Chapter 32

Nitrogen Transformations

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32.1 INTRODUCTION

Nitrogen is an important limiting element in streams (Dodds and Smith, 2016), as well as a pollutant that stimulates unwanted algal growth. Further, ammonia and nitrate can both be toxic to vertebrates, including humans, at high concentrations (Thurston et al., 1981; Carmago et al., 2005). Transport of nitrogen through streams can lead to water quality problems in coastal areas (Alexander et al., 2000), yet rivers and streams retain and process substantial amounts of nitrogen. Streams are also a globally important source of nitrous oxide (Beaulieu et al., 2011), which is one of the most potent greenhouse gasses leading to global warming.

Biotic and abiotic factors influence transformations of nitrogen into different forms in the environment. The major forms of nitrogen (N) are organic N (including proteins, nucleic acids, urea, and various other compounds) and inorganic N (Dodds and Whiles, 2010). Major gaseous forms of inorganic N include N_2 gas as a major component of the atmosphere, nitrous oxide (N_2O), and ammonia (NH_3). Dissolved inorganic ionic forms of nitrogen include ammonium (NH_4^+), nitrite (NO_2^-), and nitrate (NO_3^-).

32.1.1 Nitrogen Fluxes

All organisms require nitrogen and must take it into their cells to maintain viability and grow. Nitrogen that is taken from the environment and converted into biological molecules in the cell is referred to as assimilated. Nitrogen can be assimilated in some organic forms (e.g., many heterotrophic organisms require amino acids from their diet to synthesize proteins). Plants and microbes can also assimilate inorganic nitrogen. The pathway to assimilate inorganic nitrogen requires it to be converted to NH_4^+ before it can be converted to amino acids and other organic compounds. However, other forms can be converted to NH_4^+ before assimilation (Fig. 32.1).

In addition to *assimilatory* pathways, *dissimilatory* pathways exist where some types of nitrogen are converted to others in energy-yielding pathways. Each of the forms of nitrogen has different reduction–oxidation (or redox, the relative

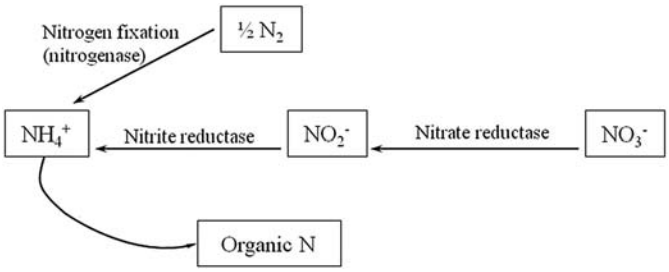

FIGURE 32.1 Assimilatory pathways of nitrogen uptake. All forms of inorganic N must be converted to ammonium. Note that use of nitrate requires more energy than ammonium and use of N_2 even more energy. *After Dodds and Whiles (2010).*

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availability of free electrons) states, and relative to the environment they are in, can yield or require energy when they are converted among forms. Specifically, the more a compound differs in oxidation−reduction state from the overall chemistry of the water it is in, the greater the potential energy it has. So, converting this compound to the same potential energy as the rest of the chemicals dissolved in the water will yield energy. Conversely, converting a compound to a form that is vastly different from the redox state of the rest of the chemicals in solution requires potential energy.

The inorganic nitrogen compounds can be ordered by redox potential, aligned from most oxidized to most reduced $NO_3^- > NO_2^- > N_2O > N_2 > NH_4^+$. This order is not too difficult to remember as the more oxygen atoms a compound has and the fewer hydrogen atoms, the more oxidized it is. Dissolved oxygen (O_2) is a key determinant of oxidation−reduction state of the water in which compounds are dissolved—if O_2 is absent, then redox is low and electrons are plentiful (because the O_2 does not react with them). Thus, the presence of O_2 in large part dictates which nitrogen transformations dominate. Given the order of oxidation−reduction state, the conditions under which various nitrogen transformations will occur can be predicted (Fig. 32.2).

The final major class of elemental flux is excretory, which is mineralization or, in the absence of O_2, sometimes referred to as ammonification. Mineralization occurs when heterotrophs break down organic N compounds that are N-rich and they need to excrete the excess nitrogen. Generally this nitrogen is excreted in the form of NH_4^+ by aquatic organisms. It is important to understand this flux because it is what keeps all the nitrogen in a system from building up as organic nitrogen and in part controls transformation dynamics. Some of the fluxes in Figs. 32.1 and 32.2 are not very important in freshwaters (e.g., ANNAMOX, Schubert et al., 2006), and so they will not be considered in detail here. For more detail on methods to measure these fluxes, please refer to Huygens et al. (2013).

When measuring rates of these N cycle processes, we distinguish between potential and actual rates as well as between gross and net rates. A potential rate is the rate in the absence of limiting factors. In the natural environment, many biogeochemical processes can be limited by any of several things, including the amount of substrate available for a reaction, the amount of enzyme present to drive a reaction, temperature, light, and other factors. So, for an individual sample, if we optimize all conditions and measure the maximum rates possible given the enzymes that are present, these are referred to as potential rates. In contrast, we can mimic the natural environmental conditions as closely as possible and these are referred to as actual rates. Any dissolved N pool will have transformations that feed into that pool and those that use up the pool. The change in the entire size of the pool (net transformation rate) will be dictated by the total rate of the reaction transforming the N out of the pool (gross rate) minus the rate of the process supplying the pool.

This chapter will cover the basic methods of (1) *actual and potential denitrification*, (2) gross and net *nitrification*, and (3) *nitrogen fixation*. These constitute standard enzyme assays or net flux estimates. We then present advanced methods that take advantage of stable isotopes as N tracers and direct measurements of changes in gas concentrations to quantify fluxes that are more difficult to trace with standard enzyme assays, in the form of direct measures of (1) nitrogen fixation and (2) *dissimilatory nitrate reduction to ammonia* (DNRA).

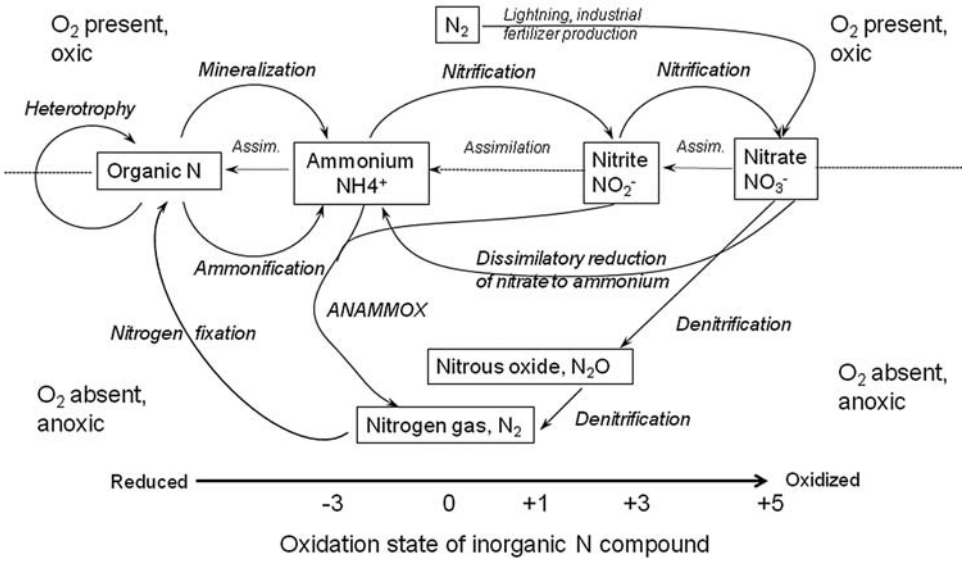

FIGURE 32.2 The general nitrogen cycle. Inorganic forms are arranged according to oxidation−reduction state with most oxidized at the right-hand side of the diagram. Some fluxes predominate in oxic conditions (top half of diagram) and others in anoxic (bottom half of diagram). *After Dodds and Whiles (2010).*

32.1.2 Small-Scale Assays for Fluxes

In this chapter, we cover common small-scale assays of various N transformation rates. These are generally "bottle" assays and may or may not scale to whole-system rates (Schindler, 1998). For a more detailed discussion of some whole-system rate measures, see Chapter 31 in this book. Readers are cautioned that bottle assays can lead to rate estimates that are different from those in the natural environment because samples must be disturbed to make such measurements, but for determining relative rates in comparative experiments, these estimates can be useful (Fig. 32.3A and B). Small incubations can diverge from in situ rates because the effect of the walls of the container reduces flow, light, and water exchange and can alter temperature. Generally incubations should be carried out in as short a time period as possible to get a reliable signal, and researchers often use a series of measures over time to verify that rates are consistent during longer incubations. Larger incubation vessels are more likely to capture heterogeneity but become more challenging to control and replicate as size increases and the more closely the operator is attempting to replicate in situ conditions (e.g., flow, temperature, light, replenishment of water).

In general, samples used to measure fluxes will be taken for the major types of substrata present in streams. Note that in this chapter we refer to the reactants that enzymes act on as substrates, but that solid materials on the bottom of the stream are substrata or substratum. Incubations in small vials are ideal for conducting many measurements in a short period of time and for comparing among different substrata (e.g., sediment, epiphytes, water column), but can be problematic for biofilms because they may require separating periphyton (see Chapter 12) from their growth substrata. Chambers can be used to measure biofilms attached to larger substrata such as wood, rocks, and organic matter, but also restrict the size the types of substrata that can be incubated and can only be run with a limited number of replicates at a time. Construction of recirculating chambers has been addressed in several papers (e.g., Dodds and Brock, 1998; Rüegg et al. 2015). Nonrecirculating chambers such as the polycarbonate containers depicted in Fig. 32.3D are ideal for incubating periphyton attached to their growth substrata and can be modified to create flow in the chambers using stir plates and bars or by attaching small pumps via Tygon tubing that circulates water across the growth substrata (Fig. 32.3C). Recirculating chambers such as that depicted in Fig. 32.3E provide the most realistic flow conditions, but they are expensive and may not be available to all researchers. Researchers are urged to consider their options for incubation containers at the onset of an experiment and select the one that is most appropriate for their substratum of interest, budget, and need for precise measurements versus replication.

