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STOICHIOMETRIC CONTROLS ON DENITRIFICATION IN HIGH NITRATE WATERSHEDS

Brian D. Grebliunas

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Watershed biogeochemistry throughout Midwestern agroecosystems has been altered through hydrologic manipulation and over-application of nitrogenous fertilizers. As a result, nitrate ($\text{NO}_3\text{-N}$) export from subsurface drainage has negative impacts on local and downstream ecosystem health. Wetland installation has proven to be a viable option for targeted management where a large proportion of $\text{NO}_3\text{-N}$ is removed through the bacterially mediated process denitrification. For denitrification to maintain high rates under prolonged $\text{NO}_3\text{-N}$ saturation, a stable supply of labile dissolved organic carbon (DOC) is required. The focus of this dissertation was to study how the stoichiometry of agricultural wetlands limits denitrification within a controlled laboratory and field-scale applications.

Denitrification rates within wetlands that retain subsurface tile drainage were limited by the availability of DOC. The limitation of DOC became more evident under high $\text{NO}_3\text{-N}$ concentrations, suggesting that wetland sediments have an insufficient pool of labile DOC to maintain elevated rates of denitrification during seasonally intense $\text{NO}_3\text{-N}$ inputs. It was then hypothesized that terrestrial DOC contributions delivered by surface water would serve as an effective DOC subsidy for denitrifying bacteria. It was

found that wetlands retaining drain tile or surface water exhibited similar changes in denitrification to 2:1 and 4:1 C:N ratios.

Bacterial production and denitrification were measured under low (1:1) and high (4:1) C:N ratios to test how bacteria allocate DOC at differing ratios. There was no change in denitrification in sediments incubated at the 1:1 ratio, while bacterial production significantly increased throughout the incubation period. The 4:1 ratio did result in a significant increase in denitrification rates and bacterial production, but bacterial production did not differ between the 1:1 and 4:1 treatments.

Changes in $\text{NO}_3\text{-N}$ removal and denitrification in response to DOC availability (1 mg/L and 10 mg/L) were observed throughout replicate 5 days. Denitrification rates increased significantly at both DOC concentrations throughout the study period. Reductions in $\text{NO}_3\text{-N}$ concentration were observed at low and high DOC availability, however significant reductions only occurred at the 10 mg/L DOC treatment wetlands.

The contribution cover crops have on WEOC in agricultural soils was tested by modeling spectral data from dissolved C fractions extracted from soil cores. Within the first years of cover crop implementation, the amount and types of WEOC did not differ between plots with or without cover crops. This also translated into similar rates of denitrification observed throughout the study period, suggesting that terrestrial denitrification was limited by C availability, similar to receiving aquatic systems.

STOICHIOMETRIC CONTROLS ON DENITRIFICATION IN HIGH NITRATE
WATERSHEDS

BRIAN D. GREBLIUNAS

A Dissertation Submitted in Partial
Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

School of Biological Sciences

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STOICHIOMETRIC CONTROLS ON DENITRIFICATION IN HIGH NITRATE
WATERSHEDS

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B.D.G.

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CHAPTER I

C:N:P LIMITATION OF DENITRIFICATION WITHIN AGRICULTURAL SURFACE
AND TILE WATER WETLANDS

Abstract

Nutrient stoichiometry within a watershed is dictated by the surrounding land use, and can play a significant role in the cycling of nutrients of concern, including $\text{NO}_3\text{-N}$. Tile drained agricultural watersheds experience high seasonal inputs of $\text{NO}_3\text{-N}$, but low $\text{PO}_4\text{-P}$ and C loads relative to surface water dominated systems. Surface water wetlands typically maintain elevated C relative to wetlands that receive tile drainage. This difference may present stoichiometric conditions that limit denitrification within receiving waterways. We investigated how C:N:P ratios impacted denitrification rates within constructed wetland sediments for incubation lengths of (0, 5, 10, and 20 days). We also tested whether denitrification rates in surface water and tile drained wetlands respond differently to C:N ratios (2:1 and 4:1). Ratios of C:N:P ($p<0.0001$) and incubation length ($p<0.0001$) had a significant effect on denitrification in tile drained wetland sediments. Denitrification responded positively to the presence of C, and the addition of C proved to be critical in maintaining denitrification at the $\text{NO}_3\text{-N}$ concentrations of 2 and 20 mg/L. Tile water and surface water wetlands were similarly limited by C:N ratios ($p=0.5010$). The 2:1 and 4:1 DOC amendments significantly increased denitrification in both wetland types ($p<0.0001$). Denitrification rates

increased with increasing C:N ratios. This result suggests the wetland sediments provide a limiting pool of labile C to maintain prolonged $\text{NO}_3\text{-N}$ removal. The terrestrial contribution of C to surface water wetlands does not appear to meet the bacterial demands required for elevated rates of denitrification. At the concentrations of $\text{NO}_3\text{-N}$ presented to tile drained wetland sediments, $\text{PO}_4\text{-P}$ did not limit denitrification. A terrestrial subsidy of C may improve wetland reduction of $\text{NO}_3\text{-N}$, but overland flow from agricultural landscapes still limits denitrification.

Introduction

Wetlands represent a useful mitigation tool to remove dissolved nutrients, particularly nitrogen (N), before entering streams or reservoirs (Gale et al. 1993; Xue et al. 1999; Lund et al. 2003). The size of wetlands relative to agricultural area plays a critical role in N removal. Effective ratios of wetland to watershed for significant N reduction span from 1:100 to 1:10 (Crumpton et al. 1993; Higgins et al. 1993). The necessary wetland size could potentially be reduced if conditions within wetland ecosystems are improved for biotic N removal. The role of carbon (C) and phosphorus (P) relative to N availability can serve as limiting factors to N removal (White and Reddy 1999; Dodds et al. 2004). In appropriate ratios, C and P availability significantly affected the rates of nitrogen transformations in low nitrogen systems, but agricultural systems represent an extreme case where C and P availability are very low relative to saturating N concentrations (Herrman et al. 2008). This stoichiometric imbalance of C:N:P may be limiting the efficiency of nitrogen conversions in constructed wetlands and receiving streams.

Intensive row crop agriculture in the Upper Midwest has altered drainage patterns and influence the concentration and stoichiometry of nutrient inputs (Raymond et al. 2012). Many Midwestern agricultural fields, particularly in Illinois, have been drained with porous corrugated plastic and clay pipe which transports water from the field directly to the streams bypassing many mitigation structures (Lemke et al. 2011). Tile water can be directed from the streams to wetlands and this water has high nitrogen concentrations and low carbon and phosphorus concentrations (Royer and David 2005; Bernot et al. 2006; Vidon et al. 2008; Tank et al. 2010). In contrast, natural wetlands receive overland flow from the surrounding watershed and this water is high in dissolved organic carbon (DOC) and low in nitrate and phosphorus. The stoichiometry can differ between surface water and subsurface water inputs due to flow paths. Surface flow paths have shortened residence times relative to water slowly percolating through upper soil profiles like that of tile drained fields (Dahm et al. 1998). Therefore, surface water typically has elevated labile DOC due to limited reduction through soil and microbial processes (Dillon and Molot 1997; Chambers et al. 2010; Griffiths et al. 2010).

Phosphorus is the primary limiting nutrient in streams and lakes, and can constrain algal and microbial production at times (Vadstein et al. 2003). The microbial processes occurring in the agricultural soils as water infiltrates to the tile immobilize carbon and phosphorus (Farahbakhshazad et al. 2008; USDA 2011). Labile DOC and $\text{PO}_4\text{-P}$ bind to fine particulate soils or are transformed through microbial processes limiting inputs of C and P (Kalbitz and Kaiser, 2007). Unequal ratios of N:P can limit denitrification in wetlands with high concentrations of $\text{NO}_3\text{-N}$ and low $\text{PO}_4\text{-P}$ concentrations, but $\text{PO}_4\text{-P}$ is not limiting in low $\text{NO}_3\text{-N}$ systems (White and Reddy

1999). In tile water during low flow, $\text{NO}_3\text{-N}$ concentrations (10 - 90 $\text{NO}_3\text{-N}$ mg/L) is significantly greater than $\text{PO}_4\text{-P}$ concentrations (0.01-1.51mg/L) suggesting $\text{PO}_4\text{-P}$ limitation of microbial processes. During seasonal increases in tile flow the elevated DOC and $\text{PO}_4\text{-P}$ concentrations are still within ranges that limit denitrification (Allison and Vitousek 2005; Eimers et al. 2008; Reinhardt et al. 2006; Sather 1992).

The role of carbon availability in particular has been shown to affect nitrogen cycling in wetlands and is now being recognized as an important in stream nitrogen cycling (Hume et al. 2002). Increased DOC inputs are associated with stimulated microbial activity and can be an important regulator of heterotrophic microbial activity. Sewage treatment plants maintain high rates of $\text{NO}_3\text{-N}$ removal by adding DOC to maintain increased C:N ratios (>2:1) and can achieve nearly 100% removal of $\text{NO}_3\text{-N}$ (Naik and Setty 2012, Cherchi et al. 2009). The reduction of $\text{NO}_3\text{-N}$ in tile water by installing woodchip bioreactors that serve as a subsurface filter (Greenan et al. 2006). The success of this method is due to the woodchips serving as a substrate for bacterial growth in addition to serving as an available C source (Jaynes et al. 2008). It is likely that availability of labile DOC within treatment wetlands may limit denitrification in wetlands (Songliu et al. 2009). If wetlands are to serve as an effective mitigation tool for $\text{NO}_3\text{-N}$ removal understanding the extent to which carbon affects denitrification potential in high nitrate systems.

The goal of this study was to assess the extent to which bacterial denitrification respond to increased carbon and phosphorus. Specifically, we created microcosms that contained sediment from an established tile drained wetland and were amended with assigned nutrient treatments. This study was followed by testing if bacterial

denitrification in sediments from constructed agricultural wetlands receiving surface water (elevated DOC) or tile water (low DOC) responded differently when treated with low (2:1) and high (4:1) C:N amendments. We hypothesized that increased carbon and phosphorus availability would increase bacterial denitrification. We also predicted that bacterial denitrification would be higher in surface water wetlands relative to tile water wetlands.

Methods

C:N:P Study

We collected sediments for the C:N:P study, from an 11-year old experimental constructed wetland complex within the Mackinaw River watershed near Lexington, IL (40° 38' 23" N, 88° 49' 18" W) in June 2011. Dissolved nitrate concentrations in drain tiles concentrations commonly exceed 40 mg NO₃-N /L in the spring. Inflow tile concentrations of DRP within our site ranged from < 0.01 mg/L - 1.2 mg/L, which is high and flux rates of this magnitude likely meet denitrification needs (Royer et al. 2006; Xue et al. 1998). Sediments were removed from the top layer (2cm) haphazardly within the wetland and homogenized into a single slurry. Sediments were stored at 4°C for 24 hours after which 20g of sediment was placed in a individual microcosms, 150ml media bottles fitted with a butyl septa screw caps. The bottle microcosms served as the experimental units in the laboratory study. An unamended 100 ml aliquot of water was added and replaced daily to each microcosm, prior to the appropriate nutrient amount.

To test the effects of C:N:P, microcosms were manipulated by adding nutrients daily, then destructively sampled at days 0, 5, 10, and 20 to observe potential changes in bacterial activity in response to different stoichiometric conditions. A total of 16

treatments consisting of different ratios as follows: added N only (0:2:0, 0:20:0), added N:P (0:2:2, 0:20:2), added C:N (10:2:0, 40:2:0, 10:20:0, and 40:2:0), and added C:N:P (10:2:2, 40:2:2, 10:20:2, and 40:20:2) were tested in triplicate (Figure 1). To create nutrient stock solutions (0.1M) we used potassium nitrate (KNO_3), glucose-C ($\text{C}_6\text{H}_{12}\text{O}_6$), and sodium phosphate (Na_2HPO_4). Nutrients were added daily to the 100 ml of overlying water which was replaced daily by using a modified 25 ml pipette that was affixed to vacuum pump to remove the overlying water, and minimize the loss of sediments within each microcosm. Stock solution was added to the newly added 100 ml of water to achieve the appropriate ratio of C:N:P for each treatment. Microcosms were flushed with nitrogen gas and incubated at 25°C set on a 24-hour dark cycle to provide favorable conditions for denitrifying bacteria. Sediments were assayed via the acetylene inhibition technique (Chan et al. 1979; Smith et al. 2006). Headspace gas samples collected throughout the assay were measured using a Shimadzu GC-2014 gas chromatograph (Porapak Q packed column; Detector temperature 300°C; oven temperature, 100°C; flow rate of carrier gas (ultrapure Nitrogen gas) 10 ml/min.).

Wetland Type Study

To test the effects of sediment type on response to CNP treatments, we did a similar microcosm study using sediments from 3 wetlands receiving surface water (Frog, Floodplain East, and Floodplain West) inputs and 3 wetlands receiving tile water (Moga, Gully, and Durbin) (Figure 2). Wetlands used in this study drain soybean and corn agricultural fields but surface water wetlands receive water through grass waterways and overland flow and is higher in DOC than tile water wetlands. Wetlands were all constructed six to seven years ago and have similar organic carbon in sediments.

To test for difference in bacterial denitrification between wetland sediments two ratios of C:N, low (2:1) and high (4:1), and an unmanipulated control were used. The maximal C:N ratio used was based upon the findings of the C:N:P study and observed to be effective in high $\text{NO}_3\text{-N}$ wastewater systems (Sobieszuk and Szewczyk 2006). $\text{NO}_3\text{-N}$ concentrations in microcosms were maintained at 10 mg/L which is a commonly observed concentration in overlying wetland water. DOC (glucose-C as $\text{C}_6\text{H}_{12}\text{O}_6$) concentrations were 20 mg/L for the low C:N treatment and 40 mg/L for the high C:N treatment. Each nutrient treatment was replicated 5 times for each time period and incubations were destructively sampled on days 0, 5, 10, and 20 (Figure 3).

Data Analysis

To examine potential differences in bacterial denitrification in response to variable ratios of C:N:P, we used a two-way fixed effects analysis of variance with the main effects being nutrient ratio and time. To test for differences in bacterial denitrification differences in the wetland type study, we tested the effects of nutrient ratios, wetland type, incubation time, and the interactions of these variables through the use of a mixed model ANOVA. The fixed effects were nutrient ratios, wetland type, and incubation time. The random effect for this model were the different wetlands, or sites, from which sediments were collected. In order to better evaluate the importance of C:N stoichiometry to denitrification rates over time, models were selected using Akaike Information Criterion (AIC_c). After running the mixed model ANOVA, variables that had a significant effect on denitrification are used as parameters to calculate AIC_c values. Using AIC_c allowed us to test which parameters (or combination of multiple parameters) account for the most variation in denitrification rates. When selecting the appropriate

model, the parameters with the lowest AIC_c value are the best fit (Burnham and Anderson 2002). Assumptions of normality and homogeneity of variances for both analyses were met without the aid of data transformations. The data analyses were performed using SAS 9.2 (SAS Institute Inc., 2008, Cary, North Carolina, USA).

Results

C:N:P Study

Denitrification changed significantly to the daily nutrient amendments ($F_{15,143}=80.44$, $p < 0.0001$). A significant increase in denitrification occurred when sediments were amended with both C and NO₃-N, and without C, no positive effects were observed (Figure 4A, 4B). Denitrification rates varied significantly over time ($F_{2,143} = 20.29$, $p<0.0001$), however the directionality of the observed changes were dependent upon the microcosm stoichiometry ($F_{30,143}=5.75$, $p<0.0001$).

Only elevated C (40mg/L) and NO₃-N (20mg/L) increased denitrification rates significantly from days 0 – 20, but similar nonsignificant trends were observed at lower C:N ratios. Without amendments of C, denitrification rates decreased throughout the course of the study. When C concentrations are low (10 mg/L) relative to NO₃-N there was no significant change in denitrification by day 20. Carbon limitation became more pronounced in the presence of elevated NO₃-N concentrations (20 mg/L) as denitrification increased relative to elevated amendments of C from 10 mg/L to 40 mg/L.

Denitrification rates did not show statistical differences between the N and N:P treatments. The similar denitrification rates with and without P suggest that the availability of P is not limiting. This also held true across low and high NO₃-N treatments, unlike C. With increasing NO₃-N concentrations from the low to high

treatment, denitrification rates decreased as the incubation progressed (Figure 4). The low treatment (2 mg/L) maintained relatively stable rates, while the high treatment (20 mg/L) decreased over time (Figure 4).

Wetland Type Study

The 2:1 and 4:1 DOC amendments resulted in denitrification rates significantly higher than that of the control ($F_{2,8}=148.09$, $p<0.0001$). Denitrification rates from surface and tile water wetland sediments did not differ between nutrient treatments ($F_{1,4}=0.55$, $p=0.5010$, Table 1) (Figure 5). Though denitrification rates did differ significantly between wetlands within each wetland type ($F_{4,5,8}=6.42$, $p=0.0246$) Denitrification rates increased rapidly in response to C:N additions, but the increase was not significant over time ($F_{4,16}=1.9$, $p=0.1592$). Maximal rates of denitrification were reached earlier in the incubation when presented with the high C:N (4:1) treatment (Figure 5). Though, the 1:1 treatment produced similar on day 20, as opposed to day 5 like the 4:1 treatment (Figure 5). A significant interaction between sites, time, and treatment nested within wetland type ($F_{16,216}=2.11$, $p=0.0090$) suggested that denitrification differs over time dependent upon nutrient availability, and that wetland type appears to affect denitrification at elevated C:N. Calculating the AICc for the observed parameters further supported that denitrification differs due to the interaction of nutrients over time across wetland types (Table 2). The lowest calculated AICc value included the parameter: treatment, day, wetland type, and site (Table 2). Having the lowest calculated value shows it fit the overall model best. This is likely attributed to drain tile wetlands maintaining significantly higher denitrification at the 4:1 treatment relative to the 2:1 treatment, while a reduced response occurred in surface water sediments.

Discussion

The results of this laboratory study suggest wetland sediments receiving elevated nitrate levels commonly observed in Central Illinois are strongly carbon but not phosphorus limited. Denitrification in control and low nitrate treatments changed only slightly over time indicating that C from sediments was sufficient to meet microbial demands. Denitrification in the presence of elevated NO_3 increased initially but then decreased over the course of the experiment suggestive that initial increase in denitrification utilized the C in the sediments but then decreased significantly as carbon availability decreased. Denitrification was not affected by the addition of phosphorus additions suggesting that P from sediments was sufficient for microbial growth. Denitrification, however, increased significantly with added carbon and as nitrate concentrations increased, denitrification continued to increase if C increased proportionally. We hypothesized that sediments from surface water wetlands might have higher carbon availability and serve as a carbon source and increased denitrification relative to tile water wetlands and may respond differently to added carbon. Denitrification in surface and tile water wetlands did not differ significantly and added carbon significantly increased denitrification but the response did not differ between wetland types. In watersheds where nitrate inputs to receiving waterways are significantly elevated, carbon availability strongly limits denitrification and even small increases can dramatically increase denitrification. The controls of carbon availability on nitrogen cycling in both wetlands and streams in agricultural areas is critically important to understand to help preserve drinking water supplies and prevent further degradation of marine environments.

C:N:P Study

Bacterial denitrification decreased over time with increased nitrate concentrations relative to controls. Denitrification did not differ with additions of phosphorus, but carbon amendments led to a dramatic increase of denitrification. Although carbon additions have been documented to lead to increased microbial activity, the extent to which bacteria were limited in agricultural watershed, whether in streams or wetlands, can be better appreciated from a study manipulating the CNP ratios that are in the ranges observed in these systems (Hill et al. 2004; Fork et al. 2014).

