CHAPTER EIGHT

Measuring Nitrification, Denitrification, and Related Biomarkers in Terrestrial Geothermal Ecosystems

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Abstract

Research on the nitrogen biogeochemical cycle in terrestrial geothermal ecosystems has recently been energized by the discovery of thermophilic ammonia-oxidizing archaea (AOA). This chapter describes methods that have been used for measuring nitrification and denitrification in hot spring environments, including isotope pool dilution and tracer approaches, and the acetylene block approach. The chapter also summarizes qualitative and quantitative methods for measurement of functional and phylogenetic biomarkers of thermophiles potentially involved in these processes.

1. Introduction

Our knowledge of the nitrogen cycle (N-cycle) has changed radically in recent years due to major discoveries, including archaeal ammonia (NH_3) oxidation at low and high temperatures (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008; Könneke *et al.*, 2005), archaeal N_2 fixation at high temperature (Mehta and Baross, 2006), anaerobic ammonium oxidation (anammox) at low and high temperatures (Jaeschke *et al.*, 2009; Strous *et al.*, 1999), and eukaryotic nitrate (NO_3^-) respiration (Risgaard-Petersen *et al.*, 2006). Until a recently, very little was known about the N-cycle at high temperature to the extent that processes such as NH_3 oxidation, nitrite (NO_2^-) oxidation, and anammox had never been addressed. Through a combination of microbial cultivation approaches, process rate measurements, and studies of phylogenetic and functional biomarkers, our knowledge of the N-cycle in terrestrial geothermal habitats is rapidly growing. Table 8.1 summarizes some of the evidence for N-cycle processes and relevant phylogenetic groups at high temperature.

Nitrification and denitrification are two important processes in the N-cycle. Nitrification is the aerobic oxidation of NH_3 to NO_3^- through a NO_2^- intermediate:

$$NH_{3(aq)} \rightarrow NO^-_{2(aq)} \rightarrow NO^-_{3(aq)}$$

No known organism can catalyze both steps of the reaction so it can be valuable to consider these two steps, and the organisms that catalyze them, separately.

The net reaction for NH_3 oxidation to NO_2^- is as follows:

$$NH_{3(aq)} + 1.5O_{2(aq)} \rightarrow NO^-_{2(aq)} + H_2O + H^+_{(aq)}(6e^-)\Delta G'_0 = -235\,\text{kJ/mol}$$

At moderate temperature, both ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) play a role in NH_3 oxidation to NO_2^-. A variety of evidence suggests that AOA are more abundant in many

Table 8.1 Summary of evidence for N-cycle processes at high temperatures, including *in situ* process rate measurements, laboratory cultures, and recovery of possible phylogenetic or functional biomarkers

Process/max. temp. (°C)	Environment	Evidence	References
N_2 fixation			
89/85	Terrestrial, YNP	PCR amplification of putative *nifH* genes; acetylene reduction	Hamilton *et al.* (submitted)
92	Marine	Pure culture, *Methanocaldococcus* sp. FS406-22	Mehta and Baross (2006)
NH_3 oxidation			
85	Terrestrial, Iceland, Great Basin	$^{15}NO_3^-$ pool dilution	Reigstad *et al.* (2008), Dodsworth *et al.* (unpublished data)
74/46	Terrestrial, YNP	Highly enriched cultures, "*Candidatus* Nitrosocaldus yellowstonii" and "*Candidatus* Nitrososphaera gargensis"	de la Torre *et al.* (2008), Hatzenpichler *et al.* (2008)
94	Terrestrial, Great Basin, Iceland	Recovery of crenarchaeol (putative biomarker of AOA)	Pearson *et al.* (2004, 2008), Reigstad *et al.* (2008)
94	Terrestrial, China, Great Basin	RT-PCR amplification of putative archaeal *amoA* transcripts	Jiang *et al.* (2010)
97	Terrestrial, "global"	PCR amplification of putative archaeal *amoA* genes	de la Torre *et al.* (2008), Reigstad *et al.* (2008), Zhang *et al.* (2008)
NO_2^- oxidation			
85	Terrestrial	$^{15}NO_3^-$ pool dilution	Reigstad *et al.* (2008)
60	Terrestrial	Activity in enrichment cultures	Lebedeva *et al.* (2005)
48	Terrestrial	Probable pure culture, "*Candidatus* Nitrospira bockiana"	Lebedeva *et al.* (2008)
57/69	Terrestrial	PCR amplification of *Nitrospira* 16S rRNA genes and *norB*	Kanokratana *et al.* (2004), Hirayama *et al.* (2005)

(continued)

Table 8.1 (*continued*)

Process/max. temp. (°C)	Environment	Evidence	References
Anammox			
85	Marine	Isotope pairing	Byrne *et al.* (2009)
43	Wastewater	Highly enriched culture	Strous *et al.* (1999)
65/52	Terrestrial	Ladderane lipids (putative biomarker); 16S rRNA genes	Jaeschke *et al.* (2009)
Nitrate reduction			
85	Terrestrial	$^{15}NO_3^-$ tracer; acetylene block; qPCR of *narG*	Dodsworth *et al.* (unpublished data)
113	Marine	Pure culture, *Pyrolobus fumarii* (NH_3 dominant product)	Blöchl *et al.* (1997)
100	Terrestrial	Pure culture, *Pyrobaculum aerophilum* (N_2O dominant product; capable of N_2 production)	Völkl *et al.* (1993)

environments (Francis *et al.*, 2005; He *et al.*, 2007; Leininger *et al.*, 2006; Nicol *et al.*, 2008; Shen *et al.*, 2008), except those impacted by high doses of anthropogenic N such as wastewater (Wells *et al.*, 2009). These results may be explained by the competitive advantage of AOA under substrate-limited conditions because AOA have a much higher affinity for NH_3 than AOB (Martens-Habbena *et al.*, 2009). Very little evidence suggests AOB are important at temperatures above 40–50 °C, although this observation needs further verification. Lebedeva *et al.* (2005) reported isolation of AOB from Garga Hot Spring in the Baikal Rift Zone that could grow up to 50 °C, and the two isolates were identified immunochemically as presumptive *Nitrosospira* and *Nitrosomonas*. In addition, *Nitrosomonas amoA* genes have been quantified in a gold mine at temperatures up to 62 °C (Hirayama *et al.*, 2005). Yet, no thermophilic isolates or enrichments capable of ammonia oxidation have been identified definitively as *Bacteria* (e.g., by 16S rRNA gene analysis). In contrast, highly enriched cultures of AOA from Garga Hot Spring and Heart Lake Hot Spring in Yellowstone National Park mediate ammonia oxidation at temperatures up to 46 and 74 °C, respectively (de la Torre *et al.*, 2008; Hatzenpichler *et al.*, 2008). Similarly, homologs of the archaeal ammonia monooxygenase alpha subunit gene, *amoA*, and the biphytanyl lipid crenarchaeol, both tentatively regarded as distinctive biomarkers of AOA, have been described at temperatures up to 97 and 87 °C, respectively (Pearson *et al.*, 2008; Reigstad *et al.*, 2008). However, both of these biomarkers are more reliably found in hot springs below 75 °C, and crenarchaeol was shown to be present at the highest concentrations relative to other archaeal lipids at around 40 °C in hot springs in the US Great Basin (Zhang *et al.*, 2006). Interestingly, crenarchaeol is much more abundant relative to other biphitanyl lipids in Great Basin hot springs, as compared with a variety of Yellowstone National Park hot springs, possibly suggesting a more important role for AOA in Great Basin geothermal ecosystems (Pearson *et al.*, 2008). Recently, two studies recovered *amoA* transcripts from hot spring sediments ranging from 44.5 to 94 °C in several springs in Tengchong, China, and the Great Basin, suggesting that AOA may be active in terrestrial springs at temperatures near boiling (Jiang *et al.*, 2010; Zhang *et al.*, 2008).

