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Research Article

Determination of Denitrification Genes Abundance in Environmental Samples

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Abstract Diversity of microorganisms involved in the biogeochemical N cycle is of fundamental interest in microbial ecology. Denitrification is a key step in the cycle by which nitrate is reduced to dinitrogen gas via the soluble nitrite and the gaseous compounds nitric oxide and nitrous oxide. The process is carried out by the sequential activity of the nitrate, nitrite, nitric oxide, and nitrous oxide reductase enzyme, respectively. The fluorescence-based quantitative real-time polymerase chain reaction (qPCR) is widely used for quantification of nucleic acids in samples obtained from numerous, diverse sources. Here, we provide a well-proven methodology for isolation of DNA from environmental samples and describe relevant experimental conditions for utilization of qPCR to assay the 16S rRNA and *nar/nap*, *nirK/nirS*, c-*nor*/q-*nor*, and *nos* denitrification genes that encode synthesis of denitrifying enzymes. The ISO 11063 standard method and MIQUE guidelines are considered with the aim to increase experimental transparency.

Keywords environmental samples; denitrifier communities; DNA extraction; DNA purification; DNA quantification; real-time PCR; relative abundance

1. Introduction

When faced with a shortage of oxygen (O_2), many bacterial species are able to switch from O_2 respiration to using nitrate or its derived nitrogen oxides to support respiration in a process known as denitrification. During this process, the water-soluble nitrate is converted into gaseous nitrogen-containing gases. These are the (a) cytotoxic and ozone-depleting nitric oxide (NO), (b) potent and long-lived greenhouse gas nitrous oxide (N_2O), and (c) the relatively inert dinitrogen gas (N_2). Because denitrification is performed by more than 60 bacterial genera, it was believed that the process would be performed exclusively by bacteria. Now there are evidences that some fungi [89, 111], archaea [118], and some Foraminifera and Gromiida [86, 98] are also able to denitrify. Moreover, nitrifiers also have genes involved in denitrification [14, 104]. A list of archaeal, bacterial, and fungal genera for which at least one denitrifying gene has been reported [84].

2. Genes and enzymes involved in denitrification

Reduction of nitrate to dinitrogen gas is carried out by the sequential activity of the enzymes nitrate, nitrite, nitric oxide, and nitrous oxide reductase, encoded by the *nar/nap*, *nirK/nirS*, c-*nor*/q-*nor*, and *nos* genes, respectively.

2.1. Respiratory nitrate reductases

The first reaction of denitrification, the conversion of nitrate to nitrite, is catalyzed by two biochemically different enzymes, a membrane-bound nitrate reductase (Nar), or a periplasmic nitrate reductase (Nap) [reviewed in [35, 93, 94, 95, 121, 122]]. Nar is a three-subunit enzyme composed of NarGHI, where the catalytic subunit NarG and the one [3Fe-4S] and three [4Fe-4S] NarH subunit are located in the cytoplasm and associate with NarI whose N-terminus faces the periplasm. Nar proteins are encoded by genes of a *narGHJI* operon. *narGHI* genes encode the structural subunits, and *narJ* codes for a cognate chaperone required for maturation and membrane insertion of Nar. In some archaea and bacteria, the NarGH subunits are on the outside rather than the inside of the cytoplasmic membrane. The Nar enzyme couples quinol oxidation with proton translocation and energy conservation, which permits cell growth under oxygen-limiting conditions [105, 137].

Nap is a two-subunit enzyme composed of the NapAB complex located in the periplasm and a transmembrane NapC component. NapA is the catalytic subunit, NapB is a diheme cytochrome c_{552}, and NapC is a c-type tetraheme membrane-anchored protein involved in the electron transfer from the quinol pool to NapAB [reviewed in [35, 88, 93, 94, 95, 121, 122]]. Up to eight different genes have been identified as components for operons that encode perisplasmic nitrate reductases in different organisms. Most bacteria studied thus far have the *napABC* genes in common. The remaining *napDEFKL* genes encode for

different proteins that are not directly involved in the nitrate reduction but in functions required for proper functioning of the enzyme. Although Nap is also linked to quinol oxidation, it does not synthesize ATP [105]. 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 [88]. *Escherichia coli* has a functional duplicate of the *narGHJI* operon named *narZYWV*, which physiologically has a function during stress response rather than anaerobic respiration.

2.2. Respiratory nitrite reductases

Two types of respiratory nitrite reductases (Nir) have been described in denitrifying bacteria, NirS and NirK [96, 97, 122, 123]. Both are located in the periplasmic space and catalyze the one-electron reduction of nitrite to nitric oxide, and neither of the enzymes is electrogenic. The best-characterized *nirS* genes clusters are those from *Pseudomonas aeruginosa* (*nirSMCFDLGHJEN*), *P. denitrificans* (*nirXISECFDLGHJN*), and *P. stutzeri* (*nirSTBMCFDLGH* and *nirJEN*). The NirK enzymes contain type I and II copper centers in the active site and is encoded by the *nirK* gene [97]. Both Nir enzymes are widespread among denitrifiers, but no evidence exists that the same specie could have both enzymes.