When added alone, $\text{NO}_3\text{-N}$ exhibited lower rates of denitrification relative to the control and declined over time (Figure 4). Elevated rates of denitrification are often observed in wetlands following pulses of increased $\text{NO}_3\text{-N}$ inputs especially in low $\text{NO}_3\text{-N}$ (or N limited) systems (<5 mg/L) (Forshay and Stanley 2005; Sirivedhin and Gray 2006; Smith et al. 2006). Increased denitrification is likely due to accumulated labile DOC within sediments where microbial demands are lower due to lower $\text{NO}_3\text{-N}$ availability. In high nitrate systems, elevated inputs of $\text{NO}_3\text{-N}$ often do not result in peaks in denitrification (Mulholland et al. 2009). When high $\text{NO}_3\text{-N}$ concentrations persist, labile fractions of DOC are depleted, resulting in conditions that limit denitrification. Spring flood pulses, however, can foster significant increases in denitrification, but early pulses of tile water have elevated DOC and $\text{NO}_3\text{-N}$. The increased denitrification in spring flood pulses can be short lived because denitrification rapidly utilizes available DOC (Zarnetske et al. 2011).

Tile inputs of $\text{PO}_4\text{-P}$ are often low relative to surface runoff, but wetland sediments offer effective binding sites leading to saturation of sediments soon after

construction. We observed no change in denitrification in $\text{PO}_4\text{-P}$ additions (Figure 4). Unlike DOC, the availability of $\text{PO}_4\text{-P}$ does not provide energy to carry out the respiration of $\text{NO}_3\text{-N}$, though it can limit bacterial growth in freshwater ecosystems (Correll 1999). Therefore, if denitrification increased with elevated $\text{PO}_4\text{-P}$, it may have been attributed to a larger bacterial population size. Aquatic systems limited by $\text{PO}_4\text{-P}$ can exhibit significant population growth of bacteria in response to low P concentrations (1-10 $\mu\text{g/L}$) but growth asymptotes at the upper end of this range (Miettinen et al. 1997). Therefore, tile inputs coupled with remobilization of P from anoxic sediments appear to be sufficient for bacteria within the benthos of agricultural waterways and P does not alter denitrification rates.

C limitation was evident in the presence of elevated $\text{NO}_3\text{-N}$ (20 mg/L) concentrations, prompting potential management issues with the remediation of tile water in constructed wetlands. Under low $\text{NO}_3\text{-N}$ (2 mg/L) and low DOC (10 mg/L), denitrification rates remained stable throughout incubation (Figure 4A). A shift from $\text{NO}_3\text{-N}$ limitation to DOC limitation was observed when an increase in DOC (40 mg/L) in the presence of low $\text{NO}_3\text{-N}$ resulted in an increase in denitrification by day 20, but was reduced relative to the elevated $\text{NO}_3\text{-N}$ treatment (20 mg/L) (Figure 4B). By design, constructed wetlands are installed to intercept a large catchment area relative to wetland size to maximize drainage retention, often resulting in extended periods of inundation (weeks to months) (Lee et al. 2002). Wetland areas that maintain pooled conditions exhibit elevated denitrification rates (and bacterial activity) relative to sediments that are temporarily inundated (Hernandez and Mitsch 2007). Coupling prolonged wetting periods with $\text{NO}_3\text{-N}$ saturation appears to have altered the demand of labile C throughout

agricultural watersheds, likely reducing the effectiveness of wetlands as a mitigation tool due to energetic constraints.

Denitrification increased significantly in the presence of elevated C, while no effect of P availability was observed. Wetlands that receive elevated $\text{NO}_3\text{-N}$ inputs with little to no allochthonous DOC to supplement what is consumed from the autochthonous pool may experience reduced rates of denitrification due to DOC limitation (Burgoon 2001; Hume et al. 2002). Establishing a matrix of emergent and submergent vegetation beds have been shown to increase denitrification, but C:N ratios of emergent macrophytes are similar to terrestrial plants leading to slow decomposition rates where any increase in plant available DOC may be overcome by saturating $\text{NO}_3\text{-N}$ concentrations (Bachand and Horne 1999). As wetlands age, the death and decomposition of aquatic plants and algae can lead to an accumulation of organic material over time (Mitsch et al. 2012). The pool of organic material within our focal wetland sediments did not maintain an adequate C:N ratio in the presence of high $\text{NO}_3\text{-N}$, even though older constructed wetlands (> seven years), as in our study, have been observed to perform in a similar manner to natural wetlands (Mustafa and Scholz 2011). A low average DOC concentration coupled with a predominantly recalcitrant pool of carbon typical of mid-successional wetlands may be interacting to limit denitrification within agricultural wetlands.

Wetland Type Study

Denitrification rates in sediments from surface and tile water wetlands did not differ significantly in their response to DOC additions (Figure 5). The type of land use within a watershed serves a strong regulatory role for the quantity and ratio of nutrients

entering the receiving water bodies, dictating those nutrients that are limiting or saturating watersheds. Surface waters entering wetlands from undisturbed uplands (prairie and forest) can have higher concentrations and more labile DOC relative to that of cropland runoff or tile drainage (Purakayastha et al. 2008). However, crop residues can contribute appreciable amounts of DOC during brief seasonal pulses of tile water (Royer and David 2005). We hypothesized that wetlands retaining surface water would exhibit substantially lower DOC limitation and higher denitrification overall. Our results suggest that denitrification rates did not differ significantly between wetland types in response to DOC additions. The uncharacteristically high inputs of $\text{NO}_3\text{-N}$ associated with row crop agriculture likely nullified the contribution of dissolved and particulate C fractions delivered in surface waters (Hussein et al. 2004; Inamdar et al. 2004).

The observed change in denitrification over time was contingent upon the C:N amendment, where the 4:1 ratio appeared to foster conditions that promoted a more rapid increase relative to the 2:1 treatment. This result suggests if brief pulses of row crop drainage had an elevated C:N, tile water may adequately enrich wetland sediments to meet the energetic demands of denitrifying bacteria. This would likely enable denitrification activity to more rapidly (≤ 5 days) respond to brief periods of $\text{NO}_3\text{-N}$ saturation (Figure 5). In addition to requiring less time to reach maximum rates of denitrification, the 4:1 treatments maintained the elevated rates for the duration of the study, whereas the 2:1 treatment required 20 days to reach similar rates. The response to the low nutrient treatment is a more prolonged, linear increase and a similar trend was observed in both surface and tile water wetlands (Figure 5). Rates of denitrification in each wetland type rapidly responded to pulses of DOC at high concentrations, suggesting

the input of minimal allochthonous DOC has the potential to dramatically increase denitrification rates in high NO_3 systems.

Our results suggest low DOC relative to $\text{NO}_3\text{-N}$ for prolonged periods will limit $\text{NO}_3\text{-N}$ reduction via denitrification (Figure 5). Low rates of denitrification due to limited DOC availability is particularly evident in newly constructed wetlands, but pulses of DOC in tile or surface water can lead to significant increases in denitrification (Song et al. 2011). The sites used for this study were 7-8 years old which is typically enough time to allow a sufficient amount of detrital accumulation and decomposition to meet heterotrophic demands (Wolf et al. 2011). In landscapes with reduced inputs of $\text{NO}_3\text{-N}$, constructed wetlands may be able to effectively reduce $\text{NO}_3\text{-N}$ to manageable concentrations. Further work is needed to investigate mechanisms to increase the availability of DOC within wetland environments, whether it comes from changes in agricultural practices or vegetation management within the wetlands themselves.

Conclusion

Within intensely farmed watersheds, autochthonous and allochthonous DOC inputs associated with row crop agriculture are insufficient to support maximal denitrification when there is excessive $\text{NO}_3\text{-N}$ entering treatment wetlands. Without supplemental DOC, wetlands receiving prolonged periods of $\text{NO}_3\text{-N}$ laden tile water may reduce the $\text{NO}_3\text{-N}$ remediation potential. Denitrification rates had a distinct negative trend when sediments were presented with only $\text{NO}_3\text{-N}$, which was most likely related to limiting heterotrophic conditions. This idea is further supported when rates of denitrification are improved with DOC additions as low as 2 mg/L over time. However, as exhibited from the wetland comparison study, inputs of DOC from surrounding

terrestrial environments does not adequately support the energy needs of heterotrophs in the presence of $\text{NO}_3\text{-N}$ concentrations commonly observed within intensively farmed regions. Further work must be undertaken to address potential methods of increasing the amount of bioavailable C in order increase the effectiveness of wetlands as $\text{NO}_3\text{-N}$ removal tools, and potentially reducing the size of treatment wetlands making them a more feasible option throughout agricultural regions.

References

- Allison, S.D., and Vitousek, P.M. 2005. Responses of extracellular enzymes to simple and complex nutrient inputs. *Soil. Biol. Biochem.* 37: 937-944.
- Bachand, P.A.M., and Horne, A.J. 1999. Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature. *Eco. Engin.* 14: 17-32.
- Bernot, M.J., Tank, J.L., Royer, T.V., and Dabid, M.B. Nutrient uptake in streams draining agricultural catchments of the midwestern United States. 2006. *Freshwater Biol.* 51: 499- 509.
- Burgoon, P.S. 2001. Denitrification in free water surface wetlands receiving carbon supplements. *Water. Sci. Technol.* 44:163-169.
- Burnham, K.P., and Anderson, D.R. 2002. Model selection and multi-model interference. Springer, New York.
- Chambers, C.P., Whiles, M.R., Rosi-Marshall, E.J., Tank, J.L, Royer, T.V., Griffiths, N.A., Evans-White, M.A., and Stojak, A. 2010. Responses of stream invertebrates to Bt maize leaf detritus. *Ecol. Appl.* 20: 1949-1960.
- Chan, Y.K., and Knowles, R. Measurement of denitrification in two freshwater sediments by an in situ acetylene inhibition method. 1979. *Appl. Environ. Microbiol.* 37: 1067–1072.
- Cherchi, C., Onnis-Hayden, A., El-Shawabkeh, I., and Gu, A.Z. 2009. Implication of using different carbon sources for denitrification in wastewater treatments. *Water Environ. Res.* 81: 788-799.
- Correll, D.L., Jordan, T.E., and Weller, D.E. 1999. Transport of nitrogen and phosphorus from Rhode River watersheds during storm events. *Water Resour. Res.* 35: 2513-2521.
- Dahm, C.N., Grimm, N.B., Marmonier, P., Valett, H.M., Vervier, P. 1998. Nutrient dynamics at the interface between surface waters and groundwaters. *Freshwater Biol.* 40: 427-451.
- Dillon, P.J., and Molot, L.A. 1997. Effect of landscape form on export of dissolved organic carbon, and phosphorus from forested stream. *Water Resour. Res.* 33: 2591-2600.

- Dodds, W.K., Marti, E., Pontius, J., Hamilton, L.S., Grimm, N.B., Bowden, R.D., Webster, J.R., and Gregory, S. 2004. Carbon and nitrogen stoichiometry and nitrogen cycling rates in streams. *Oecologia*. 140: 458-467.
- Eimers, M.C., Buttle, J., Watmough, S.A. 2008. Influence of seasonal changes in runoff and extreme events on dissolved organic carbon trends in wetland- and upland-draining streams. *Can. J. Fish. Aquat. Sci.* 65: 796-808.
- Farahbakhshazad, N., Dinnes, D.L., Li, C., Jaynes, D.B., and Salas, W. 2008. Modeling biogeochemical impacts of alternative management practices for a row-crop field in Iowa.
- Fork, M.L., and Heffernan, J.B. 2014. Direct and indirect effects of dissolved organic matter source and concentration on denitrification in northern florida rivers. *Ecosystems*. 17: 14-28.
- Forshay, K.J., and Stanley, E.H. 2005. Rapid nitrate loss and denitrification in a temperate river floodplain. *Biogeochemistry*. 75: 43-64.
- Greenan, C.M., Moorman, T.B., Kaspar, T.C., and Jaynes, D.B. 2009. Denitrification in wood chip bioreactors at different water flows. *J. Environ. Qual.* 38: 1664-1671.
- Griffiths N.A., Tank, J.L., Royer, T.D., Rose-Marshall, E.J., Whiles, M.R., Chambers, C.P., Frauendorf, T.C., Evans-White, M.A. 2009. Rapid decomposition of maize detritus in agricultural headwater streams. *Ecol. Appl.* 19: 133-142.
- Hernandez, M.E., and Mitsch, W.J. 2007. Denitrification in created riverine wetlands: Influence of hydrology and season. *Ecol. Engin.* 30:78-88.
- Herrman, K.S., Bouchard, V., and Moore, R.H. 2008. An assessment of nitrogen removal from headwater streams in an agricultural watershed, northeast Ohio, U.S.A. *Limnol. Oceanogr.* 53: 2573-2582.
- Hill, A.R., and Cardaci, M. 2004. Denitrification and organic carbon availability in riparian wetland soils and subsurface sediments. *Soil Sci. Soc. Am. J.* 68: 320-325.
- Hume, N.P., Fleming, M.S., and Horne, A.J. 2002. Denitrification potential and carbon quality of four aquatic plants in wetland microcosms. *Soil Sci. Soc. Am. J.* 66:1706-1712.
- Hume, N.P., Fleming, M.S., and Horne, A.J. 2002. Plant carbohydrate limitation on nitrate reduction in wetland microcosms. *Water Res.* 36: 577-584.

- Hussein, A.H., Rabenhorst, M.C., and Tucker, M.L. 2004. Modeling of carbon sequestration in coastal marshes. *Soil Sci. Soc. Am. J.* 68:1786-1795.
- Inamdar, S.P., Christopher, S.F., and Mitchel, M.J. 2004. Export mechanisms for dissolved organic carbon and nitrate during summer storm events in a glaciated forested catchment in New York, USA. *Hydrol Process* 18:2651-2661.
- Jaynes, D.B., Kaspar, T.C., Moorman, T.B., and Parkin, T.B. 2008. In situ and deep drain-pipe installation to reduce nitrate losses in artificially drained fields. *J. Environ. Qual.* 37: 429-436.
- Kalbitz, K., and Kaiser, K. 2007. Contributions of dissolved organic matter to carbon storage in forest mineral soils. *J. Plant. Nutr. Soil Sci.* 170: 52-60.
- Lee, E.R., Mostaghimi, S., and Wynn, T.M. 2002. A model to enhance wetland design and optimize nonpoint source pollution control. *J. Am. Water Works Ass.* 38:17-32.
- Lemke, M.A., Kirkham, K.G., Lindendaum, T.T., Herbert, M.M., Tear, T.H., Perry, W.L., and Herkert, J.R. 2011. Evaluating agricultural best management practices in tile-drained subwatersheds of the Mackinaw River, Illinois. *J. Environ. Qual.* 40: 1215-1228.
- Miettinen, IT, Vartiainen, T, and Martikainen, PJ (1997) Phosphorus and bacterial growth in drinking water. *Appl Environ Microbiol* 63:3242-3245.
- Mitsch, W.J., Zhang, L., Stefanik, K.C., Nahlik, A.M., Anderson, C.J., Bernal, B., Hernandez, M., and Song, K. 2012. Creating wetlands: Primary succession, water quality changes, and self-design over 15 years. *Bioscience* 62:237-250. doi: 10.1525/bio.2012.62.3.5
- Mulholland, P.J., Hall, R.O., Sobota, D.J., Dodds, W.K., Findlay, S.E.G., Grimm, N.B., Hamilton, S.K., McDowell, W.H., O'Brien, J.M., Tank, J.L., Ashkenas, L.R., Cooper, L.W., Dahm, C.N., Gregory, S.V., Johnson, S.L., Meyer, J.L., Peterson, B.J., Poole, G.C., Valett, H.M., Webster, J.R., Arango, C.P., Beaulieu, J.L., Bernot, M.J., Burgin, A.J., Crenshaw, C.L., Helton, A.M., Johnson, L.T., Niederlehner, B.R., Potter, J.D., Sheibley, R.W., and Thomas, S.M. 2009. Nitrate removal in stream ecosystems measured by ¹⁵N addition experiments: Denitrification. *Limnol. Oceanogr.* 54: 666-680.
- Mustafa, A., and Scholz, M. 2011. Nutrient accumulation in *Typha latifolia* L. and sediment of a representative integrated constructed wetland. *Water Air Soil Pollut.* 219:329-341.

- Naik, S.S., and Setty, Y.P. 2012. Biological denitrification of wastewater-A Mini Review on Carbon Source. ICEEBS. July 28-29. Pattaya, Thailand. pg 47-51.
- Purakayastha, T.J., Huggins, D.R., and Smith, J.L. 2008. Carbon sequestration in native prairie, perennial grass, no-till, and cultivated Palouse silt loam. *Soil Sci. Soc. Am. J.* 72:534-540.
- Reinhardt, M., Muller, B., Gachter, R., and Wehrli, B. 2006. Nitrogen removal in a small constructed wetland: An isotope mass balance approach. *Environ. Sci. Technol.* 40: 3313-3319.
- Raymond, P.A., David, M.B., and Saiers, J.E. 2012. The impact of fertilization and hydrology on nitrate fluxes from Mississippi watersheds. *Curr. Opin. Environ. Sustain.* 4: 212-218.
- Royer, T.V., and David, M.B. 2005. Export of dissolved organic carbon from agricultural streams in Illinois, USA. *Aquat. Sci.* 67: 465-471.
- Royer, T.V., David, M.B., and Gentry, L.E. 2006. Timing of riverine export of nitrate and phosphorus from agricultural watersheds in Illinois: Implications for reducing nutrient loading to the Mississippi River. *Environ. Sci. Technol.* 40:4126-4131.
- Sather, J.H. 1992. Intensive studies of wetland functions: 1990-1991 research summary of the Des Plaines River wetlands demonstration project. Technical Paper No 2. <http://www.wetlandsresearch.org/pdf/2intensiveStud.pdf>. Accessed 3 May 2012.
- Sirivedhin T., and Gray K.A. 2006. Factors affecting denitrification rates in experimental wetlands: Field and laboratory studies. *Ecol. Engin.* 26: 167–181.
- Smith, L.K., Voytek, M.A., Bohlke, J.K., and Harvey, J.W. 2006. Denitrification in nitrate-rich streams: Application of N₂:Ar and ¹⁵N-Tracer methods in intact cores. *Ecol. Appl.* 16: 2191-2207.
- Sobieszuk, P., and Szewczyk, W. 2006. Estimation of (C/N) ratio for microbial denitrification. *Environ. Technol.* 27: 103-108.
- Song, K., Lee, S-H., and Kang, H. 2011. Denitrification rates and community structure of denitrification bacteria in a newly constructed wetland. *Eur. J. Soil Biol.* 47:24-29.
- Songliu, L., Hongying, H., Yingxue, S., Jia, Y. 2009. Effect of carbon source on the denitrification in constructed wetlands. *J. Environ. Sci.* 21: 1036-1043.
- Tank, J.L., Rosi-Marshall, E.J., Royer, T.V., Whiles, M.R., Griffiths, N.A., Frauendorf, T.C., and Treering, D.J. 2010. Occurrence of maize detritus and a transgenic

insecticidal protein (Cry1Ab) within the stream network of an agricultural landscape. PNAS 107: 17645-50.

USDA Natural Resources Conservation Service. Carbon to Nitrogen Ratios in Cropping Systems. USDA NRCS East National Technology Support Center, Greensboro, N.C., January, 2011.

Vadstein, O., Olsen, L.M., Busch, A., Andersen, T., and Reinertsen, H.R. 2003. Is phosphorus limitation of planktonic heterotrophic bacteria and accumulation of degradable DOC a normal phenomenon in phosphorus-limited systems? A microcosm study. FEMS Microbiol. Ecol. 46: 307-316.

Vidon, P., Wagner, L.E., and Soyeux, E. 2008. Changes in the character of DOC in streams during storms in two Midwestern watersheds with contrasting land uses. Biogeochemistry 88: 257-270.

White, J.R. and Reddy, K.R. 1999. Influence of nitrate and phosphorus loading on denitrifying enzyme activity in everglades wetland soils. Soil Sci. Am. J.63: 1945-1954.

Wolf, K.L., Ahn, C., and Noe, G.B. 2011. Development of soil properties and nitrogen cycling in created wetlands. Wetlands 31:699-712.

Xue, Y., Kovacic, D.A., David, M.B., Gentry, L.E., Mulvaney, R.L., and Lindau, C.W. 1998. In situ measurements of denitrification in constructed wetlands. J. Environ. Qual. 28: 263-269. d

Zarnetske, J.P., Haggerty, R., Wondzell, S.M., Baker, M.A. 2011. Labile dissolved organic carbon supply limits hyporheic denitrification. J. Geophys. Res. 116:1-13.