32.2 GENERAL DESIGN

32.2.1 Basic Method 1: Denitrification—Determining Unamended Denitrification and Denitrification Enzyme Activity Rates

Denitrification is a series of dissimilatory microbial reductions of inorganic nitrogen, beginning with NO_3^- and ending with the production of N_2 gas. These reactions are essentially anaerobic respiration pathways that a wide range of organisms (from the archaea, bacteria, and fungi) are capable of performing when O_2 is not available as an electron acceptor. Determining rates of denitrification in streams and/or comparing denitrification among systems or treatments is often of great interest because NO_3^- is usually the most abundant form of inorganic N in streams and because the process can ultimately result in a loss of N from a stream as N_2 gas. Thus, denitrification is a critical process in regulating N availability and removal from ecosystems (Seitzinger et al., 2006).

Direct measurement of N_2 gas or use of isotopic techniques is often recommended to achieve actual unbiased rates of denitrification, but these methods are complex and require sophisticated equipment (see Section 32.4 on Advanced Methods). For a detailed review of different techniques used to measure denitrification, see Groffman et al. (2006). In this section, we will describe two similar methods to estimate denitrification that use acetylene gas as an inhibitor of the final step in the denitrification pathway—the reduction of N_2O to N_2. If this final step is inhibited, it is only necessary to measure the accumulation rate of N_2O in a sample to estimate denitrification. Incubation conditions can artificially stimulate synthesis of enzymes for denitrification (e.g., increase anoxic conditions or increase substrate availability), so chloramphenicol (an enzyme synthesis inhibitor) is also added to samples during incubation to limit the denitrification rate to that based on enzymes already in the collected sample.

The unamended denitrification method measures the accumulation of N_2O in samples that are incubated under anoxic conditions without substrate amendments. One criticism of this method has been that this method underestimates denitrification because acetylene also inhibits nitrification (NO_3^- production). If denitrifiers are limited by the availability of NO_3^-, a reduction in NO_3^- production can have a negative effect on the measured denitrification rate.

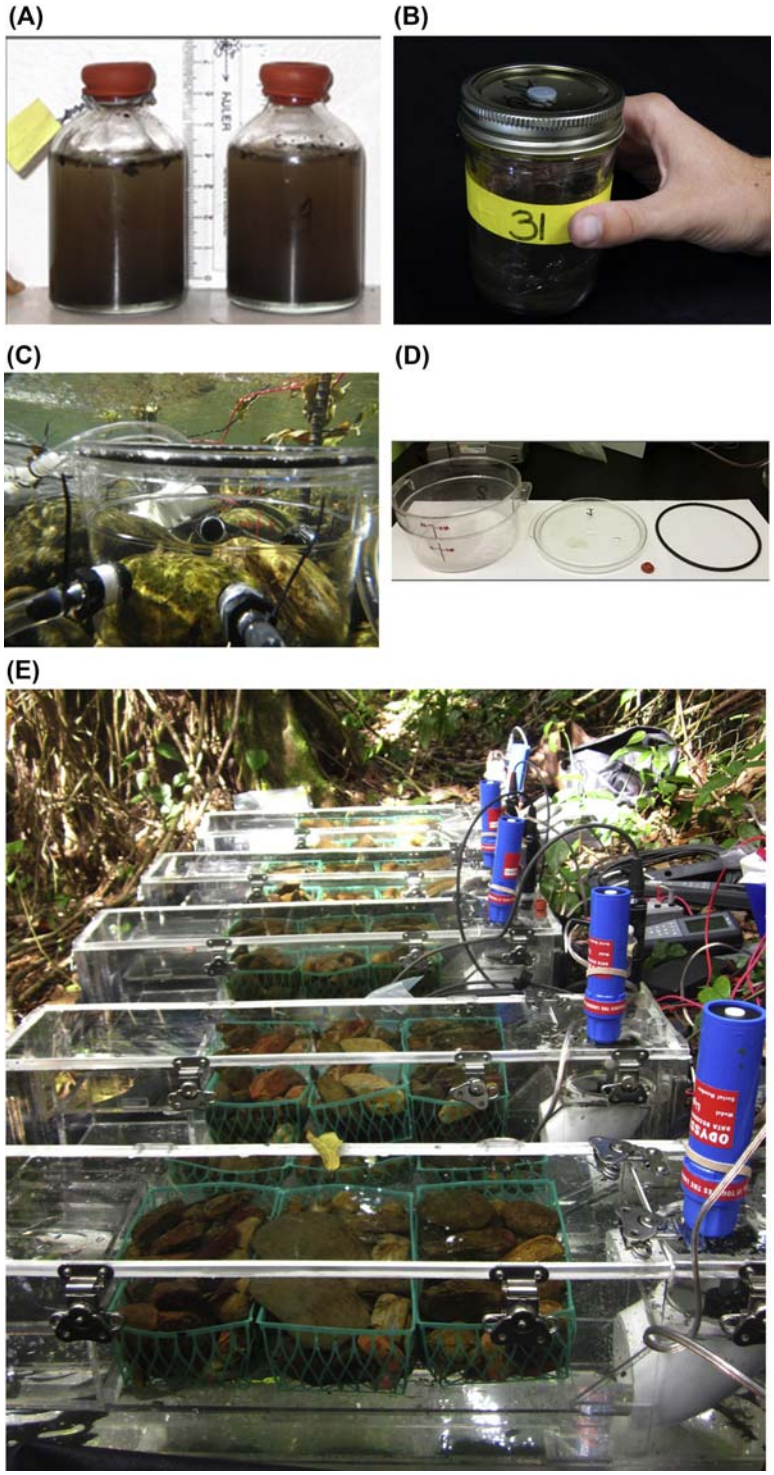

FIGURE 32.3 Examples of containers used to estimate in situ rates where gas flux needs to be determined. Serum vials (A) and canning jars (B) are ideal to incubate many replicates and/or small volumes of water or sediment, but may require separating periphyton from their growth substrata. Chambers can be circulating (C, E) or noncirculating (D). Nonrecirculating chambers such as the polycarbonate containers in (D) are ideal for incubating periphyton attached to their growth substrata and can be modified to create flow in the chambers using stir plates and bars or by attaching small pumps via Tygon tubing that circulate water across the growth substrata (C). Recirculating chambers in (E) can provide the most realistic flow conditions, but are expensive and can only be run with a limited number of replicates at a time. These chambers are being run streamside, and each has a light probe and oxygen probe attached. They can be fitted with septa for gas sampling and incubated submerged for temperature control (see Rüegg et al., 2015). *Photo credits: (A) A. Marcarelli; (B) E. Strauss; (C) M. Schenk; (D) E. Eberhard; (E) L. Koenig.*

In contrast, denitrification enzyme activity (DEA) measures denitrification potential in the presence of added substrates (i.e., glucose and NO_3^-). Thus, DEA measures full denitrification expression, that is, the potential of a sample given the extant level of denitrification enzymes (Groffman et al., 1999). DEA measurements alone are useful to show relative denitrification rates among sites or treatments. More specifically, DEA measurements can be used to identify denitrification hotspots within a stream or to compare denitrification potentials among streams. Even though DEA rates are considered "potentials," they do not always exceed ambient rates measured with more sophisticated techniques (Findlay et al., 2011). Rates measured with the DEA method should not be considered actual rates of denitrification nor be used to estimate N budgets because of the added NO_3^- and organic carbon. However, efforts have been made to combine measurements of NO_3^- availability, nitrification, unamended denitrification, and DEA to estimate actual NO_3^- flux in riverine ecosystems (Richardson et al., 2004).

32.2.2 Basic Method 2: Nitrification—Determining Gross and Net Nitrification Rates

Nitrification is a key microbial two-step transformation in the nitrogen cycle because it is the only natural pathway whereby nitrate is produced within a system. The energy gain from this aerobic chemoautotrophic process is relatively low, and rates are generally low compared to other nitrogen cycle processes. However, because the process can operate at low rates even with relatively low ammonium concentrations (Dodds and Jones, 1987), it occurs in many environments. Naturally low nitrification rates consequently necessitate longer incubation times compared to measuring rates of other processes, especially if isotopes are not used. Even though the rates are low, studies have shown nitrification rates to be influenced by important environmental factors including light, temperature, O_2, ammonium availability, pH, organic carbon availability, and C:N ratio (Strauss and Lamberti, 2000; Strauss et al., 2002). Nitrifying bacteria are generally attached to substrata within the stream, including sediment, fine particulate organic matter, and algae, and thus measurement of nitrification rates will involve incubating one or more of these materials. The methods described below can easily be modified to test the effect of different environmental conditions.

Gross nitrification is the absolute amount of ammonium converted to nitrate under oxic conditions. Gross rates can be estimated as the difference in ammonium concentrations between incubations in which nitrification was inhibited with the chemical nitrapyrin (2-chloro-6-[trichloromethyl]-pyridine) and those in which nitrification was allowed to occur. It is assumed that mineralization and ammonium assimilation are uninhibited in both incubations and that the ammonium increases in the incubations containing nitrapyrin is a result of inhibited ammonium oxidation. The method here describes a laboratory incubation of sediment and stream water placed in flasks. Modifications can be made to substitute other substrata depending on the research objective. When using sediment, studies often only use the uppermost 5 cm of sediment from the stream bottom.

Net nitrification is the change in nitrate availability through time and is the difference between gross nitrification and assimilatory/dissimilatory nitrate reduction. The net nitrification method below is similar, yet simpler, than the method for gross nitrification. Net nitrification can be measured simply as the change in nitrate concentration within a single incubation of substratum in stream water. As with gross nitrification, the method presented here will use sediment as the substratum but other substrata can be substituted depending on the objective.

