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Hot spring habitats above maximum photosynthetic temperature (73 ºC) are not well understood with respect to nitrogen (N) cycling. Few predictions have been made, and even fewer measurements of in situ activities have been reported. Thermodynamic calculations based on in situ chemical and temperature measurements will be used to predict the occurrence of the specific N-cycling reactions. In addition, these measurements in two springs will aid in an attempt to cultivate ammonia oxidizing species.

Microbial Nitrogen Cycling in Nevada Geothermal Springs

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Abstract: Hot spring habitats above maximum photosynthetic temperature (73 °C) are not well understood with respect to nitrogen (N) cycling. Few predictions have been made, and even fewer measurements of in situ activities have been reported. Thermodynamic calculations based on in situ chemical and temperature measurements were used to predict the occurrence of the specific N-cycling reactions, which were then tested. Denitrification activity was measured *in situ* via the acetylene block method. The potential for N-fixation and nitrification are tested by an attempt to amplify *nifH* and *amoA* gene sequences, respectively.

Introduction

The microbial nitrogen (N) cycle controls the supply of essential N atoms to all biological systems. However, hot spring ecosystems are not well understood with respect to N-cycling. Thermophilic microbes in geothermal hot springs take advantage of N-cycling processes for both a source of nitrogen (assimilatory reactions) and as a source of energy (dissimilatory reactions). As chemolithotrophs, they harvest energy through catalysis of redox reactions involving inorganic compounds [1]. Important N-cycling processes include: nitrification, denitrification, and nitrogen fixation. Nitrification is the oxidation of ammonia (NH_4) to nitrate (NO_3^-), which includes two steps catalyzed by different microorganisms. The first step, nitrosification, is the oxidation of NH_3 to NO_2^-. The second, nitrification, is the oxidation of NO_2^- to NO_3^-. Denitrification takes nitrogen out of the system by converting NO_3^- to N_2 and other gaseous products. Finally, nitrogen fixation is the full reduction of N_2 to NH_3. *To our knowledge, this project is the first attempt at measuring processes such as these in situ.* The goal of this project is to determine nitrogenous compound concentrations and use them in thermodynamic calculations to predict which dissimilatory reactions microbial communities are utilizing for energy. Specifically, we predict that nitrification and denitrification are key pathways through which community members of Great Boiling Springs (GBS) and Sandy's Spring West (SSW) capture free energy.

Predicted Reaction	GBS (kJ/mol e-)	SSW (kJ/mol e-)
1) $NH_4^+ + {}^3/_2O_{2\,(aq)} \rightarrow NO_2^- + 2H^+ + H_2O$ (5)	-43.8	-44.4
2) $NO_2^- + {}^1/_2O_{2\,(aq)} \rightarrow NO_3^-$ (2)	-34.9	-33.7
3) $X_{(red)} + NO_3^- \rightarrow X_{(ox)} + NO_2^-$	-18.3 to -63.3	-18.1 to -63.3

Table 1 – Change in Gibbs free energy (ΔG) of select N-cycling reactions [5]. Reactions 3 and 4 represent the most and least thermodynamically favorable nitrate reduction reactions, respectively. Other possible electron donors include H_2S, S, H_2, Fe_3O_4 and Fe^{2+}.

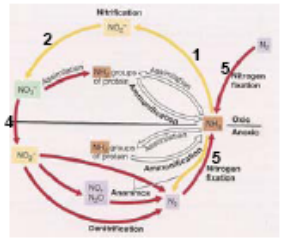


Figure 1 - The Nitrogen Cycle with tested reactions numbered. Numbers correspond with Table 1 above. Image from [3].

Methods

Denitrification rates were measured through an acetylene (C_2H_2) block experiment and subsequent analysis of nitrous oxide (N_2O) concentrations on a gas chromatography electron capture detector (Shimadzu). Our acetylene block protocol was taken and modified from [2]. The molecule acetylene has a triple bond like N_2 and has similar molecular geometry and size. When the added C_2H_2 concentration builds up intracellularly, nitrous oxide reductase (catalyzes N_2O -> N_2) becomes allosterically inhibited and thus blocks the complete reduction to N_2 (Figure 2). The microbial production of NO_3^- also ceases because ammonia monooxygenase activity is also inhibited by C_2H_2.

Nitrification

$NH3 \rightarrow X \rightarrow NO_2^- \rightarrow NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow X \rightarrow N_2$

Denitrification

Figure 2 – Coupled nitrification / denitrification pathway. A red X indicates loss of enzymatic function due to C_2H_2 addition.

