# Observations Concerning Nitrogen Cycling in a Yellowstone Thermal Soil Environment

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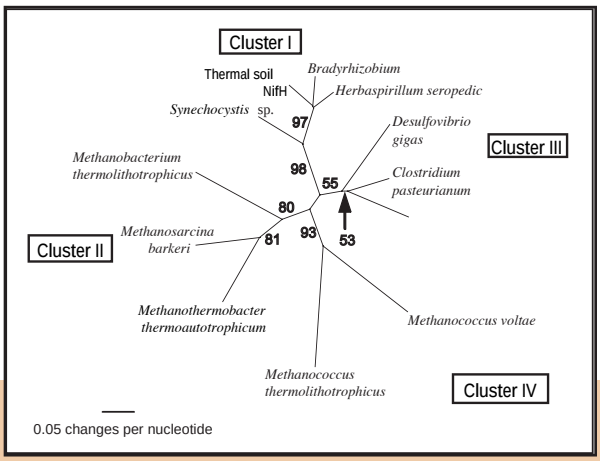

## ABSTRACT

Biogeochemical and functional gene experiments examined the nitrogen (N) cycle in geothermally heated soil (65-92°C, pH 3.9) located near the confluence of Rabbit Creek and the Firehole River in Yellowstone National Park (YNP). Nitrogen transformations examined included $N_2$ fixation, ammonification, nitrification, and denitrification. Aerobic incubations at 65°C yielded a net ammonification potential estimate of 0.19 μmoles $NH_4^+$ g-soil$^{-1}$ • h$^{-1}$. As measured at 50°C, 65°C, and 80°C, denitrification potential ranged from 0.17 to 0.54 nmol $N_2O$ • g-soil$^{1}$ • h$^{-1}$. Neither $N_2$ fixation (acetylene reduction) nor net nitrification ($NH_4^+$ oxidation) was observed at these incubation temperatures. Using total soil RNA, no RT-PCR products were obtained with primers designed for *nifH*, *amoA*, *nirK*, or *nirS* amplification. However, PCRs with the same primers and DNA extracted from the thermal soil yielded amplicons having significant homology to *nifH* and *nirK* genes. In summary, our research suggests that microorganisms present in this thermal soil are capable of some N transformations, but evidence for a complete N cycle was not obtained.



***Key Words***

denitrification
geothermal
*nifH*
nitrogen cycling
soil

## 1.0 INTRODUCTION

Most thermophilic research in Yellowstone National Park (YNP) has focused on aquatic environments, targeting either high temperature pools or springs, and their outflow channels and terraces. Examples include Dave Ward's community ecology work in circumneutral-alkaline Octopus, Mushroom, and Twin Vista Butte Springs, which are located in the Lower Geyser Basin area (e.g., Weller et al. 1992; Ferris et al. 1996; Nubel et al. 2002); and the diversity discovery work by Pace and colleagues in the Mud Volcano area (Barns et al. 1994; Hugenholtz et al. 1998). By contrast, relatively few YNP studies have examined geothermally heated soils. Most probable number techniques and direct microscopic observations were used to study *Thiobacillus* and *Sulfolobus* in YNP solfataras (Fliermans and Brock 1972), and more recently, Norris et al. (2002) studied the effect of sudden soil temperature increases on soil microbial community structure at Ragged Hills, Norris Geyser Basin. Additionally, while developing novel PCR methodology involving environmental RNA, we have documented significant archaeal and bacterial novelty and diversity in the steam-heated soil used for the current study (Botero et al. 2005). From such studies we have concluded that the microbial diversity and novelty in YNP's heterogeneous soil environments is similar to that observed elsewhere in YNP aquatic environments.

These thermal soils studies are but a few of the now numerous reports documenting microbial diversity and novelty in geothermal environments. However, there have been far fewer studies on functional processes of thermophilic microbial communities. Sulfur cycling in deep-sea vents has received significant attention, primarily because of the prominence of reduced sulfur species in the vent environment (see reviews by Karl 1995 and Reysenbach et al. 2002), but our understanding of other nutrient transformations, such as N, in thermal environments is not as advanced. *In vitro* studies of $N_2$ fixation in thermophilic cyanobacteria found optimum temperatures for nitrogenase ranged from about 45°C for *Mastigocladus laminosus* (Miyamoto et al. 1979), to 45-50°C for *Heliobacterium modesticaldum* (Kimble et al. 1995), to 55°C for *Chlorogloeopsis* (Thomsen and Cox 1993). In each case, however, nitrogenase activity was either greatly reduced or undetectable at 60°C. *In situ* measurements have documented $N_2$ fixation at up to 60°C in several different neutral or alkaline (but not acidic) YNP thermal springs (Wickstrom 1984). At present, the upper limit for $N_2$ fixation appears to be approximately 64°C (Belay et al. 1984), which is also near the upper limit for photosynthesis, suggesting that the major biological mechanisms for capturing gaseous C and N are restricted by temperature.