Table 1

Results of the mixed model ANOVA showing there is no difference in denitrification between the high and low carbon treatments when tile and surface water wetlands are compared.

Source	df	F	P
Wetland Type	1	0.55	0.5010
Error			

Table 2

Display of parameters tested, along with Akaike Information Criterion (AICc). Values within the parameters including TDS(W) highlighted bold due to lowest AICc value, showing TDS(W) to be the best fit for the model. Model parameters labeled as follows: T (treatment), D (day), S (site), and W (wetland type).

Model	AICc	Delta AICc	Exp Function	wi
TDS(W)	2373.9	0	1	0.85173
DS(W)	2378.6	4.7	0.09536916	0.08123
TS(W),DS(W)	2379	5.1	0.07808167	0.06651
S(W)	2389.6	15.7	0.000038975	0.00033
TS(W)	2390.6	16.7	0.0002364	0.0002
sum			1.174076977

Figure 1. List of C:N:P amendments applied to drain tile wetland sediments and experimental outline.

Figure 2. A map of the wetland sites used for sediment core collection within the Mackinaw River watershed, IL. The wetland denoted by an asterisk is the Gully wetland (40° 38' 23", 88° 49' 18") which was used for the C:N:P study, but also incorporated into the water source study. Sites incorporated into the water source study are denoted by circles on the watershed map as follows: surface water sites (grey) and tile water sites (black).

Tile Drained Wetlands Surface Water Wetlands

- Moga
- Durbin
- Gully

- East Floodplain
- West Floodplain
- Frog

Sediment slurry from each wetland

Slurry allocated to media
bottles and associated
treatments:

Prolonged incubation time and subset assayed on days 0, 5, 10, and 20:
N = 20 for each wetland site. For day 0, n=5 since each sample was assayed
prior to any nutrient amendment to establish a baseline rate of denitrification

Figure 3. List of sites used within each wetland category (surface water and tile drained) and experimental allocation of nutrient amendments to homogenized wetland sediments.

Figure 4. Response of denitrification to amendments of differing nutrient stoichiometry over time (5, 10, and 20). Denitrification rates measured on Day 0 were subtracted from subsequent days assayed to establish a new baseline. Graphs separated by low (A, 2 mg/L) and high (B, 20 mg/L) NO₃-N availability.

Figure 5. Shift in denitrification rates of tile (A) and surface water (B) sediments over time in response to increasing ratios of C:N. Denitrification rates presented from tile water sites are means of the Moga, Durbin, and Gully wetlands. Denitrification rates from the surface water sites are means of East Floodplain, Frog, and West Floodplain. Rates measured on Day 0 were subtracted from subsequent days assayed. Negative rates of denitrification represent a decrease in activity from Day 0. Treatments are denoted as follows: control (▲), 2:1 (■), and 4:1 (●). Wetland sediments from surface and tile water sites did not differ significantly in response to nutrient amendments.

CHAPTER II

CARBON LIMITATION OF SEDIMENT BACTERIAL PRODUCTION AND
DENITRIFICATION IN HIGH NITRATE LOW CARBON SYSTEMS

Abstract

The concentrations and ratios of carbon and nitrogen may affect bacterial denitrification through changes in enzymatic or bacterial production, but short-term laboratory and field assays may not account for changes over longer periods. Drainage from agricultural landscapes can have different ratios of C:N depending on the water source, i.e., surface runoff has elevated C:N relative to agriculture tile drainage water. We hypothesized that differences in C:N limit bacterial production and reduce denitrification rates. Sediment cores collected from surface or tile water fed wetlands were incubated in low C:N (1:1) and high C:N (4:1) ratios keeping nitrogen concentrations at 10 mg/L NO₃-N. We used replicated laboratory incubations that were destructively sampled on days 0, 1, 3, 5, 10, and 20 to represent pulsed water inputs to wetlands. Bacterial production and denitrification were estimated using [³H] leucine incorporation and the acetylene block method. Bacterial production and denitrification increased ($p < 0.0001$) in response to elevated C:N (4:1). Carbon limitation is evident during long-term incubations that would otherwise be missed during short-term laboratory incubations (≤ 24 hours).

Introduction

Constructed wetlands provide a potential mitigation tool to reduce nutrient losses from terrestrial habitats to receiving waterways, particularly in agriculturally dominated regions (Kovacic et al. 2000; Woltermade 2000). Man-made agricultural wetlands differ from natural wetlands that receive surface flow because they often have excess nitrate and low bioavailable carbon (Westhorpe and Mitrovic 2012). The availability (ratio and concentrations) of nutrients is an important regulator of bacterial population growth and enzymatic production in terrestrial and aquatic systems (Michaud et al. 2006; Spohn 2015). Understanding how bacteria respond to different nutrient stoichiometries may allow a better understanding of how bacterial denitrification in wetlands respond to watershed practices and carbon amendments (Cross et al. 2005). An increase in denitrification may be due to a short-term increase in enzymatic activity, an increase in bacterial production, or potentially both. Establishing a link between a potential increase in bacterial production in response to favorable stoichiometric conditions may better explain the response of denitrification to C additions.

The availability of dissolved organic carbon (DOC) often limits bacterial growth and activity (Maraun and Scheu 1996). Ecological stoichiometry emphasizes the importance of a balance of biologically important elements (C, N and P) (Sterner and Elser 2002). The relative quantities and forms of DOC impact how each is cycled within aquatic ecosystems, though N is typically in excess throughout agricultural watersheds due to seasonal N applications to row crop fields (Hu et al. 2007). In agricultural wetlands retaining row crop drainage, $\text{NO}_3\text{-N}$ concentrations are high relative to DOC, potentially limiting the energetic demands of heterotrophic bacteria. The sources of DOC

are from allochthonous sources (overland flow and drain tiles) and autochthonous sources (production within the wetland, e.g., macrophyte and algal). Autochthonous DOC is low in concentration but more bioavailable than allochthonous sources (C:N of 1-4:~10 mg/L) (Warner et al. 2009). Overland flow contributes the most DOC and is often in more recalcitrant forms, but NO_3 concentrations are lower than those in drainage tile (Kritzberg, et al. 2004). Drain tile DOC is low in concentration and in less bioavailable forms from slow degradation of lignin and cellulose rich crop residues (Warner et al. 2009). Subsurface drainage also maintains low DOC concentrations relative to NO_3 inputs (C:N of 1:10 mg/L). Maintaining C:N ratios of 2:1 in wetland sediments can significantly increase bacterial denitrification over time, but it is unclear whether denitrification this increase is being driven by an increase in bacterial biomass or denitrification rates (Templer et al. 2003).

The high C demand for cellular growth may further limit C availability to carry out other biotic processes, i.e. respiration (Curiel Yuste et al. 2007). A large proportion of bioavailable C may be required for growth, limiting the amount available to carry out denitrification. In addition to regulating maximal bacterial activity, stoichiometry can also affect the time it takes for bacteria to respond to brief but intense nutrient pulses. If a large nutrient imbalance must be compensated for by denitrifying bacteria, the equilibration time required to assimilate available C would likely delay the occurrence of elevated rates of denitrification, missing the highest concentrations of $\text{NO}_3\text{-N}$ early in the pulse. This would result in minimized reductions of $\text{NO}_3\text{-N}$ during seasonally critical periods. Bacteria within oligotrophic systems elicit a more immediate response to nutrient additions when compared to eutrophic waterways (Del Giorgi and Cole 1998).

Wetlands effectively address nutrient loading in watersheds dominated by different landuse types, however their relative success may be marginalized within agricultural watersheds (Lee et al. 2009). Within aquatic systems, C availability is supplied by allochthonous inputs from riparian zones and surrounding catchments in addition to autochthonous production (Kritzberg et al. 2004). However, the reliance upon allochthonous C can be reduced by increasing wetland size relative to the surrounding watershed (Hammer 1999; van der Valk and Jolly 1992). Increased C inputs may benefit heterotrophic activity, but allochthonous inputs can be in more recalcitrant forms relative to autochthonous sources which minimizes potential benefits. Enlarging wetlands also allows for prolonged water residence time and increased interaction with biotic hotspots of denitrification such as surficial sediment layers and epiphytic biofilms. However, wetlands of adequate sizes are not always feasible due to financial and land owner constraints. Therefore, understanding how the availability of C affects the removal of $\text{NO}_3\text{-N}$ within agricultural wetlands can reduce the land taken out of production.

Additions of labile DOC can lead to an immediate increase in bacterial respiration in soils, and denitrification within anaerobic wetland sediments (Lundquist et al. 1999). To appropriately test for nutrient limitation, bacterial production coupled with respiration (i.e. denitrification) should be accounted for (Alden et al. 2001). Measuring only denitrification (or soil respiration) within wetland sediments is of critical importance, but may underestimate the degree of DOC limitation (Jahnke and Craven 1995). Bacteria have the unique ability to assimilate DOC, rather than particulate fractions, therefore it is of significance to test how bacteria allocate available DOC (Cole and Pace 1995). It has been suggested that continual increases in bacterial production and denitrification in

response to a nutrient addition (> 12 hours) is strongly indicative of limitation, rather than response to a single metric (Scheu 1993).

The importance of DOC to denitrification and bacterial production has been highlighted in waste water treatment studies, but only recently has DOC been implicated as a limiting nutrient N cycling within agricultural headwater streams (Harbot et al. 2005; Rejas et al. 2005; Gudas et al. 2012). Wetlands are effective $\text{NO}_3\text{-N}$ remediation tools, but results suggest that DOC availability within agricultural wetlands is impeding higher rates of $\text{NO}_3\text{-N}$ reduction. The utilization of DOC by heterotrophic bacteria within wetland sediments for production, rather than denitrification at low DOC availability suggests preferential assimilation under limiting DOC conditions. How bacteria allocate available DOC is contingent upon overlying water C:N ratios, providing a potential explanation as to the observed limitation of agricultural wetland denitrification.

The goals of this experiment were (1) assess the effect that C:N has on bacterial production and denitrification rates within wetland sediments that receive surface water and tile drained wetland sediments and (2) to assess where there is an important interaction between each process. By measuring denitrification and bacterial production, it will be possible to observe potential preferential allocation of C and more accurately understand the extent of nutrient limitation in high NO_3 agricultural watersheds.

Methods

Sampling and Design

To test the effect of increased carbon availability on denitrification we used sediments from surface and tile fed wetlands. Sediment was collected from wetlands that retain surface water (3 sites) or drain tile water (3 sites) inputs within the Mackinaw

River watershed, a major tributary of the Illinois River, in central Illinois. C:N treatments consisted of an unamended control, a 1:1 (10 mg C /L :10 mg NO₃-N/L), and 4:1 (40 mg C/L:10 mg NO₃-N/L) ratio. Carbon was added as glucose (C₆H₁₂O₆), and NO₃-N was added as potassium nitrate (KNO₃). The 1:1 treatment is a ratio representative of spring flood pulses where initial pulses of agricultural runoff commonly have elevated DOC from crop residues decomposition. A 4:1 C:N ratio in wetlands and waste treatment plants is optimal for denitrification (Sobieszuk and Szewczyk 2006).

To estimate bacterial production and denitrification, sediment cores (top 2cm of sediment) were randomly collected from each of the tile and surface water wetland sites. Sediments were maintained at 4°C and each site was homogenized in the laboratory to create six sediment slurries. Aliquots of sediment (20g) were placed into a 120 ml media bottle with 100 ml of each nutrient treatment ration and sealed with a butyl septa cap. The overlying solution of each microcosm was siphoned off daily and refilled with the respective nutrient solution and incubated at 25°C in a dark environmental chamber. On days 0, 1, 3, 5, 10, and 20, three microcosms of each treatment from the total of 288 microcosms were destructively sampled to assess denitrification and bacterial production.

Denitrification Assay

To measure potential differences in denitrification rates between C:N treatments, sediments were subjected to the acetylene inhibition technique in triplicate (Chan et al. 1979; Smith et al. 2006). Each replicate received 50 ml of Milli-Q water and amended with 5 ml of a 0.1M solution of chloramphenicol. The headspace of each media bottle was then purged with N₂ gas for 5 minutes to create anaerobic conditions. The incubation began after 15 ml of acetylene (C₂H₂) gas was injected into the headspace and shaken to

incorporate into the soil. The addition of C_2H_2 blocks the conversion of NO_3 to N_2 gas, therefore the end product for this assay is N_2O . A 10 ml headspace gas samples was taken 15 minutes after the initial C_2H_2 injection, and once an hour for the following four hours. Denitrification rates were calculated from the production of N_2O over time per gram of dried soil. Headspace gas samples collected throughout the assay were measured using a Shimadzu GC-2014 gas chromatograph (Porapak Q packed column; Detector temperature 300 °C; oven temperature, 100 °C; flow rate of carrier gas (ultrapure Nitrogen gas) 10 ml/min.).

Bacterial Production Measurement

To estimate bacterial production, a modified anoxic microcentrifugation technique was used to determine incorporation of leucine [H^3] (Alden et al. 2001; Baath et al. 2001; Bastviken and Tranvik 2001). Leucine rather than thymidine was used because incorporation of thymidine is greatly reduced under anaerobic conditions (Bastviken and Tranvik 2001). Wetland sediment, 10g (wet weight) was combined with 30ml of low oxygen nanopure water (bubbled with N_2) in a 200ml glass media bottle with a butyl septa and purged with N_2 for 5 minutes to ensure an anaerobic headspace. Bottles were shaken at 200 rpm in a rotary shaker for 10 minutes and centrifuged in a 50ml centrifuge tube (1000 g for 10 min.). After centrifugation, 1.5 ml of supernatant was pipetted into a Discardit syringe. A 50 μ l injection of leucine solution (9.25 MBq/mol, 58.5 Ci/mmol, Perkin Elmer) was added to each syringe. The leucine solution was 1 μ l of tritiated leucine and 500 μ l of nanopure water, and mixed with unlabeled leucine to create a final concentration of 100nM leucine. The syringe serves as a controlled method to allow leucine to be injected into the test chamber while maintaining anaerobic

conditions. After the addition of leucine to each syringe, a needle was affixed and pushed into a rubber stopper to minimize O₂ exchange. Samples were stabilized in a culture tube rack and placed in a dark environment to incubate for 2 hours. At the conclusion of the incubation, samples were transferred into 2.5ml centrifuge tubes, and 50ul of 100% trichloroacetic acid (TCA) was added to halt further leucine incorporation, and centrifuged at 13000 rpm for 10 min. Three additional rinsing and centrifugation steps were performed as follows, 1.5% TCA, 80% ice cold ethanol, and 1 M NaOH as described in (Baath et al. 2001). After termination of the assay and rinsing steps, samples were transferred into 8 ml scintillation vials with 2 ml of scintillation cocktail (ScintiVerse) and analyzed on a liquid scintillation counter.

Data Analysis

A mixed model analysis of variance was used to test for the effect of nutrient ratio treatments on denitrification over time. To examine the effect of nutrient ratio treatments over time on bacterial production a mixed model analysis of variance was once again performed. For the denitrification and bacterial production ANOVA models the random effect was site (wetlands that sediment was collected from), while the fixed effects were nutrient ratio, day, and wetland type. Tukey post F-tests were used to compare differences in denitrification and bacterial production in response to C:N ratio. To test for a potential relationship between bacterial production and denitrification, a Pearson correlation was used due to the unbalanced design. Assumptions of normality and homogeneity of variances for both analyses were met without the aid of data transformations.

Results

Denitrification

Denitrification rates did not differ between drain tile and surface water sediments, suggesting that both wetland types were similarly limited by C:N treatments ($F_{1,4}=1.28$, $p=0.3216$, Table 3a). Denitrification significantly increased in response to C:N ratio ($F_{2,8}=129.11$, $p<0.0001$), and the increase occurred over time for both the 1:1 and 4:1 treatments ($F_{10,40}=30.51$, $p<0.0001$). Denitrification rates measured from the 1:1 treatment remained similar to the control over the course of the study (Table 3b). The 4:1 ratio did result in a significant increase in denitrification over time, but the increase reached maximal rates by day 10 (Figure 6, Table 3b). Although tile and surface water wetlands did not differ overall in response to C:N ratios, wetlands within each type did produce significantly different denitrification rates ($F_{4,11.5}=6.35$, $p=0.0061$, Table 3b).

Bacterial Production Measurement

Bacterial production within sediments from surface water and tile water wetlands exhibited similar limitation to the availability of C ($F_{1,7.4}=0.01$, $p=0.944$, Table 4a). Unlike denitrification rates, bacterial production increased significantly at the 1:1 and 4:1 treatments ($F_{2,13.4}=80.88$, $p<0.0001$, Table 4b), and this increase occurred over time ($F_{8,395}=12.91$, $p<0.0001$, Table 4b). The 4:1 treatment did maintain elevated bacterial production throughout the incubation relative to the 1:1 ratio, but the two C:N treatments did not significantly differ (Figure 7). These data suggested that bacterial production is strongly limited by DOC, however denitrification does not exhibit the same trend. Bacterial production, like denitrification, differed significantly between wetland sites within each wetland type ($F_{7,9.4}=$, $p=0.0129$, Table 4b) Though denitrification and

bacterial production are strongly correlated, ($p < 0.0001$, Pearson=0.372), each process has different stoichiometric demands.

Discussion

Concentrations of DOC relative to $\text{NO}_3\text{-N}$ have a significant limiting role to both bacterial denitrification and production. In the low DOC treatment, denitrification did not increase over time, yet a significant increase in bacterial production occurred. The preferential allocation of DOC to cellular growth and not energetically costly anaerobic respiration provides insight into the extent of DOC limitation (Koike and Hattori 1975). Accounting for both processes provided a more accurate assessment of the interaction of C and N, and how this interaction impacts bacterial processes regulating N transformation. The success of wetlands as $\text{NO}_3\text{-N}$ removal tools is reliant upon meeting microbial energetic demands to facilitate elevated rates of denitrification (Songliu et al. 2008). If the availability of DOC meets energetic demands with $\text{NO}_3\text{-N}$ saturation, the results suggest that denitrification rates could be significantly increased (Figure 6) within constructed wetlands, improving effectiveness.

The differential response exhibited by bacterial production and denitrification suggested that bacterial growth within wetland sediments may account for a significant proportion of DOC demand, rather than denitrification. Significant reductions in labile DOC have been shown to occur with brief incubations, however this is typically attributed to a short-term spike in activity in the presence of an allochthonous energy source, not a change in bacterial production (Kuzakov et al. 2000). The increase in bacterial production to a low DOC amendment was similar to agricultural headwater streams. Within the surficial layer of stream substrate, DOC was rapidly lost without a

concomitant increase in denitrification, suggesting that low DOC relative to $\text{NO}_3\text{-N}$ promotes assimilation as opposed to increased metabolic activity (Johnson et al. 2012). The availability of DOC is a strong regulator of bacterial enzyme activity within the stream benthos, however, at reduced DOC availability, it appeared to be utilized for growth (Hood et al. 2006; Laudon et al. 2011; Yamashita et al. 2011). There is also a link between bacterial production and particulate organic carbon (POC) within riparian soils suggesting a regulatory role within both terrestrial and aquatic habitats (Sobczak et al. 1998). Presenting a highly labile DOC source like glucose may have favored assimilation for bacterial production, but this still highlights the need for DOC to allow bacterial cellular growth (Judd et al. 2006). The increase in bacterial production also highlights the role that assimilation holds on wetland biogeochemistry and further emphasizes the role that land use maintains (Pagano et al. 2014). The coupled effects of minimal allochthonous DOC and aquatic bacterial demand is limiting the allocation of DOC for dissimilatory processes responsible for $\text{NO}_3\text{-N}$ reduction.