In the second step of nitrification, NO_2^- is oxidized to NO_3^-, a process that may also be important in high-temperature environments. The net equation for NO_2^- oxidation to NO_3^- is as follows:

$$NO^-_{2(aq)} + 0.5O_{2(aq)} \rightarrow NO^-_{3(aq)}(2e^-)\Delta G'_0 = -54\,\mathrm{kJ/mol}$$

In moderate-temperature environments, a variety of NO_2^--oxidizing bacteria (NOB) catalyze the oxidation of NO_2^- to NO_3^-, including members of the phylum *Nitrospira* and three classes of *Proteobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria*. From an ecological perspective, NO_2^- oxidation has received far less attention than NH_3 oxidation because it is generally contended that the latter is rate limiting in nature. However, this is doubtful at high temperature because some hot springs sourced with NH_3 as the dominant form of inorganic N accumulate high concentrations of NO_2^- (Costa *et al*., 2009) and AOA grow at much higher temperatures than known NOB. Few investigations have focused on thermophilic nitrite oxidation, though several lines of evidence implicate *Nitrospira* as the dominant nitrifier at moderately elevated temperature. Among cultures of *Nitrospira*, *N. moscovensis* and "*Candidatus* N. bockiana" are the most thermophilic known, with growth temperature optima (T_{opt}) of 39 and 42 °C, respectively, with the latter capable of NO_2^- oxidation and growth up to 48 °C (Ehrich *et al*., 1995; Lebedeva *et al*., 2008). However, nitrifying enrichments supporting *Nitrospira* have been reported up to 60 °C (Lebedeva *et al*., 2005) and *Nitrospira* 16S rRNA genes have been recovered from spring ecosystems from 50 to 57 °C (Kanokratana *et al*., 2004). In addition, nitrite oxidoreductase genes (*norB*) related to those from *Nitrospira* species have been amplified and quantified in Japanese gold mine samples up to 69 °C (Hirayama *et al*., 2005).

Very few measurements of rates of oxidative N-cycle processes have been done in geothermal environments. Reigstad *et al*. (2008) measured gross nitrification at 84 and 85 °C in two acidic Icelandic hot springs with high dissolved clay content using the $^{15}NO_3^-$ pool dilution technique, which yielded rates of 2.8–7.0 nmol NO_3^- N g^{-1} h^{-1} (data converted from volume using reported density). NO_3^- production was stimulated more than twofold by addition of NH_4^+ before incubation, showing that nitrification in these springs was limited by NH_3 supply. The authors have also used the $^{15}NO_3^-$ pool dilution technique to measure gross nitrification at 79–81 °C in two hot springs in the US Great Basin as described in Section 3, where rates of NO_3^- production varied from 0.5 to ~50 nmol NO_3^- N g sediment^{-1} h^{-1} (Dodsworth *et al*., unpublished data).

Denitrification is the stepwise reduction of NO_3^- to the gaseous products nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen (N_2):

$$NO^-_{3(aq)} \rightarrow NO^-_{2(aq)} \rightarrow NO_{(g)} \rightarrow N_2O_{(g)} \rightarrow N_{2(g)}$$

Denitrification is a respiratory process in which nitrogen oxides serve as electron acceptors and is contrasted with assimilatory NO_3^- reduction in that the former is coupled to energy conservation and growth, whereas the latter serves only to scavenge nitrogen for biosynthesis. A variety of thermophiles and hyperthermophiles from both terrestrial and marine geothermal habitats can respire NO_3^-, although very few studies have focused on this. Among archaea, *Pyrobaculum aerophilum*, *Ferroglobus placidus*, and *Pyrolobus fumarii* have been definitively shown to respire NO_3^- up to temperatures of 80, 95, and 113 °C, respectively, although a number of other thermophiles contain gene homologs for NO_3^- reduction pathways, including the euryarchaeon *Archaeoglobus fulgidus* (Cabello *et al.*, 2004). *P. aerophilum* uses a novel NO_3^- reductase and is capable of complete denitrification to N_2, although N_2O accumulates during growth due to a kinetically inhibited N_2O reductase (Afshar *et al.*, 1998; Cabello *et al.*, 2004; Völkl *et al.*, 1993). *F. placidus* produces NO_2^- and some NO as products during growth (Hafenbradl *et al.*, 1996); however, *in vitro* experiments with cell extracts documented N_2O production (Vorholt *et al.*, 1997). *P. fumarii* stoichiometrically reduces NO_3^- to NH_3 during growth on H_2 (Blöchl *et al.*, 1997). Among thermophilic and hyperthermophilic bacteria, NO_3^- reduction is widespread in the Thermaceae, including several species of the terrestrial genera *Thermus* and *Meiothermus* and the marine genera *Oceanithermus* (Miroshnichenko *et al.*, 2003b) and *Vulcanithermus* (Miroshnichenko *et al.*, 2003c). *Thermus* includes strains described to reduce NO_3^- fully to N_2 (Cava *et al.*, 2008) as well as strains only capable of NO_2^- production (Ramirez-Arcos *et al.*, 1998). *Meiothermus*, *Oceanithermus*, and *Vulcanithermus* are not known to be capable of reduction of NO_3^- past NO_2^-. A variety of *Geobacillus* denitrify completely to N_2 (Mishima *et al.*, 2009). In the *Aquificales*, *Aquifex pyrophilus* and *Persephonella* spp. denitrify to N_2 (Gotz *et al.*, 2002) and *Thermovibrio ruber* (Huber *et al.*, 2002) reduces NO_3^- to NH_3. The novel bacterium *Caldithrix abysii* also reduces NO_3^- to NH_3 (Miroshnichenko *et al.*, 2003a).

Although many NO_3^--respiring thermophiles exist in culture, very few studies have addressed NO_3^- reduction or denitrification in natural geothermal habitats. Burr *et al.* (2005) measured denitrification by the acetylene block approach, along with N_2 fixation, ammonification, and nitrification, in hot acidic soils at 50, 65, and 80 °C in Yellowstone National Park. N_2O production ranged from 0.34 to 1.1 nmol N_2O N g^{-1} h^{-1} with maximal activity at 65 °C. Activity was dependent on NO_3^- addition and acetylene did not significantly enhance N_2O flux, suggesting that denitrification was NO_3^--limited and that N_2O, rather than N_2, was the major denitrification product. The authors have used the acetylene block method and a ^{15}N–NO_3^- tracer method to measure denitrification rates in two hot springs in the US Great Basin, as described in Section 4 (Dodsworth *et al.*, unpublished data).

2. General Considerations for Measurement of N-Cycle Activities in Terrestrial Geothermal Habitats

The following sections describe experimental details of approaches as they have been applied to study N-cycle activities in terrestrial geothermal ecosystems. There are many variations on these general themes and the reader is advised to consult other papers before selecting the strategies that are most appropriate for their experimental system, hypotheses, and available resources (Mosier and Klemedtsson, 1994; Steingruber *et al.*, 2001; Tiedje *et al.*, 1989; Ward and O'Mullan, 2005). Regardless of the approach, the sampling strategy must be carefully conceived. Nitrification and denitrification in soils and sediments are notoriously heterogeneous because of the natural patchiness of resources that affect N-cycling such as soil moisture content, quality of substrates for biofilm formation, organic content, and the availability of N substrates (Tiedje *et al.*, 1989). Denitrification in soils is also incredibly temporally variable, correlating strongly with events that lead soils to be water saturated, such as spring thaws and rain (Tiedje *et al.*, 1989). Spatial heterogeneity is generally addressed by the soil microbiology community by using a highly replicated experimental design to distinguish within- and between-system rates (e.g., 20 replicates per sample; Mosier and Klemedtsson, 1994). However, we find this degree of replication impractical at most geothermal sites due to the relatively small sizes of geothermal features and the strict protection of springs in protected areas such as national parks. Hot spring ecosystems vary widely in terms of their basic hydrology and geochemistry. Although few, if any, springs have been investigated in detail to address spatial heterogeneity, heterogeneity is evident in many springs by the patchiness of conspicuous microbial growth and mineral precipitates. Thus, as in soils research, heterogeneity must be carefully considered. Spring size, shape, and substrate mineralogy are also important practical considerations.

Care should be taken to select the sample site and incubation site. Sample sites should have enough accessible sediment cover within reach of gloved hands to achieve the replication necessary for the experimental design. The incubation site should be of the same temperature as the sampling site and it should be adjacent to the sampling site, but in a place that does not impact the sampling site, for example, immediately downflow from the sampling site. The incubation site should be deep enough so that the liquid phase of the incubation tubes and bottles are completely submerged when sitting in racks. Alternatively, racks can be secured with wire and be suspended in the spring water. For incubation of samples, we commonly use 160 mL serum bottles (#223748; Wheaton, Millvillen, NJ, USA) or Balch tubes (Bellco 2048-00150) sealed with butyl rubber stoppers (Bellco 2048-18150) and aluminum seals (Bellco 2048-11020). Plastic test tube racks (e.g., Bel-Art 187450001) can be cut so that serum bottles fit tightly into the rack.

