1. COVER PAGE

Final Technical Report

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Principal Investigator: Frank E. Loeffler, Ph.D.
Department of Microbiology, Dept. of Civil & Environmental Engineering,
Center for Environmental Biotechnology, The University of Tennessee
Biosciences Division, Oak Ridge National Laboratory
Phone: 865-974-4933; Email: frank.loeffler@utk.edu

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Blount Hall
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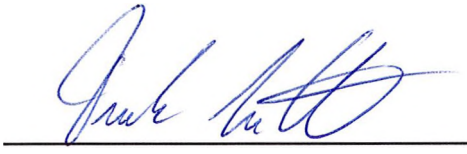
Signature:  Dr. Frank E. Loeffler, PI

2. ACCOMPLISHMENTS

What were the major goals of the project?

Background. In anoxic environments, respiratory ammonification (also known as dissimilatory nitrate reduction to ammonium, DNRA) and nitrate reduction to the gaseous products nitrous oxide (N_2O) and dinitrogen (N_2) (i.e., denitrification) are major nitrate/nitrite-consuming processes. Respiratory ammonification oxidizes more carbon (C) per mole of nitrate reduced than denitrification and generates a cation (NH_4^+), which is retained in soils and available for plants (e.g., biofuel crop production). Thus, denitrification and respiratory ammonification have profoundly different impacts on nitrogen (N) retention and greenhouse gas (CO_2, N_2O) emissions. Microbes capable of respiratory ammonification or denitrification coexist but the environmental controls over these competing nitrate/nitrite-reducing processes are largely unknown. With the current level of understanding, predictions under what environmental conditions respiratory ammonification activity predominates leading to N-retention rather than N-loss are tenuous. Further, the diversity of genes encoding the ammonium-forming nitrite reductase NrfA is poorly defined hampering the development of tools to assess and monitor this activity in environmental samples.

Another relevant knowledge gap is the fate of N_2O, a gas implicated in ozone layer destruction and climate change. The conversion of N_2O to benign N_2 is catalyzed by N_2O reductase, the characteristic enzyme system of complete denitrifiers. Thus, efforts to estimate N_2O conversion to N_2 have focused on the well-characterized denitrifier *nosZ* genes; however, our understanding of the diversity of genes and organisms contributing to N_2O consumption is incomplete. This paucity of information limits the development of more accurate, predictive models for C- and N-fluxes and greenhouse gas emissions.

The overarching goals of the project were to advance understanding of the environmental controls over nitrate/nitrite reduction pathways, and to reveal the diversity and sequence space of *nrfA* and *nosZ* genes so that the fate of nitrate/nitrate and the microbial contributions to consumption of the greenhouse gas N_2O can be more accurately assessed ands predicted.

What was accomplished under these goals?

Major Activities: The project team applied a series or cultivation and cultivation-independent experimental approaches combined with rigorous computational sequence analyses to address the existing knowledge gaps. The *Specific Objectives* of this project were to:

Objective 1. Explore the environmental parameters that control respiratory ammonification and denitrification pathways using *Shewanella loihica* strain PV-4 as a model.

Objective 2. Characterize the diversity of *nrfA* and *nosZ* gene sequences in soils and subsurface environments.

Objective 3. Apply the new knowledge and tools to investigate relevant N-cycle processes in two distinct soil ecotypes.

These experimental efforts resulted in *Significant Results and Accomplishments* and several manuscripts have been published in the peer-reviewed literature.

Objective 1. Regulatory controls of DNRA versus denitrification pathways.