32.2.3 Basic Method 3: Nitrogen Fixation

Nitrogen fixation (N_2 fixation), the transformation of nitrogen gas (N_2) to ammonium, can only be performed by certain heterotrophic bacteria, archaea, and cyanobacteria (Raymond et al., 2004). All of these organisms catalyze this reduction reaction using the enzyme nitrogenase. Even with this enzyme, the N_2 fixation reaction is energetically expensive, and nitrogenase is strongly inhibited by the presence of O_2, creating a unique challenge for N_2-fixing organisms that must expend energy and resources carrying out the fixation reaction as well as protecting the enzyme from O_2. In streams, N_2 fixation is primarily carried out by autotrophic cyanobacteria, particularly *Nostoc*, *Anabaena*, and *Calothrix* (Whitton, 2012). Species within these genera all form heterocysts, which are specialized, thick-walled cells where the N_2 fixation reaction occurs and the enzyme is protected from O_2. Although free-living, unicellular, nonheterocystous cyanobacteria have not been observed fixing N_2 in any stream to date, diatoms of the order Rhopalodiales, which host N_2-fixing cyanobacterial endosymbionts related to the unicellular *Cyanothece* sp. (Prechtl et al., 2004) are commonly found growing as epiphytes and on hard substrata in many streams.

The *acetylene reduction* assay is an indirect method for estimating N_2 fixation by measuring the activity of the nitrogenase enzyme (Stewart et al., 1967; Flett et al., 1976). The assay works because the nitrogenase enzyme recognizes the triple bond between C atoms in a molecule of acetylene (C_2H_2; H−C≡C−H) as equivalent to the triple covalent bond

between N atoms in an N_2 molecule. Nitrogenase will break one of the bonds between C atoms in acetylene, in the process of converting the molecule to ethylene (C_2H_4). Nitrogenase enzyme activity is estimated in the acetylene reduction assay by introducing acetylene to an airtight container or chamber along with the N_2 fixer of interest and measuring the amount of ethylene produced over a known time period. The rate at which ethylene gas is produced is related to the potential N_2 fixation rate.

Nitrogen fixation rates are dependent on a variety of environmental conditions that limit either the rate of the process itself (e.g., supply of enzyme cofactors, presence of O_2) or the energy available to the organisms that can be dedicated to carry out the reaction. Any of the factors that limit or constrain primary production in streams (e.g., light, nutrient supply, flow conditions) can also limit or constrain rates of N_2 fixation for autotrophic N_2 fixers. Because N_2 fixation rates in streams are particularly light sensitive, most researchers have opted to conduct acetylene reduction assays in the field, where light and temperature conditions can be easily maintained by conducting incubations in containers submerged in the study streams.

The acetylene reduction assay must be carried out in an airtight arena, and a variety of options have been used to conduct these measurements in streams, from simple to complex (Fig. 32.3). The simplest approach is to seal the substratum or biofilm of interest suspended in water into a small, glass container such as a serum vial (Fig. 32.3A). If the study is focused on autotrophic N_2 fixers such as cyanobacteria, then the chambers must be transparent and attention should be paid to the wavelengths of light transmitted through the selected material, as well as its ability to bind organic gasses such as acetylene and ethylene. Glass is ideal for light transmission and lack of reaction with organic gasses, but is not practical for large chambers. Plexiglas and polycarbonate are not reactive with organic gasses and transmit most visible light, but both absorb various wavelengths of infrared and ultraviolet light (although some ultraviolet-transmissive Plexiglas is available to build chambers; Dodds and Brock, 1998). Polyethylene and other plastics should be avoided as they can be very reactive with organic gasses such as acetylene and ethylene. The chamber or vial selected must be outfitted with at least one sampling port where septa can be placed to allow collection of gas samples using a syringe and needle.

To conduct the assay, substrata are enclosed in the chamber either suspended in or with a similar volume of overlying water. A headspace equal to about 10% of the container volume composed primarily of acetylene is introduced and the container is agitated to dissolve the acetylene in the water in equilibrium with the headspace, and then an initial headspace sample is collected to document conditions at the start of the incubation. The method used to introduce headspace can vary depending on the total volume of the chamber—for small volumes headspaces can easily be introduced using a syringe, while others have used a small balloon sealed in the chamber and then popped through a septa to introduce larger volumes of acetylene (e.g., Grimm and Petrone, 1997). Following several hours of incubation, the chamber is again agitated to equilibrate gas between the water and headspace, after which a headspace sample is collected to measure the ethylene produced during the incubation. The rate of ethylene production is typically linear for 6–8 h and then declines as the supply of acetylene is depleted. Therefore, most researchers conduct their incubations for 2–4 h, although it is strongly suggested that researchers conduct their own time-course incubations to determine ideal incubation duration to detect production of ethylene with confidence while avoiding the decline in production that occurs over long time periods. Gas samples are stored in gastight containers and subsequently analyzed for initial and final concentrations of ethylene using a gas chromatograph (GC).

32.2.4 Advanced Methods: Isotopes for Flux Rate Measurement

Additional power to detect biogeochemical processes and more refined estimates of transformation rates can be gained by incorporating the stable isotope ^{15}N into bottle rate measurements. Indeed, some N cycling processes—most notably DNRA—can only be measured using ^{15}N tracers. The advantage of increased resolution to detect biogeochemical changes using ^{15}N tracers is balanced by the challenges associated with the high costs of isotopically labeled chemicals, accessibility to instruments to measure isotopes (e.g., isotope ratio mass spectrometer, IRMS), and the need for additional training necessary to understand optimal quality control measures and contamination prevention.

Membrane inlet mass spectrometry (MIMS) is another specialized technique using a mass spectrometer (mass spec) often employed by nitrogen biogeochemists to gain additional power in measuring transformation rates, most notably for denitrification (N_2 production). MIMS can be used to also measure N_2 fixation, and most recently a method has been validated for using MIMS to measure DNRA (ox-MIMS; Yin et al., 2014). The principle of the MIMS measurement is based on how gases dissolve into water; therefore, the instrument also often referred to as a dissolved gas analyzer (available from Bay Instruments, Kana et al., 1994). In essence, gases dissolve into water based on known physics of solubility related to temperature, salinity, and relative humidity. Sample water with these dissolved gases is passed through gas-permeable tubing (silicon membrane) housed in a vacuum-tight chamber, so gases pass through the membrane into a vacuum and are then

transferred to the mass spec. The mass spec collects signals corresponding to different masses. The signals of specific atomic mass units (amu) can be related to different gaseous components, including 4 = helium; 18 = water vapor; 28−30 = dinitrogen (using isotopes $^{14,14}N$, $^{14,15}N$, $^{15,15}N$); 32 = oxygen; 40 = argon. The amplitude of the signal is proportional to the amount of the gas present, and by comparing it to a standard of known concentration (and knowing the linearity of the instrument), the mass of a certain gas present in a given sample can be calculated. Some gases are biologically inert (e.g., Ar) and can therefore be used as conservative tracers to correct for physical processes and compared to changes in biologically active gases (e.g., N_2). Once you have access to a MIMS, samples are relatively easy and cheap to collect, but can be time-consuming to process (~5 min per sample and we know of no autosampler for this equipment).

32.3 SPECIFIC METHODS

32.3.1 Basic Method 1: Denitrification

32.3.1.1 General Preparation

1. Gas chromatograph: Both denitrification methods described here require measuring N_2O gas concentrations with a GC. Instrument configurations vary widely depending on manufacturer, detectors, columns, and sampler, but the basic N_2O analysis configuration would be a GC equipped with a ^{63}Ni electron capture detector at 350°C and a GC column similar to Porapak Q 80/100. Consultation with your GC manufacturer is advisable for ideal configuration.
2. Laboratory gases: Measurement of unamended denitrification and DEA will require the use of several gases in the laboratory. Gas purity is important as noted here.
   a. GC carrier gas: Usually 95% argon−5% methane (P5) or ultrahigh purity N_2 are used as the GC carrier gas for N_2O analysis.
   b. Gas standards: Calibration of GC with gas standards of known N_2O concentration will be necessary. A good series of standards to have on hand would be 1, 10, 100, and 1000 ppm_v N_2O (balance gas is N_2). Small cylinders of these high-quality N_2O gas standards can be purchased from many specialty gas companies. Intermediate standard concentrations can be made via syringe dilutions with atmospheric air. For example, to produce a 50 ppm_v N_2O gas standard, draw 2.5 mL of atmospheric air into a gas syringe already containing 2.5 mL of 100 ppm_v N_2O. The first time you run this analysis you should use a wide range of standards from 0 to 1000 ppm_v. Eventually, you might be able to adjust the actual standard concentrations based on the level of denitrification activity in your system.
   c. Acetylene: High-quality contaminant-free acetylene gas is used to inhibit the reduction of N_2O during the incubation period. The preferred type of acetylene gas is atomic absorption grade which can be purchased from specialty gas companies. This gas can be used immediately without further purification. Industrial grade, such as welding grade acetylene can also be used but acetone and other contaminants must first be removed by bubbling gas through a concentrated H_2SO_4 trap and then a distilled/deionized water trap prior to use.
   d. Anoxic environment gas: To ensure an anoxic environment during the incubation, the samples are flushed with an O_2-free gas. Oxygen-free Ar, N_2, or He is routinely used for this purpose.
3. DEA solution: The goal of DEA analysis is to measure the full expression of denitrification enzymes present in the sample at the time of collection. This DEA solution will be added to samples before incubation to supply denitrifiers with an available source of high-quality organic carbon and NO_3^- to alleviate substrate limitation. The solution also contains chloramphenicol to inhibit new enzyme synthesis. Dissolve 1.01 g of KNO_3, 0.30 g of glucose, and 1.00 g of chloramphenicol in a 1000-mL volumetric flask with about 900 mL of deionized water. Wrap the flask with aluminum foil to protect it from light, place the flask with a Teflon stir bar on a stir plate, and stir until the chloramphenicol completely dissolves (usually requires stirring overnight). Bring the solution up to volume with deionized water and transfer the solution to a 1-L amber bottle. Store in the refrigerator. This solution may also be made without glucose and/or NO_3^- to test for C and/or N limitation. A chloramphenicol-only solution will be used for measuring unamended denitrification.
4. Incubation vessels: Just about any airtight container with septa access should be suitable. Many studies have used Erlenmeyer flasks or media bottles. An inexpensive option is canning jars sealed with standard canning rings and lids (Fig. 32.3). The canning lids will need to be hole-punched and fitted with a septum. Heating the canning lids in hot water prior to use may help soften the plastisol sealant and improve the airtight seal. It is also possible to incubate an intact sediment core in a stoppered plastic tube as long as there is septa access to the airtight headspace. If incubating intact cores, diffusion of acetylene and DEA solution can be limited. If the vessel is not glass, N_2O adsorption to the vessel should be tested before using. Regardless of vessel used, the actual headspace volume will need to be known and should be between 100 and 300 mL.

