

NITROGEN REMOVAL AND ASSOCIATED GREENHOUSE GAS PRODUCTION  
IN LABORATORY-SCALE TREATMENT WETLANDS

by

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## DEDICATION

This work is dedicated to: My family, Dr. W. Jones and all those who have inspired me to believe both in me and the powerful things that result from working with others.

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## TABLE OF CONTENTS

|                                                                                                                         |    |
|-------------------------------------------------------------------------------------------------------------------------|----|
| 1. INTRODUCTION .....                                                                                                   | 1  |
| Thesis Overview.....                                                                                                    | 14 |
| 2. PLANT INFLUENCE ON DENITRIFICATION IN TREATMENT<br>WETLANDS.....                                                     | 18 |
| Contribution of Authors and Co-Authors .....                                                                            | 18 |
| Manuscript Information Page.....                                                                                        | 19 |
| Keywords .....                                                                                                          | 20 |
| Abstract .....                                                                                                          | 20 |
| Introduction .....                                                                                                      | 21 |
| Methods.....                                                                                                            | 26 |
| Statistical Analysis .....                                                                                              | 29 |
| Results .....                                                                                                           | 30 |
| Water Quality.....                                                                                                      | 31 |
| Plant Biomass and Nitrogen Uptake .....                                                                                 | 40 |
| Discussion .....                                                                                                        | 42 |
| Conclusions.....                                                                                                        | 46 |
| Acknowledgments.....                                                                                                    | 48 |
| 3. INFLUENCE OF PLANTS AND ORGANIC CARBON ADDITION<br>ON GREENHOUSE GAS EMISSIONS FROM MODEL<br>TREATMENT WETLANDS..... | 49 |
| Contribution of Authors and Co-Authors .....                                                                            | 49 |
| Manuscript Information Page.....                                                                                        | 50 |
| Abstract .....                                                                                                          | 51 |
| Introduction .....                                                                                                      | 52 |
| Carbon Dioxide .....                                                                                                    | 53 |
| Methane.....                                                                                                            | 55 |
| Nitrous Oxide.....                                                                                                      | 56 |
| Methods.....                                                                                                            | 58 |
| Data and Statistical Analysis: .....                                                                                    | 61 |
| Results and Discussion.....                                                                                             | 62 |
| Carbon Dioxide Emissions: .....                                                                                         | 63 |
| Methane Emissions .....                                                                                                 | 69 |
| Nitrous Oxide Emissions: .....                                                                                          | 69 |
| Integration: .....                                                                                                      | 78 |
| Conclusions .....                                                                                                       | 79 |
| Acknowledgements .....                                                                                                  | 81 |

## TABLE OF CONTENTS – CONTINUED

|                                                                                                                           |     |
|---------------------------------------------------------------------------------------------------------------------------|-----|
| 4. EMPIRICAL MODELING OF PLANT INFLUENCE AND ORGANIC CARBON ADDITION ON NITROGEN REMOVAL IN TREATMENT WETLANDS.....       | 82  |
| Contribution of Authors and Co-Authors .....                                                                              | 82  |
| Manuscript Information Page.....                                                                                          | 83  |
| Abstract .....                                                                                                            | 84  |
| Introduction.....                                                                                                         | 85  |
| Rate Models: .....                                                                                                        | 89  |
| Methods.....                                                                                                              | 93  |
| Statistical Analysis:.....                                                                                                | 95  |
| Results and Discussion.....                                                                                               | 96  |
| Conclusions.....                                                                                                          | 110 |
| 5. FUTURE DIRECTIONS .....                                                                                                | 112 |
| Plant Mediated Nitrate Removal.....                                                                                       | 113 |
| Biokinetic Modeling .....                                                                                                 | 116 |
| Rhizosphere Analysis.....                                                                                                 | 120 |
| Conclusions .....                                                                                                         | 122 |
| APPENDIX A: Temperature, Plant Species and Residence Time Effects<br>on Nitrogen Removal in Model Treatment Wetlands..... | 125 |
| REFERENCES CITED.....                                                                                                     | 139 |

## LIST OF TABLES

| Table                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Page |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Table 1. Cumulative nitrogen removal over one annual cycle and above-ground plant biomass at the end of the study. ....                                                                                                                                                                                                                                                                                                                            | 36   |
| 2. Table 2. Dissolved organic carbon concentration averaged over days 3 to 15 at 4°C and 24°C. ....                                                                                                                                                                                                                                                                                                                                                   | 37   |
| 3. Table 3. Mass of total dissolved nitrogen attributed to plant uptake and microbial removal over the study period. ....                                                                                                                                                                                                                                                                                                                             | 41   |
| 4. Table 4. Mean measured greenhouse gas emissions rates and total dissolved N ion removal over a 15-day incubation and above-ground biomass at the end of the experiment as influenced by season and plant species treatment (standard error is given in parenthesis).....                                                                                                                                                                           | 64   |
| 5. Table 5. Frequency of either a zero, first or second order model best representing the time series data for a unique treatment combination based on the $R^2$ value separated by plant treatment and OC load. Each block of three model results sums to 10, representing the number of incubations analyzed, with the exception of the Phragmites OC0x and OC1x, and the Schoenoplectus OC2x, each of which had one incubation analysis fail. .... | 97   |
| 6. Table 6. Comparison of $R^2$ values between the single factor model and the k-C* model aggregated by organic carbon load and plant treatment.....                                                                                                                                                                                                                                                                                                  | 100  |
| 7. Table 7. k-C* model results summary with the rate constant at 20 °C ( $k_{20}$ ) on the left followed by the temperature coefficient $\Theta_K$ followed by the incubation residual $C^*$ and the initial concentration $C_0$ with their respective temperature coefficient. ....                                                                                                                                                                  | 102  |

## LIST OF FIGURES

| Figure                                                                                                                                                                                                                                                                                                                                                                            | Page |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1. Figure 1. Experimental set-up with low pressure blower system and headspace sample bags in place. Photo from the 24 °C sample period in 2013.....                                                                                                                                                                                                                              | 8    |
| 2. Figure 2. Reprinted from Taylor et al. (2011). Day-6 COD removal (percent) and SO <sub>4</sub> concentration (mg/L) for each species and unplanted controls at 4 °C and 24 °C. The upper 9 species had a study-long average COD removal >90% (dashed vertical line); species in the lower group averaged <90% .....                                                            | 10   |
| 3. Figure 3. Total dissolved nitrogen concentrations on incubation day six across an annual temperature seasonal cycle. Each temperature step along the x-axis represents a 45-day time increment, proceeding from summer, through fall to winter and back through spring to summer. Dashed horizontal line at 72 mg/l represents mean nitrogen feed solution concentration. .... | 32   |
| 4. Figure 4. Nitrate and ammonium concentrations across the 15-day incubation at 24 °C in the summer of 2013. Nitrogen is on the left scale, ammonium on the right. Upper panels are for OC0x, middle for OC1x and bottom for OC2x. The illustrated data represent the mean of four replicates. ....                                                                              | 33   |
| 5. Figure 5. Nitrate and ammonium concentrations across the 15-day incubation at 4 °C in the winter of 2013. Nitrogen is on the left scale, ammonium on the right. Upper panels are for OC0x, middle for OC1x and bottom for OC2x. The illustrated data represent the mean of four replicates. ....                                                                               | 34   |
| 6. Figure 6. Mean organic (DOC) and inorganic (DIC) carbon concentrations across the 15-day incubation at 4 °C in the winter of 2013. The illustrated data represent the mean of four replicates. ....                                                                                                                                                                            | 39   |
| 7. Figure 7. CO <sub>2</sub> emissions from four replicate columns with and without COD addition separated by plant species and season, winter (4°C) and summer (24°C). Headspace sampling occurred on incubation days 0-1, 2-3, 5-6, 9-10, and 14-15. The line represents sample mean and error bars represent 95% confidence intervals .....                                    | 66   |