Regulations, environmental stewardship, and safety are also important concerns for hot spring research. Terrestrial hot springs are rare resources and many are protected in public lands, such as Yellowstone National Park. The relevant regulations must be carefully considered into the experimental design and researchers are urged to follow guidelines of minimum impact research, regardless of the protective status of the research site (Spear, 2005).

Conducting research in active geothermal areas is intrinsically hazardous, due the extreme temperatures, dangerous gasses, and the potential instability and volatility of geothermal features (Whittlesey, 1995). Before any experiments are conducted, the research area should be thoroughly checked for potential hazards, including small or inconspicuous geothermal expressions that should be avoided. It is wise for researchers to minimize time spent standing or walking in close proximity (2–3 m) to springs. In addition, close attention should be paid to any changes in the activity of geothermal features, including changes in flow rate, water level, or outgassing, that could indicate an imminent "eruption" or discharge. Care should also be taken to avoid asphyxiation when working near hot springs, particularly in hot springs or fumaroles in depressions and on calm days when dense gasses (e.g., CO_2) can accumulate (Cantrell and Young, 2009; Whittlesey, 1995). In general, it is advised for the research group to make plans for dealing with potential hazards before reaching the study site, to review those plans at the research site, and to carry appropriate first aid gear in case of an emergency. Field research in geothermal areas should never be done alone.

Working with hot spring sediment and water samples under the safest of circumstances can put researchers at risk for burns. For all of the experiments described below, we find that long cuff PVC-coated gloves (e.g., Grease Monkey™, Big Time Products, Rome, GA) work well for brief manipulations at temperatures to at least 85 °C. We suggest considering sturdy rubber boots to prevent burns if researchers break through shallow crusts at hot spring margins or if the site is wet. Researchers should be aware that hot water trapped against the skin is extremely dangerous and be prepared to remove gloves, boots, socks, and other clothing without hesitation if it gets wet.

3. Methods for Measuring Nitrification in Terrestrial Geothermal Habitats

The ^{15}N–NO_3^- pool dilution technique can be used to estimate rates of gross nitrification in water and sediment samples from geothermal environments. This method involves the addition of a small amount (typically 5–10% of the total pool size) of highly enriched ^{15}N–NO_3^- to samples and monitoring the atom% ^{15}N in the NO_3^- pool over time. Flux into the NO_3^- pool by transformation of nitrogen species present in the sample at natural isotopic abundance (~0.37 atom% ^{15}N) decreases the relative ^{15}N content of the pool (thus resulting in a "pool dilution"), whereas it is assumed that processes involving consumption of NO_3^- have no effect on the isotope ratio of the NO_3^- pool. While this assumption is not strictly true, it is probably valid for short incubations and where labeled ^{15}N–NO_3^-

has been added to sufficient excess (Barraclough, 1991; Davidson *et al.*, 1991). Knowledge of the change in atom% $^{15}N–NO_3^-$ over time and the initial and final size of the NO_3^- pool allow calculation of the rate of gross nitrification, that is, production of NO_3^- (Barraclough, 1991). One advantage of this technique is that the samples are amended with only a small amount of the labeled compound; thus, *in situ* concentrations are only minimally affected.

Below we describe the preparation and field work necessary to implement the $^{15}N–NO_3^-$ pool dilution technique. We also describe the analysis of processed field samples for measurement of $NO_3^- + NO_2^-$ concentration (abbreviated as NO_x), the determination of atom% $^{15}N–NO_x$ by GC–MS, and calculation of gross nitrification rates from the resulting data.

3.1. Gross nitrification using $^{15}NO_3^-$ pool dilution approach

3.1.1. Overview

The following is a protocol for using the $^{15}N–NO_3^-$ pool dilution technique to estimate gross nitrification rates in hot spring water and sediment slurries. The protocol was designed for environments where the sediment is of a character such that slurries can be made, for example, small particle sizes and relatively little cohesion between sediment particles or aggregates. This protocol estimates gross NH_3 oxidation by monitoring the size and isotopic composition of the NO_x pool, rather than distinguishing NO_2^- and NO_3^- individually. Oxidation of NH_3 to NO_2^- will dilute the ^{15}N composition of the NO_x pool, whereas oxidation of NO_2^- to NO_3^- will have no effect on either the concentration or the isotopic composition of NO_x. Estimating NO_2^- oxidation is possible in this assay, if the concentration and isotopic composition of NO_3^- alone is monitored as well. Briefly, aliquots of spring water or a water–sediment slurry are distributed in bottles containing a predetermined amount of 98+ atom% $^{15}N–NO_3^-$ such that the resulting NO_3^- pool is 5–10 atom% ^{15}N. Sealed bottles (with ~5:1 headspace: sample volume) are incubated in the field at ambient temperatures by submersing them in spring water and the bottles are sampled over time. At each time point, samples are cooled and shaken in 1*M* KCl to extract NO_x. After extraction, samples are centrifuged, the supernatant passed through a 0.2 μm filter, and the filtrate is frozen or stored at 4 °C until analysis within 28 days (US-EPA, 1993a). Sediment samples are saved for determination of sediment dry weight. In the lab, filtrate is assayed for [NO_x] using automated colorimetry. $^{15}N–NO_x$ is determined by isotope ratio mass spectrometry (IRMS) of the ammonified NO_x pool or by coupled gas chromatography–isotope ratio mass spectrometry (GC–IRMS) of N_2O generated from the NO_x pool by *Pseudomonas aureofaciens* (Sigman *et al.*, 2001).

3.1.2. Preparation

As with all field work, it is recommended that as much preparation as possible be done prior to travel to the field site. For incubation of samples, we have used 160 mL serum bottles sealed with butyl rubber stoppers to prevent loss of liquid by evaporation, which can be excessive at high temperatures even over a short incubation time. Individual researchers may choose to use different incubation vessels depending on available resources and convenience. Incubation bottles and stoppers, as well as a large vessel for obtaining water and sediment slurry samples, should be acid washed, rinsed thoroughly, and sterilized by autoclaving. While only two time points are necessary for rate calculations, we typically prepare enough bottles for four time points (0, 3, 6, and 12–24 h incubations) with suitable replication at each time point appropriate for the experimental design. An alternative incubation approach is to prepare a single, larger flask, per replicate, and to remove subsamples from this flask over time. This has the advantage of reducing variability driven by differences between flasks, but also involves disturbing the entire sample when subsamples are removed. In our work to date, the two approaches have yielded quantitatively similar results. Using either approach, multiple time points are recommended, as they allow for more accurate determination of rates and are useful for determining whether rates are constant throughout the incubation or only for a subset of time points. A concentrated stock of 98+ atom% $^{15}N–KNO_3^-$ should be prepared, sterilized by passage through a 0.2 μm filter, and diluted to 1 m*M* or some other appropriate concentration such that attaining 5–10 atom% $^{15}N–NO_3^-$ requires addition of a small volume of the stock relative to the sample volume (e.g., 0.2% or less). If desired, a similarly concentrated solution of NH_4Cl (e.g., 500 m*M*) can be prepared for amendment of some samples with NH_4^+. For extraction of NO_3^- from samples after incubation, a 3 *M* solution of KCl should be made in a quantity of at least half the total volume of all samples to be processed. Additional equipment to bring to the field includes reagents and equipment for determination of NH_4^+, NO_3^-, and NO_2^- levels (see below); pipetmen and sterile pipet tips appropriate for adding microliter volumes; 25 mL pipets and a pipet bulb; racks suitable for suspending the sample bottles in the spring during sample preparation and incubation; aluminum crimps and a crimping tool for sealing stoppered bottles; 60 mL syringes and 23 G needles for periodically exchanging sample bottle headspace during incubation; sterile 50 mL polypropylene tubes for mixing sacrificed samples with KCl and a centrifuge compatible with these tubes to clarify sediment slurry samples; an orbital or rotary shaker for extraction of NO_3^- with KCl with racks compatible with the 50 mL tubes; syringes and 0.2 μm filters for sterilizing extracted samples; additional 50 mL polypropylene tubes for collecting the filtered, extracted samples; and wet and dry ice for cooling samples before extraction and freezing filtered samples, respectively.

