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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Microbial Nitrogen Cycle: Determination of Microbial Functional Activities and Related N-compounds in Environmental Samples

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David Correa-Galeote, Germán Tortosa and Eulogio J. Bedmar

Abstract

Nitrogen (N) is part of essential compounds such as proteins, nucleic acids, hormones, etc. Although N makes up to about 80% of the earth's atmosphere, it is not readily available for plant and animal consumption. Free-living and symbiotic microbes contain the enzyme nitrogenase which initiates the N-cycle in the biosphere by reducing dinitrogen gas to bio-available ammonia, a process called nitrogen fixation. Ammonia is subsequently oxidized to nitrate by nitrification, a two-step aerobic pathway during which ammonia is oxidized to nitrate and nitrite by the enzymes ammonia monooxygenase and nitrite oxidoreductase, respectively. Finally, nitrate is reduced to dinitrogen gas by denitrifying microorganisms, thereby closing the N cycle. Denitrification is carried out by the sequential activity of the enzymes nitrate-, nitrite, nitric oxide and nitrous oxide reductase, respectively. Ammonia can also be incorporated into cellular biomass via the glutamine synthetase–glutamate synthase and glutamate dehydrogenase pathways to form amino acids and other nitrogen compounds. After cellular death, organic nitrogen compounds are released to the environment to be mineralized by microbial activities. Widely used procedures for determination of microbial functional activities of the nitrogen cycling microorganisms and of N-compounds produced during the redox reactions of the cycle will be addressed. In addition, we will consider new methodologies being developed for further understanding of the N-cycle.

Introduction

Most of the N is in the earth's atmosphere is found as dinitrogen gas (N_2), a form which is inaccessible to eukaryotes and many bacteria. Diazotrophic microorganisms, mainly bacteria, contain the enzyme nitrogenase, which converts bio-unavailable N_2 gas to bio-available ammonium (NH_4^+). This process is called biological nitrogen fixation and initiates the N cycle in the biosphere (Fig. 9.1). Ammonium 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.

Besides its incorporation into organic nitrogen compounds, ammonium can be oxidized to nitrate (NO_3^-) by nitrifying bacteria in a process called nitrification. During nitrification the enzymes ammonia monooxygenase, hydroxylamine oxidoreductase and nitrite oxidoreductase (nitrite oxidase) oxidize ammonium to hydroxylamine (NH_2OH), nitrite (NO_2^-) and nitrate, respectively. Nitrate can be reduced to ammonia through the nitrate assimilation process by the assimilatory nitrate reductase and nitrite reductase enzymes.

Under oxygen-limiting conditions, nitrate can be reduced to N_2 via the formation of nitrite, nitric oxide (NO) and nitrous oxide (N_2O), the so called denitrification pathway. Denitrification converts nitrate to N_2, which returns to the atmosphere, thus closing the N cycle in the biosphere.

In addition to denitrification, anaerobic ammonium oxidation (anammox) converts nitrite and ammonium directly into N_2, thus

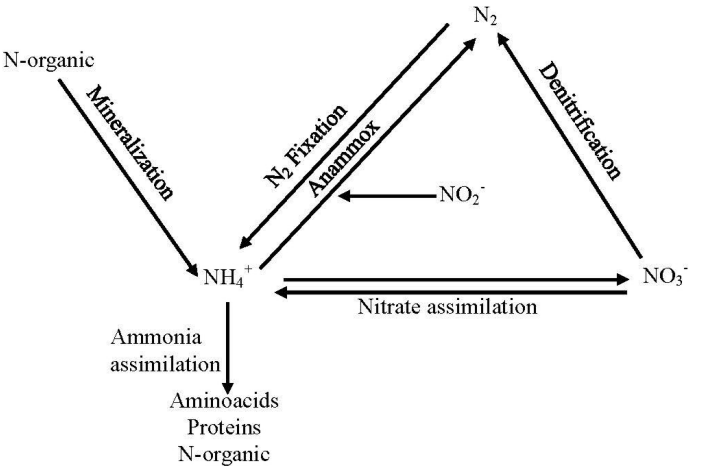

Figure 9.1 A scheme of the N-cycle.

largely contributing to production of N_2. Genome sequencing of several N-cycle organisms, the nitrite-dependent anaerobic methane oxidation (Raghoebarsing *et al.*, 2006) and hyperthermophilic N_2-fixing methane producing archaea (Mehta and Baross, 2006) are examples of the biodiversity and metabolic capacity of new nitrogen conversions within the N cycle (Jetten, 2008; van Nifrik and Jetten, 2012; Kartal *et al.*, 2012). Together these processes form the global N cycle and microorganisms are essential for maintaining the balance between reduced and oxidized forms of nitrogen (van Spanning, 2011).

Until appearance of metagenomics in the last decade, the direct determination of N-compounds and the microbial functional activities have been used as classical approaches to characterize the environmental N-cycle processes shown in Fig. 9.1. Although several methods for the study of N-cycle compounds are currently available, other new techniques are being developed due to advances in analytical technologies that are being continuously improved.

In this chapter, we present some widely-used analytical procedures for the practical study of the main N-cycle processes occurring under environmental conditions, aiding researchers to better understanding the most important reactions of the N-cycle.

Microbial functional activities of the N-cycle

Biological nitrogen fixation

Nitrogen fixation is the reduction of dinitrogen gas (N_2) to ammonium (NH_4^+), a process carried out by the enzyme nitrogenase. Nitrogenase activity can be determined according to the following protocols.

The acetylene-dependent ethylene production technique

Besides N_2 to NH_4^+, the nitrogenase complex also reduces acetylene, azide, cyanide, nitrous oxide and protons. Activity of nitrogenase can be readily detected in environmental samples using the acetylene-dependent ethylene production (acetylene reduction activity, ARA) assay (Hardy *et al.*, 1973).

Procedure

1 Weigh 25 g fresh sample (soils, sediments, etc.) and place it within a 100 ml bottle. Bottles have to be hermetically closed (e.g. by using rubber septa). Weights of the samples and volume of the bottles may vary to optimize the assay. Septa must allow injection and sampling of the internal atmosphere of the bottles. As a blank include bottles without sample.

2 Close the bottle-containing sample ($n \geq 4$).

Flush the headspace for 5 min with N_2, or the more expensive Ar or He, to create anoxic conditions.

3 Replace 10% of the internal atmosphere of the bottles by the same volume of acetylene (C_2H_2). Mix well by shaking. Commercial acetylene, or that obtained by mixing calcium carbide (CaC_2) and water (1:15 w/v), can be used.