Nitrification is typically regarded as the oxidation of $NH_4^+$ to $NO_2^-$, and of $NO_2^-$ to $NO_3^-$, by obligate aerobic autotrophs (Myrold 1998). However, some research may suggest different metabolic routes of N oxidation in thermal environments. Mevel and Prieur (1998) were unable to recover autotrophic nitrifiers from marine hydrothermal vents in laboratory enrichments above 40°C, but they were able to culture heterotrophs capable of producing $NO_2^-$ (but not $NO_3^-$) from organic matter, with peak activity occurring around 60°C and no activity above 75°C. Pel et al. (1997) were also unable to culture autotrophic nitrifiers at temperatures above 40°C, and attributed high temperature nitrification in a composting study to heterotrophic methanotrophs that simultaneously nitrified and denitrified.

Denitrification involves the sequential reduction of $NO_3^-$ to $N_2(g)$:

$$NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The enzymes sequentially involved include nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase, respectively (Myrold 1998). Research into denitrification among thermophiles has generally suggested piecemeal denitrification, i.e., thermophiles capable of some, but not all, of the denitrification steps. Gokce et al. (1989) identified a marine thermophilic *Bacillus* sp. that reduced either $NO_2^-$ or $N_2O$, and Ho et al. (1993) found that $NO_3^-$ reductase, $NO_2^-$ reductase, and NO reductase in *Bacillus stearothermophilus* were all active at 60°C. The apparent upper temperature limit for denitrification is considerably higher than that described above for $N_2$ fixation and nitrification. Chen et

al. (2002) isolated hot spring thermophiles that were able to reduce $NO_2^-$ (but not $NO_3^-$) to $N_2O$ (but not to $N_2$) at 50°C. *Thermus* and *Bacillus* isolates capable of reducing $NO_3^-$ and $N_2O$, respectively, at 70°C were cultured from thermal springs in Iceland (Hollocher and Kristjansson 1992). And, as an example of denitrification at even higher temperatures, purified nitrate reductase from the hyperthermophilic archaeon *Pyrobaculum aerophilum* displayed optimal activity at 95°C (Afshar et al. 2001).

As mentioned above, our initial investigations of microbial diversity in geothermally heated soils suggest the existence of novel and diverse archaea and bacteria (Norris et al. 2002; Botero et al. 2005), and have resulted in the isolation and characterization of novel genera (Botero et al. 2004) and species (Schäffer et al. 2004). During the course of these studies with high temperature soils, we were intrigued by the high concentrations of $NH_4$ in one location, and viewed this as an opportunity to assess whether the phylogenetic novelty (based on PCR-derived clones) might also translate into novel N cycling observations at high temperature. Here we report our observations derived from biogeochemical and functional gene experiments aimed at investigating $N_2$ fixation, ammonification, nitrification, and denitrification in one of these geothermal soils.

## 2.0 MATERIALS AND METHODS

### 2.1 Soil Description and Sampling

The soil examined in this study is located at the Fairy Falls trailhead near the confluence of Rabbit Creek and the Firehole River, YNP (Botero et al. 2005). Functional gene experiments included work with both RNA and DNA. For nucleic acid extracts, soil samples were flash frozen within minutes of sampling. When sampling for biogeochemical assays, soils were transported back to the laboratory in sterile tubes suspended in heated water contained in a Thermos® bottle. Primary dissolved inorganic constituents of a saturated paste extract were determined using either inductively coupled plasma emission spectrophotometry or ion chromatography. All analytical methods have been described previously (Page et al. 1982; Sparks et al. 1996).

