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Nitrite oxidation in geothermal springs: evidence of an upper temperature limit for thermophilic nitrite-oxidizing bacteria of 60 - 65 °C

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NITRITE OXIDATION IN GEOTHERMAL SPRINGS: EVIDENCE OF AN UPPER TEMPERATURE LIMIT FOR THERMOPHILIC NITRITE-OXIDIZING BACTERIA OF ~60 - 65 °C

by

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ABSTRACT

Nitrite oxidation in geothermal springs: evidence of an upper temperature limit for thermophilic nitrite-oxidizing bacteria of ~60 - 65 °C

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Despite growing evidence of the importance of nitrification in terrestrial geothermal environments, little is known about nitrite oxidation in these environments. In order to further our knowledge, this study combined cultivation-dependent and -independent approaches with measurements of nitrogen speciation along the outflow channels of two Great Basin geothermal springs. Enrichment cultures were inoculated with sediment slurries from sites ranging in temperature from 42 – 87 °C at the sources and along the outflows of >15 hot springs. While attempts to enrich nitrite-oxidizing bacteria (NOB) from sites ≥61°C were unsuccessful, NOB were enriched from five hot springs located in U.S. Great Basin, southwestern China, and Armenia at sites <58 °C. All enrichments analyzed contained organisms with ≥97% 16S rRNA gene identity to *Nitrospira calida*, regardless of origin, demonstrating the wide geographic range of this organism. In addition, enrichments from Armenia contained organisms with ≥97% 16S rRNA gene identity to *Nitrospira moscoviensis*. Physiological properties were similar for all enrichments, with an upper temperature limit between 60 - 65 °C and a temperature optimum of 45 - 50 °C While the rates of nitrite oxidation were significantly different for the Great Basin enrichments when compared to the Tengchong and Armenian

enrichments at 50 °C (5.4 ± 2.2, 11.4 ± 3.3, and 11.6 ± 1.7 for Great Basin, Tengchong, and Armenian, respectively), the much higher rates observed for the Tengchong and Armenian enrichments may be attributed to improved cultivation conditions for the primary enrichments.

Patterns of nitrogen speciation in water samples collected along the outflow channels of two springs (Sandy's Spring West and Rick's Hot Creek) within the Great Boiling Spring (GBS) geothermal system suggested ammonia oxidation activity at ≥75.1 °C in both springs. In contrast, nitrite oxidation activity did not appear to be present at ≥65 °C in either spring. "*Candidatus* Nitrosocaldus yellowstonii" 16S rRNA gene copy numbers were abundant in sediment samples from the outflow of both springs at ≤79.6 °C, but *Nitrospira* 16S rRNA gene sequences were only abundant at ≤57.9 °C. Thus, an apparent difference in the upper temperature limit for ammonia oxidation and nitrite oxidation exists within the GBS system, decoupling the two steps of nitrification and leading to accumulation of nitrite above ~60 °C. In addition, 16S rRNA sequences belonging to known NOB were absent from 557,076 pyrotag sequences obtained from hot springs located in the U.S. Great Basin, and Tengchong, China at temperatures ≥55 °C and no significant matches for entire NOB genome sequences were found in the ~250 Mbp of metagenomic data from GBS environmental samples at 77 to 85 °C. In addition, no significant matches for entire NOB genome sequences were found in the 557 Mbp of metagenomic data from Yellowstone National Park environmental samples obtained from 16 springs at temperatures ranging from 52.9 to 90 °C. This study presents evidence that the upper temperature limit for nitrite oxidation in geothermal systems worldwide may be similar to the upper temperature limit observed within the GBS system and demonstrates

the wide geographic range of *Nitrospira* spp. in geothermal environments. Finally, we propose that the temperature-driven decoupling of ammonia oxidation and nitrite oxidation leads to a high temperature nitrite shunt in the nitrogen cycle whereby nitrite produced by ammonia oxidation is used directly by denitrifiers in geothermal ecosystems. We propose that the high temperature nitrite shunt is complete at temperatures exceeding ~65 °C, with greater flow of nitrogen through nitrate with decreasing temperature below ~65 °C.

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CHAPTER 1

INTRODUCTION

As a critical element for the synthesis of proteins, nucleic acids, and other forms of biomass, nitrogen is essential for all living organisms. In nature, nitrogen exists mainly in the following oxidation states: -3 (NH_3), 0 (N_2), +1 (N_2O), +2 (NO), +3 (NO_2^-), +4 (NO_2), and +5 (NO_3^-). Due to chemical instability and transformations by living organisms, each oxidation state is transient. Considering the major nitrogen transformations together, the processes form an integrated cycle in which the product of one process becomes the substrate for another (Figure 1.1). Since many of the reactions are catalyzed exclusively by microorganisms, the nitrogen cycle is largely dependent on these organisms. The steps of the nitrogen biogeochemical pathway can be classified as assimilatory pathways, dissimilatory pathways, or waste pathways. Assimilatory pathways, such as N_2 fixation, ammonia assimilation, and coupled nitrate/nitrite assimilation and reduction, provide reduced nitrogen that is incorporated into cell material. In contrast, dissimilatory processes such as nitrification and denitrification produce free energy from the oxidation and reduction of nitrogenous compounds. Finally, the waste process of ammonification involves the biological removal and excretion of ammonia from nitrogen-rich compounds such as proteins or amino acids.

The availability of a suitable nitrogen source often limits primary productivity in natural environments. The largest reservoir of nitrogen on Earth is N_2, which is not readily available to most organisms. By microbial nitrogen fixation, N_2 is reduced to NH_3, thereby making the nitrogen accessible to other organisms for metabolic processes

or assimilation. NH_3 is also produced during the decomposition of organic nitrogen compounds in a process called mineralization or ammonification. The NH_3 produced is then available for nitrifying microorganisms that catalyze the oxidation of NH_3 to NO_3^- with NO_2^- as an intermediate via the two step nitrification process. During the first step of nitrification, ammonia-oxidizing microorganisms use NH_3 as an electron donor and O_2 as an electron acceptor and produce NO_2^- as a waste product. In the second step of nitrification, nitrite-oxidizing bacteria use NO_2^- as an electron donor and O_2 as an electron acceptor and produce NO_3^-. The waste products of nitrification (NO_2^- and NO_3^-) may be used as respiratory electron acceptors in environments with limited oxygen availability. A diverse group of microorganisms couple the anaerobic oxidation of organic carbon to the reduction of NO_2^- and NO_3^- producing either NH_4^+ in a process known as dissimilatory nitrate reduction to ammonium (DNRA) or the gaseous products NO, N_2O or N_2 during denitrification. In addition, members of the phylum Planctomycetes couple the oxidation of ammonia (electron donor) to the reduction of NO_2^- (electron acceptor) to produce N_2 (waste product) in anaerobic ammonia oxidation (anammox).

This cycle of coupled oxidation/reduction reactions forms an electron pool that is cycled among members of the microbial community that are often spatially separated (Reviewed in Falkowski *et al*., 2008). While oxidative processes are carried out in oxic environments, reductive processes are often limited to anoxic environments. In addition, while many of the organisms that catalyze the reduction of N species are heterotrophic and therefore require an environment with an available organic carbon source, autotrophic ammonia- and nitrite-oxidizing microorganisms fix their own carbon thereby

alleviating the need for available organic carbon. Furthermore, environmental constraints, such as low and high temperatures or pH, can cause spatial separation since some organisms are better equipped to deal with these constraints, as has been observed and manipulated in waste water treatment operations (Li *et al*., 2012).

This project focuses on nitrification. The organisms that catalyze the two steps of nitrification are distinct and, with the exception of the demonstration of a spatial-temporal co-occurrence of these organisms in some habitats (Santoro *et al*., 2010; Xia *et al*., 2011), research into the relationship between the two groups in natural environments is lacking (Hatzenpichler, 2012). Therefore, greater insight into the relationship between ammonia-oxidizing microorganisms and nitrite-oxidizing bacteria is needed. In many environments the two steps of nitrification are tightly coupled and the NO_2^- produced by ammonia oxidation is immediately consumed by nitrite oxidizers (Reviewed in Bock and Wagner, 2007). However, this coupling of the ammonia oxidation and nitrite oxidation may not be universal. For example, in waste water management low O_2 conditions are used to create a nitrite shunt where the nitrification process is shortened, ending at NO_2^- production, which is subsequently reduced by denitrifiers (Ju *et al*., 2012). In addition, NO_2^- accumulation reported in some geothermal springs may suggest a decoupling of ammonia and nitrite oxidation in high temperature environments (Costa *et al*., 2009).

Despite several indications of the importance of nitrification in terrestrial geothermal environments (Costa *et al*., 2009, Dodsworth *et al*., 2011;, Dodsworth *et al*., 2012) and the cultivation of themophilic ammonia- and nitrite-oxidizing microorganisms (de la Torre *et al*., 2008; Ehrich *et al*., 1995; Hatzenpichler *et al*., 2012; Lebedeva *et al*., 2005; Lebedeva *et al*., 2008; Lebedeva *et al*., 2011), knowledge of nitrification, and more

specifically nitrite oxidation, in these environments is still scarce. This study utilizes several approaches such as culture-dependent and -independent methods, and geochemical analyses, to study nitrite oxidation in geothermal environments. The thermal constraints as well as the organisms involved in this second step of nitrification in high temperature environments are explored.

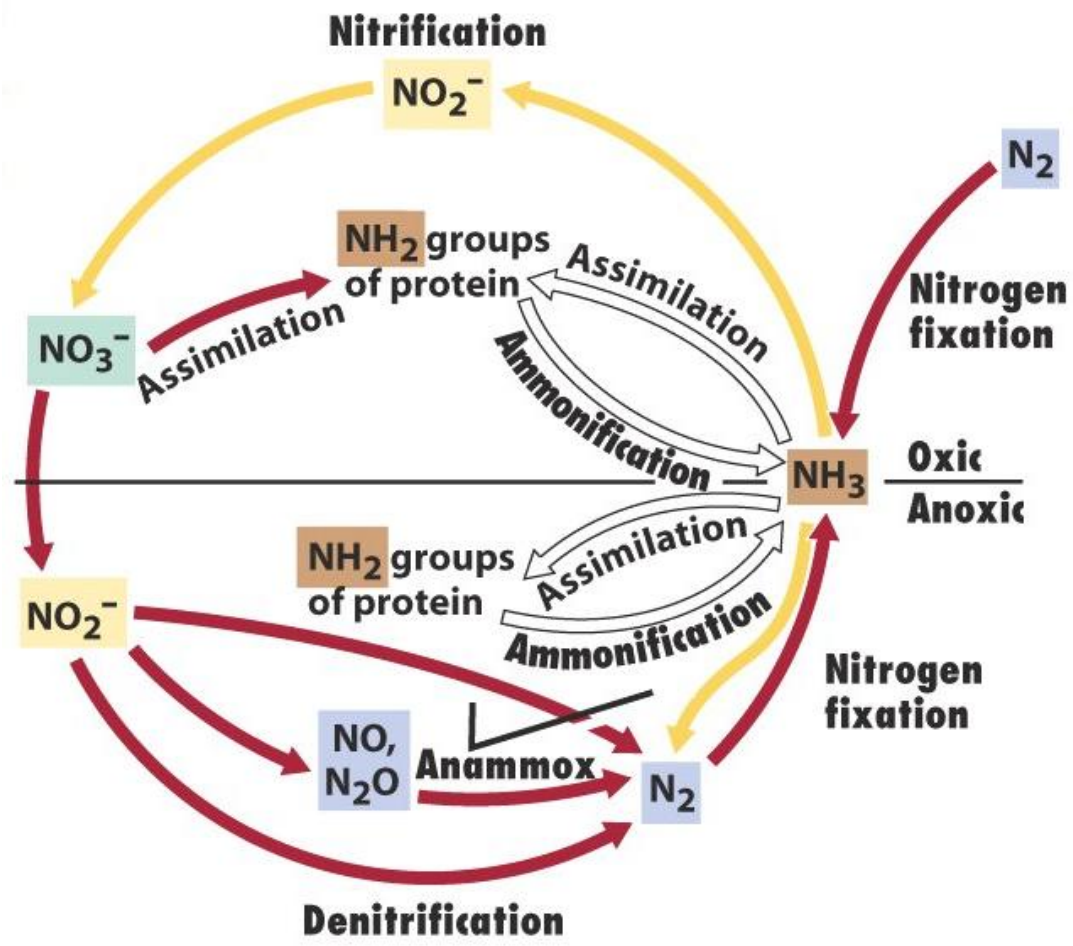

Figure 1.1. Nitrogen biogeochemical cycle. Yellow arrows indicate oxidative reactions, and red arrows indicate reductive reactions. White arrows indicate no change in oxidation state. Black line separates reactions that occur in oxic (top) and anoxic environments (bottom) (Madigan and Martinko, 2006).