2.3. Respiratory nitric oxide reductases

Three types of nitric oxide reductases (Nor) have been characterized, cNor, qNor, and qCu_ANor [reviewed in [27, 121, 122, 123, 138]]. The cNor is an integral membrane enzyme composed of two subunits, the heme c containing-NorC and NorB, which use cytochrome bc_1 complex and a soluble cytochrome c or pseudoazurin as electron donors. The qNor uses quinol or menaquinol as electron donors. The enzyme has been found not only in denitrifying archaea and soil bacteria but also in pathogenic microorganisms that do not denitrify [26] and in the Gram-positive bacterium *Bacillus azotoformans* [110]. This enzyme is bifunctional using both menahydroquinone (MKH2) and a specific c-type cytochrome c_{551} as electron donor. It was suggested that the MKH2-linked activity of qCu_ANor serves detoxification, and the c_{551} pathway has a bioenergetics function. The cNor is encoded by the *norCBQD* operon. The *norC* and *norB* genes encode subunit II and subunit I, 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 [43]. As a unique case, the Nor of *Roseobacter denitrificans* is similar to cNor but differs in that it contains copper [66].

2.4. Respiratory nitrous oxide reductase

The final step in denitrification consists of the two-electron reduction of nitrous oxide to N_2, a reaction catalyzed by the nitrous oxide reductase (Nos) located in the periplasmic space [reviewed in [121, 122, 123, 139]]. Nos is a homodimer of a 65-kDa copper-containing subunit, where each monomer is made up of the Cu_A and Cu_Z domains. The *nos* gene clusters often comprise the *nosRZDFYLX* genes. The *nosZ* gene encodes the monomers of Nos. The *nosDFYL* genes encode proteins that are apparently required for copper assemblage into Nos, although their specific role still remains unknown. The NosRX proteins have roles in transcription regulation, activation, and Cu assemblage of Nos [139].

3. Molecular markers for denitrifying bacteria

Cultivation-dependent and -independent methods have shown that denitrifiers in soils represents up to 5% of the total soil microbial community [46, 114], reaching a density of up to 10^9 cells/g of soil [2, 23, 47]. Although the diversity of denitrifiers was studied by isolating bacterial strains [33], the culture-dependent isolation techniques are limited because of the fact that only a fraction of the bacterial community is cultivable. Application of molecular methods to study microbial diversity in the environment without cultivation was also used to assess the composition of denitrifier communities in environmental samples, mainly soils, waters, and sediments. However, since the ability to denitrify is sporadically distributed both within and between different genera and cannot be associated with any specific taxonomic group, a 16S rRNA phylogeny-based approach is not possible to study denitrifiers. Therefore, existing techniques to study the ecology of this bacterial community are based on the use of functional genes in the denitrification pathway, or their transcripts, as molecular markers [reviewed in [41, 81, 83]]. Accordingly, DNA extraction followed by PCR amplification of denitrification genes is currently the most common way to quantify the denitrifier communities. For this purpose, utilization of reliable primers that allow amplification of the target genes is required. The *nirK*, *nirS*, and *nosZ* genes were the first denitrification genes to be partially amplified [7, 42, 70, 101, 133]; and primers were also developed for amplification of the *narG*, *napA*, and *nosB* genes [8, 17, 31, 37, 85]. With the exponential increase in the databases of the amount of DNA sequences corresponding to denitrification genes, new primers were developed with broader amplification range, including *nirK* [45], *nirS* [113], *nosZ* [46], *narG* [59], and *napA* [11]. A list of commonly used PCR primers for denitrification genes has been published [41]. It is expected that genome sequencing and metagenomic projects will provide new denitrification gene sequences, which could aid in designing new primersprimers as recently demonstrated [52].

4. PCR-independent analyses of denitrifier communities

4.1. Immunological assays

The structure and abundance of active denitrifiers can be determined by targeting proteins encoded by any of the denitrification genes. Antibodies have been used to detect NirK and NirS reductases in isolated denitrifiers [22,69, 127]. After removal of the antibody-labeled cells using flow cytometry, the phylogenetic affiliation of the population could be determined with 16S rRNA oligonucleotide probes. The membrane-bound NarG nitrate reductase was also proposed as a target for quantification of cells isolated from soils [63]. Although the presence of a denitrifying enzyme indicates the presence of the corresponding activity, such an activity can vary among the different species of denitrifiers. Stability of denitrifying enzymes in environmental samples is not well known as it is the time an enzyme may be detected after disappearance of its substrate.

4.2. DNA microarrays

Quantification of microbial communities can be approached using DNA microarrays based on DNA-DNA hybridization. For denitrifying bacteria, oligomer microarrays of different molecular sizes have been developed for assessing *narG*, *nirK*, *nirS*, and *nosZ* diversity and distribution [18,68,112, 115,131]. Microarray-based whole-genome hybridization has also been used as a technique to detect and identify microorganisms in environmental samples [132]. An environmental functional gene microarray to profile microbial gene transcripts was useful for assessing functional attributes of microbial communities [67].