To elucidate the environmental factors controlling nitrate/nitrite turnover in soils, a unique isolate (*Shewanella loihica*) that harbors both the respiratory ammonification and the denitrification pathways was studied. The effects of pH, temperature, C/N ratios, and nitrite/nitrate ratios were explored using batch and continuous culture experiments. For all experiments, lactate was provided as electron donor. To quantify N_2O formation as a measure of denitrification, vessels were amended with acetylene to inhibit the N_2O-to-N2 reduction step. Denitrification dominated at low C/N ratios (i.e., electron donor-limiting growth conditions), while ammonium was the predominant product at high C/N ratios (i.e., electron acceptor-limiting growth conditions). At intermediate C/N ratios, denitrification and respiratory ammonification occurred concomitantly. pH and temperature also affected NO_3^-/NO_2^- fate, and incubation above pH 7.0 and temperatures of 30°C favored ammonium formation. Reverse transcriptase real-time quantitative PCR (RT-qPCR) analyses correlated the phenotypic observations with *nirK* and *nosZ* transcript abundances, which decreased up to 1,600-fold and 27-fold, respectively, under conditions favoring respiratory ammonification. Of the two *nrfA* genes encoded on the strain PV-4 genome, $nrfA_{0844}$ transcription decreased only when the chemostat reactor received medium with the lowest C/N ratio of 1.5, whereas $nrfA_{0505}$ transcription occurred at low levels ($\leq 3.4\times10^{-2}$ transcripts per cell) under all growth conditions. At intermediate C/N ratios, denitrification and respiratory ammonification occurred concomitantly, and both $nrfA_{0844}$ (5.5 transcripts per cell) and *nirK* (0.88 transcripts per cell) were transcribed. Batch and chemostat experiments further demonstrated that NO_2^- affects pathway selection and the formation of reduced products. Strain PV-4 cells grown with NO_2^- as the sole electron acceptor produced exclusively NH_4^+. With NO_3^- as electron acceptor, denitrification predominated and N_2O accounted for ~90% of reduced products in the presence of acetylene. Chemostat experiments demonstrated that the NO_2^-/NO_3^- ratio affected the distribution of reduced products, and respiratory ammonification dominated at high NO_2^-/NO_3^- ratios whereas low NO_2^-/NO_3^- ratios favored denitrification. The NO_2^-/NO_3^- ratios affected *nirK* transcript abundance, a measure of denitrification activity, in the chemostat experiments and cells grown at a NO_2^-/NO_3^- ratio of 3 had ~37-fold fewer *nirK* transcripts per cell than cells grown with NO_3^- as the sole electron acceptor. In contrast, the transcription of *nrfA*, implicated in NO_2^--to-NH_4^+ reduction, remained statistically unchanged under continuous cultivation conditions at NO_2^-/NO_3^- ratios below 3. At NO_2^-/NO_3^- ratios above 3, both *nirK* and *nrfA* transcript numbers decreased and the chemostat culture washed out, presumably due to NO_2^- toxicity. These findings implicate NO_2^- as a relevant modulator of NO_3^- fate in *S. loihica* strain PV-4, and, by extension, suggest that NO_2^- may be a relevant determinant for N-retention (i.e., ammonification) versus N-loss and greenhouse gas emission (i.e., denitrification).

Major Conclusions

- Low C/N ratios (electron donor-limiting growth conditions) favor denitrification and N-loss.
- High C/N ratios (electron acceptor-limiting growth conditions) favor respiratory ammonification and N-retention.
- High NO_2^-/NO_3^- ratios favored respiratory ammonification
- pH above 7 and elevated temperatures favor respiratory ammonification and N-retention.

- Strain PV-4 offers a tractable experimental system to explore regulation of dissimilatory NO_3^-/NO_2^- reduction pathways.

Objective 2. Diversity of *nrfA* and atypical *nosZ* gene sequences.

A. Refined NrfA phylogeny improves PCR-based *nrfA* gene detection

Respiratory ammonification and denitrification are contrasting microbial processes in the terrestrial nitrogen- (N-) cycle in that the former promotes N-retention and the latter leads to N-loss (i.e., the formation of gaseous products). The nitrite reductase NrfA catalyzes nitrite reduction to ammonium, the enzyme associated with respiratory ammonification. Although well studied biochemically, the diversity and phylogeny of this enzyme had not been rigorously analyzed. A phylogenetic analysis of 272 full-length NrfA protein sequences distinguished 18 NrfA clades with robust statistical support (>90% Bayesian posterior probabilities). Three clades possessed a CXXCH motif in the first heme-binding domain whereas all other clades had a CXXCK motif in this location. The analysis further identified a KXRH or KXQH motif between the third and fourth heme-binding motifs as a conserved and diagnostic feature of all pentaheme NrfA proteins. PCR primers targeting a portion of the heme-binding motifs that flank this diagnostic region yielded the expected 250 bp-long amplicons with template DNA from eight pure cultures and 16 new *nrfA*-containing isolates. *NrfA* amplicons obtained with template DNA from two geomorphically distinct agricultural soils could be assigned to one of the 18 NrfA clades providing support for this expanded classification. The extended NrfA phylogeny revealed novel diagnostic features of DNRA populations and will be useful to assess nitrate/nitrite fate in natural and engineered ecosystems.

