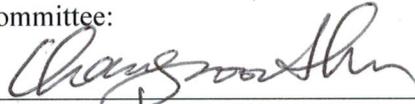
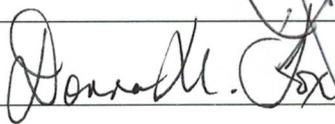
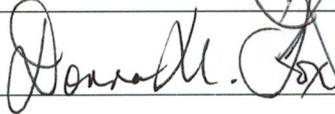


MULTIPLE CONTROLS OF CARBON AND NITROGEN PROCESSES IN FRESHWATER  
WETLANDS OF THE MID-ATLANTIC UNITED STATES WITH IMPLICATIONS FOR  
ECOSYSTEM RESTORATION AND DISTURBANCE

by

Alicia Korol  
A Dissertation  
Submitted to the  
Graduate Faculty  
of  
George Mason University  
in Partial Fulfillment of  
The Requirements for the Degree  
of  
Doctor of Philosophy  
Environmental Science and Public Policy

Committee:

	Dr. Changwoo Ahn, Dissertation Director
	Dr. Gregory Noe, Committee Member
	Dr. Kim de Mutsert, Committee Member
	Dr. Younsung Kim, Committee Member
	Dr. Albert Torzilli, Graduate Program Director
	Dr. A. Alonso Aguirre, Department Chairperson
	Dr. Donna Fox, Associate Dean, Student Affairs & Special Programs, College of Science
	Dr. Peggy Agouris, Dean College of Science

Date: 12-7-17 Fall Semester 2017  
George Mason University  
Fairfax, VA

Multiple controls of carbon and nitrogen processes in freshwater wetlands of the mid-Atlantic United States with implications for ecosystem restoration and disturbance

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at George Mason University

by

Alicia Korol  
Bachelor of Science  
Cornell University 2008

Director: Changwoo Ahn, Professor  
Department of Environmental Science and Public Policy

Fall Semester 2017  
George Mason University  
Fairfax, VA

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**DEDICATION**

*for Ricky*

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## **ABSTRACT**

### **MULTIPLE CONTROLS OF CARBON AND NITROGEN PROCESSES IN FRESHWATER WETLANDS OF THE MID-ATLANTIC UNITED STATES WITH IMPLICATIONS FOR ECOSYSTEM RESTORATION AND DISTURBANCE**

Alicia Korol, Ph.D.

George Mason University, 2017

Dissertation Director: Dr. Changwoo Ahn

Biogeochemical cycling of nitrogen and carbon supports the structure and functioning of all ecosystems and provides valued products and services essential to human well-being. Understanding the controls on these cycles and their interactions with ecological components at multiple scales predicts spatial patterns and response to environmental disturbance, guides management of these resources, and informs environmental policy. This dissertation synthesizes research conducted in three different freshwater wetlands to demonstrate interacting drivers of carbon and nitrogen processes that are important for understanding how ecosystems will respond to large anthropogenic modification.

The first two studies in freshwater mesocosms involved tests of the effects of macrophyte planting diversity on biomass production and on three nitrogen cycle processes. Empirical evidence and theory link plant diversity to greater ecosystem

functioning. Thirty-four mesocosms were planted with four functionally-distinct macrophyte species across a richness gradient (1, 2, 3, or 4 species). The elevated aboveground biomass of communities in the most species-rich mixtures at the end of the first growing season was temporary, and monocultures of a single species on average produced greater aboveground biomass compared to the most species-rich mixtures by the end of the second growing season. The suppression of biomass production in the two perennials was attributed to a dominant annual found commonly in palustrine wetlands of northern Virginia and early successional vegetation communities. The second study evaluated whether plant richness, along with biomass and tissue N content, had positive effects on soil N processing – ammonification, nitrification, and denitrification – at the end of the second growing season. Greater species richness was related to greater net ammonification potential, but lower net nitrification potential and denitrification potential. By contrast, lower biomass and/or lower tissue N content, characteristics of the two annuals, had stimulatory effects on all three nitrogen processes.

The results of the first two studies inform the design of newly-planted restored wetlands and highlight the tradeoffs between managing for multiple ecosystem functions. This research adds to the growing body of evidence suggesting that restored ecosystems may not maximize all ecosystem functions and demonstrates that establishing plant diversity in early-successional wetland plant communities may come at the expense of plant productivity and soil biogeochemical development. At the same time, the identification of mechanisms that inhibit these wetland functions suggests restoration

designs for alleviating that inhibition (*e.g.*, spatial or temporal heterogeneity in hydrologic conditions) to best promote multiple functions in restored wetlands.

The third and fourth studies were investigations of the controls of denitrification in floodplains of the Chesapeake Bay watershed. Largescale characteristics of floodplains, including hydrogeomorphic or landscape features (*i.e.*, climate, physiography, or ecosystem patterns), are of practical use for the prediction of denitrification due to emerging largescale datasets and because these characteristics may aggregate the effects of local vegetation or soil biogeochemical characteristics on denitrification. Results from the investigation of 18 nontidal floodplains indicated that the highest rates of denitrification are likely to be found in agricultural and urban watersheds with stream-floodplain hydrologic connectivity promoting sedimentation. All largescale predictors, including seasonal air temperature and channel width-to-depth ratio, explained between 43-57% of variation in the denitrification measurements and should be useful for prediction across the Chesapeake Bay watershed. In a study of TFFWs in Virginia, potential for denitrification and nitrate-limitation of denitrification were studied along two adjacent rivers that differed notably in concentrations and loads of nitrogen (Pamunkey River carries more nitrogen than the Mattaponi River). Patterns of denitrification enzyme activity (DEA) in TFFWs were found along a longitudinal gradient but not between rivers. DEA was greater in all tidal wetlands (three sites on each river) than nontidal forested wetlands (one site on each river). Further, DEA was positively related to soil organic matter, carbon, and nitrogen, and negatively related to bulk density in tidal hummocks, relationships which were all mediated by a longitudinal

gradient with highest DEA rates at the lower tidal sites. Modification to hydrogeomorphic processes from accelerated sea level rise may alter these longitudinal gradients in TFFWs.

The results of the third and fourth studies inform currently poor understanding of denitrification patterns in floodplains at landscape and regional scales. The studies both demonstrate the existence of largescale predictable patterns in denitrification that have only rarely been demonstrated in the literature. The identification of these patterns alleviate uncertainty in the process given its highly variable nature, which has stymied attempts to confidently extrapolate denitrification rates from one floodplain to another. As such, the research findings on hotspots of the process guides the prioritization of nontidal floodplains for the purposes of restoration as well as predicts how nitrogen removal capabilities of TFFWs will change given quickening sea level rise. The studies further indicate that certain characteristics of large scales (*e.g.*, emergent characteristics of the watershed) are efficient and sometimes more useful for the prediction of denitrification in floodplains than are commonly measured vegetation and soil characteristics.

Wetland ecologists are challenged to advance understanding of ecosystem functioning in the midst of complexity and uncertainty. This dissertation meets that challenge by developing quantitative relationships among ecosystem components, reflecting ecological complexity through investigations of multiple ecosystem controls, and factoring in future scenarios of anthropogenic modification. First, statistical prediction models were applied in all studies to compare competing controls and their

emergent effects on nitrogen and carbon functioning in wetlands. These statistical models provide tools for prediction and evaluation, and for developing more complicated ecosystem models. Second, the dissertation provides empirical evidence in three different wetland ecosystems of the theory that multiple, hierarchically-organized ecological components control nitrogen processing. The studies improve mechanistic understanding of the quantitative relationships between ecosystem components and nitrogen processing through the consideration of direct and indirect controls, which make the findings broadly applicable to wetlands. Finally, all studies were conducted to reduce uncertainty in ecosystem functioning given future ecosystem changes. Three studies factored in management scenarios for ecosystem restoration while a final study factored in the known threats of increasing nutrient pollution with human development and increasing rates of sea level rise. In combination, these studies provide new perspectives on the interacting controls of carbon and nitrogen processes in freshwater wetlands that inform their responses to ecosystem modification.

## CHAPTER 1 GENERAL INTRODUCTION

### **Motivations for dissertation research**

Nutrient cycling of carbon and nitrogen indirectly supports the production of all valued ecological goods and services, and further supplies specific provisioning, regulating, and cultural services that directly promote human well-being (MA 2005). Despite these benefits, the supply of these services is shrinking due to a growing human population and more loss of natural resources than their gain (Costanza *et al.* 2014). For instance, wetlands lost over half their land area in the contiguous United States in the 200 years since European settlement, a trend that continues today (Dahl 1990; Dahl 2011), reducing the provision of flood protection, water filtration, shore stabilization, and groundwater recharge, among others.

Because of the difficulty in recognizing the value of natural resources, quantitative links between ecological structure and function and ecosystem services, which directly benefit people, better communicates the tenable dependence of society on nature (*e.g.*, Compton *et al.* 2011). Such an ‘ecosystem service’ approach provides a “common language” for stakeholders to use in decision-making so that definable changes to ecosystem states are considered in alternative management scenarios (Granek *et al.* 2009). As this approach grows in interest (*e.g.*, U.S. EPA 2011), so too will the need for science. Recently, the National Research Council (2013) recommended an ecosystem service approach to assist with the natural resource damage assessment of the Deepwater

Horizon oil spill. Soon after in 2015, President Obama issued a memorandum (M-16-01) that directed federal “agencies to develop and institutionalize policies to promote consideration of ecosystem services.” These directives and guidance create an urgent challenge for ecologists to improve predictions of carbon and nitrogen cycles with greater understanding of their patterns and controls. While many supporting functions related to nutrient processing, such as soil formation or primary productivity, benefit people only indirectly and are harder to communicate, the regulation of these functions still assists managers in achieving other ecosystem services often sought through restoration.

Due in large part to regulation, restoration and conservation of wetlands and floodplains are commonplace in the United States. At a federal level, Section 404(b)(1) of the Clean Water Act requires compensatory mitigation of wetlands within U.S. waters, which is complemented by additional federal and state laws. These laws provide long-term stability to the growing restoration economic sector, in which wetland and aquatic-riparian restoration contributes the most economic activity (31%) (BenDor *et al.* 2015). Research finds that wetlands rarely achieve functional equivalency with their natural counterparts immediately after restoration, and instead, decades are needed for maturity to develop through plant succession and soil formation (Ballantine and Schneider 2009; Hossler *et al.* 2011; Moreno-Mateos *et al.* 2012; Meli *et al.* 2014). Similarly, stream restoration projects fail to recreate or sustain floodplain habitats due to difficulties modeling the stochasticity of storm flows and sediment movement (Densmore and Karle 2009). Research on all aspects of restoration, from planning, managing, and engineering,

are needed to further restoration goals to promote the highest value from ecological resources.

Research suggests that one restoration site design strategy, the initial establishment of plant community diversity, might jumpstart successful trajectories of wetland ecosystem development for the provisioning of services (Bouchard *et al.* 2007). Biodiversity has been linked to ecosystem multi-functionality (Lefcheck *et al.* 2015), and the diversity of plant communities in particular can have large consequences for ecosystem integrity by increasing ecological functioning, efficiency, and stability (Cardinale *et al.* 2012; Reich *et al.* 2012; Tilman *et al.* 2013; Handa *et al.* 2014). The benefits of plant diversity on ecosystem functioning derive from two related and co-occurring processes: the theory of complementarity suggests that greater plant diversity promotes greater ecosystem functioning, such as primary productivity, through efficient use of niches (*e.g.*, plants of different sizes, shapes, and phenological cycle); by contrast, the selection theory describes how greater diversity increases the likelihood that high yielding plants proliferate with overall net positive effects on productivity to the plant community (Loreau and Hector 2001).

Both Chapters 2 and 3 present research from an experiment testing the effect of plant diversity on ecosystem functioning in freshwater wetland mesocosms. The first study of this dissertation provides a test for the theory that macrophyte diversity promotes biomass production, a proxy for primary productivity, in addition to investigating the balance between complementarity or selection mechanisms. Though research has demonstrated the existence of these effects, the majority of studies have not been

conducted in wetlands and results in wetlands may be conditional to experimental design or species composition (Balvanera *et al.* 2006; Doherty *et al.* 2011; Shultz *et al.* 2012). The second study addresses the extended theory that plant diversity not only promotes greater plant community productivity, but also other ecosystem functions, *i.e.*, nitrogen cycling (Tilman *et al.* 1996; Grime 1998). Though plants sequester N, theory and empirical evidence suggest that plant biomass production benefits soil nutrient cycling with inputs of labile carbon that fuel microbial metabolism (Knops *et al.* 2002; Ballantine *et al.* 2014). In wetlands, the vegetation community also strongly impacts the balance of aerobic and anaerobic processes (DeMeester and Richter 2010). Because of the multiple plant community effects on nitrogen processing, the second study attempts to identify causal pathways between plant richness, biomass production, and nutrient use on nitrogen processing through the mediating effects to soil conditions (*e.g.*, nutrient pools).

Full understanding of the suite of benefits of plant diversity on ecosystem services is necessary for the realization of multiple management goals, for understanding tradeoffs in competing restoration outcomes (*e.g.*, Jessop *et al.* 2015), and for quantifying uncertainty. Research on how planting diversity affects the capacity for soil nitrogen processing in young soils compared to mature wetlands will inform the development of pre- and post-restoration objectives to remove nitrogen (*i.e.*, through denitrification), a function directly linked to human well-being. By contrast, primary productivity directly supports the provisioning, regulating, and cultural categories of ecosystem services, and may broaden the suite of ecological benefits derived from planting diversity, as tested in Chapter 3. As planning for a diverse wetland community from the start of restoration is

common to meet bioassessment standards after establishment, research linking the dual goals of an early diverse plant community with its water-cleansing capacity would inform planting strategies.

For regional-scale management, such as for the Chesapeake Bay watershed, restoration projects are carried out with considerations beyond the structural or functional characteristics of the biological community. In a highly coordinated, long-term, and transparent effort between states and the federal government (Chesapeake Executive Council 2014), state compliance with the Chesapeake Bay total maximum daily load (TMDL) standards drives the restoration and expansion of floodplain wetlands across the watershed. States are mandated under section 303(c) of the Clean Water Act to clean up waterways failing to meet the established TMDLs. For the Chesapeake Bay, many years of monitoring data were needed to build and revise a set of linked models for the entire watershed that currently sets nutrient (*i.e.*, N and P) and sediment pollution limits per state and jurisdiction (Tango and Batiuk 2013). While progress has been made on a suite of objectives, more efforts will be needed to achieve desired goals by 2025 which should impart a net economic benefit of \$22.5 billion to the region in ecosystem services if fully implemented (Harding *et al.* 2016; Phillips and McGee 2016). Conserving, restoring, and creating floodplains and wetlands are part of a comprehensive strategy to reduce these pollutants (Richardson *et al.* 2011). While TMDLs identify the universe of streams in need of water quality improvements, many considerations are involved in locating specific streams for restoration. In the 2008 joint regulation passed by the EPA and ACE, “Compensatory Mitigation for Losses of Aquatic Resources” (40 C.F.R. 230.73,

p.19594), site selection for restoration should be undertaken to broaden the positive impacts of a restored wetland on the surrounding region and increase the likelihood of long-term functioning (Environmental Law Institute 2014). Elements of this “Watershed Approach” include assessing watershed needs and regional scale outcomes, parsing suitable from available candidate sites, and site prioritization (Environmental Law Institute 2014).

The entire site selection process would benefit from information on which environmental factors promote broad-scale denitrification, the primary nitrogen transformation that permanently removes nitrogen from the biosphere. In Chapter 4, the third study of this dissertation involves the investigation of controls on patterns of the microbial process across the Chesapeake Bay watershed at regional scale. Local predictors of denitrification variability in floodplains include soil physicochemical and vegetation characteristics (Burgin *et al.* 2010, Sutton-Grier *et al.* 2013, Ballantine *et al.* 2014, Palta *et al.* 2014). Soil and vegetation characteristics, along with denitrification, are controlled by hydrogeomorphic processes shaping channels and floodplain structure, which are in turn affected by upland land use and its effects on water quality (Osterkamp and Hupp 2010, Shrestha *et al.* 2014, Harvey and Gooseff 2015, McMillan and Noe 2017). Finally, some empirical evidence and macro-system paradigms suggest climate and the underlying geomorphic characteristics of the stream network affect all of these ecosystem properties and processes (Pinay *et al.* 2007, Groffman 2012; McCluney *et al.* 2014). To assist with the prioritization of floodplains for restoration, this study focused on identifying relationships of denitrification with unique emergent characteristics (*i.e.*,

having a spatial scale of interaction greater than the biogeochemical and vegetation processes in floodplain soils) such as hydrogeomorphic, climate, and landscape geomorphic or geologic characteristics, for which there are current or emerging publicly available datasets (Carbonneau *et al.* 2012).

Chapter 5 continues the investigation of controls of denitrification in the Chesapeake Bay watershed in tidal freshwater forested wetlands (TFFWs). These wetlands and basic biogeochemical nutrient processes (*e.g.*, denitrification) are included in the Bay Estuarine Model (<http://www.chesapeakebay.net/>) for Chesapeake Bay nutrient tracking. Factors driving patterns of denitrification in these wetlands are poorly understood (Ensign *et al.* 2008; Marton *et al.* 2012; Franklin *et al.* 2017), and research at landscape scales would inform our understanding of nitrogen flows for the Bay. Longitudinal, upriver-downriver gradients, are prominent controls on hydrogeomorphic processes in estuaries as they affect the relative influence of freshwater and tidal flows, water quality, and sedimentation. The fourth study of this dissertation investigates the differences in denitrification and its limitation by nitrate in TFFWs across two rivers carrying different N loads, the Pamunkey and Mattaponi Rivers, and across a longitudinal gradient, including nontidal wetlands near the head-of-tide.

Tidal wetlands are highly susceptible to multiple disturbances from climate change (*e.g.*, warming temperatures and acidifying waters), with sea level rise and salinity intrusions threatening to diminish habitat suitability for whole plant communities. This vulnerability is heightened for the Chesapeake Bay with higher rates of sea level rise than the global average (Eggleston and Pope 2013; Boesch *et al.* 2013). A contraction of

coastal wetland acreage is expected where landforms or development prevent landward migration or surface elevation building processes, *e.g.*, primary productivity and accumulation of organic matter and sediment, failing to keep pace with rising sea levels and subsurface subsidence (Spencer *et al.* 2016; Jankowski *et al.* 2017). Freshwater tidal forested wetlands, compared to tidal marshes, may be at greater risk from the suite of changes in salinity, hydroperiod, and sedimentation because they are sensitive to infrequent storm-related salinity incursions (Middleton *et al.* 2016), they accumulate less sediment (Ensign *et al.* 2014), and their surface elevation gain is modest – though still great enough to confer resilience (Stagg *et al.* 2014). To the extent that spatial patterns can be substituted for temporal trends, understanding differences in nitrogen removal at landscape scale informs predictions of coastal wetland response to climate change and eutrophication with information on TFFW functionality and its implied consequences for water quality.

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## CHAPTER 2 DOMINANCE BY AN OBLIGATE ANNUAL AFFECTS THE MORPHOLOGICAL CHARACTERISTICS AND BIOMASS PRODUCTION OF A PLANTED WETLAND MACROPHYTE COMMUNITY

### **Abstract**

Biodiversity-ecosystem function experiments can test for causal relationships between planting diversity and community productivity. Planting diversity is routinely introduced as a design element in created wetlands, yet substantive support for the finding that early diversity positively affects ecosystem functioning is lacking for wetlands. We conducted a two-year diversity-productivity experiment using freshwater wetland mesocosms to investigate community biomass production as affected by planted macrophyte functional richness. A richness gradient of macrophytes in four emergent wetland plant functional groups was established in freshwater mesocosms for two consecutive years. Species-specific aboveground morphological traits of plant size were measured at peak growth in both years; rooting depth was measured for each species in the second year. Above- and belowground biomass was harvested after peak growth in the second year; first year aboveground biomass was estimated from morphological traits in constructed regression equations. Net richness effects (*i.e.*, both complementarity effects and selection effects) were calculated using an additive partitioning method. Species richness had a positive effect on community aboveground biomass relative to monocultures in the first year. In the second year, mean aboveground biomass was significantly reduced by competition in the most species-rich mixtures and all mixtures

underyielded relative to the average monoculture. Competition for soil resources was weaker belowground, whereby root distribution at depths >20 cm was reduced at the highest richness levels but overall belowground biomass production was not affected. Changes in species biomass were strongly reflected by variation in species morphological traits, and species above- and belowground performances were highly correlated. The obligate annual (*Eleocharis obtusa*), a dominant competitor, significantly contributed to the depression of perennial species' growth in the second growing season. To foster primary productivity with macrophyte richness in early successional communities of created wetlands where ruderal strategies are favored and competition may be stronger than species complementarity, unsystematic planting designs such as clustering the same or similar species could provide protection for some individuals. Additionally, engineering design elements fostering spatial or temporal environmental variability (*e.g.*, microtopography) in newly created wetlands helps diversify the responses of wetland macrophyte species to their environment and could allow for greater complementarity in biomass production.

## **Introduction**

Accumulating evidence suggests that the trends in local and global biodiversity loss will negatively impact important ecosystem processes within the carbon cycle (Handa *et al.* 2014; Hooper *et al.* 2012; Isbell *et al.* 2011; Reich *et al.* 2012). Between 2004 and 2009, the USA lost 62 300 acres of wetland habitat and its associated biodiversity in the conterminous USA from wetland conversion (Dahl 2011). Where

wetland habitat was or will be re-established from compensatory creation or restoration, reductions in naturally occurring diversity levels and primary production may still be sustained for decades to come (Ballantine and Schneider 2009; Dee and Ahn 2012; Stefanik and Mitsch 2012). Introducing plant structural and functional diversity early in these new wetlands ecosystems, a common strategy for restoration practitioners, may accelerate the re-initiation of carbon-related ecosystem functions, such as primary productivity (Clewell and Aronson 2008). Using manipulated biodiversity–ecosystem function experiments, we can test for causal relationships between planting diversity and primary productivity and gain insight into the facilitative and competitive interactions that drive community dynamics through species-level investigations (Naeem 2006).

Within a biodiversity–ecosystem function paradigm, positive plant diversity–productivity relationships are a product of one or more of the following mechanisms of species coexistence: niche partitioning, facilitation and the presence of species with unique traits and relative abundances (Hooper *et al.* 2005; Loreau *et al.* 2012; Tilman *et al.* 2001). Species groups that can more fully extract available resources by exploiting a greater number of niches than groups with fewer species can lead more diverse communities to higher productivity (Cardinale 2011; Gross 2007). Alternatively, interspecific processes by which species directly or indirectly facilitate the growth of neighboring species can promote greater community productivity (Bertness and Hacker 1994; de Kroon *et al.* 2012; Le Bagousse-Pinguet *et al.* 2012; Vanellander *et al.* 2009). Where species demonstrate concomitant high productivity, competitiveness and dominance, positive diversity–productivity relationships are supported by individual

functional traits (Fox 2005; Wardle 1999). The additive partitioning method by Loreau and Hector (2001) mathematically operationalizes these mechanisms into emergent effects at the community level—complementarity effects, encapsulating predominant niche partitioning and facilitative processes and selection effects, reflecting species unique influences—and has permitted greater investigation and understanding of plant diversity dynamics. Cardinale *et al.* (2007) synthesized previous research in plant communities and found that mixtures were on average 1.7 times more productive than the average monoculture due to selection effects and to equal or greater contributions of complementarity effects. Many researchers have been interested in the relative balance between these two components, particularly with an interest in finding evidence for long-term structuring forces. Theory and empirical evidence suggest that the importance of opportunistic, single-species processes should give way to more stabilizing forces as plant communities mature and reach carrying capacity (Fargione *et al.* 2007; Pacala and Tilman 2002; Turnbull *et al.* 2013; Weis *et al.* 2007). Yet, the suite of factors that affect group complementarity, such as the right combination of species, the magnitude of species or functional richness and the response variable of interest, frustrate our abilities to predict the direction of strengthening multispecies interactions (*e.g.* complementarity, interference) (Balvanera *et al.* 2006; Doherty *et al.* 2011; Hooper and Vitousek 1997). In a study using freshwater-planted mesocosms, Bouchard *et al.* (2007) found that species functional group richness positively affected community belowground biomass (BGB) while not influencing net aboveground biomass (AGB). In another wetlands mesocosm study, Schultz *et al.* (2012) found greater biomass at the highest functional group richness

level though this effect was not present for AGB in mixtures at lower richness levels. Other studies in wetlands have also demonstrated the existence of positive diversity–productivity relationships but inconsistent incremental changes from one richness level to the next (Callaway *et al.*, 2003; Sullivan *et al.*, 2007). Due in part to a paucity of research conducted in wetlands, further investigation of the nature of macrophyte diversity effects on primary productivity is needed.

Species morphological and functional traits related to growth, competition and life history strategy may help explain and formulate hypotheses on the general drivers of community-level productivity. The utility of functional trait–productivity relationships, however, is dependent on the consistency of species interactions across abiotic and biotic gradients. Both above- and belowground strategies to acquire resources determine a plant’s ability to coexist with neighbors. For instance, spatial and phenological differences in shoot or root morphology may support complementarity in realized niche space through the evasion of light or nutrient competition (Dimitropoulis and Schmid 2004; Fargione and Tilman 2005; Hutchings and de Kroon 1994; Wacker *et al.* 2009). The importance of either mode of competition depends on how plant size and form affects shading and on levels of belowground resources, but whether or not an interaction exists between plant above- and belowground competitive strategies contributes to the uncertainty in species functional performances and their effect on community productivity (Bessler *et al.* 2009; Kiaer *et al.* 2013). Similarly, morphological plasticity as observed between mixtures and monocultures may help a species preemptively acquire limiting resources (Berendse 1982; Barnes *et al.* 1990; Schmid and Bazazz 1994). A

positive change in morphological traits in the presence of neighbors, such as height, canopy area and leaf shape may indicate a competitive effect of a species and explain its resulting dominance in mixture (Gaudet and Keddy 1988); however, whether these competitive behaviors maintain functional yield levels may be species-specific (Thein *et al.* 2008). Tracking the commonality of species morphological changes and biomass allocation to community productivity can improve our understanding of selection and complementarity effects observed at the community level.

We conducted a 2-year diversity–productivity experiment with freshwater wetland mesocosms using a replacement series design to investigate changes in plant community performance across a macrophyte richness gradient. Our primary goal was to study the link between the functional performances of species and the biomass production of the community across two major axes of variation in diversity–productivity experiments: duration of study and medium of plant interaction. We first investigated whether species morphological characteristics and biomass production varied by growing season and differed above- and belowground. We chose a root core sampling method that would specifically allow us to determine the consistency in species above- and belowground functional responses. We then linked the species performance to changes at the community-level using an additive partitioning method. We finally asked whether a suite of commonly measured aboveground morphological traits (universal and specific) could be useful predictors of both species and community biomass production across a gradient of species richness. Specifically for created wetlands characterized by lower functionality in carbon-related properties (Hossler and Bouchard 2010), this study will

improve our understanding of macrophyte community productivity and its relevance to carbon processing. The practical application of our results will also inform restoration strategies intended to promote productivity in planted macrophyte communities in newly created wetlands.

## **Materials and methods**

### **Experimental Design**

The experiment was conducted using a set of 34 research mesocosms, 568 L Rubbermaid tubs with a 1.11 m<sup>2</sup> surface area each, that sit aboveground in the Ahn Wetland Mesocosm Compound at George Mason University, Fairfax campus. Mesocosms were bottom filled to 20 cm with layers of first river pea gravel and then sand, and topped with 30 cm of locally produced screened, silty-loam topsoil (1.5% total carbon and 0.11% total nitrogen composition) from the Stone Center in Manassas, VA. Retention of or amendments with topsoil are common practices in wetland creation in the Virginia Piedmont physiographic region and are implemented to augment soil nutrient pools which are often limiting in these new ecosystems (Bruland and Richardson 2004; Stauffer and Brooks 1997). Water levels were determined by precipitation events and were periodically supplemented with dechlorinated tap water in the hottest weeks of summer to maintain a minimum of 5 cm standing water.

In early May 2012, mesocosms were planted with four plugs in a linear array using a combination of four functionally distinct herbaceous wetland plant species: *Eleocharis obtusa* (Willd.) Shult. (obligate annual), *Mimulus ringens* L. (facultative annual), *Juncus effusus* L. (interstitial reed) and *Carex vulpinoidea* Michx. (interstitial

tussock) (Boutin and Keddy 1993). Species functional trait differences increase the likelihood that a species combination will use resources more efficiently and maximize community performance (Diaz and Cabido 2001). When selecting planting diversity, classifications of functional attributes can be useful tools to simplify the complexity of plant species' ecological roles on multiple scales. The planting density, appropriate for ~1 m<sup>2</sup> mesocosms, was chosen to encourage maximum growth of species and be realistic of planting schemes used for restoration (Ahn and Mitsch 2002). All plugs were of similar size at the start of the experiment. Each of the four species was assigned two monocultures, for eight mesocosms at the lowest richness level (one functional group represented: FG 1); FG 2 had all unique two-species combinations with six mesocosms; FG 3 had all unique three species combinations with 12 mesocosms; and FG 4, the highest richness level, had eight mesocosms with all species represented. Although the treatment effect of species richness was not independent of species composition, the functional performance and contribution of each species to mixture biomass production could be tracked with adequate replication. The constructed species functional richness gradient was preserved by weeding.

Freshwater herbaceous wetland plant species were selected with two criteria in mind, that they be commonly found in or commonly sowed in created mitigation wetlands in the Virginia Piedmont, and that they be classifiable within either a ruderal or interstitial functional group. Species displaying characteristics of the matrix functional group were excluded from the experiment due to their aggressive growth (Bouchard *et al.* 2007). Both ruderal species flowered in the first growing season and completely died

back aboveground in the nongrowing season demonstrating annual behavior. Reeds and tussocks are classified as interstitial perennials, a group distinguished by low percent flowering in the first year and a clumped growth form with some lateral spread (Boutin and Keddy 1993). Both *J. effusus* and *C. vulpinoidea* first flowered at the start of the second growing season. *Carex vulpinoidea*, fox sedge, was not part of the original 43 species classification but displays the morphological and phenological traits characteristic of tussocks.

### **Morphological measurements and biomass**

Morphological traits of plant size were selected for each species based on their unique growth form and were measured once each growing season in late July or early August (Table 2.1). For instance, ‘basal circumference’ was measured for *E. obtusa* in the first year because the planted plugs ‘tillered’ outward in discrete clumps, the size of which reflected increased somatic growth; in the second year, all *E. obtusa* growth was either second or third generation population growth and discrete clumps were gone. Cover was measured as the presence or absence within linked 7 cm length square quadrats. Peak biomass was used as a proxy of plant productivity and was harvested in early September 2013 in the second growing season. All AGB was cut at the soil surface and weighed by species in the compound ( $\pm 10$  g). Subsamples (~100–300 g) of the species biomass were dried at  $\leq 60^\circ\text{C}$  to a constant weight, and dry/wet ratios were used to derive the total dry mass (DM) of species biomass. Belowground sampling immediately followed aboveground harvesting. Using 7.62 cm diameter soil core samplers (steel duct pipes), one sample from the original location of each planted plug (=

4 cores per mesocosm) was taken to a depth of 30 cm and partitioned at 10 cm intervals. The cores fell within or encompassed the basal area of each planted individual. Root sections were washed and sieved to 2 mm and dried at  $\leq 60^{\circ}\text{C}$  to a constant weight (Bledsoe *et al.* 1999). Intact, recently dead tissue that clearly resembled the live tissue in shape and size (and not amorphous organic debris) was retained. Each core was predominately comprised of one species so we assigned the BGB in each core to the planted individual sampled. Our sampling scheme provides species specific information but may overstate the BGB estimates for the reed and sedge whose root densities were likely highest directly beneath their culm clusters. Community AGB and BGB, from species data aggregated to the mesocosm level, were scaled to  $1 \text{ m}^2$ , with BGB values weighted by species relative aboveground percent cover.

The productivity and morphological traits of both species and communities were of interest. At a species level, the focus of analysis was the planted individual of the species, defined here as the growth attributable to or partitioned between the four originally planted plugs per mesocosm. An individual assessment permitted examinations of trends in species-level performance across richness levels accounting for differences in species relative abundances, as well as provided a cross-walk to the partitioning of species richness effects on the basis of species relative yields in biomass production (see ‘Partitioning effects of macrophyte richness’ section). To visually compare the magnitude of species relative yields in mixtures using a common metric, we used the related calculation for proportional deviation which standardizes the change in yield by the expected value:

$$D_i = (O_i - E_i)/E_i$$

where  $D_i$  is the proportional deviation in a species biomass production on the basis of the original planting density of individuals,  $E_i$  is the expected biomass production of a species on the basis of monoculture production and the number of individuals of that species planted and  $O_i$  is the observed biomass production in mixture.

We also investigated the predictive power of species morphological traits on species and community AGB with standard multiple linear regression equations. All measured traits were included in multiple regression equations of species AGB in the second year. First-year (Yr1) peak AGB of each species was estimated from these regression equations constructed from second year (Yr2) data (See Morphological traits predict community biomass production). We pooled species data independently of richness level to estimate Yr1 AGB. The community AGB prediction equations were constructed using the most diverse mesocosms (FG 4), which had a sufficient number of replicates, using one trait per species. Traits were first screened for a significant and strong correlation (Pearson  $r > 0.7$ ) with their respective species' AGB in FG 4 mesocosms.

### **Partitioning effects of macrophyte richness**

Mixtures were assessed for differences in species interactions and resulting community performance using an additive partitioning method (Loreau and Hector 2001) where net richness effects on mixture productivities are split into complementarity and selection effects (terms 1 and 2 on the righthand side, respectively):

$$\Delta Y = N \overline{\Delta RY} \overline{M} + N \text{cov}(\Delta RY, M) ,$$

where  $\Delta Y$  = Net effect of richness on biomass yield (g DM/m<sup>2</sup>); N =Number of species;  $\Delta RY$ = Deviation from expected relative yield of species i;  $M_i$  = Monoculture biomass of species i; Cov (a,b)= Covariance. The net richness effect (NE) equals the difference between the observed and expected biomass for a mixture. Any mixture that exceeds (or falls below) the average monoculture production nontransgressively overyields (or underyields). Transgressive overyielding, in the case where mixture biomass exceeds the highest producing monoculture, is distinguished as a stronger measure of community performance (Hector *et al.* 2002a). Positive selection effects occur when species with above-average biomass in monoculture overyield (*i.e.* positive proportional deviations in biomass production); negative selection effects occur in the reverse scenario where species with below-average biomass in monoculture overyield. A positive complementarity effect indicates that, on average, resource partitioning or facilitation is significant enough to cause species overyielding and elevated community performance; a negative complementarity effect indicates that, on average, species were inhibited by their neighbors and the performance of the community suffered.

### **Data analysis**

Standard multiple regression equations using species morphological trait predictors were constructed and were used to estimate Yr1 species AGB and Yr2 community AGB. Morphological trait data were first screened for multivariate outliers, and then for multivariate normality, linearity and heteroscedasticity. Morphological traits, species AGB and BGB and community AGB and BGB were assessed for mean differences between richness levels (FGs 1–4). Partitioned richness effects (CE, SE, NE)

of species above- and belowground yields were assessed for mean differences across mixture richness (FGs 2–4). One-way analysis of variance (ANOVA) was used to determine significance of mean differences. Data were screened for normality with the Shapiro–Wilks test and homogeneity of variance with the Levene test, and with consideration of the central limit theorem and sample size. When data exhibited unequal variance, Welch (Fw) test of equality of means was used. We tested post hoc pair-wise differences with the Bonferroni and Games–Howell post hoc tests, for equal and unequal variance, respectively. Transformations were used to improve normality. All statistical tests were run in SPSS statistics software v.18 (SPSS 2009) and assessed at an  $\alpha = 0.05$ .

