Environmental regulation of tidal wetland microbial communities and associated biogeochemistry

Ember Morrissey
Virginia Commonwealth University

Follow this and additional works at: https://scholarscompass.vcu.edu/etd
Part of the Life Sciences Commons

© The Author

Downloaded from
https://scholarscompass.vcu.edu/etd/3300

This Dissertation is brought to you for free and open access by the Graduate School at VCU Scholars Compass. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of VCU Scholars Compass. For more information, please contact libcompass@vcu.edu.
ENVIRONMENTAL REGULATION OF TIDAL WETLAND MICROBIAL
COMMUNITIES AND ASSOCIATED BIOGEOCHEMISTRY

A dissertation submitted in partial fulfillment of the requirements for the
Doctor of Philosophy at Virginia Commonwealth University.

by

Ember M. Morrissey
Bachelor of Science, University of Maryland, 2007

Director: Rima B. Franklin
Associate Professor, Department of Biology

Virginia Commonwealth University
Richmond, Virginia
December, 2013
ACKNOWLEDGEMENTS

I am truly grateful to my mentors, collaborators, friends, and family for all their guidance, and support. First, and foremost, I would like to thank Dr. Rima Franklin for taking me under her wing, and imparting scientific wisdom to me. She has taught me how to turn fledgling ideas into high quality publications. Throughout my graduate study I have been continually inspired by, and strived to learn from her scientific acumen, work ethic, patience, and communication skills. Rima, I am forever in your debt.

Thanks to Dr. Scott Neubauer for giving me knowledge and insight into the field of biogeochemistry. He has been an invaluable collaborator on this work, and has been ever willing to provide assistance. I am grateful to Dr. Bonnie Brown, for guiding development of my skills in molecular biology and for sharing resources for stable isotope studies. In addition, she has provided excellent advice and has been an unwavering source of support over these last four years. I also thank my other committee members Dr. Roy Sabo and Dr. Leigh McCallister, for their time and assistance. I give special thanks to my past supervisor Dr. Steve Negus: under his guidance I fell in love with science, and his belief in me helped me gain the confidence to pursue a PhD.

In addition to my mentors, I have many coworkers and collaborators to thank. David Berrier has been my sounding board over the past four years, and his feedback has undoubtedly improved my thinking and my work. Further, his skills and assistance in the laboratory and field have been of immeasurable value. I am fortunate to have had a helpful and supportive undergraduate mentee, Joseph Morina. I am also indebted to past and present members of Franklin lab: Joseph Battistelli, Nikki Dadashian, Chansothery Dang, Shardé Dean, Jaimie Gillespie, Rana Mehr, Aaron Porter, Christine Prasse, and Caitlin Muse, all of whom have helped me in countless ways and created a wonderful environment for working and learning. Thanks to Aaron Aunins, Oliva DeMeo, and Chris Friedline for sharing their technical expertise with me.

I am grateful to the Integrative Life Sciences PhD Program, particularly Drs. Tombes and Eggleston, for the opportunities and financial support I have received over the last four years. The Department of Biology also has been supportive by providing me with a teaching assistantship for two years. Special thanks to the VCU Rice Center for the Environmental Sciences, for allowing me to conduct field work at the center and providing me with research funding via the VCU Rice Center Student Research Award. I also thank the NSF and SWS for helping fund this research, and the Philanthropic Educational Organization for providing me stipend support.

On a personal level, I thank my friends and family for all their moral support. Thanks to Susie Gifford and Julie Charbonnier whose friendship and counsel has helped me succeed in graduate school. As always, I am grateful to my family for their constant love and encouragement. Last, but certainly not least, I thank my partner Kendall Perkinson, who always believes in me, even when I doubt myself.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS .......................................................................................................................... iii
TABLE OF CONTENTS ............................................................................................................................ iv
LIST OF TABLES ..................................................................................................................................... vi
LIST OF FIGURES ................................................................................................................................... vii
ABSTRACT ............................................................................................................................................... ix

CHAPTER ONE: AN INTRODUCTION TO MICROBIAL ECOLOGY AND TIDAL FRESHWATER WETLANDS ................................................................. 1

CHAPTER TWO: RESOURCE AVAILABILITY EFFECTS ON NITRATE-REDUCING MICROBIAL COMMUNITIES IN FRESHWATER WETLANDS ............................................................ 9
   Abstract ............................................................................................................................................... 10
   Introduction ....................................................................................................................................... 11
   Methods .......................................................................................................................................... 13
   Results ............................................................................................................................................. 19
   Discussion ...................................................................................................................................... 23
   Acknowledgements ......................................................................................................................... 29

CHAPTER THREE: INTERACTIVE RESOURCE EFFECTS ON DENITRIFICATION POTENTIAL ARE MEDIATED BY COMMUNITY COMPOSITION IN TIDAL FRESHWATER WETLAND SOILS .................................................................................................................. 33
   Abstract ............................................................................................................................................... 34
   Introduction ....................................................................................................................................... 36
   Methods .......................................................................................................................................... 38
   Results ............................................................................................................................................. 44
   Discussion ...................................................................................................................................... 50

CHAPTER FOUR: USING MICROBIAL COMMUNITIES AND EXTRACELLULAR ENZYMES TO LINK SOIL ORGANIC MATTER CHARACTERISTICS TO GREENHOUSE GAS PRODUCTION IN Tidal FRESHWATER WETLANDS ............... 65
   Abstract ............................................................................................................................................... 66
   Introduction ....................................................................................................................................... 68
   Methods .......................................................................................................................................... 69
   Results ............................................................................................................................................. 77
   Discussion ...................................................................................................................................... 82
   Acknowledgements ......................................................................................................................... 89

CHAPTER FIVE: SALINITY AFFECTS MICROBIAL ACTIVITY AND SOIL ORGANIC MATTER CONTENT IN TIDAL WETLANDS .................................................................................................................. 100
   Abstract ............................................................................................................................................... 101
   Introduction ....................................................................................................................................... 102
   Materials and Methods ................................................................................................................... 104
   Results ............................................................................................................................................. 108
Discussion .......................................................................................................................... 111
Acknowledgements ............................................................................................................. 117

CHAPTER SIX: SYNTHESIS AND CONTEXTUALIZATION .............................................. 127

REFERENCES ......................................................................................................................... 133
VITA ........................................................................................................................................ 158
LIST OF TABLES

Table 3.1 Effects of organic matter treatment, nitrogen fertilization on environmental variables as well as denitrifier abundance, community composition, and potential denitrification activity........................................................................................................................................59

Table 4.1 Summary of enzymes assays methods.................................................................................................................90

Table 4.2 Statistical results evaluating treatment effects........................................................................................................91

Table 4.3 Pearson’s correlations coefficients for (r) and p-values associated with the comparison of gas production rates, microbial community attributes, and enzymatic variables ….92

Table 5.1 Site locations and mean of environmental parameters for each site.......................... 118

Table 5.2 Average enzyme activity rates for each site .......................................................................................... 119

Table 5.3 Partial correlation analysis comparing salinity to enzyme activity (Pearson’s r), bacterial abundance (Pearson’s r), and bacterial community composition (Mantel test, $r_M$) while controlling for soil OM and C:N ....................................................................................... 120

Table 5.4 Direct and partial (controlling for salinity) correlation analysis comparing enzyme activity to bacterial abundance (Pearson’s r) and community composition (Mantel test, $r_M$) ........................................................................................................................................ 121

Table 5.5 Correlations (Pearson’s r) between all evaluated parameters.................................................. 122
LIST OF FIGURES

Fig. 2.1 Treatment effects on environmental parameters ................................................................. 30

Fig. 2.2 Treatment effects on the abundance of DNF nirS and DNRA nrfA functional genes as
determined via qPCR ......................................................................................................................... 31

Fig. 2.3 Non-metric multidimensional scaling ordination diagrams derived from T-RFLP data for
the DNF nosZ and DNRA nrfA genes .................................................................................................. 32

Fig. 3.1 Treatment effects on soil moisture, redox, organic matter content, C:N, porewater NO₃⁻
concentration, and porewater NH₄⁺ concentration ........................................................................... 60

Fig. 3.2 Response of denitrifier (nirS) abundance and potential denitrification rates to
experimental treatments ......................................................................................................................... 61

Fig. 3.3 Principal coordinates analysis plots of denitrifier (nirS) community composition in
response to treatment within each sampling date .................................................................................... 62

Fig. 3.4 Principal coordinates analysis plot of denitrifier (nirS) community composition in
response to treatment over time ............................................................................................................ 63

Fig. 3.5 Path diagram displaying the full and reduced models diagramming the role of soil OM
content, NO₃⁻ availability, denitrifier community structure, and abundance in regulating
potential denitrification rates .................................................................................................................. 64

Fig. 4.1 Treatment effects on environmental parameters and microbial abundance following six
and eighteen months of in situ field incubation .................................................................................... 94

Fig. 4.2 Treatment effects on microbial community structure following six and eighteen months
of in situ field incubation ....................................................................................................................... 95

Fig. 4.3 Effects of treatment on enzyme kinetics, evaluated after eighteen months of in situ field
incubation .............................................................................................................................................. 96

Fig. 4.4 Phenol oxidase (POX) activity as affected by treatment, evaluated after eighteen months
of in situ field incubation ....................................................................................................................... 96

Fig. 4.5 Treatment effects on the production of CH₄, CO₂, and total C gas, as well as the
fraction of total C gas that is CH₄, evaluated after eighteen months of in situ field
incubation measured in anaerobic slurries ............................................................................................ 97

Fig 4.6 Conceptual model diagramming the hypothesized role of microbial community structure
and extracellular enzyme activity in wetland organic matter decomposition ................................. 99
Fig. 5.1 Map of sampling locations along four tidal rivers proximal to the Chesapeake Bay (Virginia) ................................................................. 123

Fig. 5.2 Variation in organic matter and C:N with salinity ......................................................... 124

Fig. 5.3 Correlations of extracellular enzyme activity with salinity............................................. 125

Fig. 5.4 Variation in bacterial abundance and community composition along a salinity gradient ................................................................................................................. 126
ABSTRACT

ENVIRONMENTAL REGULATION OF TIDAL WETLAND MICROBIAL COMMUNITIES AND ASSOCIATED BIOGEOCHEMISTRY

Ember M. Morrissey

A dissertation submitted in partial fulfillment of the requirements for the Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2013

Director: Rima B. Franklin
Associate Professor, Department of Biology

Microbial communities play an essential role in carrying out the biogeochemical cycles that sustain life on Earth, yet we know very little about their ecology. One question of particular interest is how environmental conditions shape microbial community structure (i.e., the types of organisms found in the community and their relative abundance), and whether such changes in structure are related to biogeochemical function. It is the aim of this dissertation to address this question via the examination of carbon (C) and nitrogen (N) cycling in wetland ecosystems, which due to their diverse hydrology have a profound influence on biogeochemical cycles.

With respect to N cycling, the community structure of denitrification- and dissimilatory nitrate reduction to ammonium (DNRA)-capable organisms was evaluated in response to changes in resource availability, specifically organic matter (OM) and nitrate (NO$_3^-$), using an in situ field manipulation. Interactive regulation of microbial community composition was exhibited in both groups, likely due to variation in C substrate preferences and NO$_3^-$ utilization.
efficiency. Subsequent experimentation considering only denitrification revealed that resource regulation of activity rates was mediated through changes in denitrifier community composition.

The resource regulation of wetland C cycling also was evaluated using an in situ OM manipulation. OM characteristics (e.g., degree of decomposition) affected microbial extracellular enzyme activity (EEA) and changed the community structure of bacteria, archaea, and methanogens. These changes were linked with carbon dioxide and methane production via a conceptual model diagramming the importance of microbial community structure and EEA in greenhouse gas production.

The investigation of C cycling in wetlands was extended to consider an important global change threat: saltwater intrusion into freshwater tidal wetlands. Bacterial community structure and EEA were examined along a natural salinity gradient. Salinity was strongly associated with bacterial community structure and positively correlated with EEA. These results suggested that salinity-induced increases in decomposition were responsible for reduced soil OM content in more saline wetlands.

This work demonstrates that microbial communities in wetlands are structured by environmental conditions including resource availability and salinity. Further, the research provides evidence that environmental regulation of important biogeochemical processes in wetlands (e.g., methanogenesis, denitrification, etc.) is mediated through changes in microbial community structure.
CHAPTER ONE:

AN INTRODUCTION TO MICROBIAL ECOLOGY AND TIDAL FRESHWATER WETLANDS

by

Ember M. Morrissey
Microbial communities are fundamental to the stability and functioning of all the Earth’s ecosystems and of great use to society. Despite the importance of these organisms, our understanding of their ecology is limited. The study of microbial communities in their natural environments has, until recently, been hindered by reliance on culture-based techniques (Forney et al. 2004). However, advances in molecular methods, specifically whole-community nucleic acid-based approaches, have revolutionized the study of microbial ecology (Xu 2006, Hirsch et al. 2010). Accordingly, this is a time of great growth within the discipline. For instance, the number of taxa (quantified as the sum of unique small subunit ribosomal RNA (16S rRNA) sequences) has risen exponentially from 400 in 1996 to ~40,000 in 2009 (Pace et al. 2009). In addition to the great increase in phylogenetic data, there have been similar advances in our ability to study microbial groups associated with a specific processes by using corresponding functional genes (Lau and Liu 2007, He et al. 2012).

With technological limitations lifting, microbial ecologists are now focused on understanding if/how the tenets of classical macro-organismal ecology apply to natural communities of microbes (Prosser et al. 2007, Fuhrman 2009, Sutherland et al. 2013). Thus far, studies suggest that many of the ecological theories established via the study of plants and animals also may apply to microbial communities (Allison and Martiny 2008, Shade et al. 2012, Macalady et al. 2013). For instance, a meta-analysis by Shade et al. (2012) concluded that microbial communities, like plant and animal communities, are sensitive to disturbance events. Of the studies synthesized in this report, sustained disturbance altered community structure in 79% (n=178) and function in 83% (n=152) of cases. Thus, these results indicate that microbial communities and the functions they perform (e.g., decomposition, nitrogen cycling, etc.) may be sensitive to environmental change. This is particularly relevant as the stability of ecosystems
around the globe faces a range of threats resulting from anthropogenic activities including climate change and pollution. Multiple scientists have posited that studying microbial communities may improve our understanding of biogeochemical processes (Schimel 2001, Allison and Martiny 2008, Strickland et al. 2009, McGuire and Treseder 2010, Todd-Brown et al. 2012). This notion is supported by significant relationships between microbial community structure and biogeochemical activity rates in a variety of ecosystems (Chan et al. 2005, Song et al. 2011, Angel et al. 2012, Beckmann et al. 2011, Parkes et al. 2012, Allison et al. 2013). The field of microbial ecology is now faced with the challenge of characterizing the wide range of community-environment and community-function relationships that result from the great diversity of microbial species and associated wealth of physiological processes (Fuhrman 2009, Gilbert et al. 2010, Fierer and Lennon 2011).

The study of microbial communities may be especially useful for understanding wetland ecology, as these systems exhibit complex biogeochemistry mediated by diverse anaerobic microbial assemblages. An understanding of microbially-mediated wetland biogeochemistry is of interest to society as these ecosystems provide important services including carbon (C) storage, water purification, and nutrient removal (Barbier 2013). Further, a predictive understanding of wetland biogeochemistry is highly applicable, as wetlands are often restored or constructed to ameliorate the loss of natural systems (EPA 2008). Wetland biogeochemistry is driven by hydrology and high plant productivity, wherein large quantities of detritus from emergent vegetation enter water-saturated soils. Microbially-mediated decomposition begins when detrital organic matter (OM) is depolymerized by extracellular enzymes; the liberated monomers/oligomers fuel heterotrophic growth (Shi, 2011). Dissolved oxygen is consumed quickly for microbial respiration, creating anoxic conditions in wetland soils. In the absence of
oxygen, microbes must resort to alternative means of energy production via the use of other inorganic terminal electron acceptors (NO$_3^-$, SO$_4^{2-}$, Fe$^{3+}$, etc.) or through fermentative metabolism. Together, these processes produce small organic acids, alcohols, H$_2$, and CO$_2$ as waste (Reddy and DeLaune 2008), which can then be utilized by methanogenic archaea who produce CH$_4$ (Megonigal et al. 2004). The anaerobic metabolisms yield less energy than aerobic respiration (Reddy and DeLaune 2008), resulting in slower microbial growth (Hoijnen et al. 1992). Further, in the absence of oxygen, decomposition of especially complex organic substrates that require enzymatic oxygenation (e.g., lignins and humics) is drastically slowed (Freeman et al. 2001). These factors combined are believed to account for the suppressed decomposition rates in wetlands compared to other systems (Gholz et al. 2000, Mitra et al. 2005), resulting in soil OM accumulation (Mitra et al. 2005, Reddy and DeLaune 2008).

Specifically, wetlands store C at a rate of approximately 830 Tg/year worldwide (Mitsch et al. 2013). Although this C storage is favorable from a climate change perspective, wetlands are also the greatest natural source of methane (CH$_4$) emissions (Susan 2007 IPCC), making these ecosystems complex players in the global C cycle.

Beyond their relevance with respect to C cycling, wetlands are also capable of removing excess nutrients, especially nitrogen (N), from surface waters (Mitsch et al. 2005, Jordan et al. 2011). Much of the N removal is attributed to denitrification (Fisher and Acreman 2004), which is the microbial reduction of nitrate (NO$_3^-$) to N$_2$ gas. However, alternate N cycling processes, including dissimilatory nitrate reduction to ammonium (DNRA), also occur in wetlands (Scott et al. 2008, Koop-Jakobsen and Giblin 2010). Although DNRA does not remove N, it does convert it to ammonium (NH$_4^+$), which is a more bioavailable and less mobile form of N. The activity of nitrate-reducing microorganisms determines whether NO$_3^-$ is removed from the ecosystem via
denitrification, retained within the system as $\text{NH}_4^+$, or whether it passes through unaffected where it can potentially cause downstream eutrophication (Howarth and Marino 2006, Conley et al. 2009).

Like so many other ecosystems, wetlands and the services they provide are threatened by global change (Neubauer and Craft 2009, Lovelock et al. 2011, Craft 2012). One of the most significant threats tidal freshwater wetlands face is saltwater intrusion (Neubauer and Craft 2009, Traill et al. 2011, Barendregt and Swarth 2013). Intrusion of saltwater into historically freshwater systems is expected to occur as a result of changes in precipitation (Smith et al. 2005) that may lead to declines in stream output (Milley et al. 2005) in combination with rising sea levels (Nakada and Inoue 2005, Wigley, 2005). These factors have the potential to shift the saltwater front farther upstream in coastal watersheds. Salinity directly impacts abiotic (Rengasamy and Sumner, 1998, Weston et al. 2010) and biological processes (Baldwin and Mendelssohn 1998, Weston et al. 2011, Marton et al. 2012,) making it a major driver of ecosystem structure and function. Consequently, even low levels of saltwater intrusion could significantly alter the biogeochemistry of historically freshwater tidal wetlands.

This dissertation comprises four complimentary investigations into the microbial ecology and associated biogeochemical cycles of tidal freshwater wetlands. Within each chapter, I investigate how microbial communities associated with important biogeochemical processes (C or N cycling) are regulated by environmental conditions with an emphasis on resource availability. Further, where possible, the investigation is extended to evaluate relationships between microbial community structure and biogeochemical function. An overarching goal of this dissertation is to demonstrate how microbial community ecology can serve as a bridge, connecting our understanding of abiotic environmental conditions and biogeochemical processes.
This approach can enhance our comprehension of, and hopefully help us attenuate, the impacts of climate change, nutrient pollution, and other factors influencing ecosystems worldwide. The specific objectives of this dissertation are as follows:

- **Investigate how resource availability (OM and NO₃⁻) affects abundance and community composition of nitrate-reducing microbial communities, specifically comparing DNRA and denitrification (Chapter 2).**

  Past research has found both soil OM and NO₃⁻ to affect the balance between denitrification and DNRA process rates (Tiedje 1988, Burgin and Hamilton 2007). However, the few studies that have simultaneously examined these processes in wetland systems tend to focus heavily on process rates and pay little regard to the underlying microbial community structure (An and Gardner 2002, Scott et al. 2008, Koop-Jakobsen and Giblin 2010). This study investigated the resource ecology of DNRA- and denitrification-capable organisms to better understand how OM and NO₃⁻ availability regulate NO₃⁻ reduction processes.

- **Determine if and how OM and NO₃⁻ resources interactively regulate denitrification activity.**

  Assess whether the effects of resources on activity rates are mediated through changes in microbial community structure (Chapter 3).

  The role of the microbial community in regulating denitrification is still unclear despite multiple investigations (Magalhaes et al. 2008, Attard et al. 2011, Song et al. 2011, Baxter et al. 2012, Philippot et al. 2013). This research built upon those prior studies by evaluating microbial community responses to resource availability in concert with
activity measurements. This allowed an assessment of the role of microbial community structure in mediating the effects of environmental resources on denitrification rates. The resources chosen, OM and NO$_3^-$, have been found to influence both the community structure (Kjellin et al. 2007, Jones and Hallin 2010, Morrissey et al. 2013a, Yang et al. 2013) and function of denitrifying organisms (Seitzinger et al. 2006, Mulholland et al. 2008, Sutton-Grier et al. 2009). However, potential interactions between these resources have rarely been investigated and could have consequences for understanding N cycling in natural and constructed wetland systems.

- **Disentangle the role of microbial community structure and extracellular enzyme activity in mediating the effect of OM resource quality on CO$_2$ and CH$_4$ production (Chapter 4).**

The production of methane is thought to be regulated by a variety of factors including vegetation, hydrology, and OM characteristics (Kettunen 2003, Bonnett et al. 2013). Specifically, OM characteristics can affect depolymerization, fermentation, and the subsequent availability of C compounds for methanogenesis (Drake et al. 2009). Despite this clear theoretical connection, few studies have experimentally tested the effect of OM characteristics on methane production in wetlands (Singh et al. 2009, Ruirui et al. 2011). Further, this study evaluated microbial community structure and extracellular enzyme activity to develop a more mechanistic understanding of how OM characteristics regulate greenhouse gas production.
Evaluate the influence of salinity on microbial decomposition and soil OM content by examining a natural salinity gradient from freshwater to oligohaline in tidal wetlands (Chapter 5).

Salinity is recognized as an important regulator of many ecosystem characteristics in wetlands, including soil OM content (Craft 2007) and decomposition rates (Roache et al. 2006, Weston et al. 2006). To better understand how salinity affects OM content and decomposition processes, I examined salinity as a regulator of microbial community structure and microbially-mediated decomposition activities (via extracellular enzyme activity rates). The findings of this work are applicable to predicting the consequences of saltwater intrusion into historically freshwater wetlands.
CHAPTER TWO

RESOURCE AVAILABILITY EFFECTS ON NITRATE-REDUCING MICROBIAL COMMUNITIES IN FRESHWATER WETLANDS

by

Ember M. Morrissey, Amy S. Jenkins, Bonnie L. Brown, and Rima B. Franklin
Department of Biology, Virginia Commonwealth University, Richmond, VA 23284 USA

Published:
Wetlands
(2013)
Abstract

Microbial communities in freshwater wetland soils process nitrate via denitrification (DNF) and dissimilatory nitrate reduction to ammonium (DNRA). Because the processes generate different end products (N-gas versus NH$_4^+$), the relative dominance of DNF versus DNRA has implications for ecosystem nitrogen cycling, greenhouse gas production, and downstream eutrophication. To examine how resource availability affects these two microbial groups, wetland soil was supplemented with labile (compost) or recalcitrant (wood) organic matter (OM) and/or potassium nitrate fertilizer. Following a three-month in situ incubation, the abundance and composition of the DNF- and DNRA-capable microbes were examined via quantitative polymerase chain reaction (qPCR) and terminal restriction fragment length polymorphism (T-RFLP) using process-specific functional genes (DNF: nirS qPCR, nosZ T-RFLP; DNRA: nrfA qPCR and T-RFLP). Denitrifier abundance was positively related to OM lability and simultaneous nitrate amendment enhanced OM effects, while DNRA abundance varied little across treatments. For both groups, community structure showed an interactive response to OM type and nitrate availability, even when abundances did not change. This work highlights the importance of considering co-varying resource gradients, and the differential responses of DNF and DNRA communities to resource manipulation provides insight into the environmental regulators of ecosystem nitrate removal in wetlands.
Introduction

It has been estimated that approximately 60% of fertilizer nitrogen (N) used in agriculture is never incorporated into plants, and instead washes out of the soil into rivers or ground waters, primarily as nitrate (Canfield et al. 2010). Freshwater wetlands are important targets for conservation due of their ability to mitigate downstream nitrogen transport via microbial nitrate reduction – particularly the processes of denitrification (DNF) and dissimilatory nitrate reduction to ammonium (DNRA) (Fisher and Acreman 2004, Ma and Aelion 2005, Erler et al. 2008, Koop-Jakobsen and Giblin 2009 and 2010). Both DNF and DNRA are anaerobic processes, typically coupled to organic matter (OM) oxidation, wherein nitrate (NO$_3^-$) is used as a terminal electron acceptor for microbial respiration to either N$_2$ and N$_2$O (DNF) or NH$_4^+$ (DNRA). The relative dominance of these two processes has implications for downstream eutrophication and greenhouse gas production (Conrad 1996, An and Gardner 2002, Fisher and Acreman 2004), but considerable uncertainty remains as to the biogeochemical regulators that determine the mechanism and extent of microbially-mediated nitrate transformations.