### 2.2 Nucleic Acid Extraction

Soil samples for both RNA and DNA analysis were collected with autoclaved spatulas and mixed approximately 1:1 with sterile distilled water in sterile disposable 50 mL tubes. Aliquots (0.5 mL) of the slurry were dispensed into sterile 2.0 mL screw cap microcentrifuge tubes and then flash frozen in liquid nitrogen. Samples were transported to the laboratory on dry ice and stored at -80°C until RNA or DNA was extracted. For RNA extraction, acid-washed glass beads (0.5 g, Sigma, 106 μm), 33.3 μL 20 % SDS, 167 μL 3% diatomaceous earth (Sigma), and 583 μL Tris-HCl buffered phenol (pH 8.0) were added to frozen soil slurry samples, which were then rapidly thawed in warm water. The sample was shaken for 45 sec on a bead mill and centrifuged at 5°C for 15 min at 14,000 x g. The aqueous layer was transferred to a fresh tube to precipitate the nucleic acids at -20°C with 3M sodium acetate and 95% ethanol. After centrifugation (14,000 x g), the nucleic acid pellet was washed with 70% ethanol and suspended in 100 μL nuclease-free water. RNA was further purified and treated with DNase using the SV Total RNA Isolation System (Promega). The relative purity and integrity of the column-eluted RNA was verified in 1% denaturing agarose gels containing formaldehyde (0.66% w/v) as the denaturant.

DNA was extracted from flash-frozen soil in 2.0 mL screw cap microcentrifuge tubes using the FastDNA SPIN Kit for Soil (Qbiogene), following the manufacturer's protocol with one modification: humics were effectively removed by several successive washes of the binding matrix-DNA complex with 5.5 M guanidium thiocyanate until the dark humic color had been removed. DNA yield was estimated by gel electrophoresis, and DNA preparations were stored at -80°C.

### 2.3 Functional Gene PCRs

Specific genes encoding enzymes involved in the nitrogen cycle were selected for reverse transcriptase (RT)-PCR and PCR amplifications. We targeted *nifH* (encodes dinitrogenase reductase) for nitrogen fixation using the degenerate primers described by Rosado et al. (1998) that are inclusive for all *Bacteria*-type *nifH* genes. For nitrification, we focused on *amoA* (codes for ammonium monooxygenase; primers described by Sinigalliano et al.,

1995). For nitrite reductase, *nirK* and *nirS* were targeted (primers nirK1F/nirK5R and nirS1F/nirS6R described by Braker et al. 1998). At the time these RT-PCRs were conducted, these primers were designed from genes coding for characterized enzymes from cultivated organisms.

RT-PCRs were carried out using Promega's Access RT-PCR System. The 50 µL reaction contained 2 µL total RNA, 10 pmol of each primer, 1X avian myeloblastosis virus (AMV)/*Tfl* Reaction Buffer, 2.5 mM $MgSO_4$, 50 mM of each dNTP, 5 U of AMV RT, and 5 U of *Tfl* DNA Polymerase. BSA (5 µg) was included as a nonspecific inhibitor-binding protein in the reactions (e.g., to sequester any remaining humics). The RNA, primers, and BSA were first incubated for 5 min at 94°C to denature the template RNA, snap cooled on ice for 5 min, followed by addition of remaining reactants. Samples were incubated at 48°C for 45 min, then the AMV RT was inactivated by incubating the samples at 94°C for 2 min. Second-strand cDNA and PCR amplification were conducted using 25 cycles of 94°C for 30 sec, 55°C for 1 min, and 68°C for 2 min, with a final cycle of 7 min at 68°C. Absence of DNA contamination was verified by conducting the same reactions without the AMV RT. In addition, template-free RT-PCR controls did not yield bands in either agarose or denaturant gradient acrylamide gels, providing evidence that the RT-PCRs were not contaminated with nucleic acids from either the BSA lots or chemical reagents used. All RT-PCRs were performed using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems).

For each of the DNA-based PCRs with the same primers used in the RT-PCRs, the 50 µL reaction mix consisted of (final concentration or amount): 50 mM KCl, 2.0 mM $MgCl_2$, 20 mM Tris-HCl [pH 8.4], 200 µM each dNTP, 2 µM each primer, 2.5 U Taq polymerase, and 0.5–5 µL of the DNA preparation. The thermal cycling protocol consisted of an initial denaturation step for 2 min at 94°C, followed by 25 cycles of: (i) denaturation at 94°C for 45 sec; (ii) annealing at 55°C for 45 sec; and (iii) extension at 72°C for 60 sec and one final extension step of 72°C for 7 min. PCR products were separated by gel electrophoresis and visualized by staining with ethidium bromide. Amplicons were cloned into pCR2.1-TOPO, and transformed into *E. coli* TOP10 as described by the manufacturer (Invitrogen). Cloned fragments were sequenced using an ABI310 DNA sequencer (Applied Biosystems), and synthetic primers complementary to the vector plasmid sequences flanking the multiple-cloning site.