4 Incubate the samples at 25°C. Incubation time depend of the acetylene reduction activity of the samples.

5 Take gas aliquots from the headspace of the bottle for injection onto the gas chromatograph. Aliquots from 100 to 1000 µl can be used.

Kinetic of ethylene (C_2H_4) production can be followed by taking samples over time. Because assays are run in closed systems, gas accumulation may cause feedback inhibition of the enzymatic activity. Accordingly, gas samples must be taken during the exponential phase of gas production. Long incubation times may result in spoiling of the samples.

When activity of the samples is low, nitrogenase activity can be determined after incubation of the samples under non-limiting N_2-fixing conditions, this is, in the presence of an excess carbon source, e.g. 10% glucose. It is to note that any available nitrogen source inhibits nitrogenase and, accordingly, nitrate concentration should be checked in the samples. Chloramphenicol can be used to prevent new protein synthesis and growth of N_2-fixing microorganisms. Under these conditions, nitrogenase activity can be detected within 0–48 h depending on ethylene production kinetic.

Ethylene can be determined by gas chromatography using a flame ionization detector (FID). The chromatograph is usually provided with N_2 as a carrier gas, and H_2 and synthetic air to make up the flame. Gas fluxes through the chromatograph as well as through the oven, injector and detector temperatures may vary depending on each chromatograph commercial brand and the type of column used for the chromatography. Concentration of ethylene in each sample can be calculated from standards of pure ethylene. A correction for dissolved ethylene in water (Bunsen solubility coefficient) has to be considered when using sediments or soil slurries. Values are usually expressed as mol C_2H_4 produced × $kg^{-}1$ (dry soil, dry sediment, etc.) × h^{-1}. Gas tight syringes should be used.

ARA can also be used to estimate rates of nitrogen fixation by pure cultures of free-living and symbiotic N_2-fixing microorganisms. For free-living cells, liquid and solid media have been defined which allow microbial growth for determination of nitrogenase activity. For symbiotic bacteria, nodules, either from roots, stems or leaves can be used.

Determination of ARA by the acetylene-dependent ethylene production technique has several disadvantages related to (a) diffusion of acetylene in the soil, especially in wet or heavy-textured soils, (b) degradation of acetylene by bacteria, (c) inhibition of other processes, for example nitrification or (d) disturbance of the soil structure when soil cores are taken. In addition, the acetylene-inhibition method does not provide information on field N_2 production rates.

Readers are referred to Burris (1974) and Hardy and Holsten (1977) to learn on general problems regarding determination of nitrogen fixation, and Vessey (1994) and Minchin *et al.* (1994) for concerns related to the use of ARA to assay nitrogenase activity in nodulated legumes. Previous methods describing the acetylene reduction assay can be found in Zechmeister-Boltenstern (1996a).

15N2 isotope determination

A direct method for determination of nitrogenase activity is based on the utilization of $^{15}N_2$. Samples are incubated with $^{15}N_2$ and O_2 for a long period of time in hermetic bottles. After the incubation, total nitrogen content and the ratios between $^{15}N_2$ and $^{14}N_2$ are determined.

Procedure

1 Weigh 10 g of environmental solid sample (soils, sediments, etc.) ($n \geq 4$) in a gas-tight bottle equipped for gases injection and withdrawal. Determine the remaining headspace volume.

2 Close the bottle. Flush the headspace with He for 5 min.

3 Substitute 50% of the headspace volume with enriched $^{15}N_2$ and 20% with O_2.
4 Seal the bottle and incubate at 25°C in the dark. Incubation time depends on nitrogenase activity, varying between 3 to more than 30 days.
5 After incubation, the ratio $^{15}N/^{14}N$ is determined by mass spectrometry.
6 Total solid nitrogen (T_N) of the samples is assayed as indicated in 'Total solid N'.
7 As a blank use a set of samples incubated without enriched $^{15}N_2$.

The isotopic composition of a sample is reported as $\delta^{15}N$ (‰) per ml:

$$\delta\,^{15}N\,(‰) = (R_{sample}/R_{standard} - 1) \times 1000$$

where $R = {}^{15}N/{}^{14}N$ ratio.

Commercial N_2 can be used as the standard for isotopic composition analyses. The $^{15}N/^{14}N$ ratio in the standard is calculated against a reference curve obtained by using internationally accepted reference materials (http://www.iaea.org).

The proportion of N derived from N_2 fixation ($\%N_{FIX}$) is calculated as:

$$\%N_{FIX} = 100 \times [1 - (A/B)]$$

where A = atom% ^{15}N excess in samples incubated with enriched $^{15}N_2$ and B = atom% ^{15}N excess in samples incubated without enriched $^{15}N_2$. atom% $^{15}N = \delta\,^{15}N\,(‰) \times 100$.

The fixed nitrogen content (FN) is calculated as:

$$FN = (\%N_{FIX} \times T_N)/100$$

The advantage of this method is that it does not imply disturbance of the soil, that N_2 production can be determined, and that an N budget can be made. The method also has some disadvantages, the most important being that (a) analysis of ^{15}N is only possible at specialized laboratories, and (b) ^{15}N is not distributed homogeneously throughout the soil. ^{15}N -labelled material can be expensive and can only be used on small plots.

Other protocols for determination of nitrogenase activity have been published by Bergensen (1980), Weaver and Danso (1994), Zechmeister-Boltenstern (1996b) and Wilson *et al.* (2012).

Ammonification/mineralization

Protease activity

Proteases are involved in the progressive cleavage of proteins to polypeptides or oligopeptides, and finally to amino acids. The activity of the different types of proteases in environmental samples can be determined using specific substrates (Ladd and Jackson, 1982), casein and N-α-benzoyl-l-argininamide being widely used.

Method based in utilization of casein as substrate

Originally developed by Ladd and Butler (1972), determination of protease activity is based on incubation of an environmental sample with excess casein as a substrate and trichloroacetic acid-soluble peptides determination using the Folin–Ciocalteu's phenol reagent.

Procedure

1 Weigh 1 g environmental solid sample (soils, sediments, etc.) ($n \geq 4$) and place in a 25-ml Erlenmeyer flask.
2 Add 2.5 ml of solution A. As a control prepare at least three tubes adding 1.3 ml of solution B. Mix the flasks briefly and close with screw caps.
3 Incubate at 50°C for 2 h on a rotatory shaker.
4 After incubation, add 1.2 ml of solution A to the control.
5 Add 1 ml of solution C to stop the reaction. Mix briefly.
6 Centrifuge at 3000 × *g*.
7 Take 1 ml supernatant into a glass tube. Add 7 ml of solution D and 1 ml solution E. Mix briefly. Incubate at room temperature for 30 min.
8 Add 1 ml of solution F. Preincubate 5 min at 37°C. Finally, incubate 30 min at room temperature.
9 Determine absorbance at 578 nm.