5. PCR-dependent analyses of denitrifier communities

5.1. Fingerprinting of denitrifier communities

Several techniques have been described to resolve PCR-amplified denitrification genes. Cloning and sequencing of the PCR amplicons offer detailed information, but more rapid analysis can be achieved using fingerprinting methods. PCR-restriction fragment length polymorphism (PCR-RFLP), terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and temperature gradient gel electrophoresis (TGGE) have been used to obtain information on the predominant populations in the denitrifier communities. All these techniques separate PCR amplicons of the same size on the basis of their nucleotide-sequence polymorphism. Comprehensive reviews on molecular methods to assess diversity of denitrifying bacteria have been published [41,83,103]. Based on the number of peaks or bands and on their relative intensity, these techniques can give estimates of both richness and evenness, but estimation of the total number of denitrifiers is neglected. To circumvent this problem, competitive PCR (cPCR) and quantitative real-time PCR (qPCR) can be used. According to MIQUE guidelines [12], the initials RT-qPCR should be used for reverse transcription-qPCR.

5.2. Quantification by PCR of denitrifier communities: cPCR and qPCR

PCR can be used for enumeration of denitrifiers using denitrifying genes as molecular markers. 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. Although most denitrification genes so far studied are present in single copies within bacterial genomes, *narG* and *nosZ* can be present in more than one copy [53,85].

Competitive PCR (cPCR) is based on the simultaneous amplification of the target DNA and a control DNA with a known concentration, the so-called competitor. They compete for the primers during amplifications. Because 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. This method has been used for quantification of cytochrome cd_1-denitrifying bacteria in environmental marine samples [70]. cPCR has also been used to quantify the *nirK* gene in soil and stream-sediment samples [90] and the *nirK* and *nirS* genes in membrane-aerated biofilms at different depths [20]. Some drawbacks of this method are its limited dynamic range, the need to screen multiple dilutions, and the requirement for a gel migration step.

Amplification of the *nirS* gene by quantitative PCR (qPCR) was first shown in 2001 [39]. In their experiments, they used the TaqMan technology and designed primers for the *Pseudomonas stutzeri nirS* gene. Due to the high specificity of this system, the primer-probe set was specific for *nirS* sequences that correspond only to *P. stutzeri* and, therefore, was of limited utility. SYBR Green is a fluorescent dye that binds non-specifically to double-stranded DNA. During PCR, the intensity of the fluorescence is detected, which results in a logarithmic increase in emission of fluorescence until the reagent become limiting. The cycle number of the PCR at which the fluorescent signal crosses the threshold receives different names: threshold cycle (C_t), take-off point (TOP), crossing point (C_p), and quantification cycle (C_q). The MIQE Guidelines [12] propose the use of the latter name for describing the fractional PCR cycle used for quantification. Because there is a positive correlation between the log of the initial DNA template concentration and the corresponding C_q, if one knows the starting amount of target DNA, a

standard curve can be constructed by plotting C_q as a function of the log of the copy number of the target DNA. The gene copy number in the DNA sample can then be determined based on its C_q. 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.

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 [9, 107, 135]. Accordingly, qPCR is currently the main method used for quantification of environmental samples, including the denitrifier communities.

Because RNA provides evidence of gene expression, qPCR techniques based on RNA extracted directly from environmental samples can be retro-transcribed to cDNA and used for qPCR. After reverse transcription, the *narG*, *napA*, *nirK*, *nirS*, and *nosZ* genes were quantified by standard qPCR [74]. Quantification of the *nosZ* was also achieved using cDNA, but *nirS* and *nirK* genes could not be amplified [25]. Using cDNA from an agricultural soil, the *nosZ* and *nirSp* (*nirS* from *Pseudomonas mandelii*) genes were quantified by qPCR, but amplification of the *nirK* and *nirS* from the total soil community, or their transcripts, was not obtained even when different primers, PCR conditions and cycling parameters were used [44]. 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.

6. DNA extraction from environmental samples

A conventional approach to evaluate the abundances of denitrifiers in environmental samples include (a) DNA extraction and purification, (b) PCR's inhibition tests, (c) Target gene quantification by qPCR, and (d) analysis of the obtained results. In this review, the term environmental sample refers mainly to soil and sediment samples.

6.1. Initial attempts for DNA isolation

Pioneer methods for soil DNA isolation used *ex situ* methodologies that included long incubations of soils in a solution made of sodium cholate and Chelex 100 resin, followed by centrifugation and passage through a Percoll gradient to separate the most dense soil particles from the floating organic matter and microorganisms [48, 117]. Further breakage of the cells by sonication and differential centrifugation allowed DNA extraction. Later, it was shown that the DNA samples obtained by this methodology were not representative of the entire DNA in the soil samples [56, 108].