32.3.1.2 Denitrification Rate Procedures (Laboratory)

Incubation Vessel Setup

1. To each incubation vessel, add 25 mL sediment and 20 mL stream water. If using sediment cores, the uppermost 5 cm of sediment from a 2.54-cm ID core would be 25.3 mL. The sediment and water volumes listed are for a vessel volume of approximately 200−400 mL; if a larger or smaller container is used, scale the materials added proportionally. If sediment and/or water volumes are changed, the volume of amendment solution added in step 2/3 will also need to be changed proportionally. Record the number of each vial and contents on a data sheet (Table 32.1).
2. For unamended denitrification samples, add 5 mL of 1 g chloramphenicol/L solution to each vessel.
3. For DEA measurement, instead add 5 mL of DEA solution.
4. Seal lid on incubation vessel. It is important that the seal is airtight and capable of withstanding both positive and negative pressure.
5. Insert a needle (c. 22 gauge) attached to vacuum line/pump into the vessel and evacuate for 90 s (Fig. 32.3).
6. Add anoxic environment gas to the vessel to a small positive pressure (c.30 kPa).
7. Repeat evacuation and gas flushing for a total of three cycles.
8. Bring vessel to atmospheric pressure by venting excess gas through a water-filled syringe (Fig. 32.3).
9. Add acetylene to sample headspace. The volume added should equal 10% of headspace. Note the time acetylene is added to each vessel; this is the start of the incubation period.

Incubation, Sampling, and Analyses

1. Place samples on an orbital shaker (175 rpm) and incubate at desired temperature for 6 h (3 h for DEA).
2. Collect and store a time series of gas samples from each vessel.
3. Using a 5-mL disposable syringe and needle (c. 22 gauge), collect a 3-mL gas sample at 1, 2, 4, and 6 h for unamended denitrification samples. For DEA samples, collect samples at 0.5, 1.0, 1.5, and 3 h. Insert needle into vessel and pump syringe 3× before collecting gas sample. *Note*: if total headspace volume of vessel is less than 100, 3 mL of acetylene should be returned to the vessel after sampling to maintain positive pressure inside the vessel.
4. Place gas sample into an evacuated 2-mL serum bottle crimp-sealed with a butyl rubber septa.
5. If N_2O concentrations will not be determined within 24 h, N_2O standards for the standard curve should also be stored in evacuated serum bottles and stored with samples. This will help account for any container effects on gas samples. For example, N_2O can bind to certain types of septa material.
6. Using a GC calibrated with N_2O gas standards, determine N_2O concentration (ppm_v) in the gas samples.

Calculation of Denitrification Rate

1. For each gas sample collected and analyzed on the GC, convert N_2O concentration from volume units to mass units. That is, convert ppm_v N_2O to μg N_2O-N/L:

$$C_m = \frac{C_v \cdot M \cdot P}{R \cdot T} \tag{32.1}$$

where C_m = mass/volume concentration (μg N_2O-N/L) in headspace; C_v = ppm_v or volume/volume concentration in μL/L; M = mole weight of nitrogen in N_2O (28 g/mole of N_2O); P = pressure in atmospheres (ATM = 760 mmHg); R = universal gas constant (0.0820575 L · ATM/K · mole); T = room temperature in Kelvin (K = °C + 273.15).

2. Calculate the total mass of N_2O-N per sample, accounting for N_2O dissolved in water and in gas phase:

$$C_T = C_m \cdot (V_g + V_l \cdot \beta) \tag{32.2}$$

where C_T = total mass of N_2O-N in vessel; V_g = headspace volume (L) in vessel; V_l = liquid volume (L) in vessel; β = Bunsen coefficient = 1.2407−0.0398 · temp + 0.0005 · temp^2; temp = incubation temperature in °C.

TABLE 32.1 Data sheet for recording data necessary for calculating denitrification rate. See also Online Worksheet 32.1.

Date:

Project:

Investigators:

Sample	Incubation Vessel ID	Treatment	Sediment Volume (mL)	Liquid Volume (mL)	Head-space Volume (mL)	Incubation Temperature (°C)	Incubation Start Time	T_1 Time	T_1 N_2O (ppm_v)	T_2 Time	T_2 N_2O (ppm_v)	T_3 Time	T_3 N_2O (ppm_v)	T_4 Time	T_4 N_2O (ppm_v)

TABLE 32.2 Data sheet for recording data necessary for calculating gross nitrification rate. See also Online Worksheet 32.1.

Date:											
Project:											
Investigators:											
Sample	Flask ID	Nitrapyrin or DMSO	Sediment Volume (mL)	Liquid Volume (mL)	Incubation Temperature (°C)	Incubation Start Time	T_0 Time	T_0 NH_4^+ (mg N/L)	T_F Time	T_F NH_4^+ (mg N/L)
1		N								
1		D								
2		N								
2		D								
3		N								
3		D								
4		N								
4		D								

3. Calculate denitrification rates in μg N_2O-N/h:

$$\text{Denitrification Rate } \left(\mu g\ N_2O\text{-}Nh^{-1}\ S^{-1}\right) = \frac{C_{Tf} - C_{Ti}}{\Delta t \cdot S} \tag{32.3}$$

where C_{Tf} = total final mass of N_2O-N (~4 h sample for unamended denitrification or ~1.5 h sample for DEA); C_{Ti} = total initial mass of N_2O-N (2 h sample for unamended denitrification or 0.5 h sample for DEA); Δt = change in incubation time (h); S = sediment characteristic in which to express rate (e.g., surface area calculated from core cross section, dry mass of sediment, ash-free dry mass (AFDM), or sediment volume).

Tips and Notes

1. Measuring a minimum of five replicate incubations per experimental unit (e.g., five incubations per site within a stream) is recommended because of heterogeneity.
2. Gas samples collected at different times may be used when determining denitrification rate; however it should be established that the increase in N_2O is linear over the time interval used. An alternative technique to calculate denitrification rate is to plot N_2O concentration against sample time and calculate denitrification rate as the slope of the line.

32.3.2 Basic Method 2: Nitrification

32.3.2.1 Gross Nitrification Procedure

Day 0 Flask Setup

1. For each sample, you will need two 125-mL Erlenmeyer flasks. One flask will contain sediment, stream water, and the nitrapyrin nitrification inhibitor solution (this flask will hereafter be referred to as the "N flask"). The other flask, the "D flask," will contain sediment, stream water, and dimethyl sulfoxide (DMSO, the solvent in the inhibitor solution). Record each flask number and contents on a data sheet (Table 32.2).
2. For each flask, place 25 mL of sediment into a labeled 125-mL Erlenmeyer flask using a plastic powder funnel. If using sediment cores, the uppermost 5 cm of sediment from a 2.54 cm ID core would be 25.3 mL as calculated for the volume of a cylinder.
3. Rinse funnel with exactly 81 mL of site water, catching all rinse water in the flask (i.e., flask should contain only 25 mL sediment plus 81 mL site water).

4. To all N flasks add 20 μL of nitrapyrin solution (1 g/20 mL dissolved in DMSO) and to all D flasks add 20 μL of DMSO. *Note*: be careful when handling DSMO;[1] while the DMSO is not toxic, it is a "super solvent" that will allow toxic chemicals dissolved in it to easily pass into the skin.
5. Tightly cover flask openings with Parafilm and shake to thoroughly mix the contents.
6. Immediately remove the Parafilm and pipette 6 mL of the slurry into a labeled 15-mL plastic centrifuge tube already containing 6 mL of 2 N KCl. This step is required to mobilize positively charged NH_4^+ ions that are ionically bound to negatively charged sediment material.
7. Cover centrifuge tubes with Parafilm. Allow NH_4^+ to extract from the samples for approximately 60 min, inverting to mix samples at least every 10 min.
8. During the NH_4^+ extraction step, initiate the nitrification incubation. Place the N and D flasks on an orbital shaker at 175 rpm. To discourage microbial contamination and facilitate aeration, loosely cover each flask with a piece of aluminum foil but do not recover flasks with Parafilm. Incubate samples for 3 days in the dark at ambient temperature.
9. Returning to the NH_4^+ extracted samples in the centrifuge tubes, filter each sample through a glass fiber filter (GFF) (e.g., Pall Type A/E or Whatman GF/C or GF/F). Brief centrifugation of samples before filtration will settle much of the sediment and ease filtration. Store filtered sample at 4°C in a clean 20-mL scintillation vial or other small bottle until analyzed for NH_4^+. If analysis will not be completed within 24 h, add 200 μL of 10% H_2SO_4 to each sample.