- Solution A: 1.05 g casein in 50 ml 0.1 M Tris-HCl, pH 8.1. Heat at 50°C until complete dissolution. Prepare daily.

- Solution B: 0.1 M Tris-HCl buffer. Dissolve 12.1 g Tris in 1 L distilled water in a volumetric flask. Adjust pH to 8.1 with 5 M HCl.
- Solution C: Mix 800 ml 0.1 M Tris, pH 8.1, and 320 ml trichloroacetic acid solution (175 g trichloroacetic acid in 1 l distilled water).
- Solution D: 3.7 g Na_2CO_3 in 100 ml distilled water.
- Solution E: 0.06 g $CuSO_4$ in 100 ml distilled water.
- Solution F: Folin-Ciocalteu solution. Mix 10 ml commercial Folin–Ciocalteu phenol reagent and 30 ml distilled water.

Solution C containing variable amounts of tyrosine (mg/l) can be used to prepare standard curves. The blue colour produced by the Folin-Ciocalteu reactive is stable at least 1.5 h.

Kinetic of peptide production can be followed by taking samples along time.

Other protocols for determination of protease activity can be found in Ladd and Butler (1972), Kandeler (1996c), Nunnan *et al.* (2000), Bonmatí *et al.* (2003) and Rejsek *et al.* (2008).

Method based in utilization of benzoyl-l-argininamide as substrate

Determination of protease activity is based on production of NH_4^+ after controlled incubation of an environmental sample with an excess N-α-benzoyl-l-argininamide as substrate.

Procedure

1 Weigh 0.5 g solid sample (soils, sediments, etc.) ($n \geq 4$) and place it in a glass incubation tube (avoid cleaning of the tubes with phosphate-containing detergents). As a control, prepare at least three tubes without N-α-benzoyl-l-argininamide.
2 Add 2 ml phosphate buffer and 0.5 ml solution C.
3 Incubate samples in a shaking water bath at 39°C for 1.5 hour.
4 Add 0.4 ml HCl 5M to samples and controls to stop the reaction, and 7.1 ml distilled water to each tube (final volume 10 ml).
5 Centrifuge at 15,000 × *g* for 15 min.
6 Screen through qualitative filter paper.
7 Measure NH_4^+ content as described in 'Ammonium determination'.

- Phosphate buffer (0.1 M, pH: 7.1): Mix 39 ml solution A (15.60 g $NaH_2PO_4 \times 2H_2O$ in 1000 ml distilled water), 61 ml solution B (17.80 g $Na_2HPO_4 \times 2H_2O$ in 1000 ml distilled water) and 100 ml distilled water. Store at 4°C.
- Solution C: 0.4977 g 30 mM N-α-benzoyl-l-argininamide in 50 ml phosphate buffer.

Buffer phosphate solutions containing variable amounts of NH_4^+ (mg/l) can be used to prepare standard curves. Results are usually expressed as mol NH_4^+/g/h. Sample weight should be checked for optimal results.

Other protocols for determination of protease activity can be found in Ladd and Butler (1972), Nannipieri *et al.* (1980), Bonmatí *et al.* (1991, 2003), Kandeler (1996c), Nunnan *et al.* (2000) and Rejsek *et al.* (2008).

Urease activity

Urease catalyses the hydrolysis of urea into ammonium and carbon dioxide (CO_2). The methodology described here is based on NH_4^+ production after controlled incubation of soils with an excess of urea as a substrate.

Procedure

1 Weigh 1 g solid sample (soils, sediments, etc.) ($n \geq 4$) and place it in a glass incubation tube (avoid cleaning of the tubes with phosphate-containing detergents). As a control, prepare at least three tubes without urea solution (add 0.5 ml distilled water instead).
2 Add 2 ml phosphate buffer and 0.5 ml solution C.
3 Incubate samples in a shaking water bath at 37°C for 2 h.
4 Add 7.5 ml distilled water to each tube (final volume 10 ml).
5 Centrifuge at 15,000 × *g* for 10 min.
6 Screen through qualitative filter paper.
7 Measure NH_4^+ content as described in 'Ammonium determination'.

- Phosphate buffer (0.1 M, pH: 7.1): Mix 39 ml solution A (15.60 g $NaH_2PO_4 \times 2H_2O$

in 1000 ml distilled water), 61 ml solution B (17.80 g $Na_2HPO_4 \times 2H_2O$ in 1000 ml distilled water) and 100 ml distilled water. Store at 4°C.
- Solution C: 6.4% urea (3.2 g urea in 50 ml phosphate buffer).

Aqueous solutions containing variable amounts of NH_4^+ (m/l) can be used to prepare standard curves. Results are usually expressed as mol NH_4^+/g/h.

Weight of the samples should be checked for optimal results.

Other protocols for determination of urease activity can be found in Nannipieri *et al.* (1980), Tabatabai (1982), Kandeler (1996d) and Öhlinger (1996).

Nitrification

Nitrification is the oxidation of ammonium to nitrate. The first step of the process is the oxidation of ammonium to nitrite which, in turn, is oxidized to nitrate. Nitrification is usually determined by measuring nitrite accumulation after addition of $NaClO_3$, which inhibits nitrite oxidation to nitrate.

Short-term nitrification assay

This method is based on incubation of the samples for a short period of time, usually no longer than 6 h. The assay uses $(NH_4)_2SO_4$ as substrate. After extraction with KCl, nitrite content is determined. Sodium chlorate is used to inhibit nitrite oxidation.

Procedure

1 Weigh 5 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it within a 100 ml Erlenmeyer flask.
2 Add 20 ml 1 mM solution A and 0.1 ml solution B. Mix and close the flasks with caps.
3 Incubate at 25°C for 5 h on an orbital shaker. As a control, at least three replicates should be kept for 5 h at –20°C.
4 After incubation, thaw the control at room temperature.
5 Add 5 ml solution C to samples and controls.
6 Mix and screen through qualitative filter paper immediately. If required, keep the filtrates overnight at 4°C.
7 Use filtrates to determine nitrite concentration as indicated in 'Ammonium determination'

- Solution A (1 mM): 0.132 g $(NH_4)_2SO_4$ in 100 ml distilled water.
- Solution B (1.5 M): 15.97 g $NaClO_3$ in 100 ml distilled water.
- Solution C (2 M): 149.12 g KCl in 1000 ml distilled water in a volumetric flask.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg/l) can be used to prepare standard curves. Nitrification is expressed as mg $NO_2^- \times$ g soil $\times$ h.