Presumably because of competition and overlapping resource needs, the balance of DNF and DNRA has been found to vary depending upon OM and nitrate availability, though scientists have yet to develop a predictive understanding of these relationships (Hill and Cardaci 2004, Scott et al. 2008, Sutton-Grier et al. 2009, Koop-Jakobsen and Giblin 2010, Nizzoli et al. 2010). Early work led to the development of a hypothesis that DNRA is favored when there is high availability of OM relative to nitrate, whereas DNF is favored under low OM to nitrate ratios (Tiedje 1988, Burgin and Hamilton 2007). This theory, however, has not been uniformly supported in subsequent examinations of wetland nutrient processing (Scott et al. 2008, Koop-Jakobsen and Giblin 2010). For example, a wetland fertilization study by Koop-Jakobsen et al.
(2010) found addition of nitrate to increase the activity of both processes by roughly equal amounts, despite the resultant change in OM to nitrate ratio. Other recent work suggests that carbon quality is also a necessary component of conceptual models that consider how OM and nitrate interactively regulate microbial nitrate-reduction in wetlands (Hill and Cardaci 2004, Burgin and Hamilton 2007, Lou et al. 2007, Dodla et al. 2008). For instance, DNF potential in wetlands has been linked with OM lability (e.g., Dodla et al. 2008) and the ratio of cellulose to lignin content (Lou et al. 2007).

Studies that simultaneously consider DNF and DNRA in wetlands have focused heavily on process rate measurements, but have rarely considered microbial community composition (An and Gardner 2002, Scott et al. 2008, Koop-Jakobsen and Giblin 2010). However, given the growing body of evidence that microbial community structure may be important for understanding ecosystem functions (Philippot and Hallin 2005, Reed and Martiny 2007, Fuhrman 2009, Dimitriu et al. 2010), an examination of the microbial ecology underlying these biogeochemical processes is warranted. In the case of DNF, both community composition and abundance of DNF-capable organisms has been found to co-vary with activity measurements (Wolsing and Prieme 2004, Magalhaes et al. 2008, Dang et al. 2009, Attard et al. 2011). In contrast, research into DNRA is limited, and very little is known about how environmental conditions affect community composition, or the relationship between community composition/abundance and process rates (Mohan et al. 2004, Smith et al. 2007a, Lam et al. 2009).

In this study, we address this knowledge gap by examining how resource availability influences the abundance and composition of DNF- and DNRA-capable organisms in a tidal freshwater wetland. *In situ* manipulations of OM type and nitrate concentration were conducted
using a modified litterbag approach, and subsequent molecular genetic analysis targeted functional genes specific to each nitrate-reduction pathway. Two types of OM were used: wood shavings, representing a more recalcitrant material with a low nutrient content, and compost, which is relatively more labile and nutrient rich (Moore et al. 2005, Antil et al. 2011). These amendments are comparable to those used to increase soil OM during wetland restoration and construction (Davis 1995, Brueland et al. 2009, Sutton-Grier et al. 2009, Warneke et al. 2011). The simultaneous addition of various levels of potassium nitrate fertilizer, mimicking porewater concentrations up to 30 mg L\(^{-1}\), provided a model system with which to study the interaction of OM quality and nitrate availability as co-regulators of wetland nitrogen cycling.

**Methods**

**Experimental Design**

This research was conducted in a 30-ha tidal freshwater wetland along the James River at Virginia Commonwealth University’s Walter and Inger Rice Center for Environmental Life Sciences, located in Charles City County, Virginia (37°19'38" N, 77°12'13" W). Experimental manipulations took place within a 10 × 10 m square plot near the center of the wetland, in an area that was dominated by obligate wetland vegetation including *Leersia oryzoides*, *Juncus effusus*, and *Polygonum arifolium*. Soils were continually saturated, with a low OM content (~ 6 %) and a C:N ratio of 14 (by mass). Soil texture was classified as silt loam, with approximately 30% sand, 55% silt, and 15% clay. Soil pH varied between 5 and 6, and cation exchange capacity was ~ 8 meq (100 g\(^{-1}\)).

The experiment was conducted using a modified litterbag approach. First, soil from 5-15 cm below the soil surface was collected from the field and homogenized in the laboratory. Soil
treatments consisted of a partial factorial design of nitrate and/or OM amendments, wherein nitrate was examined at 4 levels and OM additions were made at the lowest and highest levels of nitrate addition. Nitrate (as KNO$_3$) was added in the form of temperature-controlled slow-release fertilizer pellets (Polyon, Agrium Advanced Technologies, CAS# 7757-79-1, Loveland, CO) to achieve amendment levels of 0, 0.5, 2, and 4 mg N g$^{-1}$ wet sediment; preliminary incubations using the 4-mg N treatment yielded porewater concentrations of ~ 30 mg L$^{-1}$ following a three month field incubation. Organic amendments consisted of 30% dry weight addition of either compost (commercially available organic blend containing 26% OM, C:N=18, 0.5% total N, 0.5% P$_2$O$_5$ and 0.5% K$_2$O) or wood shavings (untreated pine, 99% OM content). Compost and wood were homogenized to ensure they were of similar particle sizes (0.2 - 5 mm diameter). Both nitrate and OM amendments were removed directly from the manufacturer’s containers, weighed, and mixed into soils using clean sterile tools. Neither amendment was sterilized because such treatment would have altered OM and fertilizer quality (e.g., heat degradation of OM, UV polymer complexation, and compromised fertilizer pellet coating).

Sediment bags (15 cm × 15 cm) were constructed using polyester thread and 0.5-mm Nitex mesh (Wildlife Supply Company, Buffalo, NY), and filled with 250 ml of control or augmented soil. Three sediment bags of each type were then buried between 5 and 15 cm below the sediment surface; this depth was necessary to ensure anoxia throughout the long-term incubation and to protect the bags from disturbance during high flow or precipitation events. Within each plot, bag placement was random and locations were marked with a flag. Sediment bags were incubated in situ from June until September 2010, at which time they were harvested, placed in air-tight plastic bags, and returned to the laboratory at ambient temperature. Three replicate sediment bags were recovered for each treatment (N=3), except the "unamended OM
with 4N," where one bag was lost. Upon reaching the laboratory, sediment bags were immediately homogenized and sub-sampled for sediment characterization; ~5 g subsamples were archived at -20°C for genetic analysis.

**Sediment and Porewater Analysis**

For each homogenized subsample, redox potential and pH were measured using a Hanna Combo pH and ORP probe (QA Supplies Norfolk, VA), and soil moisture content was analyzed gravimetrically (100 ± 5°C for 72 h). Sediment organic matter (%) was calculated as the mass loss on ignition following combustion at 500°C for 4 h. Total carbon and nitrogen content was determined using a Perkin Elmer CHNS/O Analyzer (Waltman, MA) following acidification of samples using 10% hydrochloric acid. Porewater was extracted from 50-ml soil samples by centrifugation at 3000 × g for 15 min, filtered using a 0.45-µm pore-size mixed cellulose ester syringe filter, and stored at -20°C. Porewater samples were subsequently analyzed for nitrate concentration via ion chromatography (Dionex ICS-1000, Sunnyvale CA).

**Molecular Analyses**

Whole-community DNA was extracted from 0.5-g subsamples of soil using the MoBio Power Soil DNA kit (Carlsbad, CA) and then stored at -20°C. DNA purity and concentration were analyzed using Nanodrop ND-1000 (Thermo Scientific, Willmington, DE). All DNA extracts and PCR products were verified using agarose gel (1.5%) electrophoresis and ethidium bromide staining.
Functional Gene Abundance via qPCR

Functional gene abundance was determined using quantitative polymerase chain reaction (qPCR). Triplicate reactions were performed for each sample using SYBR GreenER qPCR Supermix for iCycler (Invitrogen, Grand Island, NY) and results were reported as the log$_{10}$ of the number of gene copies g$^{-1}$ wet soil after averaging technical replicates. As part of methods development, the lack of quenching effects on qPCR analyses were verified using serial dilutions of DNA extracted from wetland samples.

For DNF, the nirS gene was targeted using the primers cd3aF (5’GTS AAC GTS AAG GAR ACS GG’3) and R3cd (5’GAS TTC GGR TGS GTC TTG A 3’) (Throback et al. 2004). Genomic DNA from Paracoccus denitrificans(Strain #17741, ATCC, Manassas, VA) was used to establish the standard curve (average efficiency=102 % and correlation coefficient $r^2$=0.99).

Reactions (25 μL) were performed with 10 ng DNA template and 0.1 μM concentrations of each primer; thermal cycling conditions were: 50°C for 2 min, 95°C for 8.5 min, and 50 cycles of 30 s at 94°C, 30 s at 56°C, and 75 s at 72°C (Biorad iCycler, Hercules, CA).

For DNRA, the abundance of the nrfA gene was quantified using the primers nrfA6F (5’GAY TGC CAY ATG CCR AAA GT 3’) and nrfA6R (5’GCB KCT TTY GCT TCR AAG TG’3) (Takeuchi 2006). Genomic DNA from Escherichia coli (Strain #11775, ATCC, Manassas, VA) was used to establish the standard curve (average efficiency 85% and correlation coefficient $r^2$=0.98).

Reactions (25 μL) were performed with 10 ng DNA template and 0.3 μM concentrations of each primer; thermal cycling conditions were: 50°C for 2 min, 95°C for 8.5 min, and 50 cycles of 20 s at 94°C, 40 s at 54.5°C, and 10 s at 72°C.
Community Composition via T-RFLP

Microbial community composition was analyzed using Terminal Restriction Fragment Length Polymorphism (T-RFLP) targeting DNF- and DNRA-specific functional genes. For DNF, the \textit{nosZ} gene was amplified using the primers Nos661F (fluorescently labeled, 5‘FAM-CGG CTG GGG GCT GAC CA A 3’) and Nos1773R (5‘ ATR TCG ATC ARC TGB TCG TT 3’) (Magalhaes et al. 2008). PCR reactions (50 µL) consisted of 0.25 μM concentrations of each primer, 100 μg BSA (bovine serum albumin; Roche Inc., Nutley, NJ), 50 ng DNA template, and GoTaq Green Master Mix (Promega, Madison WI). Cycling parameters were: 95°C for 3 min, 37 cycles of 30 s at 95°C, 30 s at 59.7°C, 90 s at 72°C, followed by 72°C for 8 min. The DNRA functional gene \textit{nrfA} was amplified using F1b (fluorescently labeled, 5 ‘FAM-GCN TGY TGG WSN TGY AA 3’) and R1b (5’TWN GGC ATR TGR CAR TC 3’) (Takeuchi 2006). Those PCR reactions (50 µL) consisted of 10 ng DNA template, 10 mM TrisHCl (pH 8.3), 50 mM KCl, 3 mM MgCl$_2$, 200 μM of each dNTP, 0.4 μM of each primer, 30 μg BSA, and 2.5 units of AmpliTaq DNA polymerase (reagents obtained from Applied Biosystems, Foster City, CA). Touchdown thermal cycling parameters were as follows: 94°C for 5 min, 30 cycles of 60 s at 95°C, 60 s at 60°C (-0.5°C cycle$^{-1}$), 90 s at 72°C, followed by 30 cycles of 30 s at 95°C, 30 s at 45°C, 90 s at 72°C with a final elongation step of 72°C for 10 min. PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen, Valencia, CA) prior to restriction enzyme digest. The \textit{nosZ} digests used 10 units of HinP1I, 1X buffer #4 (New England Biolabs, Ipswich, MA), and 130 μM spermidine (Sigma-Aldrich, St. Louis, MO); incubations were at 37°C for 6 h, followed by 20 min at 95°C. The \textit{nrfA} digests used 20 units of RsaI in 1X buffer #4 (New England Biolabs, Ipswich, MA) at 37°C for 6 h, followed by 20 min at 65°C. Digested amplicons were purified using the MinElute kit, recovered in molecular-grade water, and
detected using capillary electrophoresis with a MegaBACE 1000 DNA Analysis System. An aliquot of 70-100 ng of purified, digested PCR product was combined with 0.5 µL of MapMarker 400 ROX ladder (Bioventures, Murfreesboro, TN) plus 4.75 µL injection buffer (0.1% Tween-20). Samples were injected at 3 kV for 100 s, and electrophoresed using genotyping filter set 1 for 100 min at 10 kV. T-RFLP fragments between 70-400 bp were analyzed using Fragment Profiler software (Version 1.2; Amersham Biosciences, Buckinghamshire, UK) using a 1-bp size differential and a 40 relative fluorescent unit peak height threshold. Peaks accounting for less than 2% total sample fluorescence were removed prior to data analysis.

Data Analysis

Prior to statistical analysis, a Shapiro-Wilk test was applied to evaluate the distribution of data describing sediment characteristics, porewater nitrate concentrations, and gene copy number (qPCR). All data were normally distributed except the qPCR results, which required a log transformation and were subsequently analyzed as log\(^10\) of the functional gene copy number g\(^{-1}\) wet soil. To test for treatment effects due solely to nitrate amendment, a one-way analysis of variance (ANOVA) was performed, considering four levels of N addition: 0, 0.5, 2, and 4 mg N g\(^{-1}\) sediment (n=11, df=10), followed by a Tukey's HSD post hoc test. A two-way ANOVA was performed to evaluate potential interactive effects of nitrate amendment and OM type using a subset of samples in a full factorial design (N levels: 0 and 4 mg N g\(^{-1}\) sediment, crossed with OM treatments: unamended, added labile, and added recalcitrant; n=17, df=16). One-way ANOVAs and Tukey’s HSD post hoc test were used to identify significant differences between OM types within nitrate addition levels. Finally, t-tests were used to assess differences between
the two nitrate addition levels within OM type. Analyses were performed using the JMP statistical software (Version 8.0.2, Cary, NC; Sall, 2005) and evaluated using a 0.05 significance level.

The T-RFLP assay yielded 57 unique terminal restriction fragments (corresponding to putative taxonomic groups) for the DNF nosZ gene and 63 distinct fragments for DNRA nrfA. These results were recorded as a binary data matrix describing the presence or absence of each fragment in each sample. This matrix was then converted to a set of Jaccard coefficients that quantifies the relative similarity between each pair of samples, which was used for subsequent ordination analysis and determination of statistically significant treatment effects. Specifically, visualization of overall similarity between communities was achieved using non-metric multidimensional scaling (NMDS) performed in PAST statistical software package (Version 2.10; Hammer, 2001). One-way non-parametric multivariate analysis of variance (NP-MANOVA) was used to test for significant effects of nitrate amendment considering the four levels of addition: 0, 0.5, 2, and 4 mg N g⁻¹ sediment (n=11, df=10). Interactive effects of OM type and nitrate amendment on community composition were evaluated using the two-way NP-MANOVA in R version 2.15.0 (Oksanen et al. 2012) using the adonis function of the vegan package (N levels: 0 and 4 mg N g⁻¹ sediment, crossed with OM treatments: unamended, added labile OM, and added recalcitrant OM; n=17, df=16).

**Results**

**Individual Effect of Nitrate Amendments**

**Soil Properties**
Soils with higher amendments of nitrate had significantly greater redox potential (F=11.6, p<0.01) and OM content (F=4.8, p=0.04), and a nearly significant reduction in C:N ratio (F=4.4, p=0.05) as determined using one-way ANOVA (Fig. 2.1 A, C, E). Porewater nitrate concentrations also increased (Fig. 2.1G), but the change was not statistically significant (F=3.4, p=0.08). In contrast, soil pH (mean ± S.E.; 6.0 ± 0.1) and gravimetric moisture content (%; 46.5 ± 2.5) were not affected by these treatments (results not presented, pH: F=0.02, p=0.99; Moisture: F=0.5, p=0.69).

*Microbial Community*

Nitrate amendment did not significantly alter the abundance of either functional group (ANOVA for DNF: F=1.8, p=0.24; DNRA: F=1.2, p=0.37; Fig. 2.2 A, C) and did not exhibit a consistent impact on community composition. No nitrate-amendment effect on DNF community composition was detected (NP-MANOVA: F=1.0, p=0.40), and the effect on the DNRA community was small (F=1.4, p=0.02). Post-hoc pair-wise comparisons of the DNRA community across nitrate levels did not reveal any significant differences (all F<2.1, p>0.9).

*Interactive Effects of Nitrate Amendment and OM Type*

*Soil Properties*

No interactions among treatments were detected for the analysis of soil OM content or porewater nitrate concentration using the two-way ANOVA (OM: F=0.9, p=0.42, Porewater nitrate: F=1.7, p=0.21). However, there was a significant main effect of OM amendment on soil OM content (Fig. 2.1 D; F=62.3, p<0.01), and of nitrate amendment on porewater nitrate concentration (Fig. 2.1 H; F=14.3, p<0.01).
A significant interaction effect was observed in the two-way ANOVA of soil redox (F=6.6, p=0.01). In the absence of any added nitrate, the addition of recalcitrant OM increased redox potential relative to the unamended and labile OM types (Fig. 2.1B; F=6.7, p=0.03). In the presence of added nitrate, this differential response to OM was lost (F=3.1, p=0.13). Regardless of OM treatment, the addition of nitrate increased redox potential.

The interaction effect was not significant for soil C:N (F=3.4, p=0.07), but both nitrate addition and OM type had strong main effects (Fig. 2.1F). Specifically, nitrate amendment consistently decreased C:N (F=19.7, p<0.01) and the addition of recalcitrant OM increased C:N (F=86.2, p<0.01). No significant interactions or treatment effects were obtained for soil pH or gravimetric moisture content (results not presented; for pH: 5.8 ± 0.2, F=3.0 and p=0.06; for moisture (%): 48.4 ± 1.7, F=1.6 and p=0.23).

Microbial Community

When the combination of nitrate amendment and OM type was considered, DNF functional gene abundance was interactively affected (F=7.4, p<0.01; Fig. 2.2 B). Regardless of nitrate addition, abundance was lowest when recalcitrant OM was added and highest when labile OM was added; these differences were more pronounced when combined with nitrate addition. Within each OM treatment, t-tests revealed that nitrate fertilization significantly increased DNF abundance in the presence of labile OM (t=3.0, p=0.03) and decreased DNF in presence of recalcitrant OM (t=-2.4, p=0.04). In contrast, two-way ANOVA revealed no significant effects on DNRA functional gene abundance (all F<2.0, p>0.10), though DNRA abundance decreased when labile OM alone was added (F=10.1, p=0.01, Fig. 2.2D).
The NMDS of the T-RFLP data revealed interactive effects of nitrate and OM amendments on community composition. In ordination space, samples that are farther apart are less similar with regards to the presence/absence of terminal restriction fragments. For DNF, the greatest effect was due to OM. This can be seen in Fig. 2.3A as a distinct separation of the three OM treatments in ordination space. Within each OM type, DNF community structure shifted toward the right on Axis 1 with the addition of nitrate fertilizer, which suggests nitrate affected a consistent subset of community members regardless of OM treatment. The magnitude of the nitrate-induced shift was most dramatic in the presence of labile OM. NP-MANOVA confirmed this interaction effect was significant (F=2.9, p=0.01), as were the main effects of OM type (F=2.7, p=0.01) and nitrate addition (F=2.0, p=0.01). OM type also had an effect on DNRA community composition, with the greatest difference being due to the addition of labile material (Fig. 2.3B). Further, simultaneous addition of nitrate caused a consistent shift in DNRA community composition, this time toward the left on Axis 1. NP-MANOVA detected a nearly significant interaction between OM type and nitrate level (F=1.3, p=0.05). This manifested on the NDMS plot as a small shift in community composition due to nitrate addition to native soil and a much larger shift when nitrate was added in concert with either form of OM (Fig. 2.3B). As for DNF, main effects on DNRA community composition were significant for both OM type (F=1.7, p<0.01) and nitrate amendment (F=1.6, p=0.03).
Discussion

Effect of OM and Nitrate Addition on Soil Properties

Analysis of soil characteristics demonstrated that the experimental manipulations altered OM concentration and porewater nitrate levels in a manner consistent with expectations (Fig. 2.1), while basic soil properties like pH and moisture content remained unchanged, thus creating a unique opportunity to examine microbial community responses to altered resource environments. Quality of soil OM, as estimated by C:N, was also affected by the treatments; this was especially evident when recalcitrant OM (wood) was added to the soil, which resulted in a large increase in C:N. In the case of the labile OM addition, C:N ratio was not appreciably changed (unamended native soil C:N = 14; original compost C:N=18, which was diluted ~1/3 when treatments were prepared), though the composition and mineralization of compost OM has been shown to be distinct from native soils (Antil et al. 2011, Tuomela et al. 2000). The high-nitrate treatments had greater soil redox potential, which indicates a shift in the availability of terminal electron acceptors for microbial metabolism (Thullner et al. 2007, Reddy and DeLaune 2008). An unexpected response in the soil characteristics was a small but statistically significant increase in OM content caused by nitrate amendment (Fig. 2.1 D). Because soil percent carbon did not change as a result of nitrate fertilization (data not shown), it is likely this result was an artifact caused by the combustion of fertilization pellets during the assay for OM content. Taken together, these changes in soil OM quality and quantity, redox potential, and nitrate availability were expected to influence both the abundance and composition of the subset of the microbial community responsible for nitrate reduction.
Independent Effects of OM and Nitrate Addition on Microbial Community Composition

When only nitrate availability was manipulated, no significant differences were observed in the abundance of either DNF- or DNRA-capable organisms (Fig. 2.2 A, C). Likewise, the effect of nitrate amendment on community composition was small (Fig. 2.3). This suggests that nitrate levels were not limiting population growth of either functional group. In contrast, when soil OM was altered, DNF and DNRA communities changed in both abundance and composition. The addition of labile OM to the soil resulted in higher abundance of the DNF functional gene (Fig. 2.2 B), which is consistent with prior studies that have found labile OM to benefit DNF communities (Hill and Cardaci 2004, Ullah and Faulkner 2006, Dodla et al. 2008, Sutton-Grier et al. 2009). The simultaneous effect on composition of the DNF community (Fig. 2.3 A) demonstrates that this change in abundance was not simply the result of increased population size, but also a shift in the relative abundance of the various DNF populations. This provides evidence that genetically distinct populations of DNF-capable organisms vary in their resource preferences, and suggests that certain groups may be especially well poised to take advantage of the labile OM. In contrast, the addition of labile OM resulted in lower abundance of the DNRA functional gene (Fig. 2.2 D), which implies that DNRA microbes may be less effective competitors than DNF in the presence of labile resources. When recalcitrant OM was added, DNF abundance decreased (Fig. 2.2 B), and community composition shifted for both functional groups (Fig. 2.3). Such effects could result from decreased levels of carbon availability in the sediment that was amended with recalcitrant OM (i.e., “dilution of resources”). This reduction could depress DNF abundance by decreasing access to their preferred labile substrates. An alternative explanation is that the addition of OM may have diluted the abundance/biomass of the native microbial community, and competition during regrowth could
have influenced community structure. There is a possibility that microbial communities associated with the unsterilized OM amendments could persist and influence the abundance and composition of the functional groups measured. However, the *in situ* incubation time of three months was likely sufficient for the environmental influence on microbial communities to surpass any small initial bioagumentation or dilution effects.

The effect of OM type on the composition of DNF and DNRA communities is consistent with prior work, and suggests that the changes in community composition we observed are likely a result of selection based on differential ability of organisms to utilize the different components of the soil OM pool. Previous research has shown that both individual populations and whole communities of bacteria can have distinct substrate utilization profiles (Doutereol et al. 2010, Yadav et al. 2011), which likely occurs within the diverse set of organisms that comprise the DNF and DNRA community. For instance, Peralta et al. (2010) found both bacterial community composition (evaluated using the *16S rRNA* gene) and variations in the DNF *nosZ* gene to be structured by soil C:N and total OM. It is worth noting that, as with all molecular microbial ecology studies, our results may be biased by our choice of target functional genes and associated primer sequences. For example, not all DNF bacteria contain the *nosZ* gene (Jones et al. 2008) and there is considerable selection bias across *nosZ* primer sets (Throback et al. 2004). The primers we used, 661F and 1773R, have been employed in a variety of studies (Magalhaes et al. 2008, Krishnani 2010, Baxter et al. 2012) but some researchers have found them to be ineffective at amplifying *nosZ* sequences from particular denitrifying strains (Troback et al. 2004). Overall, we do not see this as a severe limitation in the current study due to the fact that our molecular assays detected changes in community composition and abundance *despite* these methodological limitations. Ultimately, analysis of additional genes and use of alternate primer
sets could increase the resolution of the community analysis, and may be useful in future studies to determine finer-scale controls on DNF and DNRA response to resource conditions.