Denitrification rates within wetland sediments significantly increased when C:N ratios were greater than 1:1 (Figure 6). Similar to the terrestrial soils that comprise the catchment area of the wetlands, microbial C:N within the wetland sediments are high, which further explains the need for elevated DOC to carry out high rates of anaerobic respiration, in addition to demand for cellular maintenance (Chrzanowski et al. 1996). This is counter to a study within agricultural soils where the addition of $\text{NO}_3\text{-N}$ (500mg $\text{NO}_3\text{-N/g}$ dry soil) was 100 fold higher than DOC (500 mg glucose-C/kg dry soil) but still yielded a significant increase in denitrification (Henderson et al. 2010). This may be due to a more bioavailable pool of DOC relative to the more recalcitrant forms common

within aquatic sediments (Catalan et al. 2013). Some bacteria within wetland sediments are capable of producing extracellular enzymes to reduce humic substances into more labile fractions, but low DOC concentrations are likely limiting the ability of heterotrophs to produce these enzymes, further constraining DOC availability (Van Trump et al. 2011). It may be possible for bacteria to use more recalcitrant forms of DOC under limiting conditions, potentially explaining why bacterial production increased over time within the control (Figure 7). If further degradation of recalcitrant DOC allows for a new source of bioavailable DOC, it did not appear to be in a concentration high enough to increase denitrification in the control or 1:1 ratio (Figure 7). Anaerobic respiration is far more energetically costly than aerobic respiration, this coupled with non-limiting concentrations of electron acceptors (NO_3^-), may explain why recalcitrant autochthonous DOC and low allochthonous inputs are rate limiting factors for denitrification.

Bacterial production and denitrification continually increased throughout the time course of the experiment, suggesting bacterial activity does not approach an asymptote quickly. Nutrient additions can yield a minimal bacterial response under eutrophic conditions as observed in the gradual increase in denitrification at the high C:N treatment (Sondergaard and Middelboe 1995; Kamjunke et al. 1997). This decreased response to nutrient additions is often attributed to the added nutrients becoming diluted by high background concentrations, or when in the field, intense competition with dense macrophyte stands and algae typical of eutrophic ecosystems. Therefore, brief pulses of elevated DOC typical of tile drained systems may result in a minimal $\text{NO}_3\text{-N}$ reduction.. Management to restore soil organic matter within row crop fields may serve as a potential

mechanism to improve DOC within tile drainage, though management of this type does not offer an immediate solution and restoration of DOC can be measured on a decadal time scale (McLaughlan et al. 2006). Recalcitrant forms increase more rapidly than more bioavailable fractions in agricultural fields when converted from row crops to perennial grasses, but the experimental fields were a new sink of C, likely with minimal C export.

The extent to which wetland sediments are limited by DOC was demonstrated by the positive relationship between DOC, denitrification, and bacterial production (Figs. 1 and 2). Assessing only bacterial production may have underestimated DOC limitation. The differential limitation offered a more complete explanation for the rapid DOC assimilation common in agricultural watersheds (Johnson et al. 2012). Both bacterial processes were positively correlated, but significant increases in both metrics were only observed at the 4:1 C:N ratio. The results suggested that an increase in bacterial cells do not directly lead to an increase in denitrification, and N cycling may be more reliant upon enzyme production and activity (Suberkropp et al. 2010). If an increase in bacterial cells within a sediment aliquot fostered elevated denitrification, a difference between the control and 1:1 C:N would have been more discernable with respect to denitrification (Yamada et al. 2012). Measuring bacterial production allowed us to better understand how the microbial loop within constructed wetland sediments assimilated bioavailable DOC, potentially limiting other bacterial processes. The rapid assimilation of DOC likely limits available energy for other extracellular enzyme production, beyond those used for denitrification. Some heterotrophic bacterial enzymes are capable of reducing humic acids into more bioavailable fractions, potentially limiting bacterial access to another potential pool of autochthonous DOC (Van Trump et al. 2011).

Agricultural watersheds further prove to be dynamic systems with respect to their nutritive demand when compared to other studies. Single nutrient additions, namely N, to agricultural streams suggests that watersheds are capable of becoming saturated by excess N and at times P as observed by low uptake rates across a stream reach. Additions of DOC (acetate) coupled with $\text{NO}_3\text{-N}$ to Midwestern agricultural headwater streams highlight the immediate uptake of DOC within the top 2cm of sediment (Inwood et al. 2007; Johnson et al. 2012). The rapid reduction of DOC did not support a commensurate increase in denitrification, likely because heterotrophic bacteria assimilated the amended DOC due to extreme limiting conditions. Within a laboratory setting, the interactive effects of nutrients within $\text{NO}_3\text{-N}$ saturated wetlands. Results of the LINX II project, coupled with reach scale additions throughout headwater streams would suggest that a large proportion of N reduction (and DOC) could be accounted for by autotrophic assimilation. It now appears bacterial production indirectly affects the reduction of $\text{NO}_3\text{-N}$ dependent upon the availability of DOC.

Emphasis must be placed on the important role that excess $\text{NO}_3\text{-N}$ holds on the demand for DOC, and over-application of $\text{NO}_3\text{-N}$ not only impacts bacterial use of the autochthonous pool of DOC, but affects the bioavailability of DOC throughout a wetlands catchment. Denitrification is reliant upon two primary conditions, anaerobic sediments and a readily available energy source (Burgin et al. 2010). Wetlands develop anaerobic conditions quickly, and have daily fluxes of sediment oxygen under periods of prolonged inundation. The accumulation of highly recalcitrant humic substances can dominate the available DOC pool throughout the benthic environment of wetlands. This is largely attributed to the inability for fungal degradation of lignin and cellulose that are

the dominant fraction of C within macrophytes (McLatchey and Reddy 1998; Shilla et al. 2006). To put further pressures on labile DOC, allochthonous DOC (and POC) are typically more recalcitrant relative to autochthonous pools. This is especially true within agricultural systems as the more bioavailable fractions are utilized by terrestrial heterotrophic activity prior to leaching into receiving wetlands. The recalcitrance and quantity of DOC relative to $\text{NO}_3\text{-N}$ interact to limit the activity of heterotrophic bacteria, limiting the success of constructed wetlands as nutrient remediation tools throughout agroecosystems. Increasing the availability of labile DOC will likely prove to be a difficult task within an intensely farmed watershed, but warrants further investigation as a potential mechanism of improving downstream ecosystem health and reducing financial burdens on local municipality's water treatment.

References

- Alden, L., Demoling, F., and Baath, E. 2001. Rapid method of determining factors limiting bacterial growth in soil. *Appl. Environ. Microbiol.* 67:1830-1838. doi:
- Baath, E., Pattersson, M., and Soderberg, K.H. Adaptation of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. *Soil Biol. Biochem.* 33: 1571-1574.
- Bastviken, D., and Tranvik, L. 2001. The leucine incorporation method to estimate bacterial growth equally well in both oxic and anoxic waters. *Appl. Environ. Microb.* 67: 2916-2921.
- Burgin, A.J., Groffman, P.M., and Lewis, D.N. 2010. Factors regulating denitrification in a riparian wetland. *Soil Sci. Soc. Am. J.* 74: 1826-1833.
- Catalan, N., Obrador, B., Felip, M., and Pretus, J.L. 2013. Higher reactivity of allochthonous vs autochthonous DOC sources in a shallow lake.
- Chan, Y., and Knowles, R (1979) Measurement of denitrification in two freshwater sediments by an in situ acetylene method. *Appl and Environ Microbiol* 37:1067-1072.
- Chrzanowski, T.H., Kyle, M. Elser, J.J., and Sterner, R.W. 1996. Element ratios and growth dynamics of bacteria in an oligotrophic Canadian shield like. *Aquat. Microb. Ecol.* 11: 119-125.
- Cole, J.J., and Pace, M.L. 1995. Bacterial production in oxic and anoxic freshwaters. *Limnol. Oceanogr.* 40: 1019-1027.
- Cross, W.F., Benstead, J.P., Frost, P.C., and Thomas, S.A. 2005. Ecological stoichiometry in freshwater benthic systems: recent progress and perspectives. *Freshwater Biol.* 50: 1895-1912.
- Curiel Yuste, J., Baldocchi, D.D., Gershenson, A., Goldstein, A., Mission, L., and Wong, S. 2007. Microbial soil respiration and its dependency on carbon inputs, soil temperature and moisture. *Glob. Change Biol.* 13: 1-18.
- del Giorgio, P.A., and Cole, J.J. 1998. Bacterial growth efficiency in natural aquatic systems. *Annu. Rev. Eco. Syst.* 29: 503-541.
- Hammer, D.A. 1992. Designing constructed wetland systems to treat agricultural nonpoint source pollution. *Eco. Engin.* 1: 49-82.
- Hood, E., Gooseff, M.N., Johnson, S.L. 2006. Changes in the character of stream water dissolved organic carbon during flushing in three small watersheds, Oregon.

- Hu, X., McIsaac, G.F., David, M.B., Louwers, C.A.L. 2007. Modeling riverine nitrate export from an east-central Illinois watershed using SWAT. *J. Environ. Qual.* 36:996-1005.
- Inwood, S.E., Tank, J.L., and Bernot, M.J. 2005. Patterns of denitrification associated with land use in 9 midwestern headwater streams. *J. Am. Benthol. Soc.* 24:227-245.
- Johnson, L.T., Royer, T.V., Edgerton, J.M., and Leff, L.G. 2012. Manipulation of the dissolved organic pool in an agricultural stream: Responses in microbial community structure, denitrification, and assimilatory nitrogen uptake. *Ecosystems* 15: 1027-1038.
- Kamjunke, N., Boing, W., and Voigt, H. 1997. Bacterial and primary production under hypertrophic conditions. *Aquat. Microb. Ecol.* 13: 29-35.
- Kovacic, D.A., David, M.B., Gentry, L.E., Starks, K.M., and Cooke, R.A. 2000. Effectiveness
- Kritzberg, E.S., Cole, J.J., Pace, M.L., Graneli, W., and Bade, D.L. 2004. Autochthonous versus allochthonous carbon sources of bacterial results from whole-lake ¹³C addition experiments. *Limnol. Oceanogr.* 49: 588-596.
- Kuzyakov, Y., Friedel, J.K., and Stahr, K. 2000. Review of mechanisms and quantification of priming effects. *Soil Biol. Biochem.* 32: 1485-1498.
- Laudon, H., Berggren, M., Agren, A., Buffam, I., Bishop, K., Grabs, T., Jansson, M., and Kohler, S. 2011. Patterns and dynamics of dissolved organic carbon (DOC) in boreal streams: The role of processes, connectivity, and scaling. *Ecosystems*. 14: 880-893.
- Lee, C., Fletcher, T.D., and Sun, G. 2009. Nitrogen removal in constructed wetland systems. *Eng. Life. Sci.* 9: 11-22.
- Maraun, M., and Scheu, S. 1996. Changes in microbial biomass, respiration, and nutrient status of beech (*Fagus sylvatica*) leaf litter processed by millipedes (*Glomeris marginata*). *Soil Biol. Biochem.* 28: 569-577.
- McLaughlan, K.K., Hobbie, S.E., and Post, W.M. 2006. Conversion from agriculture to grassland builds soil organic matter on decadal time scales. *Ecol. Appl.* 16: 143-153.
- Sondergaard, M., and Middelboe, M. 1995. A cross-system analysis of labile dissolved organic carbon. *Mar. Ecol. Prog. Ser.* 118: 283-294.

- Smith, L.K., Voytek, M.A., Bohlke, J.K., and Harvey, J.W. 2006. Denitrification in nitrate-rich streams: Application of N₂:Ar and ¹⁵N-Tracer methods in intact cores. *Ecol. Appl.* 16: 2191-2207.
- Songliu, L, Hongying, H, Yingxue, S, and Jia, Y (2009) Effect of carbon on the denitrification in constructed wetlands. *J Environ Sci* 21:1036-1043.
- Spohn, M. 2015. Microbial respiration per unit microbial biomass depends on litter layer carbon-to-nitrogen ratio. *Biogeosciences*. 12: 817-823.
- Sterner, R.W., J.J. Elser. 2002. *Ecological Stoichiometry: The Biology of Elements from Molecules to Biosphere*. Princeton University Press. Princeton, N.J.
- Templer, P., Findlay, S., and Lovett, G. 2003. Soil microbial biomass and nitrogen transformations among five tree species of the Catskill Mountains, New York, USA. *Soil Biol. Biochem.* 35: 607-613.
- van der Valk, A.G., and Jolly, R.W. 1992. Recommendations for research to develop guidelines for the use of wetlands to control rural nonpoint source pollution. *Eco Engin.* 1: 115-134.
- Van Trump, J.I., Wrighton, K.C., Thrash, J.C., Weber, K.A., Andersen, G.L., and Coates, J.D. 2011. Humic acid-oxidizing, nitrate reducing bacteria in agricultural soils. *ASM.* 2: 1-10.
- Yamada, N., Fukuda, H., Ogawa, H., Saito, H., Suzumura, M. 2012. Heterotrophic bacterial production and extracellular enzymatic activity in sinking particulate organic matter in the western North Pacific Ocean. *Front. Microbio.* 3: 1-13.
- Yamashita, Y., Kloeppel, B.D., Knoepp, J., Zausen, G.L., and Jaffe, R. 2011. Effects of watershed history in dissolved organic matter characteristics in headwater streams. *Ecosystems*. 14: 1110-1122.
- Jahnke, R.A., and Craven, D.B. 1995. Quantifying the role of heterotrophic bacteria in the carbon cycle: A need for respiration rate measurements. *Limnol. Oceanogr.* 40: 436-441.
- Judd, K.E., Crump, B.C., and Kling, G.W. 2006. Variation in dissolved organic matter controls bacterial production and community composition. *Ecology*. 87: 2068-2079.
- Koike, I., and Hattori, A. 1975. Growth yield of a denitrifying bacterium, *Pseudomonas denitrificans*, under aerobic and denitrifying conditions. *J. Gen. Microbiol.* 88: 1-10.

- Lundquist, E.J., Scow, K.M., Jackson, L.E., Uesugi, S.L., and Johnson, C.R. 1999. Rapid response of soil microbial communities from conventional, low input, and organic farming systems to a wet/dry cycle. *Soil Biol. Biochem.* 31: 1661-1675.
- McLatchey, G.P., and Reddy, K.R. 1998. Regulation of organic matter decomposition and nutrient release in a wetland soil. *J. Environ. Qual.* 27: 1268-1274.
- Michaud, L., Blancheton, J.P., Bruni, V., and Piedrahita, R. 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. *Aquacult. Eng.* 34: 224-233.
- Pagano, T., Bida, M., and Kenny, J.E. 2014. Trends in levels of allochthonous dissolved organic carbon in natural water: A review of potential mechanisms under a changing climate. *Water.* 6: 2862-2897.
- Shilla, D., Asaeda, T., Fujino, T., and Sanderson, B. 2006. Decomposition of dominant submerged macrophytes: implications for nutrient release in Myall, NSW, Australia. *Wetl. Ecol. Manag.* 14: 427-433.
- Sobczak, W.V., Hedin, L.O., and Klug, M.J. 1998. Relationship between bacterial productivity and organic carbon at a soil-stream interface. *Hydrobiologia.* 386: 45-53.
- Sobieszuk, P., and Szewczyk, K.W. 2006. Estimation of (C/N) ratio for microbial denitrification. *Environ. Technol.* 27: 103-108.
- Suberkropp, K., Gulis, V., Rosemond, A.D., and Benstead, J.P. Ecosystem and physiological scales of microbial responses to nutrients in a detritus-based stream: Results of a 5-year continuous enrichment. *Limnol. Oceanogr.* 55: 149-160.
- Warner, T.J., Royer, T.V., Tank, J.L., Griffiths, N.A., Rosi-Marshall, E.J., and Whiles, M.R. 2009. Dissolved organic carbon in streams from artificially drained and intensively farmed watersheds in Indiana, USA. *Biogeochemistry.* 95: 295-307.
- Westhorpe, D.P., and Mitrovic, S.M. 2012. Dissolved organic carbon mobilization in relation to variable discharges and environmental flows in a highly regulated lowland river. *Mar. Freshwater Res.* 63: 1218-1230.
- Woltermade, C.J. 2000. Ability of restored wetlands to reduce nitrogen and phosphorus concentrations in agricultural drainage water. *J. Soil Water Conserv.* 55: 303-309.

Table 3

- a.)** *Results of the mixed model ANOVA showing there is no difference in denitrification rates to C:N ratios when tile and surface water wetlands are compared.*
- b.)** *Results of the mixed model ANOVA looking at the fixed effect of time, and random effects of water source, and nutrient ratios on denitrification between surface and tile water wetlands. Numbers in bold are significant at $\alpha=0.05$.*

a.)

Source	df	F	P
Wetland Type	1	1.28	0.3216
Error	4		

b.)

Source	df	F	P
Site(Wetland Type)	4	6.35	0.0061
Treatment	2	129.11	< 0.0001
Day	5	29.17	< 0.0001
Day x Wetland Type	5	1.27	0.3137
Treatment x Wetland Type	2	0.69	0.5303
Day x Treatment	10	30.51	< 0.0001
Day x Treatment x Water	10	0.81	0.6267
Site x Day(Wetland Type)	20	1.82	0.0531
Site x Day x Treatment(Wetland Type)	40	1.20	0.2015

Table 4

- a.)** *Results of the two-way mixed model ANOVA showing there is no difference in bacterial production to stoichiometric conditions when tile and surface water wetlands are compared.*
- b.)** *Results of the two-way mixed model ANOVA looking at the fixed effect of time, and random effects of water source, and nutrient ratios on bacterial production between surface and tile water wetlands. Numbers in bold are significant at $\alpha=0.05$.*

a.)

Source	df	F	P
Wetland Type	1	0.01	0.9444
Error	7.4		

b.)

Source	df	F	P
Site(Wetland Type)	7	5.01	< 0.0129
Treatment	2	80.88	< 0.0001
Day	5	89.93	< 0.0001
Day x Wetland Type	4	1.27	0.8351
Treatment x Wetland Type	2	5.56	0.0124
Day x Treatment	8	12.91	< 0.0001
Day x Treatment x Water	6	0.76	0.6109
Site x Day(Wetland Type)	16	1.66	0.1086
Site x Day x Treatment(Wetland Type)	28	0.76	0.8062
Error	395

Figure 6. Rates of denitrification between tile drained and surface water wetlands over time. Day 0 rates of denitrification were subtracted from subsequent days to better show changes in denitrification to nutrient amendments. Negative rates of denitrification represent a decrease in activity from Day 0. Treatments presented as follows: control (▲), 1:1 (■), and 4:1 (●).

Figure 7. Changes in bacterial production throughout an extended incubation period between tile drained and surface water wetland sediments. Treatments presented as follows: control (▲), 1:1 (■), and 4:1 (●).

CHAPTER III

ROLE OF ALLOCHTHONOUS DISSOLVED ORGANIC CARBON IN

AGRICULTURAL WETLAND NITRATE REMOVAL

Abstract

The conversion of the historical prairie and wetland landscapes to agricultural production has led to a decrease in soil organic carbon (C). The modified hydrology and application of nitrogenous fertilizers throughout agroecosystems have altered watershed biogeochemistry, leading to seasonally intense N exports. Wetlands have been created to retain subsurface agricultural drainage, but low DOC inputs are limiting denitrification rates and subsequent N removal. We tested the effect of increased DOC (acetate, $C_2H_3O_2$) concentrations (low: 1 mg/L and high: 10 mg/L) on denitrification and N removal. Treatments were applied in triplicate ($n=3$) by redirecting tile flow through a pipe network to mesocosms installed within the wetland. Additions of DOC significantly reduced concentrations of NO_3-N within overlying water ($p<0.0001$). The 10 mg/L DOC treatment resulted in significantly greater NO_3-N removal than the 1 mg/L treatment. Porewater samples maintained low NO_3-N concentrations irrespective of DOC treatment or sediment depth, but elevated DOC availability did reduce porewater NO_3-N concentrations significantly ($p=0.02$). Both DOC treatments significantly increased denitrification rates ($p<0.0001$). Denitrification rates were significantly higher at the 10 mg/L treatment relative to the 1 mg/L addition, however the increase in denitrification

was not commensurate to the difference in DOC concentration. The low and high DOC additions resulted in a ~10% and ~20% reduction in $\text{NO}_3\text{-N}$ reduction within the abbreviated residence time (45min) of each mesocosm. The results suggested that a minimal increase in allochthonous DOC into constructed agricultural wetlands could result in a considerable increase in $\text{NO}_3\text{-N}$ removal.