3.1.3. Field work

The amount of $^{15}N–KNO_3^-$ to be added to attain the proper atom% $^{15}N–NO_3^-$ during incubation should be determined before sample preparation by quantifying the [NO_x] in the environment to be sampled, or by knowledge of concentrations typically found for the study system. Bulk spring water and water/sediment slurries should be considered separately, as concentrations of NO_3^- and NO_2^- are likely to differ between these habitats. It is also useful to determine the [NH_4^+], as this potentially serves as the primary "diluent" to the NO_x pool. Knowledge of NH_4^+ levels will help inform the experimenter as to whether to amend some samples with NH_4^+, and how much should be added. In the field, we routinely use a Smart2 handheld spectrophotometer and colorimetric kits for determination of NH_4^+ (#3642-SC), NO_2^- (#3650-SC), and NO_3^- (#3649-SC; all products of LaMotte, Chestertown, MD, USA). Care should be taken to correct the calculated [NO_3^-] for interference by NO_2^- as suggested by the manufacturer and this [NO_3^-] protocol may be problematic in environments in which [NO_2^-] > [NO_3^-] (Hedlund and Dodsworth, unpublished observation). Some springs may require that water is filtered prior to analysis (e.g., 0.2 μm Supor polysulfone filters (Pall)); samples for these three analyses should be assayed immediately after cooling to ~25 °C. In environments where [NO_x] is at or below the reliable detection limit (<1 μ*M*), we suggest either adding the $^{15}N–KNO_3^-$ to 0.5 μ*M* or diluting the $^{15}N–KNO_3^-$ with unlabeled KNO_3^- to 10 atom% ^{15}N and adding this solution to 10 μ*M* total KNO_3^-. It is convenient to add the appropriate amounts of $^{15}N–KNO_3^-$ and NH_4Cl to bottles prior to addition of samples.

Once sample bottles have been prepared with amendments, place them in racks in the spring water to bring the incubation bottles to ambient spring temperature before the incubation begins. Collect enough spring water for all samples in a large, sterile vessel, such as a 2 L glass flask. Add 25 mL spring water or water/sediment slurry to sample bottles by pipet. In cases where both spring water and sediment slurry will be used, collect the spring water and add it to bottles prior to preparation of the sediment slurry. This avoids unnecessary disturbance of the sediment and the unwanted collection of suspended sediment in samples intended for spring-water-only incubations. After addition of spring water to appropriate bottles, collect sediment (e.g., from the top ~1 cm of the sediment–water interface) and add to the vessel containing spring water until a desired amount of sediment is obtained. If later calculation to surface area is desired, several shallow sediment cores with known diameter can be pooled or attention can be paid to the surface area sampled. When using this protocol, we typically make the slurry at a 4:1 volume ratio of water:sediment. Seal and incubate the bottles in the racks suspended in spring water. Immediately remove bottles corresponding to the initial time point and cool on wet ice to ambient temperature. For bottles that are incubated longer than 2 h, potential O_2 limitation can be avoided by exchanging the headspace at regular intervals by flushing with 120 mL atmosphere using a 60 mL syringe and needles, or by briefly unsealing and then resealing the bottles or incubation vessels.

At each time point, remove a replicate set of bottles (or sample from the single, large incubation) and cool them on wet ice to ambient temperature ($\sim$25 °C). Decant the contents into 50 mL polypropylene tubes containing 12.5 mL of 3 *M* KCl. Seal the tubes, transfer them to a rack on a rotary or orbital shaker, and extract for 1 h with shaking at 120 rpm. If an electrical outlet is not available, the tubes may be shaken intermittently by hand for 15 min–1 h. After extraction, centrifuge the tubes containing sediment slurry samples for 10 min at $1500\times g$, and use the supernatant fraction for filtration. Alternatively, pass the sediment slurry samples through filter paper (e.g., Whatman no. 42) in a funnel if it is not practical to bring or power a centrifuge at the field site. Pass all samples through a 0.2 μm filter and collect the filtrate in 50 mL polypropylene tubes. Samples may either be frozen on dry ice or acidified to pH < 2 with H_2SO_4 and stored at 4 °C for up to 28 days before analysis (US-EPA, 1993a). Tubes containing sediment pellets should be saved and the sediment dry weight determined. Wash the sediment with water to remove excess KCl (two repetitions of resuspension in 45 mL water, followed by centrifugation as above) and dry to constant weight to determine the mass of sediment in the incubation.

3.2. Quantification and determination of atom% ^{15}N–NO_x and calculation of gross denitrification rates

3.2.1. Quantification of NO_x and NH_4^+

Samples can be analyzed for NO_x using cadmium reduction and automated colorimetry (APHA, 1992; US-EPA, 1993a). In this method, NO_3^- is reduced to nitrite by cadmium reduction; nitrite is then determined by diazotizing with sulfanilamide and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride, which forms a dye that can be measured colorimetrically. Nitrite and NO_3^- can be determined separately by first conducting the procedure with and subsequently without the cadmium reduction step.

The Berthelot reaction (Searle, 1984), or modifications thereof (e.g., Rhine *et al.*, 1998), can be used for analysis of NH_4^+, if required. In the classical approach, NH_4^+ in the sample reacts with phenol and hypochlorite, producing indophenol blue in proportion to the NH_4^+ concentration, which is measured colorimetrically (Searle, 1984). NH_4^+ can also be measured using semiautomated colorimetry (US-EPA, 1993b).

For analysis of NO_3^- plus NO_2^-, and for NH_4^+, samples can be preserved for up to 28 days by acidification to pH < 2 with sulfuric acid and storage at 4 °C. For separate analysis of NO_2^- and NO_3^-, store at 4 °C, do not acidify, and analyze within 24 h. Alternatively, samples can be frozen for long-term storage (Avanzino and Kenedy, 1993; Bremner and Keeney, 1966). To avoid interference with the cadmium column, samples should not be preserved with mercuric chloride.

3.2.2. Determination of atom% ^{15}N–NO_x

There are several procedures used for preparing aqueous NO_x samples for isotopic analysis. These procedures either convert dissolved NO_x into a solid or gaseous phase, in a form suitable for analysis by IRMS. The diffusion technique concentrates NO_x–N (as NH_4^+) onto a glass fiber filter disk, which can be analyzed by Dumas' combustion and subsequent GC–IRMS (Brooks *et al.*, 1989; Stark and Hart, 1996). The anion-exchange method traps NO_x on anion-exchange resin column followed by precipitation as silver nitrate, also suitable for analysis by combustion GC–IRMS (Chang *et al.*, 1999; Silva *et al.*, 2000). Two other approaches convert aqueous NO_x to N_2O, either chemically (McIlvin and Altabet, 2005) or biologically (Sigman *et al.*, 2001), and the N_2O is analyzed by GC–IRMS. Here, we briefly describe the diffusion and denitrifier techniques for analyzing the ^{15}N composition of NO_x.

3.2.3. Diffusion technique for ^{15}N–NO_x analysis

A volume of aqueous sample ideally containing 20 μg NO_x–N is placed in a clean (acid-washed) plastic container with an air-tight removable top. The first step of the procedure removes NH_4^+ from the sample, so the top of the container is removed to allow gas exchange (the NH_4^+ is removed as NH_3. Note: if ^{15}N determination of NH_4^+–N is also desired, this step can be modified to include a Teflon sandwich enclosed acid trap and incubation with sealed top, trapping the NH_4^+–N as described below for NO_x–N). Enough powdered magnesium oxide is added to saturate the solution (approximately 10 g L^{-1}), which increases the pH to around 9 (check), favoring phase change of NH_4^+ to NH_3, with subsequent volatilization and loss of NH_3 from the solution. The samples are incubated with moderate rotary shaking for 5–7 days, although modified procedures can reduce the required time period (Chen and Dittert, 2008). A few samples may be checked at this point to ensure NH_4^+ concentrations in the samples are below detection limits. During the incubation, prepare acid traps. First, cut Whatman glass–fiber filters into small disks, about 8 mm diameter, the size of a standard hole punch. Then, add 20 μL of 2.5 *M* $KHSO_4$ to the filter disk. Enclose the acidified disk between two layers of polytetrafluoroethylene (PTFE) tape, creating a seal around the disk by pressing the two layers of PTFE tape together using a glass test tube. To avoid accumulating ambient NH_4^+, the PTFE-enclosed acid traps should be stored in a sealed desiccator containing an open vial of H_2SO_4 or some other suitable acid. Avoid long-term storage of the acid traps.

Once the PTFE-enclosed traps have been prepared, and the preincubation of the samples is complete such that NH_4^+ has been removed, place one PTFE-enclosed acid trap into each sample container, to each add a scoop of finely ground Devarda's alloy (about 10 g L^{-1}; this metal alloy reduces both NO_2^- and NO_3^- to NH_3), and tighten the lid to ensure an air-tight seal. The sealed samples can be placed on a shaker–incubator at elevated temperature to promote rapid reduction to NH_3, which readily diffuses through the PTFE tape to the acidified filter disk, where it is trapped as NH_4^+. After 5–7 days, remove the PTFE-trap packets from the samples and place them in a sealed desiccator (with liquid acid trap and desiccant) for at least 24 h, or until analysis. On the day the samples are to be analyzed on the mass spectrometer, open the PTFE packets and retrieve the acidified filter disk using clean forceps, placing the disk into tin capsules suitable for Dumas' combustion coupled to GC–IRMS as described (Brooks *et al*., 1989; Stark and Hart, 1996). The disks are acidic and will corrode the tin if left too long, so analyze the samples immediately after they have been sealed in the tin capsules (the same day is optimal). If storage is necessary, store them in the PTFE packets in the desiccator.

