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Researching nitrite oxidation at high temperatures

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^{15}N-nitrate (NO_3^-) pool dilution experiments show that ammonia (NH_3) is oxidized to nitrate in geothermal springs up to at least 85C; however, nitrite (NO_2^-)-oxidizing microorganisms are only known to grow up to 66°C. We hypothesize that thermophilic microorganisms oxidize nitrite to nitrate at high temperatures. Alternatively, it is possible that nitrite is oxidized abiotically. We propose to test these hypotheses by setting up microbial enrichments designed to grow thermophilic nitrite oxidizing bacteria by varying incubation temperature (50, 65, 80°C), oxygen concentration (20% and 5%), and cultivation media. A negative control consisting of filtered spring water (0.1 μm) will be used to determine whether nitrite is oxidized abiotically. Enrichments will be monitored for nitrite oxidation activity by using colorimetric assays for nitrite and nitrate. Enrichments showing activity will be used as a source to try to isolate and/or identify responsible microorganisms and to study the kinetics of nitrite oxidation at high temperature.

Researching Nitrite Oxidation at High Temperatures

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Abstract

The role of nitrite oxidizing organisms in the nitrogen cycle of soils and aquatic habitats is well-established; however, it is not known whether they exist in high temperature habitats. We successfully enriched for mesophilic nitrite oxidizing bacteria from aquarium pebble and soil samples and then used an expanded approach to attempt to enrich for thermophilic organisms. To date, no activity has been demonstrated, however, the extremely slow growth of nitrifiers justifies long-term monitoring of these enrichments.

Introduction

Nitrification is a process by which microorganisms catalyze the oxidation of ammonia to nitrate via nitrite according to the following chemical equations:

$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+$ (ammonia oxidation)

$NO_2^- + 0.5O_2 \rightarrow NO_3^-$ (nitrite oxidation)

In soil and aquatic habitats, the two steps of nitrification are carried out by different types of chemolithotrophic bacteria; however, these bacteria are not known to grow at high temperature. Correspondingly, very little was known about nitrification at high temperature until recently. de la Torre et al., (2008) described an ammonia-oxidizing member of the Crenarchaeota named "*Candidatus* Nitrosocaldus yellowstonii" that grows up to 74°C. Subsequently, two groups reported related ammonia monooxygenase large subunit genes (*amo*A) and the lipid crenarchaeol, a possible biomarker for these organisms, in springs up to 90°C (Zhang, Schleper).

^{15}N-nitrate (NO_3^-) pool dilution experiments show that ammonia (NH_3) is oxidized to nitrate in geothermal springs up to at least 85°C; however, nitrite (NO_2^-)-oxidizing microorganisms are only known to grow up to 66°C. We hypothesize that thermophilic microorganisms oxidize nitrite to nitrate at high temperatures. Alternatively, it is possible that nitrite is oxidized abiotically. We have begun to test these hypotheses by setting up microbial enrichments designed to grow thermophilic nitrite oxidizing bacteria by varying incubation temperature (50, 65, 80°C), oxygen concentration (20% and 5%), and cultivation media. A negative control consisting of filtered spring water (0.1 µm) was used to determine whether nitrite is oxidized abiotically. Additional low temperature enrichments inoculated with aquarium pebbles and a soil sample were used for positive controls.

Methods

1. Sediment samples were gathered at the GBS in Gerlach, NV.
2. Bottles were inoculated with a 1 ml slurry of sediment from three different temperatures (50, 65 and 80°C).
3. Three different media were used.
   a. Medium A: Modified Lebedeva mineral medium containing 1 mM $NaNO_2$
   b. Medium B: 50/50 mix of Medium A and GBS spring water amended to 1 mM of NO_2^-
   c. Medium C: Medium A plus Wolfe's Vitamins
4. Bottles were incubated at 50, 65 and 80°C to target growth based on the inoculum temperatures.
5. Bottles contained different oxygen concentrations (1 atm air and 1:4 ratio of air:N_2)
6. Nitrite levels were measured at least once weekly using the Griess diazotization method (LaMotte).
7. All readings were done on a spectrophotometer at 540 nm.

Controls

Aquarium Enrichment

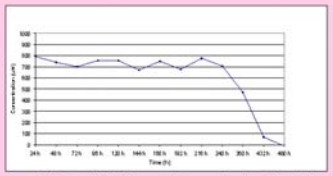


Figure 1. Nitrite oxidation by an enrichment culture inoculated with aquarium pebbles.