CHAPTER 2

CULTIVATION OF *NITROSPIRA* SPECIES FROM GEOTHERMAL SPRINGS IN CHINA, ARMENIA, AND WESTERN U.S. AT ~60 - 65 °C: IMPLICATIONS FOR GLOBAL UPPER TEMPERATURE LIMIT OF CHEMOLITHOTROPHIC NITRITE OXIDATION

Abstract

Despite its importance in the nitrogen cycle, little is known about nitrite oxidation at high temperatures. In order to bridge this knowledge gap, enrichment cultures were inoculated with sediment slurries from a variety of geothermal springs. While attempts failed to enrich nitrite-oxidizing bacteria (NOB) from >10 hot spring samples at temperatures ≥61 °C, NOB were successfully enriched from the outflow of 7 hot springs located in U.S. Great Basin, southwestern China, and Armenia at temperatures ≤57.9 °C. The stoichiometric conversion of nitrite to nitrate, chlorate sensitivity, and the loss of activity after autoclaving all confirmed biological nitrite oxidation in these enrichments. Regardless of their origin, all successful enrichments contained organisms with high 16S rRNA gene sequence identity (≥97%) with *Nitrospira calida.* In addition, Armenian enrichments also contained organisms with high 16S rRNA gene sequence identity to *Nitrospira moscoviensis*. Physiological properties of all enrichments were similar, with a temperature optimum of 45 - 50 °C and an upper temperature limit between 60 °C and 65 °C. The highest rates of NOB activity occurred with initial NO_2^- concentrations of 0.5 - 0.75 mM; however, lower initial nitrite concentrations resulted in shorter lag times. The results presented here suggest a possible upper temperature limit of 60 – 65 °C for

chemolithotrophic nitrite oxidation and demonstrate the wide geographic range of *Nitrospira* spp. in geothermal environments.

Introduction

Nitrification, a two-step process that results in the production of nitrate from ammonia, is an important component of the nitrogen cycle. In the first step of nitrification, ammonia oxidation, ammonia is oxidized to nitrite, and in the second step, nitrite oxidation, nitrite is oxidized to nitrate. Since no known organism is capable of carrying out both steps of nitrification, it can be important to consider each step separately. The majority of research on nitrification has focused on ammonia oxidation, possibly because of the excitement surrounding the recent discovery of ammonia-oxidizing archaea (Könneke *et al*., 2005), the widely held assumption that ammonia oxidation is rate limiting in nature (Kowalchuk and Stephen, 2001), and the difficulty of cultivating nitrite-oxidizing bacteria (NOB) in the laboratory.

Known chemolithotrophic NOB belong to the genera *Nitrobacter*, *Nitrococcus*, *Nitrotoga*, *Nitrospina*, and *Nitrospira* (Alawi *et al*., 2007; Ehrich *et al*., 1995; Laudelout and Van Tichelen, 1960; Lebedeva *et al*., 2008; Lebedeva *et al*., 2011; Spieck *et al*., 2006; Watson *et al*., 1986; Watson and Waterbury, 1971). While *Nitrobacter* was traditionally considered to be an important NOB, several recent studies using cultivation-independent methods have indicated that *Nitrospira* may be more abundant than *Nitrobacter* in many environments (Altman *et al*., 2004; Altman *et al*., 2003; Hovanec *et al*., 1998; Schramm *et al*., 1999). Members of the genus *Nitrospira* represent a monophyletic group of NOB within the bacterial phylum Nitrospira (Spieck and Bock, 2001). They are found in a wide variety of natural habitats such as freshwater sediments

(Altman *et al*., 2004; Altman *et al*., 2003; Stein *et al*., 2001), soils (Attard *et al*., 2010; Bartosch *et al*., 1980; Noll *et al*., 2005), marine water (Watson *et al*., 1986), and geothermal springs (Kanokratana *et al*., 2004; Lebedeva *et al*., 2005; Lebedeva *et al*., 2011). Despite the tremendous ecological importance of *Nitrospira*, knowledge about this group of NOB is limited.

All *Nitrospira* are hypothesized to be K-strategists with high substrate affinity and low maximum growth rate (Huang *et al*., 2010; Kim and Kim, 2006; Nogueira and Melo, 2006; Schramm *et al*., 1999). Consistent with this hypothesis, *Nitrospira* tend to be adapted to low oxygen (Schramm *et al*., 1999) and nitrite availability (Nogueira and Melo, 2006; Schramm *et al*., 1999) and their growth can be inhibited by nitrite concentrations as low as 1.5 mM (Off *et al*., 2010). This property might be a contributing factor to the difficulty in cultivating *Nitrospira* in the laboratory. To date, there are only five formally identified members of this genus, with several lineages detected only by cultivation-independent censuses (Lücker *et al*., 2010). *Nitrospira marina* is a marine mesophile originally isolated from the Atlantic Ocean off the Gulf of Maine (Watson *et al*., 1986). "*Candidatus* Nitrospira defluvii" is a freshwater mesophile isolated from activated sludge (Spieck *et al*., 2006). *Nitrospira moscoviensis* and "*Candidatus* Nitrospira bockiana", both isolated from heating systems, were reported to be moderately thermophilic with growth temperature optima of 39 °C and 42 °C and growth temperature ranges of 33 - 40 °C and 28 - 44 °C, respectively (Ehrich *et al*., 1995; Lebedeva *et al*., 2008). Most recently, the most thermophilic NOB isolate known, *Nitrospira calida*, was isolated from Gorjachinsk Hot Spring in the Lake Baikal area of Russia and shown to

have a growth temperature optimum of 46 - 52 °C and an upper temperature for growth of 58 °C (Lebedeva *et al*., 2011).

Despite this progress, and more substantial progress on chemolithotrophic ammonia oxidation (de la Torre *et al*., 2008; Hatzenpichler *et al*., 2008; Jiang *et al*., 2010; Pearson *et al*., 2004; Reigstad *et al*., 2008; *et al*., 2008a), very little is known about the oxidative nitrogen cycle at elevated temperatures. This study focuses on the enrichment and characterization of thermophilic NOB from geothermal springs in the U.S. Great Basin, southwestern China, and Armenia.

Methods

Sample collection

Sediment slurries consisting of the the top ~1 cm of sediment along with spring water were collected into sterile 15 mL polypropylene tubes from the source and outflow of springs located in Great Basin, U.S., Tengchong (southwestern China), and Armenia. Samples from Great Basin springs were transported to the lab without temperature control (≤ 1 week) before being inoculated into a sterile medium (described below). Sediment slurries from Armenia were kept at 4 °C before being transported to the lab without temperature control, at which time they were inoculated into sterile media. Sediment slurries from Tengchong were inoculated into duplicate serum bottles containing the enrichment medium on site with one replicate being incubated in the spring at the collection site for 3 - 5 days before being transported to the lab and the remaining replicate being maintained without temperature control until reaching the lab. In the lab all enrichments were incubated at the temperature of their collection site.

Media and cultivation

All enrichments were made using a mineral medium (modified from Ehrich *et al.*, 1995) with the following composition: 10 mg L^{-1} $CaCl_2 \bullet 2H_2O$, 5.8 mg L^{-1} $NaHCO_3$, 0.5 g L^{-1} NaCl, 150 mg L^{-1} KH_2PO_4 and 1 mL L^{-1} of the following stock solutions: $MnCl_2 \bullet 4H_2O$ (52 mg L^{-1}), H_3BO_3 (40 mg L^{-1}), $ZnSO_4 \bullet 7H_2O$ (34 mg L^{-1}), $Na_2MoO_4 \bullet H_2O$ (34 mg L^{-1}), $CuSO_4 \bullet 5H_2O$ (25 g L^{-1}), $FeSO_4 \bullet 7H_2O$ (0.97 g L^{-1}),and $MgCl_2 \bullet 6H_2O$ (47 g L^{-1}). The medium was sterilized by autoclaving 121 °C for 60 minutes.

Enrichment cultures were incubated in stoppered serum bottles with the following total and liquid medium volumes (mL): 160/40 (v/v); 160/60 (v/v); 25/10 (v/v). Primary enrichments from Great Boiling Spring (GBS; U.S. Great Basin) at 80 °C, 65 °C, or 50 °C contained 1 mM $NaNO_2$ and had a headspace composition of either full air or N_2:air (3:1, v:v). Enrichments from Armenia and China contained 0.1 mM $NaNO_2$, and had a headspace composition of N_2:air (3:1, v:v). Maintenance cultures for all springs were incubated at 50 °C. NO_2^- was monitored weekly and replenished if the NO_2^- concentration was below 10 µM. These maintenance cultures were used to inoculate enrichments for all further studies.

Rate studies measuring the consumption of NO_2^- quantitatively were used to determine the optimal conditions for metabolic activity of nitrite oxidizers in enrichment cultures. All enrichments for rate experiments were inoculated with 1% inoculum from an enrichment maintained at 50 °C with a NO_2^- concentration of 0.3 mM. The rate study enrichments were started with 0.1 mM $NaNO_2$. Two uninoculated negative controls and three inoculated enrichments were used for each condition in each study. In order to determine the optimal incubation temperature, enrichments were incubated at the following temperatures concomitantly: room temperature (24 - 26 °C), 40 °C, 45 °C, 50

°C, 55 °C, 60 °C, and 65 °C. Temperatures in all incubators were monitored with a traceable thermometer (VWR 46610-024) throughout the experiment and were within 1 °C of the target temperature. NO_2^- concentrations were determined immediately after inoculation and weekly thereafter. NO_2^- was replenished, to 0.1 mM, as needed with $NaNO_2$. After 6 (GBS and Armenian enrichments) or 8 (Tengchong enrichments) weeks, NO_2^- concentrations were increased to 0.3 mM. Samples (500 µL) were taken from each enrichment and control immediately and every 3 hours thereafter for 24 hours for NO_2^- concentration measurements.

The optimal initial NO_2^- concentration was determined in a similar manner to the optimal temperature with the following exceptions: enrichments were prepared with 0.1 mM, 0.3 mM, 0.5 mM, 0.75 mM, 1 mM, and 1.25 mM NO_2^- and incubated in the same 50 °C incubator and NO_2^- concentrations were checked immediately, after 2 weeks of incubation, and weekly thereafter for 6 weeks at which time the rate study was performed as described for the temperature optimization.

Measurement of NOB activity and inhibition

NO_3^- and NO_2^- concentrations were measured colorimetrically by diazotization with and without cadmium reduction, respectively, using commercial kits (LaMotte, Chestertown, MD, USA) and a Spectronic 20D spectrophotometer (Milton Roy, USA).

Biological nitrite oxidation was verified using three methods. For all three methods, cultures were inoculated with 1% inoculum from a culture maintained at 50°C. First, the stoichiometric conversion of NO_2^- to NO_3^- was tested. Three enrichments and two uninoculated controls were spiked with 0.1 mM $NaNO_2$ and the conversion of NO_2^- to NO_3^- was monitored. When all NO_2^- was depleted NO_3^- was measured and enrichments

were spiked with an additional 0.1 mM NO_2^-. This process was repeated until 0.9 mM NO_2^- had been consumed.

Second, inhibition of NO_2^- oxidation activity by the inhibitor ClO_3^- was tested. Ten enrichments and six controls were incubated at 50°C for 6 weeks at which time the NO_2^- concentrations were increased to 0.5 mM. Samples (500 µL) were collected from each enrichment and control every 3 hours for 30 hours and after the first 15 hours five enrichments were spiked with 5 mM $NaClO_3$. Controls included: two uninoculated bottles without $NaClO_3$, two uninoculated bottles with 5 mM $NaClO_3$, and two inoculated bottles that were spiked with 5 mM $NaClO_3$ immediately after inoculation.

Finally, to test the loss of NO_2^- oxidation activity after autoclaving, three active enrichments were incubated at 50 °C for 3 weeks at which time two of the enrichments were autoclaved. NO_2^- concentrations were monitored for an additional 4 weeks to ensure inactivity.

Identification of NOB in enrichment cultures

Cells were lysed and DNA was isolated using a FastDNA SPIN kit for Soil MP- (Biomedicals, Solon, OH) according to the manufacturer's protocol. Isolated DNA was stored at -20 °C until analysis. DNA was amplified by PCR using primers 9bF (GRGTTT GATCCTGGCTCAG) and 1512uR (ACGGHTACCTTGTTACGACTT) (Burggraf *et al*., 1992; Eder *et al*., 1999). Each 25 µL reaction contained 5 µL of 5x Go Taq buffer (Promega, Madison, Wi, USA), 400 nM dNTP (Promega), 1 µL template DNA, 400 nM each forward and reverse primers, and 0.125 U Go Taq DNA polymerase (Promega). Cycling conditions were as follows: an initial melting step of 95 °C for 3 minutes, followed by 32 cycles of 94 °C for 30 seconds, 57 °C for 30 seconds, and 72 °C for 1

minute, and a final elongation step of 7 minutes at 72 °C. Clone libraries were made using a TOPO TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Forty-four clones for each spring were sequenced by Functional Biosciences (Madison, WI) using the Sanger method. Sequences were clustered with mothur (Schloss *et al*., 2009), using the average neighbor algorithm at the 98% level and representative sequences for each cluster were identified using BLASTn (Altschul *et al*., 1990). 16S rRNA gene sequences were aligned with *Nitrospira* reference sequences obtained from the NCBI database using Clustal W within BioEdit (Hall, 2005) and PHYLIP was used to construct maximum likelihood and maximum parsimony trees with 100 bootstraps per tree (Felsenstein, 2005). Modified parameters in PHYLIP were as follows: outgroup was set to *Thermodesulfovibrio aggregans*, input order of species was randomized, and the speedier but rougher analysis was turned off.