### 2.4 Nitrogen Cycle Biogeochemical Assays

***2.4.1 Nitrogen fixation.*** Nitrogenase activity in the thermal soil was measured using the acetylene reduction assay as described previously by Weaver and Danso (1994). Ten gram (fresh wt.) samples of thermal soil were collected and immediately added to 20 mL serum bottles, which were plugged with a butyl rubber stopper and crimp-sealed with an aluminum ring. Approximately 1/10 of the headspace volume was replaced with pure acetylene. Assays were performed in triplicate, and bottles were kept between 50°C and 65°C during transport to the laboratory (starting temperature of water in the bottle was 65°C, but then cooled to 50°C during transit), where they were further incubated at 55°C for 24 hours. Ethylene concentration in the headspace was then measured by gas chromatography.

***2.4.2 Net mineralization and net nitrification.*** Net N mineralization and nitrification in the thermal soil were measured as net changes in the $NH_4^+$ and $NO_3^-$ pools, respectively. Flasks containing about 50 g soil (fresh wt.) were amended to initial target concentrations of 550 µg $NH_4^+$ and 150 µg $NO_3^-$-N per gram soil dry weight (in preliminary experiments $NO_3^-$-N was undetectable in the soil); the soil was adjusted to about 30% moisture (g $H_2O$ • $g^{-1}$ dry soil); and the soils were incubated aerobically at 65°C for 10 days. Lost moisture (gravimetric determination) was replaced daily with mixing that also kept the soil samples well aerated. Flasks were sampled periodically for $NH_4$-N and $NO_3$-N analysis by extracting soil in 2 N KCl (1 g dry soil:10 mL solution) on a shaker for 1 hour followed by filtration through Whatman No.42 filter paper. Ammonium and $NO_3$-N in the filtrate were measured by standard colorimetric methods (Bundy and Meisinger 1994). Flasks were incubated in triplicate along with autoclave-killed controls.

***2.4.3 Denitrification.*** Denitrification potential of the thermal soil was measured in laboratory incubations at

50°C, 65°C, and 80°C using the acetylene block assay (Tiedje 1994). The presence of acetylene during incubation blocks the final step of denitrification (the reduction of $N_2O$ to $N_2$) such that denitrification potential is measured as the evolution of $N_2O$. Soil samples (3 g, dry wt. basis) were transferred to 60 mL serum bottles and suspended with 30 mL sterile distilled water. Serum bottles were plugged with butyl rubber stoppers and crimp-sealed with aluminum rings. Filter-sterilized dextrose and $KNO_3$ were added through the septum (1 mM final concentration, each), the headspace (7.5 mL) was flushed with $N_2$(g), and then 1/10 volume was removed and replaced with pure acetylene. The bottles were incubated for 48 hours at the specified temperature, and the evolution of $N_2O$ was measured by gas chromatography. In addition, controls (for each temperature) included non-amended soil, amended soil without acetylene, soil amended with dextrose but not $KNO_3$, and soil amended but autoclaved.

## 3.0 RESULTS

### 3.1 Soil Characterization

The thermal soil studied is a loam (**Table 1**) located in a small depression (~1.5 m diameter; **Figure 1**) heated by an underground source of steam that has been active for at least 7 years (unpublished data). During that same time period, the pH of the thermal soil has been found to be

**Table 1. Summary of primary chemical and physical features of the soil used in this study.** Values for $NH_4$, $NO_3$, Ca, Fe, and $SO_4$ correspond to aqueous concentrations of 1:1 soil: distilled $H_2O$ extracts. $NH_4^+$ -N and $NO_3^-$ -N were determined from KCl extracts.

| Soil Feature (unit) | Value |
|---|---|
| pH | 4.0 |
| Electrical conductivity (mmhos/cm) | 0.48 |
| Cation exchange capacity (mmol/g) | 0.07 |
| Organic C (g/kg) | 54.7 |
| Total Kjeldahl N (g/kg) | 2.9 |
| $NH_4^+$ -N (mg/L) | 151-800* |
| $NO_3^-$ -N (mg/L) | 0.20 |
| Ca (mg/L) | 117 |
| Fe (mg/L) | 8 |
| $SO_4$ (mg/L) | 528 |
| Sand (%) | 55 |
| Silt (%) | 31 |
| Clay (%) | 14 |

* Range of concentrations measured from several independent samplings.


**Figure 1. Photograph of extreme thermal soil site located near the Fairy Falls trailhead parking lot.** Arrow notes the lush stand of the thermotolerant grass *Dicanthelium* growing directly adjacent to the sampling area (circled with a dashed line), which is too hot for plant growth.