**Table 2.1** Statistical results for tests of differences in morphological traits and aboveground biomass for the Sedge, Obligate Annual, Reed, Facultative Annual, and the Community between richness levels (FGs 1-4)

Species Traits	First Year				Second Year			
	Test	Statistic	df	p	Test	Statistic	df	p
<i>C. vulpinoidea</i> (S)								
Canopy Diameter (cm)	<i>F</i>	2.51	3,30	.078	<i>F</i>	45.1	3,30	<.001**
Canopy Height (cm)	<i>F</i>	1.62	3,30	.206	<i>F</i>	10.4	3,30	<.001**
Basal Circumference (cm)	<i>F</i>	2.07	3,30	.126	<i>F</i>	6.16	3,30	.002**
Cover (cm <sup>2</sup> )	<i>F</i>	.937	3,30	.435	<i>F</i>	5.34	3,30	.005**
Aboveground Biomass (g)	<i>F</i>	5.90	3,30	.324	<i>F</i>	11.1	3,30	<.001**
<i>E. obtusa</i> (OA)								
Canopy Height (cm)	<i>F<sub>w</sub></i>	.598	3,18	.625	<i>F<sub>w</sub></i>	1.22	3,18	.331
Basal Circumference (cm)	<i>F</i>	7.23	3,30	.001**	-	-	-	-
Cover (cm <sup>2</sup> ) †	<i>F<sub>w</sub></i>	1041	3,12	<.001**	<i>F<sub>w</sub></i>	36.3	3,7	<.001**
Aboveground Biomass (g)	<i>F<sub>w</sub></i>	961	3,14	<.001**	<i>F<sub>w</sub></i>	40.7	3,12	<.001**
<i>J. effusus</i> (R)								
Stem Length (cm) ‡	<i>F</i>	1.02	3,30	.397	<i>F</i>	1.31	3,30	.291
Basal Circumference (cm)	-	-	-	-	<i>F</i>	.972	3,30	.419
Stem Count	<i>F</i>	2.81	3,30	.056	-	-	-	-
Cover (cm <sup>2</sup> )	<i>F<sub>w</sub></i>	1.60	3,12	.240	<i>F</i>	12.2	3,30	<.001**
Aboveground Biomass (g)	<i>F</i>	.579	3,30	.633	<i>F<sub>w</sub></i>	9.72	3,13	<.001**
<i>M. ringens</i> (FA)								
Stem Length (cm)	<i>F</i>	5.93	3,30	.003**	<i>F</i>	12.1	3,30	<.001**
Stem Count	<i>F</i>	2.10	3,30	.121	<i>F</i>	4.40	3,30	.011*
Cover (cm <sup>2</sup> )	<i>F<sub>w</sub></i>	20.6	3,12	<.001**	<i>F</i>	67.9	3,30	<.001**
Aboveground Biomass (g)	<i>F</i>	5.90	3,30	.003**	<i>F<sub>w</sub></i>	108	3,13	<.001**
Mesocosms (C)								
Aboveground Biomass (g/m <sup>2</sup> )	<i>F<sub>w</sub></i>	.738	3,13	.548	<i>F</i>	3.68	3,30	.023*

†Square-root transformed. ‡Estimated from an average of 20 randomly selected stems. \**p* values significant at  $\alpha = .05$ . \*\**p* values significant at  $\alpha = .01$ .

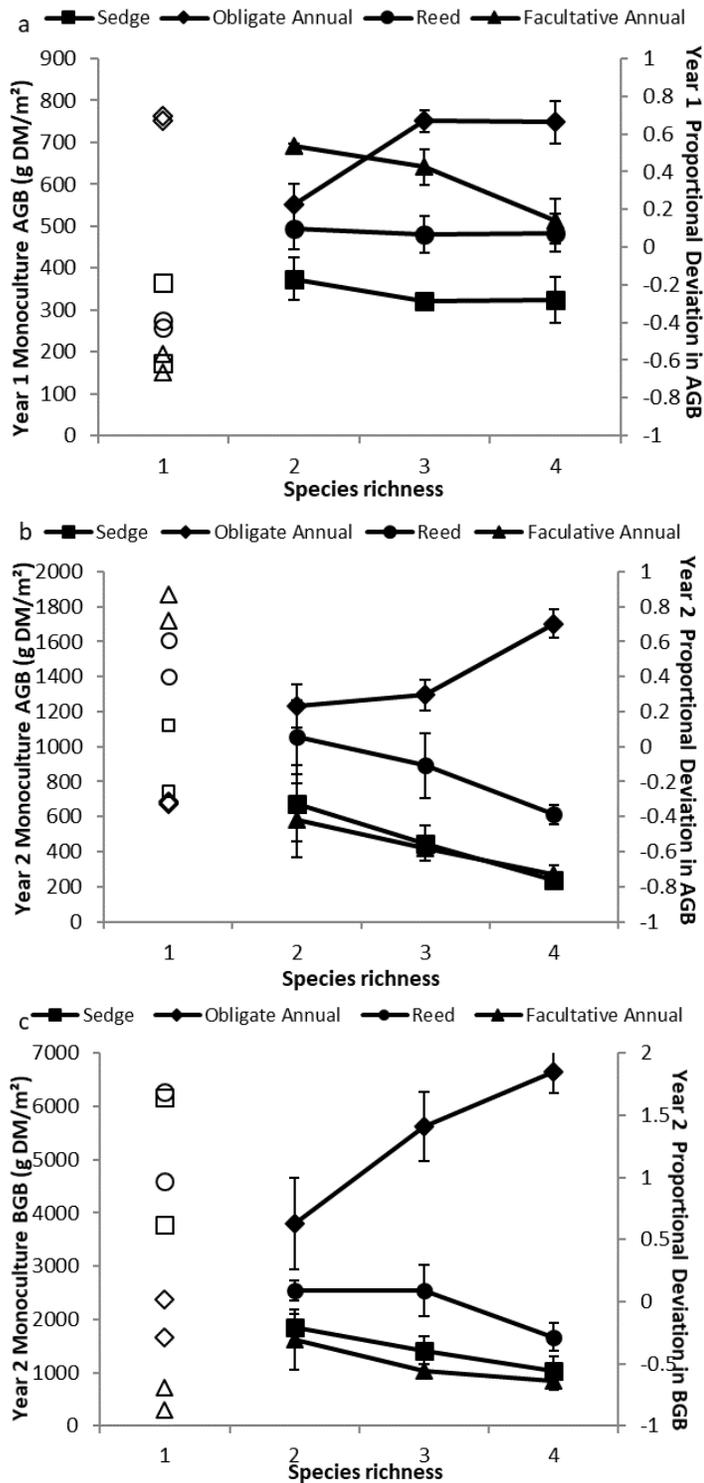
## Results

### Species morphological traits and biomass

Proportional to their original planting density, two of the four species performed better in mixture than in monoculture in the first year (Table 2.1; Fig. 2.1a). *E. obtusa* increased AGB in all mixtures, cover in FG 4, and its ‘basal circumference’ in FG 2 and

FG 4 as a result of tillering compared to in monoculture. At each richness level, *E. obtusa* also produced the most biomass of any species. *M. ringens* achieved greater AGB in FG 2 and FG 3 than in monocultures, as well as greater average stem length in FG 3 and FG 4 and greater cover for FG 2. *J. effusus* exhibited a trend-wise increase in the number of stems in FG 2 mixtures ( $P = 0.056$ ), but otherwise exhibited no substantial changes in morphological traits or AGB. *C. vulpinoidea* became slightly leaner across richness levels as it decreased in canopy diameter and AGB but these relationships were not statistically significant.

In the second year, fitness levels of all species except *E. obtusa* were adversely affected by increasing community richness (Tables 2.1 and 2.2; Fig. 2.1b and c). The AGB of *M. ringens* decreased in all mixtures compared to in monoculture, in addition to mean declines in FG 4 of stem height, stem count and cover. The BGB of *M. ringens* was also reduced in FG 4 compared to in monoculture, with a similar trend at the shallowest soil depth (0–10 cm). The disparity between *M. ringens*' AGB and BGB can be partly attributed to its extensive adventitious roots deployed above the soil surface in standing water that were counted towards AGB. *C. vulpinoidea* reduced horizontal spread in all mixtures, basal circumference in FG 4, cover in FG 3 and vertical height and AGB in FG 3 and FG 4 compared to in monoculture. *C. vulpinoidea* also exhibited a substantial decline in BGB with  $-56\%$  in FG 4 compared to monoculture, though this difference was not significant. *J. effusus*' AGB was lower in FG 4 than in monocultures, but this variation by richness level was not reflected by changes to its basal circumference, cover,



**Figure 2.1** Aboveground and belowground biomass by monoculture and proportional deviation in biomass of mixtures from monocultures. Primary axis: mesocosm biomass of species monocultures for species richness=1. Secondary axis: the mean proportional deviation,  $D_i$ , of species biomass in mixtures for species richness levels 2-4 expressed as a deviation from the expected biomass of that species on the basis of the original planting density of individuals.  $D_i > 0$  where species produced more biomass in mixture than expected from monoculture and  $D_i < 0$  where species produced less biomass in mixture than expected from monoculture. Open (primary axis) and closed (secondary axis) symbols represent *C. vulpinoidea* (sedge), *E. obtusa* (obligate annual), *J. effusus* (reed), and *M. ringens* (facultative annual) and are consistent between the two axes. Bars represent  $\pm 1$  standard error.

stem length, or BGB. The difference in the reed's root expansion at 10–20 cm depth reflects a difference between the highest two richness levels. In contrast, *E. obtusa* increased its AGB and cover in FG 4 compared to FG 1. Similarly, *E. obtusa* increased its BGB in FG 3 and FG 4 compared to in monoculture, which was reflected at 0–10 cm and 10–20 cm depths.

Species' above- and belowground responses to community richness in the second year were highly correlated (Fig. 2.1b and c). The obligate annual substantially increased its relative contribution to community biomass production from 14% (AGB) and 16% (BGB) in monoculture to 41% (AGB) and 48% (BGB) in FG 4 mesocosms, with a moderately strong correlation between AGB and BGB proportional deviations ( $r = 0.50$ ,  $P = 0.02$ ). The sedge and facultative annual both reduced their aboveground and belowground relative contributions to community biomass production, with moderately strong ( $r = 0.66$ ,  $P = 0.001$ ) and strong ( $r = 0.80$ ,  $P < 0.001$ ) correlations, respectively. The reed's contribution to community biomass production was less variable across richness levels but the correlation in proportional deviations in AGB and BGB remained strong ( $r = 0.79$ ,  $P < 0.001$ ); thus, species richness had a weak negative effect on the BGB of the reed. As such BGB:AGB ratios varied little across richness levels for the reed [ $F(3,30) = 0.820$ ,  $P = 0.493$ ], sedge [ $F(3,30) = 1.656$ ,  $P = 0.198$ ] and facultative annual [ $F(3,30) = 0.930$ ,  $P = 0.438$ ], and we found no evidence of a shift in biomass apportioning for these species when considering total biomass (Fig. 2.2). The obligate annual was the exception to this pattern with a significant change in BGB:AGB [ $F(3,30) = 3.538$ ,  $P = 0.026$ ] (Fig. 2.2). *E. obtusa* shifted its apportionment of biomass

belowground in the most diverse mixtures, where its BGB:AGB in FG 4 mesocosms (5.0) was almost twice that of its ratio in monoculture (3.1).

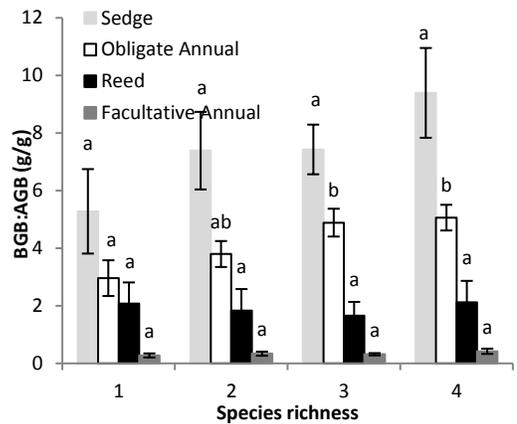
### **Community biomass**

Estimated Yr1 AGB of mesocosms did not differ by richness level (Fig. 2.3; Table 2.1). In the second year, plants achieved greater size and maturity increasing mesocosm AGB by 487 g DM/m<sup>2</sup> on average. Mean Yr2 AGB decreased with richness levels, with 530 g DM/m<sup>2</sup> less in FG 4 mesocosms than in monocultures on average (Fig. 2.3; Table 2.1). Most mesocosm BGB, 94% of roots, was distributed in the top 10 cm of soil, with 3.5% and 2.5% at 10–20 and 20–30 cm depths, respectively. A decline in root distribution to the deepest depth range was found with increasing richness (Table 2.2); FG 4 mixtures were reduced in BGB at this depth compared to FG 2 mixtures and were trend-wise but not significantly reduced by a mean (median) of 77% (64%) compared to monocultures. No differences in overall mean Yr2 BGB (>3000 g DM/m<sup>2</sup>) (Fig. 2.3) or the apportionment of AGB and BGB [ $F(3,30) = 1.476, P = 0.241$ ] were found across richness levels.

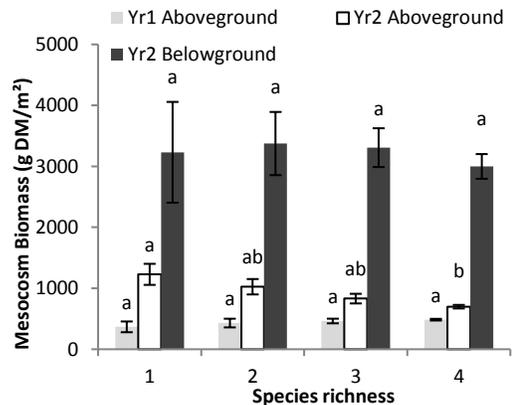
**Table 2.2 Statistical results for tests of differences in belowground biomass by depth range for the Sedge, Obligate Annual, Reed, Facultative Annual and the Community between richness levels (FGs 1-4)**

Soil Depth	Test	Statistic	df	p
<i>C. vulpinoidea</i> (S)				
≤30 cm	F	1.01	3,30	0.400
0-10 cm	F <sub>w</sub>	2.19	3,15	0.131
11-20 cm	F <sub>w</sub>	1.80	3,14	0.192
21-30 cm	F	1.42	3,30	0.256
<i>E. obtusa</i> (OA)				
≤30 cm	F	9.13	3,30	<0.001**
0-10 cm	F	13.1	3,30	<0.001**
11-20 cm	F	3.62	3,30	0.024*
21-30 cm	F <sub>w</sub>	.066	3,13	0.977
<i>J. effusus</i> (R)				
≤30 cm	F	1.01	3,30	0.400
0-10 cm	F	.764	3,30	0.523
10-20 cm	F	3.16	3,30	0.039*
20-30 cm	F	1.22	3,30	0.319
<i>M. ringens</i> (FA)				
≤30 cm	F	3.18	3,30	0.038*
0-10 cm	F	3.26	3,30	0.035*
10-20 cm	F <sub>w</sub>	.827	3,12	0.503
20-30 cm	F	.393	3,30	0.759
Mesocosms (C)				
≤30 cm	F <sub>w</sub>	.254	3,14	0.857
0-10 cm	F <sub>w</sub>	.220	3,14	0.881
10-20 cm	F <sub>w</sub>	.942	3,14	0.448
20-30 cm	F <sub>w</sub>	4.14	3,14	0.027*

\*p values significant at  $\alpha = .05$ . \*\*p values significant at  $\alpha = .01$ .



**Figure 2.2 Mean Yr2 BGB to AGB ratio for *C. vulpinoidea* (sedge, light grey), *E. obtusa* (obligate annual, white), *J. effusus* (reed, black), and *M. ringens* (facultative annual, dark grey) across species richness levels 1-4. Bars represent  $\pm 1$  standard error. Treatments not sharing a letter significantly differ at  $p < .05$ .**



**Figure 2.3 Mean estimated Yr1 aboveground biomass (white), Yr2 aboveground biomass (black) and Yr2 belowground biomass (grey) by diversity level (FGs 1-4) for mesocosms (MC). Bars represent  $\pm 1$  standard error. Treatments not sharing a letter significantly differ at  $p < .05$ .**

**Table 2.3 Species Yr2 aboveground biomass standard multiple regression equations constructed from morphological trait predictors for the sedge (*C. vulpinoidea*), obligate annual (*E. obtusa*), reed (*J. effusus*), and the facultative annual (*M. ringens*)**

Species	Regression Equation	$R^2_{adj}$	$F (df)$	$p$
<i>C. vulpinoidea</i>	$AGB_{\ddagger} = .078(CD) + .106(CH) + .085(BC) + 6.651(Cv) - 7.353$	0.85	47.46 (4,29)	<0.001**
<i>E. obtusa</i>	$AGB_{\ddagger} = .480(Cv_{\ddagger}) + 2.017$	0.60	51.14 (1,32)	<0.001**
<i>J. effusus</i>	$AGB_{\ddagger} = .012(SL) + .558(Cv) + .886$	0.57	23.00 (2,31)	<0.001**
<i>M. ringens</i>	$AGB_{\ddagger} = .007(SH) + 1.996(Cv) + .003(SC) + 1.167$	0.88	83.87 (3,30)	<0.001**

$\ddagger$ square-root transformed.  $\ddagger$   $\log_{10}$  transformed. \*\* $p$  values significant at  $\alpha = .01$ . Abbreviations: AGB= aboveground biomass (g); CD=canopy diameter (cm); CH=canopy height (cm); BC=basal circumference of clumped growth (cm); Cv=cover estimate of canopy spread (cm<sup>2</sup>); SL= mean stem length (cm); SH=mean stem height (cm); SC=stem count.

**Table 2.4 Best-fitting aboveground biomass multiple regression equations for the most species rich community (FG 4) constructed from species morphological trait predictors in the second year**

Y =	<i>C. vulpinoidea</i> + <i>E. obtusa</i> + <i>J. effusus</i> + <i>M. ringens</i> + Intercept	$R^2_{adj}$	$F (df)$	$p$
AGB $\ddagger$	$0.002(CD) + 0.192(Cv_{\ddagger}) + 0.005(BC) + 0.354(Cv) + 2.229$	0.82	8.7 (4,3)	0.053
AGB $\ddagger$	$0.009(BC) + 0.191(Cv_{\ddagger}) + 0.001(BC) + 0.004(SC) + 2.205$	0.73	5.6 (4,3)	0.094
AGB $\ddagger$	$0.002(CD) + 0.203(Cv_{\ddagger}) + 0.005(BC) + 0.002(SC) + 2.214$	0.86	12.1 (4,3)	0.034*

$\ddagger$  $\log_{10}$  transformed.  $\ddagger$ square-root transformed. \* $p$  values significant at  $\alpha = .05$ . Abbreviations: AGB=aboveground biomass (g/m<sup>2</sup>); BC=basal circumference of clumped growth (cm); CD=canopy diameter (cm); Cv=cover estimate of canopy spread (cm<sup>2</sup>); SC=stem count.

### Morphological traits as predictors of community biomass production

Most morphological traits significantly contributed to species-level multiple regression prediction equations of Yr2 AGB (Table 2.3). Cover was the most versatile predictor of AGB. Canopy and stem, height or length, were useful for all species except *E. obtusa*, who's variation in canopy height (46–50 cm) across richness levels was negligible. Species-specific predictors included canopy diameter and basal circumference for *C. vulpinoidea* and stem count for *M. ringens*. Due to the good model fit of the AGB

regression equations, morphological traits were used to estimate Yr1 AGB of species and mesocosms.

Six morphological traits met the criteria for strong and significant correlations with species AGB in FG 4 mesocosms: stem count and cover for *M. ringens*; canopy diameter, cover and basal circumference for *C. vulpinoidea*; and basal circumference for *J. effusus*. Cover was used to represent the obligate annual in the community prediction equations because it was significantly related to AGB across all richness levels. We consider three of the six possible regression equations supportive, although two with greater uncertainty ( $P \leq 0.10$ ), of the hypothesis that species individual traits can be used to predict community productivity (Table 2.4).

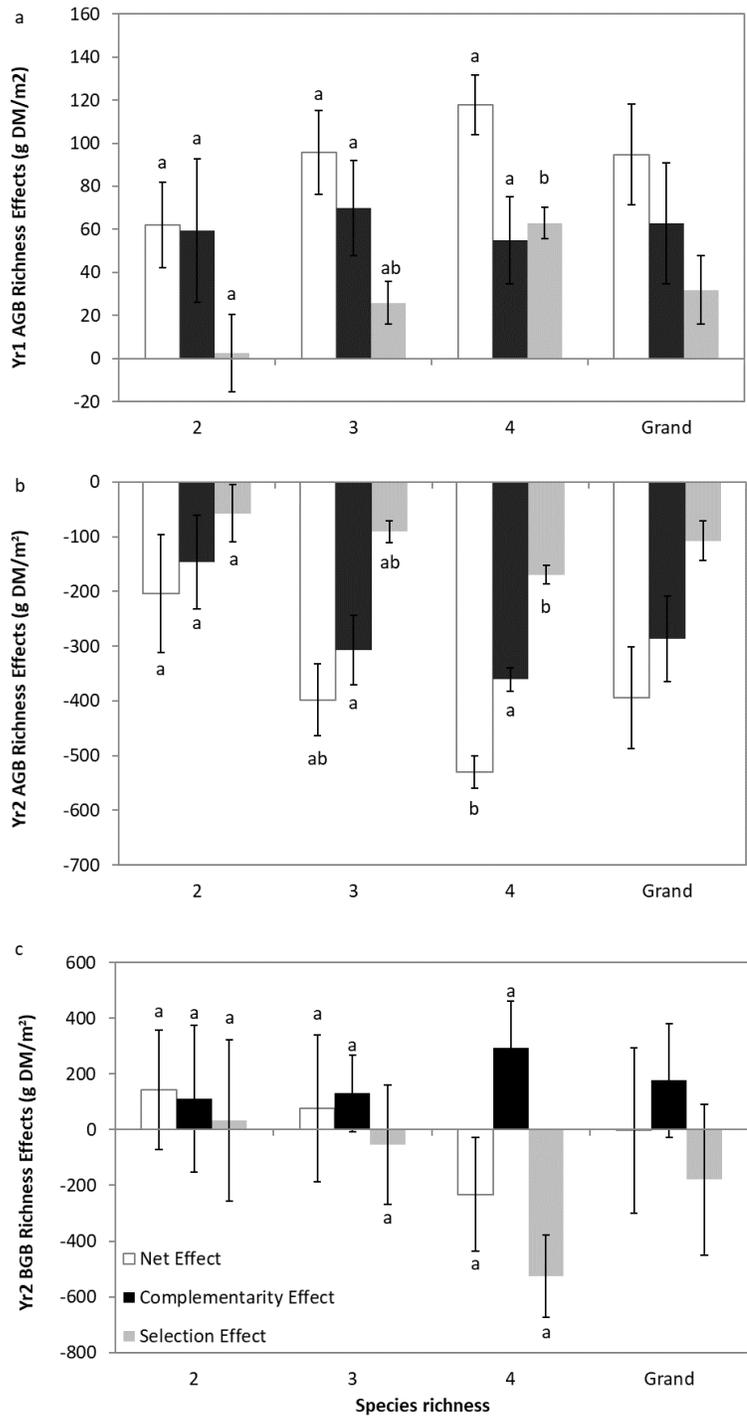
### **Partitioning richness effects**

All mean richness effects for Yr1 AGB were positive, where grand means for NE (94.8 g DM/m<sup>2</sup>), CE (62.9 g DM/m<sup>2</sup>) and SE (31.9 g DM/m<sup>2</sup>) and their 95% confidence intervals across all mixtures fell entirely above zero (Fig. 2.4a). Despite the insignificant ANOVA results for mesocosm AGB across richness levels, all mixtures but one (FG 3) nontransgressively overyielded and had higher Yr1 AGB yields than the average monoculture. Selection effects significantly increased from FG 2 to FG 4 [ $F(2,23) = 6.007$ ,  $P = 0.008$ ], at which point they were of similar magnitude to CE, while no changes across mixture richness were found for CE [ $F(2,23) = 0.111$ ,  $P = 0.895$ ] or NE [ $F(2,23) = 1.687$ ,  $P = 0.207$ ] (Fig. 2.4a). Any fluctuations around the point estimates of YR1 AGB for any of the species would not have changed the findings of a positive richness effect in the first year nor appreciably changed the strength of the effect. The obligate annual had a

large influence during the first year (Fig. 2.1a). Indeed, the three lowest yielding mesocosms at both FG 2 and FG 3 levels in the first growing season did not contain the obligate annual.

In the second growing season, all mean AGB richness effects for all richness levels were negative; grand means of NE ( $-394 \text{ g DM/m}^2$ ), CE ( $-287 \text{ g DM/m}^2$ ) and SE ( $-107 \text{ g DM/m}^2$ ) and their 95% confidence intervals all fell entirely below zero (Fig. 2.4b). A negative NE reflects lower average production in mixtures ( $-394 \text{ g DM/m}^2$ ) compared to the average monoculture. Negative interactions strengthened across mixture richness for NE [ $F(2,23) = 4.241, P = 0.027$ ] and SE [ $F(2,23) = 3.775, P = 0.038$ ], but not CE [ $\chi^2 = 4.061, P = 0.131$ ] (Fig. 2.4b). We examined the effect of *E. obtusa* on community productivity: Three mixtures, all without the obligate annual, displayed positive richness effects, while the other three mixtures with the obligate annual displayed all negative NE, CE and SE.

The overall richness effects for BGB were not significant: 95% confidence intervals for the grand means of NE ( $-3.23 \text{ g DM/m}^2$ ), CE ( $176 \text{ g DM/m}^2$ ) and SE ( $-180 \text{ g DM/m}^2$ ) all included zero (Fig. 2.3c). Neither were there significant relationships of NE [ $F(2,23) = 0.562; P = 0.578$ ], CE [ $F(2,23) = 0.307, P = 0.739$ ] or SE [ $F(2,23) = 1.663, P = 0.211$ ] for BGB across mixture richness levels (Fig. 2.4c). Although the mean BGB selection effect ( $-527 \text{ g DM/m}^2$ ) for FG 4 was significant (mean  $\pm 1.96$  standard error  $< 0$ ), its contribution to the NE was negated by the more positive CEs. The presence of the obligate annual reduced belowground total community productivity. The three mesocosms in both FG 2 and FG 3 that did not contain the obligate annual (*E. obtusa*) all



**Figure 2.4 Mean community net effect (white), complementarity effect (black), and selection effect (grey) at each mixture diversity level (FGs 2-4) and across all mixtures (Grand) for Yr1 aboveground biomass (a), Yr2 aboveground biomass (b), and Yr2 belowground biomass (c). Bars for diversity levels 2-4 represent  $\pm 1$  standard error; bars for grand mean represent 95% confidence interval. Treatments not sharing a letter significantly differ at  $p < .05$ .**

performed better on average: three non- *E. obtusa* mesocosms in FG 2 were among the four with highest BGB, and the three non- *E. obtusa* mesocosms in FG 3 all attained the highest BGB

## **Discussion**

### **Diversity–productivity relationships**

A large majority of plant diversity–productivity studies in experimental systems have found greater biomass production in more diverse (*e.g.* species and functional group richness) plant communities (Cardinale *et al.*, 2007). In our case, the slightly positive effects of plant functional richness on community biomass production in the first year were transient. We found that the positive effects of species richness in the first growing season, when mixtures produced 1.25 times greater AGB than monocultures, became negative in the second season, when monocultures produced 1.44 times more biomass than mixtures. The meta-analysis by Cardinale *et al.* (2007) revealed that the reported overall positive richness effect on productivity may mask more idiosyncratic (*i.e.* neutral to negative) results of certain underyielding mixtures at lower richness levels. The negative diversity effects found by Polley *et al.* (2003) in a study of a mixture of three annuals planted across varying densities and evenness corroborates this notion. These findings together with ours suggest that the diversity–productivity relationship is partly shaped by the number of species richness levels or the number of different communities studied. In spite of the inhibition present in our experimental mixtures, our study conforms to other established conceptual patterns from large-scale diversity–productivity experiments. One such experiment, a multi-site experiment in grasslands conducted by

Spehn *et al.* (2005), found that biomass production and diversity effects were usually lowest during the first year of the experiment and that diversity effects on AGB were stronger than BGB. Consistently, we found that the effects of richness on AGB strengthened in magnitude in the second growing season indicating that the size or maturity of plants more than the type of species interaction has a strong mediating influence on the diversity outcome. Additionally, overall richness effects were stronger aboveground than belowground in our study. Complementarity effects remained consistent across mixture richness levels and were either relatively equal to (Yr1 AGB and Yr2 BGB) or greater than selection effects (Yr2 AGB). The trends in strengthening selection effects, which occurred across richness levels in both years, relate to the performance of the obligate annual; however, *E. obtusa* had a strong but not singular influence on mixture performance. Large variation also existed for the other species' proportional yields in mixtures.

### **Species traits and interactions**

Similar to Thein *et al.* (2008), we found that species exhibited morphological plasticity in aboveground traits (*e.g.* stem and canopy height) between monocultures and mixtures and that the degree of consistency with changes in biomass production was species-dependent. Where biomass production was variable, species did respond with changes to morphology. Trait variability in the obligate annual (*i.e.* stem density) and the facultative annual (*i.e.* height) corresponded to changes in AGB in the first year. In the second year, many species' fitness levels were impaired and morphological plasticity corresponded with reductions in AGB for the facultative annual and sedge. The reed, by

contrast, maintained its basal circumference, cover and stem length while reducing overall AGB, possibly indicative of a competitive response in tolerance of competition and concomitant nutrient or resource deficiencies (Keddy *et al.* 1998).

Community richness had a weak effect on the BGB of mesocosms, whereby the most diverse mixtures distributed fewer roots at depths >20 cm than in monocultures. Plasticity in root distribution at this depth was not found at a species level, though all species exhibited a trend-wise reduction, but was an emergent attribute at the mesocosm level. In investigating whether species differences or plasticity in rooting depth in grassland plants could explain plant complementarity in belowground productivity, von Felton and Schmid (2008) found that having a sufficient volume of soil for root growth was a more important determinant of plant complementarity than soil depth, possibly due to the energetic constraints of resource extraction from deeper zones. In another study of natural root distribution and abundance in grasslands, Frank *et al.* (2010) concluded that root segregation played a minor role in species coexistence and that the majority of plant species under study were randomly distributed by depth. The lack of oxygen in the mesocosms—our wetland soils remained consistently anaerobic in the second year (authors' unpublished data)—might have contributed to less vertical root expansion overall.

In spite of decreasing rooting depth, no changes in BGB were observed at a mesocosm level. These results contrast with other studies in wetlands that have reported positive correlations between BGB and species richness (Bouchard *et al.* 2007; Callaway *et al.* 2003; Schultz *et al.* 2012), and specifically positive correlations between increasing

BGB and depth of root deployment (Bouchard *et al.* 2007). Here, we can look mechanistically at species contributions to BGB. Community richness negatively affected the BGB of the facultative annual, weakly negatively affected the sedge and reed and strongly positively affected the obligate annual. *E. obtusa* greatly expanded its BGB relative to AGB in mixtures (Fig. 2.3) and its proportional deviations belowground were almost twice that aboveground at the highest richness level (Fig. 2.1b and c). Species contributing most to BGB in the most diverse mixtures (~96% BGB), the sedge, reed and obligate annual, all exhibited greater complementarity (or weaker inhibition) belowground than aboveground.

Factors mediating biomass partitioning above- and belowground are not well understood but may be species-specific and depend on stress and medium of competition (Bessler *et al.* 2009; Burns and Strauss 2011; Kiaer *et al.* 2013). The reduction in AGB suggests that light or nutrient resources were constrained in FG 4 mesocosms. Comparisons to other natural and created freshwater wetlands indicate that the experimental mesocosms simulated soil conditions typical of created wetlands and can be classified as a low nutrient system on the basis of total carbon and nitrogen content (Bailey *et al.* 2007; Ballantine and Schneider 2009; Dee and Ahn 2012; Stauffer and Brooks 1997). While topsoil removal can be a successful approach to remediate effects of previous land use practices, such as fertilization or native plant seed bank depletion (Bakker 2013; Klimkowska *et al.* 2010), topsoil application in created and restored wetlands has been shown to have beneficial effects on the plant community (Stauffer and Brooks 1997). Thus, limitation in soil nitrogen or other nutrients might have contributed

to species above- and belowground performances in this study. Limited nutrients can promote symmetric root competition, whereby root foraging ability is linearly related to the volume of soil exploited, and may partly explain the lack of richness effects on community BGB (Frank *et al.* 2010; von Wettberg and Weiner 2003). By the same token, the obligate annual engaged in asymmetric competition by increasing its BGB:AGB ratio across the species richness gradient. A game theory model of annual plant behavior postulates that in the presence of competitors an annual will produce greater roots than optimal for reproductive fitness in a ‘tragedy of the commons’-type scenario (Gersani *et al.* 2001; O’Brien *et al.* 2005).

### **Morphological traits predict community biomass production**

Estimating peak AGB, BGB or other metrics of primary productivity using species morphological traits have a long tradition in plant ecology (Carpenter 1980; Dickerman *et al.* 1986; Mathews and Westlake 1969; Whigham and Simpson 1978; Wetzel and Pickard 1996) and can be beneficial to the ecosystem by reducing disturbance caused by destructive harvesting, particularly in the case of repeat evaluation. Biomass accumulation is a good indicator of wetland ecosystem development as it relates to plant-driven carbon processing, and can be used as success criteria in evaluating the functional maturation of compensatory wetland creation and restoration. Our biomass results comport with reported biomass values in the literature for herbaceous, emergent vegetation in freshwater nontidal marshes of similar hydrology of the current mesocosm experiment (Cole *et al.* 2001; Kao-Kniffen *et al.* 2010; Stefanik and Mitsch 2012; Wetzel and Howe 1999) and can be presumed to represent natural communities. We found tight

responses between morphological traits and AGB across a gradient of interspecific interactions holding abiotic factors constant. Traits such as cover, stem height or basal circumference contributed significantly to the equations and are easy and fast measurements to make. Counts of stem number are potentially strong predictors of AGB but can be laborious or impractical in many species for timely evaluations of plant performance.

We specifically investigated the ability to predict community biomass production using species traits. Three of the six regression equations were significant and with good model fit, demonstrating that morphological traits can be good predictors of both species and community AGB. Since more morphological traits were significant predictors of species-level AGB as generalized across richness levels, we infer that our morphological traits might be better predictors of community AGB with a greater range of species richness or abiotic variability. Our prediction equations constructed from varying morphological measures are specific to the plant community in this study but demonstrate that targeted species-dependent trait measurements may increase the accuracy of community productivity estimates. We also investigated root:shoot ratios and the explanatory power of AGB on BGB. Three of the species conserved their BGB:AGB ratios across the species richness gradient and all species had moderately strong to strong positive correlations in proportional root and shoot biomass deviations. That most of the species did not demonstrate plastic responses in biomass partitioning can be useful to managers who want to use aboveground performance or competition as a proxy for belowground interactions (Cahill 2002).

### **Competitive dominance**

Species dominance may exert a large influence on community condition through the suppression of other species, regulation of resource levels and control of nutrient cycling (Frieswyk *et al.* 2007). Multiple lines of evidence suggested that the dominance by the obligate annual partly contributed to the pervasive community interference in the second growing season. For instance, the absence of *E. obtusa* was associated with the highest BGB yields. In the most diverse mixtures, the obligate annual achieved the highest abundance and cover of any species. In the first growing season, *E. obtusa* accounted for an average of 68% of total species cover in the most diverse mixtures; in the second growing season, this number dropped to 46%, on average, compared to 18%, 12% and 7% on average for the reed, sedge and facultative annual, respectively. More evenness in cover, but not abundance, was found at FGs 2 and 3. The obligate annual also out-produced the other species in the most diverse mixtures in the second year while having below-average yields in monocultures, which suggests a trade-off in functional performance and competitive ability. We conclude that the community inhibition was not solely an artifact of having a lower yielding species in mixtures, but was primarily a result of asymmetric competition driven by *E. obtusa*.

Predictions of community productivity on the basis of species' monoculture yields are difficult in communities substantially dominated by few species, and more difficult if dominated by underyielding species, effects inconsistent with traditional sampling models (Hector *et al.* 2002a). The monoculture productivity in our study poorly corresponded to mixture productivity in the second year; instead, the functional traits related to growth rate were good indicators of a dominant competitor and of community

dominance. Dominant competitors have been shown to mitigate the positive interactions leading to higher species productivity (Engelhardt and Ritchie 2001; Hector *et al.* 2002b). Doherty *et al.* (2011) showed that species dominance across plots from a re-visited saltmarsh diversity study (Callaway *et al.* 2003) can eventually reverse early positive richness effects and lead to reduced performance in species-rich mixtures. In this study, dominance by a ruderal species suppressed community establishment from the beginning. Other wetland diversity studies using species of equivalent functional groupings to ours have nonetheless found positive diversity–productivity relationships in the second and third growing seasons of their experiments (Bouchard *et al.* 2007; Schultz *et al.* 2012). In particular, Schultz *et al.* (2012) found transgressive overyielding of diverse mixtures with a relatively small selection effect at the highest species richness level, which does not suggest species dominance and suppression of species performances. Our results may diverge from theirs because the treatment effect of richness in our study tested for differences between interspecific interactions in mixtures and intraspecific interactions in monocultures, and not for differences from interspecific interactions only. Additionally, obligate annuals were introduced to mixtures in the second growing season in Schultz *et al.*'s study to allow other functional groups to establish. Diversity effects have been found to be stronger with more numerous species (Schmid *et al.* 2009); planting multiple obligate annuals with equivalent functional abilities may have a positive effect on overall yields of young communities.