Results and Discussion

Results of thermophilic NOB enrichments

Initially, thermophilic NOB enrichments were inoculated with 50 °C, 65 °C, and 80 °C sediments from GBS and incubated at *in situ* temperature with 1 mM NO_2^- and a fully aerobic or sub-oxic atmosphere (N_2:air, 3:1, v:v). The 65 °C and 80°C enrichments remained inactive throughout the ≥1 year of incubation, but 50 °C enrichments began to show activity after 4 months of incubation. Similar lag times were observed for enrichments regardless of headspace gas composition, but in subsequent enrichments growth was more reproducible under microaerophilic conditions. In addition, when NO_2^- concentrations were reduced to 0.1 mM, lag times were reduced to ≤2 weeks. Based on

the results from GBS enrichments, all additional enrichments were microaerophilic, incubated at 50 °C, and with 0.1 mM initial NO_2^- concentration.

Using the improved NOB enrichment procedure, samples used for inocula were obtained from a variety of geothermal features in Tengchong County, Yunnan Province, China, and Armenia. Samples from Tengchong China were collected from various locations within the Rehai ("Hot Sea") geothermal field, which is a high temperature, granite-hosted system with a large diversity of geothermal features and a diverse population of thermophiles (reviewed in Hedlund *et al*., 2012), and the carbonate-hosted Ruidian system (Meixiang & Wei, 1987; Zhang *et al*., 2008b). Samples from Armenia were collected from a variety of circumneutral, carbonate-buffered springs (Mkrtchyan, 1969). While all enrichments inoculated from sites ≥61 °C were unsuccessful, enrichments from ≤53 °C samples were all successful, with the exception of one set inoculated from an acidic spring (pH 3) in Rehai and one from a spring in Armenia (Table 2.1). For successful enrichments, NOB activity became apparent within 2 weeks of inoculation.

Enrichments from the outflows of GBS (50 °C), Qiaobianrequan (48.5 °C), and Jermuk Spring (53 °C) were chosen as representative cultures from the U.S. Great Basin, China, and Armenia, respectively, for further study. Once enrichments were stable, two different approaches were employed to try to obtain axenic NOB cultures. First, optical tweezers were used to inoculate single cells from GBS and Tengchong enrichments (~90 cells total) into the same medium, autoclaved spent enrichment medium, or filtered spring water amended with 0.1 mM NO_2^-. Second, serial dilutions were used to inoculate fresh medium. To date, neither method has successfully produced pure cultures. However,

NOB are known to be extremely difficult to isolate and, to date, only three species of *Nitrospira* have been formally described as axenic species (Ehrich *et al*., 1995; Lebedeva *et al*., 2011; Watson *et al*., 1986). Additionally, "*Candidatus* Nitrospira bockiana" took up to 12 years to isolate and has not yet been described formally as an axenic culture (Lebedeva *et al*., 2008). Given the difficulty to obtain thermophilic NOB pure cultures, and recognizing both the opportunities and limitations of studying mixed microbial cultures, enrichment cultures were used to determine the optimal conditions for nitrite oxidation activity.

Evidence of biological nitrite oxidation

The predicted accumulation of NO_3^- with the depletion of NO_2^-, according to the reaction $NO_2^- + ½\ O_2 \rightarrow NO_3^-$, was used to verify NO_2^- oxidation in enrichment cultures. The approximate 1:1 stoichiometric conversion of NO_2^- to NO_3^- confirmed NO_2^- oxidation activity in all enrichments tested (Figure 2.1). In addition, since ClO_3^- is a specific inhibitor of biological NO_2^- oxidation (Belser and Mays, 1980), it was used to further confirm NOB activity. Similar results were observed for all enrichments, regardless of origin. Five mM ClO_3^- completely inhibited NOB activity in freshly inoculated subcultures and amendments of 5 mM ClO_3^- stopped NOB activity in active enrichments within five hours (Figure 2.2). Finally, autoclaving long-term enrichments led to cessation of nitrite oxidation activity.

Composition of enrichment cultures

Clone libraries revealed the presence of organisms with high (95 - 99%) 16S rRNA gene identity to *Nitrospira calida* in enrichment cultures from all three springs. 16S rRNA gene sequences for other known NOB were not found in the GBS or

Tengchong enrichments; however, the Armenian enrichments also contained close relatives of *Nitrospira moscoviensis* (Figure 2.3). The GBS enrichment contained four additional species-level groups including close relatives of *Meiothermus timidus* (99% identity), one unidentified delta proteobacterium with 84% identity to *Geobacter hephaestius*, and two different Betaproteobacteria, one with 91% identity to *Azospira restricta* and another with 89% identity to *Petrobacter succinatimandens*. The Tengchong enrichment was much less diverse with 39 of the 44 sequences identified as close relatives of *N. calida* and the remaining five sharing 93% identity with *Anoxybacillus amylolyticus*. The Armenian enrichment contained five additional species-level groups including relatives of *Anoxybacillus contaminans* (99% identity), *Ignavibacterium album* (98% identity), *Meiothermus timidus* (99% identity), *Derxia gummosa* (92% identity), and an organism with 83% identity to "*Candidatus* Chloracidobacterium thermophilum"

Physiological properties

Incubation temperatures of 45 °C and 50 °C resulted in the fastest rate of NO_2^- consumption for all enrichments (Figure 2.4). Similarly, temperatures of 40 - 45 °C for GBS enrichments and 50 °C for both Tengchong and Jermuk enrichments resulted in significantly shorter lags than other temperatures tested (Figure B1). The temperature range for NOB activity and the rate of nitrite oxidation appeared to be significantly different for the Great Basin enrichments compared to the other two enrichments during the optimal temperature experiments. However, this difference may be due to the use of improved NOB enrichment procedures for the primary Tengchong and Armenian enrichments or to differences in the other inhabitants of the co-culture. NOB activity

occurred at 60 °C in enrichments from all three springs (Figure 2.4 and 2.5); but, despite several attempts to grow enrichments from all three locations at temperatures ≥65 °C, NO_2^- oxidation activity was never observed at these temperatures. Growth at 60 °C was difficult to reproduce in all enrichments (e.g, see Figure 2.4), despite careful temperature monitoring and all attempts to maintain reproducibility. Although not conclusive, these results are in agreement with other studies (Lebedeva *et al*., 2005; Lebedeva *et al*., 2011) and suggest an upper temperature limit of 60 to 65 °C for biological nitrite oxidation.

Despite the fact that the temperature range for *Nitrospira* species can be dependent on NO_2^- concentration (Lebedeva *et al*., 2008), we did not test this dependency in our enrichments. However, we did use low (0.3 mM) NO_2^- concentrations and Lebedeva *et al*. reported that low NO_2^- concentrations (0.3 vs. 1.5 mM) resulted in a wider temperature range for "*Candidatus* Nitrospira bockiana" (Lebedeva *et al*., 2008). In addition, the temperature range did not change for *Nitrospira calida* whether 0.3 or 2.5 mM NO_2^- was used (Lebedeva *et al*., 2011). While the effect of NO_2^- concentrations on the temperature ranges was not explored, we did determine the effect of initial NO_2^- concentration on NOB activity. The experiments revealed that low concentrations of NO_2^- (0.1 - 0.3 mM) resulted in the shortest lag time for all enrichments (Figure B2). On the other hand, initial NO_2^- concentrations of 0.5 to 0.75 mM resulted in the fastest rate of nitrite oxidation (Figure 2.6). These results emphasize the importance of using low concentrations of NO_2^- when cultivating thermophilic *Nitrospira* and provide a plausible explanation for the lengthy initial lag time for GBS enrichments.

Conclusions

As evidence of their wide geographic range, *Nitrospira* species were found in enrichment cultures from geothermal features located in Great Basin, U.S., Tengchong, China, and Armenia. The sensitivity of these enrichments to both oxygen and nitrite concentrations supports the hypothesis that *Nitrospira calida* are K-strategists and emphasizes the need for low NO_2^- concentration (<1 mM) when cultivating thermophilic *Nitrospira*. Similar to previously published results (Lebedeva *et al*., 2005, Lebedeva *et al*., 2011), the upper temperature limit for growth for the nitrite-oxidizing enrichments, regardless of origin, was between 60 - 65 °C. Together the data presented here suggests an upper temperature limit for chemolithotrophic nitrite oxidation of 60 to 65 °C.

Table 2.1. NO_2^- oxidation activity in inoculated enrichment cultures.

Location	Spring	GPS location at source	pH	Temperature (°C)	NO_2^- oxidation activity
Great Basin, U.S.	GBS	N40.84139° W119.61889°	6.80	80.0	-
			7.03	65.0	-
			7.26	50.0	+
Tengchong, China	Zhenzhuquan	N24.95115° E98.43596°	4.30	91.7	-
	Diretiyanqu	N24.95396° E98.43829°	3.20	87.8	-
			2.77	74.0	-
			2.81	66.6	-
			3.09	53.0	-
	Zimeiquan	n.d.	8.98	84.7	-
	Gumingquan	N24.57060° E98.43615°	9.40	83.5	-
	Direchi	N24.95009° E98.43807°	8.29	83.0	-
			8.33	74.3	-
			8.39	69.4	-
			8.56	61.2	-
	Jinze	N23.44138° E98.46004°	6.71	80.6	-
	Gongxiaoshe	N25.44012° E98.44081°	7.29	73.8	-
	Shuirebaozha	N24.95002° E98.43728°	8.27	72.1	-
	Qiaobianrequan	N24.95044° E98.43650°	~7	57.9	+
			~7	48.5	+
	Sinter Apron	n.d.	9.00	33.6	+
Armenia	Karvachar	N40.17417° E46.27500°	7.30	56.0	+
	Jermuk	N39.96639° E45.68528°	7.05	53.0	+
			7.05	50.0	+
	Jermuk G	N39.87944° E45.77417°	6.90	53.0	+
	Arzakan	N40.68389° E44.74111	7.20	44.0	+
	Hankavan	N40.63265° E44.48463°	7.00	44.0	-

n.d. Not Determined.

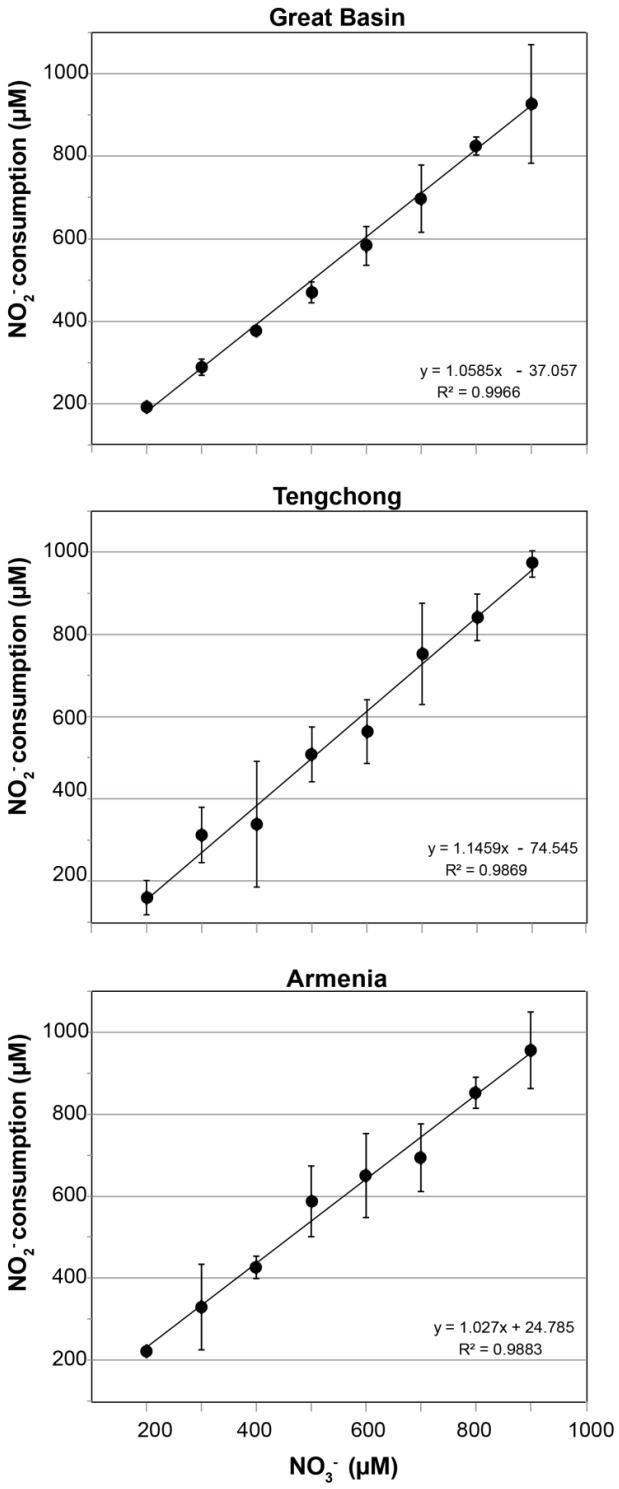

Figure 2.1. Stoichiometric conversion of NO_2^- to NO_3^- in enrichment cultures from Great Basin, Tengchong, and Armenia. NO_2^- and NO_3^- were monitored colorimetrically Errors bars, standard deviation (n=3).