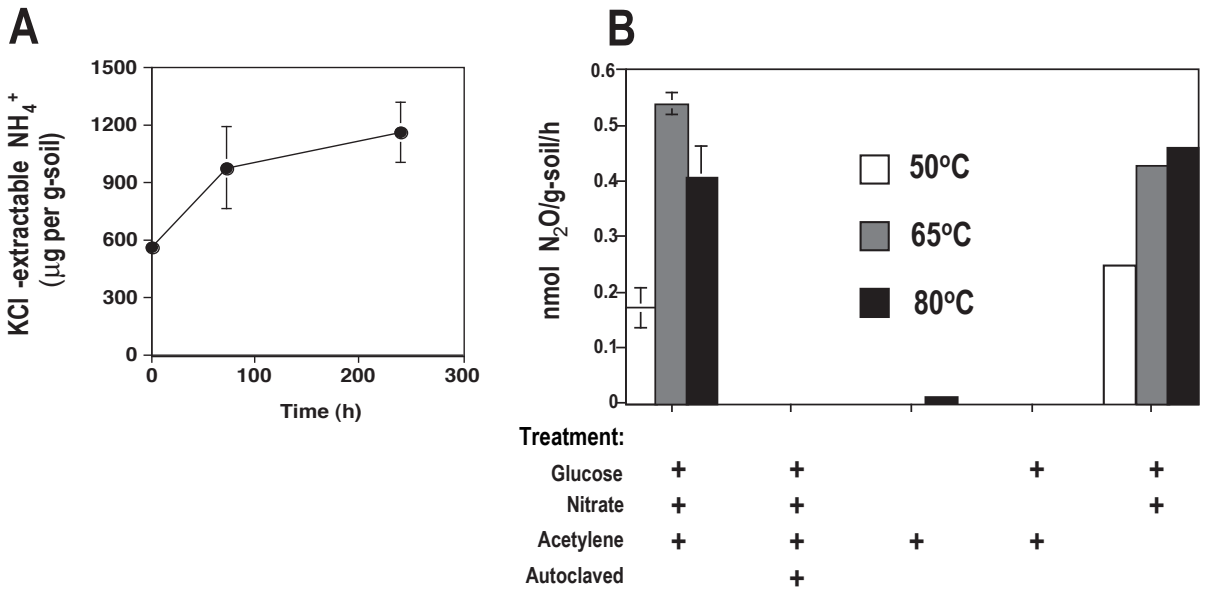


**Figure 2. Ammonification and denitrification potential as measured with *in vitro* incubated geothermal soil samples.** **A**. Ammonium accumulation as a function of time (hours) and incubation at 65°C. Data represents the mean ±1 standard error from three replicate soil samples. **B**. Nitrous oxide production *in vitro* at three different temperatures (indicated by bar color) and with varying amendments as indicated by the treatment and "+" below the panel. The treatment receiving glucose, nitrate, and acetylene was measured with three replicates (and ±1 standard error bar as shown). All other treatments were considered as controls and were measured with single replicate soil samples.

relatively constant at 3.9-4.0 (**Table 1**). Typical of many YNP thermal soils, the steam venting at this site carries $H_2S$ (strong "rotten-egg" odor) that can be abiotically and or biotically oxidized to $S^0$. Further oxidation to $SO_4^-$ by thermophilic chemolithotrophs contributes to additional acidification, resulting in low pH and high $SO_4^-$ (**Table 1**). Unpredictable changes in steam vent channels within the depression cause shifting temperature gradients and thus killing of some heat-sensitive plant species growing close to the depression (tolerant of up to ~40°C), but can also result in the proliferation of thermotolerant species such as *Dicanthelium* (Stout et al. 1997; **Figure 1**). At the time of sampling for this study, soil temperatures were 65°C at the surface, and increased to 92°C at a depth of approximately 12 cm. The soil was very wet (though not submerged), which we attributed to the continual condensation of geothermal steam near the soil surface.

Soil $NH_4^+$ concentrations ranged from 151 mg to 800 mg • $kg^{-1}$ soil (varied between sampling times in different years; **Table 1**). The high available N content may have contributed to the lush growth of *Dicanthelium* immediately adjacent to the heated soil (**Figure 1**), and presumably would offer similar nutritional benefits to the resident microbial community. Soil paste extracts did not contain elevated levels of Zn, Cu, Mo, B, Cd, Cr, Ni, As, Pb, Co, or Se (data not shown).