This blocking as shown in Figure 2 causes an increased build up of N_2O which is taken from the Balch tube headspace at different time points and stored in vials for future gas analysis. Triplicates were taken at each time point and N_2O production was normalized to sediment mass. The rate of N_2O produced per sediment mass as a function of time is thus a measure of the denitrification activity. For both springs, three different treatments were used: one without C_2H_2, another with an C_2H_2 addition (10% v/v) and finally a set with C_2H_2 (10% v/v) and 1.0 mM NO_3^- added. Without added C_2H_2, N_2O is produced in the sediment due to incomplete denitrification. Strains of *Thermus* that reduce NO_3^- to N_2O have recently been described [9] and *Pyrobaculum aerophilum* produces N_2O as the main denitrification product [8]. In the C_2H_2 blocked treatment, elevated N_2O production was observed due to the combined measurement of natural N_2O production and the accumulation of N_2O that would normally have been denitrified completely to N_2. However, since nitrification, which supplies NO_3^- to denitrifiers, is also blocked by C_2H_2, this measurement only accounts for denitrification based on the existing NO_3^- pool and should therefore be considered a lower bound on *in situ* denitrification activity. To place a maximum bound on denitrification activity, a third sample was amended with a large excess of NO_3^- (1.0 mM), resulting in a dramatic increase in the rate of N_2O production. This result indicates that denitrification *in situ* is limited by NO_3^- supply from nitrification.

Ammonia oxidation potential was determined through polymerase chain reaction (PCR) amplification for a gene encoding for a key enzyme in this pathway – ammonia monooxygenase. Its well conserved A subunit was targeted using the following two primer pairs (with standard degeneracy notation Y = C/T, H = A/C/T, D = A/G/T, N = A/C/G/T):

forward A	forward B	reverse
5'-ATH AAY GCN GGN GAY TA-3'	5'-TTY ACN GGN GTN CCN GG-3'	5'-ACY TGN GGY TCD ATN GG-3'

The enzyme nitrogenase is typically associated with N-fixation, but it also reduces other triply bonded compounds such as cyanide and acetylene. [3] The acetylene reduction assay takes advantage of the nitrogenase catalyzed reduction of acetylene to ethylene. If nitrogenase is present, then over time, acetylene will decrease as the product ethylene builds up. In the absence of nitrogenase, no ethylene production should occur. These gases were detected and quantified using a gas chromatography flame ionization detector (Shimadzu).

In addition to assaying for N-fixing activity *in situ*, a nested PCR amplification of universal sequences within the *nifH* gene was performed. This gene encodes for dinitrogenase reductase, a subunit that transfers electrons to nitrogenase to reduce N_2. Degenerate primers were based from [4] and are as follows (with standard degeneracy notation R, A/G; S, C/G; W, A/T; Y, C/T; V, A/C/G; N, A/C/G/T; B, C/G/T):

forward A	forward B	reverse
5'-GCI WTI TAY GGN AAR GGN GG-3'	5'-GGI TGY GAY CCN AAV GCN GA-3'	5'-GCR TAI ABN GCC ATC ATY TC-3'

Whether the sequence gets amplified or not will reveal whether the potential for N-fixation exists within the community. The PCR reaction conditions consisted of 40 cycles of the following conditions: 95°C for 30 seconds, 48°C for 60 seconds and 72°C for 60 seconds. Forward A was used in the first PCR reaction while the forward B primer was used in the nested reaction. The known N-fixing species *Methanococcus maripaludis* was used as a positive control.

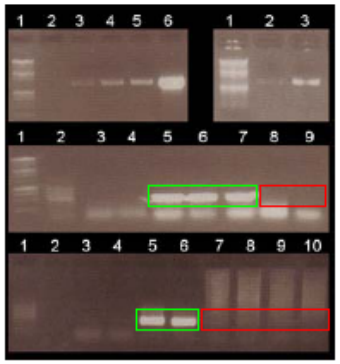


Figure 4 – Gel electrophoresis results for nifH. Lane numbers are in parentheses. All negative controls consist of the PCR master mix without added DNA. The PCR ran for gel 1 targeted the bacterial 16S rRNA gene and contains a 1kb DNA ladder (1), empty (2), a negative control (3), 1 uL of extracted GBS DNA (4), 2 uL of extracted GBS DNA (5), and 1 uL of E. coli DNA as a positive control (6). Gel 2 was designed to test the validity of SSW DNA and contains a 1kb DNA ladder (1), a negative control (2), and 1 uL of added SSW DNA (3). Gel number 3 represents the outcome of the first nifH PCR. It contains: a 1kb DNA ladder (1), a 100bp DNA ladder (2), two negative controls (3 and 4), 1 uL of 1, 10 and 100-fold dilutions of a Methanococcus maripaludis DNA stock as positive controls (5, 6 & 7), 1uL of GBS DNA (8) and 1uL of SSW DNA (9). Gel 4 shows the products of the nested PCR with a 100bp DNA ladder (1), an empty lane (2), two negative controls (3 & 4), two 1000-fold dilutions of M. maripaludis DNA stock for (5 & 6) as positive controls, two lanes of GBS DNA (7 & 8), and two lanes of SSW DNA (9 & 10). The green boxes show the positive control while the red boxes highlight the absence of the nifH gene in both habitats.