**Interactive Effect of OM and Nitrate Addition on Microbial Community Composition**

Combined addition of OM and nitrate demonstrated the ability of resources to interactively regulate microbial communities. In the DNF community, nitrate addition alone had no effect on abundance (Fig. 2.2A), but magnified the individual effects of OM type (Fig. 2.2B). This suggests that DNF abundance was more limited by OM than nitrate availability in this system, which is consistent with previous research that has similarly found microbial biomass and DNF activity to be carbon-limited in anaerobic wetland soils (Sutton-Grier et al. 2009). The effect of nitrate amendment on DNF and DNRA community composition was also greater when combined with OM addition than in isolation (Fig. 2.3). With respect to the DNF community, previous results have been equivocal, with some studies concluding that nitrate structures communities whereas others report that it is not an important environmental determinant (Jones and Hallin 2010, Peralta et al. 2010, Tang et al. 2010, Song et al. 2011). The current study helps resolve this inconsistency by emphasizing the synergistic effects of nitrate and OM type, suggesting that nitrate's effect on community composition may be strong when accompanied by particular OM characteristics (e.g., labile OM) but weak under other soil OM conditions (e.g., unamended OM; Fig. 2.3). One mechanism by which nitrate concentration could impact composition of DNF and/or DNRA organisms is by differential selection of organisms based on enzyme affinity for nitrate. Community composition shifts associated with enzyme affinity for a terminal electron acceptor have been demonstrated in other heterotrophic microbial communities (Kiesel et al. 2008).
This study garnered little evidence for direct resource competition between the DNF- and DNRA-capable microorganisms. A population increase in one group did not necessarily correspond to a decrease in the other, and abundances were not significantly correlated (Spearman’s $\rho = -0.31$, $p=0.15$). However, abundance was affected by resource availability in a manner that suggests these two groups employ contrasting metabolic strategies for resource utilization. Specifically, the abundance of DNF organisms was consistent with an ecological classification as “copiotrophs” in that they were strong competitors in environments with abundant available nutrients, and weak competitors under resource-poor conditions (Fierer et al. 2007). This preference derives from the fact that copiotrophic organisms typically have high growth rates, high maintenance requirements, and low enzyme affinity (Button 1993, Kovárová-Kovar and Egli 1998). Conversely, the pattern of DNRA abundance was more consistent with an oligotrophic classification; relative to denitrifiers, DNRA organisms thrived in resource poor environments and were not effective competitors under resource rich conditions. In addition to changes in abundance, resource manipulation resulted in differences in community composition for both groups. Further study is necessary to determine the functional significance of such changes, but microbial community composition has previously been linked to ecosystem processes rates (see Allison and Martiny 2008, and citations therein), and likely plays an important role in resource utilization associated with microbially-mediated nitrate transformations.

Implications for Wetland Restoration

The results of this study are relevant to scientists interested in the restoration of natural wetlands and the construction of artificial wetlands. In both situations, OM amendments are
commonly employed to accelerate soil development, enhance bulk density, and modulate soil moisture fluctuations in wetlands (e.g., see references in Bruland et al. 2009), all of which are important determinants in the growth and survival of colonizing vegetation and thus restoration success. Further, because organic carbon is a key substrate for many microbial processes taking place in wetlands, OM amendments are often used to enhance biogeochemical activity in newly restored wetland soils. The results presented here demonstrate that different types of OM amendments will have different consequences in terms of nitrate removal and, further, that the response of an ecosystem to OM amendment will depend on the anticipated nutrients loads from the watershed (e.g., nitrate concentration). Given that addition of OM to the surface or sediment during wetland construction is a common way to enhance nitrate removal through DNF (Fleming-Singer and Horn 2003, Burchell et al. 2007, Kadlec 2012), the current study suggests the addition of labile OM will increase denitrifier abundance. In contrast, wetlands to which more recalcitrant OM has been added may retain more of the reactive nitrogen via DNRA conversion to ammonium, thus altering nutrient availability, which could influence both plant productivity and carbon mineralization.

Conclusions

This work demonstrates that both OM and nitrate have strong yet disparate effects on DNF and DNRA community structure, and highlights the importance of evaluating resource combination effects on microbial communities in wetlands. Specifically, DNF populations are favored and DNRA populations are reduced under high resource environments, which suggests different ecological strategies may be employed by each functional group of microbes. Because
of these microbial community attributes, the types of OM additions made in wetland engineering efforts should be chosen carefully based on the ecosystem services desired.

Acknowledgements

This research was funded by a Virginia Commonwealth University Rice Center for the Environmental Sciences Student Research Award to E.M. Morrissey and A.S. Jenkins. We are grateful to the following people for field and laboratory help: David Berrier, Jaimie Gillespie, Rana Mehr, Caitlin Muse, Aaron Aunins, and Colleen Higgins. This paper is VCU Rice Center Research Contribution No. 30.
Fig. 2.1 Treatment effects (mean ± 1 standard error) on environmental parameters including: redox (A, B), OM content (C, D), C:N ratio (E, F), and porewater nitrate concentration (G, H). Panels in the left column display nitrate-amendments in the absence of added OM; letters denote significantly different subgroups as determined via one-way ANOVA and Tukey’s HSD. Panels in the right column show combined treatment effects (OM and/or nitrate amendment). Different lower case letters (or letters with a prime (‘)) represent statistically significant differences when one-way ANOVA and Tukey’s HSD were performed on OM type at nitrate amendment levels of 0 and 4 mg N g⁻¹ wet sediment, respectively.
Fig. 2.2 Treatment effects (mean ± 1 standard error) on the abundance of DNF *nirS* (A, B) and DNRA *nrfA* (C, D) functional genes as determined via qPCR. Panels in the left column display nitrate-amendments in the absence of added OM. Panels in the right column show combined treatment effects (OM and/or nitrate amendment). Different lower case letters (or letters with a prime (‘)) represent statistically significant differences when one-way ANOVA and Tukey’s HSD were performed on OM type at nitrate amendment 0 and 4 mg N g⁻¹ wet sediment, respectively.
Fig. 2.3 Non-metric multidimensional scaling (NMDS) ordination diagrams derived from T-RFLP data for the DNF *nosZ* (A) and DNRA *nrfA* (B) genes. Stress values for the 3D solutions were 0.21 and 0.29 respectively. Numbers associated with each axis title correspond to the percent of variance explained by the axis. Points are centroids ± 1 standard error; circle (O) indicates no nitrate amendment and triangle (Δ) indicates 4N nitrate amendment. In the treatments with no added OM ("unamended"), the three nitrogen levels were not significantly different, so the results were pooled into a single point. Proximity of samples on these ordination diagrams reflects overall community similarity as determined using Jaccard coefficients applied to the presence/absence of each unique terminal restriction fragment in each community profile.
CHAPTER THREE:

INTERACTIVE RESOURCE EFFECTS ON DENITRIFICATION POTENTIAL ARE MEDIATED BY COMMUNITY COMPOSITION IN TIDAL FRESHWATER WETLAND SOILS

by

Ember M. Morrissey and Rima B. Franklin
Department of Biology, Virginia Commonwealth University, Richmond, VA 23284 USA
Abstract

Microbially-mediated denitrification is widely recognized as an important means of removing excess nitrogen (N) from polluted ecosystems, particularly wetlands. However, accurate predictions of denitrification rates are not yet possible, potentially owing to complex uncharacterized interactions between denitrifier communities and environmental conditions. To better understand how the availability of organic matter (OM) and nitrate (NO$_3^-$), two of the resources most fundamental to denitrifying organisms, affect these populations and their activity, we performed an *in situ* resource manipulation in tidal freshwater wetland soils. Treatments included a full-factorial design of N fertilization crossed with OM additions, as well as appropriate no-amendment controls. Fertilization was in the form of slow-release KNO$_3$ pellets to achieve porewater NO$_3^-$ concentrations analogous to medium (~5 mg L$^{-1}$ NO$_3^-$–N) and high (~50 mg L$^{-1}$ NO$_3^-$–N) levels of nitrate pollution. Organic matter was added to double the ambient levels (%) using either plant litter or compost. Samples of each treatment were collected after 6, 9, and 12 months of incubation in July, October, and January respectively. Generally, denitrifier abundance (*nirS* copies g$^{-1}$ soil via qPCR) increased ~4 fold in response to the highest level of NO$_3^-$ fertilization, regardless of OM type or sampling date. Further, compost addition consistently increased denitrifier abundance while the plant litter amendment had little effect, most likely due to differences in the chemical structure of these substrates affecting their accessibility to denitrifiers. The community composition of denitrifiers (assessed using T-RFLP of *nirS*) was interactively regulated by both NO$_3^-$ and OM; the greatest effect was that of NO$_3^-$ addition and the associated shifts in community composition were relatively consistent across sampling dates. Denitrification potential (pDNF) rates were also strongly affected by NO$_3^-$ fertilization, which increased pDNF rates by ~10 to 100 fold at the highest level of fertilization.
(range across all OM types and months). The effect of OM addition was less pronounced (~7-fold increase) and only manifest in the absence of N fertilization. This may be due to increased mineralization of organic-N after the OM addition, following by enhanced coupled nitrification-denitrification. These results indicate that OM additions may not improve N removal in nitrate-polluted reduced wetland soils. The mechanism of NO$_3^-$ and OM effects were investigated using path analysis, which revealed that the influence of resource availability on pDNF rates was largely mediated by changes in denitrifier community composition and that denitrifier abundance was not a good predictor of activity. These results suggest that denitrification in freshwater wetlands is interactively regulated by resource availability both directly and indirectly via changes in denitrifier community composition.
Introduction

Anthropogenic disruption of the nitrogen (N) cycle is considered one of the major threats to global ecosystem functioning (Rockstrom et al. 2009, Gruber and Galloway 2008). Excess N resulting from human activities, including agriculture and fossil fuel combustion, has been implicated in the eutrophication of both freshwater and marine habitats (e.g., see Galloway and Cowling 2002, Bergström and Jansson 2006, Howarth and Marino 2006, Turner et al. 2006) and may even contribute to global climate change (Galloway et al. 2008, McCrackin and Elser 2010, Hoben et al. 2011). Denitrification is well established as an important nitrate (NO$_3^-$) removal mechanism that can help ameliorate the effects of N pollution (Schlesinger 2009). Specifically, denitrifying microorganisms transform NO$_3^-$ into gaseous N (typically N$_2$) that dissipates to the atmosphere. Wetlands are hotspots for denitrification (Fisher and Acreman, 2004) and are estimated to remove nearly 20% of reactive N inputs worldwide (Galloway et al. 2003, Jordan et al. 2011).

Numerous prior studies have demonstrated that environmental parameters such as soil texture, O$_2$ availability, redox, temperature, soil organic matter (OM) concentration, and NO$_3^-$ availability impact denitrification activity (see Megonigal et al. 2004, Xu et al. 2013, and references therein). However, despite a considerable body of research, accurate prediction of denitrification activity remains challenging (Davidson and Seitzinger 2006, Wang et al. 2013). Several scientists have suggested that studying the underlying microbial community may enhance our ability to link biogeochemical process rates to environmental conditions (Schimel 2001, Allison and Martiny 2008, Strickland et al. 2009, McGuire and Treseder 2010, Todd-Brown et al. 2012). In the case of denitrifiers, multiple studies have found associations between the structure of denitrifier communities and measures of denitrification activity (Magalhaes et al. 2012).

Denitrification is primarily performed by facultative-anaerobic heterotrophic microorganisms, making OM and NO$_3^-$ the main metabolic resources for these communities. While several studies have demonstrated that availability of these resources can impact denitrifier communities (Kjellin et al. 2007, Jones and Hallin 2010, Morrissey et al. 2013a, Yang et al. 2013) and their activity rates (Seitzinger et al. 2006, Sutton-Grier et al. 2009, Mulholland et al. 2008), there is little work that considers the potential for interactive resource regulation. In the context of Hutchinson (1957), species exhibit simultaneous fitness responses to multiple environmental gradients (dimensions). Wherein, the combination of environmental conditions under which the species is most competitive can be thought of as its multidimensional niche. This provides a basis for the expectation that microbial community structure will be interactively regulated by multiple environmental variables. As a consequence, the response of microbial community structure, and perhaps also function, to environmental variables cannot be fully understood by studying each variable in isolation. Indeed, interactive regulation of microbial community structure has been experimentally demonstrated in communities of bacteria (Castro et al. 2010, Stark et al. 2012) and denitrifiers (Morrissey et al. 2013a, Zhang et al. 2013). To the limited extent that interactive regulation of denitrification activity has been studied (Aulakh et al. 1991, Tuominen et al. 1999, Parton et al. 1996, Belmont et al. 2012), the role of community composition as a potential mediator of resources availability effects on activity rates has been largely ignored.

To address this deficiency, we conducted a long-term in situ manipulation of soil OM characteristics and NO$_3^-$ availability in a tidal freshwater wetland. We sought to determine: (1) if
and how resources important to denitrifying organisms (OM and NO$_3^-$) interactively regulate denitrifier communities and their activity rates and (2) assess whether the resource regulation of activity rates is mediated by changes in denitrifier community structure. Understanding denitrifier ecology and the role of community structure in regulating denitrification activity is applicable to predicting N removal from a variety of ecosystems including wetlands.

Methods

Experimental Design

This research was conducted in a 30-ha tidal freshwater wetland at Virginia Commonwealth University’s Walter and Inger Rice Center for Environmental Life Sciences (James River, Charles City County, Virginia; 37°20'05" N, 77°12'27" W). The study took place in a 20 × 20 m experimental plot near the center of the wetland, in a region that was continually saturated, frequently with standing water on the surface, and dominated by obligate wetland vegetation such as *Leersia oryzoides*, *Juncus effusus*, and *Nuphar luteum*. The soil had an OM content of 8%, a C:N ratio of 8 (by mass), a pH of 6.4, and the soil texture was 30% sand, 55% silt, and 15% clay.

The experiment was conducted using a modified litterbag approach as originally described in Morrissey et al. (2013a). First, in January 2011, soil from 5-15 cm below the surface was collected from a site adjacent to the experimental plot and homogenized in the laboratory. Soil was then subdivided into nine treatments with a full factorial design of N fertilization crossed with OM amendment. Fertilization with N was in the form of temperature-controlled, slow-release fertilizer pellets (KNO$_3$, Polyon CAS# 7757-79-1, Agrium Advanced Technologies, Loveland, CO) to achieve 0 (unfertilized), 0.8, and 4 mg N g$^{-1}$ wet soil. Organic
matter was either unamended, augmented with plant litter (standing dead material of the above mentioned species harvested from the field site in December 2010; OM content=92% and C:N=72), or supplemented with compost (Black Kow, Oxford, FL; organic blend with an OM content of 27% and C:N=18). The OM amendments raised the soil OM (%) to approximately double ambient levels. Prior to addition, the plant litter was dried (70°C for 5 days), chopped by hand, and then ground in a coffee grinder to be similar in particle size to the compost (0.1-5 mm in diameter).

Litter bags (12 cm × 22 cm), constructed of 0.5-mm Nitex mesh (Wildlife Supply Company, Buffalo, NY), were each filled with 400 ml (~ 215 g dry weight) of soil and incubated in situ by burying (5-15 cm depth) at random locations within the experimental plot. Care was taken to insert the bags into the ground causing as little disruption of the surrounding soil as possible. Samples were incubated for 6, 9 or 12 months and collected in July, October, and January respectively. At each sampling event, five replicate bags of each treatment were collected, as were five intact field cores. Samples were placed in airtight plastic bags, quickly transported back to the laboratory, and subdivided for immediate soil characterization (200 g), molecular genetic analyses (5 g, stored at -20°C), and potential denitrification rates (pDNF; 36 g). The field cores were analyzed only for soil characteristics and pDNF rates, and served to provide context for interpreting the experimental manipulations.

**Soil Characterization**

Soil redox potential and pH were measured using a Hanna Combo pH and ORP probe (QA Supplies, Norfolk, VA). Soil moisture (%) was determined gravimetrically (100°C for 72 h) and OM (%) was measured as the mass loss on ignition (500°C for 4 h). Total carbon and
nitrogen contents were determined using a Perkin Elmer CHNS/O Analyzer (Waltham, MA) following grinding and acidification of samples using 10% hydrochloric acid; C:N was calculated by mass. Porewater was extracted from 50-ml soil samples by centrifugation (3000 × g, 15 min), filtered using a 0.45 μm pore-size mixed cellulose ester syringe filter, and stored at -20°C until it could be analyzed for ammonium (NH₄⁺) using the indophenol colorimetric assay of Grasshoff et al. (1983) and NO₃⁻ via ion chromatography (Dionex ICS-1000, Sunnyvale CA).

**Molecular Genetic Analyses**

Whole-community DNA was extracted from 0.5-g subsamples of soil using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA) and stored at -20°C. DNA purity and concentration were analyzed using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). All DNA extracts and PCR products were verified using agarose gel (1.5 %) electrophoresis and ethidium bromide staining.

**Denitrifier Community Structure via T-RFLP**

Community structure of denitrifiers was assayed via terminal restriction fragment length polymorphism (T-RFLP) targeting the cytochrome cd1 nitrite reductase gene (nirS) gene. The assay was modeled after Wolsing and Prieme (2004) using the nirS1F and nirS6R primers. All PCR reactions (50 μl, reagents obtained from Applied Biosystems, Foster City, CA) were performed with 1X PCR Buffer, 2.5 mM MgCl₂, 200 μM of each dNTP, 1.5 μg BSA, 0.2 μM each primer, 2.5 units of AmpliTaq DNA polymerase, and 10-30 ng of template DNA. Touchdown PCR thermal cycling conditions were: 94°C for 3 min, 10 cycles of 25 s at 95°C, 30
s at 64°C (-0.5°C cycle⁻¹), 80 s at 72°C, followed by 40 cycles of 25 s at 95°C, 30 s at 58°C, 80 s at 72°C and final extension at 72°C for 7 min.

PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen, Valencia, CA) prior to restriction enzyme digest. Digests (12 µl, reagents obtained from New England Biolabs, Ipswich, MA) were conducted in 1X Buffer #4 with 1.5 µg BSA and 18 units of Hae II, and incubated for 16 h at 37°C followed by 20 min at 65°C. Restriction fragments were purified using the MinElute kit, recovered in molecular-grade water, and detected using capillary electrophoresis with a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Buckinghamshire, UK). An aliquot of 50-120 ng of purified, digested PCR product was combined with 0.3 µl of MapMarker 400 ROX ladder (Bioventures, Murfreesboro, TN) plus 4.75 µl injection buffer (0.1% Tween-20). Samples were injected at 3 kV for 100 s, and electrophoresed using genotyping filter set 1 for 100 min at 10 kV. T-RFLP fragments between 70-400 base pairs (bp) were analyzed using Fragment Profiler software (Version 1.2; Amersham Biosciences) using a 1 bp size differential and a 75 relative fluorescent unit peak height threshold. Samples were standardized by calculating peak area as a percent of the total sample fluorescence.

**Denitrifier Abundance via qPCR**

Quantitative polymerase chain reaction (qPCR) assays were performed to assess nirS denitrifier abundance using the primers cd3aF and R3cd (Throback et al. 2004). This primer set targets a segment of the nirS gene internal to the fragment amplified during T-RFLP, and yields a smaller amplicon (~425 bp) that is more amenable for qPCR. Reactions (20 µL) were performed with 10 ng DNA template and 0.1 µM concentrations of each primer; thermal cycling
conditions were: 95°C for 4 min, and 50 cycles of 30 s at 95°C, 30 s at 56°C, and 60 s at 72°C using SsoAdvanced SYBR Green qPCR Supermix (BioRad, Hercules, CA) and a BioRad CFX 96 Real-Time System. Data were analyzed using Bio-Rad CFX Manager Version 2.1. Genomic DNA from Paracoccus denitrificans (Strain #17741, ATCC, Manassas, VA) was used for the standard curve (average efficiency=96.3% and r²=0.99). Results were reported as the log\(_{10}\) of the number of gene copies per g of dry soil after averaging three technical replicates per sample.

**Potential Denitrification Rates**

Potential denitrification (pDNF) rates were assessed using an anaerobic slurry assay utilizing K\(^{15}\)NO\(_3\) similar to Thamdrup et al. (2002) and Erler et al. (2009). Immediately after sample collection, ~9 g of wet soil was placed into a 20-ml airtight vial (Catalog # 20090297, Grace Davidson Discovery Sciences, Deerfield, IL) and flushed with ultra-high purity He (Airgas Inc., Radnor, PA). Soils were then pre-incubated overnight at ambient temperature. The next morning, site water (collected from the field during sampling) was filter-sterilized, deoxygenated (He-flushed), and a 9-ml aliquot was added to each slurry. Directly afterwards, 1.8 μmoles of K\(^{15}\)NO\(_3\) (99% \(^{15}\)N, CAS# 57654-83-8; Cambridge Isotope Inc., Tewksbury, MA) was added to create a final concentration of ~100 μM within each slurry. Slurries were created in quadruplicate for each sample and one replicate was sacrificed after headspace sampling at 0, 4, 8, and 12 h of incubation. Headspace samples (0.5 ml) were collected using an airtight syringe and transferred to a He-flushed 10-ml Exetainer vial (Labco, Ceredigion, UK). Headspace samples underwent isotopic analysis for abundance of \(^{29}\)N\(_2\) and \(^{30}\)N\(_2\) by isotope ratio mass spectroscopy (IRMS; UC Davis Stable Isotope Laboratory, Stevens et al. 1993). Concentrations of \(^{29}\)N\(_2\) and \(^{30}\)N\(_2\) were determined as excess above their natural abundances and used to calculate
total N₂ production with the nitrogen isotope pairing technique (Nielsen, 1992). Dinitrogen gas production was linear over time (all r² > 0.60) and pDNF rates were calculated as the slope of these plots; rates are reported as pmoles N₂ produced per gram dry soil per h.

**Statistical Analyses**

Data were normally distributed within each population (evaluated using Shapiro-Wilks tests), with the exception of porewater NO₃⁻, denitrifier (nirS) abundance, and pDNF rates, all of which required log₁₀ transformations prior to analysis using parametric statistical techniques. In addition, geometric means are reported in the results for log₁₀ transformed variables; arithmetic means are reported for all other parameters. For each sampling event, effects of treatment (N fertilization and OM amendment) on soil characteristics, denitrifier abundance, and pDNF rates were analyzed using two-way analysis of variance (ANOVA; n=5 per group, df=44) with Tukey's HSD for post hoc comparisons. In addition, field cores were compared to control bags (i.e., unfertilized and unamended soil) using two-tailed t-tests. Analyses were performed using the JMP statistical software (Version JMP Pro 9.0.2, Cary, NC; Sall 2005) with α = 0.05.

T-RFLP results were analyzed using principal coordinates analysis (PCoA) applied to the Bray-Curtis index of similarity derived from normalized fluorescence data. Treatment effects were analyzed using a two-way non-parametric multivariate ANOVA (NP-MANOVA), again applied to the Bray-Curtis similarity index. All community analyses were conducted using the PAST Version 2.16 statistical package (Hammer 2001).

Path analysis was used to assess the relative importance of potential drivers of pDNF rates including OM, NO₃⁻, and the denitrifier microbial community. This technique allows for the evaluation of causal models wherein non-causal, indirect, and direct relationships between
predictor and response variables can be statistically tested (McCune and Grace 2002). Using experimental data, a full model of all hypothetical relationships was simplified by sequentially removing “paths” in order of probability value (largest first) until all remaining paths were significant. The model fit was evaluated using a Chi-square ($\chi^2$) test where a non-significant p-value indicates a good fit of the model to the data (Shipley 2000). The reduced path model was confirmed with stepwise multiple regression analysis. Analyses were performed with SPSS Version 21 (IBM Corp, Armonk, NY) and Amos 18 (Amos Development Corporation, Crawfordville, FL) with an $\alpha=0.05$.

**Results**

**Comparing Field Cores to Experimental Controls**

Comparisons of reference field cores to control bags (i.e., no added N or OM) showed no consistent effects of the experimental manipulation. In July and October, none of the soil characteristics significantly differed (July: all $t<|1.9|$, $p>0.11$, October: all $t<|2.4|$, $p>0.05$). In January, the only differences were that the reference field cores had a slightly lower pH than the experimental controls (mean and S.E. of 4.9±0.1 vs. 5.5±0.1 respectively), as well as slightly higher soil moisture content (%: 60.5±1.0 vs. 55.0±0.5) and OM (%: 9.0±0.3 vs. 7.9±0.2). With regards to pDNF rates (pmoles g$^{-1}$ dry soil h$^{-1}$), significant differences were observed in July (field cores: 8,089±4,167 vs. control bags: 1,528±1,259; $t=2.5$, $p=0.04$) and not in either October or January (pooled means and S.E. for October: 217±115 and January: 124±30, both $t<2.4$ with $p>0.05$).
Soil Characteristics

The response of environmental variables to N fertilization and OM amendment was assessed using two-way ANOVA (Table 3.1). Nitrogen fertilization had no impact on soil moisture while OM amendment created small but consistent changes (Fig. 3.1A). Specifically, plant litter always increased (~2.5 %) and compost always decreased (~4 %) the soil moisture relative to unamended controls. Soil moisture was generally similar across all three sampling events, ranging from 44.1 to 63.3, and averaging ~50% (mean ± S.E. for unamended controls: 52.3±0.4, plant litter: 54.8±0.3, compost: 48.2±0.3). Soil redox potential was interactively affected by the treatments across all the months sampled, wherein N fertilization increased the redox of unamended soils and those with added compost, but had less of an effect in the presence of plant litter (Fig. 3.1B). In general, redox conditions were negative and varied from a low of -104±5 mV (July, no N fertilization, compost amendment) to a high of 70±5 mV (January, 4 mg N g⁻¹ fertilization, no addition of OM). With regards to pH (results not presented), the patterns observed in July were quite distinct from those obtained in January; no data are available for October due to instrument malfunction. In July, N fertilization did not have a significant effect on pH (Table 3.1) but compost amendment marginally increased the pH (6.2±0.0) relative to the other OM types (unamended: 5.7±0.0, plant litter: 5.7±0.1). In January, there was an interaction between N fertilization and OM amendment, wherein N fertilization increased the pH of the soil when combined with either plant litter (fertilization level of 0 mg N g⁻¹: 4.7±0.1, 0.8 mg N g⁻¹: 5.0±0.2, 4 mg N g⁻¹: 5.2±0.2) or compost (0 mg N g⁻¹: 5.5±0.1, 0.8 mg N g⁻¹: 5.5±0.1, 4 mg N g⁻¹: 5.9±0.2), but not native OM (mean across all N levels: 5.6±0.1).