### 3.2 Biogeochemical Measurements

We were interested in exploring the source and fate of the very high $NH_4^+$ levels in the geothermal soil. Laboratory aerobic incubations of the thermal soil at 65°C demonstrated that organic matter mineralization (or perhaps aerobic nitrogen fixation; e.g., *Streptomyces thermoautotrophicus*, see Discussion) could contribute to the significant amounts of $NH_4$-N found in this soil. Acetylene reduction assays (65°C) with anaerobically-incubated soils failed to detect measurable $N_2$ fixation (no detectable ethylene in GC analysis, results not shown). However, aerobic

laboratory incubations resulted in significant *apparent* ammonification. Ammonium accumulation occurred at a rate of approximately 0.32 μmoles $NH_4^+ \cdot g\text{-}soil^{-1} \cdot h^{-1}$ (~ 7.68 μmoles $NH_4^+ \cdot g\text{-}soil^{-1} \cdot d^{-1}$) during the initial 3 days of incubation (**Figure 2A**), and continued to accumulate during the next 7 days of incubation, but at a lower rate of 0.069 μmoles $NH_4^+ \cdot g\text{-}soil^{-1} \cdot h^{-1}$.

We were also unable to document $NO_2^-$ or $NO_3^-$ formation in aerobically incubated thermal soil samples at any temperature examined (results not shown), suggesting that even though abundant substrate was available (see $NH_4^+$ levels, **Table 1**) for potential thermophilic nitrifiers to proliferate, meaningful nitrification likely does not occur *in situ*, even at the lower temperatures examined. Our inability to measure nitrification is consistent with low levels of $NO_3$-N (**Table 1**), which was always measured at <1 mg $\cdot$ $kg\text{-}soil^{-1}$. Anaerobic oxidation of $NH_4$ was not measured.

Denitrification ($N_2O$ formation) was observed at all three incubation temperatures in fully amended soils, but was essentially undetectable in autoclaved soil, unamended soil, or in soil amended with glucose but not $NO_3^-$ (**Figure 2B**). The addition of acetylene did not greatly affect $N_2O$ formation, suggesting that the final step in denitrification, the reduction of $N_2O$ to $N_2$, does not occur in the resident thermophile populations or is perhaps inhibited by low pH (Myrold 1998).

### 3.3 Functional Genes

The same components of the N cycle were also examined from the perspective of functional gene expression. No RT-PCR products were obtained with RNA preparations with any of the primer sets used for amplifying *nifH*, *amoA*, *nirS*, or *nirK*. However, we did verify that the RNA preparations were intact and were suitable for RT-PCR templates by showing that we could readily and reproducibly obtain strong amplicons with primers specific for *Bacteria* and *Archaea* (Botero et al. 2005).

We then examined the genetic potential for each component of the N cycle by using the same primer sets with DNA templates and PCR. PCRs using *nifH* primers yielded an amplicon of expected size (**Figure 3A**), although the reverse primer was apparently involved in significant non-specific priming as judged by the occurrence of numerous nonspecific PCR products associated with the use of that primer by itself (**Figure 3A**). Cloning and sequencing of the 365 bp amplicon revealed a gene fragment with an inferred amino acid sequence displaying excellent homology with other NifH proteins, including two conserved *nifH* family motifs (alignment not shown). Phylogenetic analysis of the inferred amino acid sequence of the cloned *nifH* fragment showed that it grouped in the *nifH* cluster I clade (Chien and Zinder, 1996; Zehr et al. 2003) comprised of the Mo-containing nitrogenases in cyanobacteria and α- and β-proteobacteria (**Figure 3B**).

PCRs with *nirK* primers also amplified gene fragments with inferred amino acid sequences sharing significant identity with several characterized nitrite reductases, including those from *Rhodopseudomonas palustris* (80% identity; 89% similarity), *Alcaligenes* sp. (75%; 87%), and *Sinorhizobium meliloti* (75%; 84%; **Figure 3C**). Attempts to PCR amplify *nirS* and *amoA* from the thermal soil DNA preparations were unsuccessful (results not shown).

## 4.0 DISCUSSION

Soils are one of the greatest sources of microbial diversity in the biosphere due to their heterogeneous chemical and physical properties (Torsvik et al. 1990). Because of such heterogeneity, geothermally heated soils contain environments that could potentially support various microbial physiologies, including transformations of N. The thermal soil studied in these experiments receives a constant source of steam, which heats the soil and also results in a high moisture content (~50% gravimetric moisture content) that would promote water film continuity between and among soil aggregates and help ensure thermal equilibrium throughout. The geothermal steam includes significant loads of $H_2S$ (Fournier 1989), which when oxidized to sulfate in the more aerobic zones of the soil profile (i.e. near the soil surface) result in significant acidification that may have important selective effects on microbial populations and impact soil chemical properties.