Location	NO3- (uM)	NO2- (uM)	NH3 (uM)
SSW	8.55	3.49	74.6
GBS 1'a	23.7	35.5	38.7

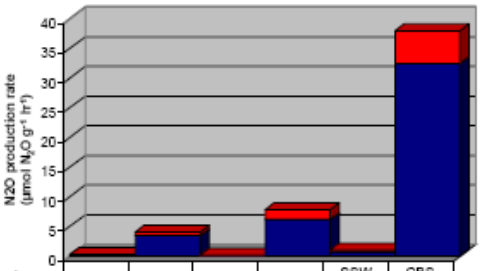
Table 2 – Concentrations of nitrogenous intermediates during Summer 2008.

Location	NO3- (uM)	NO2- (uM)	NH3 (uM)
SSW	BDL	0.21	64.1
GBS 1'a	28.0	17.9	39.4

Table 3 – Concentrations of nitrogenous intermediates during Spring 2008.

Results and Discussion

Acetylene Block Data



Units for all values are μmol N_2O g^{-1} hr^{-1}	SSW - C2H2	GBS - C2H2	SSW +C2H2	GBS +C2H2	SSW +C2H2 +NO3-	GBS +C2H2 +NO3-
Std Error Bar	0.0964	0.673	0.0306	1.66	0.352	5.41
Denitrification Rate	0.272	3.44	0.114	6.17	0.709	32.5

Figure 3 – Rates of denitrification measured by acetylene block experiments on GBS and SSW.

Both GBS and SSW share a common ammonia-laden groundwater source [10]. A lower residence time of water in SSW (5.30 minutes) [6] means that ammonia oxidizers have less time to oxidize it into nitrite and nitrate. In GBS, however, the longer residence time of 1.46 days [6] gives nitrifiers more time to convert ammonia into nitrite and nitrate. This is reflected by consistently lowered ammonia and elevated nitrite and nitrate concentrations in GBS relative to SSW (tables 2 & 3).

The slower flow in GBS allows more ammonia oxidation to occur, producing more nitrate to fuel denitrification. This could explain why denitrification rates in GBS were all significantly higher than in SSW (Figure 3). When nitrate was added, the denitrification rate increased by a factor of ~5.3 times in GBS and ~6.2 times in SSW, suggesting that nitrate is rate limiting for this metabolism. It appears that denitrification is coupled to nitrification in both habitats.

The first and nested PCR clearly show a lack of *nifH* DNA in either environment (Figure 4). In GBS and SSW, a significant amount of fixed nitrogen already exists (Table 2, 3) and so it seems logical that *nifH* is not present in either spring. Also, no ethylene peak was observed in the acetylene reduction assays. This is presumably due to a lack of the nitrogenase enzyme at both sites. On top of all this evidence, there is no evolutionary pressure to retain the ability to fix nitrogen if it is always present, especially since N-fixation consumes an energy input of 16-24 ATP [3].

As for the *amoA* PCR, a negative result suggests that ammonia oxidation is not occurring. However, close relatives to the known ammonia oxidizer "*Candidatus* Nitrosocaldus yellowstonii" [7] have been found in GBS [6] where ammonia oxidation is thermodynamically favorable as well (table 1 rxn 1). It is possible that ammonia oxidation is occurring but the PCR failed and/or the *amoA* primers did not work.

Conclusions

- Nitrogen fixation is not occurring at SSW or GBS.
- Biotic ammonia oxidation is likely occurring but has yet to be measured *in situ* in these springs.
- Nitrate availability is limiting denitrification rates at both habitats.
- Lower water flow rate allows nitrifiers to oxidize more ammonia to nitrate. The resulting increase in local nitrate concentrations stimulates denitrification and is likely why denitrification rates are much higher in GBS than SSW.

Future Directions

Additional attempts will be made at amplifying amoA gene sequences in both habitats. If successful, sequencing of these genes will be used to determine diversity. To compliment this, an *in situ* measurement of nitrification will be made using a stable ^{15}N isotope pool dilution approach.

References

Acknowledgements