Since potential nitrification of soils with pH value below 5 is very low, this method is of limited value for acid soils.

Concentration of the inhibitor $NaClO_3$ has been optimized for soils with a humus content ranging from 1.5% to 3.5%. Inhibit concentration should be optimized depending on organic matte concentration of the soils.

Ammonium oxidation is inhibited by high amounts of ammonium. Optimum substrate concentration for each sample should be checked.

Other protocols for determination of nitrification activity have been published by Schmidt and Belser (1982), Berg and Rosswall (1985) and Kandeler (1996b).

Long-term nitrification assay

This method is based on incubation of the samples up to 3 weeks at 25°C using NH_4Cl as a substrate. After extraction of inorganic nitrogen compounds with KCl solution, ammonium and nitrate concentrations are determined. The oxidation of the substrate is equivalent to the nitrification dynamics, and is expressed as percentage of the added nitrogen (Beck, 1976; Kandeler, 1996b).

Procedure

1 Weigh 10 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it into a 100 ml flask.
2 Add drop wise 1 ml solution A.
3 Adjust soil moisture to 50–60% of water-holding capacity with distilled water.
4 Close the flasks with caps and weigh them.

5 Incubate samples at room temperature for up to 3 weeks (a fixed temperature, e.g. 25°C can be used). As a control, at least 3 replicates should be kept the same time at –20°C.
6 Weigh the flasks once a week to control soil moisture content. When required, add distilled water to adjust the soil moisture content.
7 After incubation, add 50 ml KCl to both samples and controls. Shake the flasks for 30 min in a rotary shaker at about 125 rpm.
8 Screen flask contents through filter paper and measure ammonium and nitrate concentration as indicated in sections 'Ammonium determination' and 'Nitrate determination'.

- Solution A (75.7 mM): 1 g $(NH4)_2SO_4$ in 100 ml distilled water in a volumetric flask.
- Solution B (2 M): 149 g KCl in 1 litre of distilled water.

For calculation of the results there is to consider that inorganic nitrogen can be released from organic nitrogen (ammonification), and that both ammonium and nitrate can be immobilized by microorganisms during incubation.

To determine the correlation factor is used the following formula:

$$Na = (NO_3^- + NH_4^+)_X - (c^+ NO_3^- + NH_4^+)_Y$$

where:

- Na = correction factor for ammonification and immobilization during the incubation time.
- $_X$ = amount of inorganic $(NO_3^- + NH_4^+)$ nitrogen after incubation.
- $_Y$ = initial amount of inorganic $(NO_3^- + NH_4^+)$ nitrogen.
- c = amount of NH_4^+ added at the beginning of the experiment.

If Na < 0: higher ammonification than immobilization.

If Na > 0: higher immobilization than ammonification.

The nitrification turnover is equivalent to the amount of nitrogen which is released from the substrate per day and gram dry matter.

The results can be expressed as percentage loss of the initially added substrate according the following formula:

$$\%\ N/day = (NO_3^- - N_X - NO_3^- - N_Y) \times 100 / (c^+ Na) \times n$$

where:

- $_X = NO_3^-$ content after incubation.
- $_Y$ = initial NO_3^- content.
- c = amount of initially added NH_4^+.
- Na = correction factor for ammonification and immobilization during incubation.
- *n* = days of incubation time.
- %N/day = nitrification turnover.

Because nitrification in soils with high amounts of easily decomposable carbon compounds proceeds very fast, the incubation time can be reduced.

It is not recommended to increase the substrate concentration because of the toxicity of high ammonia concentration is soils.

Since nitrification in air-dried and rewetted soils starts after a lag-phase, a pre-incubation time should be considered.

Ammonification and/or immobilization during incubation could not be considered., some authors not consider these process and the results as expressed as g NO_3^- per g of sample per hour.

Other protocols for determination of long-term nitrification activity have been published by Schmidt and Belser (1982), Berg and Rosswall (1985), Kandeler *et al.* (1996b), Hu *et al.* (2002) and Li *et al.* (2011).

Alternatively, nitrification activity can be studied by measuring rates of nitrite oxidation (Belser and Schmidt, 1982). For that purpose, varying concentrations of NO_2^- are added to soils slurries along with nitrapyrin to inhibit oxidation of NH_4^+. It is recommended to follow NO_2^- disappearance by taking samples over time to calculate results during exponential phase of NO_2^- oxidation.

Nitrate assimilation

Reduction of nitrate to ammonium is catalysed by the sequential actuation of the assimilatory nitrate reductase and nitrite reductase enzymes.

Determination of nitrate disappearance

Procedure

1 Sieve the soil through a 2 mm screen.
2 Mix 10 g sample with glucose (2.5 mg of C per gram of sample) and 30 ml distilled water in a flask ($n \geq 4$).
3 Incubate the samples in a rotary shaker at 125 rev/min (to maintain aerobic conditions) at 30°C for 16–24 h conditions.
4 After incubation, add 250 µg KNO_3/g soil. As a control, at least 3 replicates should be included without addition of KNO_3.
5 Incubate at 30°C for 2 h.
6 Centrifuge10 min at 3000 × *g*.
7 To 5 ml supernatant add 15 ml 1.3 M KCl to extract NO_3^-.
8 Determine NO_3^- concentration as indicated in section 'Nitrate determination'.

Pre-incubation of the samples is recommended to stimulate microbial activity and assimilation of pre-existing ammonium and nitrate.

Other protocols for determination of nitrate assimilation have been published by Rice and Tiedje (1989) and McCarty and Bremner (1992).

Determination of nitrite production

Procedure

1 Weigh 5 g environmental sample (soils, sediments, etc.) ($n \geq 4$) into a glass tube.
2 Add 4 ml solution A, 1 ml solution B and 5 ml distilled water into the tubes. Mix and close the tubes with screw caps.
3 Incubate the tubes for 24 h at 25°C (samples). As a control, at least three replicates should be kept for 5 h at –20°C.
4 After incubation thaw the control at room temperature.
5 Add 10 ml solution C to both samples and controls. Mix and immediately filter the samples and controls through qualitative filter papers.
6 Use filtrates to determine nitrite concentration as indicated in section 'Nitrite determination'.