Introduction

Throughout agroecosystems, man-made wetlands are installed to address $\text{NO}_3\text{-N}$ loading, but considerable variation in removal rates can exist between wetland sites (Peterson 1998; Fink and Mitsch 2004; Beutel et al. 2009). Stoichiometric limitations can inhibit denitrification, leading to reduced $\text{NO}_3\text{-N}$ removal rates (LaMontagne et al. 2002). Wetlands have garnered attention as a solution to $\text{NO}_3\text{-N}$ loading, but reduced wetland sizes limit autotrophic dissolved organic carbon (DOC), and are therefore more reliant upon allochthonous DOC (Hopkinson and Smith 2004). Land use within a watershed dictates the relative amounts and forms of DOC delivered to receiving waterways (Neff and Asner 2001). The conversion of wetlands to row crop agriculture has dramatically reduced soil carbon and the subsequent leaching of DOC from agricultural fields (Mulholland 2003). The resulting imbalance of C:N within the aqueous environment impacts biogeochemical processes and $\text{NO}_3\text{-N}$ reduction (Brookshire et al. 2005).

Autochthonous DOC is often in limiting quantities, and allochthonous DOC becomes increasingly important as it can be effectively utilized to supplement heterotrophic demands (Tranvik 1998). Primary producers are an important contributor of low molecular weight autochthonous DOC, however this limited bioavailable pool is

rapidly assimilated (Bertilsson and Jones 2003). Within aquatic ecosystems, bacteria will preferentially assimilate autochthonous DOC (Kritzberg et al. 2004). Emergent and submergent vegetation communities can foster elevated heterotrophic microbial activity dependent upon the plant community composition, however the plant cell wall structure is difficult to degrade and this results in an accumulation of recalcitrant humic substances (Kellogg and Bridgham 2002; Ziegler and Fogel 2003). The continuous interaction of elevated NO_3 loads within the benthic environment of agricultural wetlands and streams presents heterotrophic bacteria with a non-limiting supply of electron acceptors under reducing conditions. The limited supply of electron donors, i.e. DOC, and predominantly recalcitrant fractions are likely limiting bacterial activity, and reduction of NO_3 . The small size of constructed agricultural wetlands relative to drainage area may be increasing the importance of allochthonous DOC inputs to meet bacterial demand.

Allochthonous DOC inputs are minimized throughout agroecosystems due to elevated soil N coupled with corn-soy bean residue management interact to decrease the pool of terrestrial DOC and a commensurate decrease in DOC entering streams (Pinney et al. 2000). The dominant proportion of DOC within Midwestern agricultural watersheds is derived from corn and soybean residues which are comprised of stalks, roots, leaves, and cobs. The high lignin, cellulose, and hemicellulose content of corn residues makes it difficult to breakdown even when incorporated into soil through light tillage (Tarkalson et al. 2008). The highly labile fractions of DOC (i.e. sugars and amino acids) that rapidly leach from corn and soybean leaves and are subsequently incorporated into soil microbes within days (Machefert and Dise, 2004; Griffiths et al. 2009). The rapid assimilation of labile fractions minimizes amount of DOC can be lost in tile

drainage when prolonged soil saturation occurs. There are specific fractions of DOC that are more susceptible to loss during periods of high soil moisture, commonly referred to as water extractable organic carbon (WEOC) (Xu et al. 2013). WEOC is characterized as the fraction of DOC present that is easily transferred into an aqueous environment and consists of a largely bioavailable and mobile portion of DOC (Zsolnay 1996). WEOC can foster elevated heterotrophic soil respiration in terrestrial systems, and improve biogeochemistry of agricultural watersheds if it is readily leached from row crop fields (Jandl and Sollins 1997).

An increase in terrestrial DOC is possible, but restoring lost soil organic matter (SOM) is a timely process, yet possible with the implementation of cover crops into a traditional crop rotation. After maturing over the fall and winter, cover crops are not harvested and the litter serves as a mechanism to restore SOM lost due to intensive row crop production (Dabney et al. 2001). Cover crops are planted post-harvest with the primary importance to retain applied nutrients over winter, but have the added benefits of reduced soil erosion, and restored soil health (Doran and Smith 1991; Battany and Grismer 2000; Dean and Weil 2009). Commonly used cover crops, such as cereal rye and tillage radish, decompose more readily than corn and soybeans, and contribute additional residues, eventually becoming part of the soil humus (Steenwerth and Belina 2008; Olson et al. 2010; Ketterings et al. 2011; USDA 2011; Peregrina et al. 2012). Allochthonous inputs often serve as an effective DOC source with the aid of fungal breakdown of recalcitrant vegetative molecules (lignin and cellulose) into more bioavailable fractions (Eriksson 1984). In some instances, DOC has not increased with cover crop implementation, but this is likely attributed to soil leaching (Stutter et al.

2007). Increased DOC, specifically WEOC, entering subsurface drainage may serve as a critical allochthonous input into mitigation wetlands. Leaf litter decay and associated pulses of nutrients can foster increased heterotrophic activity in forested headwater streams (Hood et al. 2006). Similar seasonal fluxes of nutrients post-harvest throughout agroecosystems, but due to $\text{NO}_3\text{-N}$ inputs, the energetic demands of bacteria exceed what current agricultural practices make available.

If cover crops within a corn/soybean rotation increase soil DOC over time, then continual fall cover crop planting may foster a commensurate increase of DOC within subsurface drainage. Elevated DOC inputs would likely increase $\text{NO}_3\text{-N}$ reduction from receiving wetlands since bacterial activity can rapidly increase in the presence of a newly available energy source. Additions of labile DOC have increased microbial respiration within eutrophic headwater streams, and would likely produce similar results in wetlands throughout Midwestern agroecosystems (Coleman et al. 2004; Harbott et al. 2005; Arango et al. 2007). Controlled laboratory incubations and waste water studies emphasize the importance of DOC availability on the occurrence of denitrification (Cherchi et al. 2009). Manipulating the C:N stoichiometry of mitigation wetlands, by applying treatments from successful laboratory studies, provides a mechanism to test how much additional DOC is necessary to adequately improve $\text{NO}_3\text{-N}$ removal. The importance of DOC availability is greatly diminished in low $\text{NO}_3\text{-N}$ systems, but $\text{NO}_3\text{-N}$ concentrations throughout the study region routinely exceed 10 mg/L (Martin et al. 2001; Arango et al. 2007).

The goals of this project were to: (1) examine how denitrification changes in response to the availability of DOC in low (1 mg/L) and high (10 mg/L) concentrations;

(2) observe how the $\text{NO}_3\text{-N}$ concentrations in the overlying water and in pore water across the upper sediment profile (0-5 and 5-10 cm depths) change in response to DOC amendments; (3) account for potential changes in denitrification through shifts in microbial communities throughout a simulated flood pulse.

Methods

Field and Laboratory Procedures

The site used for this study was a constructed wetland (1 acre) that drains agricultural fields under a corn/soy bean rotation. This wetland is located within an experimental subwatershed of the Mackinaw River, central IL, USA, that has been part of an ongoing study investigating the potential benefits of conservation agricultural practices within an intensely farmed and tile-drained watershed (Lemke et al. 2011). The site consists of three separate wetlands that are connected by a single 10 inch drain tile that passes through an earthen berm. The wetland complex was constructed within land that was previously used as a grass waterway. At the time of the study, this wetland complex has been in operation for 12 years.

To simulate tile inputs of DOC, a section of 7.6 cm diameter drain tile was affixed to the main tile (30.5 cm diameter) that delivers subsurface water to the inflow wetland (Figure 8). The 7.6 cm tile was affixed directly into the flow of tile water, and split with three inch drain tile wyes to redirect tile water to 9 different mesocosms installed within the wetland. Mesocosms were constructed from 208 liter rain barrels that had the lids and bottoms removed. This allowed for the barrels to be depressed ~20cm into the wetland sediment and provided access once installed to collect sediment and water samples over the course of the study. A 12 cm diameter hole was cut along the upper rim

to allow one of the branches of the tile network to be placed and maintain a steady flow of tile water and DOC addition. Allowing tile flow to serve as the conduit of nutrient delivery avoided daily pulses of nutrients common to laboratory microcosm studies.

A total of nine mesocosms were installed, and DOC treatments were injected in triplicate ($n=3$) as unamended tile water (control), low (1 mg/L), and high (10 mg/L) DOC treatments. Mesocosms were placed in areas of similar water depth and depressed into the wetland sediment to ensure each mesocosm had the same volume of water ($\sim 0.1\text{m}^3$) throughout the study. To provide DOC, acetate ($\text{C}_2\text{H}_3\text{O}_2$) was dissolved in tile water daily and stored in 208 liter rain barrels. A separate barrel was used for each DOC treatment, low (1 mg/L) and high (10 mg/L), where the low treatment stock barrel was 0.005M and the high treatment stock barrel was a 0.1M solution of acetate. The required injection rates of the concentrated acetate solutions were maintained by using a separate peristaltic pump for each of the two stock barrels. A single section of tygon tubing was placed into the pump and split three ways to inject acetate into separate mesocosms ($n=3$ per treatment). The control mesocosms did not receive any injection, only unamended tile water. By adjusting the placement of the main tile intercepting the inflow water it was possible to maintain $\sim 200\text{ml/sec}$ of flow into each mesocosm.

The acetate injection was carried out for two separate five day replicates to simulate a pulse duration similar to that of a moderate rain event. After the first five day injection was completed, the mesocosms were shifted 10 m further into the wetland and the next injection was initiated. Water samples were collected once a day over the course of the study from the overlying water and interstitial waters within the top 5cm and 10-15cm of wetland sediment through the installation of piezometers. A total of 6 overlying

water samples and 12 (6 shallow and 6 deep) interstitial water samples were collected from each mesocosm during an injection period. Concentrations of $\text{NO}_3\text{-N}$ were analyzed with a Dionex ICS-1100 Ion Chromatograph.

Sediment samples were collected prior to beginning the injection of acetate to establish a baseline rate of denitrification. After the injection began, sediments were collected on days 1 and 5 to observe potential changes in denitrification over time in response to DOC availability. Two sediment cores from the top 2cm in each mesocosm were collected on days 0, 1, and 5. Upon collection they were immediately placed on ice, and cores collected from the same mesocosm were homogenized upon getting samples to the laboratory. On the day of collection, 20g of homogenized wetland sediments were allocated in triplicate to 120ml glass media bottles and sealed with a cap containing a butyl septa. Sediments were assayed within the media bottles that following day via the acetylene inhibition technique (Chan et al. 1979; Smith et al. 2006). Headspace gas samples collected throughout the assay were measured using a Shimadzu GC-2014 gas chromatograph (Porapak Q packed column; Detector temperature 300 °C; oven temperature, 100 °C; flow rate of carrier gas (ultrapure Nitrogen gas) 10 ml/min.).

Data Analysis

A fixed-effects ANOVA was used to compare the effects of DOC concentrations on denitrification potential over time. We performed planned contrasts to test for differences in denitrification within and between DOC treatments over time. Separated fixed-effects ANOVAs were also used to test for the effect of DOC availability on the concentrations of $\text{NO}_3\text{-N}$ within the overlying and interstitial water in each mesocosm. Within the overlying water, we tested for differences in $\text{NO}_3\text{-N}$ concentrations as effected

by DOC concentration over time. With the interstitial water samples, we tested for differences in $\text{NO}_3\text{-N}$ concentration between DOC treatments over time, in addition to differences between the shallow (0-5 cm) and deep (5-10 cm) samples.

Results

$\text{NO}_3\text{-N}$ Reduction

Concentrations of $\text{NO}_3\text{-N}$ were reduced within each mesocosm that received amendments of DOC ($F_{2,107}=8.72$, $p=0.0003$). There was significant variation in $\text{NO}_3\text{-N}$ concentration over time ($F_{5,107}=3.65$, $p=0.0047$), however the interaction of DOC concentration and time was not significant ($F_{10,107}=0.57$, $p=0.8353$). Without a significant interaction, we averaged $\text{NO}_3\text{-N}$ concentration by day and found that daily flux in $\text{NO}_3\text{-N}$ concentration likely accounted for the change in concentration over time, but DOC additions (Table 5). Though concentrations of $\text{NO}_3\text{-N}$ exhibited significant variation over time, $\text{NO}_3\text{-N}$ concentrations were further reduced as a function of DOC availability (Figure 8). The 1 mg/L of DOC injection did not result in a significant decrease in $\text{NO}_3\text{-N}$ relative to unamended tile water. Mesocosms that received the 10 mg/L DOC injection did reduce $\text{NO}_3\text{-N}$ concentrations significantly relative to unamended water (Figure 8, Table 5).

Significant changes in $\text{NO}_3\text{-N}$ concentration were only observed within the overlying water samples. Concentration of $\text{NO}_3\text{-N}$ measured in pore water samples taken from the 0-5cm and 5-10cm profiles did not differ ($F_{1,35}=1.14$, $p=0.2941$). Since $\text{NO}_3\text{-N}$ concentrations did not differ between sediment depths over time ($F_{2,35}=0.62$, $p=0.5561$), we averaged the $\text{NO}_3\text{-N}$ concentrations from the two sediment profiles within each mesocosm. All treatments maintained low interstitial $\text{NO}_3\text{-N}$ concentrations (~ 1 mg/L)

throughout the course of each nutrient injection, however the high DOC injection resulted in significantly lower $\text{NO}_3\text{-N}$ on average relative to the control (Figure 9).

Denitrification

The addition of DOC to tile drained wetland sediments resulted in a significant increase in denitrification for both the low (1 mg/L) and high (10 mg/L) treatments relative to unamended tile water ($F_{2,53}=38.42$, $p<0.0001$, Figure 8). The high treatment did produce significantly higher rates relative to the low addition, however the difference was not minimal relative to the 10 fold difference in DOC concentrations between the two treatments (Figure 10). A significant interaction exists between DOC treatment and time, showing that a stable amendment of DOC promotes a continual increase in denitrification ($F_{4,53}=2.79$, $p=0.0374$). Maximal rates of denitrification were reached by day 1 under both DOC amendments (Figure 11).

Planned contrasts were used to further examine variation in denitrification in response to differing DOC concentrations over time, and the corrected test statistic for the 15 comparisons was $p=0.003$. For the control, denitrification did not change throughout the study ($p=0.4542$). Rates of denitrification for days 1 and 5 were not significantly different from day 0 at the low DOC treatment ($p=0.0045$), but the high DOC treatment did lead to a significant increase when looking at days post DOC addition ($p<0.0001$). Irrespective to DOC addition, there was an immediate positive response by denitrification, and rates within each treatment did not significantly increase from day 1 to day 5 (low: $p=0.2769$, high: $p=0.1499$). Testing differences in denitrification between treatments on each day found that rates of denitrification were similar in each mesocosm prior to the beginning the DOC injection. Denitrification rates were significantly greater

in the low treatment relative to the control on day 1 and 5 ($p=0.0007$ and $p<0.001$).

Denitrification was also significantly greater within the high DOC mesocosm relative to the control on days 1 and 5 ($p<0.0001$ and $p<0.0001$). The low and high DOC additions resulted in similar rates of denitrification on days 1 and 5 ($p=0.0380$ and $p=0.0155$).

Discussion

A minimal addition of DOC to agricultural tile drainage has the potential to improve $\text{NO}_3\text{-N}$ reduction at a watershed scale. Maintaining 1 mg/L of DOC within the overlying water significantly increased denitrification, but the increase in denitrification did not translate into significant reductions in $\text{NO}_3\text{-N}$ concentration (Figure 8). This result was likely due to the water residence time within each mesocosm (Woltermade 2000). At an inflow rate of 200 ml/sec, the total mesocosm volume was replaced within 45 minutes, limiting the amount of time water interacts with sediments. Even with limited sediment contact, the high DOC addition still had a 20% reduction in $\text{NO}_3\text{-N}$ while the low DOC addition reduced $\text{NO}_3\text{-N}$ by 11.1% (Figure 8). The mesocosm water residence time was brief relative to that of the wetland as a whole, therefore the observed reductions are likely an underestimate of wetland performance. Wetland ecosystems have greater N retention relative to that of lakes and especially rivers due to the stagnation of water movement and improved interaction of N with the benthic environment (Saunders and Kalff 2001). A reduced inflow rate would have created a water residence time more representative of the wetland as a whole, and resulted in $\text{NO}_3\text{-N}$ reductions that correlate with changes in denitrification.

Similar to the overlying water, pore water $\text{NO}_3\text{-N}$ concentrations exhibited no differences between treatments, even though denitrification rates were significantly

higher within mesocosms receiving DOC (Table 6). It is evident $\text{NO}_3\text{-N}$ is rapidly denitrified throughout the upper sediment profile, but the majority of $\text{NO}_3\text{-N}$ may have been denitrified within the top 1-3 millimeters of wetland sediment, due to the development of highly reducing conditions and elevated bacterial activity in the presence of an additional energy source (Meijer and Avnimelech, 1999).

We confirmed that injected acetate was infiltrating the upper sediment profile by tracking the movement of sodium bromide (NaBr) which was used as a conservative tracer. Sediment cores subjected to denitrification assays encompassed a deeper sediment profile (0-2 cm), including the surficial layer (1-3 mm) that was likely functioning as a denitrification hotspot. If $\text{NO}_3\text{-N}$ was not being reduced in this thin sediment surface layer, the $\text{NO}_3\text{-N}$ concentrations in pore water would likely be more reflective of the different denitrification rates. Specifically, elevated denitrification rates in the presence of DOC would maintain reduced $\text{NO}_3\text{-N}$ concentrations within pore water. Within agricultural headwater streams, DOC amendments can be rapidly assimilated within the top 2 mm of benthic substrate, but foster no change in denitrification (Arango et al. 2007). A response of this type is likely attributed to bacteria allocating labile DOC for bacterial biomass rather than respiratory activity (Grebliunas and Perry in prep). With the presence of a labile DOC source, this benthic anaerobic microlayer functions similar to epiphytic biofilms that are considered to be denitrification hotspots (Schaller et al. 2004). The wetland sediments presented with a minimal DOC (1 mg/L) in our study exhibited a more dramatic change in denitrification relative to agricultural streams, suggesting that stream environments may be limited by DOC to a greater extent than wetlands.

A minimal increase in the availability of DOC could have major implications on improving the $\text{NO}_3\text{-N}$ removal capacity of constructed wetlands. The 10 mg/L DOC treatment maintained significantly higher denitrification rates relative to the low (1 mg/L) treatment, however the increase in denitrification was not commensurate to the difference between DOC concentrations (Figure 10). Results suggest that a low ratio of DOC: $\text{NO}_3\text{-N}$ could increase the removal of $\text{NO}_3\text{-N}$ considerably. This is counter to other wetland and wastewater studies that have shown a C:N ratio greater than 1:1 to be necessary to increase denitrification rates under prolonged $\text{NO}_3\text{-N}$ saturation (Sobieszuk and Szewczyk 2010). Within wastewater systems that maintain elevated $\text{NO}_3\text{-N}$ concentrations (>80mg/L), C:N ratios greater than 0.7 have been shown to support complete denitrification, but a 1:1 ratio to as high as ~4.5:1 maintained the highest denitrification efficiency (Hong et al. 2012). Wastewater systems may require an elevated C:N ratio relative to wetland systems due to the accumulation of organic materials within wetland sediments, providing a degradable substrate during periods of elevated DOC demand (Mitsch and Gosselink 2007; Zhao et al. 2014). The supply of DOC within controlled waste water systems is contingent upon direct additions, as organic material is not created within closed reactors (Filippis et al. 2013).