Day 3 Laboratory Procedures

1. After the 3-day incubation, remove N and D flasks from the shaker.
2. Collect Day 3 samples, extract NH_4^+, filter, and prepare sample as described for Day 0 samples.
3. Analyze samples for NH_4^+-N concentration using the method of your choice. For a detailed fluorometric protocol to quantify NH_4^+-N, please see Chapter 36 in this volume.
4. If samples were acidified and stored, neutralize the acid with 200 μL of 3.6 M NaOH before NH_4^+ analysis.
5. Regardless of the method, be sure your standards account for the DMSO and KCl in the samples (i.e., all standards should be made in a matrix solution of 10 μL DMSO/100 mL 1.0 N KCl).

Calculation of Nitrification Rate

1. Express the rate in appropriate units (e.g., μg N · mL sediment^{-1} d^{-1}, or g N m^{-2} d^{-1}). Various units are obtained by using mass versus moles of nitrogen nitrified, different time units, and different sediment/substratum characteristics.
2. In general, nitrification rate can be calculated as:

$$\text{Gross Nitrification Rate} = \frac{(\Delta N - \Delta D)}{t \cdot S} \tag{32.4}$$

where ΔN = change (final−initial) in NH_4^+-N mass in the N flask during the incubation period; mass can be calculated by multiplying the concentration of NH_4^+ (mg N/L) by the volume of the sample (0.1 L); ΔD = change in NH_4^+-N mass in the D flask during the incubation period; t = actual time of incubation (e.g., days or hours); and S = sediment characteristic in which to express the nitrification rate. Examples may include surface area calculated from core cross section, dry mass of sediment, AFDM, or volume of sediment.

Tips and Notes

1. Measuring a minimum of five replicate incubations per experimental unit is recommended because of heterogeneity.
2. An incubation duration of 3 days has been shown to produce linear changes in NH_4^+-N concentration in samples from numerous stream ecosystems (Strauss et al., 2002). However, collecting more samples throughout the incubation period and regressing NH_4^+-N concentration against time for linearity over the incubation is prudent.
3. Be cautious of contamination of control flasks (D flasks) with nitrapyrin (e.g., pipette tips, bottle caps, etc.). Nitrapyrin is highly effective at inhibiting nitrification and even trace amounts can introduce significant error.

32.3.2.2 Net Nitrification Procedure

Day 0 Flask Setup

1. For each flask, place 25 mL of sediment into a labeled 125-mL Erlenmeyer flask using a plastic powder funnel.
2. Rinse funnel with exactly 85 mL of site water, catching all rinse water in the flask

1. Wear personal protective equipment including lab coat, eyewear, and gloves.

3. Tightly cover flask openings with Parafilm and shake to thoroughly mix the contents.
4. Immediately remove the Parafilm and pipette 10 mL of the slurry into a labeled 15-mL plastic centrifuge tube.
5. Place the flasks on an orbital shaker at 175 rpm. To discourage microbial contamination and facilitate aeration, loosely cover each flask with a piece of aluminum foil but do not recover flasks with Parafilm. Incubate samples for 3 days in the dark at ambient temperature.
6. Returning to the samples in the centrifuge tubes, filter each sample through a GFF (e.g., Pall Type A/E or Whatman GF/C or GF/F). Brief centrifugation of samples before filtration will settle much of the sediment and ease filtration. Store filtered sample at 4°C in a clean 20-mL scintillation vial until analyzed for NO_3^-. If analysis will not be completed within 24 h, add 200 μL of 10% H_2SO_4 to each sample.

Day 3 Laboratory Analyses

1. After the 3-day incubation, remove the flasks from shaker.
2. Collect Day 3 samples, filter, and prepare sample as described for Day 0 samples.
3. Analyze samples for NO_3^--N concentration using the method of your choice. If samples were acidified and stored, neutralize the acid with 200 μL of 3.6 M NaOH before NO_3^- analysis.

Calculation of Nitrification Rate

Net nitrification rate can be calculated as:

$$\text{Net Nitrification Rate} = \frac{\Delta NO_3^-\text{-N}}{t \cdot S} \tag{32.5}$$

where ΔN = change (final−initial) in NO_3^--N mass in the flask during the incubation period; mass can be calculated by multiplying the concentration of NO_3^- (mg N/L) by the volume of the sample (0.1 L); t = actual time of incubation; and S = substratum characteristic in which to express the nitrification rate (i.e., per unit mass, area, or volume).

Tips and Notes

1. Measuring a minimum of five replicate incubations per experimental unit is recommended because of inherent heterogeneity.
2. Since this method intentionally examines the combined effect of several processes and does not single out a particular process (e.g., nitrification alone), one should use caution in interpreting the measured rates.
3. You can also measure the change in NH_4^+-N concentration using this method as an estimate of net N mineralization. If you do this though, you will need to extract the NH_4^+ bound to sediment material before measuring NH_4^+ concentration as described in the gross nitrification method.

32.3.3 Basic Method 3: Nitrogen Fixation

32.3.3.1 General Preparation

Gas Chromatograph Configuration

1. A flame ionization detector (FID)—equipped GC is needed to analyze ethylene. Many configurations are available and it is recommended that the manufacturer of the specific brand of machine used be consulted for optimal setup.
2. Several columns can be used to analyze ethylene—the two most common are Poropak T and Hayesep T. Example GC configurations with a Hayesep T column are ultrahigh purity helium carrier at 30 psi, ultrahigh purity hydrogen to FID at 35 psi, column temperature 40°C, FID temperature 180°C. These settings result in clear separation of ethylene and acetylene peaks with retention times of about 1.5 and 3.8 min, respectively.
3. Although you do not need to know the size of the acetylene peak to calculate fixation rates, you do need to ensure that all of the acetylene gas has moved off the column before running the next sample. Ramping up the column temperature after the ethylene peak is recorded can move the acetylene through the column more quickly and shorten the analysis time per sample.
4. Similar to denitrification, you will also want to purchase high-purity, premixed standards for calculating concentrations of ethylene; 100 or 1000 ppm ethylene in He are useful concentrations that can be easily diluted to create lower concentration standards depending on rates observed in your study systems.

Site Selection, Preparation, and Incubation Decisions

1. Select the desired stream reach for the experiment, and determine the dominant substrata type(s) for the incubation. Make sure the reach has an area with water depths adequate to submerge incubating samples with light conditions/shading typical of the overall study reach.

2. Select the enclosure to be used for the incubation and determine the total volume of that container. Decide on target volumes to be used for the measurements, such that (1) substratum volume equals 30–45% of total volume, (2) water volume equals 45–60% of total volume, and (3) headspace equals 10% of total volume.
3. Procure enough acetylene for all assays. For discussion on acetylene purity see information under preparations for denitrification.

32.3.3.2 Field Procedures

Setting up a Run

1. Fixation should be run as close to the middle of the day as possible, since rates may be light-dependent and closely related to patterns in primary production. Alternately, the incubation could be repeated over a 24-h period to characterize how rates change with light availability.
2. Fill the enclosure with substratum to be measured (e.g., rocks or wood), water, and a balloon filled with acetylene, if using for a larger enclosure (e.g., chamber). Record number and contents of each container on a data sheet (Table 32.3).
3. Place enclosure in the water and seal.
4. Repeat for the desired number of replicates per site. Also set up several "blanks" with water only to account for any enclosure effects or impurities in acetylene.

Initializing the Assay

1. If using chambers, measure the temperature in the chamber by inserting a thermometer into the sampling port. Record the temperature separately for each chamber; this number is necessary to calculate gas concentrations. With smaller incubation enclosures, measure stream temperature.
2. Replace the septa underwater, making sure that there are no air bubbles.
3. Pop the balloon in the chamber by inserting a needle through the sampling port, or add the headspace by withdrawing the desired volume of water and replacing with the acetylene (may require two injection ports to pull water from one while pushing gas into another).
4. Agitate the enclosure for 20 s to equilibrate the gas dissolved in the water with that in the headspace (not necessary for recirculation chambers).

TABLE 32.3 Data sheet for in situ acetylene reduction assays to measure N_2 fixation. See also Online Worksheet 32.1.

Date:							
Project:							
Investigators:							
Chamber #	Initial Temp (°C)	T_0 Time	Final Temp (°C)	T_F Time	Headspace Volume (mL)	Water Volume (mL)	Substrate Volume (mL)

5. Collect an initial sample by bringing the headspace to the sampling port and withdrawing a sample (volume should be proportional to overall headspace volume—e.g., with a 2-L chamber and 200-mL headspace, we collect about a 10-mL sample; for a 50-mL headspace, we collect a 2- or 3-mL sample).
6. Record the initial time of sample (Table 32.3).
7. Store the initial gas sample in a gastight syringe, evacuated serum vial, or Exetainer (Labco, UK) until analysis.
8. Incubate chamber in situ for desired incubation time, typically 2–4 h depending on expected rates.

Terminating the Assay

1. At the end of the desired incubation time, collect a final gas sample by repeating steps 4–7 above.
2. After the final sample is collected, measure the temperature in each enclosure as in step 1 above.
3. Open the enclosure and measure volume of water and sediment in the chamber. If using a hard substratum, you will also want to estimate surface area to allow scaling of rates per unit area.
4. If you wish to express rates per unit biomass, process substrata for standing crop biomass (see chlorophyll and AFDM protocols in Chapter 12).

32.3.3.3 Laboratory Procedures

Analysis of Headspace Samples via Gas Chromatography

1. Ethylene concentration can be analyzed on any GC equipped with an FID.
2. On each day that the GC samples are analyzed, also run standard concentration samples of ethylene. Run standard samples before any unknown samples each day to ensure that the GC is functioning properly, then continue to analyze a standard every 10 samples to correct/monitor for instrument drift.

Calculating N_2 Fixation Rates (Based on Capone, 1993)

1. Determine the solubility correction (SC) for ethylene in aqueous phase (SC) as:

$$\mathrm{SC} = 1 + \left(\alpha \cdot \frac{A}{B}\right) \tag{32.6}$$

where α = Bunsen coefficient for ethylene at the incubation temperature, A = water volume in the chamber, and B = headspace volume; α can be determined from a chemistry handbook such as Dean (1992).