As anticipated, the addition of plant litter or compost increased the OM (%) of the soil relative to unamended controls (Table 3.1, Fig. 3.1C) by a factor of ~1.5 (July: 1.6-fold, October: 1.6-fold).
1.4, January: 1.5). Organic matter content did not differ between the compost and plant litter treatments (\%, mean and S.E. for the two groups combined; July: 15.1±0.4, October: 14.0±0.4, January: 12.8±0.2) but was always significantly greater than for the unamended soil (July: 9.3±0.5, October: 10.1±0.5; January: 8.7±0.4). Nitrogen fertilization also exerted a consistent effect on soil OM content. In soils to which high (4 mg N g\textsuperscript{-1}) levels of N were added, OM values were ~2% higher compared to unfertilized or low (0.8 mg N g\textsuperscript{-1}) levels of N. In addition to affecting the amount of OM present, the OM amendments increased the soil C:N for all the months sampled (Fig. 3.1D). The soil C:N consistently decreased as the field incubation progressed in unamended (July: 10.1±1.0, October: 7.8±9.1, January: 6.9±0.8) and plant litter added soils (July: 12.1±0.1, October: 8.6±1.1, January: 7.8±0.3). Such reductions were less pronounced in the compost treatment (July: 10.8±0.2, October: 10.6±0.9, January: 9.2±0.3). The N fertilization had inconsistent effects on the C:N; specifically, it was associated with a slight increase in July, a slight decrease in October, and had no effect in January.

In July and October, N fertilization increased the concentration of dissolved inorganic N in the porewater (Fig. 3.1E and F), while OM amendment had no significant effects (Table 3.1). The magnitude of the N fertilization effect was greatest in July, where NO\textsubscript{3}\textsuperscript{-} concentrations increased ~10 and ~85 fold for the 0.8 and 4 mg N g\textsuperscript{-1} treatments respectively while NH\textsubscript{4}\textsuperscript{+} concentrations increased ~1.8 and ~2.4 fold. In October, only the 4 mg N g\textsuperscript{-1} level of fertilization produced a significant increase, resulting in porewater NO\textsubscript{3}\textsuperscript{-} and NH\textsubscript{4}\textsuperscript{+} concentrations that were ~5 fold greater than the unfertilized controls. Similar results were observed in January in that only the 4 mg N g\textsuperscript{-1} fertilization produced a significant increase (Fig. 3.1F), though this effect varied based on OM treatment. In particular, concentrations increased in the unamended (NO\textsubscript{3}\textsuperscript{-}:...
~20 fold, \( \text{NH}_4^+ \): ~3 fold) and plant litter soils (\( \text{NO}_3^- \): ~50 fold, \( \text{NH}_4^+ \): ~12 fold), but not in the compost-amended soils.

**Denitrifier Abundance**

The abundance of denitrifiers, as assessed by \( \text{nirS} \), was affected by both N fertilization and OM treatment (Fig. 3.2A). In July, there was a general trend in that N fertilization increased \( \text{nirS} \) abundance, though post hoc tests showed that the differences were only significant for the native OM conditions and when comparing the unfertilized (0 mg N g\(^{-1}\)) soil to the highest N level (4 mg N g\(^{-1}\)). For later sampling dates, N effects were consistent across OM treatments, and the highest level of fertilization increased \( \text{nirS} \) ~5 fold in October and ~3 fold in January. Further, across all three months, soils with compost addition consistently had the highest denitrifier abundance. In contrast, the effect of plant litter relative to unamended soil was rarely significant.

**Denitrifier Community Structure**

The effects of N fertilization and OM amendment on denitrifier (\( \text{nirS} \)) community structure were analyzed with two-way NP-MANOVA, which revealed a significant interaction between these factors at all sampling dates (Table 3.1). These effects were visualized using PCoA (Fig. 3.3), which positioned samples in ordination space based on the similarity of their T-RFLP profiles. These plots revealed a consistent effect of N fertilization on community structure across all three months studied, which usually manifest as a shift to the left on PCoA Axis 1. The interaction between N fertilization and OM amendment can also be seen in these diagrams. For instance, in both July and October, the effect of N fertilization increasing from 0.8 to 4 mg N g\(^{-1}\)
corresponds to a very small shift in community structure for soils with added compost, but a
dramatic shift for those amended with plant litter. Because of this interaction, the effects of OM
type are less visually apparent in the PCoA diagram but were nonetheless significant when
assayed by post hoc one-way NP-MANOVAs. In particular, the effect of OM treatment on
community structure was statistically significant when evaluated within each N fertilization level
for each sampling date (all F>1.58, all p<0.02).

To visualize patterns in community composition across sampling events, an additional
PCoA was performed pooling the data from all three months (Fig. 3.4). As above, the effect of N
fertilization was very pronounced and generally consistent across the sampling dates, wherein
unfertilized samples cluster in the top right quadrant and N fertilization created a shift toward the
lower left quadrant of the diagram. In addition, the interactive effect with OM amendment can
be observed. For example, in the unamended soil, community composition always shifted due to
low levels of N fertilization (0.8 mg N g\(^{-1}\) level) and then again for the higher addition (4 mg N
g\(^{-1}\)). In contrast, the microbial community associated with the compost-added soils often
responded to low levels of N fertilization (0.8 mg N g\(^{-1}\) level) but showed not further change
with additional N fertilization (4 mg N g\(^{-1}\)).

**Potential Denitrification Rates**

Across all three months, pDNF rates were strongly and positively affected by N
fertilization (Table 3.1, Fig 3.2B). In July and October, this effect was consistent regardless of
OM type, and pDNF rates in the soils with 4 mg N g\(^{-1}\) fertilization were 35- to 115-fold higher
than the unfertilized soils (July and October respectively). A strong N effect was also observed in
January, but it included a significant interaction with OM treatment. In particular, the pattern for
the unamended and compost-added soils matched prior months, but, in the case of plant litter, pDNF rates were not different at low (0.8 mg N g$^{-1}$) and high (4 mg N g$^{-1}$) levels of N fertilization. Though the effect of N-fertilization on pDNF rates predominated, OM effects were apparent in the unfertilized (0 mg N g$^{-1}$) soils. Specifically, pDNF rates were higher in the compost and plant-added treatments relative to the unamended control by ~9 fold in July, ~7 fold in October, and ~4 fold in January.

**Path Analysis**

To investigate how the resource manipulations affected pDNF rates, path analysis was performed. A theory-constrained “full model” (Fig. 3.5A) was developed to represent all hypothetical relationships between the resource availability (i.e., OM content and NO$_3^-$ concentration), denitrifier community parameters (structure: PCoA Axes 1 and 2; abundance: nirS copies), and pDNF rates. Using experimental data, a reduced model (Fig. 3.5B) was produced by sequentially removing paths in order of probability value (largest first) until all paths were significant ($\alpha=0.05$). The reduced causal model explained nearly half of the variation in pDNF rates ($R^2=0.39$) combining all treatments and sampling events. Further, the model was a good fit to the observed data as determined by the model Chi-square statistic ($\chi^2=4.7$, $p=0.19$). Consistent with the ANOVA and NP-MANOVA results discussed above, which demonstrated N-fertilization effects, the path analysis supported porewater NO$_3^-$ as a driver of both pDNF rates and denitrifier community structure (PCoA Axes 1 and 2). Overall, NO$_3^-$ effects on pDNF rates were both direct and indirect (i.e., mediated through PCoA Axes 1 and 2). In contrast, soil OM was not a direct driver of pDNF, but was an indirect predictor with an effect mediated through denitrifier community structure (PCoA Axis 1). Denitrifier abundance was not a significant predictor of pDNF rates and was therefore removed from the reduced model.
Discussion

This study evaluated the role of denitrifier community structure (abundance and composition) in mediating resource regulation of denitrification activity, and specifically considered the effects of OM and NO$_3^-$.

A greater understanding of how these resources interactively regulate microbial communities and their function is relevant to understanding N cycling in natural wetlands and constructing or restoring wetlands to effectively remove N. We studied NO$_3^-$ under ambient conditions (0.5-1 mg L$^{-1}$ NO$_3^-$–N) and at two levels of N fertilization intended to represent medium and high amounts of pollution. Those concentrations (Fig. 3.1E) ranged from ~5 mg L$^{-1}$ NO$_3^-$–N, which is similar to values reported for polluted rivers (Mitsch et al. 2005) and ground waters (Santoro et al. 2006), to ~50 mg L$^{-1}$ NO$_3^-$–N, which is comparable to values reported for treatment wetlands (Lin et al. 2002, Albuquerque et al. 2009). The OM treatments included soils augmented with plant litter and compost, and were successful in increasing the OM content (%) of the soil (Fig. 3.1C). The plant litter addition was analogous to fresh detrital inputs that would typically be added to the wetland soil at the end of the growing season upon senescence, and was composed primarily of carbohydrates and lignins. This material differed in composition from the compost addition, which contained a greater fraction of humified and microbial necromass OM (Tiquia et al. 1996, Tuomela et al. 2000). The compost addition was likely similar to the native OM, which similarly undergoes humification and accumulates an increased portion of microbial necromass as it ages (Liang and Balser 2010, Throckmorton et al. 2012). In this way, the compost addition can be interpreted as an increase in C quantity without a large change in the composition of the OM, essentially mimicking older wetland soils (sensu Morrissey et al. 2013b). Another motivation for selecting compost and plant...
litter is that these OM types are often used as amendments during wetland construction and restoration (Davis 1995, EPA 2008) to enhance vegetation establishment and nutrient cycling (O’Brien and Zedler 2006, Sutton-Grier et al. 2009).

Overall, the changes in porewater NO$_3^-$ and soil OM (%) indicate that the treatments were successful in altering the microbial resource environment. Further, comparison of the field cores with the control samples (i.e., no added NO$_3^-$ or OM) showed few differences. This is evidence that the experimental manipulations had little effect on the soil environment and that the results remain applicable to unaltered wetland soils. The only treatment parameter that displayed an unexpected response was soil OM (%), which increased slightly (~2 %) in the presence of N fertilization. One possibility is that N fertilization increased the growth of root biomass in these samples, altering the OM (%). However, the effect N fertilization could be fallacious resulting from fertilizer pellet combustion during the loss on ignition assay used to quantify OM (Morrissey et al. 2013a).

In addition to the targeted changes in soil OM (%) and porewater NO$_3^-$, the treatments produced modest changes in the other environmental parameters. For example, the OM additions altered soil moisture, though never more than a few percent (Fig. 3.1A). The effect of OM amendments on soil redox was similarly small, and treatment effects were primarily due to N fertilization (Fig. 3.1B), which is consistent with the findings of McLatchey and Reddy (1998) and Morrissey et al. (2013a). This is likely a simple result of NO$_3^-$ addition increasing the concentration of oxidized compounds in the soil, thus raising the redox potential. Even at their highest (70±25 mV), redox values were well within expectations for anaerobic wetlands and appropriate for denitrification. Interestingly, fertilization effects on redox were significantly reduced in the presence of plant litter compared to the more humified OM (native and compost-
added), which suggests the chemical structure and/or bioavailability of soil OM may affect microbial utilization of terminal electron acceptors. To the extent that C:N is often used a coarse metric for OM quality, the soils were all similar and well below the range of N limitation (Reddy and DeLaune 2008). Though modest, significant differences resulted from the OM additions (Fig. 3.1D). Specifically, C:N values were elevated for OM-augmented samples due to simple mixing of the amendment, which had a higher starting ratio (compost = 18:1, plant litter = 72:1), with the relatively low C:N native soil (8:1). The effects of N fertilization on C:N were small and inconsistent.

Fertilization effects on porewater inorganic N concentrations included increased NO₃⁻, (discussed above), and higher NH₄⁺ (at the 4 mg N g⁻¹ level of fertilization). Higher NH₄⁺ concentrations could result from multiple processes. One possibility is that greater NO₃⁻ availability decreased the uptake of NH₄⁺ by plants and microorganisms (Bunch et al. 2012), although this is unlikely since NH₄⁺ is generally considered more bioavailable than NO₃⁻ (Reddy and DeLaune 2008, Bown et al. 2010). Alternatively, fertilizer NO₃⁻ may have been converted to NH₄⁺ via assimilation and re-mineralization (Guerrero et al. 1981, Reddy and DeLaune 2008), or directly through dissimilatory nitrate reduction to ammonium (DNRA; Giblin et al. 2013). DNRA is similar to denitrification in that it is a NO₃⁻ reduction pathway performed by primarily heterotrophic microbial communities (Tiedje 1988). Consequently, like denitrification, DNRA communities and their activity rates can be affected by OM and NO₃⁻ availability (Magonigal et al. 2004, Koop-Jakobsen and Giblin 2010, Morrissey et al. 2013a). However, the resource regulation of DNRA is still unclear. Scott et al. (2008) reported a negative relationship between NO₃⁻ concentration and DNRA rates, conversely Koop-Jakobsen and Giblin (2010) found NO₃⁻ fertilization to increase DNRA activity, and Morrissey et al. (2013a) found no effect of NO₃⁻
addition on the abundance of DNRA-capable organisms. It is likely that DNRA is occurring in these soils but, given these inconsistencies, it is difficult to speculate on its relative importance in either NH$_4^+$ production or NO$_3^-$ consumption.

**Denitrifier Community Structure**

In general, N fertilization increased denitrifier nirS population size (Fig. 3.2A), consistent with Kong et al. (2010) and Yuan et al. (2012), most likely due to the greater availability of NO$_3^-$ to serve as a terminal electron acceptor. An alternate explanation is that N fertilization alleviated assimilatory N limitation to growth; however, this is unlikely given the C:N ratio of these soils (Reddy and DeLaune 2008). Fertilization was also found to affect denitrifier community composition, manifesting as clear and consistent shifts on the PCoA diagrams (Fig. 3.3 and 3.4). Further, NO$_3^-$ was identified as a strong driver of denitrifier composition (PCoA Axes 1 and 2) via path analysis (Fig. 3.5B). Significant relationships between denitrifier community structure and NO$_3^-$ have been reported in other fertilization experiments (Tang et al. 2010) and along natural gradients (Jones and Hallin 2010, Baneras et al. 2012, Yang et al. 2013). One hypothesized mechanism by which NO$_3^-$ availability may impact denitrifier communities is selection based on differential ability of various denitrifying populations to scavenge for NO$_3^-$ (Morrissey et al 2013a). Another possible distinction between the communities observed under low- and high- NO$_3^-$ conditions relates to the organisms’ metabolic versatility. In environments where NO$_3^-$ is rare, organisms that can efficiently perform alternate metabolic processes (e.g., fermentation (Jørgensen and Tiedje 1993)), or use C sources that yield relatively high amounts of energy per terminal electron acceptor (Slonczewski and Foster, 2011) are likely to be more successful.
Although not as apparent on the PCoA diagrams (Fig. 3.3 and 3.4), the OM treatments also significantly altered denitrifier community structure (Table 3.1, and Fig. 3.5B). This finding is consistent with other studies that have reported relationships between soil OM characteristics and denitrification communities (Kjellin et al. 2007, Chen et al. 2010, Baneras et al. 2012, Morrissey et al. 2013a), and indicates that nirS-containing denitrifiers vary significantly in their C substrate preferences. The OM treatment also affected denitrifier abundance (Fig. 3.2A, Table 3.1). Compost consistently increased the denitrifier abundance while the effect of plant litter was variable. Given that the OM content (%) was the same in both of these treatments, this difference is not due to the amount of OM but instead the constituents of the OM pool (consistent with Warneke et al. 2011 and Morrissey et al. 2013a). The increased abundance resulting from compost addition may indicate that nirS denitrifiers are better suited to utilize the humified and microbial necromass OM in compost than the carbohydrates and lignins that dominate plant litter. The inconsistent response of nirS abundance to plant litter over the months studied may be due to changing availability of organic compounds at different stages of decomposition.

The effects of N fertilization and OM type on nirS denitrifier community structure were always interactive (Table 3.1). These results are consistent with the theory that microbial communities are structured by multi-dimensional niches (as described by Marco 2008 and Eisenhauer et al. 2013). In generally, all the explanations posited above to describe resource regulation of microbial community structure relate to the selection of individual taxa, or whole populations, based upon variations in their “trophic strategies” and the recognition that bacteria have evolved a wide range of capabilities with respect to growth and survival (Tiedje et al. 2001, Fierer et al. 2007). Trophic strategy has been found to be strongly reflected in genomic content.
(Lauro et al. 2009); therefore community-level adaptation for resource/nutrient levels could be responsible for both the PCoA and path analysis results presented here.

**Potential denitrification rates**

Across all sampling dates and OM treatments, N fertilization was the dominant driver of pDNF rates. Rates consistently increased with added N (Fig. 3.2B, Table 3.1), and pDNF had a direct causal link from NO$_3^-$ in the path analysis (Fig. 3.5B). These findings align with several other studies (e.g., Seitzinger et al. 2006, Koop-Jakobsen and Giblin 2010, Mulholland et al. 2008, Palta et al. 2013) in supporting the notion that microbial activity in anaerobic wetland soils is generally limited by the availability of terminal electron acceptors (e.g., O$_2$, NO$_3^-$, etc.). Compared to aerobic soils, there is a high rate of accumulation of otherwise bioavailable OM (Reddy and DeLaune 2008) and a presumption among researchers that OM quantity per se is rarely a limiting resource for microbial activity in the wetland soils (Ståhl 2000). The results of the present study are, by and large, consistent with these presumptions. However, this study also provides evidence of OM regulation when samples without N fertilization are considered in isolation. Specifically, in the absence of N fertilization, we observed a positive effect of OM addition on pDNF rates (Fig. 3.2B; one-way ANOVA for July: F=3.2, p=0.08; October: F=8.2, p<0.01; January: F=6.1, p=0.01) and a strong correlation with soil OM content (Pearson’s r= 0.57, all months). These results are consistent with past studies that have shown denitrification activity to be positively related to soil OM abundance (Hill and Cardaci 2004, Dodla et al. 2008) and to be stimulated following OM amendments in wetlands (Ullah and Faulkner 2006, Sutton-Grier et al. 2009). We hypothesize that our results may be driven, at least in part, by increased mineralization of organic-N due to the OM addition (Kong et al. 2010) followed by coupled
nitrification-denitrification (Seitzinger 1994). This supposition is consistent with a meta-analysis by Seitzinger et al. (2006), who reported that the majority of N used for denitrification under low NO$_3^-$ conditions ($\leq 1$ mg L$^{-1}$ NO$_3^-$–N) comes from coupled nitrification-denitrification. In contrast to the unfertilized samples, which averaged 0.85 mg L$^{-1}$ NO$_3^-$–N, nearly all the samples (~90%) to which we added N fertilizer had NO$_3^-$ concentrations in excess of 1 mg L$^{-1}$ NO$_3^-$–N, creating a scenario wherein most of the denitrified N is expected to come directly from NO$_3^-$ (i.e., uncoupled, Seitzinger et al. 2006). Our suggestion is consistent with the findings of Koop-Jakobsen and Giblin (2010), who reported that coupled nitrification-denitrification was responsible for more than half of the denitrified N under unfertilized conditions in marsh soils, but only accounted for a small fraction upon fertilization.

To evaluate whether the effects of OM (%) and NO$_3^-$ availability on pDNF rates were direct or indirect (i.e., mediated by changes in denitrifier community), path analysis was employed (Fig. 3.5). Nitrate effects on pDNF rates were strong, and demonstrated both a direct linkage as well as indirect links mediated through community composition (PCoA Axes 1 and 2). For OM, no significant direct link with pDNF was observed, but OM was identified as a driver of community composition (PCoA 1) providing a mechanism whereby it can have an indirect influence on pDNF. Taken together, these results are consistent with a scenario wherein OM and NO$_3^-$ interactivity drive denitrifier community composition and, in turn, composition affects pDNF rates. This finding contributes to a small but growing body of evidence that microbial community composition regulates ecosystem process rates (Reed and Martiny 2012, Allison et al. 2013) including denitrification (Cantarel et al. 2012 and Philipott et al. 2013). If indeed the relationships between denitrifier community structure and function reported in this and other works (Magalhaes et al. 2008, Song et al. 2011, Baxter et al. 2012, Philippot et al. 2013) are
reflective of a causal connection, further study of denitrifier community structure, and its relationship to denitrification rates will enhance our understanding of, and perhaps also ability to predict, N cycling processes.

In contrast to denitrifier community composition, nirS abundance was not a mediator of resource availability effects on pDNF rates. This finding is consistent with the many other reports that denitrifier abundance is a weak or unreliable indicator of activity (Dong et al. 2009, Henderson et al. 2010, Attard et al. 2011, Warneke et al. 2011). Given the fact that nearly all denitrifier isolates are facultative (Tiedje 1988, Zumft 1997) and that soil microorganisms exhibit variable activity states (Stenstrom et al. 2001), this is not necessarily surprising. This lack of correlation could also be compounded by the fact that DNA is present in dormant and even dead cells, which account for a large proportion of bacterial communities (Cole 1999, Bouvier and del Giorgio 2002, del Giorgio and Gasol 2008). DNA-based methods cannot distinguish live cells from dead, or active from inactive; however, simultaneous characterization of rRNA may provide a means for identifying the metabolically active fraction of bacterial communities, and recent studies have found qPCR analysis of whole-community rRNA content to be a robust indicator of activity (Freitag et al. 2010, Helbling et al 2011).

Conclusions

The results of this study suggest that denitrification activity in wetlands is regulated via a combination of denitrifier community–environment relationships. In general, NO$_3^-$ availability was a strong driver of both denitrifier community structure and pDNF rates. The additions of OM only increased pDNF rates under low porewater NO$_3^-$ concentrations, and we hypothesize this resulted from OM-enhanced N mineralization followed by coupled nitrification-
denitrification. Overall, the effects of OM (%) and NO$_3^-$ concentration on pDNF rates were largely mediated through changes in denitrifier community composition, and suggest that increased study of microbial community structure–function relationships may be valuable for scientists trying to develop a predictive understanding of biogeochemical process rates. In addition, these results have multiple implications for understanding wetland N cycling in natural and engineered systems. For example, the fact that OM addition did not stimulate denitrification when NO$_3^-$ levels were high, suggests that wetland restoration efforts that include OM additions may not enhance removal of excess NO$_3^-$ in reduced soils.

Acknowledgements

This research was funded by VCU Rice Center for Environmental Life Sciences Student Research Awards and a SWS Student Research Grant to E.M. Morrissey. Thanks to Joseph Morina, Jaimie Gillespie, Chansotheary Dang, Joseph Battistelli, Aaron Porter, Rana Mehr, and Nicki Dadashian for laboratory and field help. We are grateful to Dr. Bonnie Brown for providing the laboratory facilities used for stable isotope analyses and also to Dr. Aaron Mills and the Microbial Ecology laboratory at UVA for helping us with water chemistry analyses. A special thank you to David Berrier for being my beast of burden on many a long field day. This paper is a VCU Rice Center Research Contribution.
Table 3.1 Two-way ANOVA results (F and p) showing main effects of organic matter treatment (OM), nitrogen fertilization level (N), and the interaction between these factors (I) on environmental variables as well as denitrifier abundance (log$_{10}$ nir$S$ copies g$^{-1}$ dry soil), community composition (nir$S$ T-RFLP), and potential denitrification activity (pDNF, log$_{10}$ pmoles g$^{-1}$ dry soil h$^{-1}$) for each sampling event. Individual main effects are not presented whenever a significant interaction (I) was obtained; this is designed with a dash (–).