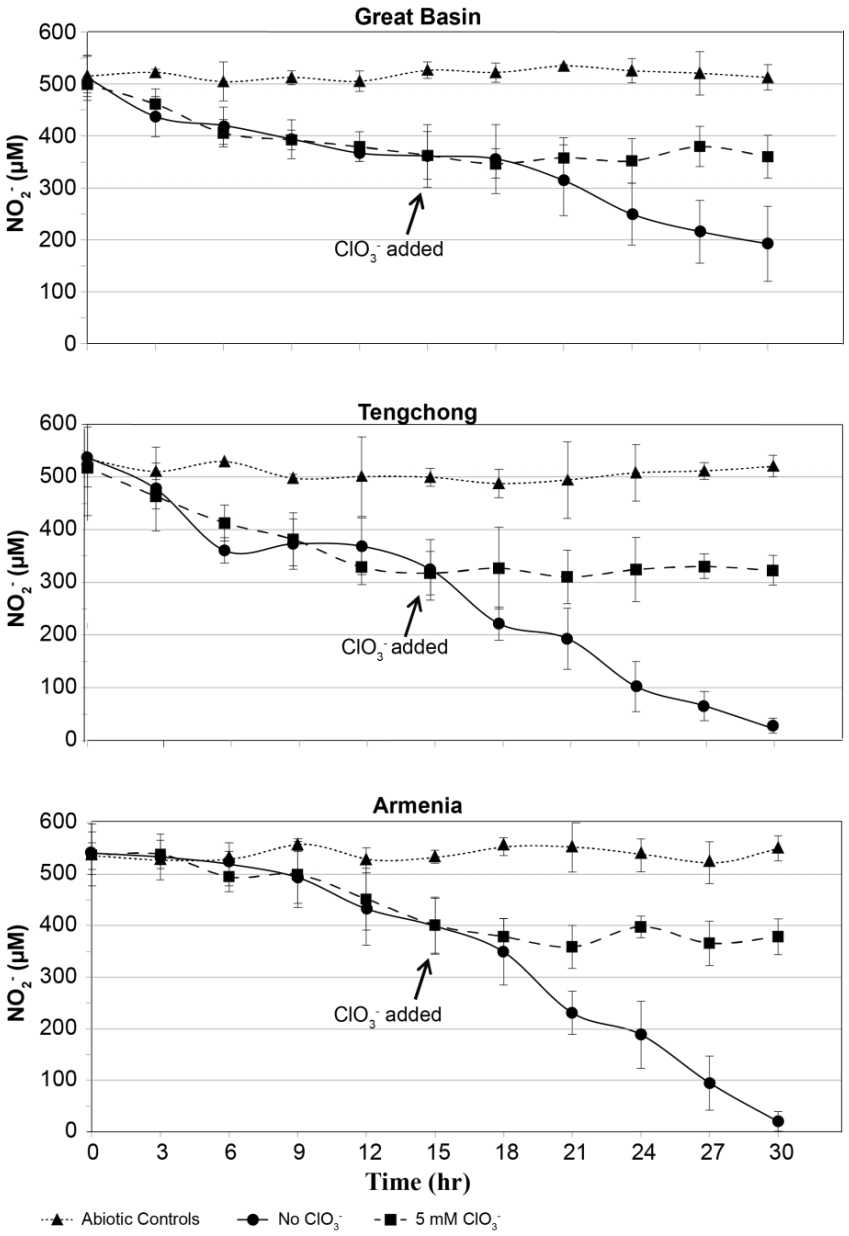

Figure 2.2. Inhibition of NO_2^- oxidation activities by ClO_3^- in enrichment cultures from Great Basin, Tengchong, and Armenia. Controls included on the graph are the uninoculated controls with ClO_3^- added. Arrows indicate addition of 5 mM ClO_3^-. Errors bars, standard deviation (n=5).

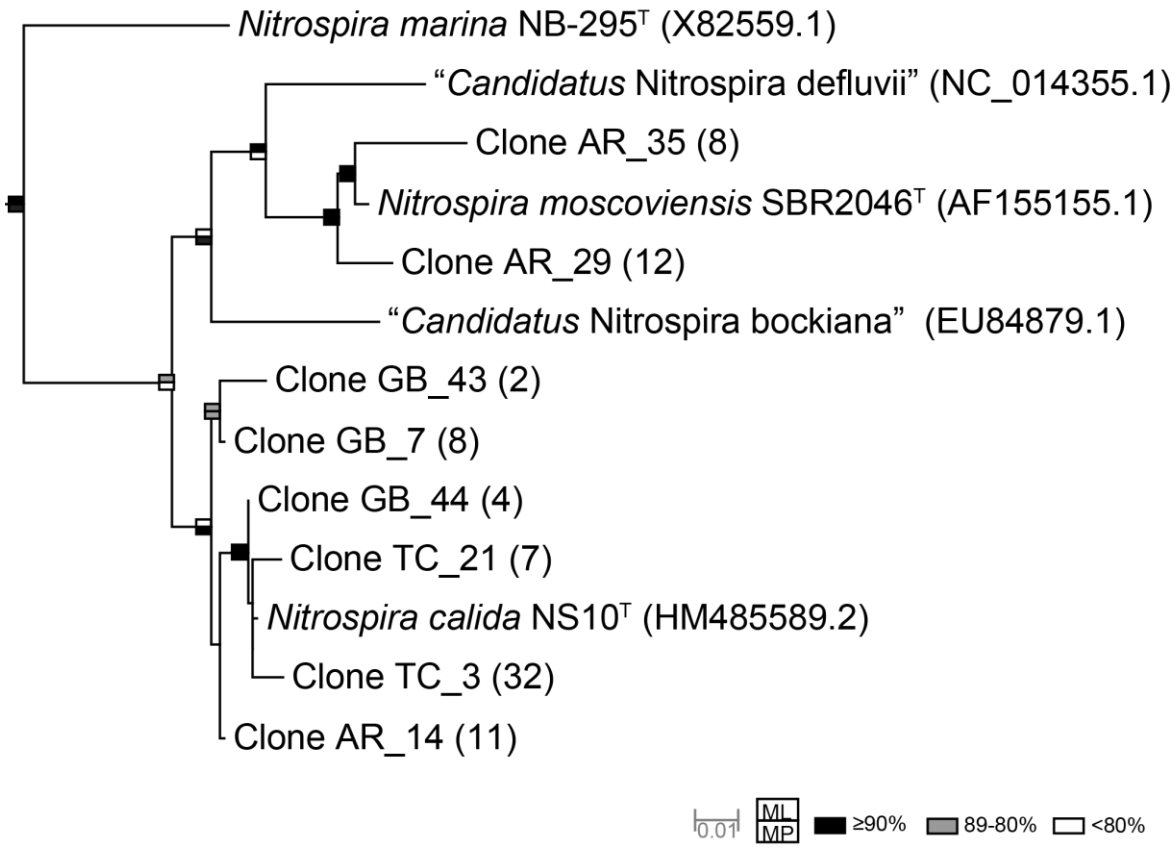

Figure 2.3. Phylogenetic tree showing the relationship between NOB in enrichment cultures from Great Basin (GB), Tengchong (TC), and Armenia (AR) and all cultivated Nitrospira species. 16S rRNA gene sequences were clustered using mothur at the 98% level. Representative OTUs are shown along with the number of clones represented in parenthesis. Accession numbers and strain designations are included for cultivated *Nitrospira* species. Bootstrap values >80% from maximum likelihood (ML) and maximum parsimony (MP) are shown.

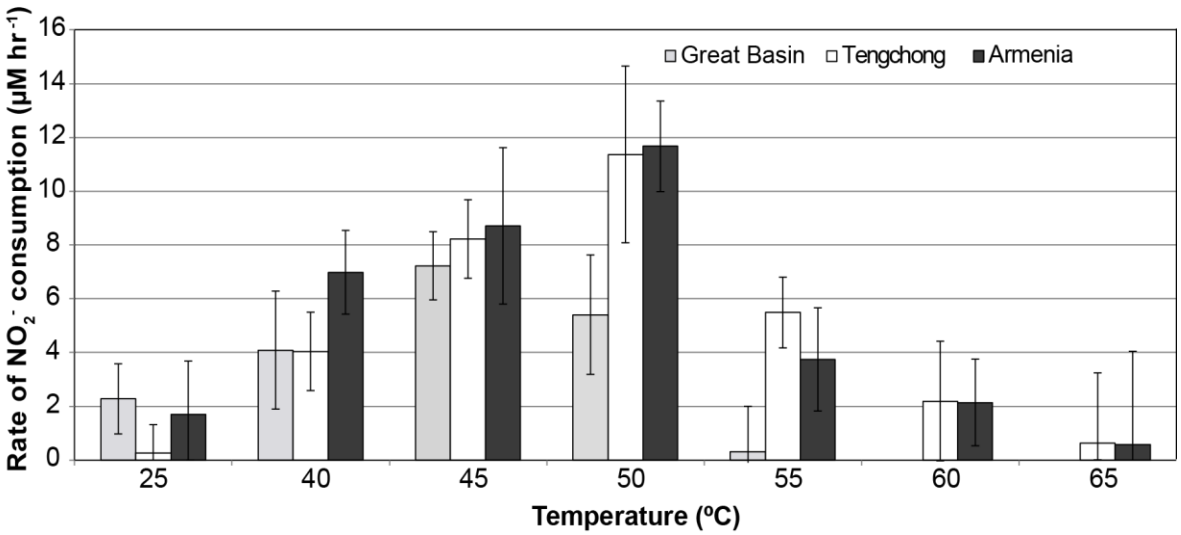

Figure 2.4. Optimization of NO_2^- oxidation activity with respect to temperature in enrichment cultures from Great Basin, Tengchong, and Armenia. NO_2^- and NO_3^- were monitored colorimetrically. Errors bars, standard deviation (n=3). * Samples incubated at room temperature (24 °C to 26 °C).

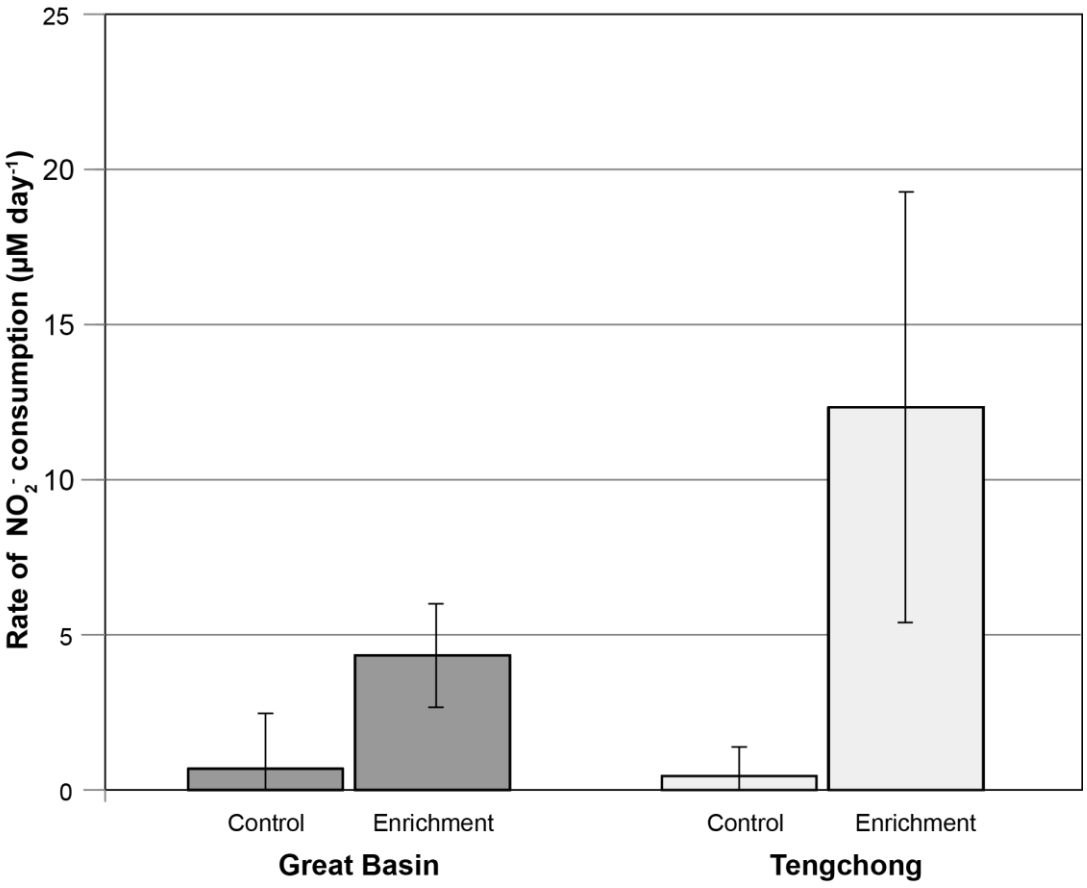

Figure 2.5. NO_2^- oxidation activities at 60 °C for Great Basin and Tengchong enrichments. NO_2^- and NO_3^- were monitored colorimetrically. Errors bars, standard deviation (n=3).