Major Conclusions

- The comprehensive NrfA sequence analysis identified novel diagnostic features shared among all known NrfA.
- An expanded PCR primer set captures the known *nrfA* gene diversity, which will be useful for assessing the prevalence and activity of potential respiratory nitrite-ammonifying populations in natural and engineered ecosystems.

B. Expanding the *nosZ* sequence space: Discovery of atypical *nosZ*

Agricultural and industrial practices more than doubled the intrinsic rate of terrestrial N fixation over the past century with drastic consequences including increased atmospheric nitrous oxide (N_2O) concentrations. N_2O is a potent greenhouse gas and contributor to ozone layer destruction, and its release from fixed N is almost entirely controlled by microbial activities. Mitigation of N_2O emissions to the atmosphere has been exclusively attributed to denitrifiers possessing NosZ, the enzyme system catalyzing N_2O to N_2 reduction. Integrated efforts using experimental and computational approaches revealed that diverse microbial taxa possess divergent *nos* clusters with genes that are related, yet evolutionarily distinct from the typical *nos* genes of denitirifers. *nos* clusters with atypical *nosZ* occur in *Bacteria* and *Archaea* that denitrify (44% of genomes), do not possess other denitrification genes (56%) or perform respiratory ammonification (i.e., dissimilatory nitrate reduction to ammonium, DNRA) (31%). Experiments

with the DNRA soil bacterium *Anaeromyxobacter dehalogenans* demonstrated that the atypical NosZ is an effective N_2O reductase, and PCR-based surveys suggested that atypical *nosZ* are abundant in terrestrial environments. Bioinformatic analyses revealed that atypical *nos* clusters possess distinctive regulatory and functional components (e.g., Sec versus Tat secretion pathway in typical *nos*), and that previous *nosZ*-targeted PCR primers do not capture the atypical *nosZ* diversity. Collectively, our results suggest that non-denitrifying populations with a broad range of metabolisms and habitats are potentially significant contributors to N_2O consumption. Apparently, a large, previously unrecognized group of environmental *nosZ* has not been accounted for, and characterizing their contributions to N_2O consumption will advance understanding of the ecological controls on N_2O emissions and lead to refined greenhouse gas flux models.

Major Conclusions

- The diversity of genes and organisms contributing to consumption of N_2O is fare greater than previously recognized.
- The contributions of organisms carrying the atypical nosZ gene must be considered in monitoring regimes and future greenhouse gas flux models.

C. Distribution and abundance of atypical *nosZ* genes in soils

Microbial activities in soils, such as (incomplete) denitrification, represent major sources of the greenhouse gas N_2O. The key enzyme for mitigating N_2O emissions is NosZ, which catalyzes N_2O reduction to N_2. Until recently, consumption of N_2O was attributed to bacteria encoding typical nitrous oxide reductase (NosZ). However, recent phylogenetic and physiological studies have shown that previously uncharacterized, functional, atypical NosZ proteins are encoded in genomes of diverse bacterial groups. The abundance and diversity of typical and atypical *nosZ* types was investigated in whole-genome shotgun metagenomes from sandy and silty loam agricultural soils that typify the U.S. Midwest corn belt. First, different search algorithms and parameters for detecting *nosZ* metagenomic reads were evaluated based on *in silico*-generated (mock) metagenomes. Using the derived cutoffs, 71 distinct alleles (95% amino acid identity level) encoding typical or atypical NosZ proteins were detected in both soil types. Remarkably, more than 70% of the total *nosZ* reads in both soils were classified as atypical, emphasizing that prior surveys underestimated *nosZ* abundance. Approximately 15% of the total *nosZ* reads were taxonomically related to *Anaeromyxobacter*, which was the most abundant genus encoding atypical NosZ-type proteins in both soil types. Further analyses revealed that atypical *nosZ* genes outnumbered typical *nosZ* genes in most publicly available soil metagenomes, underscoring their potential role in mediating N_2O consumption in soils. Therefore, this study provides a bioinformatics strategy to reliably detect target genes in complex short-read metagenomes and suggests that the analysis of both typical and atypical *nosZ* sequences is required to understand and predict N_2O flux in soils.