<table>
<thead>
<tr>
<th></th>
<th>July</th>
<th>October</th>
<th>January</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OM</td>
<td>N</td>
<td>I</td>
</tr>
<tr>
<td><strong>Environment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>30.6</td>
<td>&lt;0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>Redox (mV)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>33.6</td>
<td>&lt;0.01</td>
<td>2.25</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>66.9</td>
<td>&lt;0.01</td>
<td>14.9</td>
</tr>
<tr>
<td>C:N</td>
<td>32.6</td>
<td>&lt;0.01</td>
<td>4.8</td>
</tr>
<tr>
<td>NO$<em>3$ (log$</em>{10}$mg L$^{-1}$)</td>
<td>0.5</td>
<td>0.64</td>
<td>25.8</td>
</tr>
<tr>
<td>NH$_4$ (mg L$^{-1}$)</td>
<td>2.8</td>
<td>0.07</td>
<td>7.0</td>
</tr>
</tbody>
</table>

| Denitrifier     |    |   |   |    |   |   |    |   |   |
| Abundance       | - | - | - | - | 4.2 | <0.01 | 42.9 | <0.01 | 58.1 | <0.01 | 2.2 | 0.09 | 6.4 | <0.01 | 8.1 | <0.01 | 2.3 | 0.07 |
| Community composition | - | - | - | - | 2.7 | <0.01 | - | - | - | - | 2.0 | <0.01 | - | - | - | - | 2.2 | <0.01 |
| Activity (pDNF) | 1.4 | 0.25 | 16.9 | <0.01 | 0.9 | 0.47 | 0.7 | 0.46 | 44.7 | <0.01 | 2.3 | 0.07 | - | - | - | - | 3.1 | 0.03 |

*Results from NP MANOVA of nir$S$ T-RFLP data.
“nd” indicates no data are available.
Fig. 3.1 Treatment effects (mean ± SE, n = 5 per point) on soil moisture (A), redox potential (B), organic matter content (C), C:N (D), porewater NO$_3^-$ concentration (E, log$_{10}$ scale), and porewater NH$_4^+$ concentration (F). Nitrogen fertilization levels are presented on the X-axis and OM type is indicated by symbol (circle = unamended, triangle = plant litter, square = compost). Letters designate significant differences as determined by two-way ANOVA and Tukey’s HSD post hoc (α = 0.05) wherein capital letters denote main effects of OM type (A-C) and N fertilization (X-Z), in the case of an interaction between these factors a (*) is in the upper left corner of the graph and lower case letters denote differences.
Fig. 3.2 Response of denitrifier (nirS) abundance (A) and potential denitrification rates (B) to experimental treatments (mean ± SE, n = 5 per point). Nitrogen fertilization levels are presented on the X-axis and OM type is indicated by symbol (circle = unamended, triangle = plant litter, square = compost). Letters designate significant differences as determined by two-way ANOVA and Tukey’s HSD post hoc (α = 0.05) wherein capital letters denote main effects of OM type (A-C) and N fertilization (X-Z), and in the case of an interaction between these factors a (*) is in the upper left corner of the graph and lower case letters denote differences.
Fig. 3.3 Principal coordinates analysis of denitrifier (nirS) community composition in response to treatment. OM type is indicated by symbol (circle = unamended, triangle = plant litter, square = compost) and N fertilization is indicated by color (white = 0 (no addition), grey = 0.8, black = 4 mg N g⁻¹ wet soil).
Fig. 3.4 Principal coordinates analysis of denitrifier (*nirS*) community composition in response to treatment over time: July (unmarked), October (-), and January (*). OM type is indicated by symbol (circle = unamended, triangle = plant litter, square = compost) and N fertilization is indicated by (white = 0 (no addition), grey = 0.8, black = 4 mg N g⁻¹ wet soil).
Fig. 3.5 Path diagram displaying the full (A) and reduced (B) models of the role of soil OM(%), NO$_3^-$ ($\log_{10}$ mg L$^{-1}$ NO$_3^-$ - N), denitrifier community structure (PCoA Axes 1 and 2), and abundance ($nirS$ log10 copies g$^{-1}$ dry soil) in regulating potential denitrification (pDNF) rates ($\log_{10}$ pmoles g$^{-1}$ dry soil h$^{-1}$). Single headed arrows represent unidirectional causal relationships, and double headed arrows represent non-causal covariances. The amount of variation that can be explained by the model is indicated by the $R^2$ values associated with the response variables. Similarly standardized path coefficients ($r$) are associated with each arrow and reflect the strength of each relationship (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).
CHAPTER FOUR

USING MICROBIAL COMMUNITIES AND EXTRACELLULAR ENZYMES TO LINK SOIL ORGANIC MATTER CHARACTERISTICS TO GREENHOUSE GAS PRODUCTION IN TIDAL FRESHWATER WETLANDS

by

Ember M. Morrissey, David J. Berrier, Scott C. Neubauer, and Rima B. Franklin
Department of Biology, Virginia Commonwealth University, Richmond, VA 23284 USA

Published:
Biogeochemistry
(2013)
Abstract

To gain a more mechanistic understanding of how soil organic matter (OM) characteristics can affect carbon (C) mineralization in tidal freshwater wetlands, we conducted a long-term *in situ* field manipulation of OM type and monitored associated changes in carbon dioxide (CO$_2$) and methane (CH$_4$) production. In addition, we characterized microbial community structure and quantified the activity of several extracellular enzymes (EEA) involved in the acquisition of carbon, nitrogen, and phosphorus. Treatments included a plant litter addition, prepared using naturally-senescent vegetation from the site, and a compost amendment, designed to increase the concentration of aged, partially humified, OM. Both types of OM-amended soils had CO$_2$ production rates 40-50% higher than unamended control soils, suggesting that the added OM had inherently higher quality and/or availability than the native soil OM. Rates of CO$_2$ production were not correlated with microbial community structure or EEA except a modest relationship with cellulose breakdown via the $K_m$ of β-1,4-glucosidase. We interpret this lack of correlation to be a consequence of high functional redundancy of microorganisms that are capable of producing CO$_2$. Rates of CH$_4$ production were also influenced by OM quality, increasing by an order of magnitude with plant litter additions relative to compost-amended and control soils. Unlike CO$_2$, rates of CH$_4$ production were significantly correlated with the microbial community structure and with enzyme kinetic parameters ($V_{max}$ and $K_m$) for both carbon (β-1,4-glucosidase, 1,4-β-cellobiosidase, and β-D-xylosidase) and nitrogen acquisition (leucyl aminopeptidase). The monophyletic nature of methanogenic archaea, combined with their reliance on a small select group of organic substrates produced via enzyme-mediated hydrolysis and subsequent bacterial fermentation, provides a basis for the strong links between microbial community structure, EEA, and CH$_4$ production. Our results suggest that
incorporating microbial community structure and EEA into conceptual models of wetland OM decomposition may enhance our mechanistic understanding of, and predictive capacity for, biogeochemical process rates.
Introduction

Though wetlands account for only ~10% of terrestrial land area (Zedler and Kercher 2005), their influence on the global carbon (C) cycle is disproportionately large. For example, wetland soils store 45-70% of terrestrial organic C (Mitra et al. 2005) and are responsible for nearly 25% of global methane (CH$_4$) emissions (Conrad 2009). Carbon sequestration is enhanced in these environments due to a combination of high primary productivity and slow rates of decomposition. The quality and availability of organic material (OM), as well as an interacting suite of environmental factors (e.g., soil moisture and pH), determine the degree of OM storage versus mineralization to carbon dioxide (CO$_2$) and/or CH$_4$ (Segers 1998; Megonigal et al. 2004; Kayranli et al. 2010). Understanding how OM properties affect the balance between sequestration and mineralization is particularly relevant in the context of wetland restoration and creation, as these activities often involve OM amendments to hasten the development of organic-rich reduced soils (Davis 1995; Mitsch and Gosselink 2000).

The OM transformations that result in the production of CH$_4$ and/or CO$_2$ are driven by diverse microbial communities that depend on extracellular enzyme activity (EEA) to breakdown complex organic polymers into soluble compounds that can be transported into the cell and metabolized. Consequently, this depolymerization is the putative rate-limiting step in OM decomposition (Sinsabaugh et al. 1991) and enzyme depolymerization rates have been associated with microbial respiration in aquatic ecosystems (Arnosti and Holmer 2003; Arnosti and Jørgensen 2006; Baltar et al. 2009). In soils however, surprisingly few studies have explored the relationship between EEA and either C mineralization rates (Freeman et al. 1997; Freeman et al. 2001; Allison and Vitousek 2005) or the composition of the associated microbial communities (Kourtev et al. 2003; Gallo et al. 2004; Costa et al. 2007; Kaiser et al. 2010). Even less
consideration has been given to how these three components interact. Though some studies have found a strong relationship between microbial community composition and activity (Cleveland et al. 2007; Li et al. 2011; Goberna et al. 2012; Lazar et al. 2012), many others have not (Galand et al. 2003; Liu et al. 2011; Fromin et al. 2012). One common hypothesis for this inconsistency is that the high functional redundancy within microbial communities limits our detection of community structure-function relationships, especially when considering processes with a relatively ubiquitous distribution among taxa (e.g., respiration to CO₂, Griffiths et al. 2000; Nannipieri et al. 2003). Some have proposed that community structure only regulates “narrowly” distributed functions (Schimel 1995; McGuire and Treseder 2010), i.e., ones that are performed by only a small group of organisms with specialized physiological pathways such as methanogens.

In this study, we examined how microbial community structure and EEA regulate greenhouse gas (CO₂ and CH₄) production in wetlands receiving long-term in situ soil amendments of either plant litter or compost. This research was conducted in a recently restored tidal freshwater wetland, and the results have implications for recovering ecosystem services facilitated by OM-rich soils in impaired wetlands while simultaneously minimizing the production of CH₄.

Methods

Experimental Design

In January 2011, soil (5-15 cm depth) was collected from the middle of a 30-ha tidal freshwater wetland at Virginia Commonwealth University’s Walter and Inger Rice Center for
Environmental Life Sciences (James River, Charles City County, Virginia; 37°20'05" N, 77°12'27" W). This wetland was an impounded lake for nearly 70 years before a storm breached the dam and restored natural wetland hydrology to the site in 2006. The site was continually saturated, usually with standing water on the surface, and dominated by obligate wetland vegetation such as *Leersia oryzoidea*, *Juncus effusus*, and *Nuphar luteum*. The soil had an OM content of 8%, a C:N ratio of 10 (by mass), a pH of 6.4, and soil texture was 30% sand, 55% silt, and 15% clay. Following extensive homogenization in the laboratory, one fraction of the collected soil was amended with plant litter (standing dead material of the above mentioned species harvested from the field site in early December 2010; 99% OM content, C:N=72). A second fraction was amended with compost (Black Kow, Oxford, FL; organic blend containing 26% OM, C:N=18). The compost and litter amendments were similar in particle size (0.1-5 mm in diameter). Amendments were added to raise the soil OM content to approximately double ambient levels. A third soil fraction was unamended and served as an experimental control.

Litter bags (12 cm × 22 cm), constructed of 0.5-mm Nitex mesh (Wildlife Supply Company, Buffalo, NY), were filled with 400 ml (~ 215 g dry weight) of control or amended soil for *in situ* incubation. In January 2011, ten bags of each type were buried (5-15 cm depth) at random locations within a single 20 × 20 m experimental plot near the soil collection site. Samples were incubated for either 6 or 18 months (until July 2011 and 2012, respectively). At each sampling event, five replicate bags of each type were collected, as were five intact field cores. Samples were placed in airtight plastic bags, quickly transported back to the laboratory, and subdivided for soil characterization (200 g) and molecular genetic analyses (5 g, immediately archived at -20°C). For the 18-month sampling, subsamples were also removed for determination of CO₂ and CH₄ production (40 g, stored for 7 days at 4°C) and analysis of
extracellular enzyme activities (10 g, stored up to 5 days at 4°C). The field cores were analyzed only for soil properties and gas production rates, and served to provide context for interpreting the experimental manipulations.

**Environmental Analyses**

At both the 6- and 18-month sampling events, soil redox potential and pH were measured using a Hanna Combo pH and ORP probe (QA Supplies, Norfolk, VA). Soil moisture (%) was determined gravimetrically (100 ± 5°C for 72 h), and OM (%) was measured as the mass loss on ignition following combustion at 500°C for 4 h. Total carbon and nitrogen contents were determined using a Perkin Elmer CHNS/O Analyzer (Waltham, MA) following grinding and acidification of samples using 10% hydrochloric acid.

In addition, at the 18-month sampling, porewater was extracted and analyzed for dissolved nutrient concentrations. Briefly, water was collected from 50-ml soil samples by centrifugation (3000 × g for 15 min), filtered using a 0.45 µm pore-size mixed cellulose ester syringe filter, and stored at -20°C until it could be analyzed for: (i) ammonium (NH$_4^+$) using the indophenol colorimetric assay of Grasshoff et al. (1983), (ii) dissolved organic carbon (DOC) using a Shimazdu TOC analyzer (Columbia, MD), (iii) total dissolved nitrogen (TDN) and phosphorus (TDP) using a Skalar Sans Plus System (Buford, GA).

**Molecular Analyses**

Whole-community DNA was extracted from 0.5-g subsamples of soil using the MoBio PowerSoil DNA Isolation Kit (Carlsbad, CA) and stored at -20°C. DNA purity and concentration were analyzed using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). All DNA
extracts and PCR products were verified using agarose gel (1.5 %) electrophoresis and ethidium bromide staining.

**Microbial Gene Abundance via qPCR**

Quantitative polymerase chain reaction (qPCR) assays were performed to assess the genetic potential of the microbial communities (Smith and Osborn 2009). Assays were performed using SsoAdvanced SYBR Green qPCR Supermix (BioRad, Hercules, CA) and a BioRad CFX 96 Real-Time System; data were analyzed using Bio-Rad CFX Manager Version 2.1. Results were reported as the log\(_{(10)}\) of the number of gene copies per g of OM after averaging three technical replicates per sample and comparing to appropriate standard curves.

To estimate total bacterial abundance, the primers Eub338 and Eub517 were used to target the 16S rRNA gene (Fierer et al. 2005). Genomic DNA from Escherichia coli (Strain 11775, ATCC, Manassas, VA) was used to establish the standard curve (average efficiency=101%, r\(^2\)=0.99). Reactions (20 μl) were performed with 1.2 ng DNA template and 0.1 μM concentrations of each primer; thermal cycling conditions were: 95°C for 4 min, and 40 cycles of 30 s at 95°C, 30 s at 55.5°C, and 60 s at 72°C. The abundance of archaea was estimated using the primers Arch 967F and Arch-1060R (Karlsson et al. 2012), again targeting the 16S rRNA gene. Standard curves (average efficiency=94%, r\(^2\)=0.99) used genomic DNA from Methanococcus voltae (Strain A3, ATCC). Reactions (20 μl) used 2 ng DNA template and 0.3 μM primers; thermal cycling conditions were: 95°C for 5 min, and 40 cycles of 20 s at 95°C, 20 s at 59°C, and 30 s at 72°C. Finally, methanogen abundance was estimated using the mlas and mcrA-rev primers to target the methyl coenzyme-M reductase encoding mcrA functional gene (Steinberg and Regan 2009). As with archaea, Methanococcus voltae genomic DNA was used
for the standard curve (average efficiency=92%, $r^2$=0.99). Reactions (20 μl) had 2 ng DNA template and 0.56 μM mlas and 0.70 μM mcrA-rev primer concentrations; thermal cycling conditions were: 95°C for 5 min, and 50 cycles of 20 s at 95°C, 20 s at 59°C, and 45 s at 72°C.

**Community Structure via T-RFLP**

Microbial community structure was analyzed using terminal restriction fragment length polymorphism (T-RFLP) targeting the 16S rRNA gene of bacteria and archaea and the functional gene *mcrA* for methanogens. All PCR reactions (50 μl) were performed with 10 mM TrisHCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 20 μg BSA, and 2.5 units of AmpliTaq DNA polymerase (reagents obtained from Applied Biosystems, Foster City, CA). Bacteria PCRs including the domain-specific primers 27F (labeled with FAM) and 1492R at a concentration of 0.2 μM each (Lane 1991), 3.0 μM MgCl₂, and 1.2 ng DNA. Thermal cycling conditions were: 95°C for 3 min, 30 cycles of 45 s at 95°C, 60 s at 57°C, 120 s at 72°C, followed by 72°C for 7 min (PTC-100 Thermal Controller, MJ Research, Waltham, MA). Archaea PCRs included the primers 21F (labeled with FAM) and 958R (Cytryn et al. 2000), each at 0.2 μM, as well as 1.5 μM MgCl₂ and 4 ng DNA. Thermal cycling conditions were: 94°C for 3 min, 34 cycles of 60 s at 95°C, 60 s at 55°C, 60 s at 72°C, followed by 72°C for 7 min. The methanogen *mcrA* gene was targeted with MLf (labeled with FAM) and MLr (Smith et al. 2007b) in a reaction with 60 nM of each primer, 2 μM MgCl₂, and 4-8 ng DNA. Touchdown PCR thermal cycling conditions were: 95°C for 3 min, 6 cycles of 45 s at 95°C, 60 s at 56°C (-0.5°C cycle⁻¹), 60 s at 72°C, followed by 31 cycles of 45 s at 95°C, 60 s at 53°C, 60 s at 72°C and final extension at 72°C for 7 min.

PCR products were purified using the MinElute 96 UF PCR purification kit (Qiagen, Valencia, CA) prior to restriction enzyme digest (associated materials from New England
Digests were conducted in 1X Buffer #4 with 30 ng BSA, using either 10 units of Hha1 (16S rRNA) or 20 units of RsaI (mcrA). After digestion (16 h at 37°C, 20 min at 65°C), amplicons were purified using the MinElute kit, recovered in molecular-grade water, and detected using capillary electrophoresis with a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Buckinghamshire, UK). An aliquot of 50-120 ng of purified, digested PCR product was combined with 0.3 µl of MapMaker 400 ROX ladder (Bioventures, Murfreesboro, TN) plus 4.75 µl injection buffer (0.1% Tween-20). Samples were injected at 3 kV for 100 s, and electrophoresed using genotyping filter set 1 for 100 min at 10 kV. T-RFLP fragments between 70-400 base pairs (bp) were analyzed using Fragment Profiler software (Version 1.2; Amersham Biosciences) using a 1 bp size differential and a 15 relative fluorescent unit peak height threshold. Samples were standardized by calculating peak area as a percent of the total sample fluorescence; peaks accounting for < 1% of total sample fluorescence were removed prior to analysis.

**Extracellular Enzyme Activity (EEA)**

Soil slurries were prepared fresh each day of analysis by sonicating 1.0 g soil in 100 ml sterile deionized water (15 W for 2 min; Misonix Sonicator 3000, Farmingdale, NY). The slurries were kept on a shaker table (150 rpm) during use to prevent settling of the soil particles. The activities of five hydrolytic enzymes were measured using fluorometric assays following modified protocols from Stursova et al. (2006) and German et al. (2011) with reagents obtained from Sigma-Aldrich Co. Ltd (Table 4.1). Three technical replicates of each sample were assayed at each of ten substrate concentrations, as were three negative (no sample) controls. For the methylumbelliferone (MUB) assays, MES buffer (0.1 M, pH 6.1) was used, and quench curves
were established for each sample using a range from 0-9 nmol MUB. For the amino-4-methylcoumarin hydrochloride (AMC) assay, a Trisma buffer was used (50 mM, pH 7.8) and quench curves ranged from 0-7.5 nmol AMC.

Plates were prepared by adding soil slurry (50 µl) first, followed by substrate, and then buffer sufficient to achieve a final volume of 200 µl per well. Plates were pre-incubated at 30°C for either 1 h (for CBH, LAP, and AP) or 4 h (for BG and BX), and then read for an additional 6 h using a Synergy 2 plate reader (Biotek, Winooski, VT) programmed for 360 nm excitation and 460 nm emission wavelengths and an incubation temperature of 30°C. Activity was calculated for each sample after fitting a regression of the fluorescent reading versus MUB or AMC concentration for the corresponding quench curve. Rates were determined as the change in MUB or AMC generated in each sample during the 6 h incubation in the plate reader (each plate was read a minimum of twelve times). Technical replicates were averaged, and maximum reaction velocity (V_max) and half-saturation constant (K_m) values were calculated on Sigma Plot Version 10 (San Jose, CA) using the Michaelis-Menten hyperbola function in the regression wizard.

Phenol oxidase (POX) activity was measured colorimetrically (Sinsabaugh et al. 2003). Three technical replicates containing soil slurry (50 µl), 50 mM sodium bicarbonate buffer (pH 6.1), and l-DOPA (6.5 mM) were performed for each sample, as were triplicate no-sample and no l-DOPA controls. The plates were incubated in the dark at 30°C for 30 min and then read on the Synergy 2 at 460 nm wavelength for 6 h at 30°C.

**Anaerobic CO₂ and CH₄ Production**

Production of CO₂ and CH₄ was measured using an anaerobic slurry assay (Neubauer et al. 2005). Briefly, homogenized soil samples (7.0 ± 0.2 g) were combined with 7 ml of filtered
(glass microfiber filter GF/C; Whatman Piscataway, NJ), deoxygenated porewater in a 125-ml serum bottle under anaerobic conditions using an N₂-filled glove bag. Two technical replicates were prepared for each sample, and slurries were pre-incubated overnight (~16 h at room temperature, 23°C). The next morning, the headspace in each bottle was thoroughly flushed with N₂ to initiate a two-day experimental incubation. Gas samples (5 ml) were obtained from the headspace at 0, 8, 22, 32, and 46 h by shaking the slurry briefly, injecting 5 ml of N₂, and immediately withdrawing 5 ml of gas. Measuring headspace gas concentrations will underestimate potential production rates to the extent that gases accumulate in the slurry liquid rather than in the headspace, although this will not affect the relative comparison between our treatments since experimental conditions (pH, salinity, volumes of liquid and headspace) were similar in all bottles. Concentrations of CO₂ were measured on a LI-COR LI-7000 infrared gas analyzer (Lincoln, NE), and CH₄ was measured on a Shimadzu GC-14A gas chromatograph with flame ionization detector. All samples showed a linear increase in gas concentration over time, and production rates (nmol CO₂ or CH₄ produced per g of OM per hour) were calculated using linear regression. Median correlation coefficients were 0.97 for CO₂ and 0.99 for CH₄.

Analytical precision was ± 0.87% for CO₂ and CH₄ (mean coefficient of variation for replicate injections of CO₂ and CH₄ standards).

**Statistical Analyses**

By design, our treatments differed in their OM content. To account for this in data analysis, we normalized all microbial abundance, enzyme activity, and gas production data per gram of OM; this allowed us to focus on the effect of OM type without the confounding effect of amount. Shapiro-Wilks tests confirmed the soil properties, gas production rates, and microbial
abundance data were normally distributed within each population making them appropriate for analysis using parametric techniques. Soil properties and gas production rates for field cores and control samples were compared using a Student’s t-test (n=5 per group, df=8). Effects of treatment (control, plant litter, or compost) on environmental variables, microbial abundance, EEA, and gas fluxes were analyzed using one-way analysis of variance (ANOVA; n=5 per group, df=14) with Tukey’s HSD for post hoc comparisons. Analyses were performed using the JMP statistical software (Version JMP Pro 9.0.2, Cary, NC; Sall 2005) with a 0.05 significance level.

T-RFLP results were analyzed using Principal Coordinates Analysis (PCoA) applied to the Bray-Curtis index of similarity derived from normalized fluorescence data; the first two axes from each analysis were plotted to visualize relative similarity in community structure across samples. Treatment effects were analyzed using a Non-Parametric Multivariate ANOVA (NP-MANOVA), again applied to the Bray-Curtis similarity index. All community analyses were conducted using the PAST Version 2.16 statistical package (Hammer 2001).

For the 18-month data (July 2012), correlation analysis was performed to examine the relationships among the environmental, microbial, enzyme, and gas production data. (SPSS Statistics Version 20, Armonk, NY). Multivariate normality was confirmed using Doornik and Hansen omnibus test in PAST prior to selecting Pearson’s coefficient.

Results

Controls versus Field Samples

After 6 months, the control samples did not differ significantly from field cores for any of the soil properties (pH, redox, OM, soil moisture, C:N; all |t| < 2.0 with p>0.05). In contrast, for
the 18-month sampling, t-tests revealed significant differences for all parameters except pH (for pH: t=0.8 and p=0.45; for all others, |t| > 2.5, all p<0.05). Though the magnitude of the differences were small, redox (mV, mean ± S.E.; Control: -128 ± 13, Field: -83 ± 9), soil moisture (%; Control: 52 ± 1, Field: 60 ± 1), and OM (%; Control: 7.8 ± 0.2, Field: 9.7 ± 0.3) were all lower in the control samples; C:N was slightly higher (Control: 9.6 ± 0.2, Field: 8.4 ± 0.1). Gas flux rates (nmol g-OM\(^{-1}\) h\(^{-1}\)) were also measured for the 18-month sampling event, and no significant differences were observed for CO\(_2\) (Control: 329.8 ± 50.4, Field: 369.8 ± 120.0), CH\(_4\) (Control: 5.1 ± 0.8, Field: 40.9 ± 39.9), or total C gas production (Control: 335.0 ± 50.9, Field: 410.8 ±157.5; all t<1.0, p>0.30).

**Effects of Organic Matter Manipulation**

**Environmental Analyses**

Addition of plant litter and compost increased soil OM relative to the controls; these differences persisted throughout the study (Fig. 4.1A, Table 4.2). After 6 months, OM content in the plant litter (14.5%) and compost treatments (13.7%) was similar to the levels at the start of the study (averaged across both treatments: 14.0 ± 1.4%). However, after 18 months, average OM for these treatments decreased (Litter: 10.0%, Compost: 11.9%), but was still significantly higher than the unamended control (7.8%).

The compost and litter amendments also affected soil C:N, which was always lower in the controls (averaged by date: 9.5 ± 0.1) relative to the experimental treatments (Fig. 4.1B). At the 6-month sampling, average C:N was higher in the plant litter treatment (11.8) than in the compost treatment (10.6), reflecting a substantial decrease from the start of the study (Litter:
24.4 ± 2.4, Compost: 13.9 ± 0.2). These differences disappeared after 18 months of incubation (combined average for both treatments: 10.5 ± 0.2).

For all treatments at all times, redox potential was negative (Fig. 4.1C). After 6 months of incubation, there were modest differences between the plant litter (-54 mV) and compost treatments (-105 mV), but neither was significantly different from the control (-82 mV). After an additional year of incubation, there were no significant differences between any treatments (combined average across treatments: -135 ±6.4), though values were generally more negative than at 6-months.

