Quantifying and Qualifying Denitrifying Microbial Communities in Restored and Natural Wetlands

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Statement of Work: *Objective:* I propose to quantify and qualify the microbial communities in restored and natural wetlands on the Eastern Shore of Maryland and Delaware. Specifically I would focus on the organisms associated with denitrification process, which involves the conversion of nitrate to nitrogen gas, thereby removing a key pollutant from water. My approach is to quantify microbial biomass and to qualify genes and enzyme activity related to denitrification, in order to integrate with vegetation and soil data that has already been collected.

Background Information: Wetlands are among the planet's most productive and diverse ecosystems. Many existing wetlands were created or restored through mitigation efforts, and it is essential to determine how these wetlands function ecologically post-restoration. This will allow researchers to modify future techniques to promote maximum ecosystem functions and services. Often, when evaluating mitigation wetlands for ecological progress, common metrics include species diversity and aboveground productivity, as well as measuring soil nutrient availability. Due to limitations such as funding, time, resources and manpower, these characteristics are often looked at individually, which is not effective in evaluating ecosystem function.

One of the most prevalent ecosystem services wetlands provide is the processing of nutrients, particularly nitrogen-based nutrients. Wetlands play an integral part in the nitrogen cycle, predominantly in the nitrification of excess ammonium (NH_4^+) into nitrate (NO_3^-), and then denitrification of the NO_3^- into nitrogen gas (N_2). Arguably, the most important component in the nitrogen cycle is the microbial community (bacteria, archaea, fungi)—the microbes are the linking component between local site characteristics (plant community, soil features, hydrology, *etc.*) and nutrient dynamics. In trying to understand nutrient cycling in ecosystems, getting even a rapid assessment of the microbial community is becoming necessary (Inglett and Inglett 2013).

Recently, it has become more common for researchers to include at least a cursory assessment of the microbial biomass in wetland restorations; yet almost nothing is known about the microbial community development in restored wetlands, despite their importance in biogeochemical functions (Bruland and Richardson 2005, Meyer et al. 2008, Duarte et al. 2012, Peralta et al. 2012). Moreno-Mateos (2012) found that even restorations almost a century old did not have the "biogeochemical function" of natural wetlands. Long term soil development studies suggest that microbial communities are not restored with the system (Craft et al. 2002, Craft et al. 2003). Most studies offer the same results in terms of microbial response: not enough data to determine significance, but emerging trends show clear differences between restored and natural wetlands. Microbial communities are strong indicators of wetland function, but have not been studied enough to determine their impact on post-restoration development, and even less studies have combined these studies with other wetland characteristics such as vegetation, hydrology and soil.

With the funds from this scholarship, I would be able to conduct a suite of microbial community analyses focusing on the denitrification process, and to quantify differences between restored and natural inland, depressional wetland systems. My project seeks to rectify shortcomings from previous studies, as well as to incorporate the information found into a larger scale survey of wetland vegetation and soil characteristics. I am looking at a large suite of restored and natural isolated, depressional wetlands, where the restored wetlands range both in

age of restoration and local hydrology. I will also be sampling in several areas within each wetland to get both inter- and intra-wetland microbial variation within wetland type. By including assays of different microbial characteristics specifically relating to the denitrification community, I will be able to better define the differences between restored and natural wetland systems, and incorporate these findings, with vegetation and soil characteristics, into a post restoration conceptual model.

Study Sites: In this project, I will visit 10 restored systems and 5 natural reference systems on the Delmarva Peninsula. The reference sites are naturally occurring shallow forested depressions located in close proximity to the restored systems; they are seasonally flooded, though not hydrologically isolated. The restored wetlands were located in prior-converted cropland (farm field converted from historically wetland area); these restored systems range in size from one to ten acres (Yepsen 2012). Restored wetlands range in age between 5 and 31 years. Restoration methods were mostly conducted by soil compaction and excavation. These wetlands have already been surveyed for vegetation and soil characteristics (discussed in Work Completed). Though these wetlands are non-tidal systems, I communicated with the scholarship committee, and it was determined that these systems fit the qualifications of coastal wetlands suitable for this scholarship.