First approaches to total soil DNA isolation from environmental samples were developed by several authors using mechanical and enzymatic lysis, followed by cleaning of the crude extract and DNA precipitation. Accordingly, soils were treated with a high salt concentration-extraction buffer containing hexadecyl-trimethyl ammonium bromide (CTAB) and proteinase K. Samples were further incubated with sodium dodecyl sulfate (SDS), mixed with a mixture of clorophorm/isoamyl alcohol (24:1), precipitated with isopropanol, washed with 70% ethanol, and, finally, resuspended in milliQ (MQ) water [136]. This methodology was improved to simultaneously recover RNA and DNA from soils and sediments by homogenization of the samples in a high salt concentration-extraction buffer containing CTAB and SDS, frozen in liquid nitrogen and ground until thawed [49]. Other authors homogenized the soil samples in an extraction buffer containing PVPP and Chelex 100 resin, extracted the DNA with the same buffer supplemented with SDS, proteinase K and 10% Sarkosyl, and purified it with a mixture of phenol/clorophorm/isoamyl alcohol (25:24:1) [54]. Cell lysis was also achieved using a long treatment at 68 °C in the presence of SDS and guanidine isothiocyanate followed by precipitation with polyethylene glycol (PEG-8000) and purification with CTAB, chloroform, and ammonium acetate [30, 87].

6.2. The ISO standard 11063 Soil quality—method to directly extract DNA from soil samples

During evaluation of the effectiveness of nine DNA extraction procedures, homogenization of the samples using a bead beater disrupter and SDS in the extraction buffer gave the best results [71]. Based on these data, a method was developed and published that has been shown to provide good quantity and quality DNA [65]. In addition to good reproducibility, the method provided results for DNA extraction from diverse environmental samples, including soils from a range of origins and different physical and chemical characteristics [17, 59, 64, 79, 85]. Accordingly, this method was proposed in 2006 by the Agence Française pour la Normalisation (AFNOR) to the International Organization for Standardization (ISO). Because an ISO standard would give information on the identity and quality of each compound in the protocol, it would also provide a complete quality control for users, thus avoiding the risks associated to commercial kits. After recognition of the need for an international standard for soil DNA extraction, an action was formally agreed, and the method was evaluated by 15 independent European laboratories, 6 from France and 9 from other countries including Finland, Germany, Italy, Spain and Sweden. The amount of DNA extracted from 12 different soils was compared to evaluate both the reproducibility of the standardized method and the abundance and genetic structure of the total bacterial community. Quantification of the 16S rRNA gene abundances by quantitative PCR (qPCR) and analysis of the total bacterial community structure by

automated ribosomal intergenic spacer analysis (A-RISA) showed acceptable to good levels of reproducibility. The method has been unanimously approved by the ISO as an international standard method (ISO standard 11063) [80]. The method has also been used to extract DNA from river sediments and agricultural soil, waters, biofilms and glacier soils [10,11], polychlorinated biphenyls-contaminated sites [79], constructed wetlands [21], and technosols [40].

Essentially, the method is as follows.

(1) Sieve samples to < 2 mm. Weight 0.25 g equivalent dry weight aliquots in a 2-mL microtube and keep frozen at -80 °C until use.
(2) Thaw the samples. Add 0.5 g of 106-μm glass beads, 2 beads of 2 mm diameter, and 1 mL of homogenization buffer extemporaneously prepared (100 μL 1 M Tris-HCl (pH 8.0), 200 μL 0.5 M EDTA (pH 8.0), 100 μL 1 M NaCl, 50 μL 20% PVP 40 T, 100 μL 20% SDS, 450 μL MQ water).
(3) Homogenize the mixture by using a mini bead beater system (1.600^{-1} shaking frequency/min for 30 s). Use a shaking flask previously kept at -20 °C. Incubate for 10 min at 70 °C then centrifuge at $14.000 \times$ g for 1 min at 4 °C.
(4) 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 then centrifuge at $14.000 \times$ g for 5 min at 4 °C.
(5) Transfer the supernatant to a new 1.5-mL microtube. Add 1:1 (v/v) prechilled (-20 °C) isopropanol. Mix well by manual inversion. Incubate for at least 15 min at -20 °C then centrifuge at $14.000 \times$ g for 30 min at 4 °C.
(6) Remove the supernatant. Wash the pellet (containing the nucleic acids) with prechilled (-20 °C) 70% ethanol with precaution to avoid pellet resuspension. Centrifuge for 15 min at $14.000 \times$ g at 4 °C.
(7) Discard the supernatant and dry the pellet for 15 min at 37 °C.
(8) Resuspend the pellet in 50 μL MQ water.
(9) Prepare sample aliquots and store at -20 °C until use.

7. DNA purification

Because of the presence of PCR-inhibitory compounds in the environmental samples, DNA purification is recommended. There is to note, however, that DNA purification is not part of the ISO standard 11063.

7.1. DNA purification through PVPP and sepharose 4B columns

As a first step of purification, prepare the PVPP column as follows.