In addition to an increase in background DOC concentration, the periodicity of DOC inputs relative to $\text{NO}_3\text{-N}$ is an important factor to consider. In pulsed laboratory studies, low DOC relative to $\text{NO}_3\text{-N}$ (10 mg/L) can maintain stable rates of denitrification over time, or become rate limiting when $\text{NO}_3\text{-N}$ concentrations further increase (Grebliunas and Perry in prep). Short-term additions of nutrients can show changes in enzyme activity, but may not be the most accurate measure of nutrient limitation

(Newcomer et al. 2012). Pulsed studies may also overestimate the extent to which wetland sediments are limited by DOC. By injecting acetate at a stable rate, it was possible to significantly increase denitrification, even at low DOC concentrations relative to $\text{NO}_3\text{-N}$ (Figure 11). Studies of similar duration, but inoculate laboratory microcosms with a daily DOC spike have suggested C:N ratios 10-40 times higher are required to foster major changes in denitrification (Mousavi et al. 2014). Within tile drained watersheds, spring tile pulses can account for a large proportion of total $\text{NO}_3\text{-N}$ exported annually (Poor and McDonnell 2007). Allochthonous inputs of DOC are at times elevated during initial tile pulses after winter soil that (Royer and David 2005). The contribution of crop residues holds a great deal of potential to increase background DOC concentrations in tile drainage. If so, the increase in available energy may facilitate increased bacterial reduction of $\text{NO}_3\text{-N}$, and reduce downstream export.

When $\text{NO}_3\text{-N}$ exceeds 1 mg/L, DOC can limit denitrification rates, but the limitations appeared to be minimal within sediments studied (Figure 11) (Arango et al., 2007). Seasonal inputs of tile drainage from past monitoring are rarely below 1 mg/L, and the inflow concentration of $\text{NO}_3\text{-N}$ averaged 14.2 mg/L throughout both injection periods. It is clear that tile drained wetland sediments are limited by the availability of DOC, but this limitation may be overcome with minimal terrestrial contribution. Ratios of C:N were maintained near 1:10 and 1:1 within the low and high DOC mesocosms, respectively. Though denitrification rates significantly increased relative to the control for both DOC amendments, the reduction of $\text{NO}_3\text{-N}$ within the overlying water did not reflect a similar response (Figure 8). The 10 mg/L acetate addition may have led to saturating conditions within the associated mesocosms, potentially explaining the minimal

increase in denitrification between the low and high treatments (Figure 10). The experimental design did not allow for half saturation constants to be calculated, but denitrification appeared to become $\text{NO}_3\text{-N}$ limited at the high DOC treatment. Acetate is a labile form of DOC, and it may have been the quality of DOC driving the observed changes in denitrification (Khan and Spalding 2004).

The significant increase in denitrification to a 1 mg/L increase in DOC suggests that the degree to which agricultural wetlands are DOC limited may be overestimated. Bacterial activity will asymptote when a limiting nutrient is in excess, and will exhibit little to no response to additional inputs (Sinsabaugh et al. 2013). Within eutrophic watersheds, pulses of nutrients are met with a diminished response from bacteria and primary producers, potentially explaining the minimal. Although the injection periods were only 5 days, the rate of change in denitrification appeared to slow by the end of the experiment (Figure 11). Using bacterial respiration (aerobic or anaerobic) as a measure of nutrient limitation has been met with some criticism when looking at short-term responses (Cole and Pace 1995). The positive increase throughout the 5 day injection allowed us to take into account more than a simple spike in enzymatic activity in the presence of a newly available energy source (Calderon et al. 2000). Within agricultural wetland sediments, heterotrophic bacteria do not allocate DOC for dissimilatory processes like denitrification until cellular C:N demands have been met (Grebliunas and Perry in prep).

Tile drained wetlands are a viable option to mediate nutrient inputs from subsurface drainage, however their effectiveness as $\text{NO}_3\text{-N}$ removal tools can be greatly improved. Tile water that was not supplemented with DOC resulted in denitrification

rates that gradually decreased throughout the 5 day study period (Figure 11). The observed decrease was not significant, but the trend suggests that prolonged periods of $\text{NO}_3\text{-N}$ laden tile inflow likely diminish the ability of wetland sediments to reduce $\text{NO}_3\text{-N}$ via bacterial processes. The rate of N loss through assimilatory and dissimilatory processes in agricultural headwater streams is similarly limited. Like wetlands, the addition of an additional energy source can shorten N uptake length within N saturated streams (Herrman and Bouchard 2008). The extent to which agricultural wetland sediments are limited by DOC appears to be less than laboratory studies suggest, and heterotrophic bacterial demand may be met through small increases in allochthonous DOC input if organic materials in agricultural soils are restored. Increasing the availability of DOC within agricultural catchments would also improve stream biogeochemistry and potentially

References

- Abril, A., Casado-Murillo, N., Vazques, C., and Olivera, P. 2013. Labile and recalcitrant carbon in crop residue and soil under no-till practices in central region of Argentina. *The Open Agriculture Journal* 7:32-39.
- Arango, C.P., Tank, J.L., Schaller, J.L., Royer, T.V., Bernot, M.J., and David, M.B. 2007. Benthic organic carbon influences denitrification in streams with high nitrate concentration. *Freshwater Biol.* 52: 1210-1222.
- Aulakh, M.S., Doran, J.W., Walters, D.T., Mosier, A.R., and Francis, D.D. 1991. Crop residue and placement effects on denitrification and mineralization. *Soil Sci. Soc. Am. J.* 55:1020-1025.
- Battany, M.C., and Grismer, M.E. 2000. Rainfall runoff and erosion in Napa Valley vineyards: effects of slope, cover and surface runoff. *Hydrol. Processes* 14: 1289-130
- Bayer, C., Mielniczuk, J., Giasson, E., Martin-Neto, L., and Pavinato, A. 2006. Tillage effects on particulate and mineral-associated organic matter in two tropical Brazilian soils. *Commun. Soil Sci. Plant Anal.* 37: 389-400.
- Beutel, M.W., Newton, C.D., Brouillard, E.S., and Watts, R.J. 2009. Nitrate removal in surface-flow constructed wetlands treating dilute agricultural runoff in the lower Yakima Basin, Washington. *Ecol. Engin.* 35: 1538-1546.
- Brookshire, E.N.J., Valett, H.M., Thomas, S.A., Webster, J.R. 2005. Coupled cycling of dissolved organic nitrogen and carbon in a forest stream. *Ecology.* 86: 2487-2496.
- Corvasce, M., Zsolnay, A., D'Orazio, V., Lopez, R., and Miano, T.M. 2006. Characterization of water extractable organic matter in a deep soil profile. *Chemosphere.* 62: 1583-1590.
- Cory, R.M., and McKnight, D.M. 2005. Fluourescence spectroscopy reveals ubiquitous presence of oxidized and reduced quinones in DOM. *Environ. Sci. Technol.* 39: 8142-8149.
- Dabney, S.M., Delgado, J.A., and Reeves, D.W. 2001. Using winter cover crops to improve soil and water quality. *Commun. Soil Sci. Plant Anal.* 32: 1221-1250.

- Dean, J.E., and Weil, R.R. 2009. Brassica cover crops for nitrogen retention in the mid-Atlantic coastal plain. *J. Environ. Qual.* 38: 520-528.
- Donderski, W., and Burkowska, A. 2000. Metabolic activity of heterotrophic bacteria in the presence of humic substances and their fractions. *Pol. J. of Environ. Stud.* 9:267-271.
- Doran, J.W., and Smith, M.S. 1991. Nitrogen cycling: Role of cover crops in nitrogen cycling. In: Hargrove, W.L. editor. *Cover Crops for Clean Water*. Ankeny, IA: Soil and Water Conservation Society, pg 85-107.
- Fellman, J.B., Hood, E., Edwards, R.T., and Jones, J.B. 2009. Uptake of dissolved allochthonous organic matter from soil and salmon in coastal temperate rainforest streams. *Ecosystems.* 12:747-759.
- Fink, D.F., and Mitsch, W.J. 2004. Seasonal and storm event nutrient removal by a created wetland in an agricultural watershed. *Ecol. Engin.* 23: 313-325.
- Gale, P.M., Devai, I., Reddy, K.R., and Graetz, D.A. 1993. Denitrification potential of soils from constructed and natural wetlands. *Ecol. Engin.* 2:119-130.
- Grieve, I.C. 1985. Determination of dissolved organic matter in streamwater using visible spectrophotometry. *Earth Surf. Proc. Land* 10:75-78
- Griffiths N.A., Tank, J.L., Royer, T.D., Rose-Marshall, E.J., Whiles, M.R., Chambers, C.P., Frauendorf, T.C., Evans-White, M.A. 2009. Rapid decomposition of maize detritus in agricultural headwater streams. *Ecol. Appl.* 19: 133-142.
- Haney, R.L., and Haney, E.B. 2010. Simple and rapid laboratory method for rewetting dry soil for incubations. *Commun. Soil Sci. Plan.* 41:1493-1501.
- Jandl, R., and Sollins, P. 1997. Water-extractable soil carbon in relation to belowground carbon cycle. *Biol. Fertil. Soils* 25: 196-201.
- Kellogg, C.H., and Bridgham, S.D. 2002. Colonization during early succession of restored freshwater marshes. *Can. J. Bot.* 80: 176-185.
- Ketterings, Q.M., Kingston, J., McIlvennie, S., Long, E., Godwin, G., Gami, S. Stanyard, M., and Czymmek, K. 2011. What's Cropping Up? A newsletter for New York field crops and soils Vol. 21.
- LaMontagne, M.G., Astorga, V., Giblin, A.E., and Valiela, I. 2002. Denitrification and the stoichiometry of nutrient regeneration in Waquoit Bay, Massachusetts. *Estuaries.* 25: 272-281.

- Lewis, W.M., and Canfield, D. 1977. Dissolved organic carbon in some dark Venezuelan waters and a revised equation for spectrophotometric determination of dissolved organic carbon. *Arch. Hydrobiol.* 79: 441-445.
- Linn, D.M., and Doran, J.W. 1984. Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils. *Soil Sci. Soc. Am. J.* 48:1267-1272.
- Lund, L.J., Horne, A.J., and Williams, A.E. 2000. Estimating denitrification in a large constructed wetland using stable nitrogen isotope ratios. *Ecol. Engin.* 14:67-76.
- Machefert, S.E., and Dice, N.B. 2004. Hydrological controls on denitrification in a riparian ecosystem. *Hydrol. Earth Syst. Sci.* 8: 686-694.
- Magill, A.H., and Aber, J.D. 2000. Dissolved organic carbon and nitrogen relationships in forest litter as affected by nitrogen deposition. *Soil Biol. Biochem.* 32:603-613.
- McCutchan, J.H., and Lewis, W.M. 2008. Spatial and temporal patterns of denitrification in an effluent-dominated plains river. *Verh. Internat. Verein. Limnol.* 30:323-328.
- Menges, F. Spekwin32-optical spectroscopy software, Version 1.71.6.1, 2014. Available: http://www.effemm2.de/spekwin/index_en.html
- Mulholland, P.J. 2003. Large-scale patterns in dissolved organic carbon concentration, flux, and sources. In: *Aquatic Ecosystems: Interactivity of Dissolved Organic Matter* (Eds. Findlay, S.E.G. and Sinsabaugh, R.L.), pp. 139-159. Academic Press, San Diego, CA.
- Murphy, K.R., Stedmon, C.A., Graeber, D., and Bro, R. 2013. Fluorescence spectroscopy and multi-way techniques. *PARAFAC. Anal. Methods* 5: 6557-6566.
- Ohno, T., and Bro, R. 2006. Dissolved organic matter characterization using multiway spectral decomposition of fluorescence landscapes. *Soil Sci. Soc. Am. J.* 70: 2028-2037.
- Olson, K.R., Ebelhar, S.A., Lang, J.M. 2010. Cover crop effects on crop yields and soil organic carbon content. *Soil Sci.* 175: 89-98.
- Peregrina, F., Larrieta, C., Ibanez, S., and Garcia-Escudero, G. 2010. Labile organic matter, aggregates, and stratification ratios in a semiarid vineyard with cover crops. *Soil Sci. Am. J.* 74: 2120-2130.

- Peterson, H.G. 1998. Use of constructed wetlands to process agricultural wastewater. *Can. J. Environ. Qual.* 78: 199-210.
- Pinney, M.L., Westerhoff, P.K., Baker, L. 2000. Transformations in dissolved organic carbon through constructed wetlands. *Wat. Res.* 34: 1897-1911.
- Sarkhot, D.V., Grunwald, S., Ge, Y., and Morgan, C.L.S. 2012. Total and available soil carbon fractions under the perennial grass *Cynodon dactylon* (L.) Pers and the bioenergy crop *Arundo donax* L. *Biomass Bioenerg.* 41:233-130.
- Smith, L.K., Voytek, M.A., Bohlke, J.K., and Harvey, J.W. 2006. Denitrification in nitrate-rich streams: Application of N₂:Ar and ¹⁵N-Tracer methods in intact cores. *Ecol. Appl.* 16: 2191-2207.
- Stedmon, C.A., and Markager, S. 2005. Resolving variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using a PARAFAC analysis. *Limnol. Oceanogr.* 50:686-697.
- Steenwerth, K., and Belina, K.M. 2008. Cover crops enhance soil organic matter, carbon dynamics and microbiological function in a vineyard agroecosystem. *Appl. Soil Ecol.* 40: 359-369.
- Stutter, M.I., Lumsdon, D.G., and Cooper, R.J. 2007. Temperature and soil moisture effects on dissolved organic matter release from a moorland Podzol O horizon under field and controlled laboratory conditions. *Eur. J. Soil Sci.* 58: 1007-1016.
- Tarkalson, D.D., Kachman, S.D., Knops, J.M.N., Thies, J.E., and Wortmann, C.S. 2008. Decomposition of Bt and Non-Bt corn hybrid residues in the field. *Nutr. Cycl. Agroecosyst.* 80: 211-222.
- Tranvik, L.J. 1992. Allochthonous dissolved organic matter as an energy source for pelagic bacteria and the concept of the microbial loop. *Hydrobiologia.* 229:107-114.
- Unger, P.W., and Vigil, M.F. 1998. Cover crops effects on soil water relationships. *J. Soil Water Cons.* 53:241-244.
- USDA Natural Resources Conservation Service. 2011. Carbon to nitrogen ratios in cropping systems. Technical Report. USDA NRCS East National Technology Support Center, Greensboro, NC.
- Villamil, M.B., Bollero, G.A., Darmody, R.G., Simmons, F.W., and Bullock, D.G. 2006. No-till corn/soybean systems including winter cover crops: Effects on soil properties. *Soil Sci. Soc. Am J.* 70: 1936-1944.

- Woodmansee, R.G., and Duncan, D.A. 1980. Nitrogen and phosphorus dynamics in annual grassland. *Ecology*. 63:893-904.
- Xue, Y., Kovacic, D.A., David, M.B., Gentry, L.E., Mulvaney, R.L., and Lindau, C.W. 1999. In situ measurements of denitrification in constructed wetlands. *J. Environ. Qual.* 28:263-269.
- Zsolnay, A. 1996. Dissolved humus in soil waters. In: Piccolo, A. (Ed.), *Humic Substances in Terrestrial Ecosystems*. Elsevier, Amsterdam, pp. 171-223.
- Zarnetske, J.P., Haggerty, R., Wondzell, S.M., Baker, M.A. 2011. Labile dissolved organic carbon supply limits hyporheic denitrification. *J. Geophys. Res.* 116:1-13.

Table 5

Results of the fixed effect ANOVA looking at the effects of DOC concentration on NO₃-N concentration within the overlying mesocosm water. Numbers in bold are significant at $\alpha=0.05$.

DOC Treatment (mg/L)	Depth	Avg NO ₃ -N (mg/L)
10	Overlying Water	11.25*
1	Overlying Water	12.56
Control	Overlying Water	13.84

Table 6

Results of the two-way fixed effect ANOVA looking at the effect of treatment on average pore water (0-5 cm and 5-10 cm combined) NO₃-N concentrations. Different letters show significant differences at $\alpha=0.05$.

Treatment	NO ₃ -N (mg/L)
Control	2.22 a
Low	1.44 ab
High	1.03 b

Figure 8. Average $\text{NO}_3\text{-N}$ concentrations within all combined (0-5 and 5-20 cm) pore water samples. Changes in $\text{NO}_3\text{-N}$ are in response to the control (ambient tile water), low DOC (1 mg/L), and high DOC. Tukey multiple means was used to test for differences between treatments. Letters denote significant differences.

Figure 9. Average overall denitrification rates from both 5 day nutrient injections. Changes in denitrification are in response to the control (ambient tile water), low DOC (1 mg/L), and high DOC. Tukey multiple means was used to test for differences between treatments. Letters denote significant differences.

Figure 10. Average overall denitrification rates from both 5 day nutrient injections. Changes in denitrification are in response to the control (ambient tile water), low DOC (1 mg/L), and high DOC. Tukey multiple means was used to test for differences between treatments. Letters denote significant differences.

Figure 11. Change in average denitrification rates over a 5 day period in response to DOC availability. Treatments are denoted as follows: control($\blacktriangle$), 1 mg/L($\bullet$), and 10 mg/L ($\blacksquare$).

CHAPTER IV
CHANGES IN WATER EXTRACTABLE ORGANIC CARBON WITH COVER
CROP PLANTING UNDER CONTINUOUS CORN PRODUCTION

Abstract

Long-term row crop agricultural production has dramatically reduced the pool of soil organic carbon (C). The implementation of cover crops in Midwestern agroecosystems is primarily to reduce losses of nitrogenous fertilizers, but has also been shown to restore soil C stocks over time. If labile C within agricultural soils could be increased, it could improve soil health and if mobilized into subsurface drainage, may positively impact watershed biogeochemistry. We tested for potential differences in water extractable organic carbon (WEOC) at two different soil profiles (0-5cm and 5-20cm) between plots planted with cereal rye/tillage radish (cover crop), corn, and zero control (no vegetation) within the Illinois State University Research Farm. We also tested for potential differences in denitrification within the upper soil profile throughout the growing year. We modeled excitation emission matrices from soil cores through parallel factor analysis (PARAFAC). We found no difference in WEOC concentrations between each crop treatment ($p=0.2850$), but concentrations of WEOC were significantly lower in the 5-20cm profile than that of the upper (0-5cm) profile ($p=0.0033$). There was a significant increase in WEOC under each treatment in samples after cover crop termination. The PARAFAC model found humic and fulvic acids to be the dominant

fractions of WEOC in all soils tested. Humic and fulvic acids accounted for nearly 70% and 30% of model variation. Denitrification rates did not differ across treatments ($p=0.3520$), which is likely attributed to soil WEOC being in limiting quantities and in primarily recalcitrant fractions. Cover crops do not appear to alter soil WEOC quantity and type within the first years of planting. Restoring the availability of C within agricultural soils will not be a short term fix, and fields will likely be a net C sink, contributing minimal labile C to receiving waterways.

Introduction

The conversion of natural wetland and prairie habitats to row crop agriculture has led to long-term reductions to the amount of organic carbon (DOC) in soils (Mulholland 2003; Stanley et al. 2011). The availability of soil carbon is important for soil health to best manage nutrient availability, water holding capacity, and water filtration, and practices to restore soil carbon are being investigated. In many Midwestern agroecosystems soil organic carbon is low, recalcitrant and is relatively immobile because of farming practices (Coyne 1999; Tarkalson et al. 2008). Bioavailable carbon (i.e. sugars and amino acids) is rapidly leached from crop residues and can infiltrate lower soil profiles, but is often assimilated by soil bacteria prior to doing so (Machefert and Dise, 2004; Griffiths et al. 2009). Agricultural practices that reduce soil carbon results in a commensurate decrease in carbon lost as DOC in drainage tiles and receiving waterways altering biogeochemical processes (Pinney et al. 2000). Winter cover crops can have the potential to reduce soil loss and immobilize fall applied nitrogen, but may also increase soil organic carbon (Dabney et al. 2001). In particular,

increased carbon can lead to increase soil denitrification rates but also increased denitrification in receiving waterways (Jandl and Sollins 1997).