Major Conclusions

- Atypical *nosZ* genes are distributed in soil ecosystems.
- Atypical *nosZ* genes often outnumber their typical counterparts, emphasizing their potential role in N_2O consumption in soils and possibly other environments.

Objective 3. Apply the new knowledge and tools to investigate relevant N-cycle processes in two distinct soil ecotypes.

The spatiotemporal dynamics of microbial communities harboring typical and atypical *nosZ* genes were examined at two field sites located in Illinois. The selected field sites experience similar climatic conditions but the soils have different physical and chemical characteristics (i.e., sandy and silty soils). A three-pronged approach was applied. PCR primer sets specifically targeting either the atypical *nosZ* or typical *nosZ* (both ~1,500 bp amplions) genes were designed and validated. DNA-based T-RFLP *nosZ* profiles were obtained at five sampling times over a 1-year period (Nov 2011, Apr 2012, Jun 2012, Sept 2012, Nov 2012), and included three soil depths (0-5 cm, 5-20 cm, 20-30 cm) from three within-plot locations at both study sites. The results demonstrated that the atypical and typical *nosZ* gene diversities and abundances differed with soil depth in both the highly porous sandy soil as well as in poorly drained clayey loam. Further, both types of *nosZ* sequences demonstrated significant temporal shifts in relative abundances. Interestingly, the typical and atypical *nosZ* gene sequences did not shift in parallel over temporal scales, suggesting the microbial populations harboring the respective *nosZ* genes differ in their response to environmental factors.

Metagenomic analyses revealed the most abundant typical and atypical *nosZ* genes in both soil types, and a time-series analysis demonstrated dynamic changes of the relative abundances of the most abundant *nosZ* genes over temporal and spatial scales. Based on the metagenomic information, quantitative PCR (qPCR) assays were designed for the five most abundant typical and atypical *nosZ* genes so that absolute quantitative data can be obtained. These efforts provide horizontal and depth-resolved spatial and temporal information of *nosZ* gene dynamics. Field-based measurements demonstrated large amplitude temperature fluctuations (up to Δ30°C) in surface layer soil (< 5 cm) whereas deeper layers (20-30 cm) undergo a narrow diurnal cycle of temperature change (Δ2-3°C). The qPCR data reveal the impacts of such physical gradients on dynamics of microbial populations involved in nitrate/nitrite reduction and the reduction of N_2O.

Somewhat surprising was that the metagenome sequencing efforts provided little insight into the fungal diversity in the soils, even though cultivation-based efforts and automated rRNA intergenic spacer analysis (ARISA) demonstrated the presence of diverse fungal communities in both soil types. Fungi represent a significant fraction of the microbial biomass in soils and have been shown to produce N_2O in the mitochondria. An important distinction between fungal and bacterial denitrification is the presence of a unique gene encoding a p450 cytochrome, P450nor, responsible for reducing nitric oxide (NO) to N_2O in fungi. Unfortunately, molecular tools to determine the diversity, abundance, or activity of fungal denitrification genes in soils are largely lacking. To address this shortcoming, degenerate PCR primers targeting the known *p450nor* sequence diversity was designed and validated. The combined application of cultivation and *p450nor*-targeted PCR established the presence of denitrifying fungi and expanded the diversity of fungal P450nor sequences in the two agricultural soils with distinct physical-chemical properties. These results emphasize the value of combined cultivation-based efforts and PCR tools for assessing fungal denitrifier diversity and N_2O production potential.

The integrated analysis of metagenomic, qualitative PCR, qPCR, ARISA, cultivation and physical-chemical soil measurements (e.g., temperature, moisture) is nearly completed and several manuscripts are in preparation.