## **Implications for wetland creation**

The abilities to spread and generate more than one generation in a growing season are key traits that can be constraints on early planted wetland communities. In a disturbed community below ecological carrying capacity, early successional species can temporarily out-perform co-occurring later successional species through rapid growth (Pacala and Rees 1998). Successional niches describe a colonization–competition trade-off, or strategies to optimize seed production and establishment at the expense of growth and longevity. As an extension, here we show that strong interference and lower productivity may be expected in newly planted ecosystems where ruderal strategies are initially favored, a potentially important component shaping the relationships between diversity and community productivity. We also show that not all combinations of functionally diverse species promote community biomass production. Without replicating species within functional group richness levels, we are unable to conclude that these results are robust to community compositional changes; however, we described mechanisms responsible for negative diversity–productivity relationships that may operate in early wetland planting communities. Matthews and Endress (2010) found that site characteristics other than age in restored wetlands were better determinants of plant community succession from predominantly annuals to more clonal, perennial species. In particular, the authors found that under nutrient limiting conditions, annual or ruderal species maintained dominance in the restored communities into the fourth year.

The results of this study inform restoration practices intended to promote productivity in planted macrophyte communities in created wetlands. Whether seeded, planted, or recruited, *E. obtusa* and its ilk are likely to proliferate naturally in the first few

years until later successional species fully establish. Attempts to completely bypass this development stage by introducing perennials in the first growing season may be unsuccessful by themselves; rather, because this study demonstrated that species competed more strongly aboveground, restoration practices that alleviate uncontrollable shoot interference may be beneficial to community productivity. Less systematic planting designs such as clustering the same or similar species will vary the density and structure of plant canopies which could reduce widespread competition from monopolizing species (Twedt 2006). Alternatively, engineering design elements fostering spatial or temporal environmental variability (*e.g.* microtopography) in newly created wetlands helps diversify the responses of wetland macrophyte species to their environment (Moser *et al.* 2007) and could allow for greater complementarity in biomass production in light of competitive interference.

## **Conclusions**

We conducted a 2-year diversity–productivity experiment using freshwater wetland mesocosms to investigate biomass production as affected by macrophyte functional group richness that can be introduced as a design element in created wetlands. The positive effect of species richness on AGB was shown to be transient, and interspecific interference drove negative community dynamics in the second season. A dominant competitor, in this case a ruderal, annual species, disproportionately influenced community performance and inhibited community biomass in the second year. Good agreement was found between plant morphological trait plasticity and biomass production, as well as species above- and belowground performances across a species

richness gradient. Our study highlights the need for additional research on the functional performance of other potentially dominant species, such as matrix species not studied here, in the context of early planting designs that will help improve our understanding of plant community development and its impacts on ecosystem development in newly created wetlands.

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## **CHAPTER 3 RICHNESS, BIOMASS, AND NUTRIENT CONTENT OF A WETLAND MACROPHYTE COMMUNITY AFFECT SOIL NITROGEN CYCLING IN A DIVERSITY-ECOSYSTEM FUNCTIONING EXPERIMENT**

### **Abstract**

The development of soil nitrogen (N) cycling in created wetlands promotes the maturation of multiple biogeochemical cycles necessary for ecosystem functioning. This development proceeds from gradual changes in soil physicochemical properties and influential characteristics of the plant community, such as competitive behavior, phenology, productivity, and nutrient composition. In the context of a 2-year diversity experiment in freshwater mesocosms (0, 1, 2, 3, or 4 richness levels), we assessed the direct and indirect impacts of three plant community characteristics – species richness, total biomass, and tissue N concentration – on three processes in the soil N cycle – soil net ammonification, net nitrification, and denitrification potentials. Species richness had a positive effect on net ammonification potential (NAP) through higher redox potentials and likely faster microbial respiration. All NAP rates were negative, however, due to immobilization and high rates of ammonium removal. Net nitrification was inhibited at higher species richness without mediation from the measured soil properties. Higher species richness also inhibited denitrification potential through increased redox potential and decreased nitrification. Both lower biomass and/or higher tissue ratios of carbon to nitrogen, characteristics indicative of the two annual plants, were shown to have stimulatory effects on all three soil N processes. The two mediating physicochemical

links between the young macrophyte community and microbial N processes were soil redox potential and temperature. Our results suggest that early-successional annual plant communities play an important role in the development of ecosystem N multifunctionality in newly created wetland soils.

## **Introduction**

Wetlands promote biogeochemical pathways within the nitrogen cycle through a diversity of physicochemical properties and biological controls. Mature wetland soils feature a build-up of organic nutrient stores, specialized interactions between plants and microorganisms, and internal feedbacks buffering environmental disturbances which maximize the potential for N transformations (Mitsch *et al.* 2012; Ballantine and Schneider, *et al.* 2009; Tang *et al.* 2011). In newly created wetlands, cycling occurs in more homogenous soils and nitrogen cycling is under greater influence from the external environment (Anderson *et al.* 2005; Bruland and Richardson 2005). Plants exert a strong influence in these young soils by providing carbon subsidies, modifying soil structure, conserving N, and creating habitat for microbial communities (Morgan *et al.* 2008; Ruiz-Rueda *et al.* 2009; Forshay and Dodson 2011). The promotion of N biogeochemistry in created wetlands, such as the important soil processes of ammonification, nitrification and denitrification, requires greater understanding of how attributes of early plant communities affect multiple aspects of the soil N cycle.

Macrophytes are generally a positive long-term structuring force on soil N cycling, but the effect over shorter time-scales depends on both species individual traits and community diversity (Balvanera *et al.* 2006; Gutknecht *et al.* 2006). No clearly

positive or negative relationships have been established between species diversity and N processing in wetlands in part for this reason, as well as because of the inherent complexity of feedbacks between multiple trophic levels (*e.g.*, plants and microorganisms). Specifically for plant effects on soil N transformations, “noise” from conflicting indirect and direct effect mechanisms could contribute to the obscurity of diversity-ecosystem function relationships (Balvanera *et al.* 2006). For instance, macrophytes indirectly affect microbial N processing by regulating chemical conditions of the mediating environment (*i.e.*, soil N, carbon, oxygen, pH, and temperature) that adds variability to plant-microbe interactions (Booth *et al.* 2005; Laughlin 2011; Sutton-Grier *et al.* 2011). At the same time, plant species directly and differentially interact with soil microorganisms through resource competition and by hosting a variety of multi-functioning microbial communities in their rhizosphere (Månsson *et al.* 2009; Hu *et al.* 2014; Schlatter *et al.* 2015). Some of these effects are more immediately influential than others. To counter soil anoxia that creates strongly N-limiting conditions in wetlands, macrophytes release oxygen from their roots that has been shown to indirectly enable organic N mineralization, the aerobic production of nitrate, and the coupled anaerobic process of denitrification in adjacent soil zones (Reddy *et al.* 1989).

Two theories help explain how plant communities affect ecosystem function. The theory of species diversity indicates that multiple species promote ecosystem efficiency through greater non-additive complementary use of niche space and greater total impacts on biogeochemical cycling (Tilman *et al.* 1996; von Felten *et al.* 2009). The second theory, or the “mass ratio hypothesis”, asserts that other community functions are more

important such that positive effects on ecosystem functioning are proportional to dominant species' investments in primary production and are relatively insensitive to subordinate species impacts (Grime 1998). In the case of a diversity-ecosystem function experiment, employed for this study, these theorized mechanisms affecting ecosystem N functioning should co-occur in plant communities (Hooper *et al.* 2005; Mokany *et al.* 2008) and can be studied with investigations of manipulated plant richness alongside additional plant community attributes.

### **Mesocosm experiment**

The study was conducted in 40 aboveground mesocosms, 568 L Rubbermaid tubs with a 1.11 m<sup>2</sup> surface area each (long dimension = 147 cm, narrow dimension = 99 cm), located in the Ahn Wetland Mesocosm Compound of George Mason University, Fairfax Campus. In March 2012, mesocosms were filled with 20 cm of sand on top of river pea gravel, and topped with 30 cm of locally-produced topsoil commonly used in wetland creation in the Virginia Piedmont Physiographic Region. Water levels were influenced by precipitation events and were periodically supplemented with de-chlorinated tap-water in the hottest weeks of summer to maintain a minimum of 5 cm standing water above the soil surface (N loading rate was 1.3 µg N/m<sup>2</sup> yr in 2012 and 0.4 µg N/m<sup>2</sup> yr in 2013).

A richness gradient with four functionally distinct wetland macrophytes was established and maintained by weeding in a substitutive experimental design that varied species not plant density. Wetland macrophytes were classifiable within either a ruderal (*i.e.*, annual) or interstitial (*i.e.*, perennial) functional group: *Eleocharis obtusa* (Willd.) Shult. (obligate annual), *Mimulus ringens* L. (facultative annual), *Juncus effusus* L.

(interstitial reed) and *Carex vulpinoidea* Michx. (a sedge, herein classified as an interstitial tussock) (Boutin and Keddy 1993). In early May 2012, a combination of four similar-sized plugs were planted in a linear array in each mesocosm. Each of the four species was assigned two monocultures, for eight mesocosms at the lowest richness level; the second level had all unique two-species combinations once for six mesocosms total; the third level had all unique three-species combinations once with twelve mesocosms total; and the highest richness level had eight mesocosms with all species represented. Six mesocosms were left unplanted as an experiment control that provided baseline soil physicochemical conditions in the absence of macrophytes but in the presence of other colonizing or adventive species (*e.g.*, algae and invertebrates) and allochthonous litterfall. The shoots of the annuals died back completely over the winter between growing seasons, depositing all aboveground on the soil surface. Whereas the obligate annual is presumed to have demonstrated strict annual behavior and died completely, the facultative annual emerged early in the calendar year (approximately early March, before the official growing season in Virginia) which is taken as a sign of perennial behavior. The reed and fox sedge (*C. vulpinoidea*) remained partially green throughout the winter.

## **Methods**

### **Soil sampling and laboratory analysis**

Soil net ammonification potential (NAP) and net nitrification potential (NNP) were measured using laboratory incubations (Binkley and Hart 1989) following Hart *et al.* (1994). The procedure tracks the net change in mineralized N by measuring the initial and 28-day KCl-extractable ammonium and nitrate concentrations in soil cores. To more

closely reflect field rates, longitudinal sections of intact soil cores (*i.e.* soils were not mixed) were incubated in complete darkness in flasks partially open to the atmosphere. Soil moisture of the cores was maintained at field conditions by adding one or more drops of water each week to the surface of cores to counter any mass loss due to evaporation. In August 2013, three replicate soil samples per mesocosm were systematically collected from different areas outside of the centralized planting area down to 10 cm depth using 30 mL disposable syringes (2 cm diameter) that created suction. The soils were processed within 1-2 days of collection. Two 10 g dry-weight equivalent wet subsamples from half of each core split length-wise were each placed in 125 mL flasks, one for pre- and post-incubation extractions, respectively. Thus, the three replicates per mesocosm were not mixed, and the soils were not broken apart, prior to incubation. Day-0 soils were processed immediately while day-28 soils were incubated in the dark at 21°C. For both pre- and post-incubation extractions, soils were covered with 100 mL of 2M KCl and mixed with the extraction solution on a reciprocal shaker for an hour. The supernatant was syringe filtered to <0.45 µm and quantified for ammonium and nitrate (quantified as nitrate+nitrite) by colorimetric analysis on an Astoria-Pacific segmented-flow autoanalyzer. Net N mineralization potential (NMP) was calculated as the post incubation quantity of combined mineralized N (*i.e.* ammonium+nitrate+nitrite) less the initial quantity; the changes in ammonium and nitrate concentrations were used to calculate NAP and NNP separately.

Soil denitrification was measured as denitrification potential (DP) using the denitrification enzyme assay (Smith and Tiedje 1979; Tiedje *et al.* 1989) following

Groffman *et al.* (1999). Three soil cores were collected in each mesocosm using the same sampling method as used for NAP/NNP in August 2013. Analysis of soils occurred no later than 3 days after sampling. Soils were homogenized per mesocosm and then assessed for DP in triplicate. Field-moist soil (~25g ww) was weighed into 125 mL flasks and mixed to form slurries with 25 mL solutions of dextrose (1g/L), potassium nitrate (1.01g/L), and chloramphenicol (0.1 g/L) in deionized water. Acid-scrubbed acetylene (10 mL) was then injected into the flasks to inhibit the reduction of N<sub>2</sub>O at T<sub>0</sub>. Flasks were incubated in a reciprocal shaker and gas samples withdrawn at 45 and 105 minutes. Samples were determined for N<sub>2</sub>O (= N<sub>2</sub>O+N<sub>2</sub> production) by gas chromatography on a Shimadzu GC-8A equipped with a Supelco Hayesep Q 80/100 packed column (1 m x 1/8 in x 2.1 mm) and an electron-capture detector. Because leakage was detected in the gas-tight syringe used for sample injections towards the end of the gas chromatography runs, we only quantified DP in a subset of mesocosms comprised of five controls (unplanted control), five monocultures (richness level 1), and a total of four, seven, and four mesocosms in richness treatments 2, 3, and 4, respectively.

Physicochemical properties of the mesocosm soils that were possible correlates of N processes were measured in August 2013. Temperature readings were recorded continuously at 90 min intervals for the entire month using ibuttons (Embedded Data Systems) inserted 5-10 cm in the mesocosm soils under the plant canopy. Redox potential was measured *in situ* to 5 cm in triplicate using a handheld ORP redox meter (Extech). Soil samples were taken to 10 cm depth for additional analyses. Soil pH was measured with a Hach pH electrode in the laboratory with ~10 g of dry soil in a 1:1 soil to water

solution. Soil organic matter was measured by loss-on-ignition where soils dried to 105°C were combusted in a furnace at 550°C for 4 hours. Percent total carbon and total N of soil, and the derived mass ratio of C:N, were measured in triplicate by dry combustion using soils dried to 105°C in a Perkin Elmer 2400 Series II Element Analyzer. NAP, NNP, and DP were calculated on a dry mass basis ( $\mu\text{g N/kg soil}$ ). Soil for bulk density (cored using 59 mL metal containers) and moisture, which were collected on the same day as soils collected for denitrification, were dried at 105°C for 48 hours.

### **Biomass harvesting and tissue nutrient analysis**

Peak total (above- and belowground) biomass was harvested in the second growing season in early September 2013 after all soil sampling was completed. All aboveground biomass (AGB) was cut at the soil surface and separated by species, while four samples per mesocosm of belowground biomass (BGB) were taken from the original location of each planted plug down to 30 cm using 7.62 cm diameter steel duct pipes. Soils for nitrogen processing were collected only to 10 cm depth because 94% of BGB was found in this zone. Plant biomass, which was dried to a constant weight between 48 and 60°C, is presented as dry weight. Our sampling scheme provides species-specific information but may overstate the BGB estimates for the reed and sedge whose root densities were likely highest directly beneath their culm clusters (Korol and Ahn 2016). Community biomass, from species data aggregated to the mesocosm level, was scaled to 1 m<sup>2</sup>. Based on the assumption that the most probable location of roots was under the canopy area of their shoots (*i.e.*, where other species were not located), weighted multipliers derived from the relative aboveground percent cover were used to scale the

four individual BGB values. A subsample of the dried AGB and BGB plant tissue was ground in a steel Thomas Wiley Mini Mill and then analyzed for percent mass of carbon and N by dry combustion on a Perkin Elmer 2400 Series II Element Analyzer.

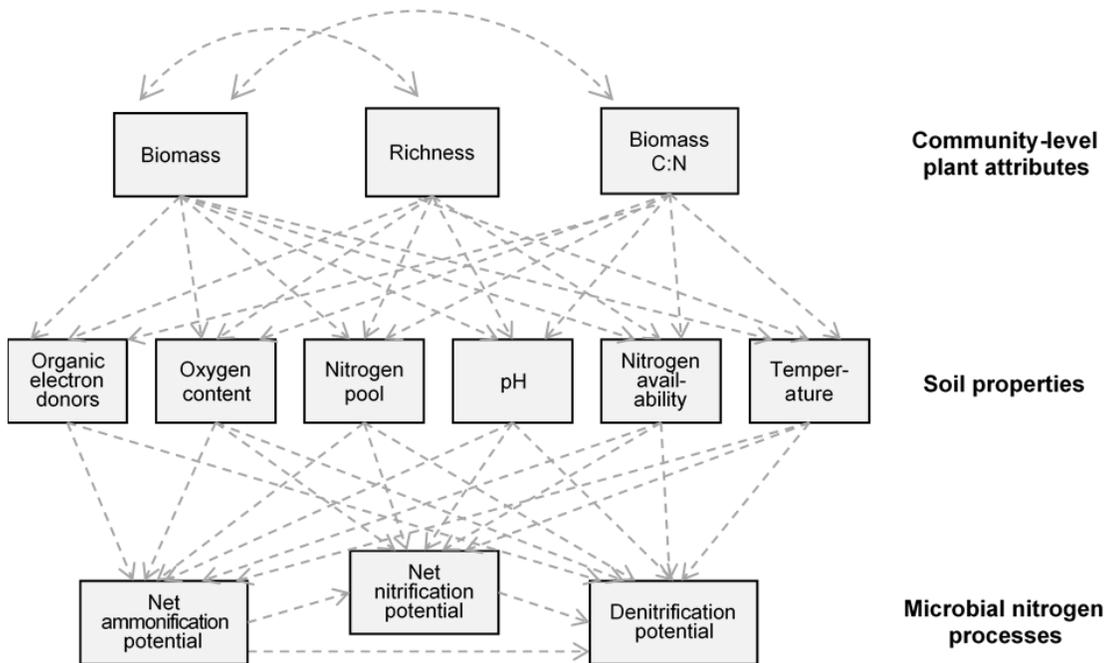


Figure 3.1 Conceptual model of hypothesized causal effects of three characteristics of the wetland macrophyte community on microbial nitrogen processing as mediated by soil physicochemical controls

### Conceptualizing macrophyte and soil ecosystem function links

Ecosystem functioning of the macrophyte and soil microbial communities are often linked without identification of the causal pathways. Our conceptual model (Fig. 3.1) displays the hypothesized hierarchical organization of plant-driven control on N cycling. Macrophytes are thought to affect N processing distally, primarily by influencing the proximate soil biogeochemical controls of microbially-mediated N mineralization and

denitrification. Physicochemical factors regulating NAP, NNP, and DP overlap but differ in regards to the metabolic needs of microorganisms. We used soil carbon and organic matter to model the availability and types of organic electron donors, soil N to model the pool of N substrate, and the soil C:N ratio to model N availability. In accordance with the mass ratio hypothesis and diversity theories, we expect a “plant effect” of rooted macrophytes on the soil properties and processes of interest to primarily reflect a combination of species functional group richness, total (above- and belowground) plant biomass, and tissue concentrations of carbon and N. To maintain focus on the relationship between plant characteristics and N processes, interactions between soil properties were not assessed.

Structural equation modeling (SEM) was used to test our conceptual model (Fig. 3.1) of the hierarchical causal network of macrophyte influence and soil physicochemical properties on soil N transformations. Direct effects were not included in the conceptual model but were necessary in constructing the final models (see section 3.4. Data Analysis). SEM, and specifically path modeling used herein, is a useful statistical technique for testing theoretical constructs of association between numerous predictor and response variables that conventional multiple regression is ill-equipped to handle without planned experimentation (Grace 2006). SEM is well-suited for experimental studies of plant diversity on ecosystem function where the confounding influence of multiple feedbacks (*e.g.*, microbial effects on plants) that weaken detection of macrophyte effects are minimized (Grace *et al.* 2007).

## **Data analyses**

Measured variables were assessed for the strength of linear relationships. All variables were linearly regressed against species richness levels, while plant and soil properties and processes were selectively assessed pair-wise for the strength of linear relationships using Pearson's correlation coefficient. Variables were screened for univariate linearity and residual normality and equal variance through visual assessment of residual scatterplots and q-q plots; Variables were transformed where necessary to improve these statistical assumptions. Univariate outliers ( $z$ -score  $> 3.29$ ) were changed to be less deviant (Tabacknik and Fidell 2013). For SEM, assumptions of multivariate linearity, normality, homoscedasticity were evaluated with scatterplots of standardized residuals and predicted values; Mahalanobis distances were calculated to identify multivariate outliers. Specifically for SEM, standardized residual covariances were screened for asymmetry and large values. Unplanted mesocosms were assessed qualitatively. Data screening and statistical tests were performed in SPSS v.18 software (SPSS 2009).

We used our conceptual model to guide the construction of one or more structural equation models for NAP, NNP, and DP, respectively. We followed a step-wise model generating procedure where functional attributes of the plant community and soil properties were first systematically assessed for statistical bivariate relationships with the N response variables; we then specified a multivariate model on the basis of theory and with the most significantly related variables where possible to produce a model most suitable to the observed data and with the highest explanatory power of NAP, NNP, and DP (Diaz *et al.* 2007). Model assessment of goodness-of-fit involved evaluation of the

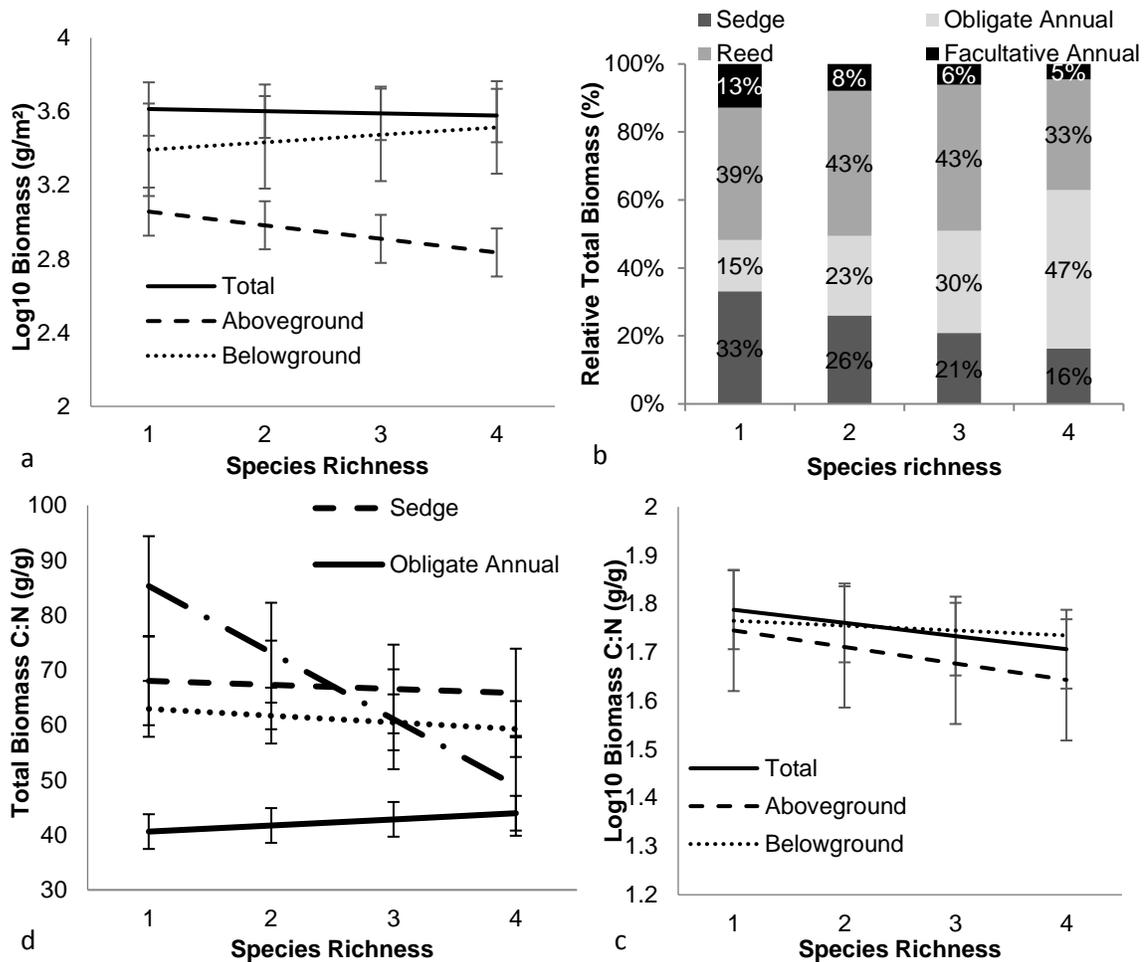
model  $\chi^2$  test, comparative fit index (CFI), and standardized root mean square residual (SRMSR) (Dimitrov 2012). We had to modify our initial models to include direct paths from plant metrics to N response processes to improve model fit. Sample-size adjusted Bayesian Information Criterion ( $BIC_{adj}$ ) was referenced in selecting the final model. Structural equation modeling was performed with Mplus v. 7.11 software (Muthén and Muthén 2013).

Missing-at-random data were present for DP, and three of the eight monoculture mesocosms (one each for *C. vulpinoidea*, *M. ringens*, and *J. effusus*) were not quantified. Due to the relatively high mean DP for the two *E. obtusa* monocultures, and the potential for bias considering the relatively lower and similar mean DP values for the other species in monoculture, missing data values for these three monocultures were estimated using multiple imputation (*i.e.* thus each species had mean DP values for two monocultures). Multiple imputation is considered the “gold standard” for estimating missing data with no subsequent loss of statistical power (Polit 2010). Multiple imputation was performed in Mplus software using all the variables considered for use in SEM and any others with significant correlation. The new variable was more conservative and used in all statistical tests (*i.e.*, correlation) and modeling (*i.e.*, SEM). Model results generated with the original (raw) and new (imputed) variable were compared for consistency.

## **Results**

### **Community and species performances**

A linear relationship between plant species richness and total biomass was not found ( $r^2=0.008$ ,  $P=0.610$ ; Fig. 3.2a); however, community AGB was negatively related



**Figure 3.2** Linear regression slopes of biomass and biomass C:N for the community and of the sedge (*C. vulpinoidea*), obligate annual (*E. obtusa*), reed (*J. effusus*), and facultative annual (*M. ringens*) across richness levels of the planted mesocosms: (a) community biomass production; (b) species relative contribution to total (above- and belowground) biomass production; (c) community biomass C:N; and (d) species total (above- and belowground) biomass C:N. Statistical tests performed on transformed data as presented. Error bars represent  $\pm 1$  standard error of the regression slope.

to species richness ( $r^2 = 0.288$ ,  $P=0.001$ , Fig. 3.2a). The reductions in AGB were not

reflected in total biomass in part because BGB remained consistent across species

richness ( $r^2=.031$ ,  $P=0.758$ ; Fig. 3.2a) and its variability around the mean was larger.

Species performance by richness levels varied (Fig. 3.2b). Relative contributions from *C.*

*vulpinoidea* (sedge) and *M. ringens* (facultative annual) to total biomass declined by an

average of -59% and -71% from monocultures to the most diverse mixtures, respectively, while the total biomass of *J. effusus* (reed) was roughly independent of richness levels. *E. obtusa* (obligate annual) exhibited a counter trend with a 156% proportional increase in biomass at the high richness levels over monocultures (Fig. 3.2b). Total (*i.e.*, above and belowground) biomass C:N was negatively related to species richness ( $r^2=0.123$ ,  $P=0.042$ ) (Fig. 3.2c), with no independent changes to the total pool of plant N ( $r^2=0.001$ ,  $P=0.856$ ) or carbon ( $r^2=0.019$ ,  $P=0.433$ ); aboveground ( $r^2=0.085$ ,  $P=0.094$ ) and belowground biomass ( $r^2=0.033$ ,  $P=0.301$ ) C:N ratios were not related to species richness. Only *M. ringens* demonstrated a change in total biomass C:N across richness treatments [ $r^2=0.627$ ,  $P<0.001$ ] (Fig. 3.2d).

The annuals markedly differed from interstitial perennials in their functional performances, which had a large influence on community functioning. From visual observation, all stems from the obligate and facultative annual, compared to portions of AGB from the reed and sedge, died back over the winter and were deposited on the soil surface. Second, the average monoculture total biomass in stands of *M. ringens* (2299 g/m<sup>2</sup>) and *E. obtusa* (2693 g/m<sup>2</sup>) were less than half that of *C. vulpinoidea* (5905 g/m<sup>2</sup>) and *J. effusus* (6940 g/m<sup>2</sup>). Total mesocosm biomass was thus negatively related to the number of annual species present ( $r^2=.334$ ,  $P<0.001$ ; Fig. 3.3a). Mesocosm biomass C:N also was influenced by the presence of annual species ( $r^2=0.199$ ,  $P=0.008$ ; Fig. 3.3b) and even more strongly by the presence of *E. obtusa* ( $r^2=0.677$ ,  $P<0.001$ ) because of the low C:N of *E. obtusa* and the stoichiometric plasticity of the *M. ringens* (Fig. 3.2d).

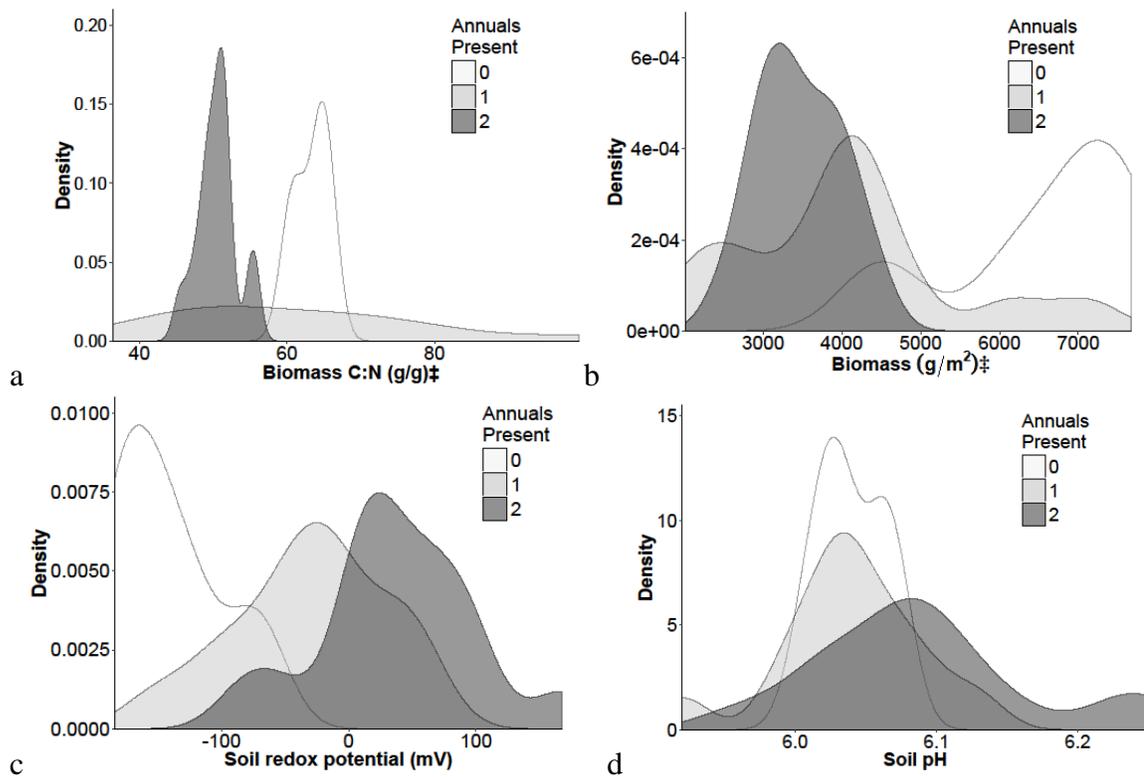
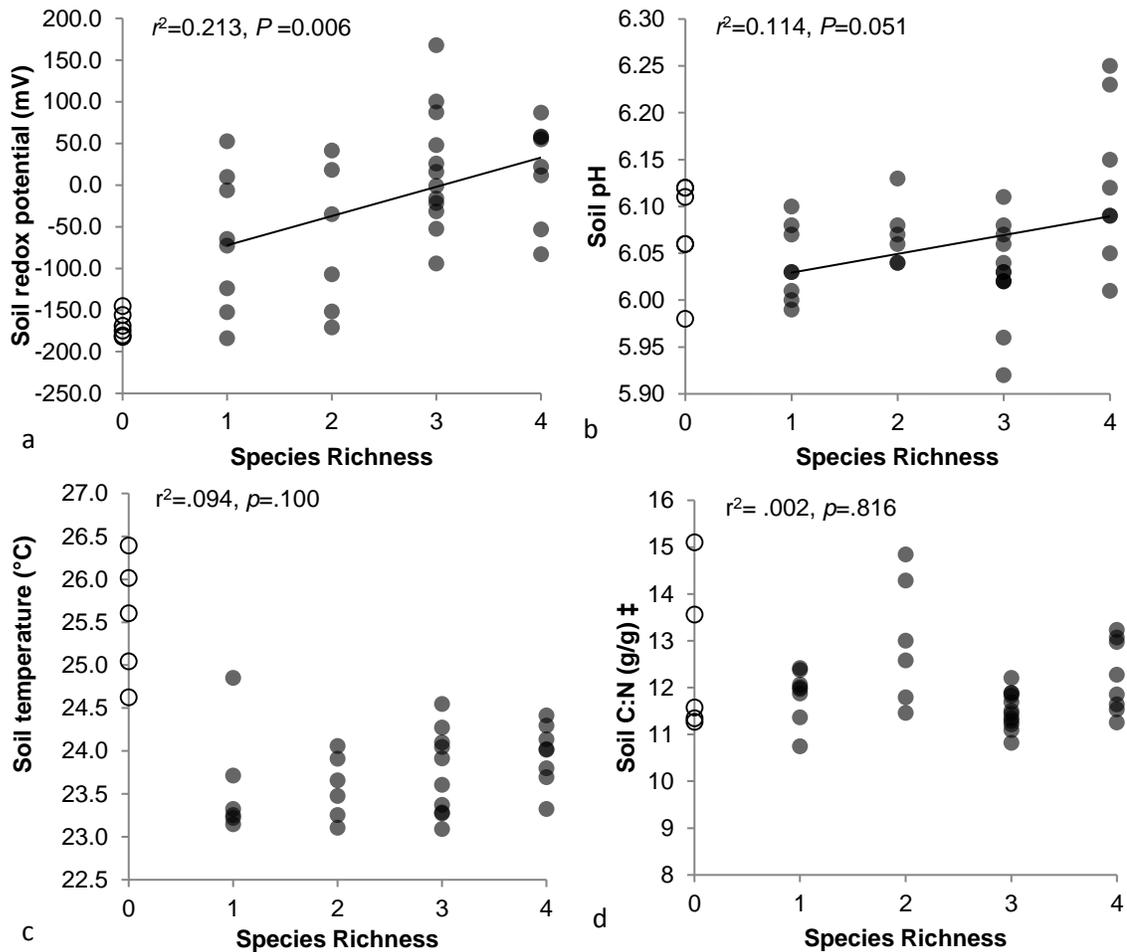


Figure 3.3 Density plots of mesocosm plant characteristics and soil properties partitioned by the number (0-2) of annual species (*E. obtusa* and *M. ringens*) present. Linear regression statistics not shown in figures. ‡Variable was log<sub>10</sub> transformed prior to statistical analysis.