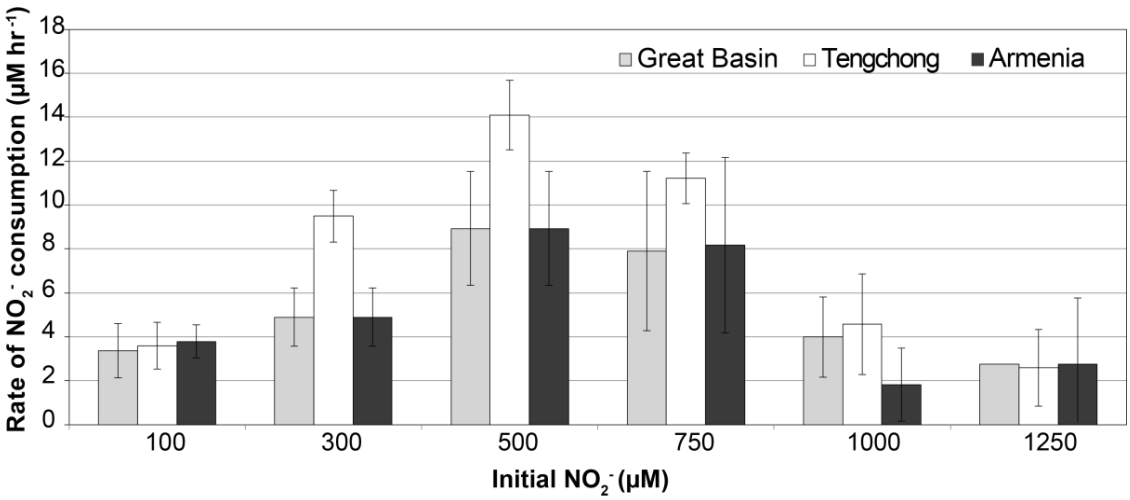

Figure 2.6. Optimization of NO_2^- oxidation activity with respect to initial NO_2^- concentration in enrichment cultures from Great Basin, Tengchong, and Armenia. NO_2^- and NO_3^- were monitored colorimetrically. Errors bars, standard deviation (n=3).

CHAPTER 3

INTEGRATED STUDY SUGGESTS DECOUPLING OF AMMONIA OXIDATION AND NITRITE OXIDATION ABOVE ~65 °C: IMPLICATIONS FOR GLOBAL UPPER TEMPERATURE LIMIT OF CHEMOLITHOTROPHIC NITRITE OXIDATION AND PROPOSAL FOR A HIGH TEMPERATURE NITRITE SHUNT

Abstract

A significant difference between the upper temperature limits for ammonia and nitrite oxidation has been suggested by previously published results, but has not been explicitly investigated. To investigate the effect of temperature on nitrification, the abundance and inferred activity of nitrifying microorganisms was determined along the outflow channels of two Great Basin hot springs. Concentrations of dissolved inorganic nitrogen from the source to ~50 °C showed apparent activity of ammonia-oxidizing archaea (AOA) at temperatures >75.2 °C and 80.3 °C for Sandy's Spring West (SSW) and Rick's Hot Creek (RHC), respectively, and an apparent upper temperature limit for nitrite oxidation activity of ~60 - 65 °C in both springs. Correspondingly, 16S rRNA gene copy numbers of the AOA "*Candidatus* Nitrosocaldus yellowstonii" were abundant at temperatures ≤79.6 °C and *Nitrospira* 16S rRNA gene sequences were only abundant at ≤57.9 °C. Clone libraries confirmed the specificity of primers used and showed a predominance of organisms with high (≥95%) 16S rRNA gene identity to either *Nitrospira calida* or *Nitrospira moscoviensis*. In addition, 16S rRNA sequences belonging to known NOB were absent from 557,076 pyrotag sequences obtained from hot springs located in the U.S. Great Basin and Tengchong, China at temperatures ≥55 °C. Furthermore, significant matches with the entire genomes of known NOB were absent from the ~250 Mbp of

metagenomic data obtained from GBS environmental samples at temperatures ranging from 77 to 85 °C, and the 557 Mbp of metagenomic data from Yellowstone National Park environmental samples at temperatures ranging from 52.9 to 90 °C. The temperature-driven decoupling of ammonia oxidation and nitrite oxidation leads to a high temperature nitrite shunt in the nitrogen cycle whereby nitrite produced by ammonia oxidation is used directly by denitrifiers in geothermal ecosystems. We propose that the high temperature nitrite shunt is complete at temperatures exceeding ~65 °C, with greater flow of nitrogen through nitrate with decreasing temperature below ~65 °C.

Introduction

Nitrification, the sequential oxidation of ammonia to nitrate via nitrite, is a key process in the nitrogen cycle. The two steps of nitrification, ammonia oxidation and nitrite oxidation, are carried out by distinct organisms. Aerobic ammonia oxidation, the first step of nitrification, was traditionally thought to be restricted to ammonia-oxidizing bacteria within the phylum Proteobacteria (Kowalchuk and Stephen, 2001). However, ammonia-oxidizing archaea (AOA) were recently discovered within the phylum Thaumarchaeota (Könneke *et al*., 2005). The second step of nitrification, nitrite oxidation, is catalyzed by nitrite-oxidizing bacteria (NOB). All cultured chemolithoautotrophic NOB belong to either the phylum Nitrospira or the alpha (*Nitrobacter*), beta (*Nitrotoga*), delta (*Nitrospina*) or gamma (*Nitrococcus*) classes of the phylum Proteobacteria (Alawi *et al*., 2007; Ehrich *et al*., 1995; Laudelout and Van Tichelen, 1960; Lebedeva *et al*., 2008; Lebedeva *et al*., 2011; Spieck *et al*., 2006; Watson *et al*., 1986; Watson and Waterbury, 1971). Both ammonia- and nitrite-oxidizing microorganisms have been reported in a variety of habitats, including: soil (Bartosch *et*

al.,2002; Nicol *et al*., 2008; Zhang *et al*, 2010), freshwater (Altmann *et al*., 2004; Altmann *et al*., 2003; Cebron *et al*.,2003; Jiang *et al*., 2009), marine (Könneke *et al*., 2005; Watson *et al*., 1986; Watson and Waterbury, 1971), and geothermal (Costa *et al*., 2009; de la Torre *et al*., 2008; Dodsworth *et al*., 2011; Hatzenpichler *et al*., 2012; Hirayama *et al*., 2005; Zhang *et al*, 2008a) environments.

Despite the wide distribution of nitrifying organisms in geothermal environments (Costa *et al*., 2009; Dodsworth *et al*., 2011; Reigstad *et al*., 2008), multiple lines of evidence suggest that the two steps of nitrification have different upper temperature limits. Ammonia oxidation has been demonstrated in co-culture at 74 °C by the thermophilic archaeon "*Candidatus* Nitrosocaldus yellowstonii" (de la Torre *et al*., 2008), and biomarkers for AOA have been recovered from environments up to 97 °C (Reigstad *et al*., 2008). In contrast, the most thermophilic NOB known, *Nitrospira calida,* is only active in pure culture up to 5Costa *et al*., 2009 °C (Lebedeva *et al*., 2011) and in enrichment culture up to 60 °C (Lebedeva *et al*., 2005). In addition, biomarkers for NOB have not been reported above 69 °C. One report has suggested that nitrite oxidation was active at 85 °C in two geothermal springs in Iceland based on the $^{15}NO_3^-$ pool dilution approach; however, that study did not distinguish between ammonia oxidation and nitrite oxidation because $^{15}NO_2^-$ and $^{15}NO_3^-$ were not distinguished (Reigstad *et al*., 2008).

Geochemical and molecular studies of the Great Boiling Spring (GBS) geothermal system in the U.S. Great Basin have highlighted the importance of nitrification in this system. The presence of NH_3 as the dominant form of dissolved inorganic nitrogen at the source of several GBS springs supports a highly active nitrogen cycle in which NH_3 is oxidized to NO_2^- at temperatures of ~80 °C (Dodsworth *et al*.,

2011). Furthermore, several studies reported on the abundance of "*Ca.* N. yellowstonii" and close relatives at <82 °C within the GBS geothermal system (Cole *et al*.,2012; Costa *et al*., 2009; Dodsworth *et al*., 2011). While DNRA and denitrification are both limited by ammonia oxidation in GBS (Dodsworth *et al*., 2011; Hedlund *et al*., 2001), metabolic interactions between microorganisms important for nitrogen cycling have not been assessed. In particular, the process of NO_2^- oxidation has been unexplored.

In this study we investigated the effect of temperature on the abundance and inferred activity of both AOA and NOB along outflow channels within the GBS geothermal system. Rates of ammonia oxidation and nitrite oxidation were calculated based on volumetric flow rates and changes in dissolved inorganic nitrogen concentration along the spring outflows. These rates were correlated with "*Ca*. N. yellowstonii" and *Nitrospira* abundance in sediment samples collected at sites along the outflow channels of two GBS hot springs.

Methods

Collection sites and samples

Sample collection was conducted in July of 2010 and September of 2012 from the source and outflow of Sandy's Spring West (SSW) and Rick's Hot Creek (RHC) which are part of the GBS geothermal system and have been described previously (Costa *et al*., 2009; Dodsworth *et al*., 2011; Huang *et al*., 2007). Temperature and pH for each sample site was measured using a handheld pH 5 meter (LaMotte, Chestertown, MD). Bulk water samples collected for inorganic chemical measurements were filtered using a 0.2 µm IC Acrodisc filter (Pall Life Sciences) and transported on wet ice to the lab. Major anions concentrations were analyzed using ion chromatography (Table A1). Sediment samples

collected for quantitative polymerase chain reaction (qPCR) were immediately frozen on dry ice, transported back to the lab, and stored at -80 °C until analysis.

Flow velocity was determined by the time it took for a neutrally buoyant 1 in. diameter rubber ball to travel a measured distance in the spring. The cross-sectional area was measured at three and four sites along the outflow channels of RHC and SSW, respectively. Volumetric flow was determined by the product of the product of the mean flow velocity and cross-sectional area.

Dissolved inorganic nitrogen concentrations and rates of metabolic activity

$\sum NH_3$ was measured by the Nesslerization method and NO_2^- and NO_3^- were measured by diazotization with and without cadmium reduction, respectively, using either a Lachat QuikChem 8000 FIA nutrient analyzer (Zellweger Analytics, Milwaukee, WI) or a Spectronic 20D spectrophotometer (Milton Roy, Rochester, NY). Rates of metabolic activity were calculated using the volumetric flow rate and changes in dissolved inorganic nitrogen (NO_2^- + NO_3^- for ammonia oxidation and NO_3^- for nitrite$^-$ oxidation) between each site.

Quantifying AOA and NOB

Qualitative PCR was performed on template DNA extracted from SSW and RHC using primer set CNY16S-F and -R (TAGCTGAAATCTATATGGCCC, ATTCTCCAGCCT TTTTACAGC), which was specific for the 16S rRNA gene of "*Ca***.** N. yellowstonii" and close relatives (Costa *et al*., 2009), and two primer sets specific for NOB. NOB-specific primers sets included: F1-norA and R2-norA (CAGACCGACGTGTGCGAAAG, CAGACCGACGTGTGCGAAAG), which were specific for the 16S rRNA gene of *Nitrobacter* (Könneke *et al*., 2005), and NSP8-F and -

R (CGGCAGTCCCCTCCGACCTT, ATGGGACGGGAAACCGTTCGGA), which were specific for the *Nitrospira* 16S rRNA gene (present study).

Quantitative PCR (qPCR) was performed on template DNA extracted from SSW and RHC using primer sets CNY16S-F and –R and NSP8-F and –R. The amplified regions were ~250 nt for the CNY16S primer set and ~189 nt for the NSP8 primer set. Standard curves were produced by isolating plasmids (pCR2.1-TOPO) containing the target sequence using a QIAprep Spin MiniPrep Kit (Qiagen). Plasmids were then quantified using a Nanodrop 1000 (Thermo Scientific, Waltham, MA) and diluted in 10-fold dilutions ranging from ~10^2 - 10^9 copies/reaction. Plasmid contained either the clone SSE_L4_B03 (for primer set CNY16S-F and –R) (Costa *et al*., 2009) or amplified *Nitrospira* 16S rRNA gene sequences from GBS (for primer set NSP 8-F and –R). All standard curve reactions were prepared in duplicate.

Sample reactions were prepared in triplicate and coupled with negative controls (no template). Reactions (25 µL) were prepared in individual wells of an iQ 96-well PCR plate (BioRad) and contained 12.5 µL of 2X PerfeCTa SYBR Green SuperMix for iQ (Quanta Biosciences, Gaithersburg, MD), 400 nM each primer, and 5 µL template DNA. The following cycling conditions were used: an initial melt cycle (95 °C for 3 min) followed by 45 cycles of melting (94 °C for 15 s), annealing (57 °C for 25 s) and extension (72 °C for 45 s), with data collection using a SYBR-490 filter enabled during the 72 °C step, followed by a melt curve 55 °C to 95 °C by 0.5 °C increments with 10 s at each step. Gene copy numbers were quantified and threshold cycles and data analysis were done using an iCycler iQ Multicolor Real-Time PCR Detection System with iCycler iQ Optical System Software v3.1 (BioRad, Hercules, CA). Standard curves were log-

linear and correlation coefficients (r^2) for regressions and amplification efficiencies ranged from 104% to 99.3% and from 0.890 to 0.997, respectively.