3.2.4. Denitrifier method for ^{15}N–NO_x analysis

P. aureofaciens is a facultative denitrifying organism, but lacks the enzyme nitrous oxide reductase, the enzyme that converts N_2O to N_2 during denitrification, so the reaction stops at N_2O. Because of the lower background of N_2O in the atmosphere compared to N_2, N_2O is a more convenient analyte for IRMS. In this method, *P. aureofaciens* is used to convert NO_x in the sample to N_2O, which is then analyzed by IRMS (Casciotti *et al*., 2002; Révész and Casciotti, 2007; Sigman *et al*., 2001). The major advantages of this method are: (1) it is fairly rapid, and, compared to the diffusion method, involves fewer steps, and (2) it can be used to measure simultaneously the isotopic composition of both N and O. The disadvantage is that the method depends on a biological enzyme system, and thus involves keeping a pure culture of *P. aureofaciens*.

P. aureofaciens cultures are grown in tryptic soy broth amended with 10 m*M* NO_3^- and 15 m*M* NH_4^+. After 4–7 days of growth, cultures are centrifuged and resuspended in NO_3^--free medium to achieve a 10-fold concentration. Eight milliliter of the concentrated suspension is added to each 20 mL vial, sealed with gas-tight septa. Each vial is purged with He for an hour. This flushing procedure is designed to promote anaerobic conditions, and to remove any residual NO_x (which will be converted to N_2O) from the broth. The flushing procedure is conveniently done using an autosampler such as the Thermofinnigan CombiPAL (Thermo Fisher Scientific, Wlatham, MA, USA), so that the purging process can be automated.

NO_x concentrations in the samples must be known, so that the appropriate volume of sample can be added to each 20 mL vial. The sample is introduced to the vial through the septum using syringe and needle.

Sufficient sample is added to obtain enough N for analysis, which for some laboratories is as little as 10 nmol (Révész and Casciotti, 2007). In our experience, higher amounts are optimal (50 nmol).

Several drops of antifoaming agent (e.g., Antifoam B Emulsion, Dow Corning, Midland, MI, USA) are added to each vial to reduce bubble formation during the reaction. The vials are allowed to incubate for 8 h, during which time NO_3^- is converted completely to N_2O. After the 8-h period, 0.1 mL of 10 N NaOH is added to each vial to stop the reaction, and to absorb CO_2, which can interfere with N_2O analysis (since CO_2 has the same masses as N_2O: 44, 45, and 46). The samples are then placed on an autosampler tray for preconcentration prior to isotope analysis. In this step, headspace is withdrawn from each vial, passed through water and CO_2 traps (Nafion drier and Ascarite or equivalent, respectively), a cryogenic purification trap (liquid N_2), a GC column, and into an open split, which interfaces with the IRMS. Typical preconcentration systems are the Thermo Scientific GasBench, SerCon Cryo-Prep, and the Isoprime Trace Gas preconcentrator. The mass IRMS is equipped with a universal triple collector suitable for masses 44, 45, and 46 (e.g., Thermo Scientific DeltaV, SerCon GEO 20-20, or IsoPrime100 IRMS). Standards of known $\delta^{15}N$ and $\delta^{18}O$ must be included in the autosampler tray, such as USGS32, USGS 34, USGS 35, and IAEA NO3. Mass ratios of 45:44 and 46:44 distinguish $\delta^{15}N$ and $\delta^{18}O$ signatures, respectively.

3.2.5. Calculation of gross nitrification rates

Once the size of the NO_x pool and the atom% ^{15}N in this pool for each time point are known, the following equations, modified from Barraclough (1991), can be used to calculate gross nitrification rates for either a constant or changing NO_x pool size. In cases where the NO_x pool size is changing over time, the gross nitrification rate, n, in units of moles per hour, can be calculated as follows:

$$n = -\theta\left(\ln\%^{15}\mathrm{N_t} - \ln\%^{15}\mathrm{N_0}\right)/\left(\ln(1 + \theta \times t/\mathrm{N_0})\right)$$

where N_0 is the initial NO_x pool size in moles, θ is the rate of change in the NO_x pool in moles per hour, $\%^{15}N_0$ and $\%^{15}N_t$ are the atom% ^{15}N–NO_x at the initial time point and at time t (in hours), respectively, and ln is the natural logarithm. If data from multiple time points are available, the slope calculated from a plot of ln (atom% ^{15}N in excess of natural abundance) versus time (in hours) can be used to replace (ln $\%^{15}N_t$− ln $\%^{15}N_0$) in the above equation, where $t = 1$. In cases where the NO_x pool size is constant, the above equation is invalid and the following one can be used:

$$n = -\mathrm{N_0}\left(\ln\%^{15}\mathrm{N_t} - \ln\%^{15}\mathrm{N_0}\right)/t$$

where the symbols have the same meaning and the slope, if multiple time points are used, can be inserted as described above. Calculations can further be normalized per gram dry weight of sediment or per milliliter of spring water, depending on whether the incubations included a sediment slurry or spring water only. In cases where $[NO_x]$ cannot be reliably determined, the pool size can be estimated at the initial time point using the atom% $^{15}N-(NO_x)$ determined for this time point and the known amount of labeled $^{15}N-NO_3^-$ added.

4. Methods for Measuring Denitrification in Terrestrial Geothermal Habitats

Many approaches for measurement of denitrification *in situ* are available to microbiologists studying geothermal habitats (Mosier and Klemedtsson, 1994; Steingruber *et al.*, 2001; Tiedje, 1994; Tiedje *et al.*, 1989). Here, we describe two of the most common, the acetylene block method and the $^{15}NO_3^-$-tracer approach. Together, these two approaches should allow the researcher to bound rates of *in situ* denitrification and quantitatively distinguish the fates of $^{15}NO_3^-$ respired in geothermal ecosystems.

4.1. Acetylene block method

The acetylene block is a simple, inexpensive, and effective approach to measure denitrification in the field that is based on the observation that acetylene inhibits the reduction of N_2O to N_2, causing an accumulation of N_2O that can be measured by GC (Balderson *et al.*, 1976; Federova *et al.*, 1967; Yoshinari and Knowles, 1976). This method is extremely sensitive at low denitrification rates since N_2O is present at a low atmospheric concentration, about 310 ppb, as compared with N_2 at about 78%. Limitations of the approach have been described in detail elsewhere (Tiedje *et al.*, 1989) but the most significant may be the inhibition of nitrification, which can lead to underestimation of denitrification rates in habitats with low NO_x concentration where denitrification is tightly coupled to nitrification. Therefore, we recommend making measurements with and without NO_3^- amendments. The latter measurement addresses the denitrification rate based on the *in situ* NO_x pool in the sediment porewater in addition to substrate diffusing into sediments from the overlying water column but will miss any contribution of NO_x from nitrification. If sufficient substrate is added, the former measurement with excess NO_3^- will provide an upper bound on denitrification, taking into account all sources of NO_x.

Coordinated experiments with either NO_3^- or NO_2^- can distinguish the relative importance of the two electron acceptors in denitrification.

A number of specific protocols for application of the acetylene block to soils or sediments have been used including the static core protocol, the gas-phase recirculation core protocol, the closed chamber protocol, sediment slurry protocols, and aqueous flow-through protocols (Mosier and Klemedtsson, 1994; Tiedje, 1994; Tiedje *et al.*, 1989). We have only used the sediment slurry approach, which is the simplest approach commonly used for aquatic sediments (Miller *et al.*, 1986; Oremland *et al.*, 1984; Sorensen, 1978). This approach may either overestimate or underestimate denitrification rates in hot spring sediments, depending on the possible stimulatory effects of sediment disruption by relieving diffusion limitations in stratified sediments or on possible inhibitory effects due to oxygen exposure during sample processing. However, the strong linear N_2O production rates without lag we have observed suggest the latter is not a major problem (Dodsworth and Hedlund, unpublished). Thus, care should be taken to minimize manipulations in the field in order to maximize efficient use of field time, mistakes due to time pressures, and potential chemical and biological contamination.