Questions, Hypotheses and Rationale: I am looking at three types of microbial analyses (biomass, gene composition, and activity) as affected by wetland type (restored and natural):

1. Question: Does microbial biomass differ between wetland types?
   a. ***Hypothesis:*** *Restored wetlands will have lower microbial biomass than natural wetland systems; older restorations will have higher biomass than younger restorations.*
      i. Rationale: Since many of the restored systems have had significant amounts of topsoil removed, the microbial community will have been altered. Systems that have had time to recover will have had time for microbial community reestablishment. There will still be a significant gap in biomass between all restored and natural sites (Meyer et al. 2008).

2. Question: How does the microbial community composition change between wetland types?
   a. ***Hypothesis:*** *Natural wetlands will have better correlation between the wetland site characteristics and the microbial community's denitrification genes than restored systems.*
      i. Rationale: These natural communities are better established, and will have tighter relationships between the plant and soil wetland characteristics and the necessary genes for nitrogen processing. (Boyle et al. 2006, Boyle-Yarwood et al. 2008).

3. Question: Does microbial enzyme activity differ between wetland types?
   a. ***Hypothesis:*** *Denitrification enzyme activity will be highest in restored systems.*
      i. Rationale: Due to the high influx of agricultural runoff to these restored wetlands and high seasonal flooding flux, the denitrification rate should be higher in restored systems. These systems also have more microtopographical variation, creating more distinct areas for aerobic and anaerobic processes to occur. This activity rate will also be corrected for the amount of biomass, so that inordinate weight is not given to one wetland type over another (Bruland and Richardson 2005, Meyers et al. 2008).

Methods: Samples will be collected in three transects in each wetland, with one plot in each hydrologic area of the depression (emergent, temporarily flooded, upland) for a total of 9 plots per wetland, 135 plots total per assay. These are the same research plots sampled in 2013 for vegetation and soil characteristics (see Work Completed). Five 5x10 cm cylindrical cores will be collected in each plot and homogenized in field, including root samples with the surrounding rhizosphere for plant community relationships. If samples will not be returned to the lab within 24 hours, preservation fluid will be added to ensure no degradation of genetic material occurs.

Microbial biomass carbon content will be quantified using the chloroform fumigation-extraction technique (Bruland and Richardson 2005, Inglett and Inglett 2013), which measures differences between total organic carbon (TOC) and microbial biomass carbon (MBC). Two 10 g oven-dried samples will be measured concurrently for each carbon measurement (TOC and MBC). The control (TOC) sample will be extracted by shaking with 0.5M K_2SO_4 for 1 hour, then filtered. The microbial (MBC) sample will fumigated by insertion of chloroform-infused cotton into the sample tube. Following treatment application, both samples will be incubated for 1 week, and subjected to the same filtration process as the control samples (shake with 0.5M K_2SO_4 for 1 hour, then filter). Chloroform will then be removed by vacuum. Both samples will be analyzed for TOC, and the difference will be the MBC. (Bruland and Richardson 2005).

The denitrification gene composition will be qualified using terminal restriction fragment length polymorphism (T-RFLP) profiles, which is a fingerprinting process (Boyle et al. 2006, Boyle-Yarwood et al. 2008). DNA extraction will be performed on 0.5 g freeze-dried samples using MO BIO's Power Soil Kit. After quantification of content, I will amplify the DNA through the polymerase chain reaction (PCR). Amplified samples will be digested with restriction enzymes, according to manufacturer specification (to be determined upon enzyme decision). Samples will then be submitted for analysis. Analysis will be aided by the Yarwood laboratory at the University of Maryland at College Park (Boyle et al. 2006).