(1) Fill about 1.2 cm of an empty micro-spin chromatography column with PVPP powder (about 95 mg) and add 400 μL of MQ water.
(2) Place the column in a 1.5-mL tube and centrifuge for 2 min at $1000 \times$ g. Discard the eluate. Add 400 μL of MQ water to the column and centrifuge for 2 min at $1000 \times$ g. At this moment, the column can be kept at 4 °C.
(3) Add the 50 μL DNA sample to the column and place it in ice for 5 min.
(4) Place the column into a new tube and centrifuge at $1.000 \times$ g for 4 min at 10 °C to recover the DNA sample. Quantify the final volume of the sample.

Further DNA purification can be obtained by using sepharose 4B columns as indicated earlier [65,80].

(1) Fill an empty micro-spin chromatography column with 1 mL of sepharose 4B solution.
(2) Place the column in a 2 mL tube and centrifuge for 2 min at $1.100 \times$ 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 $1.100 \times$ 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 $1.400 \times$ g for 5 min at 10 °C. Quantify the final volume of the sample.

After PVPP cleaning, alternatively to utilization of sepharose 4B columns, commercial kits can be used for DNA purification. Here, we described cleaning of DNA samples using the Geneclean® turbo kit (GLASSMILK®-embedded membrane, MP Bio). Following manufacturer's instructions, the procedure allows purification of DNA fragments of sizes from 0.1 kb to 300 kb. Essentially,

(1) To the DNA sample (V), add 5 volumes of salt solution and mix well by vortexing.
(2) Add the mixture to a Geneclean® cartridge and centrifuge at $14.000 \times$ g until it all has passed through the filter.
(3) Add 500 μL of the ethanol-containing Geneclean® washing solution to the cartridge and centrifuge at $14.000 \times$ g for 5 s. Discard the eluate. Recentrifuge the cartridge at $14.000 \times$ g for an additional 4 min and discard the eluate.
(4) Place the cartridge into a new 1.5-mL tube.
(5) Add 50 μL Geneclean® elution solution directly onto the GLASSMILK®-embedded membrane and incubate at room temperature 5 min.
(6) Centrifuge at $14.000 \times$ g for 1 min. Recover the eluate.

7.2. Other extraction and purification methods

The following methods are used to extract environmental DNA: (a) cation-exchange [51], (b) nitrogen-grinding [125], (c) microwave-based rupture [77], (d) Nycodenz gradient separation [6], (e) solvent-based beating [15], (f) aluminum-based extraction [78], and (g) calcium chloride [58].

Table 1: Primers used for PCR amplification of bacterial standard 16S rRNA and denitrification genes.

Primer	Primer sequence (5′-3′)	Target gene	Size of the amplicon (base pair, bp)	Reference
T7	TAATACGCATCACTATAGGG		150	Promega Corp.
Sp6	GATTTAGGTGACACTATAG			
341F	CCTACGGGAGGCAGCAG	16S rRNA*	194	[73]
534R	ATTACCGCGGCTGCTGGCA			
narG-f	TCGCCSATYCCGGCSATGTC	*narG*	174	[11]
narG-r	GAGTTGTACCAGTCRGCSGAYTCSG			
nap3F	TGGACVATGGGYTTYAAYC	*napA*	152	[11]
napA4R	ACYTCRCGHGCVGTRCCRCA			
nirK876F	ATYGGCGGVAYGGCGA	*nirK*	173	[45]
nirK1040R	GCCTCGATCAGRTTRTGGTT			
nirS4QF	AACGYSAAGGARACSGG	*nirS*	425	[113]
nirS6QR	GASTTCGGRTGSGTCTTSAYGAA			
nosZ1840F	CGCRACGGCAASAAGGTSMSSGT	*nosZ*	267	[46]
nosZ2090R	CAKRTGCAKSGCRTGGCAGAA			

*In addition to the abundance, quantification of the 16S rRNA gene allows calculation of the relative abundance of a denitrification gene as the ratio between the abundance of any denitrification gene and the abundance of the 16S rRNA gene.

In many laboratories, utilization of commercial kits for DNA isolation is also frequent. Among them are MoBIO PowerSoil DNA kit (MoBIO) [19,24,62], ultra clean soil DNA kit (Ozyme, MoBIO) [5,61,99], and fast DNA spin kit for soil (BIO 101/Q-Biogene) [29,50,55,102].

A combination of hand-made and kit methodologies have also been used [38,57,109]. This method used a Bio-101 Multimix 2 matrix tube in combination with the fast-prep FP120 bead beater disruptor. DNA extraction was performed with addition of hexadecyl-trimethyl ammonium bromide (CTAB) to the commercial extraction buffer.