Tillage practices coupled with continual corn and soy bean production has led to an overall decrease in soil organic carbon (Bayer et al. 2006). Long-term crop residue management, through the over-application of N, is conducted to increase plant biomass decomposition and reduce the labile fractions of C. Excess labile organic C can be a detriment to soil N by promoting elevated bacterial activity, and subsequent loss of N. As a result, efforts are now in place to restore the available pool of carbon to improve soil health for the benefit of increased grain yields. The decomposition of plant biomass is determined by the C:N of the residues (roots, leaves, and stalk). Common cover crops, such as cereal rye and tillage radish, have low C:N ratios, 26:1 and 20:1 respectively, relative to corn (> 60:1) and soybeans (30:1) (Ketterings et al. 2011; USDA 2011). The low C:N promotes rapid decomposition due to low lignin content and less energy required for microbial degradation, increasing C and N content in upper soil horizons (Olson et al. 2010; Steenwerth and Belina 2008; Peregrina et al. 2012). The primary goal of winter cover crops is to serve as an adaptive management technique to immobilize fall applications of N and reduce spring losses during periods of soil saturation. The stored N within the plant biomass is released upon the termination of the cover crop and remineralized to become available for the planted cash crop (Doran and Smith 1991).

Terminated cover crops rapidly leach labile fractions of DOC (and WEOC), however the amount and bioavailability of WEOC that accumulate within agricultural soils is largely unknown (Battany and Grismer 2000; Dean and Weil 2009). In some

instances, DOC has not increased with cover crop implementation, but this is likely attributed to soil leaching (Stutter et al. 2007). WEOC is characterized as the fraction of DOC present that is easily transferred into an aqueous environment and consists of a largely bioavailable and mobile portion of DOC (Zsolnay 1996). If WEOC is in elevated concentrations as a result of cover crop implementation, it will serve as a means to restore soil health (Reeves 1997). Due to this increased mobility, this fraction of DOC may account for a large proportion entering aquatic ecosystems and may control many of the biogeochemical processes in those systems.

Within corn and soy bean agroecosystems WEOC are in limited quantities due to the recalcitrant components of residues, however winter cropping systems hold the potential to increase soil organic matter, along with WEOC due to more rapid decomposition (Abril et al., 2013). Under perennial grasses such as *Arundo donax* (Giant Reed) used for biofuel production, soil organic carbon significantly increased within the upper soil profile over time (Sarkhot et al., 2012). Similar to biofuel production, implementing winter cover crops within a corn and soybean rotation will increase the plant biomass in a field and subsequent residue incorporation into field soils. Focusing on the potential contribution winter cover crops may have on fractions of WEOC is an important management question because the availability of WEOC relative to water extractable organic nitrogen in soils were a better predictor of soil respiration and foster higher rates of bacterial respiration than that of DOC (Woodmansee and Duncan 1980). If the labile fractions of WEOC leach into receiving aquatic ecosystems, higher rates of denitrification may result (Zarnetske et al., 2011).

A limited number of studies have shown that organic carbon and DOC increase with cover crop implementation. An increase in soil DOC has been observed within vineyard, and corn/soybean agroecosystems, however the types of DOC present have not been investigated (Villamil et al. 2006). The increase in soil organic matter associated with incorporating cover crop residues is a benefit to soil health, but the contribution to labile forms may be minimal due to the high lignin and cellulose content or rapid assimilation prior to leaching through soil (Dabney et al., 2001). An accumulation of humic substances would benefit soil water retention and N holding capacity of soils, but may result in a lessened response by heterotrophic bacteria in receiving waterways relative to more labile fractions (Tranvik 1992). Cover crops have the ability to increase soil porosity, potentially allowing labile fractions of WEOC to more rapidly enter subsurface drainage (Unger and Vigil 1998). If mobile fractions of DOC are increased with the incorporation of winter cover crops, identifying any bioavailable fractions within the soil profile would show the potential for increased input of labile WEOC under periods of soil saturation.

An increase in labile organic C within agricultural soils can foster elevated rates of denitrification under moist soil conditions. Terrestrial denitrification must be considered when studying allochthonous inputs of WEOC. The activity of soil bacteria mediates the quantity of WEOC that may enter tile drainage, in addition to the bioavailability. The incorporation of crop residues into agricultural soils through tillage puts plant biomass in direct contact with soil microbiota, promoting decomposition and rapid assimilation of labile WEOC fractions that are leached from plants. Elevated rates of denitrification have been observed in soils with recent additions of soybean and vetch,

both of which have low C:N ratios relative to corn, but these spikes were brief (<5 days), and rates did not differ irrespective of residue type after this initial spike (Aulakh et al., 1991). If an increase in terrestrial denitrification after long term cover crop planting were to occur, allochthonous WEOC inputs would continue to be minimal and have little positive impact on NO₃-N reduction in receiving aquatic environments.

The availability of DOC plays an important role in the dynamics of heterotrophic N processing, in aquatic systems, primarily via denitrification (Westhorpe et al. 2010). As in terrestrial systems, the presence of elevated N concentrations can shift denitrification to become C limited (Fellman et al. 2009). Denitrification is an energetically costly process and requires a continuous DOC supply in the presence of NO₃-N saturation often seen in agricultural watersheds. Increasing the soil DOC pool over time may lead to larger inputs of labile WEOC during critically important seasonal periods. If bacterial demand for DOC could be met, denitrification may be able to adequately increase during seasonally intense NO₃ inputs (McCutchan and Lewis 2008). The limited size of mitigation wetlands relative to watershed area would potentially benefit from an increase in allochthonous DOC delivery rather than wetland plant management as suggested in other studies (Kellogg and Bridgham 2002). Forested headwater streams experience increases in heterotrophic activity as a result of leaf litter decay and associated pulse of nutrients (Magill and Aber 2000). Agroecosystems experience similar seasonal fluxes of nutrients post-harvest from the decomposition of corn and soybean residues, but the DOC inputs low relative NO₃.

The objectives of the proposed studies are to test how the incorporation of cover crop residues into a crop rotation will increase the availability of water extractable

organic carbon (WEOC) throughout the upper soil profile (0-20 cm). In addition to analyzing changes in WEOC concentration, we will also investigate potential changes in WEOC quality through PARAFAC modeling of excitation-emission matrices collected through spectral analysis. We also tested how denitrification potentially responded to changes in soil WEOC over the course of the study. This provided insight to potential benefits cover crop residues may have to watershed biogeochemistry if WEOC leached into receiving waterways.

Methods

Site Description

The study of WEOC within soils under different crop types was examined in an experimental system within the Illinois State University Teaching and Agriculture Research farm near Lexington, IL, USA. The experimental cover crop system began in 2011 within an experimental field comprised of nine plots (N=9) (2023 m², half-acre), arranged in a complete randomized block design with three treatments that were each replicated three times. The crop treatments used in this study were a zero control (no corn or cover crop), control (corn), and cereal rye/tillage radish (cover crop). All treatment plots were tilled and planted within poorly drained, Drummer and El Paso silt clay loams.

Anhydrous ammonia (200 kg ha⁻¹) was directly knifed into all plots in November of 2012. During the fall of 2011, N in the form of (NH₄)SO₄ at a rate of 50 kg ha⁻¹ to promote cover crop growth. This was not necessary for the 2012 planting due to available soil N. In 2012, the tillage radish/cereal rye plots were terminated in March by applying glyphosphate to allow for remineralization of N prior to planting. Soils were

lightly tilled to incorporate residues into the upper soil profile, and to imitate practices common in the region.

Soil Collection

To test for potential differences in WEOC under different cropping systems, soil cores were collected within the same plots throughout 2013 (April-October). The spring sampling dates were April 5 and April 15 which encompassed the week before cover crop termination, along with an additional sample prior to corn planting in May. A single sample, July 15, was used during the summer growing season during a period of rapid nutrient uptake by corn plants. Two fall samples were also incorporated in the analysis which were prior to harvest (9/2) and post-harvest before soil freeze-up (10/2)

WEOC Analysis

To estimate WEOC, soil cores were randomly collected per treatment within each plot to a depth of 100 cm but two subdivided depth fractions were analyzed: 0-5cm and 5-20cm. The three subdivided cores were made into a composite sample, dried at 105°C for 24 hours, and sieved through 1 mm mesh prior to being analyzed. To measure NH_3 , NO_3 , and WEOC, a 5 g subsample was taken from each treatment and depth profile (Zsolnay 2003). Soil samples were suspended in 50 ml of 0.01M CaCl_2 , placed on a rotary shaker for 15 minutes, and followed by 10 minutes of centrifugation at 4000 rpm. The supernatant was filtered through Whatman 42 filter paper and acidified to a pH of 2 using 2M HCl (Zsolnay 2003; Lacey and Armstrong 2014).

The concentrations of DOC concentration from field soil cores were calculated from optical data using a Perkin Elmer Lambda 35 UV-Vis Spectrophotometer. From each soil extraction, 4ml of extractant was transferred into a 1 cm quartz cuvette and the

absorbance was measured at 360nm (Lewis and Canfield 1977;Grieve 1985). The specific ultra violet absorbance at 254 nm (SUVA₂₅₄) was also tested. This is a measure of recalcitrance of WEOC, the higher the SUVA₂₅₄, the more recalcitrant the WEOC is within the extractant.

To test for potential differences in the dominant fractions of C between crop treatments, fluorescence excitation-emission matrices (EEMS) of WEOC were measured on Perkin Elmer LS-55 spectrofluorometer. Synchronous scans were done across excitation wavelengths from 240 to 480 nm at 5 nm intervals and emission wavelengths of 300 to 600 nm at 0.5 nm intervals with a scan speed of 1200nm/second. By analyzing EEMS using a multivariate modeling technique, parallel factor analysis (PARAFAC), the composition of WEOC (humic-like, fulvic-like, and protein-like) can be assessed using the fluorescent characteristics of each soil extraction (Cory and McKnight 2005). After EEMs have been created for each sample, the data was exported into Microsoft Excel (2010) with the aid of Spekwin32 spectroscopy software.

Prior to the EEM scans, a 290 nm cutoff filter was applied to all samples in order to reduce second order Raleigh scattering. Spectral corrections were performed with factory-supplied data from previous instrument calibrations. Inner filter effects were accounted for with absorption corrections that were applied to the blank and sample EEMs. A sample blank (Milli-Q water) was scanned on each day of EEM analysis to subtract the fluorescence intensity from each sample to limit scatter bands (Murphy et al. 2013). Raw machine units were normalized into Raman units with the aid of the drEEM Toolbox 0.2.0 (Murphy et al., 2013). WEOC characterization was performed via PARAFAC modeling with MATLAB R2014a (The MathWorks Inc, Natick, MA, 2014).

The drEEM Toolbox 0.2.0 (<http://www.models.life.ku.dk>) was imported to MATLAB to perform the PARAFAC analysis (Murphy et al., 2013).

PARAFAC modeling allows for multiple fluorescence spectra to be overlain and decompose the data to allow for relative estimates of different components, or “types” of WEOC. By validating an appropriate number of components from the overall model, the percent contributions can be calculated from the FMax values provided in the model output. The relative contributions of each component can be applied to total WEOC within each sample to calculate what percentage of total WEOC is accounted for by each fraction of C (referred to as components) that was modeled. By accounting for the proportions of different C fractions it was possible to test how cropping systems alter the relative composition of WEOC throughout the upper soil profile.

Using PARAFAC modeling, we attempted to validate a range of 2 to 6 component models with the fluorescent EEMs measured. Only two unique components were identified across all soil extractions. A 3 component model was validated, however the core consistency values were considerably lower (37.8%). The core consistency values were compared for the 2 and 3 component models, both of which were validated via split half analysis (Stedmon and Markager 2005). The core consistency diagnostics provides a measure of how well the spectral loadings account for variation in the dataset (Ohno and Bro 2006). Core consistency values should be near 100%, the 2 component model had a much higher value (98.7%) than that of the 3 component model, and therefore the 2 component model was selected. The percent relative contributions (from FMax) of each of the two components to the overall model were then calculated to test for different relative quantities of WEOC types between crop treatments.

Soil Denitrification

The soil samples from the 0-5 cm profile were subjected to the acetylene inhibition technique to measure potential denitrification rates (Smith et al., 2006). Denitrification was only measured in the 0-5 cm soil profile as this is the zone of highest bacterial activity within terrestrial and aquatic environments. Since the soils were previously dried for prior analyses, a rewetting pretreatment was required. The length of rewetting and degree of saturation plays an important role in measuring bacterial activity. To appropriately rewet soils, all samples were rewet to the appropriate water filled pore space (WFPS). Using WFPS as the metric to reach a desired soil saturation allowed a quantifiable way to accurately rewet soils and standardize conditions across samples. Field soils were rewetted to 70% WFPS as this degree of saturation has been shown to maximize bacterial activity in terrestrial soils (Linn and Doran 1984). Soils were rewetted for 24 hours prior to being assayed for denitrification (Haney and Haney 2010).

To measure potential differences in denitrification rates between crop treatments over time, 10 g of dried soils were allocated to 150 ml glass media bottles in triplicate. After the 24-hour rewetting period, soils were assayed via the acetylene inhibition technique. Each replicate received 50 ml of Milli-Q water and amended with 5 ml of a 0.1M chloramphenicol solution. The headspace of each media bottle was then purged with N₂ gas for 5 minutes to create anaerobic conditions. 15 ml of acetylene (C₂H₂) gas was injected into the headspace and shaken to incorporate into the soil. The addition of C₂H₂ blocks the conversion of NO₃ to N₂ gas, therefore the end product for this assay is N₂O. A 10 ml headspace gas sample was taken 15 minutes after the initial C₂H₂ injection, and once an hour for the following four hours. Denitrification rates were

calculated from the production of N_2O over time per gram of dried soil. Headspace gas samples collected throughout the assay were measured using a Shimadzu GC-2014 gas chromatograph (Porapak Q packed column; Detector temperature 300 °C; oven temperature, 100 °C; flow rate of carrier gas (ultrapure Nitrogen gas) 10 ml/min.).

Data Analysis

Potential changes in WEOC concentrations at the shallow and deep soil profiles over the course of the study were tested with A fixed-effects ANOVA was used to test the effect of crop treatment, time, and soil depth on WEOC concentrations in agricultural soils (SAS Institute 9.2, 2008). A planned contrast was used to test for differences in WEOC before and after the cover crops were terminated within both soil profiles. To test for differences in soil denitrification rates between soil depths and crop type, a fixed effects ANOVA was used. Appropriate follow-up tests were performed by using a Tukey's multiple comparisons From the PARAFAC model, it was possible to calculate the percentage of variation that each model component accounted for. We used a one-way ANOVA to test for potential differences in the relative proportion of each component (FMax1 and FMax2) between crop treatments.

Results

Total WEOC

Cover crops did not have significantly higher WEOC compared to the corn and zero control plots ($F_{2,35}=1.37$, $p=0.2850$). In all treatments, a significantly higher concentration of WEOC is present in the 0-5 cm depth than that of the 5-20 cm depth profile ($F_{1,35}=12.14$, $p=0.0033$, Figure 12). WEOC concentrations varied throughout the course of the study ($F_{5,35}=3.11$, $p=0.0399$), but there was no significant interaction

between crop treatment and time ($F_{10,35}=0.4$, $p=0.9236$). Since no difference in WEOC was observed between treatments over time, a planned contrast was performed on all mean WEOC concentrations, irrespective of treatment, in the upper soil profile (0-5cm), and it was found that WEOC concentrations were significantly greater at each sampling date following cover crop termination ($F_{5,53}=7.59$, $p<0.0001$, Figure 13). At the lower soil profile, no differences in WEOC concentration were observed over time (Figure 14). When comparing all nutrient treatments at both depth profiles, there was no statistically significant difference in WEOC ($F_{2,35}=0.04$, $p=0.9563$).

PARAFAC WEOC

A two component model was validated with a high core consistency value (98.7%) and split-half analysis. Each component was characterized by single sharp peaks. Component 1 produced a maximum fluorescence peak at the excitation/emission wavelength 335/421 nm. This component exhibited spectral characteristics similar to that of ubiquitous humic acids contributed to agricultural soils from decomposing vegetation (Stedmon et al. 2003). . Component 2 was characterized by a fluorescence peak at the excitation/emission wavelength 385/470 nm (Figure 15). Component 2 closely resembled fulvic acids typical of terrestrially derived compounds previously identified in agricultural headwater streams (Stedmon and Markager 2005).

The relative amounts of humic-like versus fulvic-like compounds significantly differed from one another. The humic-like compounds accounted for 68% of model variation and fulvic-like compounds are contributing to 31% of total variation (Figure 16). Component 1 and component 2 had similar percent contributions (F_{Max1} and F_{Max2}) to the overall model for each crop treatment ($F_{2,101}=0$, $p=0.9961$). The similarity

in the relative percent of each component suggested that soils have similar amounts of humic and fulvic acids, irrespective of the type of crops planted.

Denitrification

Only soil cores collected in April and May were incorporated into the analysis. The soil samples assayed from the summer and fall collections did not produce measurable rates of denitrification because N_2O concentrations were below detection limit. The crop treatments did not have a significant effect on soil denitrification rates ($F_{2,26}=1.11$, $p=0.3520$, Figure 17). Denitrification rates exhibited no significant variation throughout the 2013 sampling period ($F_{2,26}=0.79$, $p=0.4708$). The interaction of sampling date and crop treatment was also not significant ($F_{4,26}=2.36$, $p=0.0921$).

Discussion

Within three years of cover crop implementation, cereal rye and tillage radish residues have not increased WEOC in soil relative to the other treatments (Figure 12). The lack of difference may be attributed to the relatively short time period that cover crops have been planted within this site. Restoring organic C within agricultural soils often on a decadal scale, even within restored sites where agricultural production has been halted (McLaughlan et al. 2006). Long-term studies have not focused on WEOC, but WEOC has been correlated with total soil organic C and it may be fair to assume that WEOC follows similar trends. Therefore, noticeable changes in allochthonous contributions of WEOC from cover crops may constitute a long-term benefit, and changes in stream and wetland biogeochemistry will not exhibit immediate changes.

The labile fractions of C are often leached within days of plant death and incorporation into soil or aquatic environments (Wetzel and Manny 1972; Qualls and

Haines). Within agricultural soils, labile fractions of DOC, such as amino acids and sugars, can be assimilated in less than 24 hours of release (Eilers et al. 2010). If soil sampling for this study took place closer the termination date, a pulse in WEOC may have been observed within the upper soil profile (0-5 cm). The contribution of the rapidly leach labile fractions can be minimal in that they comprise 5-10% of total DOC in terrestrial and aquatic systems (Sondergaard and Middelboe 1995). Although brief, the limited availability of labile fractions of WEOC can foster dramatic changes in bacterial activity if leached into aquatic systems (Massicotte and Frenette 2013).

Concentrations of WEOC significantly decreased from the upper (0-5 cm) to the lower (5-20 cm) soil profile (Figure 12). Although the concentrations of WEOC did not differ significantly between treatments, cover crop and corn plots maintained elevated WEOC relative to the zero control plots at both soil depths. Soil bacteria can rapidly respire and decompose labile fractions of C when available, which may account for the observed decrease in WEOC across the soil profile (Yuste et al. 2007). Increases in soil organic C are necessary to balance cellular stoichiometry in the presence of high soil N (Griffiths et al. 2012). Terrestrial soils can foster high rates of denitrification when soils become saturated, removing both C and N from fine particulate soils (Davidsson et al. 1997). The decrease in soil WEOC suggests that a large proportion of WEOC is lost as water infiltrates deeper soil layers and enters subsurface drainage.

It was initially predicted that soils within the cover crop plots would have a higher proportion of labile fractions due to the ease of residue decomposition relative to lignin-rich corn residues. Minimal differences in fluorescence spectra were observed between WEOC characterizations between each plot (Figure 15). The 2 component PARAFAC

model found WEOC to be comprised of humic-like and fulvic-like substances. For all treatments, humic-like substances (component 1) accounted for significantly more variation (~70%) in the PARAFAC model than that of the fulvic-like (component 2) fraction (30%) (Figure 15). Crop residues contribute a pulse of labile C (proteins, polysaccharides, and organic acids) after harvest or termination, but as previously stated, this brief period was likely missed in the sampling regime (Kumar et al. 2014).

