

# Abundance of *NifH* Genes in Urban, Agricultural, and Pristine Prairie Streams Exposed to Different Levels of Nitrogen Loading

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The biosphere has been highly enriched with nutrients, especially nitrogen, by human activities. In most ecosystems, nitrogen availability should be limited, but soils and aquatic ecosystems have been heavily impacted by human activities such as agriculture, recreation, and urbanization. Nutrient dynamics are important to our understanding of the processes of eutrophication and oligotrophication. Ecosystem processes drive biogeochemical cycles that influence input and losses of nutrients in the environment. In streams, the availability of nutrients, geochemical characteristics, hydrodynamics, and human activities influence the metabolic activities and structure of microbial communities. A combination of process-level and molecular microbial ecology techniques is being applied to study nitrogen fixation in small prairie streams with different nitrogen impact histories, from pristine to heavily polluted. Nitrogen fixation was measured with acetylene reduction assays. Impacted urban and agricultural streams exhibited low nitrogen fixation activity. On the other hand, nitrogen fixation was relatively high in a pristine stream at Konza prairie, particularly in leaf litter samples. Simultaneous sampling of sediments and leaf litter was performed for later molecular analyses of the nitrogen-fixing microbial guild. Direct DNA extracts were examined using real-time PCR of a nitrogenase complex gene (*nifH*) to determine the abundance of nitrogen-fixing organisms. The *nifH* gene has one of the largest non-ribosomal sequence databases from diverse uncultivated microorganisms. The construction and sequencing of PCR-based clone libraries will assess diversity and community distribution in these different streams. This study will provide a link between the abundance of *nifH* genes and nitrogen fixation activity. An understanding of the effect of nitrogen pollution on nitrogen cycling communities in small streams will increase our ability to overcome the challenges of nutrient pollution. This work is supported by Kansas NSF EPSCoR.

Quantification of nutrient dynamics is important to our understanding of processes such as eutrophication, oligotrophication, nutrient cycling and ecosystem function. In aquatic environments several factors - including nutrient availability, geochemistry, hydrodynamics, and human impact - are the main drivers of metabolic activities and microbial community composition. Nitrogen is the most significant nutrient limitation on production in aquatic ecosystems. Streams are key interfaces between terrestrial zones and downstream zones in aquatic ecosystems. A good example is hypoxic zone oxygen depletion in the Gulf of Mexico, where about 20,000 Km<sup>2</sup> have been polluted because of high concentrations of nitrogen (Rabalais, A.N. et. al., 2001). The work presented here is part of a collaborative project examining the effects of chronic nitrogen loading on urban, agricultural, and pristine stream systems as part of the Lotic Intersite Nitrogen eXperiment II (LINX II). Collaborators at Kansas State University and the University of Kansas are examining spatial and temporal distribution of <sup>15</sup>N and abundance of nitrogen reductase genes in each of the stream systems. This study uses the acetylene reduction assay to measure nitrogen-fixation rates and employs real-time PCR to examine the frequency of one of the nitrogenase complex genes (*nifH*) to determine the abundance of nitrogen-fixing organisms in each of the streams in order to contrast gene abundance and metabolic responses of N<sub>2</sub>-fixing microorganisms to chronic nitrogen loading in the three different types of Kansas streams.

Three different streams were chosen based on nitrogen load. One stream had minimal impact from human disturbance, King's Creek in the Prairie Research Natural Area, at Konza Prairie in KS. Two streams were chosen that were heavily impacted anthropogenically: one on Kansas State University campus, and one in the farms of Kansas State University, agricultural experiment station (Kemp et. al. 2001, Dodds et. al. 2000, and Dodds et. al. 2002). These impacted streams have been highly enriched with nutrients, especially nitrogen, by human activities. Surface sediment, leaf litter and algal samples were collected from each stream. Two sets of samples were collected, one for molecular analyses and the other for nitrogen fixation assay. Conversion of acetylene to ethylene by nitrogenase can be used as surrogate for nitrogen fixation. Acetylene gas (to 10%) was injected into the headspace of the jars or tubes. Samples were incubated at room temperature and analyzed via gas chromatography at 24 hours, 72 hours, 1 week, and 2 weeks after collection. The amount of nitrogen fixation was determined by normalizing