## LIST OF FIGURES – CONTINUED

| Figure                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Page |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 8. Figure 8. N <sub>2</sub> O emissions (top panel) and total dissolved nitrogen values (bottom panel) from four replicate columns with and without COD addition separated by plant species and season, winter (4°C) and summer (24°C). Headspace sampling occurred on incubation days 0-1, 2-3, 5-6, 9-10, and 14-15. Dissolved nitrogen water quality sampling occurred on days 0, 1, 3, 6, 9, and 15. The line represents sample mean with error bars represent 95% confidence intervals..... | 71   |
| 9. Figure 9. The emission ratio of the quantity of N <sub>2</sub> O emitted to the total amount of dissolved nitrogen removed by the system over the duration of the 15-day incubation. Please note difference in scale between summer and winter. ....                                                                                                                                                                                                                                          | 74   |
| 10. Figure 10. Total dissolved nitrogen removal across a batch with model approximations for the unplanted OC0x, OC1x, and OC2x treatments in the fall 18 °C temperature step. ....                                                                                                                                                                                                                                                                                                              | 99   |
| 11. Figure 11. Seasonal model of microbial removal vs. plant uptake for Carex, Phragmites, and Schoenoplectus. with OC0x on the left and OC2x on the right.....                                                                                                                                                                                                                                                                                                                                  | 108  |
| 12. Figure 12. Schoenoplectus OC2x treatment removed from column during destructive sampling. The root bound nature of the subsurface can be seen by both the mat of roots against the column wall, and the ability of the roots to self-support the gravel.....                                                                                                                                                                                                                                 | 114  |
| A1. Figure A1. Percent removal values for TN, COD, and Sulfate at Day 6 of aggregated 9 and 20-day batch lengths. Points represent mean values; vertical bars represent the maximum and minimum values. ....                                                                                                                                                                                                                                                                                     | 135  |
| A2. Figure A2. Percent removal of TN, COD, and sulfate at the final batch day for batch lengths of 3, 6, 9, and 20 days. Points represent mean values,; vertical bars represent maximum and minimum values.....                                                                                                                                                                                                                                                                                  | 137  |

## ABSTRACT

Treatment wetlands (TWs) are designed to treat domestic wastewater and water polluted from non-point sources such as agricultural runoff. Because many recent design improvements have increased aerobic removal pathways, nearly complete removal of biological oxygen demand (BOD) and oxidation of ammonium to nitrate in domestic wastewater is possible. These improvements have come at the expense of reducing the TW capacity to remove nitrate. Nitrate is also a main pollutant of concern in many non-point pollution sources. Organic carbon (OC) is a limiting factor for microbial nitrate removal and in wetlands can be supplied externally or provided by plants. Nitrate removal has the potential to release greenhouse gases (GHG) resulting in TWs being capable of acting as a net source or sink for GHG. Increasing our understanding of nitrogen removal and GHG production in TWs is the overarching goal of this project

A multi-year controlled environment greenhouse study measured water quality within 15-day incubations over annual cycles of temperature as well as greenhouse gas production at the seasonal extremes of the annual cycle. The experiment consisted of microcosms planted with either *Carex utriculata*, *Deschampsia cespitosa*, *Phragmites australis*, *Schoenoplectus acutus*, *Typha latifolia* or left unplanted. The fully factorial experiment also included three levels of OC addition, ranging from zero to two times the stoichiometric equivalent required for complete nitrogen removal.

Nitrogen removal was affected by all experimental factors; plant species, OC addition, and temperature, with plant species mediating the effects of carbon and temperature in some treatments. The three highest performing species, *C. utriculata*, *P. australis* and *S. acutus*, removed nitrogen at an annual rate exceeding  $166 \text{ g m}^{-2} \text{ yr}^{-1}$ , without OC; only *C. utriculata* showed less N removal in winter. Incubation time series analysis indicated greater total and seasonal removal capacity for these plant treatments. Total GHG emission was dominated by summer  $\text{CO}_2$  emission and varied by plant treatment and carbon load.  $\text{CO}_2$  emission correlated negatively with OC addition in the high performing species attributable to plant biomass that decreased with OC addition.  $\text{N}_2\text{O}$  production significantly increased with the addition of organic carbon and did not vary significantly by season.

## CHAPTER 1

## INTRODUCTION

Treatment or constructed wetlands (TWs) are highly engineered water quality remediation systems designed to treat collected municipal and domestic wastewater as well as polluted water from non-point sources such as agricultural and stormwater runoff. TW design has undergone many technical improvements over the last several decades, especially in regards to their ability to treat municipal wastewater with high organic carbon loads. Many of the technical design improvements in wetlands have emanated from Europe and focused on the removal of biological oxygen demand (BOD) and the oxidation of ammonium (Brix and Arias 2005, Langergraber, Leroch et al. 2009, Abou-Elela, Golinielli et al. 2013, Sani, Scholz et al. 2013). These treatment processes are primarily aerobic and remove enough pollutants to reduce acute impacts on the ecology of receiving waters. The focus on aerobic treatment potentially leaves significant quantities of nitrate in the effluent. In the regulatory environment of the United States, the removal of nitrogen including nitrate and nitrite is paramount due to the environment and health impacts associated with nitrate pollution—namely eutrophication of receiving water bodies and the potential for nitrate consumption to interfere with oxygen transport in the bodies of small children. Because of the lack of proven total nitrogen removal of some European designs, the application of TWs for municipal wastewater treatment in the US is currently limited without additional nitrate abatement. In addition, many non-point pollution sources, like agricultural runoff, contain significant quantities of nitrate. Regardless of source, the levels of nitrate in the water may be environmentally significant

but the availability of organic carbon, which can fuel microbial nitrate removal (Knowles 1982), is limited. The level of nitrate pollution that is considered significant is situationally dependent, and can vary from domestic treatment facilities that need to decrease nitrate concentrations to below the US EPA drinking water standard of  $10 \text{ mg N l}^{-1}$  to non-point source pollution with wetlands used to reduce the mass of total N loading from a watershed with concentrations of nitrate that may already be less than  $1.2 \text{ mg N l}^{-1}$  to reduce potential eutrophication. Wetlands have been used to remove nitrate from carbon limited waters, however, the full capacity of wetlands to facilitate removal is not currently well known, and amelioration of nitrate rich sources has the potential to result in significant outputs of damaging greenhouse gases (GHG) (Baker 1998, Ingersol and Baker 1998, Hume, Fleming et al. 2002, Pícek, Čížková et al. 2007, Iannelli, Bianchi et al. 2011, Groh, Gentry et al. 2015). Increasing our understanding of the holistic capacity of TWs to facilitate nitrogen removal from carbon limited waste streams is the focus of this thesis. Investigating whether and how we might best balance improvements in water quality with changes in air quality is also considered.

Nitrogen removal in TWs is achieved through the interplay between physical, plant and microbial processes. Microbial denitrification is a stepwise reduction of nitrate ( $\text{NO}_3^-$ ) to dinitrogen gas ( $\text{N}_2$ ) and is often presumed to be the primary permanent removal mechanism for nitrogen in a TW (Faulwetter, Gagnon et al. 2009). When denitrification does not run to completion, there is the potential for the release of nitrous oxide ( $\text{N}_2\text{O}$ ), a potent GHG. Denitrification is performed by heterotrophic microorganisms that couple organic carbon with oxidized nitrogen compounds as the intermediate electron acceptor.

Often water rich in nitrate have low levels of organic carbon which inhibits denitrification, lowering nitrate removal efficacy and potentially increasing the ratio of  $\text{N}_2\text{O}$  emissions to total nitrogen removed. Because the potency of  $\text{N}_2\text{O}$  over a 100 year timeframe is approximately 265 times the GHG capacity of  $\text{CO}_2$  (Myhre, Shindell et al. 2013), relatively small increases in  $\text{N}_2\text{O}$  emissions can change a wetland from a net sink of GHG to a net producer. When a wetland becomes a producer of GHG, the holistic success of the system is debatable, as improvements in water quality must be balanced against atmospheric pollution.

Dominant removal processes in TWs are influenced by physical design of the wetland. Wetland design can be characterized according to flow regimes, including free water surface, subsurface horizontal flow (SSHF) and vertical flow (VF) TWs. Early wetland designs were primarily free water surface or subsurface horizontal flow wetlands (Kadlec and Wallace 2008). In these systems, aerobic processes such as organic carbon removal and nitrification are often limited by the rate at which oxygen diffuses into the water surface and/or the rate at which plants facilitate removal. Desired loading rates outstripped many TWs capacity to remove organic carbon and nitrogen. To increase oxygen transfer, the wetland flow regime was altered to apply water in doses to the top of the wetland from which the water flows vertically down to collection pipes below. In these VF systems pulsing of the wastewater allows for aeration of the subsurface between doses, physically increasing the aerobic capacity. With VF systems, the removal of organics and ammonium can be very high, with performance expectations similar to more

traditional systems but with lower capital costs, and much lower operating costs (especially in electrical power requirements).