4.1.1. Overview

Samples from the sediment/water interface are collected with a coring device and quickly extruded under a stream of N_2 into 28 mL Balch tubes containing 5 mL of a preheated anaerobic liquid phase (e.g., filtered spring water). The headspace is flushed with N_2 for 5 min, and tubes are sealed with butyl rubber stoppers secured with aluminum crimp caps. Freshly prepared acetylene is added to a volume of 10% and the tubes are shaken for 20 s. Tubes are incubated in the hot spring under aluminum foil and sampled destructively by cooling to $\sim$25 °C, shaking vigorously, and removing a sample into an evacuated bottle. In the lab, N_2O is quantified by comparison with a standard curve by gas chromatography using a ^{63}Ni electron capture detector (GC-ECD).

As with nitrification measurements, it is best to establish linearity of N_2O production to determine the appropriate incubation time frame before doing complex experiments. Since NO_3^-/NO_2^- concentrations in geothermal springs are typically low ($<$200 μM; Holloway *et al.*, 2004; Shock *et al.*, 2010), incubations of a few hours are generally recommended over long-term experiments, particularly for experiments without NO_x amendment (e.g., 0, 2, 4, 8, and 16 h incubations).

4.1.2. Preparation

Twenty eight milliliters Balch tubes containing an appropriate liquid phase should be set up prior to the trip. We have used spring water collected from a previous trip by ultrafiltration (30 kDa molecular weight cutoff) as a medium. The ultrafiltrate is dosed with amendments, if desired (e.g., 30–1000 μ*M* NO_3^-), sparged for 30 min with N_2 in a bottle with minimal headspace, and transferred to an anaerobic chamber (e.g., Coy Type B, Grass Lake, MI). In the chamber, ultrafiltrate is added to Balch tubes (BellCo Glass, Vineland, NJ) at 5 mL per tube. Following removal from the chamber, hydrogen and other gasses are removed by three cycles of vacuum and gassing (to 1 atm of overpressure) with N_2 and pressurized to 0.5 atm of overpressure. The overpressure will enable detection of major leaks due to punctured stoppers and will ease stopper removal in the field; however, it should be recognized that pressurized tubes are a hazard and safety glasses should be used during transportation and manipulation. Finally, the tubes are autoclaved for 30 min at 121 °C. The pH of the fully prepared spring water should be checked to ensure it is close to *in situ* pH. The concentration of NO_3^- and NO_2^- should also be checked by ion chromatography or colorimetric assays, as described above. In our experience, all NO_3^-, NO_2^-, and NH_4^+ disappears in the ultrafiltrate within months of collection, presumably due to microbial consumption. Ideally, NO_3^- and NO_2^- in "unamended" ultrafiltrate should be amended to match the substrate concentrations in the hot spring water column during the time of the field experiments by adding aliquots of concentrated stock solutions following NO_3^- and NO_2^- field measurements.

As an alternative to the use of ultrafiltrate, a synthetic mineral salts medium can be prepared to simulate the hot spring water or freshly collected hot spring water can be used for the aqueous phase. However, if the latter is used, researchers should consider either sparging the spring water (e.g., with N_2) or replacing the sediment slurry approach with a static core approach because contact of the sediment microbial community with dissolved oxygen, if present, may inhibit denitrification.

Preparations should also be made for work with gasses in the field. Bottles for fresh acetylene can be prepared in the lab by adding ~1 g of calcium carbide (CaC_2, Sigma 270296) to serum bottles (e.g., Wheaton 160 mL bottles, 223748), which are subsequently stoppered, sealed, and evacuated. Acetylene is later prepared in the field by adding 1–5 mL of distilled water. Care should be taken not to add water too fast because this could explode the serum bottle. Safety glasses are required. Acetylene produced from CaC_2 contains traces of H_2, CH_4, C_2H_4, and PH_3 (Hyman and Arp, 1987), though these contaminants do not appear to influence denitrification (Tiedje *et al.*, 1989). For application of Hungate technique in the field, we use a portable field gassing manifold with 25 G needles and bent 18 G needles for gassing probes.

Other important equipment includes aluminum seals, a crimper and decrimper, cutoff and autoclaved syringes for sediment sampling, sterile spatulas for manipulating cored sediments, needles and syringes (5 or 10 mL), vacutainer needles for sampling gasses, distilled water, evacuated sample collection receptacles (e.g., Wheaton 10 mL bottles, 223739), extra racks for *in situ* incubations, aluminum foil, and sturdy gloves for working in the hot spring.

4.1.3. Field work

Measure NO_3^- and NO_2^- concentrations immediately after cooling a small spring water sample to ~25 °C and make amendments if needed. Decrimp a tube and release overpressure while minimizing oxygen exposure by constant flushing with N_2 using a cannula, following the techniques of Hungate (Hungate, 1950, 1969). Remove a sediment core from the shallows of the spring and extrude the top ~0.5 cm (or other strata if desired) directly into Balch tubes under a stream of N_2 using the techniques of Hungate. Replace the stopper, crimp, and flush for 5 min with N_2, using a needle in the stopper to allow gas to flush out. Release overpressure. Dose the tube with a 1/10 volume of acetylene (2.5 mL), shake the tube 20 s to disperse sediment and encourage acetylene solubilization, and return to the spring for incubation. Alternatively, a single anaerobic sediment slurry can be prepared for addition to all tubes.

Following the incubation period, Balch tubes are removed from the spring and cooled for ~5 min in ambient air and then in a ~25 °C water bath. The actual temperature should be noted and used to select the appropriate Bunsen coefficient, as described below. The tubes are then shaken vigorously for 30 s once every 3 min for three cycles (~10 min) to equilibrate N_2O between the aqueous phase and the headspace. A vacutainer is used to allow gas to flow to the evacuated collection vial, and 10 mL of distilled water is added to the Balch tube to alleviate the vacuum. Pressurize the collection vials to 1 atm overpressure with N_2. Collection vials receiving any liquid should be marked or discarded because microbial activity may influence N_2O concentrations during transport and storage. Transport the Balch tubes intact to the lab for determination of sediment dry weight (e.g., after filtration onto Whatman filter paper) after drying to constant weight.

4.1.4. Analysis and rate calculations

N_2O is measured in the lab as soon as possible by GC-ECD in comparison with purified standards. Here, we describe a protocol using a GC-2014 Nitrous Oxide Analyzer (Shimadzu, Moorpark, CA). If using a different GC-ECD system, we refer the reader to other descriptions of systems that have been used for N_2O measurements (Loftfield *et al.*, 1997; Mosier and Klemedtsson, 1994; Mosier and Mack, 1980). The volume needed to flush the sample loop (~5 mL with Shimadzu GC-2014) can be decreased with a short length of stainless steel tubing (~0.25 mL) to reduce the volume of sample needed to 2 mL, allowing multiple injections per sample.

The standard factory-ready protocol uses high-quality (99.999% purity) N_2 at 25 mL min^{-1} as the carrier gas. P5 (argon/methane, 95/5 v/v) at 2.5 mL min^{-1} can be used as the make-up gas. One milliliter of sample from the sample loop (injector temperature 250 °C) is injected serially onto four columns, all at 80 °C: Haysep T (80/100 mesh; 1 m), Haysep D (80/100 mesh; 4 m), Haysep N (80/100 mesh; 1.5 m), and Shimalite Q (80/100 mesh; 0.4 m). Purges are pneumatically controlled to prevent CO_2 or water from interfering with the ECD. The ECD is programmed at 325 °C, 2 nA, and a 200 ms time constant. This protocol yields a linear standard curve for N_2O concentrations up to 100 ppm (Mosier and Klemedtsson, 1994). At concentrations >100 ppm, we have found that a nonlinear equation can be applied. Alternatively, dilutions can be made prior to analysis or N_2O can be quantified by GC using a thermal conductivity detector (TCD; Ryden *et al.*, 1987).The amount of N_2O produced in any sample, X, can be calculated by the following equation:

$$X = ([\mathrm{N_2O}])(2)\left[\left(V_\mathrm{g} + \left(V_\mathrm{aq}\right)(\alpha)\right]/(24.5)(W)\right.$$

where $[N_2O]$ is the concentration of N_2O in the 10 mL collection vials (in parts per million), obtained by comparison with a standard curve; 2 accounts for the 1:1 dilution of the sample by pressurizing to 2 atm with N_2; V_g and V_{aq} are volumes (in liters) of the gas and aqueous phase in the Balch tubes, respectively; 24.5 is the liters of gas per mole using the ideal gas law at 25 °C (the constant should be adjusted if tubes were equilibrated at a different temperature); W is the dry weight of the sediment sample (in grams); and α is the Bunsen absorption coefficient of N_2O at the temperature at which the headspace sample was removed from the Balch tube. The value of α is as follows for the following temperatures: 5 °C, 1.06; 10 °C, 0.88; 15 °C, 0.74; 20 °C, 0.63; 25 °C, 0.54; 30 °C, 0.47; and 35 °C, 0.41 (Mosier and Klemedtsson, 1994; Tiedje, 1982). The resulting units are μmol N_2O/g dry weight of sediment. For experiments with spring water, replace W with the volume of the water in the Balch tube in milliliter, resulting in units of μmol N_2O/mL. To assess linearity, the amount of N_2O–N can be plotted versus time and used to calculate a slope and associated statistics.