In addition to PVPP, sepharose 4B and Geneclean® turbo kit DNA, DNA purification can be achieved using: (a) phenol [119], (b) elutip-d and sephadex G-200 columns [120], (c) cesium chloride, glassmilk and spearmine [106], (d) PVPP and Microcon-100 columns, microconcentrators [129], (e) agarose gel electrophoresis [136], (f) HR S400 spin columns fast DNA purification kit and elution through Qiagen Mini column [91], (g) Wizard DNA Clean-Up System [28], (h) AllPrep DNA/RNA mini kit [34].

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 [99], biofilms formed on estuarine rocks colonized by algae [61], the vermicompost from a fresh olive waste [124], soil-feeding mounds of nematodes or termites [28,30], and a bioreactor's biofilm [13]. 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 [116]. 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* [32].

7.3. Checking quantity and quality of the purified DNA

Quantity and quality of the DNA throughout an extraction process can be estimated by electrophoresis on 1% agarose gels in 0.5X TBE buffer [100] at 80 V. Samples (4 μL) can be supplemented with 1 μL 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).

8. Preparation of standard DNAs for qPCR

Standard DNAs were obtained after amplification from genomic DNA of the 16S rRNA, *narG* and *napA* genomic DNA from *P. aeruginosa* PAO1, *nirS* from *P. fluorescens* C7R12, *nirK* from *Ensifer meliloti* 1021, and *nosZ* from *Bradyrhizobium japonicum* USDA110. Primers used for amplification are presented in Table 1. Those primers have been used for amplification of the 16S rRNA and denitrification genes from agricultural soils [1,5,23,62, 72], forest soils [4], containers filled with wood by-products [128], rice paddy field soils [134], grassland pasture soils [82], soils from the Burgundy region [10], soil-feeding mounds of nematodes [28], technosols [40], and constructed wetlands [21].

8.1. Primers, reaction mixture and thermocycler conditions used for PCR amplification of bacterial standard 16S rRNA and denitrification genes

The primers used for the amplification of the bacterial standard for 16S rRNA and *narG*, *napA*, *nirK*, *nirS*, and *nosZ* denitrification genes are shown in Table 1.

Table 2: Thermocycler conditions for amplification of standard 16S rRNA and denitrification genes by PCR.

	Gene *narG*, *nirK*, and *nirS*	*napA*	*nosZ*	16S rRNA
Stage 1: 1 cycle	10 min at 95 °C	10 min at 95 °C	10 min at 95 °C	10 min at 95 °C
Stage 2: 6 cycles with 1 °C decrease by cycle	15 s at 95 °C 30 s at 63 °C 30 s at 72 °C	15 s at 95 °C 30 s at 61 °C 30 s at 72 °C	15 s at 95 °C 30 s at 65 °C 30 s at 72 °C	
Stage 3: 35 cycles	15 s at 95 °C 30 s at 58 °C 30 s at 72 °C	15 s at 95 °C 30 s at 56 °C 30 s at 72 °C	15 s at 95 °C 30 s at 60 °C 30 s at 72 °C	15 s at 95 °C 30 s at 60 °C 30 s at 72 °C
Stage 4: 1 cycle	10 min at 72 °C	10 min at 72 °C	10 min at 72 °C	10 min at 72 °C

Reaction mixture for PCR-amplification of standard 16S rRNA and denitrification genes:

(1) 1 to 5 ng template DNA (either 16S rRNA, *narG*, *napA*, *nirS*, *nirK*, or *nosZ*).
(2) 0.6 μmol forward primer for each gene.
(3) 0.6 μmol reverse primer for each gene.
(4) PCR buffer 1X (2.5 μL).
(5) 1.5 mmol $MgCl_2$.
(6) 0.4 mM dNTPs.
(7) 0.04 U high fidelity Taq polymerase.
(8) Add up to 25 μL MQ/ultrapure water.

The thermocycler conditions for PCR-amplification of denitrification genes are shown in Table 2.

After amplification, the PCR products were electrophoresed on agarose gels to check size and purity of amplicons, purified using any appropriate commercial kit, and cloned in pGEM-T Easy vector (Promega) following manufacturer's instructions. Plasmids were used to transform *Escherichia coli* JM109. The presence of the insert in the plasmid can be verified by PCR using T7 and Sp6 primers and further sequencing of the corresponding DNA fragments. DNA sequences will 100% match the corresponding sequences of each denitrification gene. Care should be taken to remove the DNA sequences corresponding to the polylinker regions of the plasmid, which are also amplified.

8.2. Calculation of the copy number of standard DNA

Recombinant pGEM-T easy plasmid containing insert DNA can be linearized using the restriction enzyme *Sal*I. Because other enzymes can be used to linearize the pGEM-T Easy vector, the existence of a unique cutting site in the DNA sequence can be checked by using the web site http://www.bioinformatics.org/sms2/rest_digest.html in the Sequence Manipulation Suite Program. After digestion, use any appropriate commercial kit to purify DNA. Then determine DNA concentration (ng/μL) by spectrophotometry as indicated above. To calculate the molecular weight of a DNA fragment, use the formula MW (ng/mol) = bp number $\times$ 660 g/mol $\times$ 10^9 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 (mole/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) $\times$ 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.