The presence of macrophytes almost universally improves carbon and nitrogen removal in TWs (Brisson and Chazarenc 2009). The effects of plants vary according to both physical design of the system and the plants used. Most of the research, especially species-specific research, has focused on the ability of plants to facilitate oxygen transfer into the subsurface of horizontal flow wetlands receiving organics rich wastewater and plant effects on microbial activity (Stein and Hook 2005, Vymazal 2011). In horizontal flow wetlands plant presence has been found to increase oxygen transfer and microbial activity proximal to the plant roots, resulting in system wide increases in organic removal. The research on plant inputs also helped to determine the practical limits of plants facilitating organic carbon and nitrogen removal through oxygen input and plant uptake of nutrients like nitrogen and phosphorus. As the mass of nitrogen loading to the system exceeds  $120 \text{ g m}^{-2} \text{ yr}^{-1}$ , primary removal mechanisms switch from plant to microbe dominated processes. In VF systems total nitrogen loading can exceed  $1000 \text{ g N m}^{-2} \text{ yr}^{-1}$  and plants play an important but often supporting role to the microbial communities in the subsurface by increasing the available surface area for microbial biofilm attachment, and by providing other less understood mechanisms for increasing microbial activity. Thus as TW design has improved to incorporate more physical aeration, plant specific research has focused on understanding how plants effect the subsurface microbial communities opposed to the quantification of various plant inputs.

One of the primary effects of plants in wetland systems is the introduction of organic matter and labile carbon compounds (Christensen, Panikov et al. 2003). In wetlands designed for the amelioration of nitrate polluted waters, carbon input and thus denitrification potential may be directly related to primary plant production, making the effects of plants a critical design parameter that is not currently well investigated (Iannelli, Bianchi et al. 2011). In general, as wetland modeling improves and the effects of loading and flow regime become more calculable, the selection of macrophyte species may become the next most important optimization step in wetland design (Brisson and Chazarenc 2009).

This dissertation work was performed to investigate the effects of five macrophyte plants on the remediation of nitrate-rich wastewater and associated greenhouse gas production across an annual seasonal cycle with varying amounts of additional carbon addition. The goals were to:

- Determine the seasonal capacity for wetland plants to facilitate nitrogen removal without additional carbon.
- Determine the plant, carbon loading and seasonal scenarios that minimize the ratio of  $\text{N}_2\text{O}$  production to total nitrogen removal.
- Elucidate the plant and microbial processes responsible for the water and air quality changes observed.

General experimental overview:

The above goals were addressed using a four-year controlled environment greenhouse study which measured water quality within 15 day incubations at

temperatures ranging from 4 to 24 °C, designed to mimic an annual cycle. Greenhouse gas production was measured at the seasonal extremes of the simulated annual cycle. Eighteen replicates each of unplanted controls and five plant species—*Carex utriculata* (Carex), *Deschampsia cespitosa* (Deschampsia), and *Phragmites australis* (Phragmites), *Schoenoplectus acutus* (Schoenoplectus) and *Typha latifolia* (Typha)—were used in the experiment. To test the effects of carbon addition on the removal of nitrogen, the 18 replicates of each plant treatment were broken into three carbon treatments, with additions of zero (OC0x), one (OC1x) or two (OC2x) times the stoichiometric amount of organic carbon (as sucrose) required to facilitate complete removal of nitrogen from the feed solution, assuming only nitrification and denitrification.

The three levels of organic carbon addition (OC0x, OC1x, and OC2x) were determined through an experiment using plants and infrastructure remaining from experiments originally performed by Allen et al. (2002). Nitrogen removal was tracked from three organic carbon loads added as sucrose, including zero (OC0x), twice (OC2x) and six (OC6x) times the stoichiometric requirement for complete nitrogen removal. In the planted treatments (*Carex utriculata*, *Schoenoplectus acutus* and *Typha latifolia*), the removal rates between OC2x and OC6x treatments was measurable but not significant. As a result OC2x was chosen as the highest carbon treatment for this experiment.

The fully factorial experimental design consisted of 108 microcosms made from 15-cm diameter by 30-cm deep PVC columns. These columns were refurbished from the work of Taylor et al. (2011), with the addition of approximately 4” collars on the top of the column to add freeboard above the gravel surface, expediting feeding. The columns

were arranged in a randomized block design which included six replicate blocks of three rows of six columns that contained one of each plant treatment. Each row contained a single organic carbon treatment either OC0x, OC1x or OC2x. Plant treatments were randomized within each row, and carbon load treatments were randomized within each block. The columns were attached to a constant head system designed to maintain a consistent water height in the columns regardless of evapotranspiration rates. Custom made plastic bags were fitted on four replicates of the OC0x and OC2x treatments for greenhouse gas sampling (Figure 1). Experimental design and sampling details for both the water quality and greenhouse gas measurements may be found in the methods sections of Chapters 2 and 3 respectively.



Figure 1. Experimental set-up with low pressure blower system and headspace sample bags in place. Photo from the 24 °C sample period in 2013.

The experiment was designed to build on previous mesocosm and microcosm experiments at Montana State University (MSU). The wetland group at MSU has been performing “column” experiments with wetland species since 1995. The initial experiments were designed to investigate the effects of multiple plant species over annual cycles in temperate climates. Initially three plant species—*Carex utriculata*, *Schoenoplectus (Scirpus) acutus*, and *Typha latifolia*—were investigated in great detail (Allen, Hook et al. 2002, Stein and Hook 2005, Stein, Biederman et al. 2006, Stein, Borden-Stewart et al. 2007, Stein, Towler et al. 2007, Allen, Stein et al. 2013b). These column experiments measured the removal of chemical oxygen demand (COD),

ammonium and sulfate over different incubation lengths and loading scenarios. All column experiments were run in batch hydraulic mode allowing for a focus on the kinetics of contaminant removal. These experiments have been pivotal in providing a dataset from which to calibrate biokinetic models of wetland systems. The results from these experiments concluded that plant species selection is an important factor in wetland design with effects that vary seasonally. As an extension of this work, Taylor et al. (2011) screened 19 plant species for their capacity to facilitate COD removal over an annual cycle (Taylor, Hook et al. 2011). A portion of her results can be found in Figure 2.

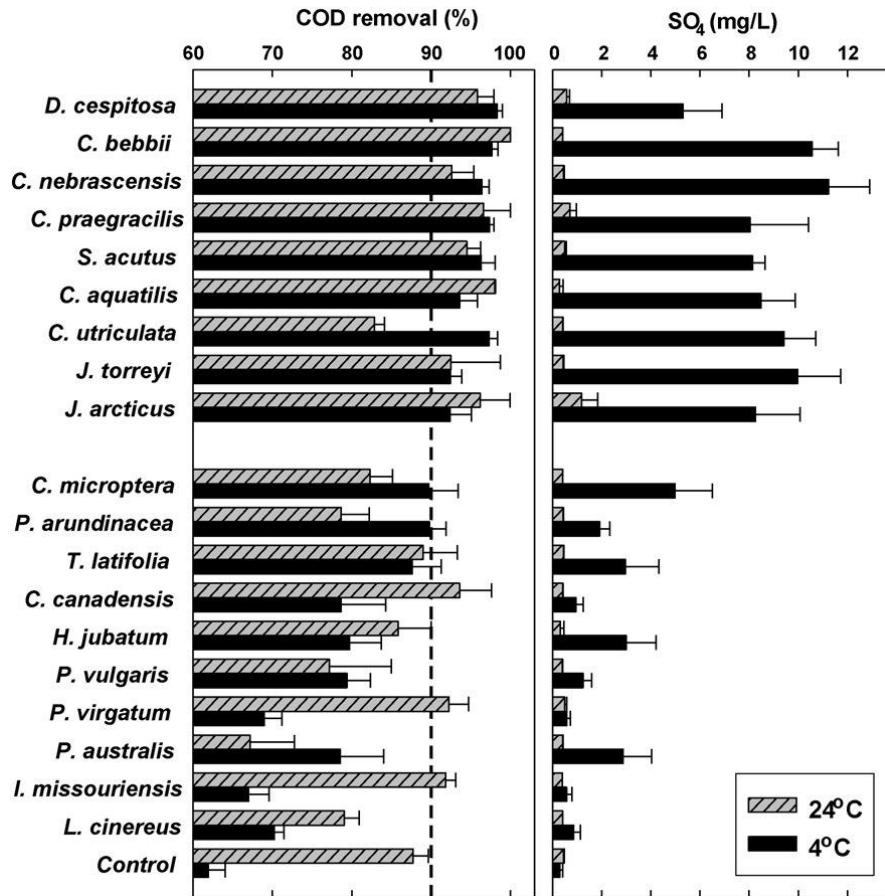


Figure 2. Reprinted from Taylor et al. (2011). Day-6 COD removal (percent) and  $\text{SO}_4^{2-}$  concentration (mg/L) for each species and unplanted controls at 4 °C and 24 °C. The upper 9 species had a study-long average COD removal >90% (dashed vertical line); species in the lower group averaged <90%

The work of Taylor et al. (2011) illustrates a variety of plant specific effects and was used as the basis for plant species selection for this body of work. As illustrated in Figure 2, the top performing species generally increased the removal of COD in the summer and to a much larger extent in the winter compared to the unplanted controls. As indicated by sulfate removal, the summer period was generally more limited by electron acceptor availability, while the winter period stayed sufficiently oxic to inhibit sulfate reduction.