4.2. $^{15}NO_3^-$ tracer approach

4.2.1. Overview

Hot spring water or anaerobic sediment slurry is amended with 98+ atom% ^{15}N–NO_3^- so that the resulting NO_3^- pool is 5–10 atom% ^{15}N and incubated in stoppered bottles *in situ* with suitable replication. Replicate bottles are sacrificed by cooling to ambient temperature (~25 °C) and shaking to equilibrate N_2 and N_2O. Gas samples are collected for

GC–IRMS to determine the amount of N_2 and N_2O and ^{15}N enrichment, and sediment slurries are extracted with KCl and used for IRMS to determine the amount of NO_x and NH_4^+ pools and ^{15}N enrichment. Changes in the pool flux and the flow of ^{15}N into different N pools can be plotted versus time to calculate rates of different NO_x reduction processes.

4.2.2. Preparation

Preparation is similar to the approaches described above. Necessary items include: incubation bottles (e.g., 160 mL serum bottles), incubation baskets, stoppers, aluminum seals, crimper and decrimper, portable gassing station and N_2 tank, 60 mL syringes and needles, evacuated gas-tight gas collection vials, concentrated stocks of N–NO_3^- and ^{15}N–NO_3^-, a 3 *M* solution of KCl of at least half the total volume of samples to be processed, 50 mL polypropylene tubes and a compatible centrifuge, an orbital or rotary shaker with racks compatible with the 50 mL tubes, syringes and 0.2 μm filters, and wet and dry ice for cooling samples before extraction and freezing filtered samples, respectively.

4.2.3. Field work

As described for the acetylene block above, we suggest using sterile, anaerobic ultrafiltrate from the spring of interest for a medium, in which case serum bottles can be amended to the *in situ* [NO_3^-] measured at the time of the experiment at 5–10 atom% ^{15}N–NO_3^-. Alternatively, an artificial medium or freshly collected spring water can be used and amended to 5–10 atom% ^{15}N–NO_3^-, with the caveats discussed above. We suggest a total aqueous volume of at least 60 mL. The prepared serum bottles are preincubated in a wire basket *in situ*. Either freshly collected sediment cores or a small amount of sediment in an anaerobic sediment slurry is added to the serum bottles. If the former is used, the bottle should be flushed for 5 min with N_2 to achieve a headspace of 1 atm N_2. The bottles are incubated for the desired time and removed from the spring to cool to ambient temperature (~25 °C). Bottles are pressurized to 2 atm N_2 and shaken vigorously for 30 s once every 3 min for three cycles (~10 min) to equilibrate N_2O and N_2 between the aqueous phase and the headspace. Safety glasses are required. The overpressure in the headspace is removed using a 60 mL syringe to fill evacuated gas sample vials. The sediment slurry is poured into three 50 mL polypropylene tubes already containing 20 mL of 3 *M* KCl and stored on ice until extraction (a few hours). KCl extraction is carried out and extracted samples are filtered and stored on dry ice, as described in Section 3.1. Sediments should be washed, dried, and weighed as described in Section 3.1 for normalization to sediment dry weight.

4.2.4. Analysis and calculations

N_2O concentrations are measured with a gas chromatograph equipped with an electron capture detector (Section 4.1). The ^{15}N composition of N_2O, if desired, is measured using the trace-gas preconcentration and IRMS procedure described in Section 3.2. NO_3^- concentrations are determined colorimetrically, and ^{15}N content of NO_3^- can be determined using the diffusion or denitrifier procedures described in Section 3.2. N_2 concentrations and ^{15}N composition can be measured by injecting headspace samples into a GC coupled to an IRMS equipped with a universal triple collector (identical to those described above, Section 3.2). Masses 30, 29, and 28 are used to determine ^{15}N–N_2.

The rate of denitrification is estimated as the accumulation of N_2 and N_2O that reflects the ^{15}N composition of the NO_3^- pool during the incubation:

$$\text{Denitrification} = \left(\left(\text{atom}\%^{15}\text{N} - \text{N}_2\right) \times [\text{N}_2] \times V\right) / \left(S \times \text{atom}\%^{15}\text{N} - \text{NO}_3{}^-\right)$$

where atom% ^{15}N–N_2 is the ^{15}N composition of N_2 gas, $[N_2]$ is the concentration of N_2 gas, atom% ^{15}N–NO_3 is the ^{15}N composition of the NO_3^- pool, expressed as atom% (either measured or estimated based on the amount of ^{15}N–NO_3^- added and the initial ambient concentrations), V is the volume of the headspace, and S is the mass of sediment (or surface area of sediment, or volume of water used in the incubation, depending on how results are to be expressed).

5. Detection and Quantification of Potential Biomarkers for Thermophilic AOA and Denitrifying *Thermus thermophilus*

Analysis of genes or transcripts involved in microbial processes of interest, so-called functional genes, can serve as valuable biomarkers to link community activity measurements and microorganisms possibly carrying out the activities. Various studies involving the authors have used conserved or degenerate primers to amplify and sequence *amoA* genes or transcripts from large numbers of hot springs using PCR or quantitative real-time PCR (qPCR), providing insights into relationships between diversity or quantity of functional groups and geochemistry or geographical location (de la Torre *et al.*, 2008; Jiang *et al.*, 2010; Reigstad *et al.*, 2008; Zhang *et al.*, 2008). More recently, we have used knowledge of the microbial community composition in well-studied springs to combine measurements of nitrification and denitrification with enumeration of specific populations using specific qPCR primers (Dodsworth *et al.*, unpublished data). Below, we describe protocols for sample collection and nucleic acid extraction and primers and procedures for diversity studies of *amoA* genes as well as specific primers and protocols for qPCR for *amoA* and 16S rRNA gene of "*Candidatus* Nitrosocaldus yellowstonii" and the nitrate reductase large subunit, *narG*, of denitrifying *T. thermophilus*.

5.1. Sample collection and nucleic acid extraction

Sediment is collected using a sterile coring device, polypropylene tube, or other sterile sampling device. For collection of planktonic cells, bulk water can be filtered through 0.1 or 0.2 μm Supor polysulfone, 25 mm diameter filters (Pall) contained in presterilized filter cartridges (Pall). Prior to freezing, individual filters are transferred to 1.5 mL polypropylene tubes containing 0.1 mL of TE (10 m*M* Tris pH 8, 1 m*M* EDTA). To maximize both the quantity and quality of extracted nucleic acids and other biomolecules, it is recommended that samples be frozen as quickly as possible after collection in the field. Flash freezing by immersion in liquid N_2 is ideal. Alternatively, samples may be frozen on crushed dry ice or by immersion in an ethanol bath cooled by dry ice. Frozen samples should be stored and transported on dry ice and transferred to an ultracold freezer (−80 °C) for storage in the laboratory until processing. If transport or maintenance of liquid nitrogen or dry ice is not practical, addition of RNA later (Applied Biosystems/Ambion, Austin, TX, USA) or a sucrose lysis buffer (SLB; 20 m*M* EDTA, 200 m*M* NaCl, 0.75 *M* sucrose, 50 m*M* Tris–HCl, pH 9) may help prevent nuclease activity for subsequent extraction of RNA or DNA, respectively (Grant *et al.*, 2006; Hall *et al.*, 2008). As a general rule, it can be useful to freeze multiple identical samples (e.g., 1.5 mL polypropylene tubes each with 1 cc of sediment or multiple filters) as this allows rapid freezing and will allow for more efficient use of the samples, either for the extraction of different analytes or for their dissemination to collaborators. In cases where biomarkers are to be analyzed alongside community activity measurements, the same fraction should be collected to enable direct comparisons.