9. Inhibition test

During environmental DNA extraction, humic acids, organic and phenolic compounds, glycogen, fats, Ca^{2+} ions, heavy metals, detergents, antibiotics, and constituents of bacterial cells can be co-extracted [130]. Because quality of the template DNA is one of the most important determinants of the sensitivity, accuracy and reliability of any PCR [75], care should be taken to avoid the presence of inhibitory compounds in the extracted DNA solution. Three potential mechanisms could inhibit PCRs: binding of the inhibitor to the polymerase, interaction of the inhibitor with the DNA, and interaction with the polymerase during primer extension. As a result, significant reductions in the sensitivity and kinetics of PCR assays can be produced. In addition, for statistical comparisons among samples, a similar PCR efficiency is required [3].

qPCR is currently the method of choice to test the presence of inhibitory compounds in DNA from an environmental sample [76]. 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.

Moreover, 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.

The absence of PCR inhibitors in the soil DNA extracts can be analyzed by mixing a known amount of standard DNA. Because this standard DNA is supposed not to be targeted in the environmental sample, the linearized

Table 3: Thermocycler conditions for qPCR inhibition test.

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

*Times and temperatures should be set according to the manufacturer's instructions. Values in Table 3 have been employed successfully with different buffers.
**Dissociation curves can be established by each laboratory. Values in Table 3 are widely used.

pGEM-T easy vector without insert can be used for this purpose with environmental DNA. Then the standard DNA can be amplified by qPCR using universal primers SP6 and T7 as described earlier [46]. A typical inhibition test can be run as follows.

9.1. Reaction mixture for inhibition test

(1) 2 ng template (environmental) DNA
(2) 1×10^7 copies of *Sal*I-digested 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 2X (containing HotStar Taq polymerase and dNTPs)
(7) Add MQ/ultrapure water up to 15 μL.

In separate wells, add (a) standard DNA and template DNA, (b) standard DNA without template DNA, and (c) qPCR control without any DNA. Add eventually mastermix. 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.

9.2. qPCR conditions for inhibition test

Thermocycler conditions are shown in Table 3.

10. Gene quantification by qPCR

10.1. Reaction mixture for qPCR

(1) 2 ng template (environmental) DNA
(2) 1 μM forward primer for 16Sr RNA and 2 μM for denitrification genes
(3) 1 μM forward primer for 16Sr RNA and 2 μM for denitrification genes
(4) 250 ng T4 Gp32
(5) 7.5 μL SYBR Green PCR buffer 2X (containing HotStar Taq polymerase, buffer and dNTPs)
(6) Add MQ/ultrapure water up to 15 μL.

10.2. Preparation of a standard DNA curve

For quantification of environmental DNA, construction of a standard curve is required. For that purpose, 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 samples. Take 2 μL from each of the 6 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. In separate wells, add reaction mixture without any DNAs.

10.3. External DNA controls

In qPCR, external DNA controls can be genomic DNA isolated from denitrifying bacteria. External DNA for the 16S rRNA, *narG*, *napA*, and *nirS* genes have been isolated from *P. aeruginosa* PAO1; *nirK* and 16S rRNA from *E. meliloti* 1021; and 16S rRNA, *napA*, and *nosZ* genes from *B. japonicum* USDA110. Since those genomes were completely sequenced, their size and the copy number of the targeted gene per genome are known. These data allow determining the expected copy number of the targeted gene per unit of weight (e.g., copy number per ng of genomic DNA). These samples can then be used to assess the reliability of the assay. A usual copy number for external DNA controls is 1×10^7.

10.4. Thermocycler conditions for bacterial 16S rRNA and denitrification genes

Thermocycler conditions for bacterial 16S rRNA and *narG*, *napA*, *nirK*, *nirS*, and *nosZ* denitrification genesare presented in Table 4.

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. New standard dilutions should be prepared for each reaction curve.

After qPCR, for each sample, the software of the q-PCR thermocycler will retrieve values of fluorescence intensity throughout the amplification cycles. At a certain cycle, the fluorescence intensity crosses over a level where the amplification enters a logarithmic growth phase. This cycle is called the quantitative (C_q). This value is inversely proportional to the log value of the initial DNA concentration in the reaction mixture. During qPCR, keep track on the background, exponential amplification, linear amplification, and plateau of each curve. Finally, a standard curve is drawn by plotting the C_q value of each standard DNA against the tenth 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

Table 4: Thermocycler conditions for quantification of 16S rRNA and denitrification genes by qPCR.