Plant inputs into the subsurface are complex and include ions, gasses such as oxygen, and a host of carbon compounds both released by the plant or resulting from the decomposition of detritus. Much of the MSU group's past research concluded that plant presence facilitated consistent removal of COD, mediating winter reductions in removal found in the unplanted treatments. High winter COD removal was correlated with elevated redox levels, which was attributed to seasonal increases in plant-mediated oxygenation rates as plants senesce and oxygen flows more freely into the subsurface (Stein and Hook, 2005). In some of the experimental plant treatments, specifically *C utriculata* and *P australis* from Taylor et al. (2011) the removal of COD was greater in the winter compared to the summer (Figure 2), and summer removal was less than the unplanted treatment. An alternative hypothesis that may address both increases in winter and decreases in summer COD removal, postulates that root oxygen loss is relatively constant over an annual cycle and that plant derived organic carbon inputs are elevated in summer, promoting heterotrophic growth and increasing the demand for electron acceptors. Active plant growth could increase labile organic carbon release via excess photosynthate from plant roots while warm temperatures increase degradation rates of plant detritus. The net effect would increase the effective subsurface COD load which may decrease the capacity of the system to remove additional COD. The reduction in treatment efficacy that correlated to the warmer months could indicate that a plant species may have been producing large quantities of excess organic carbon and would perform very well as a facilitator for nitrate removal. This introduces the hypothesis that wetland

plant species may be capable of acting as a net producer of either electron donor or electron acceptor depending on the season and overall condition of the rhizosphere.

Plant selection for this experiment was directly related to the results of previous research, primarily that of Taylor et. al (2011). Each plant was selected based on past growth success in the greenhouse environment and to represent a variety of seasonal COD removal performance results. For this experiment five plant species (with family), *Carex utriculata*, and *Schoenoplectus acutus* (Cyperaceae); *Deschampsia cespitosa* and *Phragmites australis* (Poaceae); as well as *Typha latifolia* (Typhaceae), were chosen. *Carex utriculata* was chosen for its robust growth pattern, local climate acceptability, and overall high rate of COD removal with a marked summertime reduction in COD removal efficacy. *Deschampsia cespitosa* was chosen for the overall high and seasonally independent rate of COD removal. *Phragmites australis* was chosen as an international benchmark species, the large body of existing species specific research, and the relatively poor performance in facilitating the removal of COD compared to other plants tested by Taylor et al (2011). *Schoenoplectus acutus* was chosen for the historical research at MSU and the general high rate of COD removal. Lastly *Typha latifolia* was chosen based on the widespread application of the plant in the United States, the historical context at MSU, and the middle of the road treatment efficacy in regards to COD removal. It is important to note that the performance of specific species within a genus varies, illustrated by the performance of the six *Carex* species tested by Taylor et al. (2011) (Figure 2). However, for simplicity these species are referred to by an abbreviation of their genus throughout this dissertation and can be considered a specific plant treatment.

To highlight the fact that each genus name is representing the name of a treatment, which represents a single species, the genus will not be italicized.

All plants with the exception of *P. australis* were re-planted from previous experimental stock. *P. australis* plant stock was sourced from a stand of *P. australis* near Three Forks, Montana that was identified by Carrie Taylor and used as stock in her experiments. Interestingly, a plant sample was sent to Dr. Carla Lambertini at Aarhus University, Denmark for genetic analysis and was found to be a native North American variant (C. Lambertini, personal communication). All plants were reduced to bare root stock, rinsed with de-chlorinated tap water and replanted in washed and re-sieved gravel repurposed from the experiments of Taylor et al. (2011). After replanting, artificial light and elevated temperatures were used to bolster plant growth. A plant establishment rate of over 95% was achieved as only two *Deschampsia* replicates needed to be re-planted. Re-planting occurred approximately 2 months after the initial planting. The replanting was accomplished by halving and separating two *Deschampsia* replicates with robust initial growth.

Plant establishment varied by species. *Carex* treatments qualitatively established the fastest and using data collected in the spring of 2011 were reported in initial conference proceedings as removing more nitrogen than the *Phragmites* treatments (Allen, Stein et al. 2013a). Large variations in the removal efficacy of *Phragmites* appeared to be directly correlated to the long establishment period for the plant. A consistent seasonal cycle was achieved between the summer of 2012 and 2013, the period which forms the basis of most of the water quality data presented. In the early spring of

2013, a first round of gas measurements was performed. The four replicates in each treatment block were subdivided into two groups. Two replicates received a headspace bag, and the other two were left uncovered. By breaking the four replicates into two groups it was hoped to determine if the presence of the headspace sampling bag affected water quality changes. In this first sampling, the variation in water quality data between the replicates with and without a sealed headspace was not significantly different from the variance between replicates without a sealed headspace. The variance in GHG measurements between treatment replicates was considered large, resulting in future gas measurements including four replicates of each treatment. This decision eliminated the potential for water quality comparisons between bagged and non-bagged treatments, but significantly increased the statistical power of the GHG measurements. The experiment was extended into the early spring of 2014 to acquire winter GHG data using a number of replicates consistent with the summer of 2013.

### Thesis Overview

This work encompasses the removal of aqueous nitrogen from carbon limited nitrate rich wastewater and the subsequent production of greenhouse gasses. This dissertation has been organized to include three papers set to be published, concluding with a future directions chapter as described in the subsequent outline. Much of the published work at MSU prior to this experiment has focused on the removal of COD, the dynamics of sulfate, or has provided datasets for biokinetic modeling of wetland systems. Much of the original data collected included nitrogen measurements that remained unanalyzed. As this experiment was establishing in the greenhouse, past nitrogen data

were analyzed to elucidate plant effects on nitrogen removal in past MSU experiments with carbon rich wastewater across seasons and at various loading rates. The results of this analysis are included as an appendix to illustrate the variability in plant effect across similar systems with carbon rich vs. carbon limited influent; emphasizing the hypothesis that specific plant species may be able to act as net contributors of electron donor or acceptor and where a specific species falls on that scale may be dependent on the subsurface conditions.

The major sections of this dissertation are:

Chapter 2: PLANT INFLUENCE ON DENITRIFICATION IN TREATMENT WETLANDS covers the water quality results and further details on the methods. The chapter focuses on a complete seasonal cycle encompassing one year plus 45 days from summer in 2012 through the summer 2013. Though the focus of the paper is on the analysis of nitrogen data, organic carbon and inorganic carbon data are presented and discussed along with the overall efficacy of the wetland plant species and the partitioning of removal pathways between microbial and plant uptake. This chapter will be submitted to the journal *Water Research*.

Chapter 3: INFLUENCE OF PLANTS AND ORGANIC CARBON ADDITION ON GREENHOUSE GAS EMISSIONS FROM MODEL TREATMENT WETLANDS outlines the greenhouse gas emission results focusing on the data collected in the summer of 2013 and the early spring 2014. The interaction between plant removal and microbial

removal pathways is discussed, as counter intuitive results were acquired, indicating that the addition of organic carbon (OC2x treatments) universally produced larger quantities of N<sub>2</sub>O compared to OC0x treatments, despite similar changes in water quality. This chapter will be submitted to the journal *Environmental Science and Technology*.

Chapter 4: EMPIRICAL MODELING OF PLANT INFLUENCE ON DENITRIFICATION IN TREATMENT WETLANDS applies three types of linear approximations to the time series of nitrogen removal data to determine the most likely type of kinetics: zero, first or second order. Further modeling using the k-C\* model was used to determine general removal rates and seasonal effects. Lastly, a hypothetical model for seasonal plant vs. microbial removal is proposed to account for variations in N<sub>2</sub>O production and proposes a carbon banking model for seasonal removal. This chapter will be submitted to the journal *Ecological Engineering*.