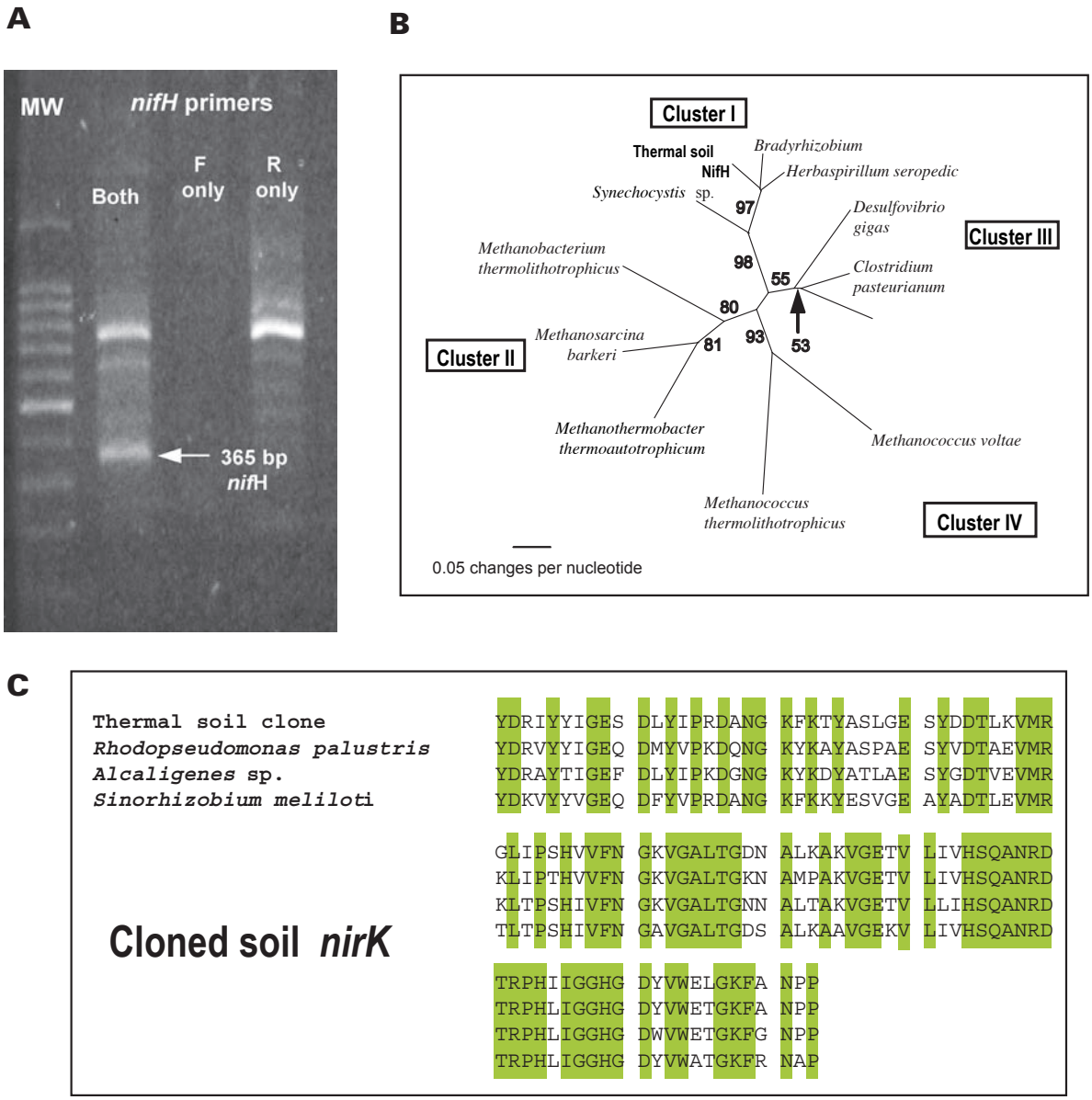


**Figure 3. PCR amplification and identification of functional genes coding for enzymes important in $N_2$ fixation and denitrification.** **A.** Agarose gel showing apparent non-specific PCR amplification with the *nifH* reverse primer and identification of a bona fide *nifH* fragment amplicon. **B.** Neighbor-joining tree showing the phylogenetic relationship of the inferred amino acid sequence of the thermal soil *nifH* PCR clone shown in panel A relative to *nifH* genes characterized in different prokaryotes and that comprise recognized *nifH* phylogenetic clusters. Bootstrap values (from 500 pseudoreplicates) are shown at each branch. **C.** Alignment of the inferred amino acid sequence of the *nirK* fragment PCR-cloned from the thermal soil total DNA extracts along with the characterized *nirK* from *R. palustris*, *Alcaligenes* sp., and *S. meliloti*. Regions of consensus amino acid sequence across all sequences are highlighted in green blocks.