Identification of NOB

Cells were lysed and DNA was isolated using a FastDNA SPIN kit for Soil (MP Biomedicals, Solon, OH). NOB 16S rRNA genes were amplified using the same primer sets as used in qPCR. Cloning was done with a TOPO TA cloning kit (Invitrogen, San Diego, CA), and NOB-specific clones were sent to Functional Biosciences (Madison, WI) for sequencing (Sanger method). The resulting sequences were clustered at the 97% OTU level using the program mothur v1.24.1 (Schloss *et al*., 2009) and the representative sequences were coarsely identified using BLASTn (Altschul *et al*., 1997).

Metagenome analysis

MUMmer v3.0 was used to evaluate the presence of known NOB genome sequences in metagenome data from GBS and Yellowstone National Park. The ~250 Mbp of GBS metagenomic data and the 557 Mbp of Yellowstone metagenomic data were queried using the default setting for MUMmer.(Kurtz *et al*., 2004). The following reference sequences were obtained from the NCBI databate: "*Candidatus* Nitrospira defluvii" NC_014355; *Nitrobacter winogradskyi*_Nb_255, NC_007406; and *Nitrobacter hamburgensis* X14, NC_007964 (Pruit *et al*., 2005) and metagenome data for GBS and Yellowstone were obtained from the Department of Energy's Joint Genome Institute (Grigoriev *et al*., 2012).

Results and Discussion

Dissolved inorganic nitrogen concentrations and calculated rates N-transformation

In order to assess zones of metabolic activity and inferred rates of nitrification, bulk water samples were collected in July of 2010 and September of 2012 at the source and at intervals throughout the outflow SSW and RHC. Sample temperature, pH, GPS location, and distance from spring source are recorded in Table 3.1. Selected ionic concentrations for samples collected during July of 2010 are shown in Table S1. In September of 2012 the volumetric flow rates were 56 ± 9.1 L s^{-1} and 157 ± 24 L s^{-1} for SSW and RHC, respectively.

Spatial patterns of dissolved inorganic nitrogen concentrations along the spring outflows were similar for both springs on both sampling dates (Figures 3.1 and 3.2). As previously noted (Dodsworth *et al*., 2011), the source waters of both SSW and RHC contained moderate concentrations of $\sum NH_3$ (59 - 97 µM) and low concentrations of NO_2^- and NO_3^- (<2 µM) (Figures 3 1 and 3.2). Decreasing concentrations of $\sum NH_3$ concomitant with increasing $NO_2^- + NO_3^-$ strongly suggested nitrifying activity along the outflows of both springs. However, ammonia and nitrite oxidation were spatially disconnected, and appeared to be defined by different temperature ranges.

Decreasing $\sum NH_3$ concomitant with increasing NO_2^- strongly suggested ammonia oxidation activity at temperatures >80.3 °C. Estimated rates of ammonia oxidation were highest between ~80 and 75 °C in both springs (0.61 ± 0.10 mol cm^{-1} s^{-1} and 0.30 ± 0.05 mol cm^{-1} s^{-1} SSW RHC) and were not significant at ≥84.6 °C in either spring (Figure 3.3). Thus, the optimal temperature for ammonia oxidation appears to be between ~80 and 75 °C within the GBS geothermal system. In addition, the thermal limit of ammonia oxidation within this system appears to be between 80.3 to 84.6 °C, which is consistent with the high abundance of "*Ca*. Nitrosocaldus" in GBS at sites ≤ 82 °C on multiple

sampling dates (Cole *et al*.,2012; Costa *et al*., 2009; Dodsworth *et al*., 2011) and measurement of relatively high rates of ammonia oxidation in GBS and SSW at 82 °C and 81 °C (Dodsworth *et al*., 2011).

In contrast, the accumulation of NO_2^- along with low NO_3^- (<1 µM) strongly suggested a lack of nitrite oxidation at temperatures >65 °C. Inferred rates of nitrite oxidation were highest between ~55 and 50 °C (0.73 ± 0.030 mol cm^{-1} s^{-1} in SSW and 0.68 ± 0.060 mol cm^{-1} s^{-1} in RHC) and were not significant at temperatures >65 °C in either spring. These results suggest an upper temperature limit between ~61 - 65 °C for nitrite oxidation within the GBS geothermal system, which differs significantly (~15 - 20 °C) from the upper temperature limit of inferred ammonia oxidation activity. The apparent thermal decoupling between the two steps of nitrification within GBS geothermal system provides a plausible explanation for the accumulation of NO_2^- reported previously in GBS at temperatures >65 °C (Costa *et al*., 2009).

Many factors, such as pH and the concentration of dissolved O_2 (DO) covary with temperature in geothermal systems (Fouke, 2011) providing possible alternative explanations for the spatial separation observed between nitrite oxidation and ammonia oxidation. However, with the exception of the source of SSW, the pH for all sites fell within the reported range supporting growth of *Nitrospira calida* (pH 7.0 - 8.8), which has been cultivated previously from this site (Table 3.1). In addition, while NOB are more sensitive to limited O_2 than AOA (Ju *et al*., 2007), NOB have been shown to persist in environments with DO levels as low as 0.8 ppm (Fussel *et al*., 2012), which is well below the DO concentrations at sites ≤79.8 °C in both springs (Table A1). While other

unmeasured physicochemical variables cannot be ruled out, temperature remains a likely factor driving ammonia and nitrite oxidation within the GBS geothermal system.

Quantification of AOA and NOB

Since "*Ca.* N. yellowstonii" and its close relatives are the only members of the AOA known to be present within the GBS geothermal system (Costa *et al*., 2009; Dodsworth *et al*., 2011), AOA abundance was determined by qPCR using "*Ca*. N. yellowstonii"-specific 16S rRNA gene primers. Gene copy numbers for "*Ca*. N. yellowstonii"-like 16S rRNA were most abundant at 71.9 °C in SSW and 79.6 °C in RHC and were not abundant ($\geq 10^6$) at temperatures ≥84.4 °C in either spring (Figure 3.1). These results delineate an upper temperature limit for "*Ca.* Nitrosocaldus yellowstonii" between 79.6 °C and 84.6 °C within the GBS geothermal system, which is in agreement with the reported lack of abundant "*Ca.* Nitrosocaldus yellowstonii" at 86.6 °C in SSW (Costa *et al*., 2009) and at temperatures >82 °C in GBS (Cole *et al*.,2012). In addition, the upper temperature limit of "*Ca.* Nitrosocaldus yellowstonii" is in agreement with the upper temperature limit for inferred ammonia oxidation activity suggesting that "*Ca.* Nitrosocaldus yellowstonii" is likely responsible for ammonia oxidation at the upper temperature limit within the GBS geothermal system.

Because the only known thermophilic NOB belong to the genus *Nitrospira* (Ehrich *et al*., 1995; Lebedeva *et al*., 2008; Lebedeva *et al*., 2011), and attempts to amplify DNA using a *Nitrobacter*-specific 16S rRNA gene primers failed, NOB were quantified using a *Nitrospira*-specific 16S rRNA gene primer set. Gene copy numbers for *Nitrospira* increased along the outflow of SSW and RHC, and were not abundant ($>10^6$) at ≥61.4 °C in either spring (Figure 3.1). However, NO_3^- concentrations in SSW in July,

2010 clearly indicate the presence of NO_2^- oxidation at ≥61.4 °C; therefore, the upper temperature limit for NO_2^- oxidation appears to be close to but slightly >61.4 °C within the GBS geothermal system.

Identification of NOB

In order to evaluate the specificity of qPCR primers used and to identify the NOB present in the samples, clone libraries were constructed from the July 2010 sediment samples that contained NOB (four samples; Table 2). 36 clones were sequenced from the NOB-abundant samples, resulting in a total of 144 clones. The sequences amplified by the NOB-specific primers were closely related to *N. moscoviensis* and *N. calida*, with a minimum identity ≥95% (Table 3.2). These results confirmed the specificity of the primers used and suggest a thermophilic clade within the genus *Nitrospira* that includes *N. moscoviensis*, *N. calida*, and yet-uncultivated relatives.

Cultivation-independent data

As an independent test of the thermal niche of *Nitrospira* and other NOB in geothermal environments, pyrotag datasets from GBS and Tengchong were scanned for 16S rRNA gene sequences belonging to known NOB. Known NOB were absent from 164,178 quality-filtered pyrotag sequences amplified from GBS (twelve environmental samples taken on four different sampling dates) at temperatures ranging from 87 – 62 °C (Cole *et al*.,2012). Furthermore, significant matches with the entire genomes of "*Candidatus* Nitrospira defluvii", *Nitrobacter winogradski*, and *Nitrobacter hamburgensis*, were absent from 250 Mbp of metagenomic data obtained from GBS environmental samples at temperatures ranging from 77 to 85 °C. Similarly, known NOB were absent from 392,899 pyrotags from 37 samples from hot springs in Rehai and

Ruidian, ranging in temperature from 55.1 - 93.6 °C and in pH from 2.46 to 9.39 (Hou *et al*., 2012). These results, along with the lack of any reports of successful NOB cultures at temperatures >60 °C, suggest an upper temperature limit for chemolithotrophic nitrite oxidation of ~60 - 65 °C.

Conclusions

The data presented here underscores the need to consider nitrite oxidation, and ammonia oxidation separately when studying nitrification. Inferred rates of metabolic activity and abundance of "*Ca*. N. yellowstonii" and *Nitrospira* suggested a distinct difference in the upper temperature limits for zones of ammonia and nitrite oxidation within the GBS geothermal system. Rates of ammonia oxidation and "*Ca*. N. yellowstonii" abundance suggested an upper temperature limit for ammonia oxidation activity of ~80 - 85 °C. In contrast, rates of nitrite oxidation and abundance of *Nitrospira* spp. suggested an upper temperature limit of ~60 - 65 °C for nitrite oxidation. In addition, 16S rRNA sequences belonging to known NOB were absent from 557,076 pyrotag sequences obtained from hot springs located in the U.S. Great Basin, and Tengchong, China at temperatures ≥55 °C. Furthermore, significant matches with the entire genomes of known NOB were absent from metagenome data obtained from GBS environmental samples at temperatures ranging from 77 to 85 °C. Together these results provide evidence of a thermal decoupling between the two steps of nitrification resulting in an accumulation of NO_2 at temperatures ≥65 °C within the GBS geothermal system.

Along with inferred activity measurements, the distribution and abundance of "*Ca*. N. yellowstonii" and *Nitrospira* spp. may offer clues about other nitrogen cycle processes within the GBS system. Because of their dependence on ammonia oxidation

within the GBS system, both DNRA and denitrification are likely to be inhibited at temperatures above >84.4 °C. While the effect of the lack of nitrite oxidation at ≥65 °C is not quite as clear, the elevated NO_2^- concentrations along with low NO_3^- may result in a nitrite shunt at temperatures >65 °C. This high temperature nitrite shunt is complete, with no flow of nitrogen through nitrate at temperatures exceeding ~65 °C. Further investigations are needed to determine the effects of this thermal decoupling on the community structure and function with the GBS geothermal system.

Table 3.1. Locations, temperatures, and pH for each sampling site.

Site	Sampling Date	Temperature (°C)	pH	GPS location	Distance From Source (m)
SSW Source	7/11/2010	84.4	6.71	n.d.	n.d.
SSW 70	7/11/2010	71.9	7.67	n.d.	n.d.
SSW 60	7/11/2010	61.4	8.11	n.d.	n.d.
SSW 50	7/11/2010	49.8	8.29	n.d.	n.d.
RHC Source	7/19/2010	92.6	7.25	n.d.	n.d.
RHC 80	7/19/2010	79.6	7.61	n.d.	17.07
RHC 70	7/19/2010	69.3	7.98	n.d.	39.93
RHC 60	7/19/2010	57.9	8.37	n.d.	103.94
RHC 50	7/19/2010	48.3	8.47	n.d.	222.81
SSW Source	9/28/2012	82.9	7.18	N40° 39.184', W119°22.499'	-
SSW 75	9/28/2012	75.2	7.29	N40° 39.180', W119° 22.494'	4.55
SSW 70	9/28/2012	70.1	7.38	N40° 39.173', W119° 22.486'	9.34
SSW 65	9/28/2012	65.3	7.51	N40° 39.168', W119° 22.470'	14.45
SSW 60	9/28/2012	60.2	7.57	N40° 39.160', W119° 22.469'	19.65
SSW 55	9/28/2012	54.8	7.67	N40° 39.158' W119° 22.465'	21.88
SSW 50	9/28/2012	49.5	7.78	N40° 39.156' W119° 22.461'	23.92
RHC Source	9/27/2012	93.9	7.02	N40° 40.438' W119° 21.854'	-
RHC 85	9/28/2012	84.6	7.32	N40° 40.450' W119° 21.855'	10.36
RHC 80	9/28/2012	80.3	7.28	N40° 40.455' W119° 21.852'	21.03
RHC 75	9/28/2012	75.1	7.34	N40° 40.461' W119° 21.848'	33.22
RHC 70	9/28/2012	70.4	7.54	N40° 40.466' W119°21.837'	51.21
RHC 65	9/28/2012	65.0	7.67	N40° 40.471' W119° 21.826'	68.28
RHC 60	9/28/2012	59.8	7.68	N40° 40.490' W119° 21.810'	111.86
RHC 55	9/28/2012	54.5	7.71	N40° 40.505' W119° 21.800'	145.69
RHC 50	9/28/2012	50.5	7.75	N40° 40'520' W119° 21.791'	173.43

n.d. = not determined

Table 3.2. OTUs from the outflows of SSW and RHC at 60°C and 50°C.