Chapter 5: FUTURE DIRECTIONS proposes extensions of this body of work including field applications to determine the scalability of these results, biokinetic models for hypothesis testing surrounding plant microbe interactions, and potential for micro-profile work on plant roots.

Appendix A1: TEMPERATURE, PLANT SPECIES AND RESIDENCE TIME EFFECTS ON NITROGEN REMOVAL IN MODEL TREATMENT WETLANDS discusses the effects of the plant species *Carex utriculata*, *Schoenoplectus acutus*, and

*Typha latifolia* with changes in season on the removal of nitrogen from high COD wastewater. A subset of the data generated in previous MSU wetland column experiments which included total nitrogen values was modeled to account for the degradation of amino-N to total dissolved nitrogen (TDN). Removal of total nitrogen was estimated with time and TDN values to analyze seasonal nitrogen removal. All plant species facilitated expedited and near complete nitrogen removal compared to the unplanted treatments across seasons. This paper was published in the journal *Water Science and Technology*.

## CHAPTER TWO

### PLANT INFLUENCE ON DENITRIFICATION IN TREATMENT WETLANDS

#### Contribution of Authors and Co-Authors

Manuscript in Chapter 2:

Author: Christopher R. Allen

Contributions: Generated physical experimental design. Performed experiment set-up, data collection, analysis and generated the initial draft of the manuscript.

Co-Author: Mark D. Burr

Contributions: Co-author on initial experiment proposal. Assisted with experimental set-up, data collection and sample analysis. Provided edits on the manuscript.

Co-Author: Anne K. Camper

Contributions: Co-author on initial experiment proposal. Advised on experimental design and analysis. Provided edits on the manuscript.

Co-Author: Jefferson J. Moss

Contributions: Assisted with experimental set-up, data collection and sample analysis. Provided edits on the manuscript.

Co-Author: Otto R. Stein

Contributions: Primary investigator on initial experiment proposal. Advised on experimental design and analysis. Provided substantial edits on the manuscript.

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## PLANT INFLUENCE ON DENITRIFICATION IN TREATMENT WETLANDS

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Keywords

Ammonium, *Carex*, constructed wetland, denitrification, *Phragmites*, nitrate, *Schoenoplectus*, season

Abstract

A controlled-environment greenhouse study examined seasonality, plant and influent organic carbon-nitrogen (C:N) ratio effects on total dissolved nitrogen (TDN) removal in batch-loaded subsurface treatment wetlands. Synthetic wastewater with an average TDN of 72 mg l<sup>-1</sup> was fed to 72 microcosms planted in monocultures of five plant species or left unplanted. Water quality was measured at 0, 1, 3, 6, 9, and 15 days within 15-day incubations at eight temperature steps ranging from 4 to 24 °C over a year-long period of seasonal growth patterns. Measured TDN was significantly affected by C:N, temperature, plant species and their interactions. *P. australis* and *S. acutus* microcosms removed virtually all of the applied 175 g N m<sup>-2</sup> yr<sup>-1</sup> load regardless of season and C:N ratio. *C. utriculata* microcosms removed all the applied TDN at warmer temperatures regardless of applied load and at a C:N ratio of 2.4 regardless of season, but removal decreased at colder temperatures for lower C:N ratios. *C. utriculata* performance was still significantly better (total removal 165.7 g N m<sup>-2</sup> yr<sup>-1</sup>) than the unplanted treatment (total removal 56.7

g N m<sup>-2</sup> yr<sup>-1</sup>). In *D. cespitosa* and *T. latifolia* treatments, increasing the C:N ratio increased the removal of TDN at all temperatures, and higher temperatures increased removal for a given C:N ratio.

### Introduction

Efficient removal of total nitrogen coupled with complete conversion of nitrogen to environmentally benign substances such as N<sub>2</sub> gas or into biomass suitable for nutrient recovery and reuse remains a challenging process in treatment wetlands (TWs). Wetland design parameters such as flow regime (horizontal, vertical, tidal etc.), flow sequencing, loading rates, support media properties and plant species all affect the efficacy of total nitrogen removal (Kadlec and Wallace 2008, Tunçsiper 2009, Langergraber, Pressl et al. 2011, Vymazal 2013). As wetland modeling improves and the effects of loading and flow regime become more calculable, the selection of macrophyte species may become the next most important optimization step in wetland design (Brisson and Chazarenc 2009), as plant selection can impact the efficacy of microbial processes and quantity of nutrient removal to standing biomass. Nitrogen removal in TWs is achieved through complicated interactions between biochemical, plant and microbial processes. For nitrogen removal to exceed 120 g N m<sup>-2</sup> yr<sup>-1</sup>, the permanent nitrogen removal processes that are thought to dominate in TWs are microbially mediated (Kadlec and Wallace 2008). Though many alternative and shortcut microbial pathways exist, the predominant microbial pathways for nitrogen removal in most TWs are assumed to be the stepwise processes of nitrification and denitrification (Faulwetter, Gagnon et al. 2009), requiring first aerobic

and then anoxic conditions. Most high-rate wetland systems such as vertical flow and intensified systems are optimized for aerobic microbial processes to remove incoming organic carbon compounds and oxidize incoming nitrogen. Though these systems can achieve total nitrogen removal rates of up to 60%, the resulting effluent typically contains low levels of organic carbon and significant quantities of nitrate (Brix and Arias 2005, Langergraber, Pressl et al. 2011, Paing, Guilbert et al. 2015). The low C:N ratio of the resulting effluent has been shown to limit denitrification, therefore inhibiting nitrogen removal (Tunçsiper 2009). Nitrogen contaminated water with low C:N ratios is not restricted to the effluent from secondary wastewater treatment systems, as agricultural runoff and tile drainage may contain significant quantities of nitrate, without significant quantities of organic carbon (Collins and Gillies 2014). Horizontal flow TWs have been proposed and tested to remediate nitrate-rich waters in both hybrid wastewater treatment wetlands and wetlands designed to treat agricultural runoff (Tanner and Kadlec 2013, Vymazal 2013, Collins and Gillies 2014). In systems with low influent C:N ratios, organic carbon input from wetland plants can fuel denitrification; however, the supplied quantity and its effect on denitrification is largely unknown.

The ability of plants to increase nitrogen removal has been shown to vary with plant species and seasonality (Kadlec and Reddy 2001, Allen, Stein et al. 2013b). The macrophytes in TWs enhance nitrogen removal by several mechanisms. Nitrogen can be directly incorporated into plant biomass, and plants can supply oxygen for nitrification as well as the organic carbon required for denitrification by either direct exudation of excess

photosynthate and other compounds from roots, or indirectly by mineralization of plant detritus (García, Rousseau et al. 2010).

Removal of nitrogen by direct plant uptake compared to microbial processes such as denitrification and assimilation increases as loading decreases (Vymazal 2005, Borin and Salvato 2012). The quantity and location (above or below ground), of nitrogen per mass of dried plant tissue varies by species, level of senescence, season, and nitrogen availability (Kroger, Holland et al. 2007, Vymazal 2011). Vymazal (2011) reported nitrogen uptake values in above ground plant biomass of emergent macrophytes from 0.04 to 88 g N m<sup>-2</sup> yr<sup>-1</sup> in natural wetlands and range between 5.3 and 58.7 g N m<sup>-2</sup> yr<sup>-1</sup> in horizontal flow TWs. Depending on the system's nitrogen loading, these values may represent a significant percentage of the incoming nitrogen. However plant biomass removal is seldom incorporated as part of TW operations; therefore, nitrogen incorporation into biomass is likely a temporary removal mechanism as plant detritus mineralizes back into the system (Vymazal 2011).