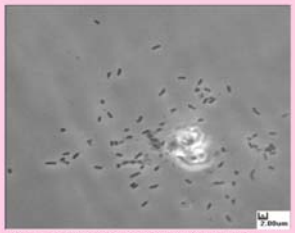


Figure 2. Bacteria found in secondary enrichment from aquarium sample.

Soil Enrichment

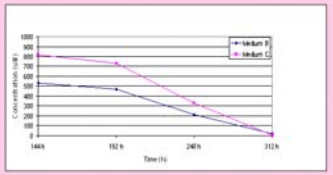


Figure 3. Nitrite oxidation by an enrichment culture inoculated with soil from the UNLV campus lawn.

Low temperature control enrichments

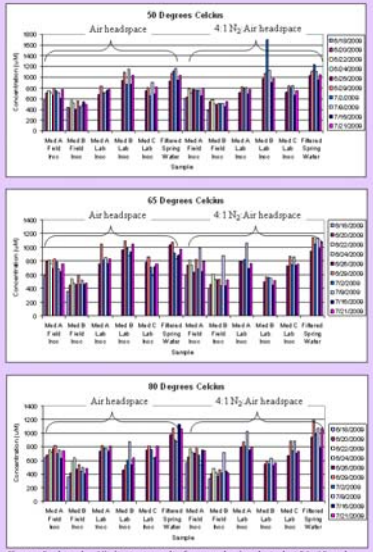
Several media known to select for nitrifiers were prepared and inoculated with samples from low temperature habitats known to have active nitrogen cycles. Primary enrichments inoculated with gravel from a freshwater aquarium showed complete nitrification within 21 days. Secondary enrichments oxidized nitrite to nitrate completely within 22 days (Fig. 1). The predominant morphotype in the culture was a pleomorphic rod- to pear-shaped organism, similar to the genus *Nitrobacter* (Fig. 2). Primary enrichments inoculated with soil from the grounds of UNLV showed complete nitrification within 13 days (Fig. 3). Secondary enrichment was not done; however, original bottles were re-seeded with NO_2^- to encourage further growth. The predominant morphotypes in the cultures were cocci- and rod-shaped organisms.

Enrichments

Figure 4. Great Boiling Spring (GBS17a) in Gerlach, NV, where samples were taken.

High temperature enrichments at GBS

The media that were used to successfully enrich mesophilic nitrifiers were inoculated with samples from a high temperature hot spring (Fig. 4). Each medium was inoculated with sediment from three different areas in the spring that maintained relatively constant temperatures of 50, 65 and 80°C. Each medium/temperature combination was incubated to favor fully aerobic and microaerophilic growth (4:1 N_2:air). Bottles were incubated in the dark at in situ temperature to help mimic their natural environment. No oxidation occurred in any of the sample bottles within the time frame of this internship (Fig. 5a, b and c); however, published doubling times of known nitrite oxidizers range from 12 to 36 hours and results have been know to take from 6 months to 1 year to occur. This project will continue throughout the upcoming school year.



Figures 5a, b and c. Nitrite test results for samples incubated at 50, 65 and 80°C. No nitrite oxidation has occurred to date.

Discussion

All control samples demonstrated nitrite oxidation indicating proper technique and methodology regarding media selection and testing procedures. Based on control results, actual project results, to date, are presumed correct. The spring water graphs show a significant flux regarding their results. This can be attributed to a variety of conditions including minor imperfections in the cuvettes or human error, such as imperfections in measuring reagents or pipetting, minute differences in reagent amounts, human error, and calibration of the spectrophotometer. Alternatively, nitrogen cycling activities such as ammonification and ammonia oxidation could lead to transient changes in nitrite concentration. Filtered spring water is the negative control for this project. It plays a very important role regarding both our original hypothesis and our alternative hypothesis. The levels in these controls have stayed relatively stable which is expected if nitrite oxidation is occurring via NOA or NOB.

Future directions

If nitrite oxidation is detected:

- Demonstrate that activity is reproducible and further stimulate growth by re-seeding primary enrichment with 1 mM NO_2^-.
- Plate on solid media and perform serial dilutions in liquid media to try to obtain a pure culture.
- Test isolates for nitrite oxidation ability.
- Identify isolates by 16S rRNA gene PCR and sequencing.
- If pure culture cannot be obtained, enrich as much as possible, identify the consortium, try to identify nitrifying organism, and characterize nitrification kinetics.

If nitrite oxidation is not detected:

- Gather fresh samples for enrichments from a variety of hot springs.
- Try a wider variety of media.

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