Major Conclusions

- Populations harboring typical and atypical *nosZ* genes undergo substantial temporal (seasonal) and spatial (depth) fluctuations.
- Temperature and soil moisture are key drivers of temporal microbial community dynamics.
- Denitrifying fungi harboring *p450nor* genes are distributed in soils but are notoriously underrepresented in metagenomic datasets.

What opportunities for training and professional development has the project provided?

The project involved undergraduate students, doctoral students and postdocs at all participating institutions. All project personnel participated in monthly project discussion via video or conference calls. Additional regular group meetings occurred at each participating institution. Presenters shared Powerpoint slides detailing research activities and findings with project personnel prior to the group meetings and received questions and feedback prior, during and after the presentation. On average, each PUNS project student and postdoc had at least four group presentations per year. To enhance the students' learning experience, the advisor met with the presenting student or postdoc prior to group meetings to discuss slide layout and content so the materials were effectively communicated. The PI and Co-PIs at each institution met regularly with undergraduate students, doctoral students and postdocs to discuss opportunities for student (travel) awards, fellowships, and possible career paths. For example, Mr. Steven Higgins was awarded a DOE-SCGSR fellowship to work for 1 year at Oak Ridge National Laboratory with Dr. Chris Schadt in the Biosciences Division on aspects directly related to the PUNCS project. All students were encouraged to apply and participate in career workshops, which are regularly offered at each of the participating institutions, and most students took full advantage of these opportunities.

Undergraduate students were provided opportunities to work with graduate students and postdocs on smaller, defined research objectives under the umbrella of the PUNCS project. Graduate students shaped their project within the PUNCS framework and had opportunities to apply for travel and subsistence support to work on clearly defined, short-term (1-2 weeks) projects at one of the collaborating institutions.

How have the results been disseminated to communities of interest?

Major research finding have been published in the peer-reviewed literature and several more manuscripts are in preparation. In addition, the presentation of research findings at conferences and workshops contributed to the broad dissemination of the new information. The involvement of a USDA collaborator facilitated the distribution of relevant information to the farming community. Such outreach activities are relevant because agricultural production is a major source of atmospheric N_2O.

3. PRODUCTS

Manuscripts in Preparation

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Welsh, A., J. Chee-Sanford, L. Connor, F. Löffler, and R. Sanford. 2013. Diversity of NrfA in agricultural soils with depth, position in the field, season and soil type reveals varying populations capable of mediating dissimilatory nitrate reduction to ammonia (DNRA). 5th Annual Argonne Soil Metagenomics Meeting, October 2-4, 2013, Bloomingdale, IL.

Posters 2013

Nissen, S., X. Liu, K. Chourey, K. M. Ritalahti, R. Hettich, S. Pfiffner, and F. E. Löffler. Identification of a c-type cytochrome involved in Mn(IV) reduction in *Anaeromyxobacter dehalogenans* strain 2CP-C. ASM 2013, General Meeting, American Society for Microbiology, Denver, CO, USA, May 18-21, 2013.

Chee-Sanford, J. C., A. K. Welsh, L. M. Connor, F. E. Löffler, and R. A. Sanford. Spatial and temporal changes in distribution of typical- and atypical nitrous oxide reductase (*nosZ*) genes in two contrasting agricultural soils. Abstract 2446. Poster presented at the 113th General Meeting of the American Society for Microbiology, Denver, CO, USA, May 18-21, 2013.

Yoon, S., R. A. Sanford, K. M. Ritalahti, and F. E. Löffler. Regulation of nitrate/nitrite reduction pathways in *Shewanella loihica* strain PV-4. Abstract 696, Poster presented at the 113th General Meeting of the American Society for Microbiology, Denver, CO, USA, May 18-21, 2013.

Orellana, L. H., S. Higgins, J. Chee-Sanford, R.A. Sanford, F.E. Löffler, and K. T. Konstantinidis. Detecting nitrous oxide reductase (*nosZ*) genes in metagenomes: method development and

application to midwestern soils. Abstract 352, Poster presented at the 113th General Meeting of the American Society for Microbiology, Denver, CO, USA, May 18-21, 2013.