Denitrification is generally considered the dominant nitrogen removal mechanism in TWs (Faulwetter, Gagnon et al. 2009), and is a four-reaction microbially-mediated heterotrophic process converting NO<sub>3</sub><sup>-</sup> to N<sub>2</sub> via intermediates: NO<sub>3</sub><sup>-</sup> → NO<sub>2</sub><sup>-</sup> → NO → N<sub>2</sub>O → N<sub>2</sub> (Knowles 1982). Denitrification requires an electron donor like organic carbon for each reaction, so a lack of carbon negatively impacts treatment efficacy and/or increases the relative production of the intermediate product nitrous oxide, a potent greenhouse gas (Kampschreur, Temmink et al. 2009). During their annual lifecycle, wetland plants provide labile organic carbon in the form of exudates during active

periods and the slow release during decay (García, Rousseau et al. 2010, Iannelli, Bianchi et al. 2011), as well as ions and gasses such as oxygen (Hawkes, DeAngelis et al. 2007). Much work has been done to quantify the amount of oxygen that plants introduce to the rhizosphere, its effects on root-associated microorganisms, and the resulting effect on treatment (Armstrong 2000, Colmer 2003, Bezbaruah and Zhang 2004, Taylor, Hook et al. 2011). While plants aerate the subsurface, less is known about various species' ability to introduce electron donors into the subsurface. Estimates of organic carbon exudation rates for several species resulted in the potential to reduce  $9.4\text{--}26.7 \text{ g NO}_3^- - \text{N m}^{-2} \text{ yr}^{-1}$  in one study (Zhai et al., 2013), while others have found that the net input of labile organic carbon compounds is species specific, resulting in different levels of microbial enzymatic activity in the rhizosphere (Salvato, Borin et al. 2012, Zhai, Piwpuan et al. 2013). The total input of labile organic carbon from plant decomposition depends on the TW system design and how easily the plant material can be biodegraded. Most wetland species contain 40-45% carbon by mass; however, the ratio of lignin to cellulose varies and the more ligand within plant tissue, the more difficult it is to biodegrade (Hume, Fleming et al. 2002). The degradation of plant biomass and its availability to microbes in the subsurface also varies with the design of the wetland, as subsurface flow wetlands limit organic carbon input by above-ground plant degradation because the biomass is physically separated from the water by an unsaturated gravel layer.

Compared to unplanted conditions, the presence of wetland plants mitigates the negative effects of cold temperature on COD and ammonium removal in TWs (Stein and Hook 2005, Taylor, Hook et al. 2011, Allen, Stein et al. 2013b). The presence of wetland

plants can increase cold season nitrate removal (Sirivedhin and Gray 2006, Bastviken, Weisner et al. 2009), despite a known reduction in denitrification rates at cold temperatures; however, some studies have shown winter nitrate removal to be very limited without organic carbon addition (Gagnon, Maltais-Landry et al. 2010). Stein and Hook (2005) postulated that observed species-specific seasonal trends in COD removal, sulfate concentrations and oxidation-reduction potential (Eh) were a result of increased oxygenation in winter, potentially resulting from reduced root oxygen demand due to winter dormancy or senescence / dehydration of plants which has been shown to increase oxygen supply via hollow root aerenchyma and open stomates (Colmer 2003). However, the observed patterns could also be attributed to increased plant-induced carbon inputs in summer by either increased rates of photosynthate release or increased decomposition rates of subsurface detritus.

This study investigates the effects of plant species, temperature/season and the ratio of influent organic carbon to nitrogen on nitrogen removal and greenhouse gas emission in model subsurface flow wetland microcosms. To simulate partially treated wastewater entering a sub-surface horizontal flow TW, the total nitrogen load was partitioned into 75% nitrate and 25% ammonium. Three levels of organic carbon, including zero organic carbon, allowed investigation into the plants' implied capacity to act as net producers of organic carbon fueling nitrogen removal via denitrification. The practical goal was to find cold weather appropriate wetlands species that can enhance total nitrogen removal and minimize greenhouse gas production in the second stage of a hybrid wetland system which combines a horizontal flow wetlands after a vertical flow

wetlands. Five plant species representing varying levels of performance for COD removal and possible differences in rootzone oxidation (Taylor, Hook et al. 2011) were chosen to represent a range of expected performance in this study. This paper reports the water quality results.

### Methods

The experiment was performed in a climate-controlled greenhouse located in Bozeman, Montana (45.7° N, 111.0° W) between November 2010 and September 2014. In October 2010, 18 replicates of five plant species (with family), *Carex utriculata*, and *Schoenoplectus acutus* (Cyperaceae); *Deschampsia cespitosa* and *Phragmites australis* (Poaceae), and *Typha latifolia* (Typhaceae), were planted into 15-cm diameter by 40-cm deep PVC columns. Each plant species represents an experimental treatment with the capacity to effect the subsurface environment which may result in variations in treatment efficacy and in similar systems these effects have been shown to be species specific (Taylor et al. 2011). For simplicity all plant treatments will be referred to using the genus name, non-italicized to emphasize the naming convention and the potential variation between species in a genus. All columns, including 18 unplanted controls, were filled to a depth of 30 cm with gravel consisting of approximately 10% mica, 10% aluminum silicates, and 80% quartz and a sorption capacity estimated to be around 190 meq kg<sup>-1</sup>. Both gravel and plants were re-purposed from a previous experiment (Taylor et al. 2011) with the exception of *P. australis*, which was harvested from a nearby riparian area. Gravel from the previous experiment was thoroughly washed and mixed, then re-sieved to pass a number 4 sieve (4.76 mm) and be retained on a number 8 sieve (2.38 mm).

Plants were trimmed to bare roots and rinsed in de-chlorinated tap water before replanting. All columns were connected to a watering system that maintained a constant water level regardless of evapotranspiration. Artificial lighting (16 hours a day) and elevated temperatures (18 °- 24 °C), in conjunction with a reduced-strength synthetic wastewater helped facilitate plant development. In July, 2011 the use of artificial lighting was discontinued and an annual temperature profile mimicking a northern climate was initiated. The temperature was changed on 45-day increments from 24 °C, to 18 °C, to 12 °C, to 8 °C, to 4 °C and stepwise back to 24 °C following the same pattern. In October, 2011 the use of full strength synthetic wastewater began.

The full strength synthetic wastewater contained 18 mg  $\text{NH}_4\text{-N l}^{-1}$  and 54 mg  $\text{NO}_3\text{-N l}^{-1}$ , one of three levels of organic carbon (OC) supplied as sucrose, macronutrients P, Mg, K, Ca, and S as well as micro-nutrients consistent with previous experiments (Taylor, Hook et al. 2011). Every 15 days the TW columns were gravity drained and approximately 1.8 liters of synthetic wastewater was added to each column to initiate an incubation, with three incubations per temperature step. The 18 replicates were subdivided into three treatment groups of six replicates. Each group received either zero organic carbon (OC0x) or one of two levels of organic carbon representing once (OC1x) or twice (OC2x) the stoichiometric equivalent required to remove all nitrogen by classic denitrification. These feed solutions represented OC:N mass ratios of zero, 1.2 and 2.4 and COD:N of zero, 2.7 and 5.5 g COD per g N, respectively. Six groups of 18 columns, each containing one replicate of each combination of OC load and plant treatment were organized in a randomized block design.

Beginning in January 2012, 30 ml water samples were taken at the geometric center of the gravel bed via a permanently installed sample tube. Collection occurred during the third incubation of each temperature and seasonal setting (8 per year) on incubation days 0, 1, 3, 6, 9 and 15. Incubation day zero samples were collected within 30 min of feeding the columns, with all subsequent samplings at the approximate same time of day as the initial feeding. Samples were collected from the center four of the six replicate blocks, totaling 72 columns. The samples were filtered at the time of the collection through a 0.2  $\mu\text{m}$  filter, refrigerated and analyzed within 5 days or frozen for long term storage prior to ion analysis. All samples were analyzed for dissolved concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$  and  $\text{HPO}_4^{2-}$  using ion chromatography (Metrohm USA.); pH as well as approximately 500 total randomized COD measurements were taken, representing every sample day from the first three sample incubations at 24, 18 and 12  $^{\circ}\text{C}$  (HACH company). Filtered samples taken at both temperature extremes (4  $^{\circ}$  and 24  $^{\circ}\text{C}$ ) were also analyzed within five days of collection for dissolved inorganic and dissolved organic carbon (Skalar Formacs). Regular data recording also included the automated collection of temperature and oxidation-reduction potential (redox, Eh) measurements. Routine sampling and data collection ended in September of 2013 (after 16 months), however, regular wastewater feedings occurred until a final destructive sampling of biomass at 24  $^{\circ}\text{C}$  in September of 2014.