2. Calculate the quantity of ethylene in the sample as:

$$\mathrm{ethylene(nmol)} = \left(\frac{\mathrm{Peak\ Height_{sample}}}{\mathrm{Peak\ Height_{standard}}}\right) \cdot C_{\mathrm{standard}} \cdot B \cdot \mathrm{SC} \tag{32.7}$$

where $\mathrm{Peak\ Height_{sample}}$ = ethylene peak height in sample, $\mathrm{Peak\ Height_{sample}}$ = ethylene peak height in a standard, C_{standard} = concentration of that standard in nmol mL^{-1}, B = headspace volume in mL (volume of headspace in assay vessel in mL), and SC = solubility correction as described above.

3. Calculate the rate of fixation in the chambers as:

$$\mathrm{Ethylene\ fixation\ rate}\left(\frac{\mathrm{nmol}}{t}\right) = \frac{(\mathrm{ethylene_{final}} - \mathrm{ethylene_{initial}})}{t} \tag{32.8}$$

where $\mathrm{ethylene_{final}}$ and $\mathrm{ethylene_{initial}}$ = ethylene concentrations in the chamber at the beginning and end of the incubation and t = duration of the incubation period (commonly reported in hours).

4. Convert the ethylene fixation rate to the N_2 fixation rate assuming a ratio of 3 moles of ethylene produced for every 1 mole of N_2 gas potentially fixed (but see N-fixation literature for discussions of the appropriateness of this conversion factor and alternates).
5. Scale the fixation rate per substratum area or unit biomass as desired.

Tips and Notes

A time series of incubations is suggested for early runs in a particular system. This will allow for determination of the minimum incubation time to detect activity as well as an incubation length where rates remain linear.

32.3.4 Advanced Method 1: Using ^{15}N to Measure DNRA

32.3.4.1 General Preparation

Analytical Considerations (Prepare >1 Month Prior to Experiment)

1. Plan to make $^{15}NO_3^-$ solution at an appropriate concentration for your field site. Solution can be made from $K^{15}NO_3$ or $Na^{15}NO_3$.[2] It is easiest to dose out the appropriate amount of ^{15}N as a solution (by volume), as opposed to measuring out very small masses.
   a. Nitrate concentrations are often difficult to impossible to measure in sediments due to the low concentrations and fast removal rates. Therefore, addition of any $^{15}NO_3^-$ may stimulate removal rates, as well as DNRA or denitrification rates. The additional $^{15}NO_3^-$ should be added at concentrations small enough to minimize this stimulation, but large enough to get measurable signal. In general, the labeled nitrate should not boost the concentration of the ambient nitrate by more than 10%.
   b. Isotopes are always expressed relative to a standard, which is a notation that takes some getting used to. If you are not familiar with using isotopes, we highly recommend a general reference to start (e.g., Kendall and Caldwell, 1998; Michener and Lajtha, 2008; see also Chapter 23). Isotopic enrichment is usually expressed one of two ways, as atom percent or as delta notation. Enrichment of an experiment is calculated as (from Steingruber et al., 2001):

$$\varepsilon = \left[NO_3^-\right]_a - \frac{\left[NO_3^-\right]_b}{\left[NO_3^-\right]_a} \tag{32.9}$$

   where $\left[NO_3^-\right]$ = nitrate concentration of the solution after (a) and before (b) the $^{15}NO_3^-$ tracer addition. Enrichment of ^{15}N can be expressed two ways:
      i. Atom percent = (mass of heavy isotope)/(mass of all isotopes). In the case of N:

$$\text{Atom \% } ^{15}N = \left[^{15}N/(^{14}N + {}^{15}N)\right]\cdot 100 \tag{32.10}$$

      ii. Delta notation (δ, expressed in ‰):

$$\delta‰ = (R_{sample}/R_{standard} - 1)\cdot 1000 \tag{32.11}$$

   where R = ratio of the heavy to light isotope in the sample or standard (see also Chapter 23).
2. Gather syringes of various sizes (1, 5, 10, 20, 60 mL) depending on the volume of your assay bottle and the amount of tracer you need to add. You will also need assay bottles (e.g., 60-mL Nalgene or 120-mL Wheaton bottles). It is best to use bottles that do not have any potential for background ^{15}N contamination and create an airtight seal. You will also need access to a shaker table large enough to hold all of your assay bottles and diffusion bottles.
3. After your assay is complete, quickly process the resulting samples to measure for $^{15}NH_4^+$ and $^{15}NO_3^-$. Quick turnaround depends on having the following materials assembled and ready to use:
   a. Diffusion bottles—these are a separate set of bottles in which your diffusions will be done (see Section 32.3.4.3 step 7). Diffusions require a vessel with a tight-fitting lid, which can withstand some pressure buildup without gas escape.
   b. Acid traps—see details in Section 32.3.4.3 step 7b.
4. Finally, determine ahead of time where you plan to send your samples for analysis by IRMS. It is best to contact the contract lab well ahead of time to ask about their backlog and turnaround times. It is also good to ask other researchers about the reliability of the laboratory chosen for analyses. Pick one that has analyzed similar samples before, has well established quality control and quality assurance procedures, and can meet your time requirements for sample turnaround. This often is not the least expensive analytical laboratory (see also Chapter 23 for recommendations).

32.3.4.2 Field Procedures

1. If you collect substrata directly into your assay bottles, you need to take steps to make the bottles anoxic in the field (e.g., adding water to the sediments so they do not become oxidized, or exchanging the bottle headspace with an anoxic gas such as He or N_2).
2. Alternatively, you can collect intact cores from your study site and carefully transport them back to the laboratory. Ideally cores are broken down under anaerobic conditions (e.g., Coy anaerobic chamber or "glove box"). If a glove box is not available, portable glove bags can be used and are less expensive (e.g., Aldrich AtmosBag, Sigma–Aldrich).

2. The 98 atom% $Na^{15}NO_3^-$ can be purchased from Sigma–Aldrich (item # 364606) at ~US $100/g, which can be enough for a large number of bottle assays. Stable isotopes can be backordered for months, so be sure to place your order well ahead of the experiment.

32.3.4.3 Laboratory Procedures

Adding ^{15}N to Incubations

1. Determine how much of your substratum to add to an assay bottle (e.g., 10 grams of wet sediment). Record the wet mass and also be sure to estimate the dry weight by drying a subsample and calculating the percentage of water in the substratum. Keep the substratum as anoxic as possible, either by putting it in a glove box or by adding deoxygenated water (e.g., site water sparged with an inert gas such as He) to the sample immediately after weighing it out.
2. Add enough tracer to each assay bottle to reach a desired predetermined final NO_3^- concentration. The decision of how much ^{15}N to add depends on the goals of the study and the estimated rates. For example, if your goal is to measure as close to ambient rates as possible and you are working with a substratum you expect to be very anoxic, you would want to add as little ^{15}N as necessary to meet the minimum detection limits of the instrument that will analyze your samples, which varies by lab and by instrument (e.g., a MIMS can measure ^{15}N, but is much less sensitive than an IRMS and thus requires greater enrichment). Nitrate is often undetectable in anoxic pore waters; thus, adding any nitrate will artificially elevate the rates being measured.
3. Incubate bottles for a predetermined time period. Again, how long to incubate the bottles depend on the predicted rates. If you expect you are working with a substratum that will yield high rates, it is best to incubate for as short of a period as possible. If your rates are completely unknown, we strongly advise running a "pilot" batch of samples wherein you incubate assays for different time periods to determine the shortest possible incubation time that will yield measurable results for the IRMS. While this may sound time intensive, it can be time well spent, as too short of an incubation could result in an entirely unusable (and expensive) data set. Incubate the bottles under as similar conditions to the field collection as possible (e.g., light and temperature); this often requires environmental chambers.
4. Once the incubation is complete, separate the water and the sediment fractions. This is often done by centrifugation. The $^{15}NH_4^+$ will be in both the water fraction and sorbed to the sediments; $^{15}NH_4^+$ can be released from the sediments by exchanging it with 2 M KCl (as a final concentration; be sure to account for the volume of water in your sediment that may dilute a straight 2 M solution of KCl; Robertson et al., 1999).
5. Measure the NH_4^+ concentration of the KCl solution and of the water fraction. If analyzing a KCl sample, be sure your standards are also prepared using KCl as a matrix.
6. Once you know the concentration of NH_4^+ in your samples, you can estimate the volume you need to reach the minimum mass requirements of the IRMS (or other mass spectrophotometer) used to analyze the samples. It is advisable to not diffuse your entire sample volume. Samples can be frozen indefinitely. If working with contract labs, samples can go missing (in the mail or through other means) or be dropped, and thus, if you have diffused your entire sample, you may need to redo the experiment. Once you know the volume of the samples you will need to diffuse onto acid traps, you can proceed to the diffusion method. Again, the volume will likely vary by sample, so be sure to calculate the volume for the full range of NH_4^+ concentrations in your samples.
   a. Example calculation: Your bottle contains 50 mL of sample at 0.5 mg/L NH_4^+. The total mass of NH_4^+ is 0.2 mg (0.156 mg as N or 156 μg N). The IRMS requires a minimum of 20 μg N per sample. Diffusing 10 mL (1/5th) of sample would result in 31.2 μg N from the resulting IRMS sample, well above the minimum detection limit, but also leaving four-fifths of the sample remaining should it need to be reprocessed.
7. The final step before sending samples off for IRMS analysis involves transferring the dissolved NH_4^+ onto an acidified filter "trap" so that it can be analyzed as a solid. The alkaline headspace diffusion method increases the pH of the sample water with the addition of magnesium oxide (MgO), thereby converting the dissolved NH_4^+ into gaseous ammonia, which can be trapped onto an acidified filter either floating on or suspended above the water. The dried filter containing trapped ammonia is packed into combustion capsules for analysis on an IRMS interfaced with an elemental analyzer (EA). Diffusion vessels require a vessel with a tight-fitting lid, which can withstand some pressure buildup without gas escape.
   a. Record volumes and concentrations of samples to determine the efficiencies of the conversion and trapping processes. Also use a "standard" of NO_3^- solution at known concentration, similar to the concentration of the samples (to calculate conversion efficiency), blanks (deionized water), and controls (deionized water with reagents to check for reagent contamination).
   b. Add a filter packet to each diffusion bottle. Filter packets are made from two pieces of 2.54-cm Teflon tape with an acidified (25 μL of 2.5 M $KHSO_4$) Whatman GF/D GFF (1 cm diameter) sandwiched between the tape. The tape covers are then pressed together where they overlap to make a seal using an item with a diameter slightly larger than 1 cm (e.g., a scintillation vial). A weak seal can allow the alkaline solution to contact the filter during the diffusion process, thereby neutralizing the acid and compromising the sample (Fillery and Recous, 2001). Alternatively,

filter packets can be suspended above the water in the headspace by a small hook glued to the inside lid of the bottle. This requires more preparation (e.g., building the diffusion hooks), and care should be taken to not use materials that off-gas NH_4^+, as may be the case with some plastics or glue.