Representative Sequence	# in OTU[a]	Cultured microorganism whose 16S rRNA gene has the highest BLASTn hit to the OTU representative sequence		
		Organism name and strain	Accession #	id.[b]
SSW 60 °C				
SSW_60_23	18	*Nitrospira moscoviensis* SBR2046	AF155155.1	97%
SSW_60_10	14	*Nitrospira calida* NS10	HM485589.1	96%
SSW_60_36	4	*Nitrospira calida* NS10	HM485589.1	95%
SSW 50 °C				
SSW_50_13	14	*Nitrospira moscoviensis* SBR2046	AF155155.1	97%
SSW_50_31	12	*Nitrospira calida* NS10	HM485589.1	96%
SSW_50_23	9	*Nitrospira calida* NS10	HM485589.1	95%
SSW_50_27	1	*Nitrospira moscoviensis* SBR2046	AF155155.1	96%
RHC 60 °C				
RHC_4_20	28	*Nitrospira calida* NS10	HM485589.1	98%
RHC_4_32	8	*Nitrospira calida* NS10	HM485589.1	95%
RHC 50 °C				
RHC_5_30	19	*Nitrospira calida* NS10	HM485589.1	95%
RHC_5_17	11	*Nitrospira moscoviensis* SBR2046	AF155155.1	97%
RHC_5_37	3	*Nitrospira moscoviensis* SBR2046	AF155155.1	96%
RHC_5_24	3	*Nitrospira calida* NS10	HM485589.1	96%
RHC_4_35	1	*Nitrospira calida* NS10	HM485589.1	97%

[a] Number of sequences in the 16S rRNA gene library represented by the OTU.

[b] Percent identity to the OTU representative sequence.

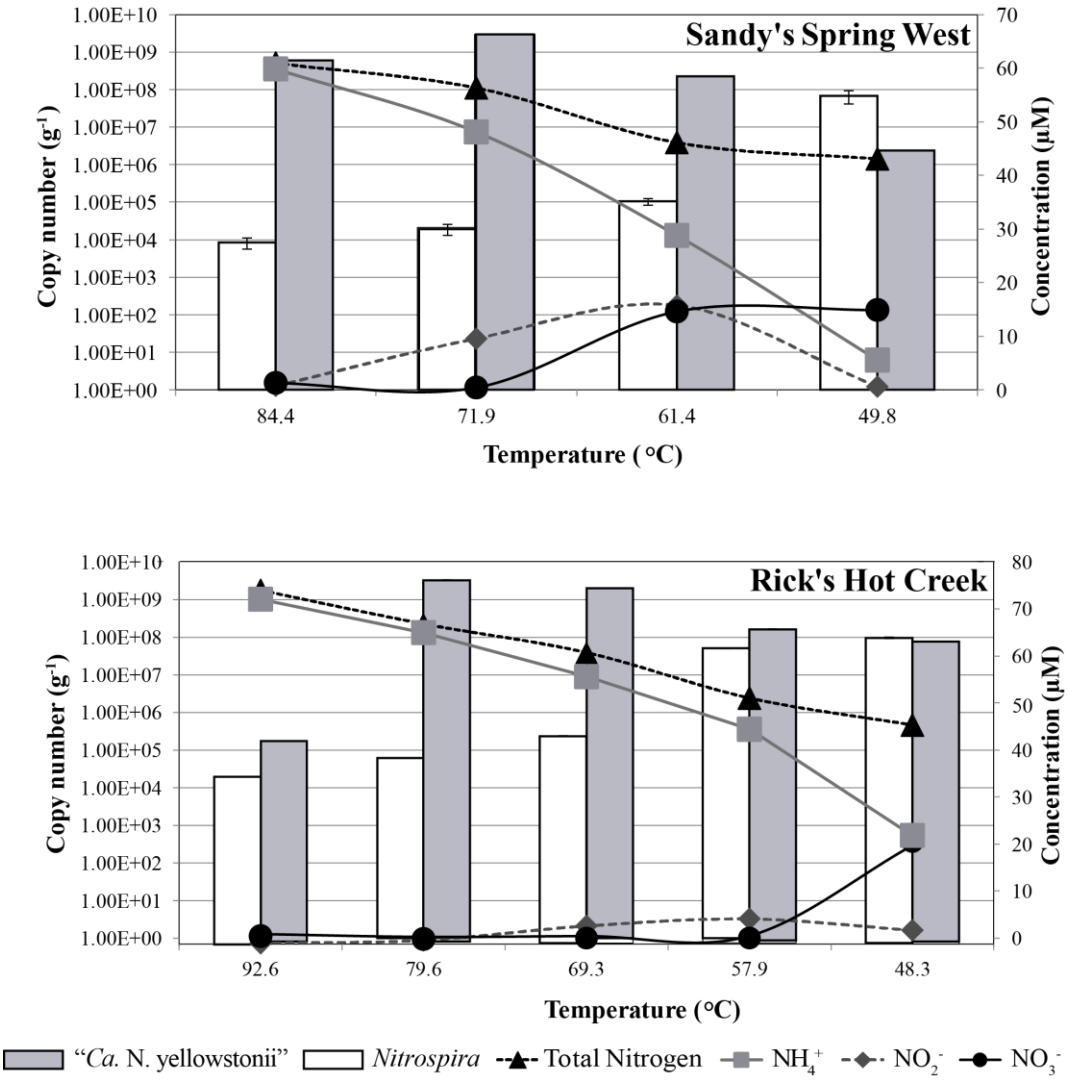

Figure 3.1. Abundance of "*Ca*. N. yellowstonii" and *Nitrospira* and concentrations of dissolved inorganic nitrogen along geothermal outflow channels of SSW and RHC in July 2010. Ammonia, nitrite, and nitrate were measured colorimetrically and 16S rRNA gene copy numbers were measured using qPCR.

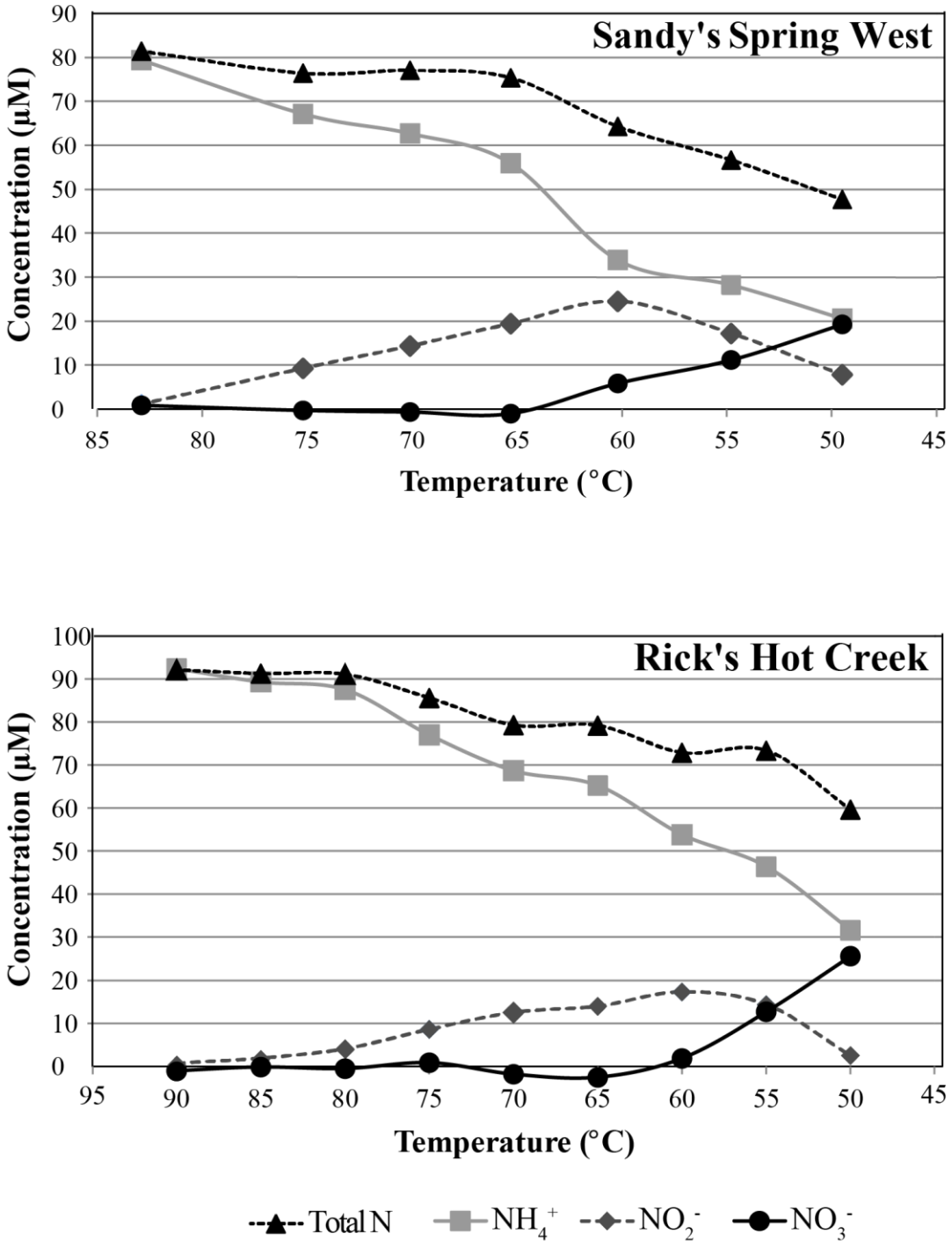

Figure 3.2. Concentrations of dissolved inorganic nitrogen along geothermal outflow channels of SSW and RHC in September 2012. ΣNH_3, NO_2^-, and NO_3^- were measured colorimetrically.

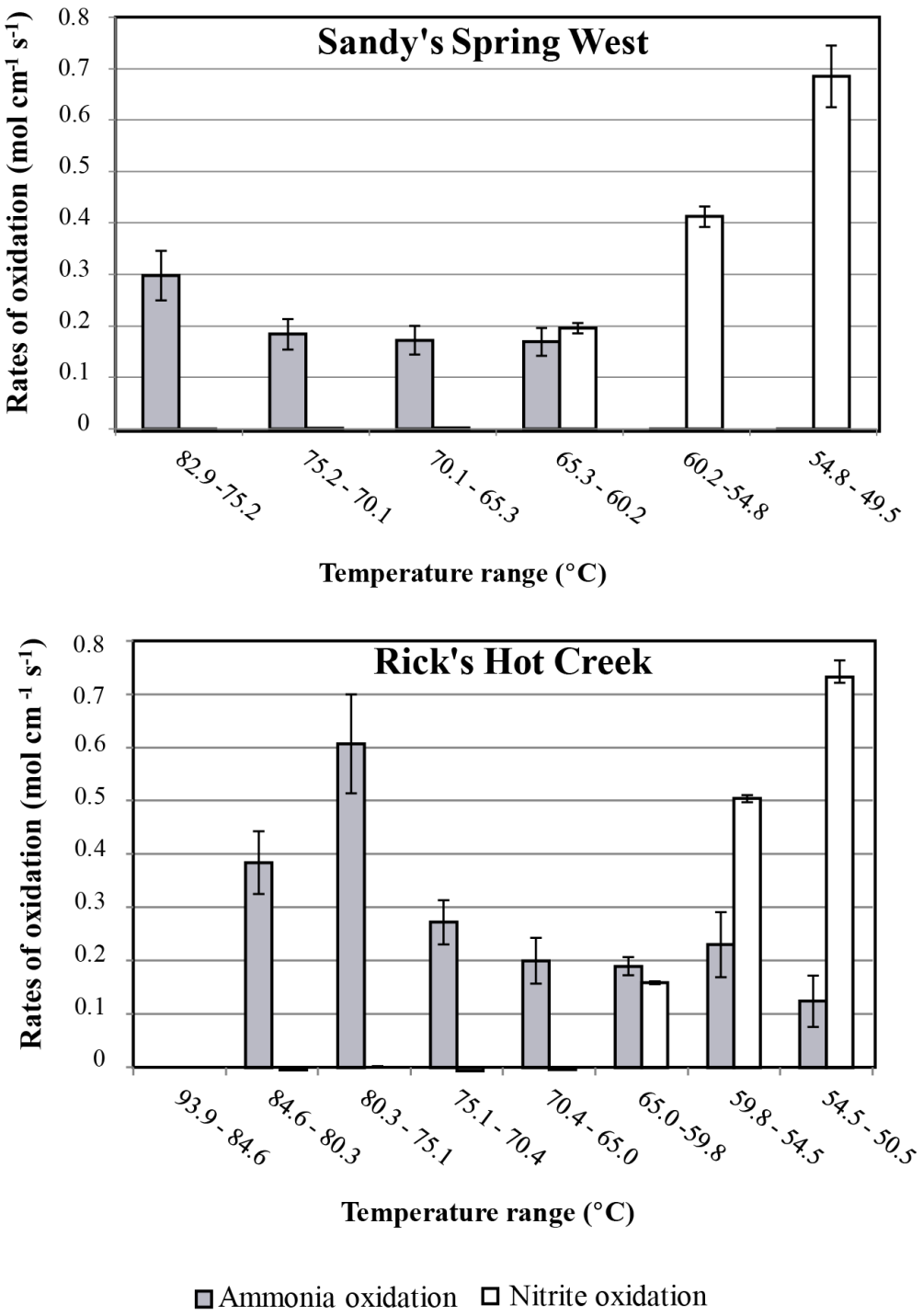

Figure 3.3. Inferred rates of metabolic activities along the outflow of SSW and RHC. Rates of ammonia and nitrite oxidation were calculated using the flow rates and changes in NO_2^- + NO_3^- (ammonia oxidation) and NO_3^- (nitrite oxidation).