Soil moisture was consistently lower in the compost-amended soils (%; 6-months: 47.4 ± 0.5, 18-month: 50 ± 1.2) relative to the plant litter (6-months: 56 ± 1.1, 18-month: 52.1 ± 0.9) and control soils (6-months: 55.0 ± 2.1, 18-month: 57.1± 2.0) (Table 4.2). For pH, there were significant treatment effects at the 6-month sampling event only; pH was higher in the compost (6.1± 0.1) compared to the control (5.7 ± 0.1) or plant litter amendment (5.6 ± 0.1). There were no significance differences in porewater chemistry for any of the parameters (mg L⁻¹, mean ± S.E.; DOC: 5.9 ± 0.9, TDN: 0.50 ± 0.08, TDP: 0.06 ± 0.01, NH₄⁺: 0.18 ± 0.04).

**Microbial Abundance and Community Structure**

For all three groups, abundance was lowest in the compost-amended soils, where it changed little over time (gene copies g-OM⁻¹; averaged across both sampling events for Bacteria: 17.9 × 10⁹, Archaea: 1.4 × 10⁹, Methanogens: 1.2 × 10⁹ (Fig. 4.1 D-F)). Bacterial abundance was ~5-fold higher in the control and litter-added soils for the 6-month sampling, and not significantly different at the 18-month sampling. For both times, archaea abundance in the control and litter-added soils was similar and ~3-fold higher than in the compost. For
methanogens, significant differences were detected across all treatments for the 6-month sampling (gene copies g-OM$^{-1}$; Control: $4.3 \times 10^9$, Litter: $12.9 \times 10^9$, Compost: $1.6 \times 10^9$).

Abundance was slightly lower for all three treatments at the 18-month sampling, and the control and litter-added soils were no longer different (gene copies g-OM$^{-1}$; Control and Litter: $2.2 \times 10^9$, Compost: $0.9 \times 10^9$).

Treatment effects on microbial community structure were visualized using PCoA (Fig. 4.2 A-C) and statistical significance was evaluated using NP-MANOVA (Table 4.2). For all three groups and both sampling events, community structure in the plant litter treatment was significantly different from that in the control and the compost addition. Generally, the compost treatment did not significantly alter microbial community structure relative to the control; the only exception is for the archaea community at the 18-month sampling.

*Extracellular Enzyme Analysis*

Treatment effects varied depending on the substrate tested and the kinetic parameter of interest (Table 4.2, Fig. 4.3 and 3.4). In general, the addition of plant litter corresponded to an increased $V_{\text{max}}$ relative to the control, which was statistically significant for BG (200% higher), BX (550%), and LAP (50%). Conversely, the addition of compost suppressed $V_{\text{max}}$ relative to the control, though the trend was only statistically significant for AP (~25% decrease). For $K_m$, fewer treatment effects were observed, and there were never any differences in $K_m$ between the control and compost soils. In the presence of plant litter, $K_m$ was significantly lower for CBH (by 75% vs. control) and LAP (by 15%). The activity of POX was reduced by 10% (relative to controls) in the litter-amended soils and by roughly 40% when compost was added.
Anaerobic CO$_2$ and CH$_4$ Production

Potential rates of CH$_4$ and/or CO$_2$ production in anaerobic slurries increased in response to the OM additions (Table 4.2, Fig. 4.5). The rate of CH$_4$ production did not change significantly for compost but increased ~10-fold for plant litter. Rates of CO$_2$ production in the compost and plant litter soils were 40-50% higher than rates in the controls, with no significant differences in CO$_2$ production between the two. Relative to the controls, total C gas production (CO$_2$ + CH$_4$) increased by 40% in the compost treatment and by 70% in the plant litter treatment (Fig. 4.5C). Methane accounted for ~2% of the total C gas production in the control and compost treatments, but that fraction increased to ~15% in the treatment with the addition of plant litter (Fig. 4.5D).

Correlation Analysis

When C gas production rates were correlated with the environmental variables, only the relationship between soil C:N and CO$_2$ (r=0.61, p=0.01) was significant (other results not presented; all r<0.50, p>0.08). Similarly, microbial abundance did not show a strong relationship to gas production, except for the modest positive correlation of methanogen abundance and CH$_4$ rates (Table 4.3). To examine how microbial community structure and gas production were linked, correlation analysis was performed using the scores from each PCoA. In each case, the first axis describing bacterial, archaeal, and methanogen community structure was strongly correlated with CH$_4$ production (Table 4.3), and no significant correlations were obtained for any of the second axes (not presented; all r<0.39 and p>0.15). For EEA, CH$_4$ production was significantly positively correlated with $V_{\text{max}}$ for BG, CBH, BX and LAP, and negatively
correlated with $K_m$ for CBH and LAP (Table 4.3). The only significant correlation with CO$_2$ production was for $K_m$ of BG ($r$=-0.60, $p=0.02$).

Several aspects of the microbial community were correlated with one another, including a significant relationship between the abundance of all three microbial groups (Table 4.3). Similarly, bacterial, archaeal, and methanogen community composition were correlated when each PCoA 1 was considered. Significant correlations were found between microbial community composition and EEA for $V_{max}$ for BG, BX and LAP, and with $K_m$ for CBH and LAP. In general, correlations were highest between EEA and bacterial or archaeal community composition, and were significant less often for methanogens.

**Discussion**

The loss of natural wetland ecosystems is often mitigated by construction or restoration of wetlands elsewhere in the watershed (EPA, 2008). As a means of improving soil quality and promoting plant productivity, OM amendments are regularly included in these mitigation projects (Davis 1995; Mitsch and Gosselink 2000). Our study complements other research that has examined how this practice affects soil characteristics, redox gradients, vegetation, and nutrient cycling (O’Brien and Zedler 2006; Bruland et al. 2009; Sutton-Grier et al. 2009), and demonstrates that amendments can alter rates of C mineralization and induce shifts in microbial community structure and function. Furthermore, because our manipulation produced long-term changes in soil OM and C:N with relatively limited effects on other soil parameters (e.g., redox, pH, and soil moisture), we were able to isolate how OM characteristics can affect C biogeochemistry and identify key microbial drivers and feedbacks to the multi-stage process of
decomposition. The similarities between measurements on control soils and intact field cores suggest that the results remain applicable to unaltered wetlands soils.

The amendments used in this study differ considerably in their biochemical composition. Compost, although derived from plant materials, undergoes a humification process that yields chemically-complex OM with few residual plant polymers and increased microbial necromass (Tiquia et al. 1996; Tuomela et al. 2000). Compost may be similar to native soil OM, which is also considered to have a significant portion of C of microbial origin (Simpson et al. 2007; Liang and Balser 2010; Throckmorton et al. 2012). In general, microbial necromass contains a larger fraction of proteins and lipids than does plant litter, and has only a small fraction of the carbohydrates and lignins dominant in plant litter (Nelson and Baldock 2005; Simpson et al. 2007; Throckmorton et al. 2012). Thus, while nutrient availability may have varied between the control and compost-amended soils, the chemical composition of the OM was probably more similar between these treatments and distinct from that in the litter-amended soil (that is, microbially-dominated vs. plant-dominated).

**Treatment Effects and Carbon Gas Production**

Overall, we found that both the plant litter and compost-added treatments exhibited higher potential rates of anaerobic C gas production relative to unamended soils on a per gram-OM basis (Fig. 4.5), which indicates that a greater fraction of the OM was mineralized compared to the control soil. One explanation for this result is that the OM in the plant litter and compost amendments was more labile than the existing soil OM. Additional factors at play include the potential for enhanced decomposition of native material through “priming” (Blagodatskaya and Kuzyakov 2008; Nottingham et al. 2009) or the possibility that some of the native soil OM was
physically inaccessible (e.g., via sorption onto mineral surfaces), which could limit
decomposition regardless of inherent lability (Kalbitz et al. 2000).

We did not identify any significant relationships between either soil environmental
conditions or porewater chemistry and any of the following: microbial community composition,
abundance, EEA, or C gas production, excepting a small positive correlation between C:N and
CO₂. Given the narrow range of C:N for our treatment soils (Fig. 4.1B), it is likely that C:N is
not the driver of decomposition rates \textit{per se} but instead a co-variant associated with finer-scale
OM characteristics such as the degree of humification or OM lability as discussed above. The
general lack of correlation between environmental variables and either the soil microbial
community or biogeochemical response metrics suggests environmental conditions were not
major divers of the observed treatment effects. It further supports our assertion that differences
in C gas production were the result of microbial responses to OM type and not an unintended
consequence of treatment on abiotic soil characteristics.

\textit{Role of Microbial Communities}

\textit{Extracellular Enzyme Activity}

Microorganisms can detect substrates in their environment and regulate enzyme
production accordingly to balance resource needs with metabolic costs (Bhat and Bhat 1997;
Shackle et al. 2000; Allison and Vitousek 2005; Allison et al. 2011; Shi 2011). In this study, we
found considerable evidence that OM type (i.e., polymer availability) can influence EEA. For
example, in the plant litter treatment, enzymes that target compounds abundant in plant litter
(e.g., cellulose and hemicellulose) were elevated. This response was observed for $V_{\text{max}}$ of all of
the C and N hydrolytic enzymes we measured, and was statistically significant for BG, BX, and
LAP (Fig. 4.3 and Table 4.2). Microbial adjustments of EEA are also evident in the compost treatment, where the reduced $V_{\text{max}}$ of AP was likely a response to decreased P limitation. The compost we used contained 0.2% P, and microorganisms generally produce fewer acquisition enzymes for nutrients that are readily available (Sinsabaugh and Moorhead 1994; Allison and Vitousek 2005). Similarly, POX decreased approximately 50% following compost addition, likely due to lower lignin content in compost (Sinsabaugh 2010).

Our results suggest that this variation in EEA across OM types may be due, at least in part, to changes in microbial community structure. Specifically, the decrease in $K_m$ associated with CBH and LAP in the plant litter treatment indicates the synthesis of isoenzymes with higher substrate affinity, which reflects more efficient allocation of resources (Marx et al. 2005; Stone et al. 2012). Although multiple isoenzymes are known to occur within an individual organism (Esser et al. 2013), shifts in isoenzymes are also consistent with changes in microbial community composition (Farrell et al. 1994; Martinez et al. 1996; Tabatabai et al. 2002). The significant correlations between $K_m$ of CBH and LAP with bacteria PCoA 1 and archaea PCoA 1 (Table 4.3) further support our conceptual model that community structure can influence enzyme activity (Fig. 4.6). We anticipate that most of the EEA in our soils is of bacterial origin, as bacterial abundance was ~10-fold greater than that of archaea (average bacteria:archaea ratios 16S rRNA gene ratio at the 18 month sampling, with a range from 4 to 21). Although enzyme parameters were correlated with archaea in our study (Table 4.3), and archaea have been demonstrated to produce extracellular enzymes in marine sediments (Lloyd et al. 2013), we suggest that these groups were not significant producers of enzymes in our system. This assertion is based on the fact that most of our archaea were likely methanogens (average archaea 16S rRNA:methanogen mcrA ratio was 1.3, with a range from 1.0 to 1.7), and methanogens
exclusively use fermentation products and CO₂ for as their carbon source (Thauer et al. 2008, Reddy and De Laune 2008). Thus, it is unlikely they would expend resources to produce enzymes for carbon polymer breakdown (e.g., BG, CBH, BX, and POX) to liberate products they cannot directly utilize. Instead, we propose that the correlations we observed between EEA and archaea/methanogens are indirect based on methanogen consumption of fermentation productions affecting upstream pathways of organic carbon breakdown (Fig. 4.6).

Microbial Community Structure

We demonstrated that the plant litter amendment supported a distinct microbial community compared to the control and compost-added soils (Fig. 4.2A-C), and propose these differences developed in response to C substrate availability as mediated by EEA (Fig. 4.6). The initial mechanism for this OM effect is selection for a distinct set of heterotrophs capable of directly metabolizing the unique oligomers and monomers generated from EEA on plant litter. Given the current knowledge on wetland soil microbiology (Reddy and DeLaune 2008; Wüst et al. 2009), we anticipate a large fraction of these organisms are fermentative bacteria. Fermentation generates acetate and other simple organic acids that support methanogens, the main archaea in our system. Thus we hypothesize the plant litter addition directly affected bacterial community structure (similar to Nemergut et al. 2010), and resulted in greater availability and altered composition of fermentation end products (e.g., acetate vs. propionate, Uz and Ogram 2006). Then, because many methanogen genera can use only a specific subset of fermentation products (Garcia et al. 2000), this altered substrate availability was the indirect mechanism for the observed change in community structure of methanogens (and archaea).
These changes in community structure have the potential to impact C mineralization rates and the balance of CO\(_2\) and CH\(_4\) production.

**Microbial Regulation of C Gas Production**

If polymer breakdown is the rate-limiting step in decomposition, there should be a positive correlation between EEA \(V_{\text{max}}\) and C mineralization (e.g., Schimel and Weintraub 2003). In this study, no such relationships were observed for the CO\(_2\) production rates (Table 4.3). This may be partly due to the particular suite of enzymes we considered. Although commonly tested in soils, BG, BX, and CBH are fairly selective for plant polymers, and thus may not be as responsive to the availability of microbial necromass or humified material, which potentially dominated our control and compost treatments. Nonetheless, our results are consistent with the work of Freeman et al. (1997; 1998), who similarly found that BG activity did not correlate with CO\(_2\) production in wetland soils. The production of CO\(_2\) was also unrelated to microbial community structure, similar to the work of Bell et al. (2005) and Fromin et al. (2012). This may be because CO\(_2\) is generated by a plethora of microbial species with diverse metabolic strategies, creating considerable functional redundancy in natural communities (Botton et al. 2006; Griffiths et al. 2000).

In contrast to CO\(_2\), we did observe strong relationships between CH\(_4\) production, microbial community composition, and EEA, which we hypothesize are mediated through OM and bacterial community effects on the abundance, composition, and activity of methanogens (Fig. 4.6). Because methanogenesis is a fairly well-conserved function, performed by a monophyletic group of organisms (Garcia et al. 2000) that can utilize a limited range of organic substrates, there is relatively low functional redundancy associated with CH\(_4\) production and the
contribution of individual species to overall ecosystem function should be more important (Allison and Martiny 2008; McGuire and Treseder 2010). We were able to identify two terminal restriction fragments (T-RF) in our data associated with the genus Methanosarcinales (after Smith et al. 2007b) and found their relative abundance was positively correlated with CH$_4$ production (Spearman correlation; T-RF 95 bp: r=0.62, p=0.01; T-RF 179 bp: r=0.56, p=0.02). These results suggest that specific taxa of methanogens may be strong drivers of CH$_4$ production and are consistent with several other recent studies (Beckmann et al. 2011; Angel et al. 2012; Parkes et al. 2012). Additional research into the ecological and physiological attributes of these community members could further enhance our understanding of ecosystem-scale methane dynamics.

**Conclusions**

Our results have been used in conjunction with current knowledge on wetland decomposition to develop a conceptual model that incorporates microbial community structure and EEA to expand our understanding of CO$_2$ and CH$_4$ production rates (Fig. 4.6). Models such as this may be particular helpful in understanding methanogenesis, since rates of CH$_4$ production were strongly correlated with microbial community structure and multiple enzyme kinetic parameters. Relationships of enzyme activity and microbial community composition with CO$_2$ production were considerably more tenuous. This may be a consequence of the numerous microorganisms, substrates, and metabolic pathways associated with anaerobic CO$_2$ production (see Megonigal et al. 2004).

This work has direct implications for wetland restoration as plant litter and compost produced disparate changes in C gas production. Both OM sources increased total rates of
anaerobic C mineralization relative to unamended soils, but only the plant litter additions increased rates of CH$_4$ production (by roughly an order of magnitude). Similar results have also been reported for rice paddy soils (Singh et al. 2009; Ruirui et al. 2011), suggesting that the incorporation of highly decomposed OM amendments such as compost may help with wetland restoration (Stauffer and Brooks 1997; Sutton-Grier et al. 2009) while minimizing production of the greenhouse gas CH$_4$.

**Acknowledgements**

This research was funded by the VCU Rice Center for Environmental Life Sciences Student Research Award to E.M. Morrissey. Thanks to Joseph Morina, Jaimie Gillespie, Chansotheary Dang, Joseph Battistelli, Aaron Porter, Rana Mehr, and Olivia De Meo for laboratory and field help. Sincere thanks to the anonymous reviewers whose feedback greatly enhanced this manuscript. This paper is contribution #38 from the VCU Rice Center for Environmental Life Sciences and contribution #1677 from the University of South Carolina’s University of South Carolina’s Belle W. Baruch Institute for Marine and Coastal Sciences.
Table 4.1 Summary of enzymes assays used in this study, their natural substrates and products, as well as artificial substrates and concentration ranges used for determination of enzyme kinetics. The phenol oxidase assay was colorimetric and measured reaction velocity at only one substrate concentration.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Abbreviation</th>
<th>Target Molecule → Product</th>
<th>Artificial Substrate¹(Sigma Aldrich #)</th>
<th>Enzyme Commission #</th>
<th>Assay Conc. (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,4-glucosidase</td>
<td>BG</td>
<td>Cellulose → Glucose</td>
<td>4-MUB-β-D-glucopyranoside (M3633)</td>
<td>3.2.1.21</td>
<td>2-800</td>
</tr>
<tr>
<td>1,4- β-cellobiosidase</td>
<td>CBH</td>
<td>Cellulose → Disaccharide</td>
<td>4-MUB-β-D-cellobioside (M6018)</td>
<td>3.2.1.91</td>
<td>2-800</td>
</tr>
<tr>
<td>β-D-xylosidase</td>
<td>BX</td>
<td>Hemicellulose → Xylose</td>
<td>4-MUB-β-D-xylopyranoside (M7008)</td>
<td>3.2.1.37</td>
<td>2-800</td>
</tr>
<tr>
<td>Leucyl aminopeptidase</td>
<td>LAP</td>
<td>Polypeptides → Leucine</td>
<td>L-leucine-7-AMC (L2145)</td>
<td>3.4.11.1</td>
<td>1-600</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>AP</td>
<td>Phospho-monoesters → Phosphate</td>
<td>4-MUB-phosphate (M8883)</td>
<td>3.1.3.1</td>
<td>1-600</td>
</tr>
<tr>
<td>Phenol oxidase</td>
<td>POX</td>
<td>Lignin → Oxidized lignin</td>
<td>3,4-dihydroxy-L-phenylalanine (D9628)</td>
<td>1.10.3.2</td>
<td>6500</td>
</tr>
</tbody>
</table>

¹ MUB = methylumbelliferone; AMC = amido-4-methylcoumarin hydrochloride
Table 4.2 Statistical results evaluating treatment effects. Analysis of microbial community structure was performed using NP-MANOVA; all other parameters were analyzed using ANOVA.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>July 2011 (6 months)</th>
<th>July 2012 (18 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Environmental variables</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil pH</td>
<td>9.1</td>
<td>0.004 *</td>
</tr>
<tr>
<td>Redox</td>
<td>6.8</td>
<td>0.01 *</td>
</tr>
<tr>
<td>Moisture</td>
<td>11.8</td>
<td>0.001 *</td>
</tr>
<tr>
<td>OM</td>
<td>50.5</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>C:N</td>
<td>16.1</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Porewater</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TDN</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TDP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Microbial community</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abundance †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>30.5</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Archaea</td>
<td>12.7</td>
<td>0.01 *</td>
</tr>
<tr>
<td>Methanogens</td>
<td>21.5</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Structure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria</td>
<td>6.3</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Archaea</td>
<td>8.2</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Methanogens</td>
<td>3.2</td>
<td>&lt; 0.001 *</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V POX</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$V_{max}$ BG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BX</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$K_m$ BG</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CBH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BX</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LAP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gas production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO$_2$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CH$_4$</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fraction CH$_4$</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

† Measures were log$_{10}$ transformed prior to analysis.
* Statistically significant with $\alpha = 0.05$
- No data available
### Table 4.3 (page 1 of 2). Pearson’s correlations coefficients for (r, above and bolded) and p-values (below) associated with the comparison of gas production rates, microbial community attributes, and enzymatic variables at the July 2012 (18-month) sampling.

<table>
<thead>
<tr>
<th>Category</th>
<th>Parameter</th>
<th>Gas</th>
<th>Abundance</th>
<th>Community Composition $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CH$_4$</td>
<td>CO$_2$</td>
<td>Bacteria</td>
</tr>
<tr>
<td>Gas</td>
<td>CH$_4$</td>
<td>-</td>
<td>0.42</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>CO$_2$</td>
<td>0.11</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td>Abundance</td>
<td>Bacteria</td>
<td>0.37</td>
<td>0.54</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Archaea</td>
<td>0.14</td>
<td>0.84</td>
<td>0.03 $^*$</td>
</tr>
<tr>
<td></td>
<td>Methanogens</td>
<td>0.03 $^*$</td>
<td>0.94</td>
<td>0.01 $^{**}$</td>
</tr>
<tr>
<td>Composition $^1$</td>
<td>Bacteria</td>
<td>&lt;0.01 $^{**}$</td>
<td>0.21</td>
<td>0.03 $^*$</td>
</tr>
<tr>
<td></td>
<td>Archaea</td>
<td>&lt;0.01 $^{**}$</td>
<td>0.06</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Meth</td>
<td>&lt;0.01 $^{**}$</td>
<td>0.46</td>
<td>0.34</td>
</tr>
<tr>
<td>Enzymes</td>
<td>POX $^2$</td>
<td>0.53</td>
<td>0.17</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>BG $V_{max}$</td>
<td>0.01 $^{**}$</td>
<td>0.19</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>CBH $V_{max}$</td>
<td>0.04 $^*$</td>
<td>0.79</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>BX $V_{max}$</td>
<td>&lt;0.01 $^{**}$</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>LAP $V_{max}$</td>
<td>&lt;0.01 $^{**}$</td>
<td>0.42</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>AP $V_{max}$</td>
<td>0.86</td>
<td>0.17</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>BG $K_m$</td>
<td>0.42</td>
<td>0.02 $^*$</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>CBH $K_m$</td>
<td>0.01 $^{**}$</td>
<td>0.46</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>BX $K_m$</td>
<td>0.20</td>
<td>0.77</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>LAP $K_m$</td>
<td>0.01 $^{**}$</td>
<td>0.27</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>AP $K_m$</td>
<td>0.18</td>
<td>0.31</td>
<td>0.58</td>
</tr>
</tbody>
</table>

$^1$ Correlation reported for PCoA 1; no significant results obtained for PCoA 2 (all p $\geq$ 0.10)

$^2$ POX reaction velocity only recorded at one substrate concentration, as described in the methods.

* Statistically significant with 0.01 < p $\leq$ 0.05

** Statistically significant with p $\leq$ 0.01
Table 4.3 (page 2 of 2). Pearson’s correlations coefficients for (r, above and bolded) and p-values (below) associated with the comparison of gas production rates, microbial community attributes, and enzymatic variables at the July 2012 (18-month) sampling.