c. After the addition of a filter packet, add 50 g of NaCl and 3 g of MgO/L of sample directly into the diffusion vessel, sealing the vessel immediately. Both salts should be precombusted (450−500°C). Shake for 2 weeks at 40°C or 3 weeks at room temperature to ensure the complete trapping of ammonia on the filter.

d. Recovery is checked using standards of an appropriate concentration to match the samples. Also, to correct for chemical contaminants, include reagent blanks containing only the NaCl and MgO and, if appropriate, Devarda's alloy.

e. The measurement of $^{15}NO_3^-$ is a slight variation on the above diffusion method. The only difference is that the NH_4^+ is first driven off and the remaining $^{15}NO_3^-$ is reduced to NH_4^+ with Devarda's alloy.

i. Calculate sample volume based on desired mass, as in step 6a above.

ii. Add 5 g of NaCl and 3 g of MgO to the sample, boil briefly with a stir bar until volume is ~100 mL. Heating and stirring will speed the conversion of NH_4^+ to NH_3 (gas) and drive it out of the water phase.

iii. Once the NH_4^+ conversion is complete, only NO_3^- remains in the sample. Add 0.5 g of Devarda's alloy to the sample, along with another 0.5 g of NaCl and 0.5 g of MgO. Immediately place a filter packet in the bottle and seal it. Cap the bottle tightly.

iv. Place bottles at 60°C for 48 h. Remove from oven and shake for 7 days to facilitate full transfer of NH_4^+ (from the reduced NO_3^-) onto the filter packet. Remove filter and process for IRMS analysis.

f. The methods summarized here were used by the Lotic Intersite Nitrogen eXperiment (LINX) group. The LINX protocol is more detailed and is available for download at http://andrewsforest.oregonstate.edu/data/abstract.cfm?dbcode=AN006. Several variants of this method are published and none has clearly emerged as superior (e.g., Holmes et al., 1998; Herman et al., 1995; Diaconu et al., 2005). New methods to measure $^{15}NH_4^+$ continue to be proposed (e.g., Gardner et al., 1995; Yin et al., 2014) and may eventually replace the diffusion method.

8. Once filters are dried (in a desiccator for ~3−4 days), pack filters into tins suitable for elemental analysis. Place into a 96-well plate or similar tray and make a label key.
9. Send samples to an IRMS lab. Include sample, standards, controls, and blanks.

Calculating DNRA Rates

1. Convert isotope ratios into mole fractions (MF)

a. The data file you receive from the contract lab should contain the delta value ($\delta^{15}N$, expressed as ‰) and the mass of sample (μg N).

$$^{15}\text{N MF} = \frac{[((\delta^{15}\text{N}/1000)+1)\cdot 0.0036765]}{[1 + ((\delta^{15}\text{N}/1000)+1)\cdot 0.0036765]} \quad (32.12)$$

This conversion will result in a very small number. For 0 per mil (0‰) enrichment, the calculation should result in a ^{15}N MF = 0.003663.

2. Multiple MF by N pool sizes

a. As an example, we have a sample at 50‰ enrichment, which converts to an MF = 0.003845. We measured the N concentration in this sample as 200 μg NH_4^+/L or 156 μg N/L, which converts to 11.14 μmol N/L. Therefore, 11.14 μmol N/L * 0.003845 = 0.04 μmol ^{15}N/L. This can be multiplied by the appropriate volume to convert from concentration to mass of ^{15}N in the assay bottle.

3. Convert to a rate (flux)

a. Divide by the incubation time to convert the mass to a rate. The final units will be in μmol ^{15}N/h.

4. More complete calculations are available in the LINX2 protocols, referenced above. These describe how to correct for the reagent blanks and for any addition of nitrate to the sample, as may be needed to reach the detection threshold of the IRMS (known as "spikes").

Tips and Notes

1. A time series of incubation is suggested for early runs in a particular system. This will allow for determination of the minimum incubation time to detect activity as well as an incubation length where rates remain linear.
2. Practice making diffusion filter packets prior to use. These should be inspected well with a light from behind to ensure there are no holes but also that there is a good seal (Teflon tape pressured together is clear).

32.3.5 Advanced Method 2: Using MIMS to Measure Net N_2 Flux

Net N_2 flux is a balance between producing processes (denitrification) and consuming processes (N fixation). Net N_2 flux can be quantified by measuring N_2/Ar ratios in natural environments (Laursen and Seitzinger, 2002; McCutchan et al., 2003) or in experimental enclosures (e.g., assay bottles) using MIMS. MIMS allows for simple, rapid, and selective analysis of dissolved air gas concentrations dissolved into water samples. MIMS can also be paired with ^{15}N enrichment to more specifically measure N cycling processes, including denitrification, particularly when paired with the isotope pairing technique (e.g., Risgaard-Petersen et al., 2003). For simplicity, we will focus on the easier-to-measure net N_2 flux in a bottle assay. Since the goal is to measure potentially small changes in N_2 concentration against a *very* large background pool of atmospheric N_2 (78%), contamination is a major obstacle and concern with any MIMS method. Excessive caution in guarding against any air entry, once an experiment is started, is the surest way to produce useable results. Remember—air exposure or bubbles are your worst enemy! Complete the incubations and transfer steps underwater, as is possible, to help ensure that samples are not compromised by contact with the atmosphere.

32.3.5.1 Assay Procedure

1. Set up bottle assays as described for other protocols in this chapter, noting the mass of substratum and volume of water added to each bottle. Fill each bottle to full capacity—eliminate any air bubbles in the bottles. You also need a set of "blank" or "control" bottles that only contain water and do not have any substrata.
   a. As with all bottle assays, the timing of this assay is highly dependent on the expected rate of the process; if you expect very "fast" net N_2 flux, you will incubate these for a shorter period of time.
   b. If you want to speed up the reaction or are more interested in "potential" rates as opposed to near-ambient rates, the addition of NO_3^- to the assay bottles will likely enhance N_2 fluxes by increased denitrification.
   c. If you are not going to artificially stimulate N_2 fluxes, we highly recommend running some pilot tests prior to your full-scale experiment to determine target incubation times and rates.
2. Sampling times: Samples are collected before and after the assay.
   a. Collect $t = 0$ sample (beginning sample) from your control and experimental assay bottles. Ideally, since these are starting from the same source water, they should be identical in gas composition. Incubate bottles on a shaker table for the predetermined time.
   b. Collect your $t =$ final samples from the control and experimental assay bottles. Record the temperature of the water bath at the end of the experiment.
3. Sample collection: MIMS samples are typically collected into 12-mL Exetainer vials. When filling the vials, use a small diameter piece of Tygon (or Viton) tubing to extend down to the bottom of the Exetainer, allowing you to fill the Exetainer from the bottom up.
   a. This action minimizes entrainment of atmospheric gasses (potential source of contamination), which can disrupt the dissolved gas signatures.
   b. Overflow the tube ~3×, and slowly lift the tubing out of the vial taking care to replace the volume of the tubing with water so there is no headspace in the vial.
   c. Once the tubing is out, there should be a semicircle of water over the rim of the vial. Additional detail and pictures are available in Burgin et al. (2013).
4. Sample preservation: Preserve sample with 200 µL of a 50% zinc chloride ($ZnCl_2$) solution (50% W/V; dissolve 50 g in 100 mL deionized water). Add the aliquot of $ZnCl_2$ to the Exetainer placing the tip of the pipette under the water surface. Quickly cap the Exetainer and flip the vial upside down to mix the heavy $ZnCl_2$ solution into the water. You should also check for bubbles that may have formed around the cap seal. Any samples with bubbles should be discarded and resampled.
   a. Caution: $ZnCl_2$ is corrosive and acutely toxic, so it should be handled with extreme caution and wearing appropriate personal protective equipment! The reaction dissolving zinc chloride in H_2O is exothermic, so beware of heating in containers. $ZnCl_2$ is also harmful to aquatic environments so be extra cautious when using it in the field; waste bottles should not be discarded in the field. All needles used during the procedures should be disposed off following standard sharps disposal procedures.
5. Sample storage: Once samples are collected, keep them as cool as possible, preferably in a refrigerator at 4°C. Keeping gases cooler than the temperature at which they are collected helps to keep the gases in solution. If the samples are allowed to warm up, significant bubble formation can occur, resulting from degassing. As an added protection, if samples are to be shipped or will be stored for long periods, it is best to keep the samples underwater. Keeping samples underwater

limits extreme temperature fluctuations and also limits any potential evaporation that can occur near the seal of the cap. In general, ~40–50 samples can be placed into a 1-L Nalgene bottle, or similar container. If samples are placed in water, sample IDs should be written with a thick Sharpie and covered over with Parafilm so that the sample ID is not rubbed off in transit. Alternatively, white electrical tape works well in place of standard label tape and will stay on underwater.

   a. Place all bottles in a large water-filled container (e.g., plastic box), ensuring that the bottles are completely submerged, particularly at areas where gas might leak (e.g., seals or lids). Place a lid on the box to keep water from splashing out. Measure the temperature of the water bath; ideally, keep a recording thermometer in the water bath for the duration of the experiment. If that is not available, record the temperature whenever samples are collected. Temperature is a key driver of gas solubility and is necessary for determining the dissolved N_2 and Ar.