The elevated C:N ratio and high lignin content of corn residues contribute to the slow decomposition and serve as a starting material for the formation of humic substances in agricultural soils (Kogel-Knabner 2002). Humic acids are formed from the combination of polyphenolic and carboxylic groups and largely resistant to bacterial degradation due to their complex aromatic structure (Amador et al. 1989; Stevenson 1994). Relative to corn residues, cereal rye and tillage radish have lower C:N ratios, but did not affect the relative percentage of humic acids within the associated test plot (Mary et al. 1996). The prevalence of humic acids within the experimental plots may have been due to previous agricultural practices rather than recent incorporation of cover crops into the annual rotation (Hong et al. 2010). Continual corn production prior to this study appeared to reduce the relative quantity of WEOC within the study soils, as observed by the zero control. The complexity of humic acids reduces their usage by soil bacteria, however humic acid-oxidizing bacteria can be prevalent within soils, and may be accounting for the significant reduction observed within the upper soil profile (Van Trump et al. 2011). Although humic acids occupy a greater percentage of total WEOC than fulvic acids, the relative increase humic acid concentration in cover crop plots was minimal.

Under periods of organic C limitation, bacteria that are members of the *Acidobacteria*, *Firmicutes*, *Betaproteobacteria*, and *Caulobacteriales* (along with other phyla) can oxidize humic acids into more readily usable compounds (Van Trump et al. 2011). Component 2 of the PARAFAC model represented fulvic-like compounds which were likely formed from the bacterial degradation of humic acids into the less aromatically complex compounds. Although fulvic acids were not the dominant fraction of WEOC, they are highly soluble in water and may be exported from agricultural soils to a greater degree than humic acids (Guimaraes et al. 2013). The ease of transport coupled with increased bioavailability holds the potential that the contribution of fulvic acids from crop residues may have some benefit to aquatic heterotrophic bacteria. Unfortunately the role of fulvic acids as an electron donor for denitrifying bacteria is largely unknown, the increased availability of a degradable DOC source may be an effective energy source under prolonged C limitation.

The availability of WEOC may also be limiting the occurrence of denitrification within the agricultural soils. Throughout the study period there are considerable fluxes in available NO_3 but minimal changes in denitrification potential over time. In field soils, elevated soil NO_3 often translates to increased denitrification rates under moist soil conditions (Bakken et al. 2012). The availability of applied N within each plot appeared to shift organic C to be the rate limiting nutrient. Throughout the study period, each treatment plot maintained similar WEOC concentrations, and denitrification rates appeared to reflect the lack of change in WEOC availability. Agricultural soils have been suggested to serve as a net C sink, which is attributed to the high demand by soil microbiota due to artificially high soil N availability (Morgan et al. 2010).

There was sufficient WEOC to facilitate the occurrence of denitrification within the experimental plots and may account for the reduction of WEOC concentrations within the top 5 cm of soil. A small percentage of the observed WEOC loss is likely due to bacterial assimilation, but bacterial activity is responsible for a large proportion organic C reduction within soils (Fang et al. 2014). The similarity in denitrification rates across each crop treatment may also be explained by the fractions of WEOC available. Humic acids can be used as an energy source to facilitate bacterial respiration, but the structural diversity of terrestrially derived humic acids can lead to low or variable activity (Lu et al. 2013). Denitrification would likely spike immediately after cover crop termination due to the pulse amino and fatty acids leached from cover crop residues, but this increase would be brief and not accounted for with the sample frequency.

Within the first years of cover crop implementation, the availability of WEOC and dominant fractions suggest there will a minimal change in allochthonous C inputs. The lack of difference in denitrification between treatments may offer potential insight as to how allochthonous organic C from early cover crops would potentially alter agroecosystem biogeochemistry. The mobile fractions of C are primarily in recalcitrant forms, minimizing the usage by heterotrophic bacteria within receiving waterways (Ramirez et al. 2012). Although recalcitrant fractions of WEOC can be degraded into more usable forms within aquatic environments, the change in concentrations are insufficient to drive significant changes in aquatic denitrification (Greblunas and Perry in prep). The allochthonous contribution of recalcitrant DOC may be of some benefit in that bacteria have been found to use the recalcitrant fractions for growth under strong C limitation (Kritzberg et al. 2006). In some instances, photochemical degradation can also

convert humic acids into more bioavailable fractions in aquatic environments (Anesio et al. 2006). Wetlands within agricultural landscapes have minimal canopy coverage, potentially allowing recalcitrant fractions of DOC to be reduced via this photochemical pathway and fostering elevated rates of denitrification if the pool of terrestrial organic C in soils were increased over time. An increase in labile DOC would be ideal to create conditions for a rapid bacterial response to seasonal pulses of NO_3 with elevated rates of denitrification (Greblunas and Perry in prep). Although cover crops are capable of increasing soil organic C and improving soil health, the impact on the biogeochemistry within intensely farmed watersheds may be minimal until fields assimilate a sufficient pool of organic C and begin to export appreciable amounts of organic C.

References

- Abril, A., Casado-Murillo, N., Vazques, C., and Olivera, P. 2013. Labile and recalcitrant carbon in crop residue and soil under no-till practices in central region of Argentina. *The Open Agriculture Journal* 7:32-39.
- Amador, J.A., Alexander, M., and Zika, R.G. 1989. Sequential photochemical and microbial degradation of organic molecules bound to humic acid. *Appl. Environ. Microb.* 55: 2843-2849.
- Anesio, A.M., Granelli, W., Aiken, G.R., Kieber, D.J., and Mopper, K. 2005. Effect of humic substances photodegradation on bacterial growth and respiration in lake water. *Appl. Environ. Microbiol.* 71: 6267-6275.
- Aulakh, M.S., Doran, J.W., Walters, D.T., Mosier, A.R., and Francis, D.D. 1991. Crop residue and placement effects on denitrification and mineralization. *Soil Sci. Soc. Am. J.* 55:1020-1025.
- Bakken, L.R., Bergaust, L. Liu, B., Frostegard, A. 2012. Regulation of denitrification at the cellular level: a clue to understanding of N₂O emissions from soils. *Philos. T. Roy. Soc. B.* 82: 87-98.
- Battany, M.C., and Grismer, M.E. 2000. Rainfall runoff and erosion in Napa Valley vineyards: effects of slope, cover and surface runoff. *Hydrol. Processes* 14: 1289-1304.
- Bayer, C., Mielniczuk, J., Giasson, E., Martin-Neto, L., and Pavinato, A. 2006. Tillage effects on particulate and mineral-associated organic matter in two tropical Brazilian soils. *Commun. Soil Sci. Plant Anal.* 37: 389-400.
- Cory, R.M., and McKnight, D.M. 2005. Fluourescence spectroscopy reveals ubiquitous presence of oxidized and reduced quinones in DOM. *Environ. Sci. Technol.* 39: 8142-8149.
- Coyne, M.S. *Soil Microbiology: An Explanatory Approach*. Delmar Publishers, Albany.
- Dabney, S.M., Delgado, J.A., and Reeves, D.W. 2001. Using winter cover crops to improve soil and water quality. *Commun. Soil Sci. Plant Anal.* 32: 1221-1250.
- Davidson, E.A., Belk, E., and Boone, R.D. 1998. Soil water content and temperature as independent or confounded factors controlling soil respiration in a temperate mixed hardwood forest. *Glob. Change Biol.* 4: 217-227.
- Dean, J.E., and Weil, R.R. 2009. Brassica cover crops for nitrogen retention in the mid-Atlantic coastal plain. *J. Environ. Qual.* 38: 520-528.

- Doran, J.W., and Smith, M.S. 1991. Nitrogen cycling: Role of cover crops in nitrogen cycling. In: Hargrove, W.L. editor. *Cover Crops for Clean Water*. Ankeny, IA: Soil and Water Conservation Society, pg 85-107.
- Eilers, K.G., Lauber, C.I., Knight, R., and Fierer, N. 2010. Shifts in bacterial community structure associated with inputs of low molecular weight carbon compounds to soil. *Soil Biol. Biochem.* 42: 896-903.
- Fang, H., Cheng, S., Wang, Y., Yu, G., Xu, M., Dang, X., Li, L., and Wang, L. 2014. Change in soil heterotrophic respiration, carbon availability, and microbial function in seven forests along a climate gradient. *Ecol. Res.* DOI 10.1007/s11284-014-1194-6.
- Fellman, J.B., Hood, E., Edwards, R.T., and Jones, J.B. 2009. Uptake of dissolved allochthonous organic matter from soil and salmon in coastal temperate rainforest streams. *Ecosystems*. 12:747-759.
- Grieve, I.C. 1985. Determination of dissolved organic matter in streamwater using visible spectrophotometry. *Earth Surf. Proc. Land* 10:75-78
- Griffiths, B.S., Spilles, A., and Bonkowski, M. 2012. C:N:P stoichiometry and nutrient limitation of the soil microbial biomass in a grazed grassland site under experimental P limitation or excess. *Ecol. Proc.* 1: 6-16.
- Griffiths N.A., Tank, J.L., Royer, T.D., Rose-Marshall, E.J., Whiles, M.R., Chambers, C.P., Frauendorf, T.C., Evans-White, M.A. 2009. Rapid decomposition of maize detritus in agricultural headwater streams. *Ecol. Appl.* 19: 133-142.
- Guimaraes, D.V., Gonzaga, M.I.S., da Silva, T.O., da Silva, T.L., da Silva Dias, N., Silva Matias, M.I. 2013. Soil organic matter pools and carbon fractions in soil under different land uses. *Soil Till. Res.* 126: 177-182.
- Haney, R.L., and Haney, E.B. 2010. Simple and rapid laboratory method for rewetting dry soil for incubations. *Commun. Soil Sci. Plan.* 41:1493-1501.
- Jandl, R., and Sollins, P. 1997. Water-extractable soil carbon in relation to belowground carbon cycle. *Biol. Fertil. Soils* 25: 196-201.
- Kellogg, C.H., and Bridgham, S.D. 2002. Colonization during early succession of restored freshwater marshes. *Can. J. Bot.* 80: 176-185.

- Ketterings, Q.M., Kingston, J., McIlvennie, S., Long, E., Godwin, G., Gami, S. Stanyard, M., and Czymmek, K. 2011. What's Cropping Up? A newsletter for New York field crops and soils Vol. 21.
- Kogel-Knabner, I. 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biol. Biochem.* 34: 139-162.
- Kritzberg, E.S., Cole, J.J., Pace, M.M., and Graneli, W. 2006. Bacterial growth on allochthonous carbon in humic and nutrient-enriched lakes: Results from whole-lake ^{13}C additions. *Ecosystems*. 9: 489-499.
- Kumar, S., Nakajima, T., Mbonimpa, E.G., Gautam, S. Somireddy, U.R., Kadono, A., Lal, R., Chintala, R., Rafique, R., and Fausey, N. 2014. Long-term tillage and drainage influences on soil organic carbon dynamics, aggregate stability and corn yield. *Soil Sci. Plant Nutr.* 60: 108-118.
- Lacey, C.G., and Armstrong, S.D. 2014. In field measurements of nitrogen mineralization following fall applications of N and the termination of winter cover crops. *Air Soil Water Res.* 7: 53-59.
- Lewis, W.M., and Canfield, D. 1977. Dissolved organic carbon in some dark Venezuelan waters and a revised equation for spectrophotometric determination of dissolved organic carbon. *Arch. Hydrobiol.* 79: 441-445.
- Linn, D.M., and Doran, J.W. 1984. Effect of water-filled pore space on carbon dioxide and nitrous oxide production in tilled and nontilled soils. *Soil Sci. Soc. Am. J.* 48:1267-1272.
- Lu, Y., Bauer, J.E., Canuel, E.A., Yamashita, Y., Chambers, R.M., and Jaffe, R. 2013. Photochemical and microbial alteration of dissolved organic matter in temperate headwater streams associated with different land use. *J. Geophys. Res.* 118: 1-15.
- Machefert, S.E., and Dice, N.B. 2004. Hydrological controls on denitrification in a riparian ecosystem. *Hydrol. Earth Syst. Sci.* 8: 686-694.
- Magill, A.H., and Aber, J.D. 2000. Dissolved organic carbon and nitrogen relationships in forest litter as affected by nitrogen deposition. *Soil Biol. Biochem.* 32:603-613.
- Mary, B., Recous, S., Darwis, D., and Robin, D. 1996. Interactions between decomposition of plant residues and nitrogen cycling in soil. *Plant Soil.* 181: 71-82.

- Massicotte, P., and Frenette, J. 2013. A mechanistic-based framework to understand how dissolved organic carbon is processed in a large fluvial lake. *Limnol. Oceanogr.* 3: 139-155.
- McCutchan, J.H., and Lewis, W.M. 2008. Spatial and temporal patterns of denitrification in an effluent-dominated plume river. *Verh. Internat. Verein. Limnol.* 30:323-328.
- Morgan, J.A., Follett, R.F., Allen, L.H., Del Grosso, S., Derner, J.D., Dijkstra, F., Franzluebbers, A., Fry, R., Paustian, K., and Schoeneberger, M.M. 2010. Carbon sequestration in agricultural lands of the United States. *J. Soil Water Conserv.* 65: 6-13.
- Mulholland, P.J. 2003. Large-scale patterns in dissolved organic carbon concentration, flux, and sources. In: *Aquatic Ecosystems: Interactivity of Dissolved Organic Matter* (Eds. Findlay, S.E.G. and Sinsabaugh, R.L.), pp. 139-159. Academic Press, San Diego, CA.
- Murphy, K.R., Stedmon, C.A., Graeber, D., and Bro, R. 2013. Fluorescence spectroscopy and multi-way techniques. *PARAFAC. Anal. Methods* 5: 6557-6566.
- Olson, K.R., Ebelhar, S.A., Lang, J.M. 2010. Cover crop effects on crop yields and soil organic carbon content. *Soil Sci.* 175: 89-98.
- Ohno, T., and Bro, R. 2006. Dissolved organic matter characterization using multiway spectral decomposition of fluorescence landscapes. *Soil Sci. Soc. Am. J.* 70: 2028-2037.
- Peregrina, F., Larrieta, C., Ibanez, S., and Garcia-Escudero, G. 2010. Labile organic matter, aggregates, and stratification ratios in a semiarid vineyard with cover crops. *Soil Sci. Am. J.* 74: 2120-2130.
- Pinney, M.L., Westerhoff, P.K., Baker, L. 2000. Transformations in dissolved organic carbon through constructed wetlands. *Wat. Res.* 34: 1897-1911.
- Qualls, R.G., and Haines, B.L. 1992. Biodegradability of dissolved organic matter in forest throughfall, soil solution, and stream water. *Soil Sci. Soc. Am. J.* 56: 578-586.
- Ramirez, K.S., Craine, J.M., and Fierer, N. 2012. Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes. *Glob. Chang. Biol.* 18: 1918-1927.

- Reeves, D.W. 1997. The role of soil organic matter in maintaining soil quality in continuous cropping systems. *Soil Till. Res.* 43: 131-167.
- Sarkhot, D.V., Grunwald, S., Ge, Y., and Morgan, C.L.S. 2012. Total and available soil carbon fractions under the perennial grass *Cynodon dactylon* (L.) Pers and the bioenergy crop *Arundo donax* L. *Biomass Bioenerg.* 41:233-130.
- Sondergaard, M., and Middelboe, M. 1995. A cross-system analysis of labile dissolved organic carbon. *Mar. Ecol. Prog. Ser.* 118: 283-294.
- Stanley, E.H., Powers, S.M., Lottig, N.R., Buffam, I., and Crawford, J.T. 2011. Contemporary changes in dissolved organic carbon (DOC) in human-dominated rivers: is there a role for DOC management? *Freshwater Biol.* 57: 26-42.
- Stedmon, C.A., Markager, S., and Bro, R. 2003. Tracing dissolved organic matter in aquatic environments using a new approach to fluorescence spectroscopy. *Mar. Chem.* 82: 239-254.
- Stedmon, C.A., and Markager, S. 2005. Resolving variability in dissolved organic matter fluorescence in a temperate estuary and its catchment using a PARAFAC analysis. *Limnol. Oceanogr.* 50:686-697.
- Steenwerth, K., and Belina, K.M. 2008. Cover crops enhance soil organic matter, carbon dynamics and microbiological function in a vineyard agroecosystem. *Appl. Soil Ecol.* 40: 359-369.
- Stevenson, F.J. 1994. *Humus chemistry*. 2nd ed. Wiley-Interscience, New York.
- Stutter, M.I., Lumsdon, D.G., and Cooper, R.J. 2007. Temperature and soil moisture effects on dissolved organic matter release from a moorland Podzol O horizon under field and controlled laboratory conditions. *Eur. J. Soil Sci.* 58: 1007-1016.
- Tranvik, L.J. 1992. Allochthonous dissolved organic matter as an energy source for pelagic bacteria and the concept of the microbial loop. *Hydrobiologia.* 229:107-114.
- Unger, P.W., and Vigil, M.F. 1998. Cover crops effects on soil water relationships. *J. Soil Water Cons.* 53:241-244.
- USDA Natural Resources Conservation Service. 2011. Carbon to nitrogen ratios in cropping systems. Technical Report. USDA NRCS East National Technology Support Center, Greensboro, NC.

- Van Trump, J.I., Wrighton, K.C., Thrash, J.C., Weber, K.A., Andersen, G.L., and Coates, J.D. 2011. Humic acid-oxidizing, nitrate-reducing bacteria in agricultural soils. *Microbiology*. 2: 1-9.
- Villamil, M.B., Bollero, G.A., Darmody, R.G., Simmons, F.W., and Bullock, D.G. 2006. No-till corn/soybean systems including winter cover crops: Effects on soil properties. *Soil Sci. Soc. Am J.* 70: 1936-1944.
- Westhorpe, D.P., Mitrovic, S.M., Ryan, D., and Kobayashi, T. 2010. Limitation of lowland riverine bacterioplankton by dissolved organic carbon and inorganic nutrients. *Hydrobiologia*. 652: 101-117.
- Wetzel, R.G., and Manny, B.A. 1972. Decomposition of dissolved organic carbon and nitrogen compounds from leaves in an experimental hard water stream. *Limnol. Oceanogr.* 17: 927-931.
- Woodmansee, R.G., and Duncan, D.A. 1980. Nitrogen and phosphorus dynamics in annual grassland. *Ecology*. 63:893-904.
- Yuste, J.C., Baldocchi, D.D., Gershenson, A., Goldstein, A., Misson, L., and Wong, S. 2007. Microbial soil respiration and its dependency on carbon inputs, soil temperature and moisture. *Glob. Change Biol.* 13: 1-18.
- Zarnetske, J.P., Haggerty, R., Wondzell, S.M., Baker, M.A. 2011. Labile dissolved organic carbon supply limits hyporheic denitrification. *J. Geophys. Res.* 116:1-13.
- Zsolnay, A. 1996. Dissolved humus in soil waters. In: Piccolo, A. (Ed.), *Humic Substances in Terrestrial Ecosystems*. Elsevier, Amsterdam, pp. 171-223.

Figure 12. Mean WEOC concentrations of all soil cores collected throughout sampling period. Bars are separated by crop treatment and soil core depth: hollow (0-5cm) and grey (5-20cm).

Figure 13. Change in WEOC concentration within the upper soil profile (0-5cm) throughout the study period. Crop treatments denoted by lines as follows: zero control (dashed black), corn (grey), and cover crop (black).

Figure 14. Change in WEOC concentration within the lower soil profile (5-20cm) throughout the study period. Crop treatments denoted by lines as follows: zero control (dashed black), corn (grey), and cover crop (black).

Figure 15. Excitation-emission matrices of the two components identified from the PARAFAC model. Components separated by letter to denote the different crop treatments as follows: A (zero control), B (Corn), and C (Cereal rye/Tillage radish).

Figure 16. Mean average percent contribution of each component by the PARAFAC model from soils of each crop treatment. Significant differences denoted by an asterisk ($p < 0.05$).

Figure 17. Overall mean denitrification rates of all sampling dates. No significant differences were observed between crop treatments.

Figure 18. Mean denitrification rates by crop treatment over time. Crop treatments are denoted as follows: Zero control (no fill), corn (grey), cover crop (diagonal).