ethylene production according to a conversion factor 3.8 moles of acetylene reduced = 1 mol of N<sub>2</sub>-reduced (Jensen, B. et al., 1983). DNA was extracted from sediment, leaf litter and algal samples using the protocol of Bürgmann et al. 2001 with some modifications. Real-time PCR was performed using a Smart Cycler® II System (Cepheid Technology). Real-time PCR was performed using SYBR® *Premix EX Taq*<sup>TM</sup> (Perfect Real Time TaKara Mirus Bio) in accordance with the manufacturer's instructions. The primer set used for this analysis was UNIFF forward and ZNIFR reverse (Ueda et al. 1995; Zehr et al. 1995). The three different types of stream each year showed particular characteristics that made each stream an independent environment. The acetylene reduction assay showed high variability in nitrogen fixation rates among the three different stream types each year, rates from 0.004 to 0.009 pmol N<sub>2</sub> reduced/g sample/h in 2003-urban stream, from 0.002 to 0.003 pmol N<sub>2</sub> reduced/g sample/h in 2004-urban stream, from 0.004 to 0.01 pmol N<sub>2</sub> reduced/g sample/h in 2003-agricultural stream, from 0.002 to 0.005 pmol N<sub>2</sub> reduced/g sample/h in 2004-agricultural stream, from 0.003 to 0.08 pmol N<sub>2</sub> reduced/g sample/h in 2003-pristine stream, and from 0.009 to 0.07 pmol N<sub>2</sub> reduced/g sample/h in 2004-pristine stream. The streams that have been anthropogenically affected showed lower nitrogen fixation rates than the reference streams. *nifH* gene copies/gram sample (abundance) were found in all different types of streams, at rates from 1.0E03 to 1.0E06 in urban streams, from 7.0E03 to 1.0E07 in agricultural streams, and from 9.0E03 to 1.0E06 in pristine streams. Nitrogen enrichment of the biosphere is an issue of global concern. Enrichment of fresh water is leading to a process of cultural eutrophication, hypoxia and anoxia in the water column of aquatic environments. This problem may put human health at risk besides the increased cost of treatments to purify water for human consumption (Gregory, F.M., et al. 2002). Quantification of organisms such as diazotrophs may provide new approaches to study how populations interact within the environment and how the metabolic activities are controlled. Studies developed by Piceno and Lovell in 2000 suggest that N<sub>2</sub>-fixing organisms are stable even when the N availability has been increased over the long term. Microbial communities are highly structured in specific environments, but changes in the environment impact the activity levels of microorganisms in a microbial community. Nitrogen addition to the environment might be a factor selecting against N<sub>2</sub>-fixing microorganisms because of energy costs (Bagwell, C. et al. 2000).

- The acetylene reduction assay showed high variability in nitrogen fixation rates among the three different stream types each year.
- Nitrogen fixation appeared to be much higher in the leaf litter fractions in 2003 and in the sediment fractions in 2004.
- N<sub>2</sub> fixation activity appears to be negatively correlated with nitrogen loading in the streams.
- N<sub>2</sub>-fixation activity was not directly correlated with *nifH* gene abundance in this environment.
- The abundance of *nifH* genes ranged between 1E+03 to 1E+07 gene copies in all streams.
- The difference between *nifH* gene abundance and bacteria and archaea 16S rRNA gene abundance suggests that for every 1,000,000 microorganisms only 1 is a N<sub>2</sub>-fixing organism.
- *nifH* gene may not give a selective advantage in these microbial communities under normal conditions (Jenkins et al. 2004)

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