Welsh, A.K., J. C. Chee-Sanford, L. M. Connor, F.E. Loeffler, and R.A. Sanford. Are microbial communities in agricultural soils homogeneous at different depths and spatial scales? Abstract 2460, Poster presented at the 113th General Meeting of the American Society for Microbiology, Denver, CO, USA, May 18-21, 2013.

Welsh, A.K., J.C. Chee-Sanford, L.M. Connor, F.E. Löffler, and R.A. Sanford. 2013. Towards Predictive Understanding of Nitrogen Cycling in Soils: The Role of Ammonia-forming Nitrite Reductase (NrfA). Genome Science Program Contractor-Grantee Workshops, Washington DC, February 25-27, 2013.

Orellana, L.H., S. Higgins, J. C. Chee-Sanford, R.A. Sanford, F.E. Löffler, and K.T. Konstantinidis. 2013. Detecting Nitrous Oxide Reductase (nosZ) Genes in Metagenomes: Method Development and Application to Midwestern Soils. Genome Science Program Contractor-Grantee Workshops, Washington DC, February 25-27, 2013.

Posters 2012

Ritalahti, K.M., R.A. Sanford, S. Yoon, A.K. Welsh, J.C. Chee-Sanford, L.-M. Rodríguez Rojas, K.T. Konstantinidis and F.E. Löffler. 2012. Toward predictive understanding of nitrogen cycling in soils. Poster presented at the 14th International Symposium on Microbial Ecology, Copenhagen, Denmark, August 19-24, 2012.

Yoon, S., C. Cruz-Garcia, K.M. Ritalahti, R.A. Sanford, and F.E. Löffler. 2012. Environmental controls of nitrate/nitrite reduction pathways in *Shewanella loihica* PV-4. Abstract 12-A-4817-GM-ASM. In Abstracts of the 112th General Meeting of the American Society for Microbiology, San Francisco, CA, USA, June 16-19, 2012.

Welsh, A.K., J.C. Chee-Sanford, L.M. Connor, F.E. Löffler, and R.A. Sanford. 2012. New tools for detection of the *nrfA* gene responsible for dissimilatory nitrite reduction to ammonia in the environment. Abstract 12-GM-A-3211_ASM. In Abstracts of the 112th General Meeting of the American Society for Microbiology, San Francisco, CA, USA, June 16-19, 2012.

Sanford, R.A., A.K. Welsh, K.T. Konstantinidis, J.C. Chee-Sanford, K.M. Ritalahti, S. Yoon, and F.E Löffler. 2012. PUNCS: Towards predictive understanding of nitrogen cycling in soils. Abstract 160, DOE Genomic Science - Systems Biology for Energy and Environment. DOE Genomic Science Awardee Meeting X, February 26-29, 2012.

Posters 2011

Sanford, R.A., J.C. Chee-Sanford, L.M. Connor, and F.E Löffler. Expanded diversity of bacterial *nosZ* genes and corresponding functional acitvity beyond denitrifiers revealed in different agricultural soils. 3rd Annual Argonne Soil metagenome workshop, October 5-7, 2011.

Invited Oral Presentations

Löffler, F.E. Systems Biology Approach for Managing Groundwater Resources. Center for Applied Geochemistry, University of Tübingen, Tübingen, Germany. November 25, 2015.

Konstantinidis K. T. Accurate detection of target genes in shotgun metagenomes: method development and application to N cycle. International Symposium on Frontiers in Soil Microbiology. Oct 25-27, 2015. Beijing, China.

Konstantinidis K. T. Opening the black box of soil microbial communities with metagenomics. Univeristy of Georgia, Department of Crop and Soil Sciences. Athens, GA. Oct. 24th, 2014.

Orellana, L.H. Accurate detection and quantification of target genes in shotgun metagenomes: Method development and application to the nitrogen cycles genes. 7th Annual Argonne Soil Metagenomics Meeting. Lisle, IL, October 22, 2015.

Orellana, L.H. Seasonal and spatial changes in nitrogen cycle potential in Midwestern agricultural soils as revealed by Metagenomics. 6th Annual Argonne Soil Metagenomics Meeting, St. Charles, IL, October 2, 2014.

