding nitric oxide reductase)	Geets *et al*. (2007)
NxrB 1R	CCGTGCTGTTGAYCTCGTTGA		
hzsB 396F	ARGGHTGGGGHAGYTGGAAG	*hzsB*[4] (encoding hydrazine synthase)	Wang *et al*. (2012b)
hzsB 742R	GTYCCHACRTCATGVGTCTG		

[1]16S rRNA gene from populations of *Nitrobacter* sp. for the quantification of the nitrite-oxidizing bacteria (NOB).
[2]16S rRNA gene from populations of *Nitrobacter* sp. for the quantification of the nitrite-oxidizing bacteria (NOB).
[3]*nxrB* gene (nitric oxide reductase gene, also abbreviated as *norB*) from *Nitrobacter* sp.-like population ($nrxB_{Nitrosobacter}$-bearing communities).
[4]*hzsB* gene (hydrazine synthase gene) for quantification of anammox population.

cycle. This is the case for the newly described set of primers, nosZ-II-F and nosZ-II-R (Jones *et al.*, 2013), whose utilization resulted in finding of a much larger diversity of denitrifying bacterial and archaeal populations carrying a *nosZ* gene.

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