CHAPTER 4

CONCLUSIONS

The results presented here demonstrate the wide geographic range of *Nitrospira calida* in terrestrial geothermal systems. Organisms with high (>95%) 16S rRNA gene identities to *Nitrospira calida* were found in both SSW and RHC. Similarly, enrichment cultures from geothermal springs located in geothermal springs in Tengchong, China, and Armenia all contained organisms that were closely related to *Nitrospira calid*a (≥97% 16S rRNA identities). The physiological properties were similar for all enrichments characterized, regardless of their origin, with an optimal initial NO_2^- concentration of 500 - 750 µM, and an optimal temperature for nitrite oxidation activity of 45 - 50 °C

Data obtained from characterization of enrichment cultures, geochemical analyses, and qPCR all consistently indicated an upper temperature limit for thermophilic NOB of 60 - 65 °C. While attempts to enrich for NOB from environments with temperatures >61 °C were unsuccessful, enrichments were successfully inoculated from 5 springs located in Great Basin, U.S., Tengchong China, and Armenia at sites <58 °C. In addition, while active at 60 °C, successful enrichments never showed any activity when incubated at temperatures >60 °C. Measurements of dissolved inorganic nitrogen concentrations in two GBS springs showed NO_2^- accumulation along with consistently low NO_3^- (<2 µM) suggesting a lack of nitrite oxidation activity at temperatures >60 °C. Furthermore, inferred rates of *in situ* nitrite oxidation activity were not significant at temperatures >65 °C. Consistent with these results, *Nitrospira* 16S rRNA gene copy numbers were not abundant at temperatures >57.9 °C in either spring. Similarly, 16S rRNA sequences belonging to known NOB were absent from 557,076 pyrotag sequences

obtained from hot springs located in Great Basin, US and Tengchong, China at temperatures ≥55 °C. In addition, the 250 Mbp metagenomic data from GBS environmental samples at temperatures of 77 - 85 °C, and the 577 Mbp metagenomic data from Yellowstone environmental samples at temperatures of 52.9 - 90 °C did not contain any significant genome matches to known NOB.

In contrast to results for NOB, the data presented here suggests an upper temperature limit for AOA of 75 - 84 °C. Inferred rates of ammonia oxidation activity were significant at temperatures <84.6 °C and "*Ca*. N. yellowstonii" 16S rRNA genes were abundant at ≤84.4 °C. Since NH_3 is in its most reduced oxidation state, ammonia oxidation is required to provide substrate for reductive N processes. Therefore, these results indicate that the dissimilatory N cycling processes would not function within the GBS geothermal system at temperatures >82 °C (Figure 4.1). In addition, these results suggest a spatial and thermal (~15 - 20 °C) difference between the two steps in nitrification within the GBS geothermal system. While the effects of this thermal decoupling on other N cycling processes within this system have yet to be explored, it is possible that a high temperature nitrite-shunt (as shown in Figure 4.1) exists within these geothermal environments. Further research is needed to determine whether this decoupling is ubiquitous in high temperature environments. In addition, the greater implications of this decoupling of nitrification in terms of community structure and function needs to be addressed.

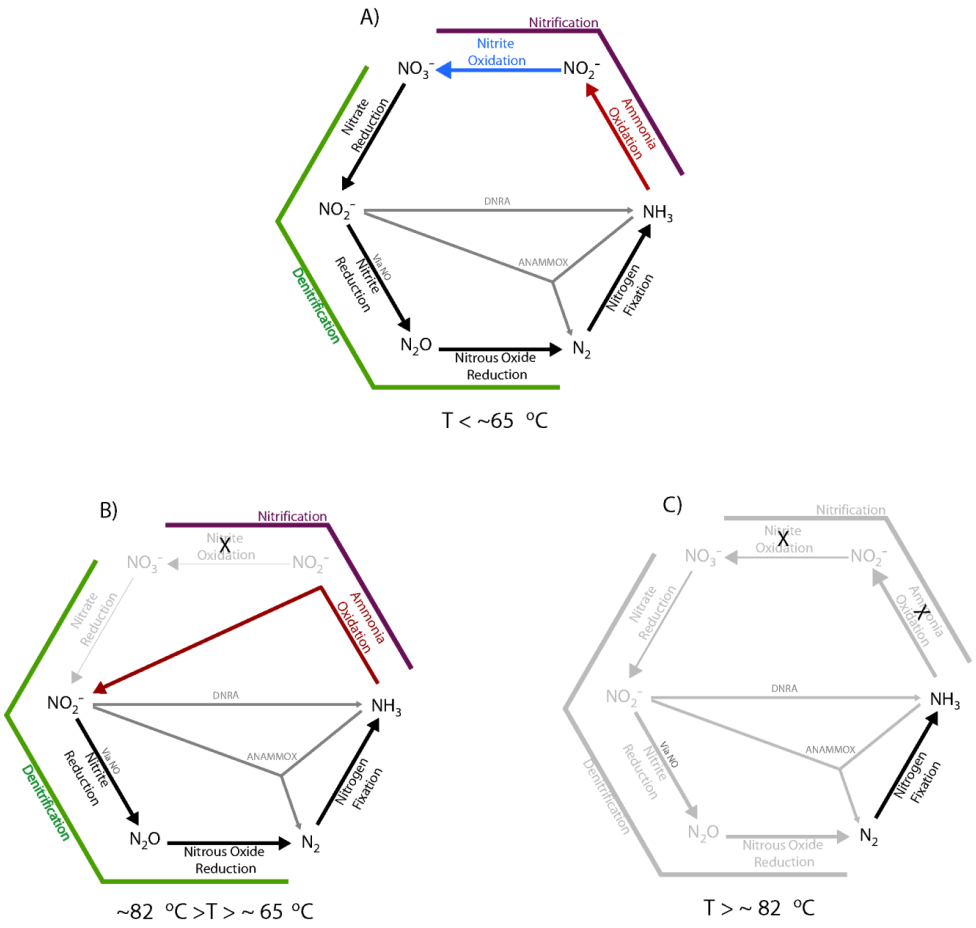

Figure 4.1. Conceptual model of nitrogen cycle activities in GBS system springs at temperatures: A) <65 °C B) ~65 – 82 °C C) >~82 °C.

APPENDIX A: ADDITIONAL TABLES

Table A.1. Concentrations of major anions and O2 along SSW and RHC outflow systems in July, 2010.

Site	Sampling Date	Temperature (°C)	Fluoride (µM)	Chloride (mM)	Bromide (µM)	Sulfate (mM)	O_2 (ppm)
SSW Source	7/11/2010	84.4	350.00	61.6	65.06	3.94	0.40
SSW 70	7/11/2010	71.9	104.95	63.3	60.79	4.1	1.80
SSW 60	7/11/2010	61.4	b.d.	63.8	57.07	4.1	3.00
SSW 50	7/11/2010	49.8	451.04	65.4	51.22	4.2	5.20
RHC Source	7/19/2010	92.6	b.d.	61.52	82.31	3.8	0.00
RHC 80	7/19/2010	79.6	151.81	63.2	69.36	4.0	1.20
RHC 70	7/19/2010	69.3	b.d.	65.1	68.79	4.1	1.80
RHC 60	7/19/2010	57.9	319.52	65.2	64.15	4.1	2.22
RHC 50	7/19/2010	48.3	18.19	66.3	58.44	4.2	2.80

b.d. is below detection

APPENDIX B: ADDITIONAL FIGURES

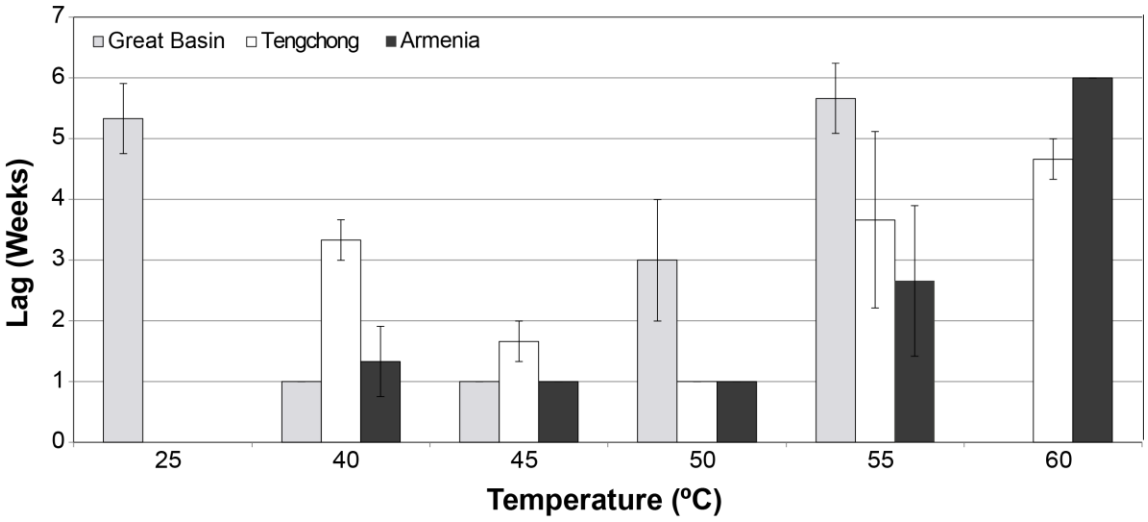

Figure B.1. Lag of NO_2^- oxidation activity with respect to temperature in enrichment cultures from Great Basin, Tengchong, and Armenia. NO_2^- was monitored colorimetrically. Errors bars, standard deviation (n=3). * Samples incubated at room temperature (24 °C to 26 °C).

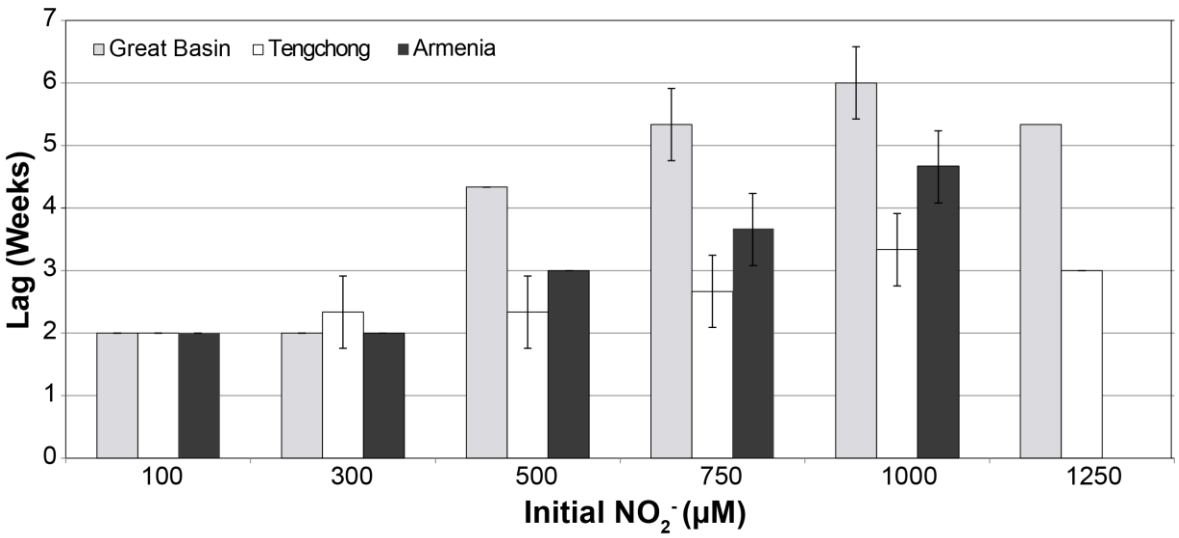

Figure B.2. Lag of NO_2^- oxidation activity with respect to initial NO_2^- concentration in enrichment cultures from Great Basin, Tengchong, and Armenia. NO_2^- was monitored colorimetrically. Errors bars, standard deviation (n=3).

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