6. Experimental design: As described, this design employs a simple two-point rate calculation ($t = 0$ and $t =$ final). More complicated and likely more accurate rates can be measured by (1) collecting multiple samples from the same assay bottle over time or (2) destructively harvesting replicate assay bottles at different time points in an experiment.
   a. If the first approach is used, an assay bottle with a septum needs to be employed so that water can be injected into the assay bottle to remove a sample for MIMS analysis. The gas signature of the water injected into the ongoing assay should also be accounted for in subsequent calculations.
   b. If the second approach is used, you should take care that the assay bottles are all as homogeneous as possible and incubations are started at the exact same time, as minor differences between bottles can cause complications for the eventual rate calculations.

32.3.5.2 Running MIMS Samples

1. Each MIMS is uniquely built from different components. If running your samples in-house, follow the instructions specific to your instrument.
2. If sending samples to another lab for analysis, use the following precautions:
   a. Keep Exetainers underwater to decrease risk of contamination.
   b. Keep Exetainers and water bath at a cooler temperature than at which they were collected to decrease risk of degassing due to warming. It is best to add multiple ice packs to the cooler and ship the samples overnight.

32.3.5.3 Calculations (Adapted From Kana et al., 1998)

1. Calculate N_2 and Ar concentrations:

$$N_2 \text{ concentration} = \frac{\text{MIMS signal}_{\text{sample}}}{\text{MIMS signal}_{\text{standard}}} \cdot \text{solubility of } N_2 \tag{32.13}$$

   where "MIMS signal" = mass signal output from the mass spectrometer for the standard water and the solubility of N_2 is for the temperature, salinity, and barometric pressure of the standard, determined from the solubility tables, such as those in Colt (2012). The same formula is used for Ar.
2. Calculate the N_2 flux from the N_2:Ar:

$$N_2 \text{ flux} = (N_2\text{: Ar } T_0 - N_2\text{: Ar } T_f) \cdot \text{Ar}_{\text{pred}} \tag{32.14}$$

   where T_0 = starting time point, T_f = final time point, and Ar_{pred} = predicted Ar concentration at saturation, which can be found in a solubility table, as in Colt (2012).
3. Calculate Net N_2 flux:

$$\text{Flux}\left(\mu\text{mol/area}^2 \text{ time}\right) = (N_2 \text{ flux} \cdot \text{water volume})/(\text{surface area} \cdot \text{incubation time}) \tag{32.15}$$

   Units should be specific to how you measure surface area and time for the incubation. Net fluxes can be either positive (denitrification driven) or negative (N fixation driven), but cannot tell you which process is dominant or larger than the other.

32.4 QUESTIONS

32.4.1 Denitrification

1. Compare the unamended and the potential rates of denitrification. Did they differ? If so, why?

2. How do the denitrification rates you measured compare to those in other streams and rivers using similar methods? For comparison, see rates in Martin et al. (2001), Richardson et al. (2004), and Findlay et al. (2011).
3. What other factors besides availability of nitrate and organic carbon might affect denitrification rates?
4. Denitrification is often considered a beneficial process in relation to coastal eutrophication. Why would that be true?
5. In some systems, high rates of denitrification might decrease overall N:P ratios. Why might this be a concern in some ecosystems?

32.4.2 Nitrification

6. How do the nitrification rates you measured compare to those in other streams and rivers measured using similar methods? For comparison, see rates in Kemp and Dodds (2002), Strauss et al. (2002, 2004), and Starry et al. (2005).
7. The incubation period for nitrification is much longer than that for denitrification. Why is this so? What can be inferred about the biology of nitrifying bacteria based on the long incubation time?
8. We provided methods for measuring net and gross nitrification. Why would you want to measure one or the other process?
9. How can nitrification (NO_3^- production) limit denitrification when one process is aerobic and the other is anaerobic? In other words, how can both processes be occurring in the same place?

32.4.3 Nitrogen Fixation

10. What was the N_2 fixation rate that you measured for your study reach?
11. Which taxa do you think were responsible for the nitrogen fixation in your reach?
12. How do the N_2 fixation rates you measured compare to those in other streams? How about those measured in other ecosystems? For comparison, see tables in Grimm and Petrone (1997) and Marcarelli et al. (2008).
13. How might changing the nutrient or light availability alter rates of N_2 fixation? What other environmental factors could be important for controlling rates of this process?
14. Why are isotopic methods necessary when there are small pools of the measured compound that turn over quickly?
15. Why do ^{15}N methods make it easier to detect rates of denitrification when there is so much N_2 in the atmosphere and dissolved in most streams?

32.4.4 DNRA and N_2 Flux

16. How do the DNRA rates you measured compare to rates measured in other studies? See Nogaro and Burgin (2014) or Washbourne et al. (2011) for comparison.
17. What are the processes that contribute to net N_2 fluxes, as measured using the MIMS method described herein? How can you tell which process is dominant in your system?
18. How do the net N_2 fluxes you measured compare to other studies that do not use isotopic enrichment (e.g., Grantz et al., 2012; Deemer et al., 2011)? How do the net N_2 fluxes compare to studies that measure denitrification using ^{15}N tracers (e.g., Mulholland et al., 2008)?

32.5 MATERIALS AND SUPPLIES

32.5.1 Denitrification

Field Materials

- Sediment corer and utensils
- Plastic bags for sediment
- Plastic bottles for stream water
- Thermometer
- Cooler with ice
- Labeling tape and permanent marker
- Data sheet, clipboard, pencil(s)

Lab Materials

- Data sheet

Incubation vessels with septa (see details in General Preparation)
Labeling tape and permanent marker
Plastic powder funnel
Adjustable pipettes and tips
Graduated cylinders
Disposable syringe (5 mL)
Syringe needles (c. 22 gauge)
Vacuum pump
Tubing
Orbital shaker
Glass serum bottles (2 mL) with butyl rubber septa and aluminum seals
Crimper
Gases (see details in General Preparations)
Volumetric flasks
Potassium nitrate (KNO_3)
Glucose ($C_6H_{12}O_6$)
Chloramphenicol (Sigma−Aldrich CAS # 56-75-7)

32.5.2 Nitrification

Field Materials
Sediment corer and utensils
Plastic bags for sediment
Plastic bottles for stream water
Thermometer
Cooler with ice
Labeling tape and permanent marker
Data sheet, clipboard, pencil(s)
Lab Materials
Data sheet
Labeling tape and permanent marker
Glass Erlenmeyer flasks (125 mL)
Adjustable pipettes and tips
Graduated cylinders
Orbital shaker
Plastic centrifuge tube (15 mL)
Centrifuge
Parafilm
Aluminum foil
Syringe filter apparatus
Glass fiber filters (e.g., Pall Type A/E or Whatman GF/C or GF/F)
Glass scintillation vial (20 mL)
Volumetric flasks
Potassium chloride (KCl)
Dimethyl sulfoxide (DMSO)
Nitrapyrin (2-chloro-6-[trichloromethyl]-pyridine; Sigma−Aldrich CAS # 1929-82-4)
Sulfuric acid (H_2SO_4)
Sodium hydroxide (NaOH)

32.5.3 Nitrogen Fixation

Data sheet and clipboard, pencil(s)
Chambers and septa for injection port(s)
Balloons or syringes as needed to introduce acetylene headspace

Acetylene gas—either in a compressed air tank or generated from calcium carbide
Syringes and needles for collecting gas samples
Thermometer
Gas storage vials or Exetainers (two per chamber)
Large (2 L minimum) graduated cylinder for measuring volume of water and substratum
Rite-in-the-rain paper for tracing surface areas of substrata
Other assorted field materials—sharpies, label tape, etc.

32.5.4 Dissimilatory Nitrate Reduction to Ammonia

Field Materials
Sediment corer and utensils
Plastic bags for sediment
Plastic bottles for stream water
Thermometer
Cooler with ice
Labeling tape and permanent marker
Data sheet, clipboard, pencil(s)
Lab Materials
Data sheet or lab notebook
Labeling tape and permanent marker
Wheaton or Nalgene bottles for assay
$^{15}NO_3^-$ solution
Adjustable pipettes and tips
Syringes (various sizes)
Orbital shaker
Plastic centrifuge tube (15 or 50 mL)
Centrifuge
Diffusion bottles
Acid trap filter packs
Glass scintillation vials (20 mL)
Potassium chloride (KCl)
Sodium chloride (NaCl)
Magnesium oxide (MgO)
Sulfuric acid (H_2SO_4)
EA tins and 96-well plate for organizing/storing samples

32.5.5 N_2 Flux

Field Materials
Sediment corer and utensils
Plastic bags for sediment
Plastic bottles for stream water
Thermometer
Cooler with ice
Labeling tape and permanent marker
Data sheet, clipboard, pencil(s)
Lab Materials
Data sheet or lab notebook
Labeling tape and permanent marker
Wheaton or Nalgene bottles for assay
Exetainers
Tygon or Viton tubing for sample transfer between assay bottle and Exetainer
Luer-lok syringes (various sizes)

Luer-lok connectors for creating tubing transfer component
Zinc chloride (as sample preservative)
Pipette and tips
Gloves and appropriate waste disposal
Electrical tape or Parafilm for preserving sample labels
Storage containers with good watertight seals (Lock & Lock is a good brand)

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