Löffler, F. E. Foundation for Environmental Biotechnology: Background and Principles. United Nations University, Biotechnology Programme for Latin America and the Caribbean (BIOLAC), Facultad de Química, Universidad de la República, Montevideo, Uruguay. September 22 - October 3, 2014.

Löffler, F. E. Environmental Controls over NO_3^-/NO_2^- Fate. United Nations University, Biotechnology Programme for Latin America and the Caribbean (BIOLAC), Facultad de Química, Universidad de la República. Montevideo, Uruguay. Facultad de Química, Universidad de la República. Montevideo, Uruguay, September 22 - October 3, 2014.

Orellana, L.H. Detecting nitrous oxide reductase (*nosZ*) in soil metagenomes: method development and implications for the nitrogen cycle. 1st Annual Southeastern Biogeochemistry Symposium, Atlanta, GA, April 5, 2014.

Konstantinidis K. T. Expanding the bioinformatic toolbox for the analysis of complex metagenomes. 2nd International Thünen Symposium on Soil Metagenomics. Braunschweig, Germany, December 11-13, 2013.

Löffler, F. E. Nitrogen cycling - New Twists on an Old Tale. 21st International Symposium on Environmental Biogeochemistry (ISEB21), Wuhan, China, October 13-18, 2013.

Orellana, L.H. Detecting nitrous oxide reductase (*nosZ*) genes in soil metagenomes: method development, temporal patterns and implications for the nitrogen cycle. 5th Annual Argonne Soil Metagenomics Meeting, Bloomingdale, IL, October 4, 2013.

Löffler, F. E. New Insights into Nitrogen Cycling in Soils. China-US Workshop on Advances in Environmental Microbiology and Biotechnology. Institute of Soil Science, Chinese Academy of Sciences, Nanjing, China. June 1, 2013.

Löffler, F. E. New Insights into Nitrogen Cycling in Soils. Chinese Academy of Science, Institute of Applied Ecology, Shenyang, China, May 29, 2013.

Löffler, F. E. 05/24/2013. New Insights into Nitrogen Cycling in Soils. China Ecological Forum (CEF), Chinese Ecosystem Research Network (CERN), China-US Ecopartnership for

Environmental Sustainability. Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Science, Beijing, China, May 24, 2013.

Löffler, F. E. 21/02/2013. Towards Predictive Understanding of Nitrogen Cycling in Soils (PUNCS). Genome Science Program Contractor-Grantee Workshops, Washington DC, February 25-27, 2013.

Löffler, F. E. 26/02/2013. Towards Predictive Understanding of Nitrogen Cycling in Soils (PUNCS). Genome Science Program Contractor-Grantee Workshops, Washington DC, February 25-27, 2013.

Löffler, F. E. 02/21/2013. Nitrogen cycling - New Twists on an Old Tale. University of Minnesota, MN. February 21, 2013.

Löffler, F. E. 04/03/2012. Identification of c-Type Cytochrome Activity Biomarkers. Biochemical Society Meeting, April 2-4, 2012, University of East Anglia, UK.

4. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Performers at the University of Tennessee

Name	Project role	Month worked	Contributions
Frank Loeffler	PI	Part-time	Project oversight, coordination of efforts, experimental design, student guidance, reporting, manuscript preparation
Samiha Ahsan	Undergraduate student	Part-time	Cultivation, analytical measurements, PCR
Jenny Onley	Graduate student	Full-time	Physiological studies with *Anaeromyxobacter dehalogenans*
Steven Higgins	Graduate student	Full-time	Genome sequencing and bioinformatics analyses; primer design, p450nor gene analysis
Lingzhi (Linda) Liu	Visiting professor	12	Design of *nosZ*-targeted qPCR assays; quantification of *nosZ* in soil samples
Silke Nissen	Postdoc	Part-time	Cultivation, qPCR assays
Kirsti Ritalahti	Research Assistant Professor	Part-time	Student guidance, qPCR assays
Sukhwan Yoon	Postdoc	Full-time	Chemostats, kinetic experiments, qPCR, growth yield experiments, manuscript preparation