### Soil physicochemical properties

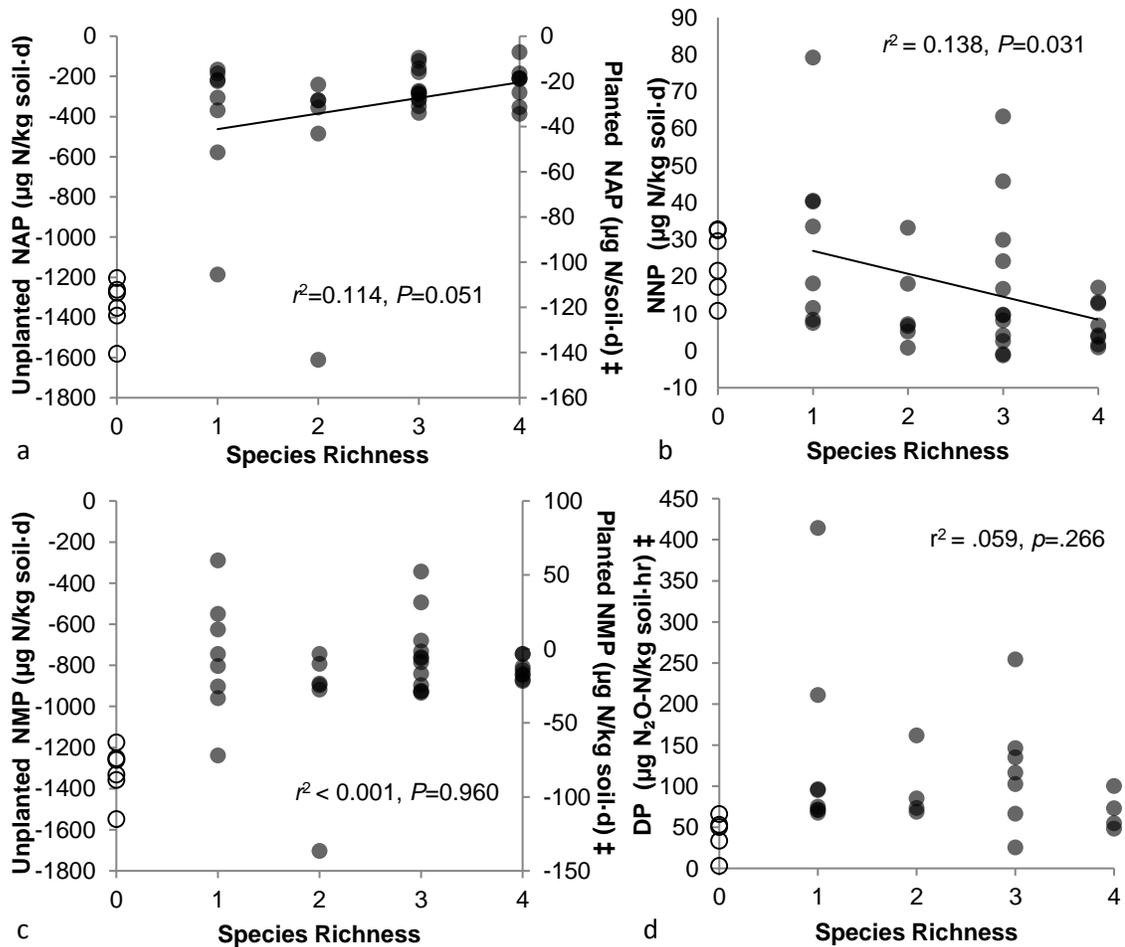
The soils of planted mesocosms had higher redox potentials than the unplanted controls due to plant root oxygenation (determined using iron sulfide (FeS) agar probes in June 2013; unpublished data). The facultative and obligate annuals had higher soil redox potentials on average (2 and -6 mV, respectively) than the reed and sedge (-98 and -170, respectively) in monocultures, which explained part of the positive correlation between soil redox potential and planted species richness (Fig. 3.4a). The higher soil redox is also likely due to the greater spatial spread of roots by the more spatially dispersed and numerous annual plants within the mesocosm. Soil pH of the planted mesocosms was



**Figure 3.4** Scatter plots of mesocosm (a) soil redox potential, (b) soil pH, (c) soil temperature, and (d) the ratio of soil carbon to nitrogen across the planted species richness gradient (black circles) and the unplanted controls (empty circles). Statistical results pertain to the planted mesocosms only. Statistically significant linear regression slopes at  $\alpha = .05$  are displayed. ‡Variable was log10 transformed prior to statistical analysis.

also positively associated with species richness (Fig. 3.4b), and both redox ( $r=0.723$ ,  $P<0.001$ ) and pH ( $r=0.334$ ,  $P=0.049$ ) were segregated on the basis of annual species (Figs. 3.3c, d). In contrast, soil temperature (Fig. 3.4c), organic matter (range: 5.2 to 5.9%;  $r= -0.109$ ,  $P=0.540$ ), carbon (range: 1.1 to 1.5%;  $r= -0.010$ ,  $P=0.955$ ), N (range: 0.09 to 0.12%;  $r= .035$ ,  $p=.845$ ), and C:N (Fig. 3.4d) were not affected by the planted richness level, though temperatures were higher by  $0.5^{\circ}\text{C}$  in mesocosms with the obligate

annual ( $r=0.498$ ,  $P=0.005$ ). Mesocosm biomass was negatively correlated with redox potential while biomass C:N was negatively correlated with pH and temperature (Table 3.1).



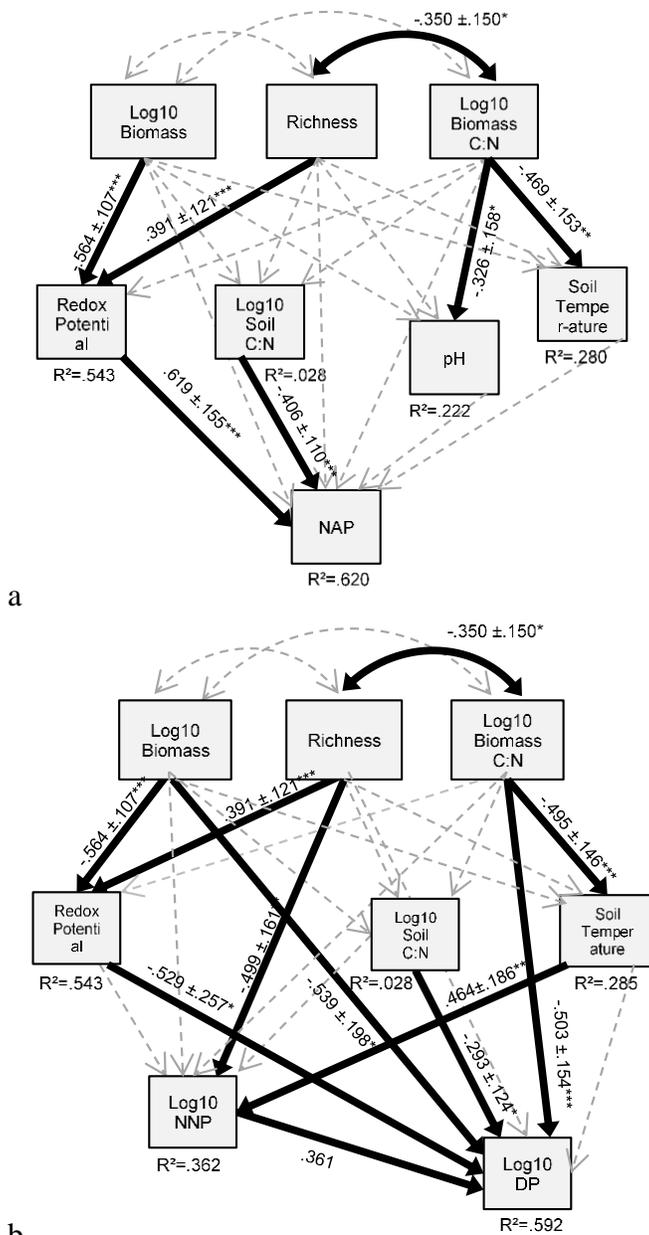
**Figure 3.5** Scatterplots of nitrogen processing in mesocosms across the species richness gradient (solid circles) and unplanted controls (open circles) for (a) net ammonification potential, (b) net nitrification potential, (c) net mineralization potential, and (d) denitrification potential. Statistical results pertain to the planted mesocosms only. Statistically significant linear regression slopes at  $\alpha = .05$  are displayed. ‡Variable was  $\log_{10}$  transformed prior to statistical analysis.

### **Net Ammonification (NAP), Net Nitrification (NNP), and Denitrification Potential (DP)**

All NAP values were negative, with unplanted mesocosms an order of magnitude more negative than the negative rates for the planted mesocosms (Fig. 3.5a). NNP was largely positive, with roughly similar ranges of rates between unplanted and planted mesocosms (Fig. 3.5b). As the sum of NAP and NNP, NMP was negative in the unplanted mesocosms and bridged negative and positive rates in the planted mesocosms without a significant trend by richness (Fig. 3.5c). Because a majority of NMP rates were negative (median = -13.2  $\mu\text{g N/kg soil}\cdot\text{d}$ ), either not all ammonium loss could be due to nitrification, suggesting some uptake of ammonium into microbial biomass, or some nitrate was concurrently lost to denitrification. Higher species richness had a positive effect on NAP, reducing the intensity of ammonium immobilization, and a negative effect on NNP, reducing the net production of nitrate (Fig. 3.5a,b). Plants had a positive effect on DP relative to the unplanted mesocosms, but DP was not correlated with planted species richness (Fig. 3.5d).

### **Structural equation models**

Two final SEM models were chosen, one for NAP and the other for NNP and DP, that maximized explained variance in the three N processes and minimized the  $\text{BIC}_{\text{adj}}$ . Regression coefficients remained significant ( $P < 0.05$ ) across the different models considered. Both models had good fit: for the NAP model, the  $\chi^2$  test of model fit (*i.e.*, test of the degree of difference between observed and model implied covariance matrices) was not significant ( $\chi^2=5.77$ ,  $df=6$ ,  $P=0.45$ ), CFI = 1.00 (CFI > 0.93 is evidence of good fit), and SRMR = 0.093 (SRMR > 0.10 is a poor fit) (Fig. 3.6a); for the NNP and DP



**Figure 3.6** Structural equation models of the causal effects of plant community biomass, richness, and C:N mass content of biomass on (a) net ammonification potential (NAP) and (b) net nitrification potential (NNP) and denitrification potential (DP). Statistically significant (\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ) standardized partial regression coefficients  $\pm 1$  standard error are shown with solid lines and insignificant standardized partial regression coefficients are not shown but represented with dotted lines. Curved arrows are equal to the standardized bivariate correlations between variables.

model, the  $\chi^2$  test of model fit was also not significant ( $\chi^2=0.721$ ,  $df=3$ ,  $P=0.87$ ), CFI = 1.00, and SRMR = 0.065 (Fig. 3.6b). Three soil properties, redox potential, C:N, and temperature, were conserved across both models because of their correlation with plant metrics and their control over multiple N processes. Because of the negative correlation between species richness and biomass C:N (equal to their bivariate correlation; Table 3.1), a single causal relationship is implied for these exogenous variables but their effects on soil processes were assessed independently. In both models, redox potential was higher in mesocosms with more species and in mesocosms with lower biomass, and soil temperature increased with decreasing biomass C:N (Fig. 3.6). While soil redox was well explained by both models ( $R^2=0.543$  for both models), soil C:N, temperature, and pH were not well-explained by either the NAP model ( $R^2=0.028$ ,  $R^2=0.280$ , and  $R^2=0.222$ , respectively) or the NNP-DP model ( $R^2=0.028$ ,  $R^2=0.285$ , and  $R^2=0.222$ , respectively). Soil total carbon, total N, and organic matter were either of low significance or insignificant as mediating soil physicochemical properties and were not modeled. No meaningful change in model fit ( $\chi^2=0.714$ ,  $df=3$ ,  $P=0.87$ ; CFI = 1.00, and SRMR = 0.065) and no change in the identified significant pathways were found if the original DP variable was used in place of the imputed variable; only the strengths of the partial regression coefficients were marginally altered.

Net ammonification potential was directly related to soil C:N and redox, and was indirectly related to richness and biomass through soil redox, with 62% of its variation explained by the model (Fig. 3.6a, Table 3.2). Higher NAP was found at lower soil C:N ratios but higher soil redox potentials. The two compound pathways from species

**Table 3.1 Selected Pearson correlation coefficients (r) for the relationships between macrophyte biomass attributes, soil properties, and nitrogen transformations for the planted mesocosms in the second growing season**

Nitrogen Processes			Macrophytes		Soil Properties							
NAP	NNP‡	DP‡	BM‡	BMCN‡	OM	C	N‡	CN‡	redox	temp	pH	
<u>NAP</u>	-0.019	.171	-.315	-.399*	-.315	-.055	.361*	-.377*	.631*	.176	.280	
	<i>p</i> =.913	<i>p</i> =.436	<i>p</i> =.069	<i>p</i> =.019	<i>p</i> =.070	<i>p</i> =.759	<i>p</i> =.036	<i>p</i> =.028	<i>p</i> <.001	<i>p</i> =.361	<i>p</i> =.108	
		<u>NNP‡</u>	.416*	.129	.000	.063	-.202	-.010	-.228	-.175	.130	-.199
			<i>p</i> =.048	<i>p</i> =.468	<i>p</i> =.999	<i>p</i> =.722	<i>p</i> =.251	<i>p</i> =.957	<i>p</i> =.194	<i>p</i> =.322	<i>p</i> =.501	<i>p</i> =.260
		<u>DP‡</u>		-.232	-.459*	-.138	-.279	.236	-.421*	.006	-.120	-.193
			<i>p</i> =.286	<i>p</i> =.028	<i>p</i> =.531	<i>p</i> =.198	<i>p</i> =.278	<i>p</i> =.046	<i>p</i> =.980	<i>p</i> =.626	<i>p</i> =.378	
			<u>BM‡</u>	.218	.035	-.201	-.249	-.023	-.612*	.016	-.166	
				<i>p</i> =.215	<i>p</i> =.843	<i>p</i> =.254	<i>p</i> =.156	<i>p</i> =.899	<i>p</i> <.001	<i>p</i> =.932	<i>p</i> =.350	
			<u>BMCN‡</u>	-.086	-.086	.038	-.131	.156	-.316	-.417*	-.419*	
				<i>p</i> =.628	<i>p</i> =.628	<i>p</i> =.831	<i>p</i> =.460	<i>p</i> =.378	<i>p</i> =.069	<i>p</i> =.024	<i>p</i> =.014	

‡ Variable was log10 transformed. \*Correlation significant at  $\alpha = .05$ . Abbreviations alphabetically: BM = total mesocosm biomass; BMCN = total mesocosm biomass C:N; C = carbon; CN = carbon:nitrogen mass ratio; DP = denitrification potential; NAP = net ammonification potential; NNP = net nitrification potential; OM = organic matter; redox = redox potential; temp = mean August soil temperature.

**Table 3.2 Standardized effects from the two structural equation models. Total, direct, and indirect effects  $\pm$  1 standard error of the three plant explanatory characteristics, richness, biomass C:N, and biomass, on net ammonification potential, net nitrification potential, and denitrification potential**

	Richness	Biomass C:N	Biomass
Net Ammonification Potential			
Total Effects	.216 $\pm$ .156 <sup>†</sup>	-.264 $\pm$ .158 <sup>†</sup>	-.231 $\pm$ .149
Total Direct	-.043 $\pm$ .133	-.163 $\pm$ .138	.105 $\pm$ .142
Total Indirect	.259 $\pm$ .128*	-.101 $\pm$ .135	-.336 $\pm$ .137*
Net Nitrification Potential			
Total Effects	-.425 $\pm$ .153**	-.177 $\pm$ .168	.130 $\pm$ .158
Total Direct	-.499 $\pm$ .161**	.088 $\pm$ .179	.195 $\pm$ .183
Total Indirect	.074 $\pm$ .119	-.265 $\pm$ .124*	-.065 $\pm$ .142
Net Denitrification Potential			
Total Effects	-.272 $\pm$ .194	-.490 $\pm$ .164**	-.171 $\pm$ .152
Total Direct	.107 $\pm$ .275	-.503 $\pm$ .154***	-.539 $\pm$ .198**
Total Indirect	-.379 $\pm$ .182*	.013 $\pm$ .133	.369 $\pm$ .190*

\**P* value significant at  $\alpha=.05$ . \*\**P* value significant at  $\alpha=.01$ . \*\*\**P* value significant at  $\alpha=.001$ . <sup>†</sup>Effects statistically inconsistent with bivariate correlation coefficients.

richness  $\rightarrow$  redox potential  $\rightarrow$  NAP (0.242 $\pm$ 0.097,  $P=0.013$ ) and biomass  $\rightarrow$  redox potential  $\rightarrow$  NAP (-0.349 $\pm$ 0.115,  $P=0.002$ ) were statistically significant and contributed to the overall indirect effects of richness and biomass on NAP. Insignificant total effects of species richness and biomass C:N on NAP in the model were inconsistent with significant bivariate relationships (Table 3.1) between the variables. Reciprocal suppression can occur when two explanatory variables are themselves related irrespective of the effects on a predicted variable. The loss of power in the model coefficients may derive from the shared variance between richness and biomass C:N as well as the mediating effect of redox potential on the richness-NAP relationship.

Net nitrification potential and denitrification potential were related to both soil properties and plant attributes (Fig. 3.6b, Table 3.2). NNP was positively correlated with soil temperature, while higher DP was correlated with higher NNP, lower soil C:N, and

lower redox potential. Species richness had a moderately strong direct negative effect on NNP with a total negative effect consistent with the bivariate correlation coefficient between the variables (Table 3.1). Biomass C:N also had an indirect negative effect on NNP mainly through the compound pathway biomass C:N → temperature → NNP but also influenced by the compound pathway biomass C:N → soil C:N → NNP ( $b=-0.259$ ,  $P=0.035$ , for combined indirect effects). Denitrification potential was affected by all three plant community metrics either directly or indirectly through the cumulative effects on soil properties. As an extension of the negative effects on NNP, species richness had a significant negative indirect effect on DP through the cumulative effects of two compound pathways: richness → NNP → DP and richness → redox → DP ( $b=-0.386$ ,  $P=0.033$ , for combined indirect effects). Biomass, in contrast, had positive indirect effects on DP through the cumulative effects of redox and NNP, though this was accompanied by a direct negative relationship with DP: biomass → redox → DP and biomass → NNP → DP ( $b=0.369$ ,  $P=0.052$ , for combined indirect effects). Biomass C:N had a moderately strong direct and resulting overall negative effect on DP. The explanatory power of the model was higher for DP (59.2%) than for NNP (36.2%).

## **Discussion**

### **Macrophyte performance in response to plant community richness**

Species richness was an important determinant of plant community evenness and biomass quality. Complete occupancy of the most species-rich mesocosms was a result of the obligate annual's high abundance and the greater spatial spread of both obligate and facultative annuals. Higher biomass quality (lower C:N), which can positively influence

litter decomposition (Espershsütz *et al.* 2013), was promoted at higher richness levels because of greater annual plant contributions to biomass. In other terrestrial and wetland studies, diverse plant communities have often been found to have lower biomass quality and higher N use efficiency than less diverse communities (Sullivan *et al.* 2007; Fornara and Tilman 2009; Oelmann *et al.* 2007; Pasari *et al.* 2013). Prolific growth has been attributed to a dilution effect in plant N biomass, where more productive individuals have higher C:N ratios because they theoretically invest in more structural, C-rich, tissue as they grow taller and compete for light (Ågren 2008; Abbas *et al.* 2013). This hypothesis holds partly true for the facultative annual where it displayed highest biomass C:N at highest fitness levels, except this was found in monoculture and thus was not related to interspecific interactions. Because of the elemental plasticity of the facultative annual and the low biomass C:N concentrations of the obligate annual, our results do not support the conclusion that ruderal species have lower tissue N concentrations (McJannet *et al.* 1995) but rather that plant elemental compositions differ by species and respond variably to resource availability and community interactions (Güsewell and Koerselman 2002; Novotny *et al.* 2007).

### **Macrophyte community effects on soil physicochemical properties**

The presence and specific attributes of the macrophyte community affected the non-nutrient physicochemical properties of the young soils. As shown by the comparison between the planted and unplanted soils, macrophytes raised redox potentials through root oxygenation and reduced temperatures through shading. Control of these properties in the planted mesocosms was further linked to the structure and characteristics of the

plant community. Soil temperatures were lowest under communities with lower quality biomass, and namely those without the obligate annual, possibly from heavier shading. Soil redox levels and pH were highest in communities with greater species richness and communities of lower biomass (reflective of the annuals). Greater temperatures and redox levels have large effects on soil biogeochemical processing by promoting faster nutrient cycling, litter decomposition, and soil organic carbon mineralization, though greater temperatures also exacerbate oxygen or nutrient limitations. Many ion concentrations, states, and reactions are functions of soil pH. Because pH usually increases in flooded, anoxic soils, the opposite positive associations found between pH and both species richness (due to the presence of annuals) and redox potential likely resulted from greater organic matter decomposition and proton ( $H^+$ ) consumption in a weakly oxic environment (Reddy and DeLaune 2008).

In contrast, the macrophyte community did not alter the bulk soil nutrient pools of N, carbon, or organic matter, or the proportion of C:N. These findings were unexpected considering that the two annual species died back either partially or fully at the end of the first growing season. Additionally, relative growth rate, often higher in annual plants, has been found to be one positive indicator of root exudates release of labile carbon (Zhai *et al.* 2013; Cantarel *et al.* 2015). On the other hand, this study did not examine changes in the make-up of organic material (*e.g.*, water-extractable organic carbon) or concentrations of inorganic nitrogen which might be more responsive to variations in plant community type (Espershütz *et al.* 2013). Only the effect on pH and that the two heterotrophic N processes – NAP and DP – tracked strongly with the presence of annuals

(as measured by total biomass and total biomass C:N) provides some indication that changes in soil organic matter or N pools occurred on a finer scale than could be detected in this study. Empirical and theoretical evidence does suggest that attributes of the microbial community are primary controls on short-term organic matter and N pools that decouple the influence of plants (Cole *et al.* 2001; Knops *et al.* 2002; Robertson and Groffman 2015).

### **Macrophyte community effects on nitrogen processing**

In our 2-year study, greater macrophyte richness and higher biomass quality, which were positively correlated, each uniquely promoted NAP, while greater species richness inhibited NNP. Because of the opposing trends, species richness had no overall effect on NMP. Our results suggest a degree of nitrogen limitation, where the average combined change in the mineralized N pool was negative, all NAP was negative, and the magnitude of rates for the most part were lower than those reported for freshwater wetlands (Dick and Gilliam 2007; van Hoewyk *et al.* 2000; Hanson *et al.* 1994; Fellman and D'Amore 2007). Net negative mineralization rates reflect low dissolved N availability resulting from a possible combination of microbial immobilization, slower respiration, or nitrate consumption. A study by Zhu *et al.* (2012) that used experimental treatment wetlands receiving domestic wastewater offers a counterpoint that may speak to the importance of nutrient availability. Across 16 species, the authors found that greater richness of wetland plants augmented soil N mineralization, nitrification, and ratio of net nitrate produced to the total pool of N mineralized (nitrate + ammonium) from in situ soil cores over four weeks. In our study, we infer that the predominant

microbial uptake of ammonium was due in part to the consistently low redox potentials in all mesocosms, which possibly contributed to smaller initial dissolved ammonium pools. The markedly greater rates of immobilization in the unplanted mesocosm soils with the lowest redox potentials and lack of plant influence highlights how root oxygenation strongly promotes microbial respiration. Immobilization may also have been promoted by the abrupt alteration in oxygen availability to the previously anoxic soils: N demand grew in the incubations as microbial activity and microbial biomass N increased. Some oxygen limitation persisted in our whole-core incubations and nitrate consumption from denitrification would also have reduced rates of NNP and NMP.

Studies have identified multiple mechanisms underlying the positive relationships between species richness and N mineralization. Species complementarity in a diverse community can increase microbial biomass and N mineralization through greater productivity and faster decomposability of plant biomass (van der Krift and Berendse 2001; Cong *et al.* 2014). Alternatively, certain species may “prime” N mineralization in their rhizosphere by releasing carbon compounds that increases the need for mineralized N (Finzi *et al.* 2015; Mueller *et al.* 2015). Here, the SEM model indicated that higher redox potentials, which can increase rates of microbial respiration, directly regulated the positive NAP trend across species richness, a potentially important mechanism in wetlands for both N and carbon cycling. The higher biomass quality at higher species richness would have also increased ammonification as the greater proportion of nitrogen to carbon in organic matter stimulates net microbial release of mineralized ammonium.

Ammonium production and oxygen availability, both of which co-varied positively with species richness, are two primary facilitators of nitrification (Bodelier *et al.* 1996). Yet in our study, NNP was uncorrelated with NAP and was inhibited at higher species richness. The similarity between the monocultures and the unplanted mesocosms, both highest in NNP on average, would suggest that the trends in NNP relate to the build-up of ammonium substrate in the mesocosms of lower species richness and redox potentials. Under anoxic conditions, ammonium accumulates in soils from nitrification inhibition, lower diffusion and volatilization, lower microbial demand, and greater clay fixation (Reddy *et al.* 1984; Schneiders and Scherer 1998). A lack of correlation between NAP and NNP was also found by Wolf *et al.* (2013) in young created wetlands. This outcome may reflect the consequence of measuring more veiled net flux rates, but incubating the soil cores whole might have contributed to preserving microsite complexity that spatially segregated ammonification and nitrification processes (Schimel and Bennett 2004). Temperature, another primary facilitator of nitrification, positively mediated the effect of higher biomass quality on NNP (Forshay and Dodson 2011; Laughlin 2011). Higher temperatures are known to increase microbial activity and have been shown to stimulate surficial oxygen production from algal photosynthesis (Christensen and Sørensen, 1986).

Due to the low explanatory power of our model for predicting NNP, the dominant regulatory factors of nitrification were not identified by this study. The invariance of NNP between the planted and unplanted mesocosms suggests that NNP was largely suppressed. Competition for ammonium from heterotrophic microbes and plant roots has

been known to inhibit nitrifiers (Verhagen *et al.* 1994; Arth and Frenzel 2000). The obligate annual was found to proliferate its roots at higher species richness levels (Korol and Ahn 2016), a plant strategy used to increase nutrient uptake particularly under interspecific competition (Nacry *et al.* 2013), which would have increased the delivery of labile carbon exudates and uptake of soil nitrogen. Further, greater microbial respiration and readily decomposable litter (*e.g.*, the obligate annual was a particularly good indicator of biomass C:N) could have intensified the soil oxygen demand along the richness gradient that limited the potential for nitrification during the incubations (Lee *et al.* 2009). The suppression of NNP was not present in a study of soil incubated under fully oxic conditions with added ammonium: NNP was higher in wetland soils with emergent vegetation than in bare sediment and NNP was strongly correlated with redox potential (Soana and Bartoli 2014).

Plant community characteristics exerted the most control on DP, with the majority of variability explained by biomass and biomass C:N. Denitrification potential was directly stimulated in mesocosms with lower biomass, one indicator of the presence of annuals, and with higher qualities of biomass and soil organic matter, indicators of the availability of organic carbon and N. The concomitant soil additions of decomposable biomass with low C:N and potentially greater rhizodeposition from the annuals would have increased bioavailable organic carbon resources for heterotrophic denitrifiers. Ballantine *et al.* (2014) demonstrated that denitrification potential in restored freshwater wetlands was positively related to topsoil amendments with relatively low C:N content. Direct additions of glucose, the most labile form of organic carbon, has been shown to

increase microbial N stores, decrease dissolved soil N, and decrease N content in plants (Schmidt *et al.* 1997).

While DP was directly promoted by lower biomass quantity and greater quality, lower biomass and greater richness concomitantly suppressed DP indirectly through higher redox potentials (*i.e.* root oxygenation). While some soil oxygenation facilitates coupled nitrification-denitrification (Burgin and Groffman 2011), high levels of root oxygenation suppresses the process. The inhibition of DP through this mechanism stands in contrast to the elevation of DP in planted mesocosms compared to unplanted mesocosms which lacked rhizosphere oxygenation and plant-derived carbon inputs. Because two of the three highest DP rates were found in the monocultures of the obligate annual, which also exhibited relatively high redox levels, rhizosphere oxygenation in the anoxic mesocosm soils was one important positive driver of DP. As the two annuals increased the redox potential in more species-rich mesocosms from greater root oxygen leakage, increased microbial respiration may have also promoted heterotrophic activity and a demand for ammonium that inhibited the autotrophic nitrifier community.

Support for a direct relationship between species richness (or diversity) and denitrification potential in wetlands is lacking (Bouchard *et al.* 2007; McGill *et al.* 2010). Sutton-Grier *et al.* (2011) found an insignificant direct relationship between functional diversity and DP but determined that background concentrations of N, organic matter, and soil moisture were statistically significant mediators to the relationship. Our study corroborates this finding of joint control of DP and identifies specific mechanisms for young wetland soils. Specifically, higher redox potentials suppressed DP and possibly

diminished nitrate pools, while lower productivity but higher biomass quality associated with the annuals promoted carbon-coupled N cycling. Our denitrification rates were similar to other rates reported for freshwater marshes with mineral soils and for diversity-ecosystem function studies (McGill *et al.* 2010; Sutton-Grier *et al.* 2011; Wolf *et al.* 2011; Ballantine *et al.* 2014). An interesting avenue for future research would be to test the richness-DP relationship in wetlands under soil conditions (*i.e.*, greater nitrogen, organic matter, or flood pulses) that could support higher DP rates (*i.e.*, up to one order of magnitude) that have been reported for some mineral freshwater wetlands (Jordan *et al.* 2007).

### **Model insights and interpretations**

An overarching advantage of the SEM approach was illustrating the tested multiple hierarchical, direct and indirect relationships among variables that would be difficult in the absence of a visual model. Our results provide insights into the relative strength and mediating mechanisms of plant effects that have rarely been demonstrated in wetlands. Plant-soil feedbacks depend on these initial plant-induced soil changes and understanding these mechanisms are important for further research on the development of plant-driven N functionality in created wetlands. The modification of our conceptual model to incorporate direct effects indicated that certain mechanistic details of plant effects on soil N processes were not captured by this study. In particular, our analysis raises research questions about the structural controls on NNP in wetlands and on nutrient forms and concentrations in the rhizosphere. Since the conclusions of this diversity study were derived from four species, two annuals and two perennials, planted at moderate

densities, similar research in wetlands is needed across different species composition, species density, and soil conditions.

Did higher species richness levels promote ecosystem multifunctionality within the N cycle? The negative trends in this study would suggest the opposite, seemingly contradictory to recent understanding that multiple species are more likely to maintain multiple ecosystem processes (Lefcheck *et al.* 2015). Here, greater species richness promoted one but not all three N functions measured in this study, and was not associated with biomass production. In a meta-analysis of biodiversity studies conducted in grasslands, Isbell *et al.* (2011) concluded that pairs of mixtures comprised of more unique species could together promote ecosystem functions better than mixtures with fewer unique species. That we found richness to both promote soil oxidation and NAP but at the same time have inhibitory effects on NNP and DP suggests that more community composition heterogeneity in early wetlands may be better suited to maximize all three of these ecosystem N functions.

At the same time, the presence of annuals, which explained a main axis of variability in decreasing biomass quantity and increasing quality, was found to positively affect all three of the N processes. The various mechanisms of higher redox potential, temperature, carbon inputs from decomposable litter, and the quality of litter all suggest that the annuals promoted greater carbon processing. This finding provides evidence that species composition differentially regulates the effects of plant carbon on the microbial use of N in young ecosystems and finds support of a carbon-flow mechanism of macrophyte stimulation of N cycling. Allowing annual plants to establish in young

created wetlands as a natural strategy to boost soil carbon and N development may be cost-effective and more reliable than planting a mix of early and late successional plants (*i.e.*, annual and perennial) that may maximize plant productivity but may not promote soil biogeochemical cycling (Mitsch *et al.* 2012; Schultz *et al.* 2012). As microbial N demand and release (*e.g.*, denitrification) increase in response to greater inputs of plant carbon, environmental conditions supporting natural N flux, such as hydrologic connectivity or pulses, would benefit soil N functionality in often N-poor created wetlands (Ballantine and Schneider 2009; Wolf *et al.* 2013).

## **Conclusions**

In a 2-year plant richness-wetland N functioning study, multiple characteristics of a planted macrophyte community regulated soil N cycling as measured by net N transformation potentials. Species richness positively impacted NAP, but directly and indirectly inhibited NNP and DP, respectively. Lower biomass quantity and tissue C:N, two characteristics that strongly related to the morphological and phenological traits of the annuals, more consistently stimulated N cycling with positive effects on all three N processes. Our structural equation model revealed that the community characteristics of lower tissue C:N and lower biomass, related to the potential for greater decomposable biomass inputs from the annuals, increased NAP via higher redox potential, increased NNP through higher temperature, and increased but also decreased DP through inhibition from higher redox potentials. Both redox and temperature were significant mediators of the plant community-soil N functioning relationships. Other soil physicochemical properties – pH, soil C:N, and soil N – that were associated with either the macrophyte

community or N processing but not both, were possibly exhibiting slower mediating influences (*i.e.*, effects and responses) on ecosystem functionality that could grow more important with time. Localized plant effects in the rhizosphere on soil carbon or inorganic N may have been more pronounced than overall changes in bulk soil stocks in these young systems and suggests similar processes at created wetland sites. Our findings highlight the utility of a priori annual-perennial functional group classifications in predicting ecosystem responses to planting schemes in created wetlands. Permitting early successional, annual plant-dominated communities to establish through an active management approach (*e.g.*, mitigating the influence of volunteer species) may be a successful restoration strategy that could improve the development of soil biogeochemical cycles and important wetland services such as denitrification.

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## CHAPTER 4 CONTROLS OF DENITRIFICATION IN NONTIDAL FLOODPLAINS OF THE CHESAPEAKE BAY WATERSHED, USA

### **Abstract**

Not all floodplains have the same capacity to remove excess nitrogen through denitrification, and managers could use information on predictors of denitrification to select and prioritize floodplains for restoration. Many local biological and soil physical components of floodplains relate well to denitrification but lack regional-scale datasets for practical use. By contrast, many datasets are currently available or are anticipated to become available for hydrogeomorphic, physiographic, and climate (largescale) characteristics, which include geographic mapping tools for characterizing riverscapes. Therefore, we investigated control of denitrification by these largescale characteristics in nontidal floodplains of the Chesapeake Bay watershed, as well as by local floodplain soil and herbaceous vegetation characteristics. Soil denitrification was measured seasonally (spring and summer) in the laboratory using acetylene-based techniques under an anoxic environment. Experimentally-determined nitrogen and carbon limitation and seasonality contributed to spatiotemporal denitrification variability. Linear largescale predictors of the denitrification measurements included stream nitrogen and phosphorus concentrations (+), channel width-to-depth ratio (+), floodplain sedimentation (+), forested (-) and urban (+) land cover of the catchment, and seasonal air temperature (-). Three predictors, forested land cover (strongly related to agricultural land cover) and urban land cover of

the catchment, and floodplain sedimentation were related to the most number of denitrification measurements, including ones in both seasons. Using the local floodplain soil and herbaceous vegetation characteristics as statistical mediators in path analysis, greater non-forested catchment land cover indirectly affected denitrification through greater floodplain soil nitrate, total phosphorus, and herbaceous aboveground biomass. Additionally, greater floodplain sedimentation indirectly affected denitrification through greater soil pH, total phosphorus, and potential carbon mineralization. Due to the number of relationships found with the denitrification measurements and the mechanistic understanding contributed by the path modeling results, hotspots of floodplain denitrification at the regional scale should be found in urban and agricultural catchments where river-floodplain hydrologic connectivity promotes sedimentation. Though explanation of variation in the denitrification measurements was improved with the soil biogeochemical and herbaceous vegetation predictors, the reach-scale hydrogeomorphic and catchment predictors explained 43-57% of the variation and should be useful for prediction of denitrification in floodplains of a watershed.

*Key words:* denitrification enzyme activity, denitrification potential, land cover, nitrogen, phosphorus, nutrient sedimentation, geomorphology, soil

## **Introduction**

Denitrification removes large portions of diffuse anthropogenic nitrogen (N) from the biosphere and mitigates the negative impacts of excess nitrogen on ecosystem functioning in local and downstream environments (Fowler *et al.* 2013). Because of the high potential for denitrification in floodplains, management efforts to conserve aquatic

resources seek to promote the process in floodplains at the scale of watersheds (U.S. EPA 2010). Conservation efforts include reverting floodplains to unmanaged (*e.g.*, non-cropped) ecosystems, restoring degraded floodplains with reconnected hydrologic connectivity and greater stabilization, and prioritizing restoration sites in a landscape to maximize benefits to the surrounding watershed (*e.g.*, a watershed approach) (Zedler 2003, Opperman *et al.*, 2010, Chabot *et al.* 2016). Current uncertainty in identifying floodplains with high rates of denitrification due to environmental heterogeneity across rivers precludes use of criteria on nitrogen removal rates for site selection of these restoration projects and creates a need for studies on the predictors of denitrification conducted at large spatial (*e.g.*, regional) scales (Heffernan *et al.* 2014, Van Groenigen *et al.* 2015).