<table>
<thead>
<tr>
<th>Category (cont’d)</th>
<th>Parameter (cont’d)</th>
<th>Enzyme Activity</th>
<th>K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>POX ( \text{V_{max}} )</strong></td>
<td></td>
</tr>
<tr>
<td>Gas</td>
<td>CH_4</td>
<td>0.18</td>
<td>0.66 **</td>
</tr>
<tr>
<td></td>
<td>CO_2</td>
<td>-0.37</td>
<td>0.36</td>
</tr>
<tr>
<td>Abundance</td>
<td>Bacteria</td>
<td>0.16</td>
<td>0.28</td>
</tr>
<tr>
<td></td>
<td>Archaea</td>
<td>0.75 **</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Methanogens</td>
<td>0.67 **</td>
<td>0.48</td>
</tr>
<tr>
<td>Composition ¹</td>
<td>Bacteria</td>
<td>-0.22</td>
<td>-0.58 *</td>
</tr>
<tr>
<td></td>
<td>Archaea</td>
<td>0.06</td>
<td>0.64 **</td>
</tr>
<tr>
<td></td>
<td>Meth.</td>
<td>-0.38</td>
<td>-0.37</td>
</tr>
<tr>
<td>Enzymes</td>
<td>POX ²</td>
<td>-</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>BG     ( \text{V_{max}} )</td>
<td>0.49</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CBH    ( \text{V_{max}} )</td>
<td>0.16</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>BX     ( \text{V_{max}} )</td>
<td>0.38</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
<td></td>
<td>LAP    ( \text{V_{max}} )</td>
<td>0.04 *</td>
<td>&lt;0.01 **</td>
</tr>
<tr>
<td></td>
<td>AP     ( \text{V_{max}} )</td>
<td>0.01 **</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>BG     ( K_m )</td>
<td>0.84</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>CBH    ( K_m )</td>
<td>0.47</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>BX     ( K_m )</td>
<td>0.19</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>LAP    ( K_m )</td>
<td>0.07</td>
<td>0.05 *</td>
</tr>
<tr>
<td></td>
<td>AP     ( K_m )</td>
<td>0.47</td>
<td>0.01 **</td>
</tr>
</tbody>
</table>

¹ Correlation reported for PCoA 1; no significant results obtained for PCoA 2 (all p ≥ 0.10)
² POX reaction velocity only recorded at one substrate concentration, as described in the methods.
* Statistically significant with 0.01 < p ≤ 0.05
** Statistically significant with p ≤ 0.01
Fig. 4.1 Treatment effects on environmental parameters (A, B, C) and microbial abundance (D, E, F) following six (July 2011) and eighteen (July 2012) months of in situ field incubation. Treatment effects were evaluated using one-way ANOVA within each sampling event; data are presented as means ± S.E, n=5 per group. For all the graphs, lowercase letters denote statistically significant subgroups within the 6-month data set; letters with ‘ were used for the 18-month data. Treatments are color-coded and the symbols for the six month data are distinguished with a (+).
Fig. 4.2 Treatment effects on microbial community structure following six (July 2011) and eighteen (July 2012) months of \textit{in situ} field incubation. Patterns in microbial community structure were visualized using PCoA and statistical significance was evaluated by NP-MANOVA. Data are presented as means ± S.E, n=5 per group, and the percent of variance explained by each axis is provided. Statistically significant subgroups are designated using lowercase letters for the 6-month data set, and letters with a ’ were used for the 18-month data. Treatments are color-coded and the six month data are distinguished with a (+) on the symbol.
Fig. 4.3 Effects of treatment on enzyme kinetics, evaluated after eighteen months of *in situ* field incubation (mean ± 1 S.E., n=5 each; sampled July 2012): (A) maximum reaction velocity ($V_{max}$) and (B) half-saturation constant ($K_m$). Lowercase letters denote significant differences as determined via one-way ANOVA and Tukey’s HSD.
Fig. 4.4 Phenol oxidase (POX) activity as affected by treatment, evaluated after eighteen months of *in situ* field incubation (mean ± 1 S.E., n=5 each; sampled July 2012). Lowercase letters denote significant differences as determined via one-way ANOVA and Tukey’s HSD.
Fig. 4.5 Treatment effects on the production of CH$_4$ (A), CO$_2$ (B) and total C gas (C), as well as the fraction of total C gas that is CH$_4$ (D), evaluated after eighteen months of *in situ* field incubation measured in anaerobic slurries (mean ± 1 S.E., n=5 each; sampled July 2012). Lowercase letters denote significant differences as determined via one-way ANOVA and Tukey’s HSD.
Fig 4.6 Conceptual model diagramming the hypothesized role of microbial community structure and extracellular enzyme activity in wetland organic matter decomposition. Microbially mediated flows of carbon are represented as thick arrows beginning with polymers and concluding with the terminal decomposition end products CO$_2$ and CH$_4$ (after Megonigal et al. 2004). Interactions between microbial structure and carbon pools/flows are designated as supported, not supported, or not tested in the current study.
CHAPTER FIVE

SALINITY AFFECTS MICROBIAL ACTIVITY AND SOIL ORGANIC MATTER CONTENT IN TIDAL WETLANDS

by

Ember M. Morrissey, Jaimie L. Gillespie, Joseph C. Morina, and Rima B. Franklin
Department of Biology, Virginia Commonwealth University, Richmond, VA 23284 USA

Published:
Global Change Biology
DOI: 10.1111/gcb.12431
(2013)
Abstract

Climate-change associated sea level rise is expected to cause saltwater intrusion into many historically freshwater ecosystems. Of particular concern are tidal freshwater wetlands, which perform several important ecological functions including carbon sequestration. In order to predict the impact of saltwater intrusion in these environments, we must first gain a better understanding of how salinity regulates decomposition in natural systems. This study sampled eight tidal wetlands ranging from freshwater to oligohaline (0-2 ppt) in four rivers near the Chesapeake Bay (Virginia). To help isolate salinity effects, sites were selected to be highly similar in terms of plant community composition and tidal influence. Overall, salinity was found to be strongly negatively correlated to soil organic matter content (OM%) and C:N, but unrelated to the other studied environmental parameters (pH, redox, and above- and below-ground plant biomass). Partial correlation analysis, controlling for these environmental covariates, supported direct effects of salinity on the activity of carbon-degrading extracellular enzymes (β-1,4-glucosidase, 1,4-β-cellobiosidase, β-D-xylosidase, and phenol oxidase) as well as alkaline phosphatase, using a per unit OM basis. Since enzyme activity is the putative rate-limiting step in decomposition, enhanced activity due to salinity increases could dramatically affect soil OM accumulation. Salinity was also found to be positively related to bacterial abundance (qPCR of the 16S rRNA gene) and tightly linked with community composition (T-RFLP). Further, strong relationships were found between bacterial abundance and/or composition with the activity of specific enzymes (1,4-β-cellobiosidase, arylsulfatase, alkaline phosphatase, and phenol oxidase) suggesting salinity’s impact on decomposition could be due, at least in part, to its effect on the bacterial community. Together, these results indicate that salinity increases microbial decomposition rates in low salinity wetlands, and suggests that these ecosystems may experience decreased soil OM accumulation, accretion, and carbon sequestration rates even with modest levels of saltwater intrusion.
Introduction

Climate change is predicted to alter the global hydrological cycle in many ways. For example, rising sea levels (Nakada and Inoue 2005, Wigley 2005, Church and White 2006), reduced precipitation in watersheds (Smith et al. 2005) with resulting declines in stream flow (Milley et al. 2005), and global increases in water consumption (Gleick 2003) may result in widespread saltwater intrusion into freshwater coastal ecosystems. Of particular concern is the impact of increased salinity on tidal freshwater wetlands (TFW), where it has been shown to drive changes in microbial metabolism (Weston et al. 2011, Neubauer et al. 2013), nutrient cycling (Weston et al. 2006, Marton et al. 2012), plant community composition (Sharpe and Baldwin 2012) and primary production (Baldwin and Mendelssohn 1998). Taken together, these changes may significantly alter the carbon (C) biogeochemistry and organic matter (OM) storage capacity of freshwater wetlands (Craft 2007, Loomis and Craft 2010). Wetlands store an estimated 45-70% of all terrestrial C (Mitra et al. 2005), making them important targets for conservation and major players in the global C cycle (Mcleod et al. 2011). One of the reasons for the high C sequestration rate of wetlands is that decomposition slows in water-saturated anaerobic soils (Reddy and DeLaune 2008). Microbial decomposition of soil organic C and plant detritus begins with extracellular enzyme-mediated hydrolysis of complex substrates into monomers and oligomers that can be directly used for metabolism (Shi 2011). This enzymatic hydrolysis has been proposed by many researchers to regulate decomposition rates (Sinsabaugh 1991, Sinsabaugh and Moorhead 1994, Schimel and Weintraub 2003, Freeman et al. 2004, Allison and Vitousek 2005) and has been tied to rates of soil respiration in multiple ecosystems including wetlands (Sinsabaugh and Findlay 1995, Freeman et al. 1998, Margesin et al. 2000).
Elevated salinity has been reported to both increase (Weston et al. 2006, Craft 2007), and decrease decomposition activity rates in wetland ecosystems (Rejmánková and Houdková 2006, Roache et al. 2006, Neubauer 2013). These inconsistencies highlight the need for a more mechanistic understanding of how salinity affects decomposition, which can best be achieved by simultaneously studying extracellular enzymes, the proximal agents of decomposition, as well as the microorganisms responsible for enzyme production. Several prior studies have documented a salinity effect on extracellular enzyme activity (EEA) in both aquatic (Mulholland et al. 2003, Nausch et al. 1998, Cunha et al. 2000) and soil ecosystems (Rietz and Haynes 2003, Rejmánková and Sirova 2007, Neubauer et al. 2013). Likewise, an effect of salinity on microbial community structure has been found by scientists considering both environmental gradients (Casamayor et al. 2002, Blum et al. 2004, Crump et al. 2004, Asghar et al. 2012) and experimental manipulations (Langenheder et al. 2003, Mandeel 2006). However, very few studies have simultaneously studied the effects of salinity on microbial communities and the enzymes they produce (Rietz and Haynes 2003, Pinckney et al. 2011). This sort of information is essential if we are to understand naturally-occurring patterns in soil C across estuarine systems and develop a predictive understanding of how saltwater intrusion will influence decomposition and C biogeochemistry in wetlands.

The current study provides insight into the potential consequences of saltwater intrusion into freshwater ecosystems by investigating changes in microbial decomposition processes and soil OM content along a natural salinity gradient. Specifically, this work examines variation in bacterial community structure and EEA in tidal wetlands ranging from fresh to oligohaline in the Chesapeake Bay watershed (Virginia). In an attempt to isolate the effect of salinity, sampling
locations were close enough in proximity that weather, land-use, tidal influence, and underlying lithology were likely similar, and site selection targeted nearly identical plant communities.

**Materials and Methods**

**Site Description and Sampling**

Samples were collected during a two-week period in June 2010 from eight tidal wetlands in Virginia (Fig. 5.1), varying in salinity from completely fresh (~0 ppt) to oligohaline (~2 ppt). At each wetland, an area dominated by *Peltandra virginica* (min 75% above-ground biomass) was located and a 10 ×10 m² plot was established. Within each plot, five sampling stations (1 m² each) were randomly selected with the caveat that the minimum separation distance between stations was 3 m. All living plant material found within each 1-m² area was harvested to determine above-ground biomass (AGB, clipped to the soil surface). In addition, a 60-cm³ plastic syringe was modified to form a miniature soil corer and used to collect soil for quantifying below-ground biomass (BGB). Lastly, samples of surface soil (0-10 cm) were placed in air-tight plastic bags and returned to the laboratory on ice, at which point each sample was homogenized and sub-divided for analysis of soil properties, enzyme activity (~50 g stored at 4°C), and bacterial community structure (~ 5 g stored at -20°C).

**Soil Properties**

In the lab, soil was immediately analyzed for pH and redox potential (Hanna Combo pH and ORP probe, Smithfield, RI), OM concentration (% as loss on ignition, 425°C for 12 h), and C and N content (Perkin Elmer Series II CHNS/O Analyzer 2400, Waltham, MA). In addition, porewater was collected from 5-ml soil samples by centrifugation (3000 × g for 15 min), filtered
using a 0.45 µm pore-size mixed cellulose ester syringe filter, and stored at -20°C until it could be analyzed for the concentration of chloride via ion chromatography (Dionex ICS-1000, Sunnyvale CA). Salinity (ppt) was then calculated as described by Bianchi (2006).

**Plant Biomass**

The plant material harvested from each station was dried (70°C for 7 days) to estimate AGB as kg of dry plant material per m². BGB was determined by submerging each of relevant soil cores in 35 mL of tap water (24 H) prior to straining through a No. 45 metal mesh (U.S.A. Standard Testing Sieve, A.S.T.M. E-11 specification, Fisher Scientific, Waltham MA). Roots were collected by hand, dried (95°C for 96 H), and BGB was calculated as mg of dry roots per cm³ of soil.

**Enzyme Activity**

Rates of EEA were determined within one week of sampling using the fluorimetric and colorimetric microplate assays described in Neubauer et al. (2013) and a Synergy 2 plate reader (Biotek, Winooski, VT). Briefly, enzyme activity associated with breakdown of cellulose (β-1,4-glucosidase (BG) and 1,4-β-cellobiosidase (CBH)), hemicellulose (β-D-xylosidase (BX)), and lignin (phenol oxidase (POX)), as well as the release of phosphorus (alkaline phosphatase (AP)) and sulfur (arylsulfatase (AS)) from organic molecules, was measured. All substrates and reagents were obtained from Sigma-Aldrich Co. Ltd (St. Louis, MO). Methylumbelliferone (MUB) linked assays relied on the following substrates: 4-MUB β-D-glucopyranoside (BG), 4-MUB β-D-cellobioside (CBH), 4-MUB-β-D-xylopyranoside (BX), 4-MUB-phosphate (AP), and 4-MUB-sulfate (AS) and incubations of either 1 (CBH, AP) or 4 hrs (BG, BX, AS) at 30°C with
gentle agitation. POX activity was measured colorimetrically via the oxidation of L-DOPA after a 30 min incubation using the methods of Sinsabaugh et al. (2003).

**Bacterial Abundance**

Whole-community DNA was extracted from 0.25-g subsamples of soil using the Mo Bio PowerSoil DNA Isolation Kit (Carlsbad, CA) and then stored at -20°C. DNA purity and concentration were analyzed using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE). All DNA extracts and PCR products were verified using agarose gel (1.5%) electrophoresis and ethidium bromide staining.

Bacterial abundance was estimated using the quantitative polymerase chain reaction (qPCR) approach outlined in Fierer et al. (2005); specifically, we targeted the 16S rRNA gene using the Eub338 and Eub517 primers and analytical details presented in Morrissey et al. (2013b). Assays utilized SsoAdvanced SYBR Green qPCR Supermix (BioRad, Hercules, CA) and the BioRad CFX 96 Real-Time System; data were analyzed using Bio-Rad CFX Manager Version 2.1. Samples were run in triplicate and averaged; standard curves were established using genomic DNA from *Escherichia coli* (Strain #11775, ATCC, Manassas, VA; average efficiency=101%, r²=0.99).

**Bacterial Community Structure**

Bacterial community structure was analyzed using terminal restriction fragment length polymorphism (T-RFLP) targeting the 16S rRNA gene of bacteria and the protocols described in Morrissey et al. (2013b). Briefly, PCRs included 1.2 ng of template DNA and the domain-specific primers 27F (labeled with FAM) and 1492R at a concentration of 0.2 μM each. PCR
products were digested using Hha1 (New England Biolabs, Ipswich, MA) and detected via capillary electrophoresis using a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Buckinghamshire, UK). T-RFLP fragments between 70-400 base pairs (bp) were analyzed using Fragment Profiler software (Version 1.2; Amersham Biosciences, Buckinghamshire, UK) with a 1-bp size differential and a 40 relative fluorescent unit peak height threshold. The T-RFLP results were recorded as a binary data matrix describing the presence or absence of each terminal restriction fragment in each sample.

Statistical Analyses

Shapiro-Wilks tests confirmed that soil pH, redox potential (mV), AGB (kg m\(^{-2}\)), and BGB (mg cm\(^{-3}\)) data were normally distributed. A natural log (ln) transformation was necessary for salinity (ppt), all enzyme activity measures (µmoles g-OM\(^{-1}\) hr\(^{-1}\)), and bacterial abundance (copies g-OM\(^{-1}\)); transformed values were utilized in all subsequent statistical analyses. Site differences were analyzed using one-way analysis of variance (ANOVA; n=5, df=39, \(\alpha=0.05\)) with Tukey's HSD for post hoc comparisons. Direct and partial correlations (Pearson’s r, n=40) were used to evaluate relationships between bacterial abundance, enzyme activity, and environmental variables; statistical significance was established using a sequential Holm-Bonferroni adjustment to correct for multiple comparisons (Holm, 1979). All ANOVAs and Pearson’s correlations were performed using the JMP statistical software (Version JMP Pro 9.0.2, Cary, NC; Sall, 2005).

The binary matrix of T-RFLP data was converted to a set of Jaccard coefficients, which were used for all statistical analyses that considered bacterial community composition. Visualization of overall similarity between samples was achieved using non-metric
multidimensional scaling (NMDS, 2-dimentional solution) and site differences were analyzed using one-way non-parametric multivariate analysis of variance (NP-MANOVA; n=5, df=39, α=0.05). Mantel and partial Mantel tests (r_M) were performed to compare changes in bacterial community composition (Jaccard similarity matrix) to changes in environmental and enzyme variables (Euclidian distance matrices) All community composition analyses were performed in PAST statistical software package (Version 2.10; Hammer, 2001).

Results

Site differences

Sites varied significantly with respect to porewater salinity, from fresh (0.03 ppt at JR) to brackish (1.85 ppt at CC, Table 5.1). Likewise, soil characteristics (pH, redox, %OM, and C:N) and macrophyte biomass (AGB and BGB) exhibited significant differences between sites, though all parameters were within the natural ranges expected for these types of wetlands (Reddy and DeLaune, 2008, Barendregt et al. 2009).

The activity of all enzymes varied significantly across sites (Table 5.2, rates in µmoles g-OM^{-1} hr^{-1}). For the hydrolytic carbon-degrading enzymes (CBH, BG, and BX), rates were lowest at JR (CBH=0.13, BX=0.03) and/or WL (BG=0.09, BX=0.03) and highest (~ 5-times greater) at SH (mean: CBH=0.75, BG=0.36, and BX=0.12). This pattern was also observed for POX (lowest at JR (mean: 31) and highest at SH (mean: 1,071); ~35-fold increase) and AP (lowest at JR (mean: 0.22) and highest at SH and GM (mean: 1.39); ~6-fold increase). Activity of AS also varied ~6-fold from the site with the lowest activity (BC=0.08) to the site with the highest activity (YC=0.45).
Bacterial abundance differed significantly across sites (Table 5.1), ranging from the lowest at BC ($8.3 \times 10^9$ copies g-OM$^{-1}$) to the highest at GM ($35.3 \times 10^9$ copies g-OM$^{-1}$, ~4-times greater). The community structure of bacteria also differed across sites (NP-MANOVA F=7.32, p<0.01); all pairwise comparisons between sites yielded p<0.03.

**Correlation Analysis**

**Salinity:** Salinity was negatively correlated with soil OM content ($r=-0.65$, p<0.01) and C:N ($r=-0.50$, p<0.01, Fig. 5.2) but unrelated to the other environmental variables (all $|r|<0.29$, p>0.07). Salinity exhibited strong positive correlations with EEA (Fig. 5.3; all $r>0.58$, all p<0.01) and bacterial abundance (Fig. 5.4; $r=0.56$, p<0.01). Similarly, salinity was the strongest environmental correlate with bacteria community structure ($r_M=0.32$, p<0.01). This relationship can be visualized on the lower panel of Fig. 5.4 where increasing salinity is associated with a positive shift on the NMDS Axis 1, and a negative shift on Axis 2. This is reflected by a salinity vector that represents the strength and direction of the correlation of salinity with each NMDS axis.

**Soil OM:** In addition to the relationship with salinity, soil OM was strongly correlated with C:N ratio ($r=0.67$, p<0.01), but unrelated to the other environmental parameters (all $|r|<0.26$, p>0.09). Further, OM was negatively correlated with enzyme activity rates (Pearson’s r: CBH =-0.91, BG=-0.65, BX=-0.80, POX=-0.73, AP=-0.90, AS=-0.91; all p<0.01) as well as bacterial abundance ($r=-0.64$, p<0.01). Changes in soil OM were not significantly related to changes in bacterial community composition following the Holm’s Bonferonni correction ($r_M=0.13$, p=0.04).
**Other environmental variables:** The only other significant relationships among environmental variables were between redox and pH ($r=-0.73$, $p<0.01$) and between AGB and both pH ($r=-0.47$, $p<0.01$) and redox ($r=0.52$, $p<0.01$). In addition to salinity and OM (discussed above), EEA was correlated with C:N (Pearson’s $r$: CBH=$-0.65$, BG=$-0.42$, BX=$-0.46$, POX=$-0.48$, AP=$-0.71$, AS=$-0.74$; all $p<0.01$), but unrelated to all other measured environmental variables. The only other significant correlation for bacterial abundance was with AGB ($r=0.46$, $p<0.01$). Bacteria community structure was unrelated to all other environmental variables (all $r_M<0.12$ and $p>0.05$). Full correlation results are presented in Table 5.5.

**Partial Correlation Analysis**

To determine the extent to which salinity was a direct driver of EEA and the bacterial community, a partial correlation analysis was performed to control for the main environmental covariates (%OM and C:N; Table 5.3). All enzymes remained significantly correlated with salinity after controlling for C:N ($r>0.35$, $p<0.04$); similarly, relationships remained significant after controlling for OM ($r>0.32$, $p<0.05$) except for AS rates. Bacterial abundance remained significantly correlated with salinity after controlling for C:N but not OM. The association between salinity and community structure was robust and remained highly significant after controlling for covariates with salinity (both $r_M>0.29$ with $p<0.01$).

Relationships between the bacterial community and EEA were also examined. Bacterial abundance was significantly correlated with all enzymes except BG, and three of these relationships remained significant after controlling for covariation with salinity via partial correlation (Table 5.4). Further, the composition of the bacterial community was significantly
related to CBH, POX, and AP, even following a partial Mantel test to account for any shared relationship with salinity.

**Discussion**

The aim of this study was to examine changes in bacterial community structure and function (EEA) along a salinity gradient from fresh to oligohaline in order to better understand how salinity regulates soil OM decomposition and storage in wetlands. The role of salinity as a driver of ecosystem processes in tidal freshwater wetlands is particularly important in light of the potential for climate-change associated saltwater intrusion. Generally there have been few studies that focus on this range of salinity (Poffenbarger et al. 2011, Neubauer 2013, Sharpe and Baldwin 2013), even though the transition from fresh to oligohaline is what many historically freshwater systems are likely to experience as a consequence of sea level rise in the coming decades (Woodroffe and Wallace 2012).

The negative relationship between soil OM and salinity exhibited in the current work is consistent with Craft (2007), who found soil organic C (%) to be negatively related to salinity in tidal wetlands across the conterminous United States. Specifically, for the 63 studies surveyed in Craft’s work, soil bulk density and organic C content were consistently lower in saltier marshes than their freshwater counterparts regardless of geographical region. We consider three non-exclusive possible explanations for this pattern: (1) enhanced tidal influences that increase OM export and/or sediment deposition in saltier marshes, (2) decreased plant production and/or altered community composition, which changes the quality and quantity of OM deposited on the marsh surface, and (3) increased decomposition rates at more saline sites.
Tidal range has been shown to be a strong regulator of aqueous export of total organic C and suspended solids from marsh ecosystems (Childers et al. 2000) and could potentially affect mineral sediment deposition (Chmura and Hung 2004). Consequently, a greater tidal range at the more saline wetlands could contribute to the lower levels of soil OM at these sites. Although not directly measured in the current study, we expect the differences in tidal range across the sites we sampled were not sufficient to explain the OM patterns. For instance, tidal range predictions by NOAA on the James River (20 year average from http://tidesandcurrents.noaa.gov) indicate that the mean tidal range near our most saline site is only a few inches greater than for the most upriver freshwater site (2.26 vs 2.15 ft). In addition, soil texture (determine by the hydrometer method using one aggregate sample per site; results not presented) did not correlate with salinity (considering % sand, silt, and clay, all p>0.29), which suggests no consistent changes in sediment deposition along the salinity gradient of our sites.

The second explanation for the salinity-OM relationship we observed – i.e., the effect of saltwater on plant production and/or community composition – was also not well supported. We saw no correlation between above- or below-ground plant biomass and either salinity or soil OM content, and plant production differed little across sites (Table 5.1). This is likely due to the relatively narrow salinity range we considered, as prior work has shown decreased plant productivity (McKee and Mendelssohn 1989, Wieski et al. 2010) as well as differences in below-ground biomass allocation (Neubauer et al. 2005) along broader salinity gradients. Aside from biomass production, salinity could influence plant community composition and diversity (Latham et al. 1994, Tuxen et al. 2011, Wieski et al. 2010). Because various plant species differ in polymer composition (Kögel-Knabner 2002), this could affect the quality of the litter deposited on the marsh surface and potentially alter rates of OM accumulation. Although this
possibility cannot be completely ruled out in our study, a large variation in litter quality is not expected due to the fact that site selection targeted plots that were dominated by a single perennial species (*Peltandra virginica*).

Given the similar tidal regimes and plant communities at each of our sites, the third of our possible explanations for the salinity-OM relationship – increased decomposition under more saline conditions – is best supported by our results. In particular, we documented strong relationships between salinity and enzyme activity (Fig. 5.3), soil OM content (Fig. 5.2), and microbial community structure (Fig. 5.4) that typify enhanced decomposition. This could result from a combination of factors whereby salinity: (i) increases the bioavailability of organic substrates, (ii) facilitates enzyme activity, and (iii) stimulates changes in microbial community structure. Further, we propose that salinity’s influence in this study derives largely from the effect of increased ionic strength on the molecular stability and sorption of both enzymes and their organic substrates. With regards to OM, increased ionic strength can make organic particles more accessible by disrupting soil microaggregates (Rengasamy and Sumner 1998) and increasing the number of macropore spaces in the soil profile (Brady and Weil 2004) potentially affecting soil aeration (Blackwell et al. 1990, Kühne et al. 2012). Ionic strength could also affect solute concentrations of organic compounds by decreasing their sorption to soil particles (Reemstma et al. 1999, Mavi and Marschner 2012). Both of these processes would increase microbial access to organic substrates and, as past studies have found enzyme activity rates to be positively related to target molecule availability (Shackle et al. 2000, Allison and Vitousek 2005, Morrissey et al. 2013b), this could explain the higher levels of enzyme activity we observed at more saline sites (Fig. 5.3). Similarly, the abundance of cations in seawater can affect inorganic N exchange and P sorption (Portnoy and Giblin 1997, Weston et al. 2006, Weston et al. 2011),
allowing higher salinity to liberate otherwise limiting nutrients (Weston et al. 2010). It is important to point out that the electrostatic interactions described above are interactively affected by other environmental parameters (e.g., pH) and soil physicochemical properties like surface area. For example, increased pH can limit the binding capacity of clay compounds, leading to decreased humic acid sorption (Vermeer et al. 1998, Abgate and Mansi 2003). All these effects are also somewhat dependent on the chemical nature of the C compounds involved. In particular, the behavior of humic substances, which constitutes 50–80% of the natural OM (Shaker et al. 2012), will be governed by the relative abundance of functional groups like carboxyls, hydroxyls, and phenolics.