Nucleic acid extraction is performed on samples immediately after their removal from storage at −80 °C. There are a variety of protocols and commercially available kits that can be used to extract nucleic acids from environmental samples (Purdy, 2005), including geothermal environments (Herrera and Cockell, 2007; Mitchell and Takacs-Vesbach, 2008). For extractions of DNA and RNA, we and others (Costa *et al.*, 2009; de la Torre *et al.*, 2008; Jiang *et al.*, 2009, 2010; Reigstad *et al.*, 2008; Vick *et al.*, 2010) have had success with the FastDNA® SPIN Kit for Soil and FastRNA® Pro Soil-Direct Kit, respectively (MP Biomedicals, Solon, OH, USA; formerly made by Bio101 and Q-biogene). Both of these commercially available kits utilize a benchtop FastPrep Instrument (MP Biomedicals; formerly made by Bio101/ThermoSavant and Q-biogene) for cell disruption and sample homogenization. These kits allow rapid and reproducible DNA or RNA extractions from a variety of sample types (e.g., sediments, microbial mats, biomass collected on filters). Prepared DNA and RNA should be stored at −20 and −70 °C, respectively, until use. It is best to freeze samples in multiple aliquots to avoid potential loss of nucleic acids due to repeated freeze–thaw cycles. To prepare crude RNA samples for use in RT-PCR, DNA is digested by treatment of the sample with RNase-free DNase I (Takara, Japan). The DNase-treated samples are then checked for potential genomic DNA contamination by PCR amplification with primer sets specific for archaeal and bacterial 16S rRNA and archaeal *amoA* genes according to Jiang *et al.* (2010). DNA-free RNA samples are reverse transcribed into cDNA using the Promega AMV reverse transcription system (Promega Corporation, Madison, WI) as previously described (Jiang *et al.*, 2009).

5.2. PCR and qPCR

PCR and qPCR are useful techniques for detection and quantification, respectively, of AOA in geothermal environments, especially in samples that contain relatively low biomass, which might not yield sufficient amounts of lipids or other biomarkers. Many studies have used primers specific to the archaeal *amoA* to detect and/or quantify AOA in a variety of environments (de la Torre *et al.*, 2008; Francis *et al.*, 2005; Jiang *et al.*, 2009; Leininger *et al.*, 2006; Zhang *et al.*, 2008). Primers designed by Francis *et al.* (2005) are most commonly used and have led to amplification of extremely diverse putative *amoA* genes and transcripts from a wide variety of geothermal habitats (Jiang *et al.*, 2010; Zhang *et al.*, 2008). However, these primers are minimally degenerate and the forward primer has two mismatches near the 3′ end with the *amoA* sequence of "*Ca.* N. yellowstonii." Thus, de la Torre *et al.*, (2008) modified the forward primer to be more degenerate. However, we have recently found that neither the original primer pair of Francis nor the modified primer pair of de la Torre were successful in amplifying *amoA* genes from certain high temperature (>73 °C) spring sources in the US Great Basin, despite the high abundance of 16S rRNA genes >98% identical to the AOA "*Ca.* N. yellowstonii" in clone libraries in some of these hot springs (Costa *et al.*, 2009). We therefore designed a more degenerate set of primers (DegAamoA-F and DegAamoA-R; Table 8.2), which targets a region conserved between "*Ca.* N. yellowstonii" and all *amoA* cluster IV sequences, as described by de la

Torre *et al.* (2008). PCR using these primers yielded product of the predicted size, ~450 bp. Sequences obtained from this product were aligned with the putative *amoA* gene from "*Ca.* N. yellowstonii," and nondegenerate primers specific for this set of sequences for use in qPCR were designed (CNY amoA-F and CNY amoA-R, Table 8.2). We recommend using an approach similar to that described above to attempt to obtain *amoA* sequences for primer design from geothermal environments in cases where other less degenerate primer sets fail to yield product. In general, this type of result also mandates that some caution be used in interpreting results of studies dependent on functional gene PCR, particularly when the study site has not been investigated using a metagenomics approach.

The following is a protocol optimized for primer sets specific for the 16S rRNA gene (CNY 16S-F and -R; Table 8.2) and putative *amoA* (CNY amoA-F and -R) of "*Ca.* N. yellowstonii" and close relatives detected in US Great Basin hot springs (Costa *et al.*, 2009). This is designed for use with an iCycler iQ Multicolor Real-Time PCR Detection System (BioRad, Hercules, CA, USA), using SYBR Green to detect PCR product. For each template, reactions should be prepared in triplicate and coupled with negative controls containing no template. Standard curves are obtained by using linearized plasmid containing the target sequence as template in 10-fold dilutions ranging from ~10^2 to 10^7 copies/reaction. Prepare individual reactions (25 μL) in individual wells of a iQ 96-well PCR plates (223-9441, BioRad): PerfeCta SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD, USA), 12.5 μL; 2.5 μL each of forward (F) and reverse (R) primer (4 μ*M*); 2.5 μL template DNA; and 5 μL sterile, nuclease-free water. The following cycling conditions are used: an initial melt cycle (95 °C for 3 min) followed by 45 cycles of melting (94 °C for 15 s), annealing (58 °C for 15 s), and extension (72 °C for 35 s), with data collection using a SYBR-490 filter enabled during the 72 °C step, followed by a melt curve 55–95 °C by 0.5 °C increments (10 s each step). Threshold cycles are calculated and data analyses are performed using version 3.1 of the iCycler iQ Optical System Software (BioRad). This same protocol can be used for quantification of *amoA* transcripts by using cDNA generated from RNA, as described in Section 5.1, as template and including RNA samples not treated with reverse transcriptase as a negative control.

We have used a similar qPCR protocol for *narG* using primers TnarG-F and TnarG-R (Table 8.2), which target the plasmid-borne *T. thermophilus* HB8 *narG* sequence, as well as *T. thermophilus* isolates from Great Basin hot springs (Hedlund, unpublished data). These primers have several mismatches and are not expected to amplify *narG* from *Meiothermus* or other genera. Currently, no other *Thermus narG* sequences are available for primer design. We urge reevaluation of the primers as additional *Thermus narG* sequences become available.

Table 8.2 Primers targeting putative *amoA* from AOA, 16S rRNA genes of "*Ca*. N. yellowstonii," and putative *narG* from denitrifying *Thermus thermophilus* strains

Primer name	Sequence (target position in "*Ca*. N. yellowstonii" *amoA* or *T. thermophilus* HB8 *narG*)	Application	Reference
Arch-amoAF	5′ STA ATG GTC TGG CTT AGA CG (-3-17)	PCR	Francis *et al*. (2005)
Arch_amoA_F	5′ AAT GGT CTG GST TAG AMG (-1-17)	PCR	de la Torre *et al*. (2008)
Arch-amoAR	5′ GCG GCC ATC CAT CTG TAT GT (616-635)	PCR	Francis *et al*. (2005)
DegAamoA-F	5′ ATH AAY GCN GGN GAY TA (73-89)	PCR	This study
DegAamoA-R	5′ ACY TGN GGY TCD ATN GG (502-518)	PCR	This study
CNY amoA-F	5′ ATA TTC TAC TCY GAC TGG ATG (91-111)	qPCR	This study
CNY amoA-R	5′ TAT GGG TAK CCT AAG CCT CC (265-284)	qPCR	This study
CNY 16S-F	5′ TAG CTG AAA TCT ATA TGG CCC	qPCR	This study
CNY 16S-R	5′ ATT CTC CAG CCT TTT TAC AGC	qPCR	This study
TnarG-F	5′ GGG TCT GGT TCA TCT GGC (2024-2041)	qPCR	This study
TnarG-R	5′ TTC CTG TAG ACC ACC TCC (2151-2168)	qPCR	This study

6. Closing Remarks

Recent advances in our understanding of nitrogen cycling processes at high temperatures have sparked new interest in the field and highlight how little is known regarding these processes in geothermal environments. Some terrestrial hot springs may harbor large populations of AOA (Costa *et al.*, 2009), but their relative contribution to primary production and the energy budget in these systems are not understood. Although nitrite oxidation apparently occurs at temperatures up to 85 °C (Reigstad *et al.*, 2008), the organisms responsible for this process at these temperatures are not known. Furthermore, although a diverse array of thermophilic and hyperthermophilic *Bacteria* and *Archaea* are capable of nitrate reduction, their relative contributions to this process *in situ* are unknown. The authors hope that the application of existing techniques for quantifying nitrification and denitrification to terrestrial geothermal ecosystems, as described in this chapter, will help further the understanding of the N-cycle in these systems and the microorganisms responsible for these processes.

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