Controls on denitrification can be categorized into direct and indirect regulators of the microbial N transformation. The proximal, mechanistic controls include the availability of nitrate and organic carbon, the local absence of oxygen, and the physicochemical limitations determined by pH and temperature, all of which can influence rates of denitrification quickly on the scale of minutes (Burgin *et al.* 2010, Ballantine *et al.* 2014, Robertson and Groffman 2015). Beyond these immediate controls, distal controls further regulate the process through indirect interactions that occur over longer periods of time and by driving variability in denitrification at larger spatial extents (Wallenstein *et al.* 2006). Many ecosystem components are frequently reported as predictors of N processing that provide evidence for distal control of denitrification in floodplains (Groffman *et al.* 1992, Ashby, Bowden, and Murdoch 1998, Sutton-Grier *et*

*al.* 2013). For instance, denitrification rates are related to soil texture which indirectly influences soil moisture and nutrient (e.g., nitrate and carbon) content (Pinay *et al.* 2000), and the vegetation community, which influences soil biogeochemical cycling such as through additions of organic carbon or changes to temperature (Korol *et al.* 2016). The combined effects of these and other direct and indirect soil and vegetation controls of floodplains result in high spatiotemporal variability in denitrification (Vidon *et al.* 2010). Though most commonly studied, these ecosystem components represent only the local environmental characteristics of floodplain ecosystems affecting soil N processing.

Hydrogeomorphic (*e.g.*, river flow and sediment movement), physiographic (*e.g.*, land form and cover), and climate processes further exert influence on denitrification variability (Boyer *et al.* 2006). No correct class of ecological characteristics (*i.e.*, properties and processes) exists for explaining denitrification patterns across floodplains, but spatially emergent characteristics of the floodplain and channel, or characteristics that influence the riverscape may be helpful for two reasons. First, the growing availability of geospatial datasets of hydrogeomorphic and catchment characteristics (*e.g.* size, land cover, climate metrics) that describe the river-floodplain ecosystem (Carbonneau *et al.* 2012) could enable extrapolation of floodplain denitrification over broad spatial extents useful to N modeling and management. Second, predicting denitrification with these largescale emergent controls should be more efficient because they can integrate the multiple effects, non-linearities, and long-term interactions of the local controls (Kulkarni *et al.* 2014).

River flow, groundwater levels, sediment movement, and in-stream nutrient cycling (*i.e.*, hydrogeomorphic processes) predominantly regulate the biogeochemical cycling and vegetation community of floodplain ecosystems (Noe 2013). Pertinent to denitrification, hydrologic connectivity between river and floodplains, involving material exchange from groundwater or surface water, promotes nutrient cycling and creates a large potential for anaerobic processes in floodplains relative to upland areas (Hill *et al.* 2000, Hefting *et al.* 2004, Duncan *et al.* 2013). Short pulses of high denitrification rates can occur after flooding due to reduced redox potential, elevated dissolved organic carbon, and nitrate inputs (Forshay and Stanley 2005, Shrestha *et al.* 2014); while long-term pulses can occur in zones with higher groundwater levels, longer residence times, and sediment inputs (Hefting *et al.* 2004, McPhillips *et al.* 2015, McMillan and Noe 2017). At reach or segment scales of a river network, floods and sediment supply to the floodplain are influenced by the underlying geomorphic template of the stream valley (e.g., stream slope, fine sediment storage, and bedrock constraint). These local hydrogeomorphic processes are in turn spatially nested within hydrogeomorphic processes of the larger river basin shaped by physiographic and climatic landscapes (Lowrance *et al.* 1997, Winter 2001, McCluney *et al.* 2014).

One notable landscape characteristic, upland human land use, degrades reach-scale hydrogeomorphic processes of river basins and modifies natural floodplain nitrogen cycling (Groffman *et al.* 2002, Allan 2004). Concentrated areas of urban or agricultural development can alter flooding regimes and increase nutrient and sediment inputs to surface water (Noe and Hupp 2005, Gellis *et al.* 2009). Urban streams in particular are

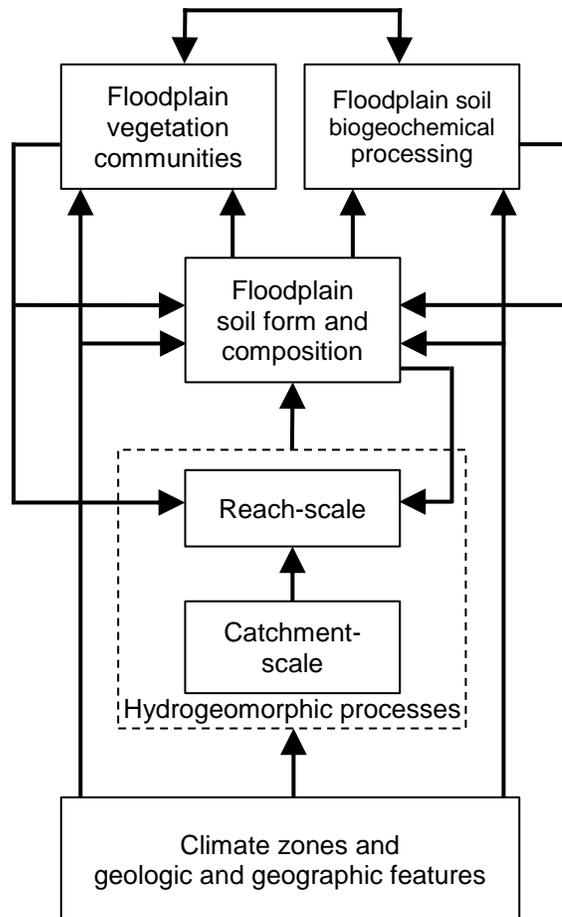
often characterized by more powerful and flashier discharges after storm events, which can increase the frequency of high energy, short duration floodplain inundation (Hupp *et al.* 2013, Hopkins *et al.* 2015). Highly energetic storm flow further lowers base flow and riparian groundwater tables and can increase channel-floodplain disconnection through greater channel incision (Walsh *et al.* 2005, Schwartz *et al.* 2012). Both agricultural and urban land use can be responsible for the majority of nitrogen flowing into coastal waters and thus gradients in river N loads across a watershed should strongly impact patterns of denitrification in nontidal floodplains (Howarth *et al.* 2002, Ator *et al.* 2011).

Our goal for this study was to investigate spatial controls on denitrification in nontidal floodplains of the Chesapeake Bay (CB) watershed (Fig. 4.1), which in entirety drains more than 165,000 km<sup>2</sup> across the mid-Atlantic United States. Soil and groundwater flow vary greatly across the watershed's five major physiographic provinces from the local influences of topography, climate, and parent material and a patchwork of differing lithologies (Bachman *et al.* 1998, Sherwood *et al.* 2016). In addition, continental position acting together with physiographic region (e.g., altitude) give rise to numerous ecoregions in the watershed within the broader humid temperate climate (Bailey 2009). An expert panel of the Chesapeake Bay Program, a multi-entity effort to restore water quality to the CB, recommended more research conducted in floodplains throughout the CB watershed (Berg *et al.* 2014). For this study, our primary objective was to investigate “largescale” controls of denitrification (Fig. 4.1), defined as the hydrogeomorphic characteristics of river reaches, and morphometric, land use, and physical characteristics of catchments (*i.e.*, the sub-basins of the CB watershed). We

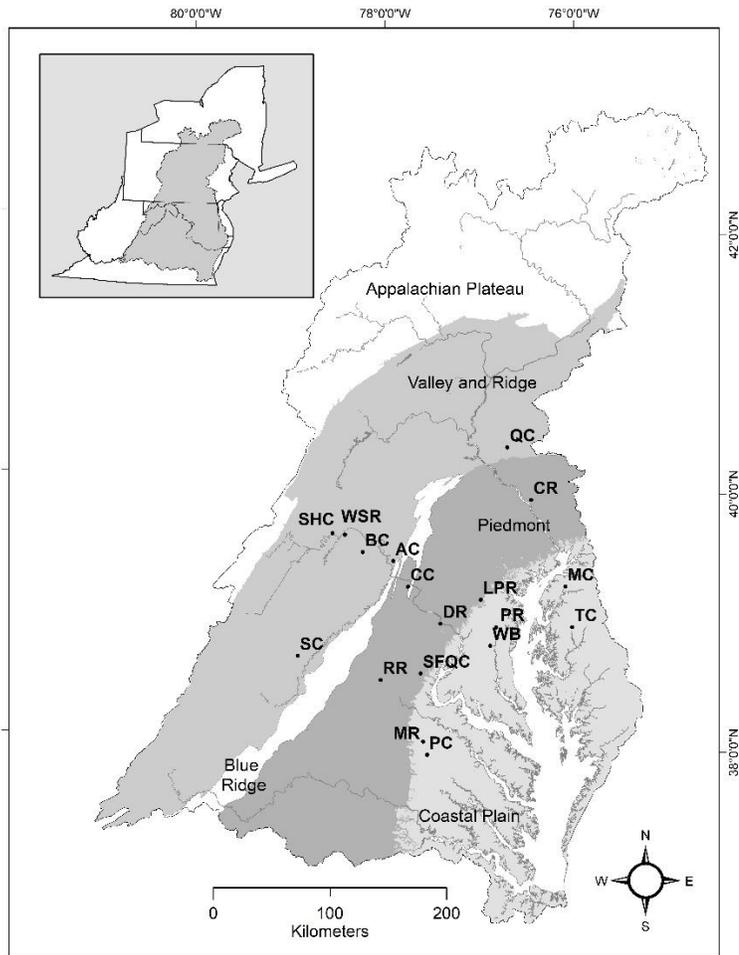
considered hierarchical spatial frameworks, where catchment-scale hydrogeomorphic processes drive reach-scale hydrogeomorphic processes (Thorp *et al.* 2006; Fig. 4.1), and used a statistical approach that evaluated shared variation along largescale predictors of denitrification.

Our second and third objectives sought to provide greater theoretical and practical support to findings from our first objective. Due to the correlative results in the first objective, our second objective was to determine whether relationships of denitrification with local, soil or vegetation characteristics could explain relationships with the (largescale) reach-scale hydrogeomorphic and catchment characteristics (Fig. 4.1). We used short-term, plot-scale measurements of soil and vegetation properties to test for statistical mediation as these properties are also strongly controlled by hydrogeomorphic, physiographic, and climatic processes (Thorp *et al.* 2006, Noe 2013). Alternatively put, we asked whether largescale controls directly or indirectly influence denitrification through their influence on local characteristics? We used path models that account for these multiple causal effects, as well as the reciprocal effects that vegetation and the form and composition of soil (*e.g.*, texture) have on reach-scale hydrogeomorphic processes by moderating sediment flux and soil moisture (Noe 2013, Harvey and Gooseff, 2015). Our third objective was to evaluate the explanatory power of the collective largescale predictors and compare that with the explanatory power of the collective local predictors to inform statistical models of denitrification at regional scale. Finally, we examined the effect of season on our outcomes to determine the temporal consistency of spatial controls on denitrification. We expected that the patterns in denitrification would follow

reach-scale hydrogeomorphic and catchment characteristics that exert strong control over the proximal controls on denitrification: characteristics related to more hydrologic connectivity, nitrogen delivery, or soil carbon accumulation and higher pH in floodplains.



**Figure 4.1. Conceptual model of the expected controls of denitrification and other biogeochemical processes in floodplains. Floodplain ecosystems are comprised of the physical habitat, vegetation community, and biogeochemical processes, which all interact spatially within the limits of the floodplains. Hydrogeomorphic processes create interactions between the channel and floodplain components, and reach-scale processes are mediated by the morphometric and composition of the physical habitat and the structure and activity of the vegetation community. Biogeochemical mediation of hydrogeomorphic processes are considered negligible for this study and are not considered. Floodplain components and hydrogeomorphic processes are all influenced by climate zones and geologic and geographic features.**



**Figure 4.2. Eighteen nontidal floodplain sites within three physiographic provinces of the Chesapeake Bay watershed. Site acronyms: Antietam Creek (AC), Back Creek (BC), Catoctin Creek (CC), Conestoga River (CR), Difficult Run (DR), Little Patuxent River (LPR), Mattaponi River (MR), Morgan Creek (MC), Patuxent River (PR), Polecat Creek (PC), Quittapahilla Creek (QC), Rappahannock River (RR), Sideling Hill Creek (SHC), Smith Creek (SC), South Fork Quantico Creek (SFQC), Tuckahoe Creek (TC), Warm Springs Run (WSR), Western Branch (WB).**

## **Methods**

### **Floodplain sites and plots**

Eighteen forested floodplains associated with the USGS Chesapeake Floodplain Network and adjacent USGS Nontidal Network of river gage and load stations in the CB watershed were selected for study (Fig. 4.2). Floodplains span the lower central region of the watershed ( $37^{\circ}58' - 40^{\circ}21' \text{N}$ ;  $75^{\circ}56' - 78^{\circ}39' \text{W}$ ), all within daily driving range of George Mason University (Fairfax, VA). Sites were selected to capture broad-scale heterogeneity in drainage area, land use, and geology and topography of the CB watershed (Fig. 4.2, Table 4.1). For physiographic heterogeneity, six sites were selected in each of the Coastal Plain, Piedmont, and Valley and Ridge physiographic provinces. Two sites each were selected to have high percentages of either of three land cover classes – urban, agricultural, and forested – for each of the three physiographic provinces. For hydrogeomorphic heterogeneity, sites were associated with a range in catchment size ( $10^1 - 10^3 \text{ km}^2$ ). Data on soil physicochemical properties were available for 30 USGS Chesapeake Floodplain Network sites in the Ridge and Valley and Piedmont provinces at the time of our study design, which we clustered into subgroups to help select this study's sites across a range of soil conditions (Noe, unpublished data).

At each site, a floodplain transect was established on one bank running perpendicular from the stream to the base of the toe slope. Four sampling point locations (= plots) were established at unique geomorphic features of the floodplain, including the natural levee and the base of the toe-slope, and two additional locations such as depressions or areas of abrupt change in vegetation or elevation. Due to the narrowness

and homogeneity of the floodplain cross-section for two sites (*i.e.*, Antietam Creek, MD, and Warm Spring Run, WV), one of the plots in the middle of the four was placed on the opposite bank, behind the levee, ensuring greater variation in sampling. For denitrification measurements in August only (see below), a fifth plot was sampled at 9 out of 18 sites across the watershed.

### **Field sampling for denitrification and floodplain soil and vegetation characteristics**

Measurements of denitrification and soil and vegetation characteristics of the floodplain occurred in spring and summer of 2016. In May and again in August, we collected soils for two denitrification rate measurements and physicochemical properties (*i.e.*, bulk density, moisture, pH, N, C, organic matter, and extractable NO<sub>3</sub>, NH<sub>4</sub>, orthophosphate and their ratios) and processes (*i.e.*, potential carbon mineralization flux). In July, we collected soils for a nutrient limitation experiment of denitrification and all measurement or sampling of above- and belowground vegetation occurred. In each sampling month, soils were retrieved during a contiguous 7- to 9-day period and refrigerated each evening. We sampled for vegetation and soils perpendicular to the floodplain transect within 5 m of the denitrification plot. Soil cores (2.1 cm diameter), with the exception of root biomass cores, were collected to 10 cm because the highest rates of denitrification were expected to this depth.

**Table 4.1. Characteristics of 18 floodplain sites selected for study associated with U.S. Geological Survey stream gage and load stations**

USGS gage #	Location	Physiographic Province <sup>1</sup>	Catchment area (km <sup>2</sup> ) <sup>2</sup>	Catchment % forested land cover <sup>3</sup>	Catchment % urban land cover <sup>3</sup>	Catchment % agricultural land cover <sup>3</sup>	Floodplain % soil N <sup>4</sup>	Floodplain soil pH <sup>4</sup>
01619500	Antietam Creek, MD	RV	729	32	13	54	0.26	7.64
01614000	Back Creek, WV	RV	607	77	4	18	0.13	6.04
01638480	Catoctin Creek, VA	PD	234	33	6	60	0.12	6.07
01576754	Conestoga River, PA	PD	1213	24	25	48	0.19	7.17
01646000	Difficult Run, VA	PD	151	37	57	2	0.09	5.62
01593500	Little Patuxent River, MD	PD	102	20	66	10	0.18	5.18
01674000	Mattaponi River, VA	CP	668	70	4	14	0.18	4.80
01493500	Morgan Creek, MD	CP	32.7	2	1	91	0.14	5.12
01594440	Patuxent River, MD	CP	908	33	32	27	0.23	5.80
01674182	Polecat Creek, VA	CP	127	68	8	14	0.15	5.17
01573160	Quittapahilla Creek, PA	RV	201	16	31	51	0.32	7.60
01664000	Rappahannock River, VA	PD	1605	61	3	35	0.18	5.82
01610155	Sideling Hill Creek, MD	RV	270	81	2	17	0.10	4.84
01632900	Smith Creek, VA	RV	249	47	8	45	0.32	7.63
01658500	S. Fork Quantico Creek, VA	PD	23.7	87	2	1	0.11	4.70
01491500	Tuckahoe Creek, MD	CP	224	15	1	68	0.06	5.99
01613030	Warm Springs Run, WV	RV	21.7	60	28	11	0.19	6.56
01594526	Western Branch, MD	CP	239	31	51	11	0.16	6.04

Notes: <sup>1</sup> CP = Coastal Plain, PD = Piedmont, RV = Ridge and Valley

<sup>2</sup> USGS National Water Information System (2016)

<sup>3</sup> Chesapeake Conservancy (2017)

<sup>4</sup> This study

Three soil measurements were measured in the field or prior to 2016. For continuous soil temperature measurements, iButton thermistors (Embedded Data Systems) were deployed at the end of May to three plots (levee, next adjacent to levee, and base of toe-slope) to a depth of 5-10 cm, recording at 90 min intervals, and retrieved during the August collection (beginning of month). Mean maximum daily soil temperatures were calculated for June and July. Soil bulk density, total P (mg per g dry soil; microwave assisted strong acid digestion followed by ICP-OES analysis) and the percent of particles smaller than 63  $\mu\text{m}$  (laser diffraction analysis) were measured in previous years (2013-2015) at six locations (5 cm depth) among the two transects at each site; one of those transects was sampled for the measurements of this study.

#### **Datasets for reach-scale hydrogeomorphic and catchment characteristics**

We used datasets on the following reach-scale hydrogeomorphic characteristics: stream discharge and nutrient concentrations, floodplain sedimentation, channel and floodplain morphometry metrics, and stream slope. Mean three-year discharge ( $\text{m}^3/\text{s}$ ) for water years 2014, 2015, and 2016 was retrieved from the USGS National Water Information System (USGS 2016). Mean stream concentrations (mg/L) of total nitrogen and phosphorus, measured up to three times per month, were retrieved for the same three-year period from the Chesapeake Bay Program Water Quality Database (CB Program 2012). Water quality data were screened for duplicate measurements and non-detectable values. Between 2013-2015, channel and floodplain morphometric metrics were measured at two transects lying roughly 100 m apart; means were calculated for a total floodplain width (bank to toe-slope, both sides), channel width (measured at top of bank),

bank height, channel width-to-depth ratio (calculated ratio of channel width and bank height), and entrenchment ratio (calculated ratio of floodplain to channel widths). Long-term net floodplain vertical deposition ( $\text{cm yr}^{-1}$ ) using the dendrogeomorphology method (Hupp *et al.* 2016) was also measured previous to this study (mean period of measurement, on the basis of tree age = 45 years; mean number of trees sampled per site = 11) and calculated as a sedimentation ( $\text{g m}^{-2} \text{yr}^{-1}$ ) using site-specific soil bulk density (0-5 cm). River surface water slope (%) was measured using the Floodplain and Channel Evaluation Toolkit [FACET] to process 3-m LiDAR-derived DEMs of digital stream reaches (1.5 km in length, on average) (Sam Lamont, NOAA, unpublished data; Hopkins *et al.* 2017).

Catchment characteristics were chosen to reflect catchment-scale hydrogeomorphic processes and the regional-scale physiographic and climatic variability that could affect hydrogeomorphic processes. The datasets for catchment characteristics included catchment land cover and morphometry, and sample-site physiographic province, stream elevation, air temperature, and precipitation. For this study, we defined the following land cover types using a  $1 \text{ m}^2$  resolution dataset created for the CB watershed: (1) forested = “tree canopy”, (2) agriculture = “low vegetation” + “barren”, and (3) and urban = “impervious roads” + “impervious surfaces” + “tree canopy over impervious surfaces” (Chesapeake Conservancy 2017). Catchment area and stream elevation were retrieved from the USGS National Water Information System (USGS 2016). Physiographic provinces were identified from Bachmann *et al.* (1998). We summarized daily maximum air temperature and total daily precipitation records

modelled for each floodplain site (PRISM Climate Group 2017) at two time scales: seasonally (spring: March-May; summer: June-July) and for the year up to sampling (September 2015 – August 2016).

### **Denitrification measurements**

We made denitrification measurements in the week immediately following soil collection. Denitrification was measured with laboratory incubations using more than one technique but always in a 10% acetylene/N<sub>2</sub> headspace to inhibit N<sub>2</sub>O reduction to N<sub>2</sub> (Knowles 1990). In all, we made seven measurements of denitrification from every plot. All gas samples were held in 10 mL glass vials with aluminum caps and butyl rubber septa for a maximum of 2 days prior to analysis for N<sub>2</sub>O using electron capture gas chromatography and a Hayesep Q 80/100 packed column.

In May and August, we measured denitrification enzyme activity (DEA) from nitrate- and carbon-amended, saturated soil slurries. Our procedure follows Groffman *et al.* (1999). Soil-solution mixtures were formed with 25 g field moist soil (from triplicate soil cores) in 25 mL media comprised of KNO<sub>3</sub> (1.01 g/L), dextrose (1.80 g/L), chloramphenicol (0.1 g/L), and deionized water. Slurries were bubbled with N<sub>2</sub> prior to incubations to remove oxygen. Gas samples were withdrawn at 45 and 105 minutes after the injection of acetylene. We expressed DEA rates as  $\mu\text{g-N kg}^{-1}\text{hr}^{-1}$ , on the basis of dry soil after adjusting for soil moisture.

We measured denitrification potential in static, whole cores either at field moisture (for May and August samplings) or at saturation (July sampling for a nutrient limitation experiment) (Groffman *et al.* 1999). Extracted whole soil cores were placed in

acrylic tubes (20.3 x 2.2 cm inner diameter) in the field and held capped and refrigerated in the lab until analysis. Caps on the tubes were replaced with rubber stoppers prior to flushing the headspace with N<sub>2</sub>. For denitrification potential from field moist soil (DP), cores were incubated in the dark after acetylene addition and sampled at 4 and 8 hours. We demonstrated that N<sub>2</sub>O production was constant over the 8-hour incubation period with laboratory trials on saturated and unsaturated cores from one of our sites with low soil N (Difficult Run, VA), and thus not inhibited by NO<sub>3</sub> availability. A nutrient limitation test was conducted with saturated static cores and three treatments: saturation (DP<sub>s</sub>), saturation + nitrate (DP<sub>n</sub>), and saturation + nitrate + carbon (DP<sub>nc</sub>). Nutrient solutions were prepared with the same KNO<sub>3</sub> and dextrose concentrations used for DEA measurement. Cores were submerged in 15 mL of solution prior to headspace adjustment, and sampled at 4 and 8 hours after the start of incubation. Denitrification potential in field moist cores and saturated measurements were expressed by soil volume as  $\mu\text{g N m}^{-3} \text{ hr}^{-1}$ .

### **Floodplain soil and vegetation measurements**

Soil gravimetric moisture, measured from one dedicated soil core, and bulk density (BD), measured using a slide hammer and plastic sleeves (9.9 x 4.7 cm), were measured immediately within the week of sampling. Soils were dried at 105 °C for 48 hours for moisture, expressed as g g<sup>-1</sup>, and over 72 hours for BD, expressed as g cm<sup>-3</sup>.

Triplicate soil cores per plot were specifically retrieved for organic matter (OM), total carbon (C), total nitrogen (N), and pH, and were air dried after the soil collection period. Soils were then ground and sieved to less than 2 mm. For OM, measured by mass loss on ignition, ~2 g soil subsample was dried to 105 °C for 24 hours prior to ignition in

a muffle furnace at 550 °C for 4 hours ( $\text{g g}^{-1}$ ; Nelson and Sommers 1996). For C and N, a separate subsample of soil was dried over 105 °C for 24 hours immediately prior to analysis of 12-18 mg of soil using dry combustion in an elemental analyzer (% of dry soil; Perkin Elmer 2400 Series II). These measurements and the prior soil P measurements were used to calculate soil nutrient mass ratios: C:N, C:P, and N:P. Soil pH was measured with a hand-held sensor in a 1:2 ratio of soil to deionized water (Robertson *et al.* 1999a). Soil slurries were shaken vigorously and allowed to settle for 30 minutes prior to taking measurement of the solution above the soil.

Two soil cores per plot were retrieved for potential carbon mineralization in the spring only and were refrigerated until analysis. We used short-term measurements with field moist soils as an index of “immediately-available” carbon given soil moisture (Robertson *et al.* 1999b). We placed 20 grams of dry-weight equivalent field moist soil (mean water-filled pore space of soils = 74%) into 360  $\text{cm}^3$  glass jars and let the soils equilibrate to room temperature overnight. We measured  $\text{CO}_2$  flux ( $\text{mmol-CO}_2 \text{ kg-dw}^{-1}\text{hr}^{-1}$ ) over 2 minutes, twice per plot, using a LI-COR 8100A automated soil gas flux system adapted for use with small chambers (Craft *et al.* 2003).

For measurements of soil  $\text{NH}_4$ ,  $\text{NO}_3$ , and orthophosphate (SRP: soluble reactive phosphorus), salt extractions (Robertson *et al.* 1999b) on dedicated duplicate soil cores per plot occurred no later than 3 days following collection from each floodplain site. Six grams of field moist soil was mixed with 40 mL of 2M KCL on a shaker table for an hour then allowed to gravity separate for 30 minutes. Supernatant was filtered through an Acrodisc syringe filter with a Supor 0.45  $\mu\text{m}$  membrane and measured photometrically

on a discrete analyzer (SEAL analytical, AQ2) along with 2M KCl blanks and an external standard (ERA, Arvada, Colorado, USA). Measured concentrations of N or P were expressed as  $\mu\text{g-N g-dw}^{-1}$  or  $\mu\text{g-P g-dw}^{-1}$ .

We measured three characteristics of the plant community: herbaceous cover, herbaceous aboveground biomass (AGB), and belowground biomass (BGB). Total percent cover of the herbaceous plant community (and woody plants < 1 m tall) was measured in 1 x 2 m grids using 11 cover classes (Tiner 1999). Cover class midpoints per plot were used for further statistical analysis. Aboveground herbaceous biomass was harvested within a 1 m<sup>2</sup> grid and placed in paper bags. Soil cores (6.4 cm diameter; 30 cm depth) for analysis of belowground biomass (Bledsoe *et al.* 1999) were held in plastic bags and refrigerated until roots  $\geq 2$  mm were washed and sieved from the soil. Above and belowground biomass was dried at 60 °C to constant mass before weighing. Biomass was calculated by area as  $\text{g-dw m}^{-2}$ .

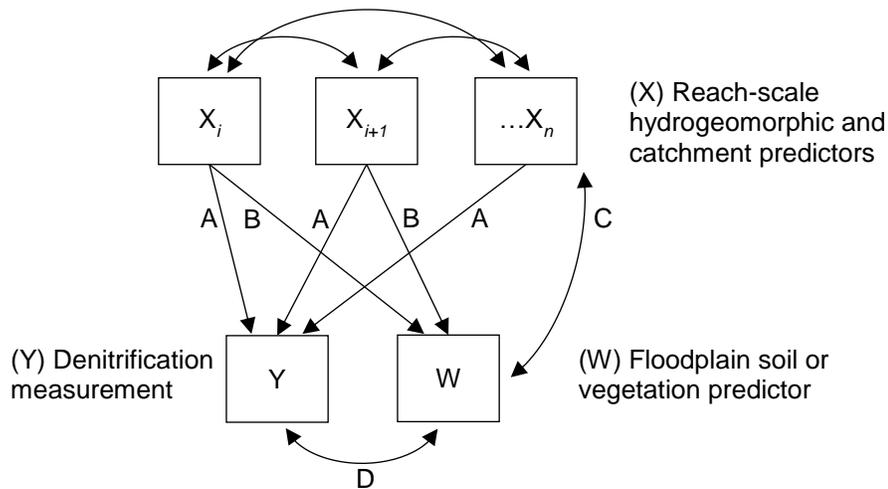
### **Data analysis**

We evaluated all linear relationships between denitrification measurements and the explanatory variables using generalized least squares linear models with restricted maximum likelihood to test model fit (Zuur *et al.* 2009). Mean values were calculated for plots at the site level for each sampling month (N = 18). Logarithmic (base 10) transformations were used on variables to reduce outliers, the spread of data over multiple orders of magnitude, or skew. Transformations were applied consistently across seasons on the following: DEA, AGB, BGB, soil NO<sub>3</sub>, NH<sub>4</sub>, and SRP, stream concentrations of N and P, stream discharge, and floodplain sedimentation. We explicitly

modeled spatial correlation structures where necessary to account for spatial dependency in the residuals of the linear regression model. The largest model improvement in Akaike's Information Criterion with the inclusion of a structure (*i.e.*, Gaussian, rational, and spherical) was tested for significance (*i.e.*, a violation of spatial independence) with a likelihood ratio test. For the two categorical variables, physiographic province and season, we evaluated mean differences in denitrification rates with either the one-way analysis of variance or paired *t*-test, respectively. Specifically for our first objective to investigate reach-scale hydrogeomorphic and catchment controls, we sought to determine whether two or more largescale variables were measuring one underlying causal effect on the denitrification measurements. Thus, we evaluated associations between statistically significant largescale predictors of the denitrification measurements using Pearson correlation coefficients. In one instance of spatially dependent variables, we modeled and interpreted partial regression coefficients on denitrification measurements.

For our second objective, we applied path analysis to our results from the first objective, the statistically significant largescale (reach and catchment) predictors, in combination with the statistically significant local (soil and vegetation) predictors. We evaluated the partial linear effects of the largescale predictors on the denitrification measurements as well as the possible explanations for those partial effects using the local predictors. Models were constructed for each denitrification measurement made in May and August because concurrent soil and vegetation measurements were also available, which was not the case for July DP measurements. The general structure of models (Fig. 4.3) followed our conceptual model (Fig. 4.1) and included causal pathways (partial

regression coefficients) from largescale predictors to a denitrification measurement and to a floodplain soil and vegetation predictor to determine the unique effects (effect controlling for other variables' effects; a partial effect) on each of the two response variables. For any vegetation or soil composition predictor and their mediating effect on reach-scale hydrogeomorphic variables, we used a reciprocal pathway (partial correlation) that controls for all other effects in the model instead of a causal pathway (Fig. 4.3). A reciprocal pathway was also used between the denitrification measurement and soil and vegetation predictor to capture the correlation after controlling for the effects of the largescale variables (Fig. 4.3). Though no causal relationship was defined in the model between the denitrification measurement and the local predictor, certain directional-appropriate outcomes were interpreted as causal and supported an overall interpretation of mediation. Correlations among the largescale variables are identical to bivariate linear correlations (Fig. 4.3). For each denitrification measurement and its one or more largescale predictors, we constructed multiple models for each floodplain soil and vegetation predictor separately. Multiple  $R^2$  presented for the path models are not adjusted by sample size, and are not comparable to the adjusted values presented for other analyses.



**Figure 4.3.** Statistical model to test for the partial causal effects of multiple reach-scale hydrogeomorphic or catchment (largescale) predictors on a denitrification measurement through paths (A), and on a floodplain soil or vegetation predictor of Y causally through paths (B) or causally on but with a reciprocal effect through path (C), and the resulting partial correlation path (D) between Y and W after controlling for the effects of the largescale predictors.

To assess the relative explanatory power of the one or more largescale predictors on each denitrification measurement, our third objective, we evaluated the total and unique variation explained by the reach-scale hydrogeomorphic and catchment predictors compared to the floodplain soil and vegetation predictors using partial redundancy analysis. For use on a single response variable, partial redundancy analysis is a variant of multiple regression that clearly communicates the unique and shared explanatory contribution of two or more groups of predictors on a response variable using semipartial  $R^2$  (Legendre and Legendre 2012). Largescale characteristics can be useful for denitrification prediction where they are indicative of broad functional attributes of the floodplain that integrate the multiple relationships of denitrification to its proximal and local biogeochemical controls. The same variables used for path modeling were used for redundancy analyses; characteristics not statistically related to denitrification

measurements were excluded to avoid artificially inflating adjusted  $R^2$  values. Unique variation (the only statistically testable fraction) was tested for significance by permutation. Moderate to severe multi-collinearities (variance inflation factors  $> 10$ ) within each set of predictors were reduced by data reduction through principal component analysis. With the use of principal component scores as predictors, no severe multicollinearity (variance inflation factors  $> 15$ ) was found across the two sets of predictors. Path modeling was conducted in Mplus v7.11 (Muthén and Muthén, 2013). All other statistical analyses were conducted in R v3.3.2 (R Core Team 2016). Statistical significance was defined at  $\alpha = 0.05$ .

## Results

### Variability in denitrification measurements across sites and seasons

Variability of denitrification was associated with the availability of N and C and followed patterns of floodplain soil and vegetation characteristics. Rates of DEA in spring ( $7.2\text{-}1113 \mu\text{g-N kg}^{-1} \text{hr}^{-1}$ ), and summer ( $9.7\text{-}1192 \mu\text{g-N kg}^{-1} \text{hr}^{-1}$ ), and rates of DP in spring ( $0.1\text{-}166.4 \mu\text{g-N m}^{-3} \text{hr}^{-1}$ ) and summer ( $2\text{-}38.6 \mu\text{g-N kg}^{-1} \text{hr}^{-1}$ ) spanned 2-3 orders of magnitude. We inferred nutrient limitation of DP for each site where amended mean rates were larger in value (directionally) than unamended mean rates. Denitrification was limited by  $\text{NO}_3$  in 15 of 18 floodplains ( $\text{DP}_n > \text{DP}_s$ ), including solely by  $\text{NO}_3$  in one floodplain ( $\text{DP}_{nc} = \text{DP}_n$ ); limited jointly by  $\text{NO}_3$  and C at 14 sites ( $\text{DP}_{nc} > \text{DP}_n > \text{DP}_s$ ); and limited by a combination of  $\text{NO}_3$  and/or C at all sites ( $\text{DP}_{nc} > \text{DP}_s$ ) (Fig. 4.4). One or more denitrification measurements were positively related to soil pH, moisture, potential C mineralization, OM, C, N, P, C:N,  $\text{NO}_3$ , and SRP, and herbaceous

AGB; DP in August was negatively related to BD (Table 4.2). The calculation of DEA on a dry weight basis involves soil moisture and thus moisture was not assessed as a predictor of DEA (Table 4.2). No denitrification measurements were related to mean maximum daily June or July soil temperature, soil NH<sub>4</sub>, soil N:P or C:P ratios, herbaceous plant cover, belowground biomass, or the percent of silt and clay in the soil (Table 4.2).

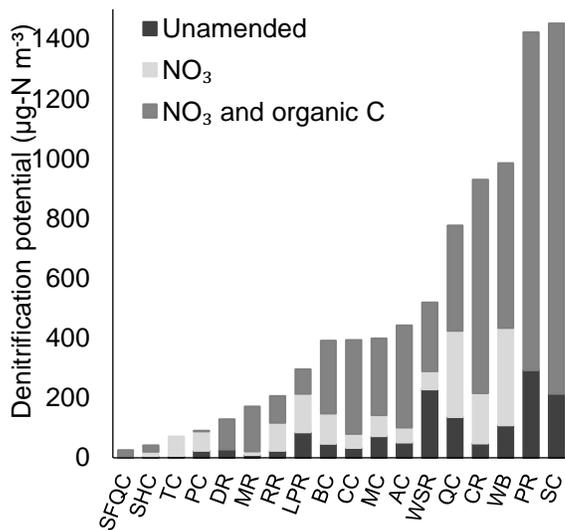


Figure 4.4. Gains in denitrification potential with two nutrient amendments. Site acronyms: Antietam Creek (AC), Back Creek (BC), Catoctin Creek (CC), Conestoga River (CR), Difficult Run (DR), Little Patuxent River (LPR), Mattaponi River (MR), Morgan Creek (MC), Patuxent River (PR), Polecat Creek (PC), Quittapahilla Creek (QC), Rappahannock River (RR), Sideling Hill Creek (SHC), Smith Creek (SC), South Fork Quantico Creek (SFQC), Tuckahoe Creek (TC), Warm Springs Run (WSR), Western Branch (WB).

Rates of DP were lower in summer than spring ( $t = -3.94$ ,  $P = 0.001$ ), along with lower soil moisture ( $t = -3.5$ ,  $P < 0.01$ ), NH<sub>4</sub> ( $t = -10.8$ ,  $P < 0.001$ ), and OM ( $t = -4.05$ ,  $P < 0.001$ ). At the same time, floodplains in summer had higher soil NO<sub>3</sub> ( $t = 5.94$ ,

$P < 0.001$ ), SRP ( $t = 7.05$ ,  $P < 0.001$ ), pH ( $t = 2.3$ ,  $P < 0.05$ ) and temperature (22.9,  $P < 0.001$ ). Season had no effect ( $P > 0.05$ ) on DEA, soil nutrient ratios, bulk density, C, and N. The remaining floodplain soil characteristics were measured only once.