Enzyme activity will be analyzed using the denitrification enzyme assay (DEA) (Bruland and Richardson 2005, Boyle et al. 2006, Stephanie Yarwood pers. comm.). 20 g of fresh soil will be placed in 133 cm^3 jars fitted with syringe-accessible lids and containing necessary buffers (glucose, NO_3^-, PO_4^{3-}) for substrate availability. The jars will be made anaerobic by flushing jars with N_2 gas, and injected with acetylene to inhibit N_2O-reductase activity. Jars will then be shaken for a total of 120 minutes with gas samples will be taken at 0, 30, 60, 90 and 120 minutes. Samples will be analyzed for N_2O content using gas chromatography. Point values and N_2O flux rates will be used to calculate denitrification potential (Bruland and Richardson 2005).

Summary of Work Completed: My Master's project has thus far focused on the vegetation characteristics of these wetlands. The project is a collaboration under the USDA's Conservation Effects Assessment Project (CEAP) for Wetlands, which is divided between two labs at the University of Maryland at College Park (UMD), and funded by the USDA-BARC office in Beltsville, Maryland. My project focuses on vegetation, consisting of mirrored above and below ground characteristics: composition, productivity, and nutrients. The soil component, conducted by a fellow graduate student at UMD, quantifies the following characteristics: bulk density, iron reduction, soil compaction, decomposition, water table level, soil temperature, and soil profile. With such tight collaboration, these two components create a comprehensive look at the between the soil and vegetation characteristics of these wetlands, and provide substantial information towards the development of a comprehensive model.

With my Master's program now half completed, the vegetation characteristics field work has been completed, as well as much of the lab component. During four seasonal trips to these 15

wetlands, I completed the following field methods: collection of seed bank for greenhouse analysis; installation and subsequent removal of root ingrowth cores; standing field species composition analysis; soil sample collection; and herbaceous biomass collection. Concurrently, I also ran the following laboratory analyses: analysis of seed bank composition; laboratory identification of unknown plants from field; and processing of biomass samples for nutrient analysis. Processing of root ingrowth cores and nutrient analysis of all biomass and soil samples collected (including nitrogen content) is being completed this spring.

Benefits to Wetlands: Microbial communities truly define the functionality of wetlands. Wetlands provide one ecosystem service that, particularly in areas of high agricultural production, is becoming more and more essential: removal of excess nutrients. Microbes in particular play a crucial role in the processing of excess ammonium (NH_4^+) and nitrate (NO_3^-) due to the anaerobic zones present in soil below standing water (Mitsch and Gosselink 2007). Especially in the Chesapeake Bay, having local wetlands decrease the eutrophication level decreases the need for large scale intervention. By completing this study, I will be able to better understand the excess nutrient conversion potential of these restored systems.

In particular, this study is advancing a relatively new field of knowledge by allowing for the incorporation of multiple field components in one large-scale study. The study of microbial communities is still relatively novel, especially when tightly correlated to the plant and soil characteristics. Considering the importance of microbes in nutrient processing, it is becoming necessary to include relevant assays in large scale ecosystems surveys. This becomes particularly important in the field of wetland restoration ecology due to the implications it has on improving ecosystem services. By understanding the microbial community dynamics across restoration variations in relation to the other characteristics of the wetlands, future restoration efforts will maximize ecosystem functions and services beneficial to local communities and watersheds.

Use of Scholarship Funds: This scholarship would fund the analysis of these microbial assays. The analyses, will be time consuming and costly; however, this information is crucial to the full development of a conceptual model, as well as increasing the body of literature of microbial community ecology in restored wetland systems. With the funds from this scholarship, I should be able to conduct the full suite of analyses, including biomass, presence of functional genes and denitrifying activity and create that extra depth of understanding in the functionality of these wetland systems. I predict the following expenses, for a total of $4940:

Budget: Procedure	Samples	Approximate Cost per Sample	Materials Cost	Total
Chloroform Fumigation	135	$6	$600	$1410
T-RFLP	135	$14	$900	$2790
DEA	135	$4	$200	$740

Outputs and Outreach: The microbial community aspect is a large missing piece of the post-restoration wetland development puzzle. By incorporating different components of microbial community dynamics, the integrated ecosystem functions of these wetlands be better understood for future restoration application. This will also produce better educators to the community, and help local wetland users learn more about these diverse and complex systems.