Acetylene reduction assays showed no evidence for N fixation in this thermal soil and were consistent with lack of *nifH* cDNA amplicons from RNA templates. However, the genetic potential for $N_2$ fixation was detected based on a partial *nifH* amplicon derived from DNA templates. Whether the cloned *nifH* was from the resident thermophile community, or from non-thermophiles that could potentially have been washed in from the surrounding low temperature soils (but then heat killed), is not known. The temperatures recorded at this site (65–92°C) are higher than those at which $N_2$ fixation has generally been documented (Miyamoto et al. 1979; Wickstrom 1984; Belay et al. 1984; Thomsen and Cox 1993; Wahlund and Madigan 1993), and perhaps offers an explanation for our inability to detect acetylene reduction or to RT-PCR amplify *nifH*. In addition, the high native $NH_4^+$ concentrations would likely inhibit $N_2$ fixation, both at the transcriptional level and as an enzyme inhibitor as documented for *H. modesticaldum* (Kimble et al. 1995). Alternatively, $N_2$ fixation of the type observed for the *S. thermoautotrophicus* nitrogenase may contribute N to this soil. In contrast to the inhibitory effects of $NH_4^+$ on expression and function of most nitrogenase enzymes, the *S. thermoautotrophicus* nitrogenase has been reported to be insensitive to both $NH_4^+$ and $O_2$, will not reduce acetylene, and is active at least as high as 65°C (Ribbe et al. 1997). This might account for some of the $NH_4^+$ occurring in this particular soil, and would have been undetectable using the acetylene reduction employed in this study.

Another potential source of the $NH_4^+$ in this soil may be the steam itself, as ammonia is often found in YNP geothermal steam (Fournier 1989). However, laboratory aerobic mineralization experiments showed that even in the absence of a geothermal steam source, $NH_4^+$ levels doubled during the 10-day incubation (**Figure 2A**), showing that microbial activity could contribute significantly to the $NH_4$ pool. This soil contained significant amounts of organic carbon (5.5% total organic C), which may derive from the infrequently introduced bison dung (unpublished observations), or alternatively may come from microbial biomass turnover. Mineralization of these organic matter sources may contribute to the ammonification observed in this soil.

We were also unable to amplify *amoA* from the thermal soil RNA or DNA. Although clearly not conclusive evidence (e.g., primer design may have been inappropriate), this agrees with the negative results obtained with our attempts to document nitrification activity. Neither $NO_2^-$ nor $NO_3^-$ accumulation was observed in the aerobic incubations and is consistent with the relatively low concentrations of $NO_3^-$ measured in this soil (**Table 1**). The source of the low levels of $NO_3^-$ in this soil is not known. If it is generated from heterotrophic mineralization of organic matter similar to that described by Mevel and Prieur (1998), then it occurs at rates slower than what we could detect in our assays.

*In situ* denitrification activity could also contribute to maintaining relatively low $NO_3^-$ levels in this high temperature soil, immediately consuming any products of nitrification (Pel et al. 1997). Significant denitrification potential was observed (**Figure 2B**), and is consistent with the observed soil conditions, including high moisture content, elevated temperature contributing to reduced $O_2$ solubility, and a potential source of electron donors (high organic C). While *nirK* sequences could be recovered from PCRs using DNA templates (**Figure 3B**), we were unable to document *in situ nirK* expression using RT-PCR with the same primers and purified soil RNA. Failure to RT-PCR amplify functional genes from soil nucleic extracts was not necessarily due to poor RNA template preparations, as the same RNA preparations were used for amplifying the rRNA cDNA clones. Thus, we tentatively conclude that expression of the targeted genes either was not occurring in the soil or was at undetectably low levels at the time of sampling. It is also possible that N functional genes present in this soil were not suitable targets for the primer sets employed. We are currently developing transcriptomics techniques aimed at more directly assessing prokaryote gene expression *in situ*.

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