**Table 4.2. Regression coefficients for the linear relationships between floodplain soil and vegetation explanatory variables and denitrification response variables<sup>1</sup> by month of sampling**

<i>Explanatory Variables</i>	DEA (May)	DEA (August)	DP (May)	DP (August)
pH	0.46**	0.39**	21.9	0.55 <sup>g</sup>
Gravimetric moisture	na	na	114	29.7* <sup>r</sup>
Bulk density	-0.49	-0.78	-45.4	-16.8* <sup>g</sup>
Potential C mineralization	3.96**	nm	302*	nm
Organic matter	0.14*	0.19**	9.02	3.44*** <sup>g</sup>
C	0.32*	0.29**	19.4	1.63 <sup>r</sup>
N	5.32**	5.28**	340	76.8* <sup>s</sup>
P	1.37**	1.45***	64.9	7.94 <sup>g</sup>
C:N	0.17*** <sup>g</sup>	0.04	8.10	0.40 <sup>g</sup>
C:P	-0.10	-0.01	-1.99	0.08
N:P	-1.86	-0.18	-59.7	2.69
Ammonium	0.05	0.17	34.6	8.86 <sup>g</sup>
Nitrate	1.03***	1.55***	80.8***	17.3* <sup>r</sup>
Soluble reactive P	0.35*	2.01	7.84	3.36 <sup>g</sup>
Herbaceous aboveground biomass	1.02	1.10*	96.3	3.97 <sup>g</sup>
Herbaceous plant cover	0.02	0.01	0.88	-0.04
Belowground biomass	-0.77	-0.71	-75.4	-1.53 <sup>g</sup>
Silt and clay	0.00	0.01	0.22	0.18 <sup>r</sup>
June temperature, mean daily maximum	0.11	na	-0.50	na
July temperature, mean daily maximum	na	0.03	na	0.52

Notes: Asterisks denote statistical significance: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

Letters indicate the type of correlation structure applied to the model to eliminate spatial dependency in the residuals: g = Gaussian, r = rational, s = spherical.

Missing data acronyms: na = not applicable; nm = not measured

<sup>1</sup> Denitrification enzyme activity (DEA) is expressed as  $\mu\text{g-N kg}^{-1} \text{hr}^{-1}$  and denitrification potential (DP) is expressed as  $\mu\text{g-N m}^{-3} \text{hr}^{-1}$ .

### **Relationships of reach-scale hydrogeomorphic and catchment characteristics with denitrification measurements**

The seven denitrification measurements were linearly related to multiple largescale characteristics (Table 4.3): positive predictors of denitrification were floodplain sedimentation rates, stream total N and P concentrations, and catchment urban land cover; negative predictors were catchment forested land cover, seasonal daily maximum air temperature, and channel width-to-depth ratio (statistical test: linear regression with model fit by restricted maximum likelihood). For catchment land cover, DEA responded more linearly to changes in % forested land cover than to % urban land cover (Fig. 4.5). No denitrification measurements were linearly related to channel or floodplain width, bank height, stream slope, stream discharge, stream elevation, catchment area, catchment agricultural land cover, annual daily maximum air temperature, or physiographic province (Table 4.3).

**Table 4.3. Regression coefficients for the linear relationships between reach-scale hydrogeomorphic and catchment explanatory variables and denitrification response variables<sup>1</sup> by month of sampling**

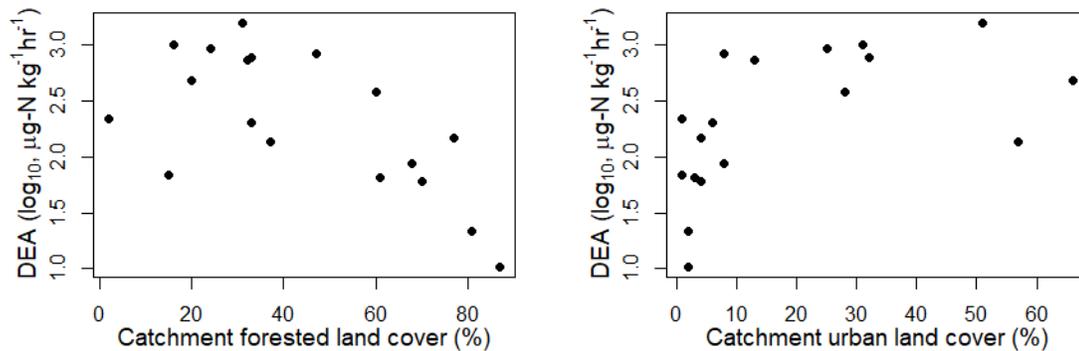
<i>Explanatory Variables</i>	DEA (May)	DEA (August)	DP (May)	DP (August)	DP <sub>s</sub> (July)	DP <sub>n</sub> (July)	DP <sub>nc</sub> (July)
<b>Reach-scale hydrogeomorphic</b>							
Channel bank height	0.27	0.33	12.3	-3.50 <sup>g</sup>	0.17	0.19	0.25
Channel width	0.00	0.01	-1.80	-0.11 <sup>g</sup>	-0.00	0.00	0.01
Channel width-to-depth ratio	-0.03	-0.04	-8.32 <sup>**</sup>	-0.18 <sup>g</sup>	-0.04	-0.03	-0.02
Entrenchment ratio	-0.05	-0.04	-2.61	0.35 <sup>d</sup>	-0.04	-0.04	-0.06
Floodplain width	-0.00	-0.00	-0.36	-0.02 <sup>g</sup>	-0.00	-0.00	-0.00
Stream slope	-85.4	-140	9660	1648 <sup>g</sup>	34.1	-3.59	-67.7
Stream elevation	0.00	0.00	0.26	-0.01 <sup>g</sup>	0.00	0.00	0.00
Stream discharge	0.31	0.33	-27.4	-1.66 <sup>g</sup>	0.12	0.24	0.30
Stream N concentration	0.89 <sup>*</sup>	0.69 <sup>*</sup>	45.0	7.20 <sup>g</sup>	0.54	-0.56	0.56
Stream P concentration	1.42 <sup>**</sup>	1.13 <sup>**</sup>	64.0	7.42 <sup>g</sup>	0.78	0.76 <sup>*</sup>	0.85
Floodplain sedimentation	0.00 <sup>*</sup>	0.00 <sup>*</sup>	0.01 <sup>*</sup>	-0.00 <sup>g</sup>	0.00	0.00	0.00
<b>Catchment</b>							
Catchment forested land cover	-1.57 <sup>**</sup>	-1.30 <sup>*</sup>	-113 <sup>*</sup>	-35.0 <sup>**</sup>	-1.15 <sup>*</sup>	-1.12 <sup>*</sup>	-0.99 <sup>*</sup>
Catchment urban land cover	1.61 <sup>*</sup>	0.98	161 <sup>*</sup>	4.74 <sup>g</sup>	1.38 <sup>*</sup>	0.84	0.84
Catchment agricultural land cover	0.63	0.78	18.2	19.4 <sup>g</sup>	0.40	0.67	0.57
Catchment area	0.00	0.00	-0.05	-0.00 <sup>g</sup>	0.00	0.00	0.00
Daily max. air temperature, spring	-0.20 <sup>*</sup>	n/a	-13.6	n/a	n/a	n/a	n/a
Daily max. air temperature, summer	n/a	-0.19	n/a	-3.79 <sup>r</sup>	-0.35 <sup>*</sup>	-0.27	-0.27
Daily max. air temperature, full year	-0.18	-0.11	-13.2	-1.68 <sup>g</sup>	-0.16	-0.14	-0.15
Physiographic province <sup>2</sup>	0.39	0.17	0.19	1.10	0.73	0.83	0.46

Notes: Asterisks denote statistical significance: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$

Letters indicate the type of correlation structure applied to the model to remove spatial dependency in the residuals: g = Gaussian, r = rational, s = spherical.

<sup>1</sup> ANOVA  $F$  values presented for physiographic province

<sup>2</sup> Denitrification enzyme activity (DEA) is expressed as  $\mu\text{g-N kg}^{-1} \text{ hr}^{-1}$  and denitrification potential (DP) is expressed as  $\mu\text{g-N m}^{-3} \text{ hr}^{-1}$ .



**Figure 4.5.** Scatterplots of catchment land cover (forested and urban) with denitrification enzyme activity (DEA) in spring.

Among the reach-scale hydrogeomorphic and catchment predictors linearly related to denitrification measurements, we found multiple instances of correlation among predictors (Table 4.4). We inferred that forested land cover caused gradients in N and P stream concentrations and their effects on denitrification (Table 4.4). To eliminate collinearity in further statistical tests, we used catchment forested land cover as a proxy for stream N and P concentrations. For the positive correlation between catchment forested land cover and spring air temperature (Table 4.4), we assessed whether the variables had unique effects (*i.e.*, effect controlling for another variable's effect) on the denitrification measurements apart from geographic overlap. With partial regression coefficients, only forested land cover was a significant predictor of spring DEA (data not presented). In other words, once the variation explained by forested land cover was accounted for, the remaining variation in spring DEA explained by spring temperature was not significant ( $P > 0.05$ ). In spite of this result, the daily maximum air temperature

was interpreted as a predictor and used for further analysis because summer daily maximum air temperature (not correlated with forested land cover, so no partial regression coefficients were calculated) was a significant predictor of  $DP_s$  in the summer (Table 4.3). The negative correlation between stream N concentration and spring air temperature was interpreted as an effect of catchment forested land cover and not assessed further (Table 4.4).

**Table 4.4. Correlation matrix for reach-scale hydrogeomorphic and catchment predictors<sup>1</sup> of denitrification measurements**

	Forest	Urban	Stream N	Stream P	Channel WD	Sediment.	Air Temp. Spring
Urban	x						
Stream N	-0.84 <sup>***</sup>	x					
Stream P	-0.83 <sup>***</sup>	x	0.78 <sup>***</sup>				
Channel WD	0.4 <sup>†</sup>	x	x	x			
Sediment.	x	x	x	x	x		
Air Temp. Spring	0.48 <sup>*</sup>	x	-0.59 <sup>**</sup>	x	x	x	
Air Temp. Summer	x	x	-0.44 <sup>†</sup>	x	x	x	0.86 <sup>**</sup>

Notes: Notation of statistical significance: x denotes  $P > 0.1$ ; <sup>†</sup> $P < 0.1$ ; <sup>\*</sup> $P < 0.05$ ; <sup>\*\*</sup> $P < 0.01$ ; <sup>\*\*\*</sup> $P < 0.001$

Abbreviations: Forest = catchment forested land cover, urban = catchment urban land cover, stream N = stream N concentration, stream P = stream P concentration, WD = width-to-depth ratio, sediment. = floodplain sedimentation, air temp. = maximum daily air temperature.

<sup>1</sup> Predictors are characteristics that are linearly related to at least one denitrification measurement (Table 3).

### **Mediating effects of floodplain soil and vegetation predictors**

We constructed path models with the predictors that were found to be significantly related to the denitrification measurements (i.e. Tables 4.2 and 4.3). Those predictors that had been modeled with spatial correlation structures could be included in the redundancy analysis because the spatial correlations were statistically explained using the largescale predictors: for spring DEA, including either sedimentation or daily maximum spring temperature as a covariate in the regression model removed the significant spatial correlation with C:N; for summer DP, including forested land cover in the regression model removed the significant spatial correlations with moisture, BD, OM, N, and NO<sub>3</sub>.

Path modeling revealed a plausible set of explanatory mechanisms for the effects of reach-scale hydrogeomorphic and catchment characteristics on denitrification measurements (Tables 4.5 and 4.6). Catchment forested land cover had direct partial effects on DEA (paths A) in spring and summer, and floodplain soil NO<sub>3</sub>, soil P, and AGB were mediators of these relationships (AGB in summer): partial correlation between DEA and soil NO<sub>3</sub>, soil P, and AGB were significant (paths D), as were the effects of forested land cover on soil NO<sub>3</sub>, soil P, and AGB (paths B) (Table 4.5). Thus, greater non-forested land cover promoted greater soil DEA, NO<sub>3</sub>, P, and AGB in floodplains, and separate from these effects, DEA positively tracked with NO<sub>3</sub>, P, and AGB in floodplain soils. In spring and summer, floodplain sedimentation also had a direct partial effect on DEA at the same time that sedimentation had an effect on soil P, pH, and potential C mineralization, which in turn were all partially correlated (positively)

with DEA (Table 4.5). On DP, catchment forested land cover, floodplain sedimentation, and channel width-to-depth ratio had significant partial effects in spring and or summer (Table 4.6). DP was positively and partially correlated to potential C mineralization in the spring, and both variables were affected by floodplain sedimentation.

### **Partitioning explained variation among local and largescale predictors**

Using redundancy analysis, we partitioned variation of each May and August denitrification measurement between floodplain soil and vegetation (local) predictors and reach-scale hydrogeomorphic and catchment (largescale) predictors (Fig. 4.6). The same predictors used for path modeling were used for redundancy analysis. Within the set of local predictors, we reduced severe collinearity using principal component scores that captured the bulk percentage of total variation between the variables: soil potential C mineralization, OM, C, and N in spring (91%); soil OM, C, and N in the summer for DEA (95%) or soil OM and N in summer for DP (97%); and soil pH and SRP in spring (92%). Largescale predictors explained 43-57% of variation in denitrification measurements (unique and shared variation with the local predictors), with 22-30% of DP uniquely explained ( $P < 0.05$ ) in spring and summer (Fig. 4.6). Floodplain soil and vegetation predictors explained between 27-77%, with 15% of DEA uniquely explained ( $P < 0.05$ ) in spring (Fig. 4.6). Shared variation between the largescale and floodplain soil and vegetation predictors ranged from 15-46% (not statistically testable), while unexplained variation was greater in the summer (41-43%) than spring (13-32%) (Fig. 4.6).

**Table 4.5. Standardized coefficients from path models (Fig. 4.3) for partial effects of reach-scale hydrogeomorphic and catchment (largescale) predictors<sup>1</sup> on denitrification enzyme activity (DEA) (path A) and an individually-modelled<sup>2</sup> floodplain soil or vegetation (local) predictor<sup>3</sup> (Paths B or C)<sup>4</sup>, and resulting partial correlation between denitrification enzyme activity and the local predictor (Path D)**

Largescale Predictors <sup>5</sup>	Path A: DEA	Paths B,C,D	NO <sub>3</sub>	SRP	P	pH	Pot. C min.	OM	C	N	C:N	AGB
<b>Spring</b>												
1. Floodplain sedimentation	0.36*	B: D:	0.28 0.71***	0.26 0.72***	0.00* 0.03*	0.40* 0.66***	0.53** 0.59***	0.26 0.65***	0.21 0.68***	0.30 0.72***	-0.07 0.44*	n/a
2. Catchment forested land cover	-0.43**	B: D:	-0.45** 0.71***	-0.19 0.72***	-0.98*** 0.03*	-0.23 0.66***	-0.08 -0.59***	-0.06 0.65***	-0.03 0.68***	0.01 0.72***	-0.15 0.44*	n/a
3. Catchment urban land cover	0.19	B: D:	-0.02 0.71***	-0.54** 0.72***	-0.62** 0.03*	-0.33 0.66***	-0.25 -0.59***	0.08 0.65***	0.00 0.68***	-0.01 0.72***	0.10 0.44*	n/a
4. Daily maximum spring air temperature	-0.20	B: D:	-0.34 0.71***	-0.48*** 0.72***	-0.00 0.03*	-0.55** 0.66***	-0.27 -0.59***	-0.03 0.65***	-0.18 0.68***	-0.17 0.72***	0.05 0.44*	n/a
<b>Summer</b>												
5. Floodplain sedimentation	-0.42**	B/C: D:	0.33 0.57***	n/a	0.19 0.52**	0.32 0.56**	n/a	0.27 0.67***	0.30 0.53**	0.33 0.61***	n/a	0.03 0.39*
6. Catchment forested land cover	-0.51**	B: D:	-0.53** 0.57***	n/a	-0.69*** 0.52**	-0.31 0.56**	n/a	-0.21 0.67***	-0.22 0.53**	-0.26 0.61***	n/a	-0.42* 0.39*

Notes: Notation of statistical significance: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

<sup>1</sup> Variables linearly related to DEA in either spring or summer (Tables 2 and 3) were modelled, otherwise "n/a".

<sup>2</sup> The spring and summer models were applied to each floodplain soil and vegetation predictor individually.

<sup>3</sup> All floodplain soil and plant predictors were measured seasonally with the exception of AGB and P. Abbreviations: SRP = soluble reactive phosphorus, Pot. C min. = potential carbon mineralization, OM = organic matter, GM = gravimetric moisture, AGB = herbaceous aboveground biomass.

<sup>4</sup> The path between floodplain sedimentation and herbaceous AGB (Path C) was modeled as a reciprocal effect (partial correlation).

<sup>5</sup> The following paths modelled between largescale predictors were statistically significant ( $P < 0.05$ ): 2-3 (-) and 2-4 (+).

**Table 4.6. Standardized coefficients from path models (Fig. 4.3) for effects of reach-scale hydrogeomorphic and catchment (largescale) predictors<sup>1</sup> on denitrification potential (DP) (Paths A) and an individually-modelled<sup>2</sup> floodplain soil predictor (Paths B)<sup>3</sup> and resulting partial correlation between denitrification potential and the soil predictor (Path D)**

Largescale predictors <sup>4</sup>	Path A: DP	Path	NO <sub>3</sub>	Pot. C min.	OM	N	GM	BD
<b>Spring</b>								
1. Floodplain sedimentation	0.30*	B:	0.27	0.54**	n/a	n/a	n/a	n/a
		D:	0.58***	0.42**				
2. Channel width-to-depth ratio	-0.39*	B:	-0.05	0.04	n/a	n/a	n/a	n/a
		D:	0.58***	0.42***				
3. Catchment forested land cover	-0.17	B:	-0.58**	-0.21	n/a	n/a	n/a	n/a
		D:	0.58***	0.42***				
4. Catchment urban land cover	0.29	B:	0.04	-0.20	n/a	n/a	n/a	n/a
		D:	0.58***	0.42***				
<b>Summer</b>								
5. Catchment forested land cover	-0.70***	B:	-0.57***	n/a	-0.25	-0.30	-0.29	-0.16
		D:	0.30		0.45*	0.22	0.65***	-0.53**

Notes: Notation of statistical significance: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

<sup>1</sup> Variables linearly related to DEA in either spring or summer (Tables 2 and 3) were modelled, otherwise “n/a”.

<sup>2</sup> The spring and summer models were applied to each floodplain soil and vegetation predictor individually.

<sup>3</sup> All floodplain soil and plant predictors were measured seasonally. Abbreviations: Pot. C min. = potential carbon mineralization; OM = organic matter; GM = gravimetric moisture; BD = bulk density.

<sup>4</sup> The following relationships between largescale predictors were statistically significant ( $P < 0.05$ ): 3-4 (-).

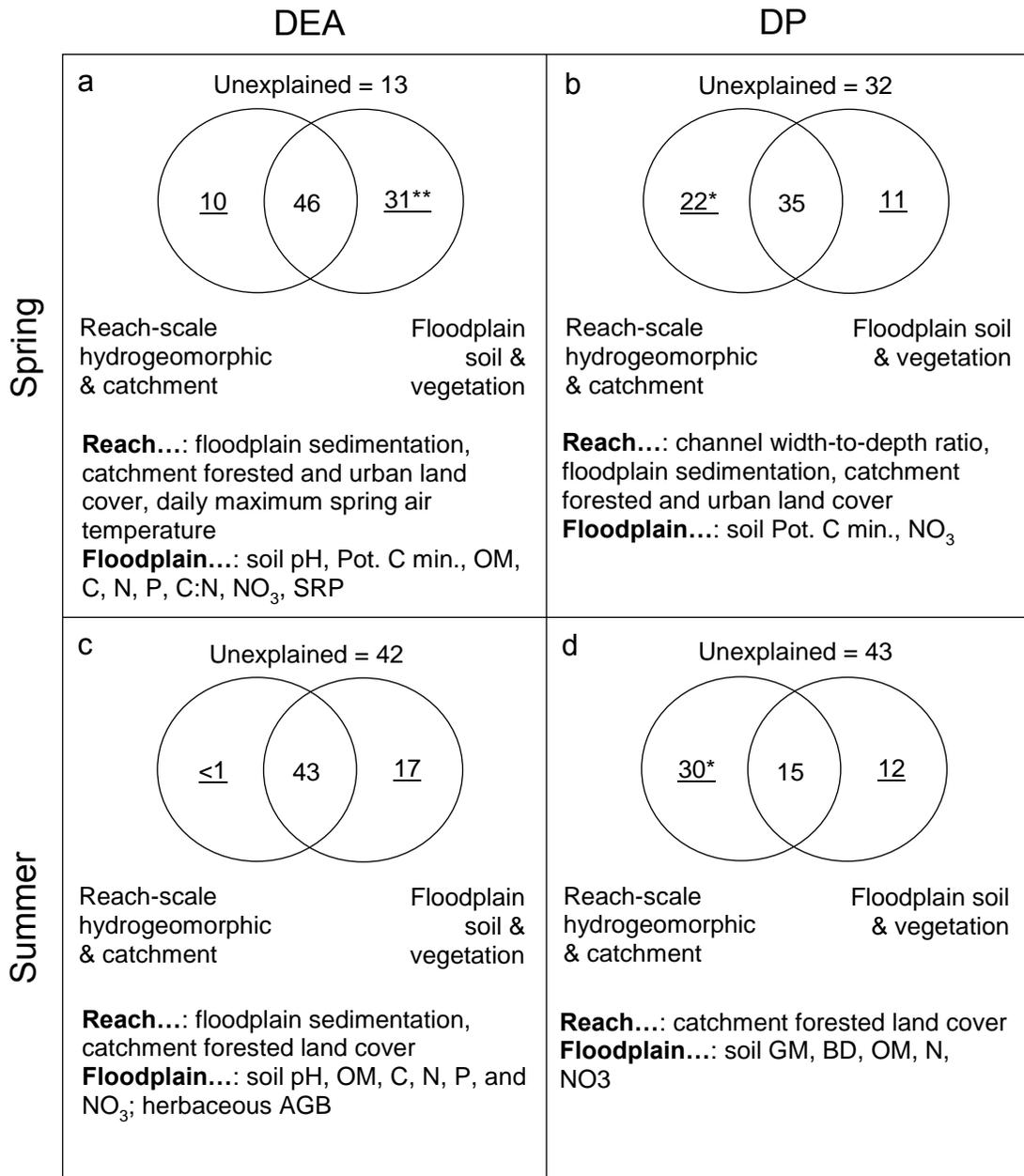


Figure 4.6. Partitioned percent variation of DEA and DP variables for May and August by groups of predictors. Unexplained percent variation outside circles. Underlined values are statistically testable, non-shared fractions: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Abbreviations: Pot. C min. = potential carbon mineralization, OM = organic matter, SRP = soluble reactive phosphorus, GM = gravimetric moisture, AGB = aboveground biomass, BD = bulk density.

## Discussion

### Variability in denitrification across sites and seasons

Floodplain denitrification across a large, diverse regional landscape was largely limited by  $\text{NO}_3$  or combined  $\text{NO}_3$  and C, as demonstrated experimentally with amendments to saturated soil cores. Only in three floodplains (all with catchments high in either agricultural or urban land cover) was denitrification not limited by nitrate, which is consistent with previous findings of nitrate-limitation of denitrification and the theory that nitrogen availability often limits ecosystem functioning in floodplains (Forshay and Stanley 2005, Schilling and Lockaby 2005, Antheunisse *et al.* 2006). Two floodplains, both on the Coastal Plain with sandy soils and high entrenchment ratios ( $>8$ ), were almost entirely limited by nitrate. One of these floodplains, Tuckahoe Creek, showed little soil development with the lowest soil C, N, OM, potential C mineralization, C:P, N:P, and moisture, and the highest soil BD, across seasons. The other floodplain, Polecat Creek, was notable for the highest belowground biomass (and below-average organic matter and nutrient stores). Most floodplains (14 of 18) showed both  $\text{NO}_3$  ( $\text{DP}_s < \text{DP}_n$ ) and C limitation ( $\text{DP}_n < \text{DP}_{nc}$ ), indicating predominantly joint regulation at regional scales. The regression results corroborate this conclusion, where nitrogen and carbon-related soil floodplain characteristics (*i.e.*,  $\text{NO}_3$ , N, OM, Pot. C. min, C:N) along with moisture, were in aggregate the most consistent predictors (all positive) of the four denitrification measurements. While this study did not examine isolated C limitation (in the absence of nitrate amendment), Waters *et al.* (2014) found that denitrification potential was primarily C limited in some forested and especially herbaceous riparian soils in urban catchments near Baltimore, MD. Long-term flooding of alluvial soils stymies soil profile

development and the accumulation of organic C by the erosion of organic litter and burial by new mineral sediment (Cabezas and Comin 2010, Saint-Laurent *et al.* 2016). Our data indicated that denitrification was sensitive to microbial respiration, an index of labile carbon availability, in the spring, at the same time that DEA was positively related to C:N ratios of the soil suggesting C limitation. Certain denitrification measurements were positively related to soil P or SRP, but the relatively low median ratios of soil C:P (39:1) and N:P (3:1) compared to the relative microbial demand for P (C:N:P = 60:7:1) suggests C and N were more limiting than P (Cleveland and Liptzin 2007).

Large environmental changes in the Chesapeake Bay watershed from spring to summer were expected to influence floodplain denitrification. Stream baseflow was substantially lower in August than May at all sites. Herbaceous vegetation had also grown thicker and taller by August (and exceeded 2 m in height at Quittapahilla Creek). We found season was an important determinant of variability in denitrification potential, floodplain nutrient availability, and of the strength of relationships between denitrification with local and largescale characteristics. Average DP was lower in the summer, concurrent with lower soil moisture,  $\text{NH}_4$ , and OM; and higher soil temperatures,  $\text{NO}_3$ , SRP, and pH. Denitrification responds exponentially to changes in water-filled pore space within a threshold of 60-80% (Machefert and Dise 2004), and median water-filled pore space dropped from spring (75%) to summer (64%). Two soil characteristics measured in both May and August, SRP and C:N, were (positive) predictors only in spring. Wetter soils in May would have stimulated potential C mineralization (Wilson *et al.* 2011; Shrestha *et al.* 2014), and C demand in turn would

have increased the importance of soil C:N. By summer, soil organic matter stores were smaller and labile C pools may also have more evenly inhibited denitrification rates across sites (weakening denitrification – C:N relationships). Senescence of ephemeral spring vegetation was first observed in July and thus increases in water-soluble labile C from the senescence of spring vegetation was less likely to have contributed to the results in May. For SRP, lower availability in spring would strengthen the relationship to DEA.

### **Influence of reach-scale hydrogeomorphic characteristics on denitrification**

Channel dimensions can be useful indicators of difficult to measure hydrologic and geomorphic processes (Schenk *et al.* 2013). In the spring, small width-to-depth ratio of stream channels (more incised) was a linear predictor of high DP, with direct but no mediating effects on DP identified (path modeling). Channel geometry is influenced by the composition of the stream banks, bedrock control of channel incision, and the stability and slope of the stream relative to discharge (Rosgen 1994, Gordon *et al.* 2004). In this study, greater channel width-to-depth ratio was associated with lower annual stream flow ( $r = 0.54$ ,  $P < 0.05$ ) and smaller drainage area, with three of the five lowest ratios found in the smallest catchments (21.7 – 32.7 km<sup>2</sup>). Catchment drainage area was not predictive of denitrification, in contrast to other studies that have found changes in floodplain biogeochemistry along longitudinal gradients of streams from headwaters to larger rivers (Arp and Cooper 2004, Noe *et al.* 2013). Greater channel width is associated with greater annual peak bankfull discharges (Parrett and Johnson 2004), and for floodplains in the CB watershed, likely more frequent or energetic spring flooding with higher winter and spring baseflow. Spring flooding can export inorganic N and P from

floodplains, and particularly  $\text{NO}_3$ , a primary predictor of denitrification potential in spring (Noe and Hupp 2007). This would explain why channel width-to-depth ratio was only associated with denitrification potential measured in field moist, whole soil cores.

Though water fluctuations in floodplains leach mineralized nutrient forms (Bechtold *et al.* 2003), greater hydrologic connectivity between the stream and floodplain increases sedimentation of soil and organic material and dissolved nutrient inputs (Steiger and Gurnell 2002, Noe and Hupp 2005,). In turn, nutrient sedimentation and dissolved inputs have large influences on floodplain biogeochemical cycling (Wassen and Olde Venterink 2006, Noe *et al.* 2013, McMillan and Noe 2017). With floodplain sedimentation related positively to both denitrification measurements and to DEA rates across seasons, our data indicate that sedimentation is an important control on denitrification across the Chesapeake Bay watershed. Similar findings reported by McMillan and Noe (2017) further support this conclusion.

Our path models identified greater soil P, pH, and potential C mineralization as explanations for the effect of sedimentation on denitrification in the spring. This study and others have found that denitrification positively tracks with levels of floodplain P (Ashby *et al.* 1998; McMillan and Noe 2017), the relative availability of which can limit general nutrient processing in floodplains (Schilling and Lockaby 2005). Floodplains sequester large quantities of phosphorus from streams, which is largely in particulate form adsorbed to sediment, and they can serve as sinks for SRP during flooding. Compared to all sources of floodplain P (*e.g.*, overland or subsurface flow), flood deposition of particulate P contributes the most towards floodplain P retention (Noe and

Hupp 2007; Hoffman *et al.* 2009). Sedimentation had an effect on both P and pH, which have previously been found to be positively related and indicative of zones of a floodplain that receive the greatest sedimentation (Kaase and Kupner 2016). Soil pH is a proximal control on denitrification activity (*e.g.*, growth or metabolism), where a pH of 6-8 promotes the highest rates of denitrification with severe limitation occurring below 5 (Saleh-Lakha *et al.* 2009): eight sites had pH within the 6-8 range and three sites had pH below 5. The higher concentrations of nutrients (*e.g.*, base cations) that accompany high sedimentation, as well as labile organic matter and greater hydrologic connectivity, might buffer soil pH levels from long-term microbial decomposition of organic matter and would raise potential C mineralization with greater labile C stores (*e.g.*, dissolved organic C) (Reddy and DeLaune 2008). Thus, the positive associations between denitrification measurements, potential C mineralization, and pH, as affected by floodplain sedimentation, suggests that pH is positively affected by greater microbial heterotrophic respiration generally, of which denitrification contributes a small part (Meronigal and Neubauer 2009). Our measurement of potential C mineralization was taken on field moist soils and captures microbial respiration as affected by site moisture levels. Because floodplain areas with greater sedimentation are more hydrologically connected (*e.g.*, Noe *et al.* 2013), the positive effect of floodplain sedimentation on soil denitrification through the index of labile carbon may have partly been due to a contributing effect of greater soil moisture promoting short-term microbial decomposition (Wilson *et al.* 2011).

### **Influence of catchment characteristics on denitrification**

Forested land cover was a consistent predictor of denitrification measurements.

For six of those measurements, denitrification was markedly elevated in catchments with less than 60-80% forested land cover; for the summer measurements of DP, marked increases were in catchments with less than 60% forested cover. The path models identified soil NO<sub>3</sub>, soil P, and herbaceous AGB as positive mediators of the causal effect of forested land cover on DEA. McMillan and Noe (2017) found that dissolved inputs of NO<sub>3</sub> to floodplain soils, from overbank flooding and overland flow, was positively related to denitrification, and its spatial gradients across floodplains partially differed from sedimentation patterns. In the same way, the positive effect on soil P in non-forested catchments, which was separate from the effect of sedimentation, likely included P from overland flow and dissolved P in floodwater. Anthropogenic land use has previously been linked to reduced nitrogen limitation of plants and greater soil phosphorus levels in floodplains (Antheunisse *et al.* 2006). Greater NO<sub>3</sub> and soil P likely promoted a larger and more diverse herbaceous vegetation community (herbaceous cover ranged from 41-97%) which could favor denitrification such as from greater inputs of labile carbon to the soil (*e.g.*, Korol *et al.* 2016). Forested and agricultural land cover were inversely linearly related ( $r = -.66$ ;  $p < .01$ ), but not to urban land cover, which suggests the largest contributor to the control of denitrification by forested land cover came from agricultural land use.

Isolating the individual effects of urban or agricultural land cover type presents analytical challenges for inherently covarying land cover classes. The strong relationships of denitrification with forested land cover in the Chesapeake Bay watershed make

intuitive sense as both urban and agricultural land cover are both large sources of anthropogenic nutrients to the Chesapeake Bay (Ator *et al.* 2011). Furthermore, catchments with high urban and agricultural land cover overlap spatially at a regional scale in the CB watershed, with high stream nutrient concentrations in the north and east while greater forested land cover and lower stream nutrients are found to the south and west (Langland *et al.* 2013). As a result, below-average denitrification rates were consistently found at the four sites in southern Virginia with catchment forested land cover  $\geq 61\%$ . The lack of linear relationships of agricultural land cover with denitrification measurements was surprising for the CB watershed. Two study sites with highly agricultural catchments were located on the Eastern Shore (the Delmarva Peninsula), which exhibits nationally high rates of nitrogen and phosphorus inputs to catchments and surface- and groundwater (Denver *et al.* 2004). However, dense agricultural land cover is not unique to the Coastal Plain, and the greater variability in denitrification with agriculture might be due to the confounding influence of largescale hydrogeomorphic variability found across the watershed (e.g., entrenchment ratios, though not predictive of denitrification, were strongly related to physiographic location in the watershed); this differs from more geographically clustered urban land use. Notably, low sediment yields in streams are found in heavily forested catchments and those of flat topography on the Eastern Shore (Langland *et al.* 2013), which would reduce material exchange between streams and floodplains. A second reason for the lack of relationships (or weak relationships undetectable by the sample size in this study) may have been due

to the confounding influences of urban land cover on the percentage classes of agricultural land cover.

Urban land cover was a positive predictor of denitrification measurements particularly in the spring and early summer (July). When modeled with other largescale predictors, however, catchment urban land cover had unique negative effects on soil P and SRP only. Hogan and Walbridge (2007) found depressed soil P levels and lower P sorption capacities in floodplains in highly urbanized watersheds (*i.e.*, 24.8-37.6% impervious surface cover), compared to moderately urbanized watersheds, attributable to hydrologic modification and deposition of crystalline Fe. By this standard, seven of the eighteen sites of this study were located in ‘high’ or ‘very high’ urbanized catchments (25-66%) and would have been susceptible to habitat modifications inhibiting P accumulation. Further resolution of urban and agricultural land cover (*e.g.*, with focus on spatial arrangement of cover in a catchment, or indices of stream-floodplain integrity) would likely better discriminate land cover effects on denitrification in floodplains. Denitrification measurements were markedly elevated at urban land cover of about 10% (Fig. 4.5), with pronounced lower rates at lower % urban cover, which indicates very little urban land cover of catchments can have large effects on floodplain denitrification.

Spring and early summer denitrification measurements were inversely related to the maximum daily air temperature summarized by season (spring and summer). Daily maximum temperatures in May (20.4 – 22.1 C) across all sites were slightly more variable than in July (30.6 – 31.9 C). Warmer temperatures increase rates of microbial activity, microbial decomposition of organic matter, mineralization of organic nitrogen

and phosphorus, and are associated with lower pools of labile organic carbon (Keddy and DeLaune 2008), the latter of which would explain our results. An index of labile carbon was an important positive predictor of denitrification in the spring (related to both denitrification measurements). Highest denitrification rates were also found at intermediate soil temperatures (15-20 °C) in a largescale study of alluvial soils across Europe (Pinay *et al.* 2007). No partial effects of spring temperature on DEA were found for the spring path models, presumably due to its shared variance with catchment forested land cover, but spring temperature did have negative effects on soil SRP and pH. Greater microbial activity that depletes organic matter stores with warmer temperatures would increase soil acidity. Greater statistical power might be necessary to resolve the separate mediating effects of spatially-dependent climate metrics and land cover on denitrification in the CB watershed.

