Geochemical Evidence for Denitrification in a Yellowstone National Park Hot Spring. H. E. Hartnett[1,2], S. Romaniello[1], B. Johnson[1], M. Kyle[3], T. Anderson[4], A. D. Anbar[1,2], J. Elser[3], and E. Shock[1,2]. [1]School of Earth and Space Exploration, Arizona State University, Tempe AZ, 85287-1404, [2]Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ, 85287-1406, [3]School of Life Sciences, Arizona State University, Tempe, AZ, 85287, [4]Department of Biology, University of Oslo, Oslo, Norway.

Introduction: Nitrogen (N) is a key nutrient element for all known forms of life. Microbial metabolisms have evolved 'recipes' that require N as well as carbon, C; phosphorus, P; iron, Fe; and other elements in specific stoichiometric ratios. The requirement for N is such that organisms will expend significant amounts of metabolic energy to obtain N when it is in short supply. Therefore, both autotrophs and heterotrophs have evolved numerous metabolic strategies for obtaining and utilizing N. While our understanding of these metabolic strategies in aquatic and marine systems has advanced markedly, our ignorance of these biogeochemical processes in extreme environments (e.g., hot springs) is almost complete.

Biogeochemical processes have been explored in high-temperature ecosystems (terrestrial hot springs and marine hydrothermal vents) from both geochemical and biological perspectives. Geochemical approaches provide information about the reactions that are possible, the chemical concentrations in and isotopic compositions of the fluids, and occasionally, rates of reactions. Biological studies (both organismal and molecular) have made significant progress with respect to describing the types and diversity of organisms present in hydrothermal systems but are limited in their ability to determine what microbes and metabolisms are active in a given location at a specific time. Laboratory studies using indirect acetylene block techniques have demonstrated the presence of a variety of N-transformation reactions in both hot springs and thermal soils from the Yellowstone hydrothermal region [1], [2]; but, there are few, if any, direct N-process rate measurements.

We have conducted a series of geochemical rate measurements for metabolic reactions involved in nitrogen cycling in Yellowstone National Park hot springs. These stable-isotope tracer-addition experiments were designed to track the fate of specific nitrogen species into various pools (biomass, dissolved and gaseous species). The N-cycle pathways of interest included nitrogen fixation, ammonia oxidation and nitrate reduction. These experiments were coupled with detailed measurements of hot spring geochemistry and will be able to be compared with results of genomic analyses that are also underway.

Methods: Field incubations using ^{15}N-tracer additions were conducted during the summer 2009 field season in Yellowstone National Park. At each hot spring water and sediment or biofilm was collected, subdivided into three treatments and amended with either $^{15}NO_3^-$, $^{15}NH_4^+$, or $^{15}N_2$. One third of the bottles in each treatment were poisoned with mercuric chloride to inhibit biological activity (killed controls). Amended samples were distributed into 125 ml serum bottles, capped, and incubated at the in situ temperature in the hot spring or the hot spring outflow channel. Initial samples were collected immediately upon distributing samples into bottles (~1h) and final samples were collected after 3 to 5 hours of incubation. Subsamples of water were collected without headspace, fixed and returned to the laboratory for subsequent analysis of $^{15}N_2O$ and $^{15}N_2$ by continuous-flow isotope ratio mass spectrometry.

Results and Discussion: The stable-isotope label experiments permit specific pathways in the nitrogen cycle to be traced based on the labeled species being metabolized and the labeled products being formed. For example, denitrification is the name given to the heterotrophic reduction of nitrate (NO_3^-) to nitrogen gas (N_2) coupled to the oxidation of organic carbon. In simplified form it follows this reaction:

$$4NO_3^- + 5\text{“}CH_2O\text{”} \rightarrow 2N_2 + 3H_2O + 4HCO_3^- + CO_2,$$

where “CH_2O” is a generalized organic carbon molecule. The reaction proceeds in a stepwise fashion and the NO_3^- is converted into a series of intermediate compounds on the way to N_2 ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Thus, if an addition of ^{15}N-labelled NO_3^- is found to produce ^{15}N-labelled N_2O (nitrous oxide) the inference is that denitrification is occurring in the experiment. Another pathway that can be examined this way is nitrification, the chemoautotrophic conversion of NH_4^+ to NO_3^-:

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + H_2O + 2H^+.$$

This reaction proceeds via a consortium of microbes and one of the byproducts of the reaction is N_2O. So, if $^{15}N_2O$ is produced in the incubation with added $^{15}NH_4^+$ we infer that nitrification is an active pathway.

Results from a slightly acidic hot spring (pH ~5, T = 55 ºC) indicate the production of $^{15}N_2O$ in incubations with $^{15}NO_3^-$ addition (Fig. 1a), but not in incuba-

tions with $^{15}NH_4^+$ (Fig. 1b). The nitrogen isotope composition of the N_2O also became enriched in ^{15}N over the course of the incubation period.

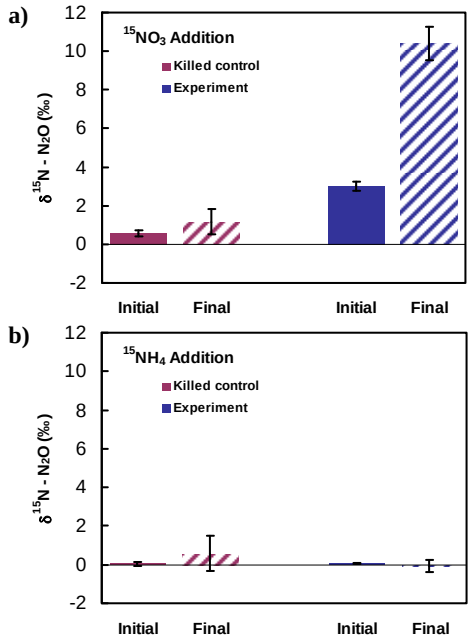

Fig. 1. Isotopic composition of nitrous oxide, $\delta^{15}N$-N_2O (‰), produced in incubations amended with $^{15}NO_3^-$ (a) and with $^{15}NH_4^+$ (b). Significant amounts of labeled N_2O were produced with $^{15}NO_3^-$ addition relative to poisoned controls. There was no detectable $^{15}N_2O$ produced in any of the $^{15}NH_4^+$ additions.

Taken together, these results indicate that denitrification was occurring in this spring and that no nitrification could be detected. Nitrate concentrations in the spring were relatively low (0.7 μM kg^{-1}), consistent with the presence of reactions that consume NO_3^-. In previous years the spring has also shown low but measurable amounts of dissolved organic carbon (82 μM kg^{-1}), a necessary component for heterotrophic denitrification.

Summary and Implications: To our knowledge these are the first direct measurements of denitrification in Yellowstone hot springs. When these results are coupled with ongoing analyses of $\delta^{15}N$ in the biomass, the N_2 gas, and the dissolved N-species they will provide one of the most complete pictures of N-cycling in hot springs to date. These results provide geochemical evidence that can guide genomic and metabolomic studies. They will also inform future studies investigating the detailed elemental requirements for specific enzymes in the denitrification pathway (e.g., Mo in nitrate reductase, or Cu in nitrite reductase) in high-temperature aquatic ecosystems.

References: [1] Holloway, J. et al. (2004) *Water Rock-Interaction, Proc. 11th Intl. Symposium on Water-Rock Interaction.* eds. Wanty and Seal, Balkema NY, pp145-148. [2] Burr. M. D. et al. (2005) *Geothermal Biology and Geochemistry in Yellowstone National Park: Proc. Thermal Biol. Inst. Workshop.* eds. Inskeep and McDermott, Montana St. UP, pp 171-182.