The largest output from this project is the completion of a comprehensive conceptual model of post restoration wetland development for future USDA use and distribution. With the vegetation and soil components close to finished, I am beginning to develop the model

components and relationships; however, the microbial component is a key connection between the two. Along with the model, I am planning to create a Life-Cycle Analysis for more wide-spread distribution; this will be intellectually accessible to the local community, providing information and educative materials to local users of these wetland systems. I will also be completing a manuscript detailing my findings, as well as preparing conference talks, including for the annual SWS meeting.

An outcome I strive for is the development of better accessibility for community members to interact intellectually with these wetland systems. By adapting the conceptual model into an easily understood Life-Cycle Analysis (LCA), landowners and community members visiting these wetlands will be able to access and understand these wetlands function. An LCA diagrams the relationships between many characteristics of an ecosystem while both educating but not overwhelming the reader. An easily distributable and understandable life-cycle analysis of these wetlands will not only include the components that are familiar (plants and animals, e.g.), but also begins to delve into those that are not quite as well known (soil dynamics, microbial communities, nutrient fluxes). This way, everyone benefitting from these beautiful wetlands can appreciate not only their aesthetic values, but also their functional values and services.

References:

Brinson, M.M., F. R. Hauer, L.C. Lee, W.L. Nutter, R.D. Rheinhardt, R.D. Smith, and D. Whigham. 1995. A Guidebook for Application of Hydrogeomorphic Assessments to Riverine Wetlands. Wetlands Research Program, Technical Report WRP-DE-11. U.S. Army Corps of Engineers, Vicksburg, MS.

Boyle, S.A., J.J. Rich, P.J. Bottomley, K. Cromack Jr., and D.D. Myrold. 2006. Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon. Soil Biology and Biogeochemistry 38: 870-878.

Boyle-Yarwood, S.A., P.J. Bottomley, and D.D. Myrold. 2008. Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon. Environmental Microbiology 10(11): 2956-2965.

Bruland, G.L. and C.J. Richardson. 2005. Hydrologic, edaphic and vegetative responses to microtopographic reestablishment in a restored wetland. Restoration Ecology 13: 515-523.

Craft, C., S. Broome, and C. Campbell. 2002. Fifteen years of vegetation and soil development after brackish-water marsh creation. Restoration Ecology 10(2): 248-258.

Craft, C., P. Megonigal, S. Broome, J. Stevenson, R. Freese, J. Cornell, L. Zheng, and J. Sacco. 2003. The pace of ecosystem development of constructed *Spartina alterniflora* marshes. Ecological applications 13(5): 1417-1432.

Duarte, B., J. Freitas and I. Caçador. 2012. Sediment microbial activities and physic-chemistry as progress indicators of salt marsh restoration processes. Ecological Indicators 19: 231-239.

Inglett, P.W., and K.S. Inglett. 2013. Biogeochemical changes during early development of restored calcareous wetland soils. Geoderma 192: 132-141.

Moreno-Mateos, D., M.E. Power, F.A. Comìn, R. Yockteng. 2012. Structural and Functional Loss in Restored Wetland Ecosystems. PLoS Biology 10(1): 1-9.

Meyer, C.K., S.G. Baer, and M.R. Whiles. 2008. Ecosystem across a chronosequence of restored wetlands in the Platte River valley. Ecosystems 11: 193-208.

Mitsch, W.J. and J.G. Gosselink. 2007. Wetlands. Wiley, Hoboken (NJ).

Peralta, A.L., J.W. Matthews, D.N. Flanagan, and A.D. Kent. 2012. Environmental factors at dissimilar spatial scales influence plant and microbial communities in restored wetlands. Wetlands 32: 1125-1134.

Sutton-Grier, A.E., M. Ho, and C.J. Richardson. 2009. Organic amendments improve soil conditions and denitrification in a restored riparian wetland. Wetlands 29: 343-352.

Yepsen, M.M. 2012. Vegetation patterns in depressional restored, natural reference, and prior-converted wetlands in the USA mid-Atlantic Coastal Plain. MS thesis. University of Maryland, College Park, MD. 96 pp.