### **Partitioning variation among local and largescale predictors of denitrification**

Local biogeochemical and vegetation controls are known to explain large portions of variability in denitrification, but their relationships at broad scales may change and not be as useful or practical as a predictive tool as those controls of denitrification with spatial interactions over larger scales (Merrill and Benning 2006, Kulkarni *et al.* 2014). Our redundancy analysis results indicated that the soil and vegetation predictors of denitrification explained a larger portion of variability in the denitrification measurements (up to 77%) compared to the reach-scale hydrogeomorphic and catchment predictors (up to 57%). In the spring, floodplain soil predictors also explained unique variation in denitrification measurements not explained by the largescale predictors. Even

so, the 43-57% of variation explained by the reach-scale hydrogeomorphic and catchment predictors represents roughly half the variation in denitrification that should be useful for prediction of denitrification across the CB watershed. Largescale predictors explained unique variation only for the two DP measurements (variation not explained by local predictors), which were related to fewer soil and vegetation characteristics than DEA; only DEA was positively related to soil P, SRP, pH, C, C:N, and AGB, suggesting these soil characteristics might be contributing variation to DP that was uniquely explained by the largescale predictors. Merrill and Benning (2006) found that variation in denitrification potential was better explained by riparian ecosystem type, an integrated largescale predictor, than by soil physicochemical properties across a catchment. Progress in denitrification prediction will likely be made when denitrification's local controls are also linked to hydrogeomorphic characteristics and largescale characteristics of river catchments.

## **Conclusions**

The need to understand patterns in denitrification, an important ecological function at regional scales, will continue to grow with the implementation of watershed N management. Both reach-scale hydrogeomorphic and catchment characteristics were predictors of unique variability in our denitrification measurements made in the Chesapeake Bay watershed. Of those, catchment forested land cover was the most consistent predictor (negatively) of all seven denitrification metrics measured across spring and summer, while catchment urban land cover and floodplain sedimentation were the next two most consistent predictors (both positively) of denitrification metrics either

in spring and/or summer. The effects of percent forested land cover on denitrification most strongly reflected inversely covarying patterns with agricultural land cover as well as anthropogenic gradients in stream concentrations of N and P. We showed that greater percent non-forested land cover positively affected floodplain soil NO<sub>3</sub>, total P, and herbaceous aboveground biomass, which in turn was positively related to denitrification enzyme activity across seasons. The effects of floodplain sedimentation on both denitrification measurements were indirectly linked to either higher floodplain pH, P, and/or potential C mineralization. We did not identify any mediating effects of urban land cover on denitrification metrics. On the basis of this mechanistic information, hotspots of floodplain denitrification should be found in catchments of high non-forested land cover and reaches with high rates of nutrient sedimentation because combined they promote multiple proximal controls on denitrification. Thus, floodplains in agricultural and urban catchments of the CB watershed are removing more NO<sub>3</sub> from streams that have greater stream N loads than more forested catchments. For restoration purposes, increasing stream connection and promoting nutrient sedimentation to floodplains in urban or agricultural catchments should remove more unwanted N through denitrification (as well as N and P storage). For prediction purposes across the CB watershed, variation in denitrification measurements was best explained with the use of all local and largescale predictors (57-87%) but was still well explained by the combined reach-scale hydrogeomorphic and catchment predictors (43-57%).

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## **CHAPTER 5 LONGITUDINAL PATTERN OF DENITRIFICATION IN TIDAL FRESHWATER FORESTED WETLANDS ALONG TWO RIVERS**

### **Abstract**

Denitrification exhibits spatial heterogeneity at landscape scales. For tidal freshwater forested wetlands (TFFWs), prominent soil biogeochemical, vegetation, and hydrogeomorphic patterns exist between rivers and along a longitudinal continuum, and little research has examined whether these gradients affect denitrification. We measured denitrification and ecosystem attributes in hummocks and hollows of three TFFW sites (upper, middle, and lower) along both the adjacent Pamunkey and Mattaponi Rivers, to investigate denitrification potential (DP) and denitrification enzyme activity (DEA) by river and site. We also included a nontidal forested floodplain site to compare with tidal sites. Longitudinal position and microtopography affected denitrification, but not river. Tidal sites had greater DEA than nontidal sites, inferred to be caused by greater moisture in all tidal sites. Within tidal wetlands, DEA in hummocks was positively associated with soil organic matter, N, and C, with the highest rates in lower tidal sites, demonstrating a longitudinal pattern of denitrification in TFFWs dependent on microtopography. Compared to hollows, hummocks supported greater DP measured after soil aeration with a simulated low tide, likely because hummocks also had greater potential C mineralization and turnover. Modification to hydrogeomorphic processes from sea level

rise may alter these longitudinal and microtopographic gradients of denitrification in TFFWs.

Key words: denitrification enzyme activity, denitrification potential, microtopography, nitrate, nontidal, soil biogeochemistry

## **Introduction**

Tidal freshwater forested wetlands (TFFWs), commonly found along tidal rivers at the upstream end of estuaries, provide important biogeochemical source/sink functions for their estuaries. These water quality functions include sequestering N through burial and permanently removing N through denitrification, the predominant microbial anaerobic nitrate removal process for tidal wetlands (Ensign *et al.* 2008, Scott *et al.* 2008, Tzortziou *et al.* 2011, Morris *et al.* 2016, Zheng *et al.* 2016). These and other ecosystem functions in TFFWs are threatened by two primary landscape-scale disturbances: river nutrient enrichment through watershed land use intensification, and hydrogeomorphic (*e.g.*, tidal fluctuations) and ecogeomorphic modifications (*e.g.*, vegetation community shifts) resulting from accelerated sea-level rise (Ozalp *et al.* 2007, Ensign *et al.* 2014, Paerl *et al.* 2014, Stagg *et al.* 2014, Middleton *et al.* 2016). Knowledge of spatial patterning of denitrification along landscape-scale gradients in TFFWs is poor and the identification of denitrification's spatial controls and locations with high rates of the process will help predict how future environments will impact N cycling in TFFWs (Ensign *et al.* 2008, Marton *et al.* 2012, von Korff *et al.* 2014, McCluney *et al.* 2014).

Hydrologic, geomorphic, and vegetation community development processes in TFFWs are strong controls of soil biogeochemical cycling, as evidenced by the saturated,

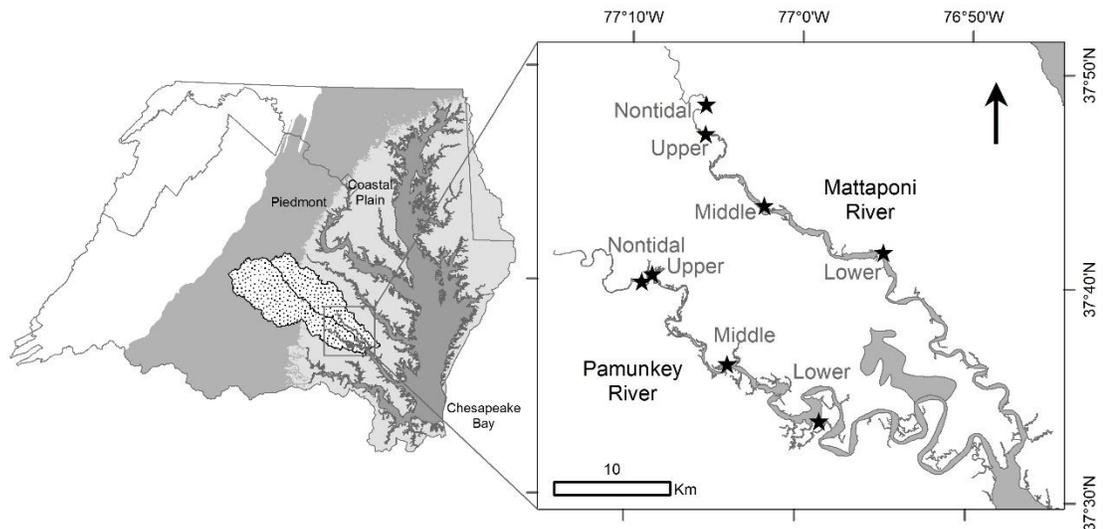
organic soils of TFFWs, which interact with the immediate physicochemical regulators of the denitrification process (*e.g.*, nitrate, carbon, oxygen, pH) (Connor *et al.* 2007, Groffman 2012, Krauss *et al.* 2012). Studies show that many of these processes exhibit longitudinal gradients (*e.g.*, Czwartacki 2013) and thus are implicated in the longitudinal differences found in soil physicochemistry (*e.g.*, organic matter decomposition) (von Korff *et al.* 2014). Hydrologic processes foremost create a prominent salinity gradient within estuaries with direct effects on biogeochemical N cycling (Larsen *et al.* 2010). Because low levels of salinity (less than ~2 ppt) also affect soil respiration, plant productivity, community composition, and hydrogeomorphic processes of the wetland, salinity may also indirectly affect denitrification by mediation of other biogeochemical processes (Krauss and Whitbeck 2012, Cormier *et al.* 2013). In addition to salinity, relative distance from the head and mouth of the estuary affects the balance between fluvial and tidal hydroperiods: less tidal influence at the most upriver locations affects water table levels, inundation, and the composition of sediment in these wetlands (Czwartacki 2013, Ensign *et al.* 2013). Hydroperiods can also vary by longitudinal position due to autogenic soil building processes, or to the mediating effect of hummock-hollow formation on tidal influence (Krauss *et al.* 2012, Stagg *et al.* 2016). These vegetation feedbacks on hydrogeomorphic controls are related to greater sedimentation in downriver tidal forest-to-marsh transition zones and to greater rates of nutrient processing (Noe *et al.* 2013, Ensign *et al.* 2014, Noe *et al.* 2016). Species composition of forested communities further vary by spatial positioning along tidal rivers as influenced by a mix of hydrological patterns, salinity, and soil characteristics (Anderson and Lockaby 2011,

Johnson *et al.* 2015). Longitudinal gradients are one source of landscape-scale variability, while watershed differences in upland land cover and landscape physiography are another (Thoms and Parsons 2002). As nutrient delivery limits ecosystem processes in TFFWs (Ozalp *et al.* 2007), variation in nutrient loading between rivers may cause predictable variation in denitrification in TFFWs.

Denitrification rates are commonly nitrate limited in wetlands and respond to gradients in N availability (Seitzinger 1988, Hopfensperger *et al.* 2009). Nitrogen availability boosts denitrification rates while also indirectly structuring the denitrifier community through effects on the ecosystem (Wallenstein *et al.* 2006, Xiong *et al.* 2017). Nitrate increases in TFFW soils with uptake from surface water and from *in situ* microbial production through the aerobic process of nitrification. The relative balance between the two sources in their coupling to denitrification in part depends on the magnitude of river N loading (McKellar *et al.* 2007, Koop-Jakobsen *et al.* 2010) that can greatly vary on the basis of anthropogenic enrichment. The delivery of river nitrogen to soils further depends on the hydrologic connection between wetland soils and tidal inundation. For instance, incomplete porewater turnover limits direct nitrate pumping into soil sediments (Neubauer *et al.* 2005a), and microtopographic features (*e.g.*, hummocks) limit the influence of direct tidal inundation (Day *et al.* 2007). These hydrogeomorphic differences also affect nitrification, which is inhibited in wetlands where water turnover slows and anoxia builds (Grimm *et al.* 2003, Palmer *et al.* 2009). In tidal wetlands, soil porewater turnover slows with greater tidal inundation magnitude or incomplete tidal recession. Studies of denitrification in TFFWs have not investigated resource limitation

of denitrification, and particularly the sources and variation of nitrate availability, which may support predictions of denitrification through greater mechanistic understanding of its biogeochemical controls.

To investigate spatial patterns of denitrification in TFFWs, the goal of our study, we used a multi-measurement approach to assess both the potential for denitrification and indices of nitrate limitation of denitrification (*i.e.*, potential for nitrification and the effect of short-term tidal fluctuations on denitrification potential). Focusing on TFFWs at estuarine and landscape scales, our four objectives to study potential denitrification were to (1) determine the effect of differences in river N, making use of a natural experiment between two tributaries of the York River (an estuary) in Virginia, (2) determine the effect of longitudinal, riverine-estuarine gradients, including nontidal wetlands above the head of tide, (3) determine the widespread but local effects of hummock and hollow microtopography, and (4) provide context to our previous results by quantifying patterns and/or evaluating denitrification associations with soil physicochemical properties (e.g., bulk density or organic matter). We hypothesized that potential denitrification would increase in rivers with greater nitrogen loads, and would increase with greater nutrient pools and soil anoxia along a downstream longitudinal gradient. At the same time, we also expected that nitrate limitation may regulate some of these overall trends and that denitrification would positively follow areas with greater nitrification such as hummocks or sites with lower water levels.



**Figure 5.1.** Map of tidal forested wetland study sites on the Pamunkey and Mattaponi Rivers, their watersheds spanning the Piedmont and Coastal Plain physiographic provinces, and their location in Virginia within the lower Chesapeake Bay watershed, USA.

## Methods

### Study sites and sampling scheme

We studied tidal and nontidal forested wetlands along the alluvial Mattaponi (166 km) and Pamunkey (150 km) Rivers (Virginia, U.S.A). The rivers are located within a subtropical climate on the Coastal Plain, but drain watersheds that extend into the Piedmont. Together the adjacent rivers flow southeast and comprise the primary tributaries of the York River Estuary beginning at West Point, VA, that eventually joins the Chesapeake Bay. The Mattaponi River watershed (2,359 km<sup>2</sup>) discharges 14.3 m<sup>3</sup> s<sup>-1</sup> above the head of tide (USGS gage 01674500, water years 1997-2016) from a mix of 79% natural vegetation, 16% agriculture, and 3% developed land cover; the Pamunkey River watershed (3,812 km<sup>2</sup>) discharges 26.5 m<sup>3</sup> s<sup>-1</sup> (USGS gage 01673000) from a mix

of 73% natural vegetation, 22% agriculture, and 3% developed land cover (Homer *et al.* 2011, WBD 2013). Long-term (2006-2015) mean NO<sub>3</sub> concentrations are higher in the nontidal Pamunkey River (0.25 mg N/L) than nontidal Mattaponi River (0.16 mg N/L) (CB Program 2012), with over 2 times greater mean annual load on the Pamunkey calculated for 1985-2012 (Chanat *et al.* 2015).

Four forested floodplain wetland sites on each river were established along a freshwater estuarine gradient including a nontidal forested floodplain wetland close to the fluvial-tidal wetland transition, an upper (upriver) and middle TFFW, and a lower (downriver) TFFW that is converting to marsh possibly due to low-level salinization (Figure 5.1). All measurements were made at nontidal sites and the upper and middle tidal sites, and a subset of measurements was made at the lower tidal site (Figure 5.1). Hummock-hollow microtopography was present at tidal sites and nascent hummock-hollow formation was present at the Pamunkey nontidal site. Hummocks were berms or discrete habitat patches in the wetland comprised of dense mats of soil, moss, and roots from woody and herbaceous vegetation, which resisted compression if walked upon, while hollows comprised the zones of depression around the hummocks where predominantly herbaceous vegetation was observed to grow and soil was saturated and easily compressible. While sampling at low tide, all or part of hollows were inundated while hummocks were generally exposed to air and soils were quick to drain from high tide inundation (many hummocks were observed inundated at higher tide). Hummocks are elevated above hollows by 10-60 cm (Kroes *et al.* 2007, Dubberstein and Connor 2009, Courtwright and Findlay 2011).

Six 1 m<sup>2</sup> plots were established at each site, at least 3 m apart, capturing 3 hummocks and 3 hollows for tidal wetlands; for nontidal wetlands, 3 plots were placed in levees (or early hummocks when present) and 3 plots in depressions or sloughs. Plots were within 100 m of stream edge. We visited the sites in September 2016 for soil collection and deployment of long-term soil oxygen sensors. Soils for laboratory analyses of denitrification and soil biogeochemical properties were collected during periods of low tide, and placed on ice until subsequent refrigeration later the same day. Either one large soil core (7.6 cm diameter) or two smaller cores (4.8 cm diameter) as a composite were retrieved to 5 cm depth, per soil measurement procedure as outlined below. The surficial 5 cm of soil was expected to support the highest nitrification and oxygen fluctuations affecting denitrification in the tidal environments.

### **Surveys of soil denitrification enzyme activity, denitrification potential, net nitrification potential, and nitrate concentration**

Denitrification enzyme activity captures a long-term potential of denitrifiers to denitrify (Groffman *et al.* 1999) and was measured on soils from nontidal, upper, middle, and lower tidal sites. Soils were mixed coarsely from a dedicated larger core, or were cut into small segments (which were held together by roots) for hummocks. In 125 mL flasks, slurries were formed with 30 g of soil and 30 mL of media containing N (1.01 g/L) and C (1.80 g/L) and chloramphenicol (0.1 g/L). We flushed slurries with N<sub>2</sub> prior to twice pressurizing and depressurizing the headspace through rubber stoppers. The start of the incubation was marked by injecting 10 mL of acid-scrubbed acetylene into the headspace, slightly pressurizing it. Gas samples (2 mL into glass vials with aluminum crimp-top caps and butyl rubber septa) were retrieved from flasks placed on a shaker

table after 45 and 105 minutes. Gas samples were quantified for N<sub>2</sub>O within 48 hours of collection using a Shimadzu GC 8-A gas chromatograph with electron capture detection and a packed column (Hayesep Q 80/100). Rates of N<sub>2</sub>O + N<sub>2</sub> production were expressed per dry mass of soil using concurrently measured soil gravimetric moisture (method: dried 4 days to constant mass at 60 °C).

In addition to DEA, we measured denitrification potential (DP) (Groffman *et al.* 1999) as experimentally affected by tidal inundation to ascertain landscape gradients in nitrate or other biogeochemical limitation. Rates of DP were not measured from lower tidal sites to avoid the confounding effects of salinity. We used a 2x2 factorial experiment (factor 1: low tide drawdown, factor 2: high tide saturation) with 4 final treatments comprised of a pre-measurement, dry period of either an anoxic or oxic incubation followed by a period of saturation with or without nitrate amendment under anoxic conditions during which denitrification measurement took place. For the pre-measurement incubation period, we exposed ~30 g of field moist soil, from dedicated soil cores, to a simulated low tide for 6 hrs in either an oxic (ambient air) or anoxic (N<sub>2</sub>) environment in the dark. To incubate soils anoxically, flasks were capped with rubber stoppers and flushed with N<sub>2</sub> at high flow for 5 min and left slightly pressurized. At the end of the dry treatment, soils were amended with 30 mL of media (wet treatment), but not mixed to a slurry to preserve macropore structure, and incubated for another 4 hours in the dark for a simulated high tide during which denitrification potential was measured. Media for the second treatment consisted of either deionized water or river-specific concentrations of long-term average nitrate (in the form of KNO<sub>3</sub>). The headspace above

soils and media was flushed with N<sub>2</sub> for 3 minutes, then brought to atmospheric pressure, and finally slightly pressurized with 10 mL of acetylene. Gas samples (2 mL) were taken at 2 and 4 hours after mixing the headspace with a syringe. The rest of the procedure was identical to methods used to sample, quantify, and express DEA.

**Table 5.1. Denitrification potential ( $\mu\text{g-N kg}^{-1} \text{hr}^{-1}$ ) measurements**

Description	Wetland sites	Headspace, dry 6-hr, 1 <sup>st</sup> incubation	Media, wet 4-hr, 2 <sup>nd</sup> incubation	Calculation
Effect of high-tide nitrate delivery	Tidal	Air or N <sub>2</sub>	water or nitrate	= nitrate trt – water trt
High tide	Tidal	Air or N <sub>2</sub>	nitrate	
Nitrate limitation	Nontidal & Tidal	N <sub>2</sub>	water or nitrate	= nitrate trt – water trt
Non-nitrate limitation	Nontidal & Tidal	N <sub>2</sub>	nitrate	
Effect of low-tide aeration	Tidal	air or N <sub>2</sub>	water or nitrate	= air trt – N <sub>2</sub> trt

Differences among the experimental treatments on tidal soils were used to measure the response of DP to simulated tidal fluctuations (Table 5.1). We estimated the effect of high tide on DP by comparing DP treatments with and without nitrate amendment, while we estimated DP at high tide using just the nitrate-amended treatments. To estimate the effect of low-tide aeration (as it would affect DP at high tide), we compared DP treatments pre-incubated under an oxic and anoxic environment. Differences among the experimental treatments using both tidal and nontidal soils were interpreted differently. To compare nutrient limitation of DP between nontidal and tidal

soils, we estimated nitrate limitation of DP by comparing treatments with and without nitrate amendment that were pre-incubated under an anoxic environment. Non-nitrogen limitation of DP was measured using only the treatment group with nitrate amendment after an anoxic pre-incubation period (Sigunga 2003). For all DP rate changes with nitrate amendment, negative rates were converted quantitatively to zero.

To assess the potential for nitrate production during low-tide aeration of tidal soils, as well as post-flood aeration in nontidal soils, we measured the effect of aeration on net nitrification potential during 7-day laboratory incubations (Hart *et al.* 1994, Roy and White 2013). Soils from dedicated soil cores were incubated under oxic conditions to determine the potential for nitrate production, and were paired with soils incubated under anoxic conditions, to use as a control. Net nitrification potential with aeration, which was measured on both tidal (upper and middle) and nontidal soils, was calculated as the difference between the net nitrification potential rates incubated oxically and anoxically. Soils were held for ~10 days before analysis. We placed 4 g wet mass-equivalent samples of homogenized soils into flasks for each treatment: 2 replicates for time zero extraction, and 1 replicate for extraction after a day 7 incubation treatment. Soils for initial extractions were mixed well with 50 mL of a 2 M KCl solution and placed on a shaker table for an hour. Soils were then left to gravity separate for 30 minutes and then 20 mL were syringe-filtered to 0.45  $\mu\text{m}$  (Acrodisc Supor membrane). On the same day, and prior to these initial extractions, soils for incubation were prepared similarly and incubated in the dark. Soils incubated oxically were left partially open to ambient air, and evaporated water was replenished by mass after 4 days. Soils incubated anoxically were flushed for 5

minutes with N<sub>2</sub> and left slightly pressurized with rubber stoppers; we repeated the flushing procedure after 4 days to reduce pressure build-up and maintain more consistent headspace concentration across soils; soils maintained pressure throughout the incubation and thus oxygen penetration was negligible. On day 7, we extracted soil nitrate as we had done with the day-0 soils. Extracts were measured for NO<sub>3</sub>+NO<sub>2</sub> on a discrete analyzer (SEAL analytical, AQ2). Discrete analyzer runs included 2 M KCl blanks and an external standard (ERA, Arvada, Colorado, USA) evaluated with a 10% tolerance.

### **Surveys of soil physicochemical properties**

With the exception of soil moisture, soil physicochemical properties were measured at all tidal and non-tidal sites. We collected soils for bulk density using a slide hammer soil sampler with a cylindrical metal retaining liner (5 cm length x 4.8 cm diameter). Soils for bulk density and separate soil samples for moisture were dried to constant weight at 60 °C to measure mass loss on drying per volume liner or sample. We chose a low temperature to allow time for water evaporation while reducing the potential for N volatilization. Two measurements of bulk density were eliminated from analyses due to apparent measurement error.

We measured soil %C and %N in an element analyzer (Perkin Elmer 2400 Series II) and %organic matter in a furnace. Collected soils were dried for 3 days at 55 °C and then laid to air dry further after being ground to 2 mm and separated from large roots. Immediately prior to analyses, 1-3 g of soils were completely dried at 105 °C for 2 hours. Soil for C and N was placed into capsules for dry combustion and quantification by gas

chromatography, and soil for organic matter was combusted at 550 °C for 4 hours and calculated by mass lost to ignition.

Potential aerobic C mineralization was measured with a LI-COR 8100A automated soil gas flux system adapted for benchtop analysis. We followed the procedure by Robertson *et al.* (1999) which calls for soil moisture to be raised to 60% water-filled pore space. For the tidal wetlands, most soils needed to be dried to 60% water-filled pore space so we first brought all soils to a common moisture level by drying them for three days at 55 °C (Franzluebbers 1999). We then ground the soils to 2 mm and measured their bulk density. We raised moisture levels on 20 g of this soil using the new bulk density measurements and a calculation assuming total porosity = 2.65 g cm<sup>-3</sup>. Soils were incubated in the dark for 21 days, partially covered, with moisture loss replaced weekly. Two replicates of CO<sub>2</sub> flux per plot were measured over 2 minute periods. Rates of flux were expressed per dry mass of soil. Potential C turnover was calculated as potential C mineralization normalized by soil %C.

Long-term pore water conductivity ( $\mu\text{S cm}^{-1}$ ) and site water level were recorded at 20-minute intervals from a sonde (Solinst Levelogger model 3001) installed in a slotted surficial groundwater well in each of the eight sites. Records were taken over 10 months on the Pamunkey River and over 12-18 months on the Mattaponi River that bracketed soil sampling. Salinity (ppt) was calculated assuming 1 dS m<sup>-1</sup> = 640 ppm dissolved ions in water.

Long-term soil oxygen was measured only on the Mattaponi River at 5 locations: at one nontidal location between a levee and slough plot; and in a hummock and a hollow

of both upper and middle tidal sites. Sensors, calibrated in the laboratory, were inserted 3 cm into the ground and recorded oxygen levels within a protective wire mesh cage extending 3.5 cm deeper into the soil. Oxygen sensors (Apogee Instruments SO-110) and data loggers (Pace Scientific XR5-SE-100mV) recorded soil %O<sub>2</sub> levels for roughly 8 weeks until the end of November 2016.

### **Data analysis**

Statistical tests were performed to evaluate group differences of denitrification variables among rivers, longitudinal positions, and microtopography, and to test for associations of denitrification variables with soil characteristics at  $\alpha = 0.05$ . Certain variables were transformed to improve normality or reduce the influence of outliers: DEA, DP at high tide, non-N limited DP, potential C mineralization, nitrate, and the effect of aeration on net nitrification potential were log<sub>10</sub> transformed; effect of high-tide nitrate on DP, N-limitation of DP, and effect of low-tide aeration on DP were square root transformed.

For tidal wetlands, we evaluated the effect of river, longitudinal position, and hummock/hollow microtopography using factorial analysis of variance (ANOVA) and Type II sums of squares. Post-hoc pairwise comparisons using Tukey's Honestly Significant Difference test were interpreted for interaction effects. To support our study objectives, only the river\*microtopography and site\*microtopography interactions were generally reported for the associated soil biogeochemical measurements, though all interactions were evaluated. We also assessed river differences on nontidal soils for DEA, aeration of net nitrification potential, and nitrate to help support the effect of river N

availability on tidal soils. For instance, are differences in nitrogen concentrations large enough to affect denitrification potential, without the confounding effects of tidal pulsing? To compare with effects on tidal soils, we used ANOVA to assess the effect of river availability of N (*i.e.*, nitrate delivery from overbank flooding, groundwater fluctuations, or surface flow) on DEA, aeration of net nitrification potential, and nitrate in nontidal soils. Correlations between DEA and soil biogeochemistry measurements in tidal wetlands were tested with Spearman Rank correlation.

To compare nontidal and tidal soils, we used a mixed effect multilinear model fit by loglikelihood, with site and river as fixed effects and microtopography as a random effect (Field *et al.* 2012). Nontidal site means were compared to those of each tidal site using non-orthogonal contrasts and planned comparisons.

## **Results**

### **Denitrification potential variables measuring effects of tidal inundation**

Three DP measurements in tidal soils were calculated across two low-tide or high-tide factor levels to represent natural variability in the range of soil oxygenation at low tide or the range of nitrate concentration at high tide: the effect of high-tide on DP, and high-tide DP, were measured after either an oxic or anoxic dry low-tide, while the effect of low-tide aeration on DP was measured with either a nitrate unamended or amended high tide. We assessed these treatment groups for statistical differences using the *t* test: the effect of high-tide nitrate delivery on DP did not differ by low-tide aeration treatment groups ( $t = -0.49$ ,  $df = 45.8$ ,  $P = 0.626$ ); high-tide denitrification potential also did not differ by low-tide aeration treatment groups ( $t = -1.87$ ,  $df = 32.0$ ,  $P = 0.07$ ); and the effect

of low-tide aeration on DP was similar across high-tide nitrate treatment groups ( $t = -0.45$ ,  $df=29.6$ ,  $P = 0.655$ ). We pooled these groups for presentation and statistical analyses for assessing the effects of river, longitudinal positions, and microtopography.

**Table 5.2. Factorial ANOVA for tidal wetland soil measurements**

Response Variable <sup>1</sup>	Statistic	River	Site	MT	River*Site	River*MT	Site*MT	River*Site*MT
DEA <sup>2</sup>	<i>F</i>	0.19	2.01	1.84	0.69	0.61	1.76	0.33
	<i>P</i>	0.665	0.156	0.187	0.513	0.441	0.191	0.720
adjDP <sub>HT</sub>	<i>F</i>	3.10	4.06	0.06	1.98	9.07	11.4	1.10
	<i>P</i>	0.086	0.051	0.807	0.167	<b>0.004</b>	<b>0.002</b>	0.301
DP <sub>HT</sub>	<i>F</i>	2.50	4.76	0.129	1.26	5.44	3.35	0.17
	<i>P</i>	0.122	<b>0.035</b>	0.722	0.269	<b>0.025</b>	0.07	0.681
nitrate	<i>F</i>	13.7	5.84	6.87	3.03	18.7	15.7	1.89
	<i>P</i>	<b>0.002</b>	<b>0.028</b>	<b>0.019</b>	0.101	<b>&lt;0.001</b>	<b>0.001</b>	0.188
NNP	<i>F</i>	1.68	2.37	0.17	0.04	0.24	1.01	4.22
	<i>P</i>	0.213	0.143	0.687	0.844	0.629	0.330	0.057
adjDP <sub>LT</sub>	<i>F</i>	7.10	0.33	4.72	0.22	2.42	1.43	0.163
	<i>P</i>	<b>0.017</b>	0.571	<b>0.045</b>	0.645	0.139	0.249	0.220

Notes: *P*-values in boldface are statistically significant (< 0.05)

<sup>1</sup> DEA: denitrification enzyme activity; adjDP<sub>HT</sub>: effect of high-tide nitrate on DP; DP<sub>HT</sub>: denitrification potential at high tide; NNP: net nitrification potential; adjDP<sub>LT</sub>: effect of low-tide aeration on denitrification potential.

<sup>2</sup> Additionally measured at lower tidal site.

### Effects of river N differences: tidal Mattaponi vs. Pamunkey Rivers

Differences in river N availability (Pamunkey > Mattaponi) had impacts on soil nitrate, the effect of high-tide nitrate delivery on DP, and DP at high tide, depending on microtopography (Table 5.2). Hollows in the Pamunkey River tidal wetlands had greater soil nitrate, lower stimulation of DP with high tide nitrate delivery, but also lower DP with high-tide nitrate (Table 5.2; Figure 5.2). Therefore, nitrate limitation of

denitrification was lower in Pamunkey hollows, not because of larger nitrate pools, but because of some additional limitation (*e.g.*, carbon) evidenced by DP measured with high-tide nitrate addition. In contrast to hollows on the Pamunkey, hummocks on the Pamunkey River had less soil nitrate than on the Mattaponi River (Figure 5.2). River had no effect on tidal DEA, effect of aeration on net nitrification potential, or the effect of low-tide aeration on DP (Table 5.2).

We compared the nontidal soils of each river to evaluate the effect of river N availability on N processing without daily tidal influence. Nontidal soils of the Pamunkey River had higher DEA ( $F = 12.92$ ;  $P = 0.005$ ), net nitrification potential given aeration ( $F = 7.69$ ;  $P = 0.020$ ), and nitrate ( $F = 12.81$ ,  $P = 0.005$ ) compared to the Mattaponi River (Figure 5.2). These nontidal soil differences were not due to differences in nontidal soil moisture by river ( $F = -0.05$ ;  $P = 0.866$ ).

### **Longitudinal patterns within and across nontidal and tidal wetlands**

Within tidal wetlands, hummock microtopography mediated longitudinal effects on soil nitrate, the effect of high-tide nitrate delivery on DP, and high-tide DP (Table 5.2). Middle tidal hummocks had greater nitrate than upper tidal hummocks (Figure 5.2). Concurrently, high-tide nitrate had less stimulatory effect on DP in middle tidal hummocks than upper tidal hummocks (Figure 5.2). Denitrification potential at high tide was also overall lower in middle tidal sites than upper tidal sites, suggesting a reason for the high nitrate in these soils, and this effect was only meaningful in hummocks (Figure 5.2). Neither DEA, net nitrification potential given aeration, nor the effect of low-tide aeration on DP differed longitudinally within tidal wetlands (Figure 5.2).

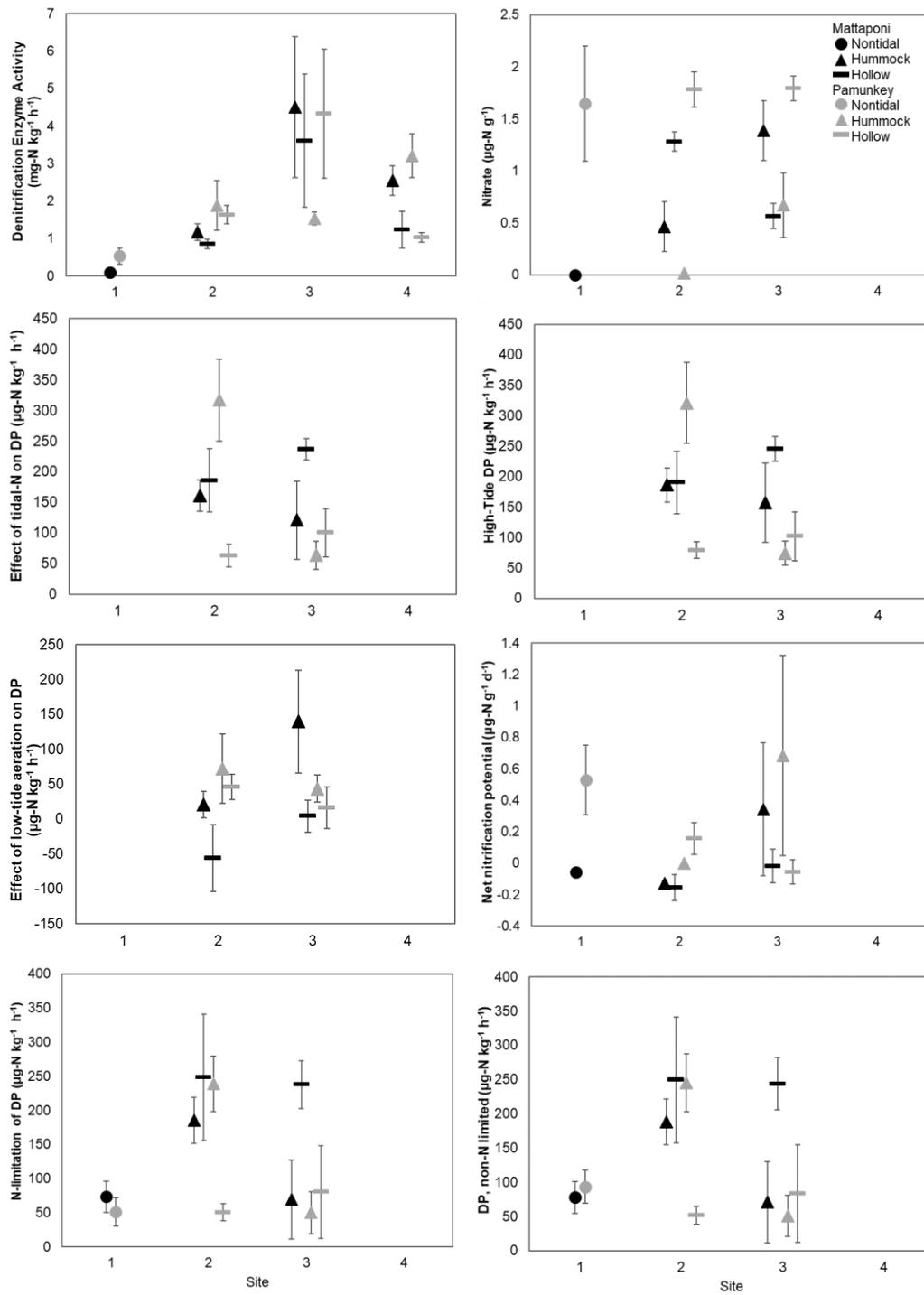


Figure 5.2. Mean ( $\pm$  standard error) measurements by river, tidal site (1: nontidal, 2: upper, 3: middle, 4: lower), and microtopography where present (sites 2,3,4)

Compared to nontidal soils, tidal soils had greater DEA (Table 5.3; Figure 5.2). Upper tidal soils also had greater nitrate limitation of denitrification potential than nontidal soils, but the difference was only consistent for upper tidal hummocks (and not upper tidal hollows) of both rivers and both microtopographic positions along the Mattaponi River (Table 5.3; Figure 5.2). Tidal and nontidal soils did not differ in soil nitrate, the effect of aeration on net nitrification potential, or denitrification potential with nitrate addition (Table 5.3; Figure 5.2).