Collaborators at the University of Illinois, Urbana-Champaign

Name	Project role	Month worked	Contributions
Robert Sanford	Co-PI	Part-time	Coordination of efforts at Illinois, experimental design, help with field sampling, student/postdoc guidance, reporting, culturing and new isolate characterization, hosting student collaborators from the University of Tennessee and Georgia tech for sampling and microcosm experiments, manuscript preparation.
Allana Welsh	Postdoc	Full-time	NrfA phylogeny, NosZ phylogeny, molecular methods development, manuscript preparation.
Stephanie Napieralski	Graduate student	Part-time	Experimental design, method development for RNA extraction, manuscript preparation.
Ryan Riessen	Undergraduate student	Part-time	*nosZ* clone libraries, field site characterization and in situ measurements.

Collaborators at the USDA-ARS and the University of Illinois, Urbana-Champaign

Name	Project role	Month worked	Contributions
Joanne Chee-Sanford	Co-PI	Part-time	Managed site location instrumentation, provided laboratory space for collaborators from Tennessee and Georgia Tech, experimental design, *nosZ* and community analysis, isolation and cultivation, manuscript preparation, supervised sample collection and DNA extraction, storage and archiving of DNA (>600 samples), supplied DNA to collaborators.
Lynn Connor	USDA technician	Part-time	Performed DNA extractions and community analysis from >600 samples.

Collaborators at the Georgia Institute of Technology

Name	Project role	Month worked	Contributions
Konstantinos Konstantinidis	Co-PI	Part time	Oversaw bioinformatics analyses and metagenomics/metatranscriptomics work. Student guidance and reporting.
Luis Orellana	Graduate Student	Full time	Performed metagenomic-based assessment of *nosZ* diversity and dynamics in soils.
Luis Miguel Rodriguez-R	Graduate Student	Part time	Developed bioinformatics algorithms (Nonpareil and ROCker) to assess target gene diversity and coverage in metagenomes.
Despina Tsementzi	Graduate Student	Part time	Developed metatranscriptomics protocols and applied them to environmental samples.
Minjae Kim	Graduate Student	Part time	Helped with the metatranscriptomics work.
Rachel Poretsky	Post-doc	Part time	Helped with the metatranscriptomics work and 16S rRNA gene analysis from metagenomes.
Eric Johnston	Graduate Student	Part time	Helped with metagenomics dataset assembly and comparative analysis.
Janet Hatt	Research Scientist	Part time	Performed sequencing of DNA and RNA samples from soils.

5. IMPACT

The outcomes of the PUNCS project significantly impact our understanding of N- and associated C-cycling in soils and sediments, and the processes that control nitrous oxide (N_2O) emissions to the atmosphere. Knowledge of the environmental parameters that control nitrate fate in soils enables predictive understanding of N-retention versus N-loss in soils and may lead to improved soil management. The expanded knowledge of the *nrfA* and *nosZ* sequence space forms a basis for the design and application of more comprehensive, quantitative tools for monitoring microbial activities affecting nitrate/nitrite turnover and N_2O emissions. Organisms with atypical *nosZ* genes were demonstrated to be widely distributed and abundant in soils. Importantly, organisms with atypical NosZ exhibit significantly higher affinity to N_2O indicating that the relative activity of bacteria with typical versus atypical NosZ control N_2O emissions and determine a soil's N_2O sink capacity. These findings advance our understanding of the diversity of microbes and functional genes involved in the N-cycle and provide the means (e.g., gene sequences) to study N_2O fluxes to the atmosphere and associated climate change. The improved process understanding along with enhanced monitoring tools will allow a larger research community to generate comprehensive datasets required to generate Earth System Models with higher predictive power.

6. CHANGES/PROBLEMS

Minor adjustments to experimental procedures and the sequence of experimental efforts were required to respond to research findings and other new information. Overall, the project stayed on track and the overarching goals of the PUNCS project were effectively achieved.

7. SPECIAL REPORTING REQUIREMENTS

None

8. BUDGETARY INFORMATION

The lead institution (University of Tennessee) and the collaborating institutions (University of Illinois at Urbana-Champaign, USDA-ARS, and the Georgia Institute of Technology) have expended their funds according to the expenditure plan.