To accommodate gas-sampling equipment used for concurrent research, any biomass taller than approximately 80 cm or detritus easily disassociated from the plant was removed and saved prior to gas sampling at the temperature extremes. Total above

ground plant biomass was collected from two replicates of each plant treatment at OC0x and OC2x in September 2013 and March 2014 and from the remaining columns at the end of the experiment in September 2014. All sampled biomass was dried at 40 °C, weighed, and ground for nutrient analysis. Nutrient analysis was performed using a total carbon and nitrogen combustion-based analyzer (LECO Corporation). Total plant nitrogen uptake was estimated by averaging the total nitrogen content for the four destructively sampled replicates for each plant treatment at both OC0x and OC2x levels. The percentage of nitrogen removed by plant uptake during the experiment was calculated by comparing the quantity of nitrogen incorporated into biomass to the total nitrogen measured as removed from the wastewater, assuming consistent nitrogen removal within a temperature step.

### Statistical Analysis

Treatment effects on day-6 TDN were analyzed statistically using linear mixed effects models (function `lme()` in package `nlme`) in the program R version 3.1.2. TDN data were log-transformed for analysis. The focus was on day 6 as temporal variation was generally minimal thereafter, and day 6 is consistent with our previous experiments (Stein and Hook 2006, Taylor, Hook et al. 2011). The three main effects tested were: plant treatment (6 levels: unplanted, *Carex*, *Deschampsia*, *Phragmites*, *Schoenoplectus*, and *Typha*), carbon load (3 levels: OC0x, OC1x, OC2x), and temperature (5-levels: 24, 18, 12, 8, and 4 °C). Variation among replicates within a specific OC load and plant treatment were accounted for as random effects and the models were weighted by the inverse of the inter-replicate variance. Organic carbon load and temperature were treated as ordered

factors. An initial model evaluated random effects for replicate and fixed effects for plant treatment, OC load and temperature, and considered the three two-way interactions and the three-way interaction between them. A “two-way model” considered the same random and fixed effects but only the three two-way interactions. Because other factors often interacted with species, an “intra-species model” was used to test the two-way interaction between carbon load and temperature within each plant treatment. For comparisons of plant biomass and plant nitrogen content, plant treatment specific linear models were used with either sampling season (winter/summer) or OC load as main effects.

### Results

Obtaining adequate plant establishment and determining seasonal behavior was considered paramount, as short duration studies performed during the establishment of plants may not reflect long-term behavior (Brisson and Chazarenc 2009). Plant growth and establishment time varied; *Carex*, *Deschampsia*, *Schoenoplectus* and *Typha* qualitatively grew more rapidly early in the experiment while *Phragmites* took longer to establish. Early establishment of *Carex elata* and *Typha latifolia* compared to *Phragmites australis* has been previously observed (Borin and Salvato 2012); and a full-scale *P. australis*-planted wetland system did not achieve steady state until three growing seasons (Langergraber, Pressl et al. 2011). Unreported water quality measurements taken prior to April 2012 were influenced by plant growth differences. A repeating species-specific seasonal pattern was evident by the end of the second growing season. The data reported herein is from one complete annual cycle from 24 °C to 4 °C to 24 °C (August 2012 to September 2013).

### Water Quality

Total dissolved nitrogen (TDN), the summation of nitrate, nitrite and ammonium, represents the vast majority of nitrogen in the controlled experiments with only dissolved nitrogen inputs. Figure 3 summarizes annual patterns of TDN removal in terms of concentration measurements taken on day six at each temperature. The first 8 °C temperature step data from Fall 2012 is missing due to equipment failure during the water quality analysis for day 6 of that incubation. Figures 4 and 5 show concentrations as a function of time within an incubation at the temperature extremes, 24 °C and 4 °C, respectively. Table 1 summarizes annualized TDN removal rates.

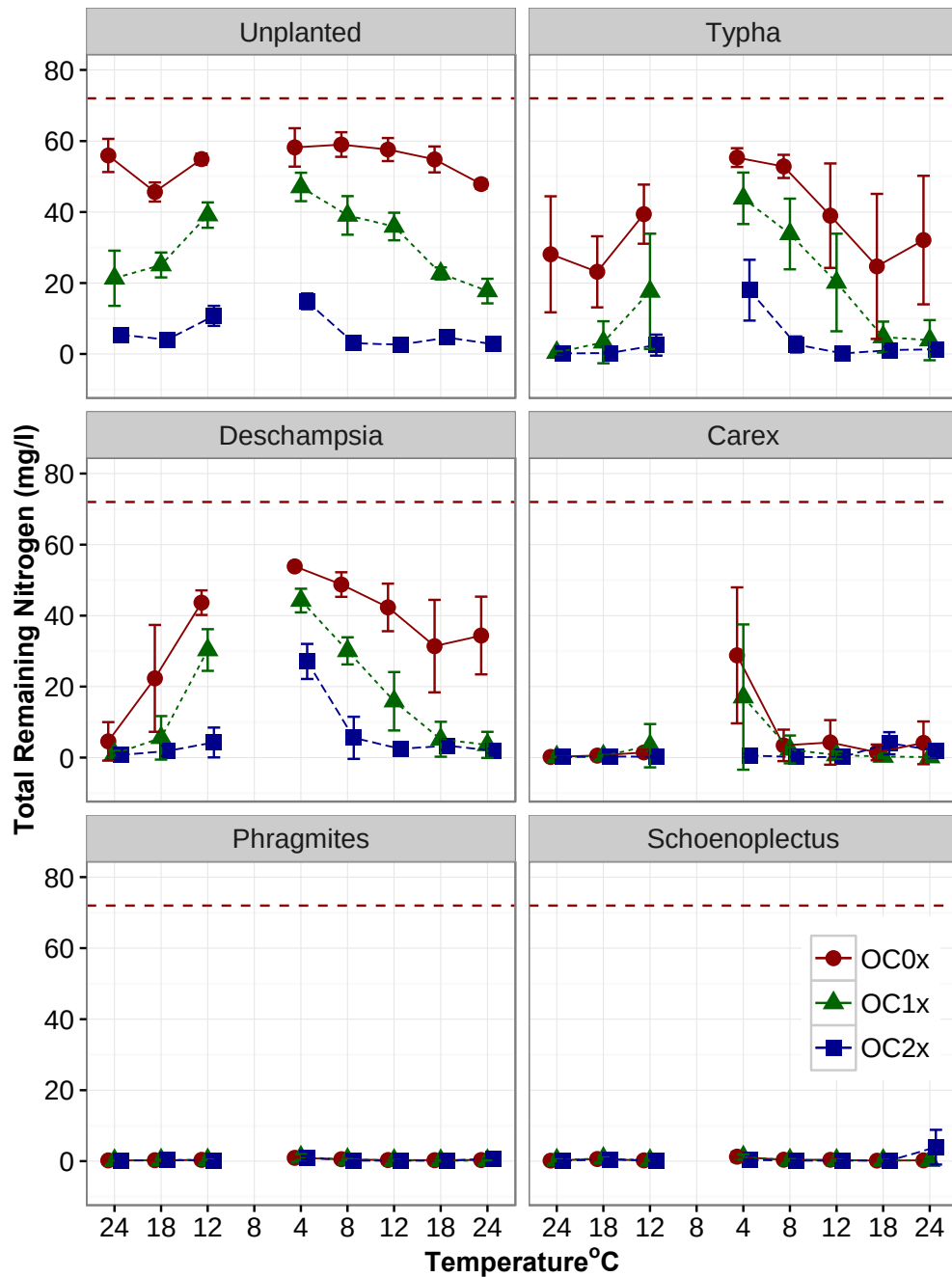


Figure 3. Total dissolved nitrogen concentrations on incubation day six across an annual temperature seasonal cycle. Each temperature step along the x-axis represents a 45-day time increment, proceeding from summer, through fall to winter and back through spring to summer. Dashed horizontal line at 72 mg/l represents mean nitrogen feed solution concentration. Missing 8 °C data in fall due to analytical equipment error.