Besides ionic strength effects on organic substrate availability, it is also necessary to consider how changes in salt concentration may directly impact extracellular enzymes. With regards to sorption, several studies have documented a decrease in mobility and catalytic activity when enzymes are adsorbed to clay surfaces (Fusi et al. 1989, Quiquampoix 1987, Tietjen and Wetzel 2003), and such immobilization reduces the probability of a contact between enzyme and substrate (Lammirato et al. 2010). However, scientists’ understanding of how these proteins interact with mineral surfaces is limited, especially when considering confounding factors such as solution pH and enzyme molecular weight. Since it appears that the majority of the extracellular enzymes are adsorbed to soil particles (Kandeler 1990, Gianfreda and Bollag 1994, Violante et al. 1995, Lipson and Nasholm 2001, Nannipieri et al. 2002), this is an essential area for future research if we are to develop a predictive understanding of how C mineralization is affected by saltwater intrusion.

Changes in salinity also have the potential to impact extracellular enzyme activity by influencing molecular stability and protein confirmation states. Most laboratory studies show a
detrimental effect of salt on enzyme activity, but these assays use purified enzymes in solution and only a select group of substrates (Das et al. 1997, Fang et al. 2010). Work in soils similarly shows high salt concentrations inhibit enzyme activity, but these studies target hypersaline conditions well beyond the range relevant to our study (e.g., Tripathi et al. 2007, Yun et al. 2010, Pan et al. 2013). The limited reports from wetland soils are inconsistent. Even within a single study, results vary by enzyme, and there is evidence that activity can simultaneously be enhanced, suppressed, and unaffected by salinity (Chambers et al. 2013, Saviozzi et al. 2011, Jackson and Vallaire 2009, Neubauer et al. 2013, Wu et al. 2008). There are several noteworthy differences between the studies cited above and our own, in which we found salinity significantly enhanced enzyme activity for a diversity of substrates (e.g., breakdown of both labile and recalcitrant compounds, acquisition of C, N, and P). First, the research efforts differed dramatically in their potential for plant effects. Our experimental design minimized the influence of salinity-induced differences in plant biomass and community composition, which was a significant co-variant in much of the prior work. Second, our study compared sites with relatively modest salinity (0-2 ppt); this contrasts, for example, the work of Chambers et al. (2013) that compared “fresh” (0.5 ppt), “brackish” (13 ppt), and “saline” (26 ppt) conditions. Future studies need to consider a broad range of exposure levels as ecosystem responses to salinity are highly non-linear. Third, our study represented field conditions integrating prolonged exposure to varying salinity levels. Studies like Jackson and Vallaire (2009) assessed the response to short-term (55 days) increases in laboratory microcosms. In this context, our findings suggest that long-term shifts in C cycling may not be consistent with short-term disturbance-type responses, as hypothesized by Neubauer et al. (2013), and highlight a need for in situ work combined with manipulative studies.
Combined, these lines of reasoning help explain the strong correlation we observed between salinity and enzyme activity and provide several possible mechanisms by which salt water intrusion could directly influence decomposition rates. Salinity effects could also be indirect, and mediated through changes in microbial community structure. In this study, we observed a consistent shift in bacterial abundance and community composition with increased salinity (Fig. 5.4) and found these parameters to be correlated with the activity of several extracellular enzymes (Table 5.4). This could indicate that the community structure (i.e., abundance and composition) regulates, at least in part, organic polymer breakdown and thus could constrain decomposition rates. Several prior studies have similarly documented a link between extracellular enzyme activity and changes in composition of the soil microbial community (e.g., Gallo et al. 2004, Costa et al. 2007, Morrissey et al. 2013b). The shifts in bacterial community structure we observed may be in part due to the physiological effects of increased saltwater, and numerous recent studies have demonstrated a link between salinity and microbial community composition (Bouvier and del Giorgio 2002, Langenheder et al. 2003, Langenheder and Ragnarsson 2007, Berga et al. 2012). Some researchers have even reported that salinity is the most important factor determining the distribution patterns of microorganisms across the globe (Lozupone and Knight 2007, Auguet et al. 2010). Besides ionic strength, saltwater intrusion brings with it specific ions that may affect microbial functional groups. For instance, elevated sulfate can stimulate the growth of sulfate-reducing bacteria, who then outcompete the methanogens typically found in freshwater wetlands (Weston et al. 2006, Chambers et al. 2011, Weston et al. 2011).

In conclusion, salinity was found to be strongly related to bacterial community structure and decomposition activity in tidal wetlands ranging from fresh to oligohaline (Fig. 5.3 and 4).
The results of this study suggest that via abiotic and/or microbial processes, salinity is stimulating extracellular enzyme activity. This increased enzyme activity could reflect increased decomposition rates, contributing to the negative relationship between salinity and soil OM content in these ecosystems (Fig. 5.2). If saltwater intrusion does increase soil OM decomposition; previously freshwater wetlands may face reduced soil OM accumulation leading to lower accretion rates (Callaway et al. 1997, Craft 2007). Consequently these ecosystems may have more difficulty keeping pace with sea level rise than previously anticipated, potentially leading to ecosystem loss and large releases of stored C (DeLaune and White 2011). More research on the mechanisms underlying salinity’s regulation of enzyme activity and decomposition is sorely needed if we are to quantitatively predict salinity-induced changes in C cycling in tidal wetlands.

Acknowledgements

This work was funded in part by the VCU Howard Hughes Medical Institute Summer Scholars program awards to Morina and Gillespie. Thanks to Amy Jenkins-Lederer, Caitlin Muse, Aaron Porter, Jamie Bourne, John Furry, Ed Crawford, Ariana Johns, and Susan Chong for field and laboratory assistance. We would like to acknowledge Cyrus Brame and Merry Maxwell of the US Fish and Wildlife Service, Bob Greenlee of the Virginia Department of Game and Inland Fisheries, and Jim Perry of the Virginia Institute for Marine Sciences for helping us with site access. Special thanks to Christine Prasse for assistance in enzyme methodologies and David Berrier whose constructive comments improved this manuscript.
Table 5.1 Site locations and mean (± SE) of environmental parameters for each site. Values with the same superscript letter are not significantly different from each other (ANOVA and Tukey’s HSD post hoc test with α=0.05; salinity and bacterial abundance were natural log (ln) transformed values prior to testing).

<table>
<thead>
<tr>
<th>Site</th>
<th>GPS (N)</th>
<th>GPS (W)</th>
<th>Porewater salinity (ppt)</th>
<th>Soil properties</th>
<th>Plant biomass</th>
<th>Bacterial abundance (× 10^9 copies g-OM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>pH</td>
<td>Redox (mV)</td>
<td>OM (%)</td>
</tr>
<tr>
<td>JR</td>
<td>37°16'27&quot;</td>
<td>77°09'18&quot;</td>
<td>0.03 ± 0.01 a</td>
<td>6.1 ± 0.1 b</td>
<td>-11 ± 5 b</td>
<td>37.4 ± 2.9 a</td>
</tr>
<tr>
<td>WL</td>
<td>37°43'60&quot;</td>
<td>77°00'96&quot;</td>
<td>0.04 ± 0.01 a</td>
<td>5.6 ± 0.2 b</td>
<td>309 ± 26 a</td>
<td>35.8 ± 1.7 a</td>
</tr>
<tr>
<td>YC</td>
<td>37°19'64&quot;</td>
<td>76°52'26&quot;</td>
<td>0.15 ± 0.03 b</td>
<td>6.4 ± 0.1 a</td>
<td>-96 ± 9 b</td>
<td>10.4 ± 2.0 b</td>
</tr>
<tr>
<td>MC</td>
<td>37°16'78&quot;</td>
<td>76°53'38&quot;</td>
<td>0.16 ± 0.01 b</td>
<td>6.2 ± 0.2 ab</td>
<td>-65 ± 28 b</td>
<td>18.2 ± 0.6 b</td>
</tr>
<tr>
<td>BC</td>
<td>37°18'76&quot;</td>
<td>76°51'89&quot;</td>
<td>0.17 ± 0.02 b</td>
<td>6.2 ± 0.1 ab</td>
<td>23 ± 19 b</td>
<td>34.2 ± 5.0 a</td>
</tr>
<tr>
<td>GM</td>
<td>37°38'14&quot;</td>
<td>76°51'39&quot;</td>
<td>0.54 ± 0.04 c</td>
<td>5.6 ± 0.1 a</td>
<td>255 ± 58 a</td>
<td>15.3 ± 0.4 b</td>
</tr>
<tr>
<td>SH</td>
<td>37°33'02&quot;</td>
<td>76°53'31&quot;</td>
<td>0.88 ± 0.08 cd</td>
<td>6.2 ± 0.2 ab</td>
<td>-64 ± 31 b</td>
<td>14.2 ± 0.6 b</td>
</tr>
<tr>
<td>CC</td>
<td>37°15'08&quot;</td>
<td>76°42'60&quot;</td>
<td>1.85 ± 0.11 d</td>
<td>6.4 ± 0.2 a</td>
<td>-73 ± 22 b</td>
<td>16.4 ± 0.7 b</td>
</tr>
</tbody>
</table>

§Above-ground biomass
†Below-ground biomass
Table 5.2 Mean (± SE) enzyme activity rates for each site. Values with the same superscript letter are not significantly different from each other (ANOVA and Tukey’s HSD post hoc test on natural log (ln) transformed values, α=0.05).

<table>
<thead>
<tr>
<th>Site</th>
<th>CBH</th>
<th>BG</th>
<th>BX</th>
<th>POX</th>
<th>AP</th>
<th>AS</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR</td>
<td>0.13 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.03 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>WL</td>
<td>0.15 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.03 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>YC</td>
<td>0.46 ± 0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.28 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.10 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>365 ± 26&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.87 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.00&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MC</td>
<td>0.41 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.18 ± 0.03&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>222 ± 39&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.84 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24 ± 0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>BC</td>
<td>0.20 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.07 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>295 ± 101&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>GM</td>
<td>0.61 ± 0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.26 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>501 ± 74&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1.39 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SH</td>
<td>0.75 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.12 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1071 ± 238&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.39 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CC</td>
<td>0.50 ± 0.06&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.23 ± 0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.09 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>250 ± 39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.10 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.36 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Table 5.3 Partial correlation analysis comparing salinity to enzyme activity (Pearson’s r), bacterial abundance (Pearson’s r), and bacterial community composition (Mantel test, rM) while controlling for soil OM and C:N.

<table>
<thead>
<tr>
<th>Partial correlation controlling for</th>
<th>OM</th>
<th>C:N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CBH</td>
<td>0.52</td>
<td>&lt;0.01 *</td>
</tr>
<tr>
<td>BG</td>
<td>0.33</td>
<td>0.04 *</td>
</tr>
<tr>
<td>BX</td>
<td>0.44</td>
<td>&lt;0.01 *</td>
</tr>
<tr>
<td>POX</td>
<td>0.38</td>
<td>0.02 *</td>
</tr>
<tr>
<td>AP</td>
<td>0.55</td>
<td>&lt;0.01 *</td>
</tr>
<tr>
<td>AS</td>
<td>-0.05</td>
<td>0.74</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abundance</td>
<td>0.26</td>
<td>0.11</td>
</tr>
<tr>
<td>Composition</td>
<td>0.30</td>
<td>&lt;0.01 *</td>
</tr>
</tbody>
</table>

* Significant with p<0.05
Table 5.4 Direct and partial (controlling for salinity) correlation analysis comparing enzyme activity to bacterial abundance (Pearson’s $r$) and community composition (Mantel test, $r_M$).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Bacterial abundance</th>
<th>Bacteria Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>Partial</td>
</tr>
<tr>
<td></td>
<td>$r$</td>
<td>$p$</td>
</tr>
<tr>
<td>CBH</td>
<td>0.62</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>BG</td>
<td>0.31</td>
<td>0.05</td>
</tr>
<tr>
<td>BX</td>
<td>0.44</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>POX</td>
<td>0.39</td>
<td>0.01*</td>
</tr>
<tr>
<td>AP</td>
<td>0.63</td>
<td>&lt;0.01*</td>
</tr>
<tr>
<td>AS</td>
<td>0.60</td>
<td>&lt;0.01*</td>
</tr>
</tbody>
</table>

* Significant with $p<0.05
Table 5.5 Correlations coefficients (Pearson’s r, top right half of the matrix) and p-values (lower left half) between all evaluated parameters. Enzyme (µmoles g-OM^{-1} hr^{-1}) and bacterial abundance (16S rRNA gene copies g-OM^{-1}) data were natural log transformed (ln) prior to analysis. Bacterial community composition was represented using a Jaccard similarity matrix derived from 16S rRNA T-RFLP; correlations presented below are from associated Mantel tests (rM). Abbreviations are as follows: CBH = 1,4- β-cellobiosidase, BG = β-1,4-glucosidase, BX = β-D-xylosidase, AS = arylsulfatase, POX = phenol oxidase, AP= alkaline phosphatase , BGB = below-ground plant biomass, and AGB = above-ground plant biomass.

<table>
<thead>
<tr>
<th>Environmental Properties</th>
<th>Enzyme activity</th>
<th>Bacterial Community</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Salinity</td>
<td>pH</td>
</tr>
<tr>
<td>Salinity (ln ppt)</td>
<td>-</td>
<td>0.25</td>
</tr>
<tr>
<td>pH</td>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td>Redox (mV)</td>
<td>0.07</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>OM (%)</td>
<td>&lt;0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>C:N</td>
<td>&lt;0.01</td>
<td>0.37</td>
</tr>
<tr>
<td>BGB (mg cm^{-3})</td>
<td>0.74</td>
<td>0.01</td>
</tr>
<tr>
<td>AGB (kg m^{-2})</td>
<td>0.51</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Enzymes:

<table>
<thead>
<tr>
<th>CBH</th>
<th>BG</th>
<th>BX</th>
<th>AS</th>
<th>POX</th>
<th>AP</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01</td>
<td>0.54</td>
<td>0.15</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.35</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.53</td>
<td>0.07</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.19</td>
<td>0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.35</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.20</td>
<td>0.09</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.30</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.53</td>
<td>0.44</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.51</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.64</td>
<td>0.29</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Bacteria:

<table>
<thead>
<tr>
<th>Abundance</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.01</td>
<td>0.56 0.60 0.02</td>
</tr>
<tr>
<td>&lt;0.01</td>
<td>0.05 0.04 0.16</td>
</tr>
</tbody>
</table>
Fig. 5.1 Map of sampling locations along four tidal rivers proximal to the Chesapeake Bay (Virginia). The lowest salinity sites (white symbols, ppt < 0.1) are Walkerton Landing (WL) and James River National Wildlife Refuge (JR). Intermediate salinity sites (grey symbols, 0.1< ppt < 0.4) are Yarmouth Creek (YC), Blackstump Creek (BC), and Morris Creek (MC). The most saline sites (black symbols, ppt > 0.4) are Gleason Marsh (GM), Sweet Hall Marsh (SH) and College Creek (CC).
Fig. 5.2 Variation in (a) organic matter and (b) C:N with salinity (natural log (ln) scale). Correlation results are shown in the lower right corner (Pearson’s r, both p<0.01). Data points are colored by salinity (white <0.1 ppt, grey = 0.1< ppt < 0.4, and black >0.4 ppt).
**Fig. 5.3** Variation in extracellular enzyme activity (rates in μmoles g-OM⁻¹ hr⁻¹) with salinity; both parameters are presented using a natural log (ln) scale. Correlations results are shown in the lower right corner of each panel (Pearson’s r, all p<0.01). Data points are colored by salinity (white <0.1 ppt, grey = 0.1< ppt < 0.4, and black >0.4 ppt). Enzyme abbreviations are as follows: (a) CBH = 1,4-β-cellobiosidase, (b) BG = β-1,4-glucosidase, (c) BX = β-D-xylosidase, (d) AS = arylsulfatase, (e) POX = phenol oxidase, and (f) AP = alkaline phosphatase.
Fig. 5.4 Variation in (a) bacterial abundance and (b) community composition with salinity. Bacterial abundance and salinity axes use a natural log (ln) scale. Community composition is displayed using an NMDS ordination diagram (stress = 0.20) wherein the vector presents the relationship of salinity with each axis. Correlation results for salinity with abundance (Pearson’s, r) and composition (Mantel test, rM) are shown in the top left corner. Data points are colored by salinity (white <0.1 ppt, grey = 0.1< ppt < 0.4, and black >0.4 pp)
CHAPTER SIX:

SYNTHESIS AND CONTEXTUALIZATION

by

Ember M. Morrissey
The field of microbial ecology currently is struggling to understand how microbial communities are influenced by their environments, and whether such influences on community structure have consequences for ecosystem function (Prosser 2012). The work presented in this dissertation addresses these questions via a combination of manipulative and observational studies in tidal wetland ecosystems. While the objectives of each chapter were strongly rooted in wetland ecology, my findings demonstrate that studying microbial communities can enhance our understanding of biogeochemical processes, a conclusion that is relevant across ecosystems. The results of my work indicate that both whole communities and functional groups are sensitive to environmental perturbations (e.g., salinity increases, nitrate pollution, etc.), and that these changes often translate into altered biogeochemical activity rates.

In this dissertation, I determined that a wide variety of environmental factors, including soil organic matter (OM) characteristics and salinity, had a significant influence upon the structure of bacteria and archaea communities (Fig. 4.2 and 5.4) and also upon functional groups such as methanogens (Fig 4.2) and denitrifiers (Fig. 2.3 and 3.3). Generally speaking, these relationships are consistent with the literature. For instance, soil OM has been shown to influence microbial communities in a variety of ecosystems (Bååth et al. 1995, Bastida et al. 2013, Merilä et al. 2010, Nemergut et al. 2010, Wessén et al. 2010) including wetlands (Li et al. 2010, Peralta et al. 2013). However, relationships between environmental variables and microbial communities may be expected to vary depending upon a variety of factors including the ecosystem type, the microbial community of interest (e.g., bacteria, methanogens, etc.), and the scale of examination (Franklin and Mills 2003, Fierer and Jackson 2006, Fuhrman 2009). For instance, pH has been strongly correlated with changes in microbial community structure across large spatial scales and environmental gradients (over a pH range of ~3-9; Fierer and Jackson 2006, Lauber et al. 2009).
However, no significant relationships between pH and microbial community structure were detected in the current work, wherein variations in pH and spatial separation were comparatively small (pH ranged from ~ 5-7). In contrast to pH, salinity has been found to exhibit strong relationships with bacterial community structure over small (fresh to oligohaline; Fig 5.4) and large (fresh to hypersaline; Lozupone and Knight, 2007) gradients. These variations in microbial community-environment relationships highlight the need for a mechanistic comprehension of how environmental variables cause changes microbial communities. Such knowledge would allow scientists to predict the circumstances under which any given environmental variable will be a strong regulator of microbial community structure. Many studies to date rely on correlative analysis to establish community-environment relationships (e.g., Fig 5.4, Bernhard et al. 2005, Hartman et al. 2008, Gilbert et al. 2009). Such results should be interpreted cautiously, as covariance can result from non-causal means (e.g., lurking variables, spatial autocorrelation, etc.). To avoid this quandary, many microbial ecologists have begun to focus on manipulative controlled experiments (such as Chapters 2-4). These studies aid in establishing the causal influence of a given environmental variable on microbial community structure (Jin et al. 2010, Nemergut et al. 2010, Lane et al. 2012, Hopkins et al. 2013). However, because environmental variables interact at a hierarchy of nested scales, even these studies are confounded by the possibility of complex indirect effects, making it difficult to establish the mechanisms by which environmental variables influence community structure. For instance, in Chapter 3, I reported that the addition of plant litter increased the moisture content of the soil (Fig. 3.1). Therefore the changes in microbial community structure associated with plant litter addition may have been partially mediated by changes in soil moisture, even though I concluded that the major driver was the augmentation of soil OM. Future research in microbial community–environment
relationships would benefit from focusing on establishing mechanisms and, given the tremendous complexity of microbial communities, this will likely require a combination of field, microcosm, and modeling approaches.

Despite the difficulty that can sometimes accompany interpreting microbial community–environment relationships, the abundance of these relationships in the literature indicates that microbial communities are sensitive to environmental conditions (see reviews by Wallenstein et al. 2006, Fischer et al. 2009, Ollivier et al. 2010, Guo et al. 2013). But the question remains as to if/when/how these responses have consequences for microbial community function. In my research, I found that the community structure of functional groups (Fig 3.5, and Table 4.3) and the total bacterial community (Table 4.3 and Table 5.4) exhibited significant relationships with biogeochemical processes. Specifically, the community structure of a given functional group (i.e., denitrifiers or methanogens) was related to the corresponding activity rates (i.e., potential denitrification and methanogenesis, respectively). These findings are consistent with reports in the literature (Beckmann et al. 2011, Song et al. 2011, Angel et al. 2012, Parkes et al. 2012) and suggest that studying the ecology of functional groups may enhance our ability to predict the associated biogeochemical process rates (Wilmes and Bond 2006). In addition to functional groups, the study of whole communities using phylogenetic approaches remains popular (Schütte et al. 2008, Tringe and Hugenholtz 2008). Connecting whole communities to microbial functions is complicated by the fact that many processes (e.g., denitrification, methanotrophy, nitrogen fixation, etc.) are only performed by a subset of the community, while others (e.g., cellulose decomposition, CO₂ production, etc.) are so broadly distributed that they often are viewed as nearly intractable. Despite this complication, even the structure of whole communities has been associated with biogeochemical process rates (Monson et al. 2006, Cleveland et al. 2007,
Carrino-Kyker et al. 2012), including those only performed by a subset of the community (Table 4.3, Nugroho et al. 2009, Peralta et al. 2010). Associations between whole community structure and specialized processes may result from functional groups being affected by the activities of the greater microbial community. For instance, in the current work, whole communities of bacteria and archaea were associated with rates of methanogenesis (a process only performed by a subset of archaea). These relationships were attributed to syntrophic connections between bacteria and methanogens. Specifically, bacterial activities (e.g., fermentation) impacted methanogen communities and their activity rates via the availability of substrates for methanogenesis (Fig. 4.6). Further, study of syntrophic associations, inhibitory affects, quorum sensing, and other interactions between microbial groups can help us understand how the structure of the entire microbial community influences ecosystem process rates.

The findings reported in this dissertation are in agreement with a growing body of evidence that microbial community structure is frequently associated with rates of biogeochemical activity. Whether examined via functional groups or whole communities, most relationships between community structure and function in the literature, as in the current work, remain largely correlational (Magalhaes et al. 2008, Peralta et al. 2010, Carrino-Kyker et al. 2012). With observational studies, it is nearly impossible to obtain clear evidence of a causal relationship between community composition and ecosystems process rates. To establish causal linkages, experimental studies must manipulate the community structure while controlling for environmental conditions (e.g., see Reed and Martiny 2007). Establishing this link is an emerging area of study in microbial ecology, and there is early evidence that community structure does affect function (Allison et al. 2013, Reed and Martiny 2013, Philippot et al. 2013). However, much more study is needed, as the degree to which microbial structure regulates
function may vary across biogeochemical processes, as well as over temporal and spatial scales. For instance, ecological theory would predict that community structure should be more important for processes that have narrow phylogenetic distribution and consequently low functional redundancy (McGuire and Treseder 2010).

As there are still major areas of uncertainty in how microbes respond to their environments and how these responses translate into altered biogeochemical function, it remains difficult to incorporate microbial communities into conceptual and quantitative models of ecosystem function. Despite these difficulties, researchers already are assessing the utility of aspects of microbial community structure and ecology in modeling biogeochemical processes (e.g., Fig 3.5, Fig 4.6, Schimiel and Wientrab 2003, McGuire and Treseder 2010, Wieder et al. 2013). Although these models are inarguably rudimental, they are representative of how microbial ecology can advance our understanding of ecosystem function by serving as a bridge between abiotic environmental conditions and biogeochemical activity.
REFERENCES


Angel R, Claus P, Conrad R (2012) Methanogenic archaea are globally ubiquitous in aerated soils and become active under wet anoxic conditions. ISME J 6: 847-862


abundance and diversity drive potential denitrification after changes in land uses. Glob Chang Biol 17: 1975-1989


Bruland GL, Richardson CJ, Daniels WL (2009) Microbial and geochemical responses to organic matter amendments in a created wetland. Wetlands 29: 1153-1165


Environmental Protection Agency (2008) Compensatory mitigation for losses of aquatic resources. Federal Register Vol.73 No. 70: 19594-19705


Helbling DE, Ackermann M, Fenner K, Kohler HPE, Johnson DR (2011) The activity level of a microbial community function can be predicted from its metatranscriptome. ISME J, 6: 902-904


Latham PJ, Pearlstine LG, Kitchens WM (1994) Species association changes across a gradient of freshwater, oligohaline, and mesohaline tidal marshes along the lower Savannah River. Wetlands 14: 174-183


McKee, KL, Mendelssohn, IA (1989). Response of a freshwater marsh plant community to increased salinity and increased water level. Aquat Bot 34: 301-316


Morrissey EM, Berrier DJ, Neubauer SC, Franklin RB (2013b) Using microbial communities and extracellular enzymes to link soil organic matter characteristics to greenhouse gas


VITA

Ember Michelle Morrissey was born September 9th, 1985 in Cumberland, Maryland. She graduated from Fort Hill High School in Cumberland, Maryland in 2003. She received a Bachelor of Science in Biology from The University of Maryland in 2007. After two years working as laboratory specialist for Dr. Steve Negus, she returned to academia, entering graduate school at Virginia Commonwealth University in 2009. During her time in graduate school, she received multiple awards and grants including a VCU Rice Center Student Research Award, an SWS Student Research Grant, an NSF Doctoral Dissertation Improvement Grant, and a Philanthropic Educational Organization Scholar Award. Ember intends to pursue a career in environmental research, starting as a postdoctoral scholar for Dr. Bruce Hungate at Northern Arizona University.