	Genes *narG*, *nirK*, and *nirS*	*napA*	*nosZ*	16S rRNA
Stage 1*: 1 cycle	10 min at 95 °C	10 min at 95 °C	10 min at 95 °C	10 min at 95 °C
Stage 2*: 6 cycles with 1 °C decrease by cycle	15 s at 95 °C 30 s at 63 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	15 s at 95 °C 30 s at 61 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	15 s at 95 °C 30 s at 65 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	
Stage 3*: 35 cycles	15 s at 95 °C 30 s at 58 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	15 s at 95 °C 30 s at 56 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	15 s at 95 °C 30 s at 60 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)	15 s at 95 °C 30 s at 60 °C 30 s at 72 °C 30 s at 80 °C (data acquisition)
Stage 4**: dissociation stage (melting curve: 30 cycles with 0.5 °C increase by cycle)	15 s at 95 °C 15 s at 80 °C 15 s at 95 °C	15 s at 95 °C 15 s at 80 °C 15 s at 95 °C	15 s at 95 °C 15 s at 80 °C 15 s at 95 °C	15 s at 95 °C 15 s at 80 °C 15 s at 95 °C

*Times and temperatures should be set according to the manufacturer's instructions. Values in Table 4 have been employed successfully with different buffers.
**Dissociation curves can be established by each laboratory. Values in Table 4 are widely used.

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 spread sheet application and run appropriate statistical analyses.

When running inhibition tests, absence of inhibition is considered when differences in C_q values are ± 1 cycle. Should inhibition be detected, repurification of the sample DNA is required.

11. Other primers for qPCR

Several research groups have developed different sets of primers for qPCR amplification of the bacterial 16S rRNA and denitrifying genes (Table 5). Accumulation in the databank of complete sequences from bacterial genome projects and from newly isolated denitrifying bacteria will help to design and increase sensitivity or new denitrification primers.

Table 5: Other primers used for PCR amplification of bacterial standard 16S rRNA and denitrification genes.

Primer	Primer sequence (5′-3′)	Target gene	Reference
519F	GWATTACCGCGGCKGCTG	16S rRNA	[116]
907R	CCGTCAATTCMTTTRAGTTT		
1055f	ATGGCTGTCGTCAGCT	16S rRNA	[19]
1392r	ACGGGCGGTGTGTAC		
1960m2f	TAYGTSGGGCAGGARAAACTG	*narG*	[59]
2050m2r	CGTAGAAGAAGCTGGTGCTGT		
narG328f	GACAAACTTCGCAGCGG	*narG*	[92]
narG497r	TCACCCAGGACGCTGTTC		
V16	GCNCCNTGYMGNTTYTGYGG	*napA*	[126]
V17	RTGYTGRTTRAANCCCATNGTCCA		
F1aCu	ATCATGGTSCTGCCGCG	*nirK*	[29]
R3Cu	TTGGTGTTRGACTAGCTCCG		
nirK517F	TTYGTSTAYCACTGCGCVCC	*nirK*	[16]
nirK1055R	GCYTCGATCAGRTTRTGGTT		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	[16]
nirS950R	GCBACRCGSGGYTCSGGATG		
nirS2F	TACCACCCSGARCCGCGCGT	*nirS*	[19]
nirS3R	GCCGCCGTCRTGVAGGAA		
nirSsh2F	ACCGCCGCCAACAACTCCAACA	$nirS_{Pm}$[1]	[44]
nirSsh4R	CCGCCCTGGCCCTGGAGC		
forward	ACAAGGAGCACAACTGGAAGGT	$nirS_{Ps}$[2]	[39]
reverse	CGCGTCGGCCCAGA		
cnorBPF	CATGGCGCTGATAACGGG	$cnorB_P$[3]	[24]
cnorBPR	CTTIACCATGCTGAAGGCG		

Table 5: To be continued.

Primer	Primer sequence (5′-3′)	Target gene	Reference
cnorBBF	AIGTGGTCGAGAAGTGGCTCT	$cnorB_B$[4]	[24]
cnorBBR	TCTGIACGGTGAAGATCACC		
nirS263F	TGCGYAARGGGGCANCBGGCAA	*nirS*	[16]
nirS950R	GCBACRCGSGGYTCSGGATG		
nosZ1F	WCSYTGTTCMTCGACAGCCAG	*nosZ*	[44]
nosZ1R	ATGTCGATCARCTGVKCRTTYTC		
Forward	AGAACGACCAGCTGATCGACA	*nosZ*	[19]
Reverse	TCCATGGTGACGCCGTGGTTG		
nosZ-F-1181	CGCTGTTCITCGACAGYCAG	*nosZ*	[60]
nosZ-R-1880	ATGTGCAKIGCRTGGCAGAA		

[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 for populations of *P. mandelii* and closely related strains ($cnorB_P$-bearing communities).
[4] *norB* gene for populations of *Bosea*, *Bradyrhizobium*, and *Ensifer* spp. ($cnorB_B$-bearing communities).

Using the new set of primers, nosZ-II-F (CTIGGICCIY-TKCAYAC) and nosZ-II-R (GCIGARCARAAITCBGTRC) [52], a much larger diversity than that previously reported for bacterial and archaeal populations carrying a *nosZ* gene has been detected [36, 53]. Quantification of the new nosZ-II population in different environmental samples revealed that its relative abundance is similar to that found when the conventional *nosZ* primers (Table 1) were used.

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