- Solution A: 0.9 mM 2,4-dinitrophenol (DNP) solution
- Solution B: 25 mM KNO_3 (2.53 g KNO_3 in 1 litre of distilled water in a volumetric flask.
- Solution C: 4 M KCl (298.24 g KCl in 1 litre of distilled water in a volumetric flask.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg/l) can be used to prepare standard curves. Activity is expressed as µg NO_2^-/g/h.

It is recommended to follow NO_2^- production kinetic by taking samples over time to calculate results during exponential phase of NO_2^- production.

To reduce the possible presence of lag phase, samples can be pre-incubated overnight with DNP.

Prior to any analysis, an estimation of the optimum amount of inhibitor should be tested as DNP concentration may vary from 5 to 300 µg DNP (Abdelmagid and Tabatabai, 1986).

Other protocols for determination of nitrate assimilation have been published by Kandeler *et al.* (1996a) and Deiglmayr *et al.* (2004).

Denitrification

Denitrification is associated with the production of N_2, N_2O and NO by environmental samples. The most frequently used measurement method to assay denitrification is the determination of N_2O production by gas chromatography. The ^{15}N-labelling technique can also be used to detect N_2O and N_2. NO can also be determined by using a chemiluminescent analyser.

Determination of N2O production

Assessment of denitrification is hard to study because of the difficulties in quantifying its gaseous end products (N_2O and N_2) and high spatial and temporal variability (Groffman *et al.*, 2006, 2009; Philippot *et al.*, 2009; Hallin *et al.*, 2009; Bru *et al.*, 2011; Keil *et al.*, 2011). Although molecular methods (Philippot and Hallin, 2006) have

contributed to understanding of denitrification, gene expression, denitrifier community composition and enzyme activities could not be easily related with the simultaneous production and emission of denitrification products (Wallenstein *et al.*, 2006; Čuhel *et al.*, 2010). Methodological problems continue hampering our understanding of denitrification at site, from landscape to continental scale, as well as the controls and magnitude of net N_2O losses (Butterbach-Bahl *et al.*, 2011).

This method is based on the inhibition of the nitrous oxide reductase, the enzyme reducing N_2O to N_2, by acetylene at 0.1–10% concentration (Balderston *et al.*, 1976; Yoshinari *et al.*, 1977).

Procedure

1 Weigh 25 g fresh sample (soils, sediments, etc.) ($n \geq 4$) and place it within a 100 ml bottle. Bottles have to be hermetically closed (e.g. by using rubber septum). Weights of the samples and volume of the bottles may vary to optimize the assay. Septa must allow injection and sampling of the internal atmosphere of the bottles. As a blank include a bottle without sample.
2 Close the bottle-containing sample. Then, evacuate and flush the headspace four or five times with N_2, or the more expensive Ar and He, to create anoxic conditions.
3 Replace 10% of the internal atmosphere by the same volume of acetylene. Commercial acetylene, or that obtained by mixing calcium carbide (CaC_2) and water (1:15 w/v), can be used. Mix well by shaking.
4 Incubate the samples at 25°C. Incubation time depend of the denitrification activity of the samples.
5 Take gas aliquots from the headspace of the bottle for injection onto the gas chromatograph. Aliquots from 100 to 1000 µl can be used. Gas-tight syringes should be used.

Kinetic of N_2O production can be followed by taking samples along time. Because assays are run in closed systems, gas accumulation may cause feedback inhibition of the enzymatic activity. Accordingly, gas samples must be taken during the exponential phase of gas production. Long incubation times may result in spoiling of the samples.

When activity of the samples is low, denitrifying enzymatic activity can be determined after incubation of the samples under non-limiting denitrifying conditions, this is, in the presence of an excess carbon and nitrogen sources. Under these conditions, addition of chloramphenicol to prevent new protein synthesis and, consequently, growth of denitrifying microorganisms can be used. The procedure is the same as indicated above, except that 25 ml of a sterile solution containing 1 mM KNO_3, 1 mM glucose and 1 g/l chloramphenicol is added to the samples. Under these conditions, N_2O production is usually detected within 24–48 h.

N_2O can readily be detected by gas chromatography, using either a thermal conductivity detector (TCD) or the more sensitive electron conductivity detector (ECD). The chromatograph is usually provided with N_2, or the more expensive Ar and He, as carrier gas. Carrier gas flux through the chromatographic column as well as oven, injector and detector temperatures may vary depending on the commercial brand of the chromatograph and of the type of column used for chromatography.

Concentration of N_2O can be calculated from standards of pure nitrous oxide. A correction for dissolved N_2O in water (Bunsen solubility coefficient) has to be considered. Values are usually expressed as mol N_2O produced per kg (soil, sediment, etc.) per hour.

Other protocols for determination of N_2O production have been published by Smith and Tiedje (1979), Tiedje (1982) and Šimek *et al.* (2000, 2004).

Determination of N2 production

In addition to N_2O formation, denitrification can be estimated analysing production of N_2, the end product of the process.

Procedure

The procedure is the same as indicated above for N_2O production, except that acetylene is not added to the bottles where samples are incubated.

N_2 can be detected by gas chromatography, using either a FID or a TCD, but N_2 cannot be

used as the carrier gas. Concentration of N_2 can be calculated from standards of pure N_2. Values are usually expressed as mol N_2 produced per kg-(soil, sediment, etc.) per hour.

During studies on denitrification, N_2 production can be determined by analysing N_2O production in parallel environmental samples incubated with and without acetylene (Philippot *et al.*, 2009). N_2 concentration is then estimated as the difference between the N_2O produced in the presence and in the absence of acetylene, respectively. In addition, the ratio $[N_2O/(N_2O+N_2)]$ represents an estimation of the nitrous oxide reductase activity in the samples.

The isotope 15N-labelled method

^{15}N-labelled substrates such as nitrate and ammonium can be measured using mass spectrometry. The advantage of this method is that it does not imply disturbance of the soil, that N_2 production can be determined, and that an N budget can be made. The method also has some disadvantages, the most important being that 9a) analysis of ^{15}N is only possible at specialized laboratories, (b) only denitrification from ^{15}N is measured, and (c) ^{15}N is not distributed homogeneously throughout the soil. ^{15}N -labelled material can be expensive and can only be used on small plots. The readers are referred to Baggs (2008) for a comprehensive review on stable isotope techniques for determination of N_2O in soils.

Determination of NO production

This method is based on the determination of NO fluxes of an environmental sample incubated within a dynamic flow through chamber. After the incubation the NO concentration is determined by chemiluminescence (Parrish *et al.*, 1987; Pilegaard *et al.*, 1999).