**Table 5.3. Factorial mixed effect ANOVA for nontidal and tidal wetland soil measurements with microtopography as random effect**

Response Variable <sup>1</sup>	Statistic	Site	River <sup>2</sup>	Site*River <sup>2</sup>
DEA <sup>3</sup>	<i>L. Ratio</i>	16.79	4.26	10.37
	<i>P</i>	<b>&lt;0.001</b>	<b>0.039</b>	<b>0.016</b>
dDP <sub>N</sub>	<i>L. Ratio</i>	6.34	4.19	0.59
	<i>P</i>	<b>0.042</b>	<b>0.041</b>	0.746
DP <sub>N</sub>	<i>L. Ratio</i>	5.54	0.895	1.58
	<i>P</i>	0.063	0.344	0.454
nitrate	<i>L. Ratio</i>	1.45	4.65	11.18
	<i>P</i>	0.485	<b>0.031</b>	<b>0.004</b>
NNP	<i>L. Ratio</i>	7.72	5.55	2.33
	<i>P</i>	0.257	<b>0.019</b>	0.312

Notes: *P*-values in boldface are statistically significant (< 0.05)

<sup>1</sup> DEA: denitrification enzyme activity; dDP<sub>N</sub>: nitrate limitation of DP; DP<sub>N</sub>: non-N limitation of denitrification potential; NNP: net nitrification potential.

<sup>2</sup> Main effect of river and interaction not interpreted for this study.

<sup>3</sup> Additionally measured at lower tidal site.

### Effects of hummocks and hollows of tidal wetlands

In addition to the mediating effect on nitrate and DP measurements, hummock-hollow microtopography impacted the effect of low-tide aeration on DP (Table 5.2).

Across river and longitudinal position, low-tide aeration of DP stimulated hummocks

more than hollows, with greater differences on the Mattaponi River (Figure 5.2).

Microtopography had no effect on DEA or the effect of aeration on net nitrification potential (Table 5.2).

### **Spatial patterns in soil biogeochemical characteristics**

Soil biogeochemical properties in tidal wetlands did not differ by river (all  $P > 0.05$ ) (Table 5.4). In nontidal wetlands, the Pamunkey River had greater potential C mineralization ( $P = 0.006$ ) and potential C turnover ( $P = 0.010$ ), and no difference in organic matter, N, C, bulk density, or moisture (all  $P > 0.05$ ) compared to the Mattaponi River (Table 5.4).

Compared to tidal soils, all nontidal soils were lower in moisture ( $P = 0.043$ ) and higher in bulk density ( $P < 0.001$ ) (Table 5.5). Nontidal sites were also lower than middle and lower tidal sites for N, C, and organic matter ( $P < 0.009$ ), and nontidal sites were lower than middle tidal sites for potential C mineralization ( $P = 0.024$ ) (Table 5.5). Nontidal and tidal soils did not differ for potential C turnover ( $P = 0.133$ ) (Table 5.5).

All soil biogeochemical characteristics exhibited longitudinal patterns within tidal wetlands (all  $P < 0.05$ ) with the exception of moisture ( $P > 0.05$ ). Middle and lower tidal soils differed from upper tidal soils with lower bulk density and higher soil organic matter, N, and C (Table 5.5). Significant patterns in organic matter were further dependent on site or microtopography: in hummocks, upper < middle < lower tidal sites; in hollows, upper < middle and lower tidal sites, and middle > lower tidal sites (Table 5.5). Potential aerobic C mineralization was higher in lower tidal soils compared to upper

tidal soils (Table 5.5). Middle tidal soils were lower in potential C turnover than lower tidal soils, and neither differed from upper tidal soils (Table 5.5).

Compared to hollows, hummocks had lower moisture, greater bulk density, greater potential C mineralization, and greater potential C turnover (all  $P < 0.05$ ) (Tables 5.4 and 5.5). Hummock and hollow microtopography did not affect organic matter, N, or C concentrations (all  $P > 0.05$ ) (Tables 5.4 and 5.5).

The highest soil porewater salinity level recorded for sites during the measurement periods (10-18 months, variable by site) was 0.61 ppt with the exception of the Mattaponi lower tidal site, which surpassed 1 ppt (Table 5.6). Water levels were recorded over the same measurement periods. Median water levels in nontidal sites were higher on the Mattaponi River than the Pamunkey River (Table 5.6). In tidal sites, the Mattaponi upper tidal site had the highest median standing water level (9 cm) while the Pamunkey upper tidal site had the lowest (-3 cm) (Table 5.6). Water levels dropped farther below ground on the Pamunkey than on the Mattaponi for each pair of sites (Table 5.6). In the two weeks leading up to the sampling date of each site, there were no river or longitudinal trends across sites in water levels: on the Mattaponi, water levels were generally lower than tidal sites, while the same was mostly true for the Pamunkey except that water levels were lower in the upper tidal site than the nontidal site (Table 5.6).

Five soil oxygen sensors were deployed at 3-cm depth to three sites on the Mattaponi River (non-, upper, and middle tidal). The two sensors in tidal hollows were deployed at low tide with standing water above the soil surface and almost consistently recorded 0% for the monitoring period (data for upper tidal site not shown) presumably

due to undrained soils during low tides. The sensors in the tidal hummocks were not deployed directly under water, and were assumed to be inundated less frequently than the sensors in the hollows and to drain rapidly at low tide. The sensor in the lower tidal hummock remained at < 1% with slight variability indicative of high microbial oxygen demand (Figure 5.3). The remaining two sensors, to the upper tidal hummock and the nontidal site, revealed higher long-term soil oxygen levels (> 10%) that dropped with a watershed-driven river flood (Figure 5.3).

### **Correlations of DEA with soil biogeochemistry within tidal wetlands**

We assessed correlations of DEA with soil biogeochemistry separately for hummocks and hollows in tidal soils. In hummocks, DEA was positively correlated with N, C, and organic matter, and negatively correlated with bulk density (Figure 5.4). The lack of correlation between DEA and either potential C mineralization or potential C turnover (Figure 5.4) in hummocks indicates that the correlations between DEA and organic matter or C were not related to microbial activity. DEA was not correlated with soil biogeochemistry in hollows: N ( $r_s = 0.06$ ;  $P = 0.823$ ), C ( $r_s = -0.01$ ;  $P = 0.964$ ), organic matter ( $r_s = -0.01$ ;  $P = 0.984$ ), bulk density ( $r_s = 0.25$ ;  $P = 0.345$ ), potential C mineralization ( $r_s = -0.09$ ;  $P = 0.711$ ), and potential C turnover ( $r_s = -0.18$ ;  $P = 0.484$ ) (see Appendix A for plots). Relationship between DEA and moisture were not assessed because the calculation of DEA adjusts for moisture.

**Table 5.4. Mean ( $\pm$  standard error) soil physicochemical properties<sup>1</sup> of rivers by microtopography**

Micro-topography	River	OM (%)	N (%)	C (%)	BD (g cm <sup>-3</sup> )	GM (g g <sup>-1</sup> )	Pot. C min. (mmol-C kg <sup>-1</sup> d <sup>-1</sup> )	Pot. C turnover (mol-C mol-C <sup>-1</sup> d <sup>-1</sup> )
Nontidal <sup>2</sup>	Mattaponi	10.9 $\pm$ 0.38	0.23 $\pm$ 0.02	4.02 $\pm$ 0.31	0.77 $\pm$ 0.06	0.85 $\pm$ 0.24	2.84 $\pm$ 0.13	0.0009 $\pm$ 0.0000
	Pamunkey	13.8 $\pm$ 1.91	0.38 $\pm$ 0.07	5.54 $\pm$ 1.15	0.65 $\pm$ 0.09	0.81 $\pm$ 0.12	5.30 $\pm$ 0.84	0.0012 $\pm$ 0.0001
Hummock	Mattaponi	43.7 $\pm$ 7.50	0.22 $\pm$ 0.02	3.20 $\pm$ 0.36	0.27 $\pm$ 0.04	3.20 $\pm$ 0.36	20.0 $\pm$ 5.79	0.0008 $\pm$ 0.0000
	Pamunkey	34.4 $\pm$ 5.21	0.38 $\pm$ 0.11	2.50 $\pm$ 0.33	0.28 $\pm$ 0.03	2.50 $\pm$ 0.33	16.7 $\pm$ 3.65	0.0013 $\pm$ 0.0001
Hollow	Mattaponi	42.9 $\pm$ 7.34	0.25 $\pm$ 0.03	5.02 $\pm$ 0.45	0.18 $\pm$ 0.04	5.02 $\pm$ 0.45	7.24 $\pm$ 1.17	0.0009 $\pm$ 0.0001
	Pamunkey	33.1 $\pm$ 3.68	0.38 $\pm$ 0.10	4.25 $\pm$ 0.45	0.21 $\pm$ 0.02	4.25 $\pm$ 0.45	8.04 $\pm$ 2.05	0.0011 $\pm$ 0.0002

Notes: <sup>1</sup> Abbreviations: soil organic matter (OM), total N, total C, bulk density (BD), gravimetric moisture (GM), potential aerobic C mineralization (Pot. C min.), potential aerobic C turnover (Pot. C turnover)

<sup>2</sup> Levee, flat, and slough

**Table 5.5. Mean ( $\pm$  standard error) soil physicochemical properties<sup>1</sup> of nontidal and tidal sites by microtopography**

Micro-topography	Site	OM (%)	N (%)	C (%)	BD (g cm <sup>-3</sup> )	GM (g g <sup>-1</sup> )	Pot. C min. (mmol-C kg <sup>-1</sup> d <sup>-1</sup> )	Pot. C turnover (mol-C mol-C <sup>-1</sup> d <sup>-1</sup> )
Levee, flat, and slough	Nontidal	12.3 $\pm$ 1.02	0.31 $\pm$ 0.04	4.78 $\pm$ 0.61	0.71 $\pm$ 0.06	0.83 $\pm$ 0.13	4.07 $\pm$ 0.55	0.0011 $\pm$ 0.0001
	Upper	16.3 $\pm$ 1.61	0.37 $\pm$ 0.06	5.67 $\pm$ 0.79	0.37 $\pm$ 0.03	1.86 $\pm$ 0.34	5.56 $\pm$ 0.98	0.0012 $\pm$ 0.0002
Hummock	Middle	47.5 $\pm$ 6.56	1.37 $\pm$ 0.18	23.4 $\pm$ 3.64	0.28 $\pm$ 0.03	3.11 $\pm$ 0.40	16.1 $\pm$ 2.62	0.0009 $\pm$ 0.0001
	Lower	53.4 $\pm$ 2.83	1.55 $\pm$ 0.08	27.1 $\pm$ 2.02	0.19 $\pm$ 0.01	3.58 $\pm$ 0.22	33.4 $\pm$ 5.22	0.0015 $\pm$ 0.0001
Hollow	Upper	19.2 $\pm$ 0.83	0.49 $\pm$ 0.07	7.33 $\pm$ 0.90	0.30 $\pm$ 0.03	3.58 $\pm$ 0.46	3.66 $\pm$ 0.45	0.0006 $\pm$ 0.0000
	Middle	50.4 $\pm$ 7.43	1.36 $\pm$ 0.19	25.6 $\pm$ 4.24	0.17 $\pm$ 0.03	5.05 $\pm$ 0.57	7.09 $\pm$ 0.65	0.0004 $\pm$ 0.0000
	Lower	44.4 $\pm$ 3.20	1.43 $\pm$ 0.10	23.6 $\pm$ 1.52	0.16 $\pm$ 0.01	5.28 $\pm$ 0.45	12.2 $\pm$ 2.34	0.0006 $\pm$ 0.0001

Notes: <sup>1</sup> Abbreviations: soil organic matter (OM), total N, total C, bulk density (BD), gravimetric moisture (GM), potential aerobic C mineralization (Pot. C min.), potential aerobic C turnover (Pot. C turnover)

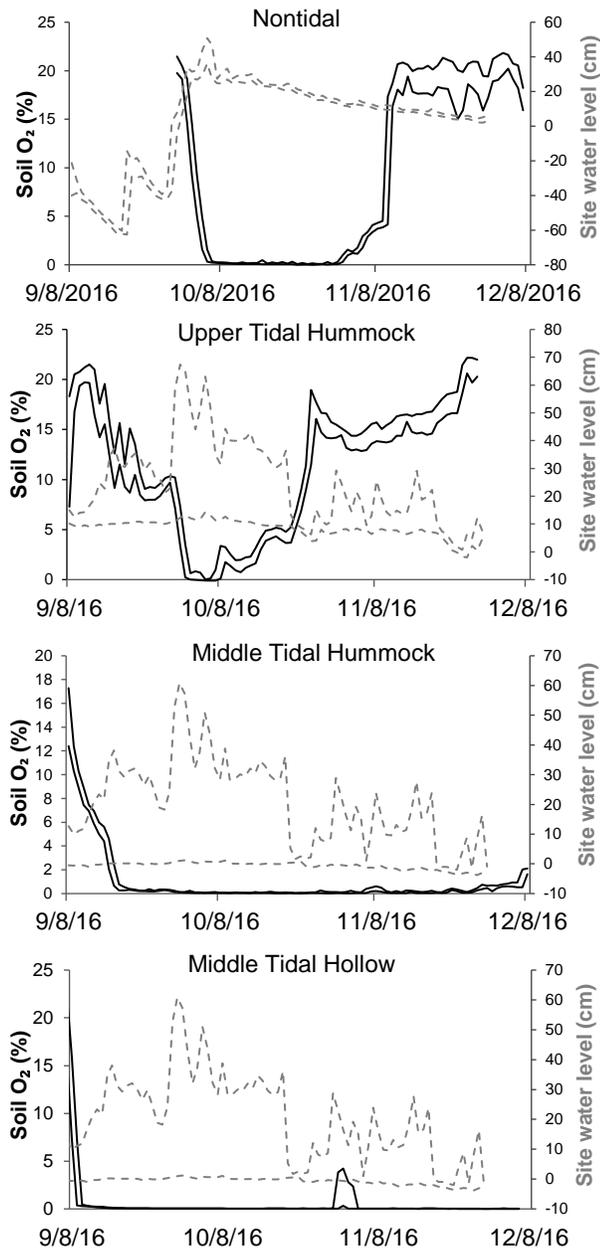
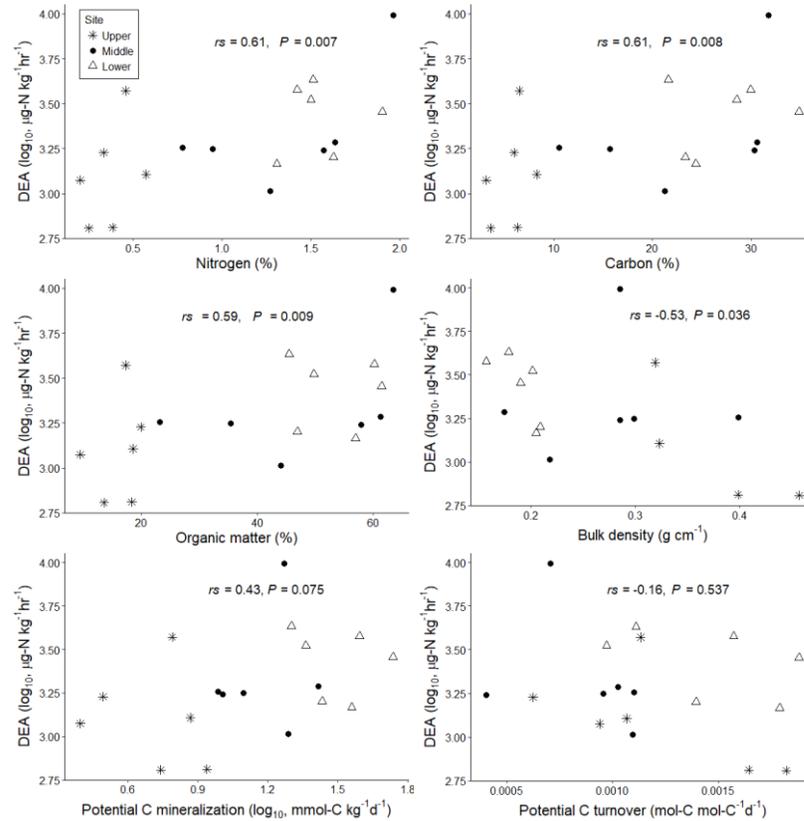


Figure 5.3. Daily minimum and maximum soil oxygen levels (black) measured on the Mattaponi River juxtaposed with daily minimum and maximum site water levels (gray)



**Figure 5.4. Associations of denitrification enzyme activity (DEA) and soil biogeochemical properties in tidal hummocks with Spearman rank correlation statistics**

## Discussion

This study investigated landscape-scale patterns in denitrification and its limitation by nitrate that inform our understanding of N cycling in TFFWs. We expected greater nitrogen loading in a river to stimulate denitrification in TFFWs (Megonigal and Neubauer 2009), and we expected denitrification to increase with longitudinal position along a downstream gradient known to affect a suite of wetland characteristics (*e.g.*, Ensign *et al.* 2013). Though denitrification was nitrate limited, greater nitrate concentrations on the Pamunkey River caused additional biogeochemical limitations and inhibited the potential for denitrification in tidal hollows, demonstrating nitrate

concentrations are insufficient by themselves to stimulate denitrification potential at landscape scales. We found instead that greater soil moisture and soil organic matter-related nutrient concentrations along a longitudinal gradient were linked to increasing denitrification potential rates. All results were affected by eco-hydrogeomorphic processes that create hummock-hollow microtopography, which also directly affected denitrification potential, microbial activity, and potential carbon mineralization in tidal sites.

### **Effect of tidal river N on potential denitrification**

Differences in river total nitrogen and nitrate concentrations and loads between two adjacent rivers (Chanat *et al.* 2015, this study) created a natural experiment to test the effect of watershed loading of N on tidal denitrification. Mean annual loads of nitrogen on the Pamunkey River (1750 mg/L) are larger than the Mattaponi River (769 mg/L) (Chanat *et al.* 2015). As a result, mean long-term nontidal nitrate concentrations for the Pamunkey River are also higher than the Mattaponi River (0.25 vs 0.16 mg-N/L, respectively), as well as tidal concentrations measured near the sampled middle tidal sites (0.22 vs 0.18 mg-N/L, respectively) (CB Program 2012). These nitrate concentrations are indicative of a moderate influence from anthropogenic N (Langland *et al.* 1995; Shields *et al.* 2008) which should stimulate N cycling for tidal wetland soils. At the same time, these concentrations are within a range where denitrification rates are still nitrate limited, as shown by studies in freshwater aquatic sediments (Inwood *et al.* 2005, Arango *et al.* 2007) and where denitrifiers may still rely predominantly on nitrate production from nitrifiers (Seitzinger *et al.* 2006, Hopfensperger *et al.* 2009, Jantii *et al.* 2011).

Experimental nitrate limitation of denitrification using nitrate amendment is commonly reported for tidal wetlands, as are relationships of denitrification with nitrate availability (Koop-Jakobsen *et al.* 2010, Morrissey and Franklin 2014, Pastore *et al.* 2016). Almost all our denitrification potential rates (>90% of measurements) were stimulated by nitrate amendment.

River identity had an effect on wetland soil nitrate concentrations that depended on microtopography. Soil nitrate concentrations in hollows were greater on the Pamunkey River. Hollows are usually inundated with standing water throughout most of the tidal cycle, and their shallow soils are strongly influenced by stream dissolved and particulate matter due to tidal pumping (Zheng *et al.* 2016). Hollows should reflect river nitrate concentrations where nitrate demand by the wetland ecosystem has been met. This appears the case only for the Pamunkey soils which had very similar levels of nitrate in hollows at the upper and lower tidal sites. Nitrate concentrations in hummocks showed the opposite pattern, with greater nitrate concentrations on the Mattaponi than on the Pamunkey River attributed to lower soil moisture and/or nitrate delivery to Pamunkey soils. The upper tidal site on the Pamunkey River likely received less dissolved nitrate with lower water levels than the analogous Mattaponi soils (Table 5.6), while the middle tidal hummocks on the Pamunkey River had lower soil moisture, along with lower organic matter, carbon, and nitrogen (all  $P < 0.001$ ) than the paired site on the Mattaponi River.

Though higher N availability on the Pamunkey River stimulated DEA and NNP in nontidal sites, river N availability had no effect on DEA or NNP in tidal sites. We

showed that denitrification potential in hollows at high tide (with nitrate amendment) was not higher on the Pamunkey River with greater nitrogen availability. Instead, denitrification potential in hollows at high tide was higher on the Mattaponi River with lower nitrogen availability, and denitrification potential was limited by nitrate more in hollows of the Mattaponi than the Pamunkey. DP was measured using river-specific average concentrations of nitrate, whereby Pamunkey soils were incubated with slightly more nitrate than Mattaponi soils yet their rates with nitrate amendment were lower. Thus, the potential for denitrification was similar across rivers at the same time that rates did not linearly follow gradients in N because of competing biogeochemical controls.

Carbon is a mechanistic control on denitrification, and both carbon and nitrate commonly limit denitrification in wetlands. Despite large stores of soil C in tidal wetlands, particularly in organic soils, rates of labile C production where nitrate is abundant can limit denitrification (Reddy *et al.* 1982). Dissolved organic carbon is exported to estuaries from tidal wetlands (Tzortziou *et al.* 2011), and in tidal hollows, labile C may not accumulate in large quantities in shallow soils due to tidal flushing (Neubauer *et al.* 2005b). Thus, low labile C concentrations at the time of sampling and present during 8-hr anoxic incubations for denitrification potential might have been depleted faster from greater initial C demand for microbial respiration with greater nitrate. Our data did not suggest that greater N availability systematically reduced the quantity of soil C or labile C on the Pamunkey River across longitudinal position (Wallenstein *et al.* 2006); however, river differences in nutrient loading may have impacted other soil biogeochemical properties not measured by this study. Not only does

the Pamunkey River have greater long-term nitrogen loading, but it also has greater long-term phosphorus loading (Chanat *et al.* 2015) and higher soil pore-water conductivity. Long-term nitrogen and phosphorus loading in terrestrial environments has been linked to lower soil cation exchange capacity, soil acidification, and changes to plant diversity (Bowman *et al.* 2008). Greater soil acidity may have contributed to the higher soil pore-water ion concentrations on the Pamunkey River and may have altered soil biogeochemical suitability for denitrification potential.

### **Patterns along a watershed to estuary gradient in tidal wetlands**

Within the tidal reaches of both rivers, we found a longitudinal gradient in soil physicochemical properties whereby upper tidal sites differed from middle and lower tidal sites (Table 5.5): upper tidal soils had lower organic matter, C, and N, and higher bulk density than the middle and lower tidal soils. These differences could reflect a greater fluvial sediment influence on the upper tidal sites: Kroes *et al.* (2007) found that lower tidal freshwater and nontidal forested floodplains on the Pocomoke River, MD, had relatively equal vertical accretion rates, but tidal sedimentation was comprised of much greater organic material as a result of different inundation processes and sources of sediment. Thus, the loading and composition of mineral sediment delivered to the tidal freshwater wetlands likely differed by the degree of fluvial influence (watershed loading) on the upper vs. middle and lower TFFW. These longitudinal gradients along the Mattaponi and Pamunkey rivers is consistent with the hypothesis that only TFFWs near the head of tide accumulate greater terrigenous sediment from watershed floods (Ensign *et al.* 2013).

Longitudinal patterns were found for DP at high tide, and the effect of high-tide nitrate delivery on DP, which coincided with patterns in soil nitrate. Compared to upper tidal hummocks, high-tide DP was lower in middle tidal hummocks, where nitrate limitation of DP was also lower and soil nitrate concentrations were higher. The trend in both DP measurements was strongest in hummocks of the Pamunkey River and weak on the Mattaponi River. Similar to the differences in high-tide DP across rivers, low high-tide DP in TFFW middle tidal hummocks suggests limitation by soil biogeochemistry other than nitrate. Greater high-tide DP in upper tidal hummocks, and thus nitrate reduction, is a likely driver of the coincident lower soil nitrate concentrations. The lowest water levels were recorded on the Pamunkey upper tidal site compared to the other sites (Table 5.6) for the preceding months, which may have indirectly stimulated denitrification potential in hummocks through more aerobic biogeochemical processing.

Mean denitrification enzyme activity did not change longitudinally between tidal wetland sites; however, the relationships of DEA with soil physicochemical properties were mediated by longitudinal position in addition to microtopography. Associations between DEA and soil properties were statistically significant only in hummocks and non-existent in hollows. The trend in hummocks followed a longitudinal gradient, with the highest rates of DEA in middle and especially lower tidal hummocks that had greater organic matter, N, and C concentrations, and lower bulk density. Both middle and lower tidal sites also supported the nine highest individual rates of DEA. Von Korff *et al.* (2014) found positive associations between denitrification and the quantity of organic

matter in TFFWs and their channels along two rivers in NC, though the trends were seasonally dependent and suggestive of mediation by carbon quality.

In addition to gradients in soil biogeochemical properties, spatial patterns in sedimentation and salinity might have affected denitrification. Lower tidal sites are likely distinct from middle and upper tidal sites in the amount of nutrient deposition that occurs via sedimentation due to greater river sediment availability and greater wetland herbaceous biomass (Ensign *et al.* 2014, Noe *et al.* 2016). Lower tidal sites also likely endure larger or longer salinity intrusions than the upper tidal sites (Table 5.6). Freshwater tidal wetlands are vulnerable to storm- and drought-related saltwater surges that elevate soil salinities and cause stress and mortality for woody species (Anderson and Lockaby 2013). Higher salinities may indirectly benefit denitrification from greater plant N inputs into the soil from early leaf senescence (Brinson *et al.* 1985) or to greater labile biomass from decomposing herbaceous plants, which has been attributed to the higher net rates of ammonification in salt-stressed forested wetlands (Noe *et al.* 2013). In hummocks, four of the six highest rates of DEA coincided with relatively high rates of potential C mineralization (Fig. 4). Higher salinities within forested or herbaceous freshwater wetlands have been associated with greater denitrification potential and gene abundance (Marton *et al.* 2012, Franklin *et al.* 2016).

Strong gradients in organic matter content could affect denitrifiers in hummocks both directly and indirectly by promoting gradients in oxidation reduction potentials as suggested by our long-term soil oxygen data. The one soil oxygen sensor in a middle tidal hummock on the Mattaponi recorded very low soil oxygen levels (< 1%) that were driven

by high soil oxygen demand, while much higher soil oxygen levels were recorded in the hummock on the Mattaponi upper tidal river. Middle tidal hummocks had over 4 times soil C and roughly 3 times soil N and organic matter than upper tidal hummocks.

Velinsky *et al.* (2013) reported positive relationships between N<sub>2</sub> production and soil oxygen demand in tidal wetlands.

DEA was unrelated to soil physicochemical properties in hollows, and DEA was noticeably lower in hollows than in hummocks at the salt-impacted, lower TFFW sites (Figure 5.4, Appendix A). By comparison, Arango *et al.* (2007) found a positive association between denitrification and substratum organic matter in freshwater streams over the same large range of organic matter found in this study. One reason for the lack of relationships between DEA and soil physicochemical properties in hollows may be due to greater vertical sediment accretion in hollows than in hummocks (Ensign *et al.* 2014). Recently buried plant litter with greater sediment accumulation constitutes low quality carbon which provides less stimulatory effect on denitrification compared to more humified material (Morrissey and Franklin 2014); high C:N ratios of undecomposed plant litter can increase competition for ammonium and suppress nitrification (Strauss and Lamberti 2000, Starry *et al.* 2005, Megonigal and Neubauer 2009). A second reason for the lack of relationships found in hollows might be the longer period of interaction with salinity compared to the higher elevation hummocks during brief periods of salinity intrusion to the lower TFFW sites. Liu *et al.* (2017) found that N<sub>2</sub>O flux was inhibited by low levels of salinity under constantly flooded conditions but not under fluctuating soil moisture conditions. The adverse effects of H<sub>2</sub>S on nitrification (Joye and Hollibaugh

1995, Dollhopf *et al.* 2005) would be more severe under the more consistently inundated hollows; however, others have found positive effects of or associations between salinity and nitrification in tidal streams or forested wetlands (Magalhães *et al.* 2005, Noe *et al.* 2013). Third, higher concentrations of sulfate in the lower tidal hummocks could stimulate carbon mineralization through sulfate reduction which would reduce labile carbon stores to the detriment of denitrifiers (Weston *et al.* 2011).

### **Patterns across nontidal to tidal wetlands**

Tidal wetlands had greater DEA than nontidal wetlands, which was more pronounced on the Mattaponi River that had lower nontidal rates than on the Pamunkey River. Soil oxygen levels measured in one sensor at the nontidal Mattaponi floodplain wetland were above 15% O<sub>2</sub> during baseflow conditions, which would have an inhibitory effect on denitrification (Burgin *et al.* 2010, Duncan *et al.* 2013). On the Pamunkey River, the low nontidal DEA rates occurred despite relatively high concentrations of NO<sub>3</sub> and NNP, adding to evidence that tidal inundation promotes denitrification (Verhoeven *et al.* 2002). Because all tidal sites differed from nontidal sites with greater soil moisture and lower bulk density, but not with nutrient pools or processes, tidal inundation likely primed denitrification enzyme activity in tidal soils through greater saturation and anoxia.

For net nitrification potential, variability across sites was apparently affected more by low nutrient availability than the influence of tidal inundation. Water flow aerates shallow sediments in streams where nitrification can oxidize ammonium and rival rates in terrestrial environments (Bernhardt *et al.* 2002, Starry *et al.* 2005). In tidal wetlands, longer residence time of water and high soil oxygen demand can lower oxygen

levels in summer to levels inhibiting nitrifiers (Ensign *et al.* 2008, Jantii *et al.* 2011), though these differences in oxygen availability may have been less important for our study because measurements were made in September at the end of summer. Our highest rates of net nitrification potential were measured in the nontidal wetlands of the Pamunkey River and in middle tidal hummocks of both rivers, all with less influence of tidal inundation than tidal hollows, but also with elevated nitrate or organic matter concentrations compared to other sites. Availability of  $\text{NH}_4$  is a primary regulator of nitrification (Gribsholt *et al.* 2005, Magalhães *et al.* 2005).

### **Effect of hummock and hollow microtopography**

A simulated dry, aerated low tide promoted denitrification potential during a subsequent simulated high tide, with a larger increase in denitrification potential in hummocks than hollows. Previous studies have demonstrated that dry-wet cycles produce greater  $\text{N}_2\text{O}$  flux and microbial respiration in tidal forested wetlands soils (Krauss *et al.* 2012, Liu *et al.* 2017). Because potential C mineralization and C turnover, but not the effect of aeration on net nitrification potential were affected by hummock-hollow microtopography, we conclude that the effect on DP resulted from increased labile C availability and increased carbon mineralization activity. Though we did not find a difference in the potential for nitrification, Noe *et al.* (2013) found that TFFW hummocks in South Carolina and Georgia tidal rivers had higher rates of field-measured net nitrification, as well as greater microbial biomass nutrient (*i.e.*, N, C, and P) content than hollows. Our study further links microbial activity during low-tide to denitrification at high tide when soils are re-inundated: thus, nutrient mineralization at low-tide is more

important in hummocks for high-tide denitrification in hummocks than in hollows. In hollows, the initial stages of tidal flooding may stimulate aerobic microbial activity when floodwaters have greatest dissolved oxygen and are moving fastest (Knights *et al.* 2017). Our long-term soil oxygen sensors on the Mattaponi demonstrated that soil oxygen levels in hollows remained low due to sustained high water levels, whereas soil oxygen levels were higher and/or showed greater variability in hummocks due to less frequent inundation. In particular, high oxygen levels (>10%) were measured in the upper tidal hummock at baseflow. Thus, these data support the conclusion that aerobic microbial respiration is greater in hummocks than hollows.

## **Conclusions**

Understanding the existence and causes of spatial patterns of denitrification in TFFWs will increase confidence in our ability to predict ecosystem change from increasing land use intensification and sea level rise. Greater N availability on the Pamunkey River, adjacent to the Mattaponi River, was expected to have direct positive effects on denitrification in TFFWs. Instead, we found that greater N availability in Pamunkey River tidal hollows led to greater non-N soil limitation. These results suggest that increases in nitrogen availability (similar to the differences between the two rivers in this study) will not impact the potential for denitrification in TFFWs. Second, we examined spatial patterns of denitrification in four locations across a longitudinal watershed to estuary gradient including nontidal sites and forested tidal freshwater sites affected by salinization and marsh encroachment. Tidal soils had greater moisture and lower bulk density than nontidal soils, and middle and/or lower tidal soils were higher

than upriver (upper) tidal sites in organic matter, N, C, and potential C mineralization (also lower in bulk density). We inferred that the higher moisture levels caused the elevated DEA in tidal wetlands compared to nontidal wetlands, the strongest spatial pattern in DEA found. Longitudinal position of wetlands along the tidal rivers also mediated the relationship between DEA and soil physicochemical properties in hummocks. Sea level rise will thus increase denitrification in nontidal soils that convert to tidal freshwater, and increased rates of sea level rise that affect hydrogeomorphic processes, such as sedimentation and salinity, could alter current spatial gradients in existing TFFWs. Hummock-hollow microtopography, which affects soil biogeochemistry by regulating the effect of tidal inundation on soils, was present across tidal sites and soil oxygen levels were higher in hummocks than hollows. Besides mediating the effects of river and longitudinal position on denitrification, hummocks had positive effects on DP at high tide from greater potential C mineralization and turnover at low-tide. Future studies of TFFWs should consider the influence of this ecogeomorphological feature on ecosystem processes.

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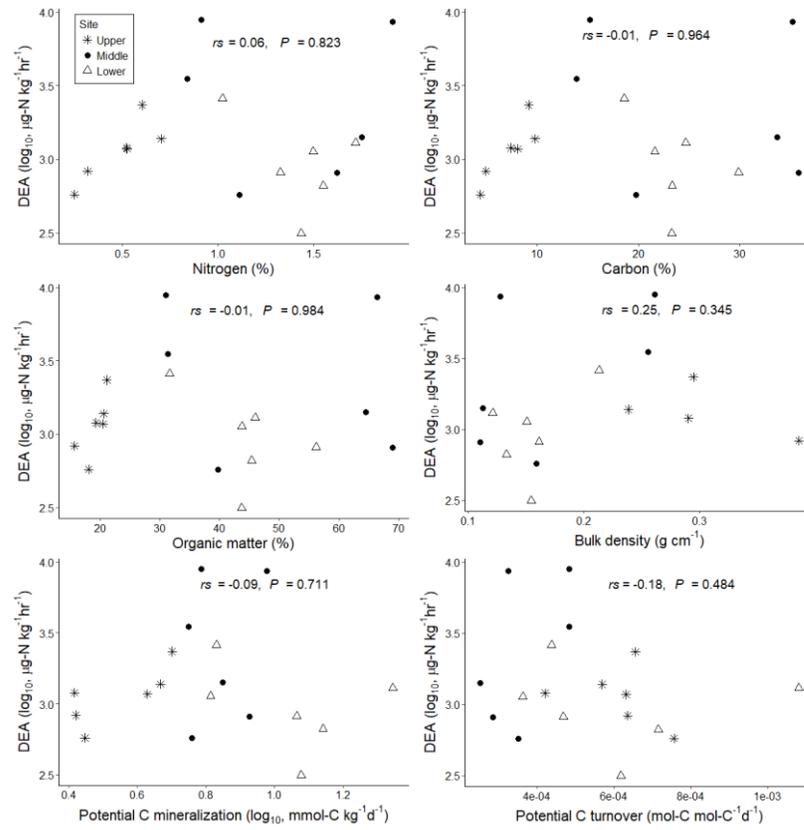
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## Appendix A



Associations of DEA with soil biogeochemical properties and processes in tidal hollows with correlation statistics

## **BIOGRAPHY**

Alicia Korol graduated valedictorian from the 2004 class of Ilion High School, located in the Mohawk Valley of upstate New York. She received her Bachelor of Science from Cornell University (Ithaca, NY) in 2008 for her study of the biological sciences with a concentration in ecology and evolutionary biology. She was employed as a biologist by the Environmental Protection Agency (Arlington, VA) for three years prior to beginning graduate study. At George Mason University (Fairfax, VA), Alicia worked as a graduate research assistant (2011-2014) in the Environmental Science and Policy Department and graduate teaching assistant (2014-2016) in the Biology Department. She was honored for her teaching and received the Elaine Joyce Outstanding Graduate Student Teaching Award. She was granted scholarships from the Cosmos Foundation Young Scholars Program and Society of Wetlands Scientists for her dissertation research.