Procedure

1 Weigh 10 g of environmental sample (soil, sediment, etc.) ($n \geq 4$) and place it into a 100 ml flask.
2 Place the flask inside a stainless-steel chamber provided with a gas-tight lid, inlet and exhaust ports for gas flushing, and a sampling port.
3 The chamber can be introduced in a water bath to control the temperature and reduce potential gas diffusion into the chamber.
4 Replace the internal atmosphere of the chamber by thorough flushing with an N_2-free gas (either He or Ar) to create anoxic conditions. Flush with the N_2-free gas until the original sample atmosphere is replaced. Overpressure should be avoided.
5 Take 40 ml of the head space of the chamber into an airtight bag pre-filled with 2 l of pure N_2.
6 Determine the NO concentrations with a $NO\text{-}NO_2\text{-}NO_x$ chemiluminescent analyser. To obtain a well-represented NO concentration of the gas stored in the bag, a sample flow of 600 ml/min and 3 minutes of continuous measurement is required to get a stable output signal.

Kinetic of NO production can be followed by taking samples along time (Veldkamp and Keller, 1997). Incubation times as long as 3 weeks have been recommended (Pilegaard *et al.*, 1999).

Concentration of NO can be calculated from standards of pure nitric oxide.

Values are usually expressed as flux of NO as follows:

- $F = (V_{head} \times \Delta C_i^* \times M)/(M_{ds} \times MV \times 103) \times (273/273+T)$
- F = NO emissions in µg/h/kg.
- V = volume of the headspace (ml).
- ΔC_i^* = Change in NO concentration (ml/m^3).
- M = atomic weight of the N in NO (14 g/mol).
- M_{ds} = dry weight of environmental sample.
- MV = molar volume of NO at 273 K and 1013 hPa (l/mol).
- T = incubation temperature.

Other protocols for determination of NO emission have been published by Parrish *et al.* (1987), Pilegaard *et al.* (1999) and Wang *et al.* (2011).

Determination of some N-compounds relative to the N-cycle

Total solid N

Total solid N (T_N) refers to each inorganic (NH_4^+, NO_3^-, NO_2^-) and organic N (amino acids, proteins and other organic compounds) contents in solid samples (soils, plants, sediments, etc.). The Kjeldahl (wet digestion) and the Dumas (dry digestion) methods are widely used for determination of T_N.

Determination of TN: the Kjeldahl method

The Kjeldahl method is a two-step process by which organic N of a sample is digested (oxidized) into NH_4^+ by acidic digestion with H_2SO_4. Ammonium salts produced can be collected and dissolved with a strong alkali. The ammonium produced can be distilled, dissolved in acid solution, and finally titrated with caustic soda to indirectly measure nitrogen. In his original method, Kjeldahl used KSO4 to raise the boiling point of the acid and Hg as catalyst to speed the digestion. For the back titration process of the released ammonium, he used a solution of boric acid. According to the sample characteristics, diverse modifications of the original Kjeldahl method have been introduced in order to solve the recovery of refractory heterocyclic compounds or molecules containing N–N and N–O linkages (Du Preez and Bate, 1989; Bremner and Mulvaney, 1982; Watkins *et al.*, 1987; Domini *et al.*, 2009).

A protocol to carry out Kjeldahl method in the laboratory requires a Kjeldahl Steam Distillation system with a block digester (available commercially).

Procedure

- Dry the sample at 60°C for 48 h.
- Homogenize the sample by grinding to ≤ 0.5 mm.
- Weigh 1 g sample and place it into the block digester tube.
- Add about 5.0 g of catalyst mixture (K_2SO_4-$CuSO_4$ × 5 H_2O-Se, 100:10:1 w/w ratio) and 15 ml H_2SO_4, and swirl carefully.
- Set temperature in the block digester at 370°C for 3–5 h until complete digestion.
- Cool the sample at room temperature.
- Add 15 ml distilled water and transfer the sample into a steam flask containing 10 ml 10 N NaOH.
- Distilled the sample to recover ammonium released.
- Determine ammonium as indicated in section 'Ammonium determination'.

The Kjeldahl method is widely used in industrial, agricultural, and food analysis. A major disadvantage of the method is that it only converts organic-N (mainly proteins) and some NO_3^- into NH_4^+. It is difficult to automatize and consumes relatively high amounts of sample.

Determination of TN: the Dumas method

This methodology is based on combustion of samples (900–1020°C) in the presence of oxygen to obtain N-compounds which are further reduced catalytically into N_2. Usually, the process is automatically run by the s- called (macro/micro) Elemental Analysers, of which several brands are commercially available.

Procedure (for preparation of the samples for the analyser)

- Dry the sample at 60°C for 48 h.
- Homogenize the sample by grinding to ≤ 0.5 mm.
- Weigh (1–50 mg) the sample and place it into a tin capsule and close it.
- Subject the sample to analysis in an Elemental Analyser.

Before use, calibrate the elemental analyser with standard N-compounds provided by the manufactures according to the expected N content of the samples to be analysed.

The Dumas method is fast and clean. It requires just a small amount of sample and causes no environmental hazards. It has been successfully used for T_N determination in soils, plants, and other proteinaceous samples (Kirsten and Hesselius, 1983; Simonne *et al.*, 1997; Wiles *et al.*, 1998; Jung *et al.*, 2003).

In solid samples, $T_N = N_{INORGANIC} + N_{ORGANIC}$, where $N_{INORGANIC}$ is mainly $NH_4^+ + NO_3^- + NO_2^-$. $N_{ORGANIC}$ is calculated indirectly using this equation, and it refers to nitrogen of organic molecules, such as proteins, amino acids, etc.

Regardless of the methodology used, T_N can be expressed as % or as part per million of N in relation to the dry weight of the solid sample.

An indirect N determination of crude protein content can be done by multiplying $N_{ORGANIC}$ by 6.25, a value which refers the experimental average N content in proteins.

In addition to Kjeldahl and Dumas methods, the near infra-red (NIR) spectroscopy has been described as a promising technology for T_N determination in plants (Gitelson *et al.*, 2003) and soils (Russell, 2003).

Determination of total dissolved N

Total dissolved nitrogen (T_{DN}) refers to N in liquid samples containing organic and inorganic N compounds. In addition to liquid samples, T_{DN} can be analysed in solid samples after extraction by mechanical means or piezometry (Randall *et al.*, 1997; Kalbitz *et al.*, 2000; Jones and Willett, 2006; Krause *et al.*, 2009; Hood-Nowotny *et al.*, 2010).

Prior to determination, N-compounds in solid samples can be extracted as follows:

- Mix the solid sample with water (1:20 w/v) and shake in a water bath for 2 hours at 25°C.
- Centrifuge at 15,000 × *g* for 20 min.
- Filter the supernatant through qualitative filter paper or 0.45 µm pore size appropriate membranes.

The weight to volume ratio can be modified to increase the content of dissolved N in the samples.

Liquid samples and liquid-extracted samples from solid materials can now be used for T_{DN} determination. For liquid samples, the Kjeldahl method is not adequate as urea cannot be determined properly (Solorzano and Sharp, 1980).

If the Dumas method is to be used, the liquid sample is added to an inert absorbent, e.g. diatomaceous earth, to avoid sample evaporation.

The most accurate analytical method for T_{DN} is based on a high-temperature catalytic oxidation (Merriam *et al.*, 1996). Liquid samples are combusted, converted into NO which further reacts with ozone to form nitrogen dioxide (NO_2), which is detected with a nitrogen-specific chemiluminescence detector coupled to an automatic liquid Analyser. This technique is now accepted as the most precise and efficient technique for T_{DN} determination (Álvarez-Salgado and Miller, 1998).

Before use, calibrate the automatic liquid Analyser with standard N-compounds provided by the manufactures according to the expected N content of the samples to be analysed.

T_{DN} is usually expressed in mg/l for liquid samples and in part per million of N in relation to the dry weight of the solid sample.

In liquid samples, $T_{DN} = D_{INORGANIC} + D_{ORGANIC}$, where $D_{INORGANIC}$ is mainly $N\text{-}NH_4^+ + N\text{-}NO_3^- + N\text{-}NO_2^-$. $D_{ORGANIC}$ can be calculated indirectly using this equation.

Determination of dissolved inorganic N

Dissolved inorganic nitrogen (D_{IN}) refers to NH_4^+, NO_3^- and NO_2^- found in either solid or liquid samples. After extraction, samples should be kept at –20°C to prevent NH_4^+, NO_3^- and NO_2^- assimilation or chemical modifications.

Ammonium determination

Indirect method

NH_4^+ can be readily determined using an indirect, colorimetric method based on Berthelot's reaction (Weatherfourn, 1967; Patton and Crouch, 1977).

Procedure

1 Add 1.6 ml reactive A, 0.8 ml reactive B, 0.8 ml reactive C (see below), and 6.4 ml distilled water to 0.4 ml liquid sample.
2 Mix well by vortexing or shaking. Keep at least 45 min in darkness until appearance of blue colour.
3 Measure absorbance at 660 nm.

- Reactive A: (freshly prepared): 7.81 g sodium

salicylate and 25 mg sodium nitroprusside. Add distilled water to fill up to 100 ml in a volumetric flask.
- Reactive B: 4.0 g NaOH and 0.5 g of sodium dichloride isocyanurate. Add distilled water to fill up to 100 ml in a volumetric flask (pH 13).
- Reactive C: 9.33 g sodium citrate and add distilled water to fill up to 100 ml in a volumetric flask.

Aqueous solutions containing variable amounts of $(NH_4)_2SO_4$ (mg/l) can be used to prepare standard curves.

Direct methods

Ion chromatography (Michalski, 2006; Michalski and Kurzyca, 2006) and the use of an ammonium ion-selective electrode (Bakker, 2004; Bakker and Qin, 2006) are two alternatives for determination of NH_4^+ in most liquid samples. The former is a sensitive and accurate technique with good reproducibility and versatility (e.g. it offers the possibility of simultaneous detection of some other anions). A review on types of samples, columns and eluents has been published by Michalski and Kurzyca (2006). A conductometric detector is usually employed for ion chromatography. The latter is fast and accurate, usually well-suited for portable field applications, with the disadvantage that the electrode may respond to other anions with similar physical properties.

Nitrate determination

Indirect methods

These assays are based on chemical reduction of NO_3^- to NO_2–. Then nitrite is determined as indicated in section 'Nitrite determination'. Several reducing agents have been investigated, and copperized Cd and Zn are widely used, with efficiencies for NO_3^- to NO_2^- conversion approaching 100% (Fanning, 2000). Commercial kits have been developed for *in situ* application during field determinations of nitrate content in liquid samples that are easy to use following manufacturer instructions.

Under laboratory conditions, nitrate in liquid and solid samples can also be determined after chemical reduction. Reduction columns were first described by Wood *et al.* (1967) and can be purchased or prepared in the laboratory.

A. Preparation of the column

1 Weigh 25 g 40–60 mesh commercial Cd granules and wash them with 6N HCl. Rinse thoroughly with distilled water.
2 Mix the granules with 100 ml 2% $CuSO_4$ solution until blue colour partially fades (5–10 minutes).
3 Decant the granules. Mix them with fresh $CuSO_4$ solution until a brown colloidal precipitate begins to develop.
4 Wash with distilled water to remove the precipitated Cu.
5 Insert a glass wool plug into the bottom of a glass column (15–20 cm long) and fill with water. Add copperized Cd granules to produce a column 13.0–18.0 cm. Pour distilled water until exceed the height of the granules to prevent the entrapment of air.
6 Wash the column with 200 ml solution A.
7 Activate the column by passing through it at least 100 ml solution C at a rate of 5–10 ml/min.

- Solution A: Dissolve 13 g NH_4Cl and 1.7 g ethylene diaminetetraacetate (EDTA) in 750 ml distilled water in a volumetric flask, adjust to pH 8.5 with concentrated NH_4OH and dilute to 1 L distilled water. Finally, dilute 300 ml solution to 500 ml with distilled water.
- Solution B: Dissolve 7.21 mg KNO_3 in 1 litre of distilled water in a volumetric flask.
- Solution C: Mix solution A and solution B in 3:1 proportion.

B. Sample reduction

1 Extract nitrate from solid environmental samples as indicated in section 'Direct methods' below.
2 Filter liquid samples through qualitative filter paper or 0.45 μm pore size appropriate membranes.
3 Add 75 ml solution C to 25 ml sample. Mix gently and pour into the column.
4 Collect the eluate at a rate of 5–10 ml/min. Discard the first 25 ml. Collect the remaining

75 ml into a clean flask. Determine nitrite concentration as described in 'Nitrite determination' within 15 minutes after reduction.

Aqueous solutions containing variable amounts of either $NaNO_3$ or KNO_3 (mg/l) can be used to prepare standard curves. Reduce standards as described for samples. Compare at least one nitrite standard to a reduced nitrate standard at the same concentration to verify reduction column efficiency. Also determine any contaminant nitrite in the samples.

Nitrate concentration is expressed as mg of NO_3^- per gram of sample.

There is no need to wash columns between samples. If columns are not to be reused for several hours or longer, pour 50 ml solution A on to the top of the column and let it pass through the system. Then, add 100 ml more, close the Cu-Cd column and store.

Crutchfield and Grove (2011) have described a Cd reduction microplate method for nitrate determination. Other protocols for nitrate determination by the Cd reduction method have been published by Wood *et al.* (1967), Jones (1984), APHA Standard Methods (1995) and Gal *et al.* (2004).

Direct methods

High-performance liquid chromatography (HPLC) (Thayer and Huffaker, 1980) and ion chromatography (Stratford, 1999; Kissner and Koppenol 2005; Michalski and Kurzyca, 2006) are two alternatives for determination of nitrate and nitrite in most liquid samples. A comprehensive review on HPLC and ion chromatography applications, including types of columns, eluents, detectors and matrix samples has been previously published (Michalski and Kurzyca, 2006).

Prior to determination, NO_3^- in solid samples can be extracted as follows:

1 Mix the solid sample with water (1:20 w/v) and shake in a water bath for 2 hours at 25°C.
2 Centrifuge at 15,000 *g* for 20 min.
3 Filter the supernatant through qualitative filter paper or 0.45 µm pore size appropriate membranes.

The weight to volume ratio can be modified to increase the NO_3^- content in the samples.

Procedure (for HPLC)

1 To prepare the eluent, dissolve 0.1049 g LiOH in 1000 ml Milli-Q water (2.5 mM) in a volumetric flask. Gas the eluent with N_2 for 10 min to avoid dissolved CO_2 interference. Alternatively, a CO_2 trapping agent such as ascarite can be used. A borate/gluconate buffer can also be used as an eluent.
2 Filter the eluent and the samples through 0.45 µm pore size appropriate membrane.
3 Aqueous solutions containing variable amounts of either $NaNO_3$ or KNO_3 (mg/l) can be used to prepare standard curves.
4 Subject the samples to HPLC. Absorbance can be determined at 220 nm.

Nitrate ion-selective electrodes have been developed that can be used for laboratory and field nitrate determinations. They are commercially available.

Nitrite determination

Indirect method

The classical method for nitrite identification and quantitative determination is the Griess reaction (Griess, 1864), which uses diazotation and coupling to form a purple dye by adding the sulphanilamide-naphthylethylene diamine dihydrochloride reagent (Snell and Snell, 1949; Nicholas and Nason, 1957).

Procedure

1 Filter the sample through 0.45 µm pore size membrane.
2 Add 0.4 ml reactive A to 20 ml sample and mix well by gentle shaking for 5 minutes.
3 Add 0.4 ml reactive B to the mixture. Mix well by gentle shaking.
4 Keep in darkness, at least for 30 minutes, until colour development.
5 Measure absorbance at 540 nm.

- Reactive A: 1 g sulfonamide ($C_6H_8N_2O_2S$) in 100 ml 10% HCl (10 ml of HCl in 90 ml distilled water).

- Reactive B: 0.1 g N-(1-naphthyl)-ethylenediamine dihydrochloride ($C_{12}H^{14}N_2$, NNEDA) in 100 ml distilled water.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg/l) can be used to prepare standard curves. Results are usually expressed as mg NO_2^-/l.

Direct methods

Nitrite determination can be assayed by HPLC as described for nitrate detection in section 'Nitrate determination – direct methods'. Prior to determination, NO_2^- in solid samples has to be extracted as indicated in section 'Nitrate determination – Direct methods'.

Aqueous solutions containing variable amounts of either $NaNO_2$ or KNO_2 (mg/l) can be used to prepare standard curves.

Other spectroscopic methods, including UV/visible, chemiluminescence, fluorimetric, Infrared (IR), Raman and molecular cavity emission, have been reviewed by Moorcroft *et al.* (2001). Also, examples of HPLC and ion chromatography applications for their determination, including type of columns, eluents, detectors and matrix samples are reviewed and discussed in Michalski and Kurzyca (2006).

Determination of gaseous N compounds

During the denitrification pathway N_2O and N_2 are produced whose determination can be accomplished by gas chromatography using thermal conductivity (TCD) and electron capture (ECD) detectors after separation of the samples through either packed or capillary chromatographic columns. Protocols for NO, N_2O and N_2 determination are explained in section 'Denitrification'.

Photoacoustic infrared spectroscopy has been developed for continuous measurement of NH_3 and N_2O in environmental samples (Osada *et al.*, 1998). This methodology is based on the detection of acoustic waves that result from absorption of infrared radiation of the sample. It is, however, an expensive technology for routine analysis.

Mass spectrometry, mainly membrane inlet mass spectrometry (MIMS), allows detection of gases in liquid samples (Srinivasan *et al.*, 1997), with the major advantage of being able to detect parts-per-trillion of NO, N_2O and N_2 (Lloyd *et al.*, 1996; Kana *et al.*, 1998; Kim *et al.*, 1999), and the disadvantage of being an expensive technology.

Future trends and directions

Molecular methods have greatly contributed to the understanding of processes involved in the microbial N-cycle. On one side, targeting functional genes at the DNA level allows for their detection and analysis of diversity, providing inferences to which genes are functionally important in the environment. On the other side, analysis of gene expression targeting mRNA provides evidence of actual activity. However, measurement of microbial N-cycle-related activities and N-derived compounds in environmental samples is often hard to study because of the difficulties to quantify the end products resulting from the microbial activities. As an example, this is the situation when studying the assessment of denitrification in environmental samples. Maybe because of the problems to quantify its gaseous end products (N_2O and N_2) or to the high spatial and temporal variability of the process (Groffman *et al.*, 2006, 2009; Philippot *et al.*, 2009; Hallin *et al.*, 2009; Bru *et al.*, 2011; Keil *et al.*, 2011), total N losses from arable soils due to denitrification have been estimated to be in the range of 22–87 Tg N/year (Hofstra and Bowman 2005). Thus, methodological problems continue hampering our understanding of the N-cycle related pathways. Although available, many techniques are rather expensive and cannot be afforded by ordinary laboratories, and this without considering the special facilities and installation that complex apparatus and equipment require for their functioning. The advancement of scientific knowledge requires the development of new methodologies for simple, cheap and effective estimation of the parameters you wish to analyse.

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