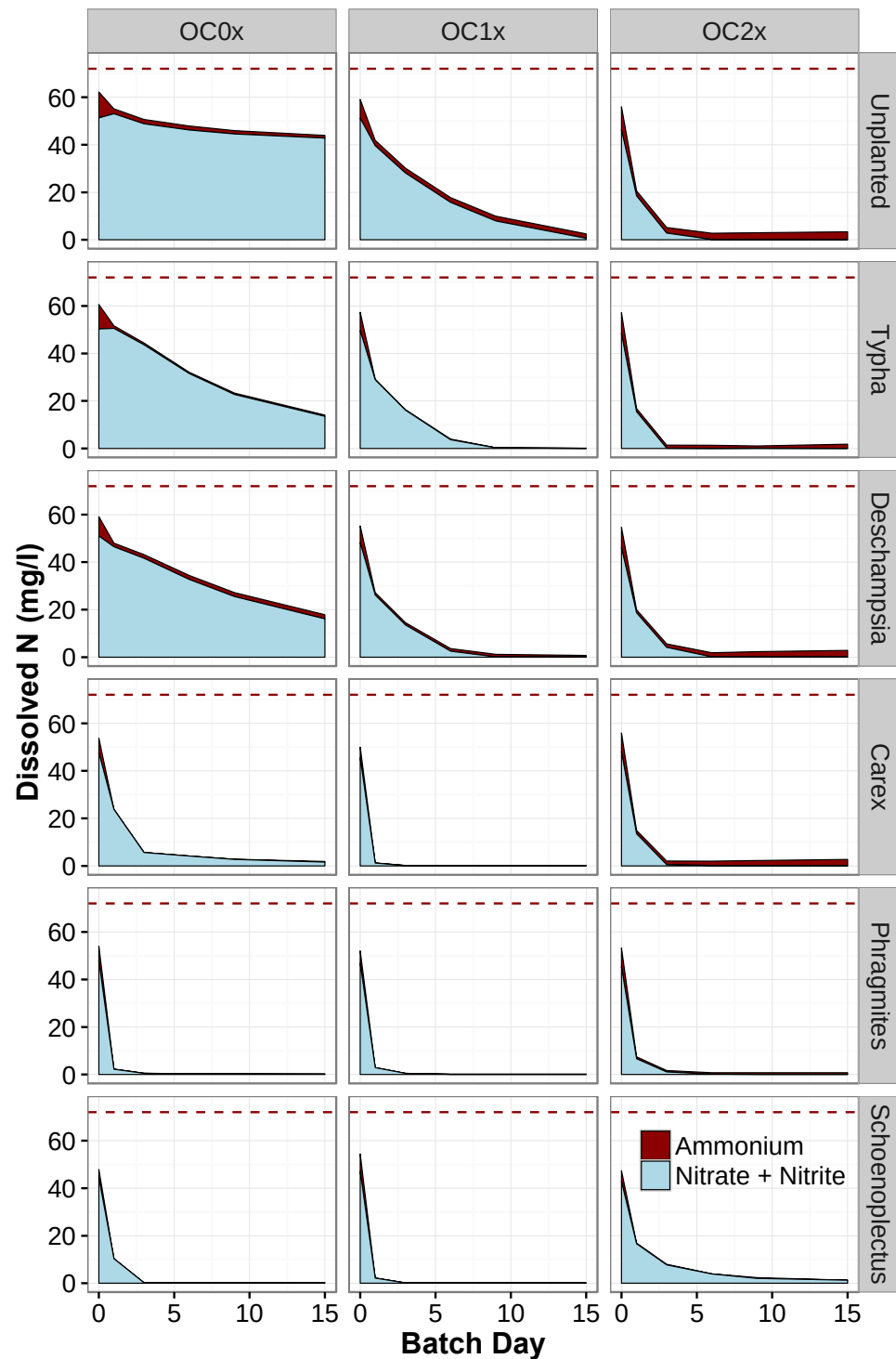


Figure 4. Nitrate plus nitrite and ammonium concentrations across the 15-day incubation at 24 °C in the summer of 2013. The panels increase in carbon addition from left to right and are grouped vertically according to plant treatment. The illustrated data represent the mean of four replicates.

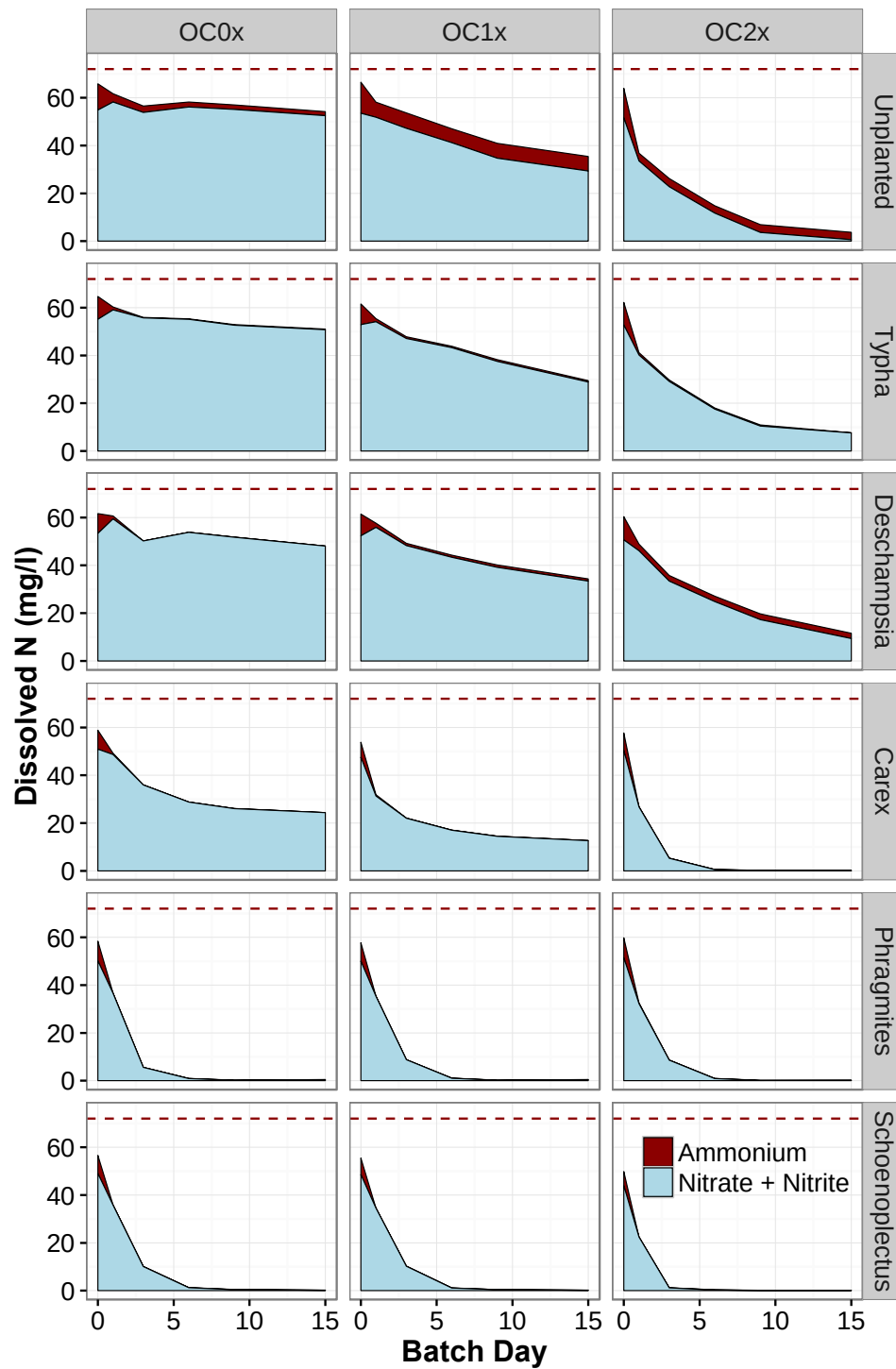


Figure 5. Nitrate and ammonium concentrations across the 15-day incubation at 4 °C in the winter of 2013. The panels increase in carbon addition from left to right and are grouped vertically according to plant treatment. The illustrated data represent the mean of four replicates.

Summation of the oxidized nitrogen species nitrate and nitrite (hereafter referred to as nitrate for simplicity as nitrite was generally negligible) comprised the majority of the measured TDN, especially in planted treatments (Figures 4 & 5). Measured TDN at incubation day 6 was significantly affected by plant species, OC load and temperature. The initial statistical model did not show a significant 3-way interaction. There were strongly significant interactions between all factors in the two-way model;  $p$  values were  $< 0.0001$  for the interaction between plant treatment and carbon load, 0.005 for plant treatment and temperature, and 0.002 for carbon load and temperature. The lowest day 6 TDN values were observed in *Phragmites* and *Schoenoplectus* regardless of temperature and OC load. In the other plant treatments and in the unplanted columns, measured TDN generally decreased with increasing carbon load and at higher temperatures (Figure 3).

The two-way statistical model showed that *Phragmites* and *Schoenoplectus* columns performed significantly better with respect to nitrogen removal than the unplanted columns ( $p = 0.005$ ), with consistently low levels of TDN across all OC loads and temperatures and with an annual removal rate exceeding the approximate annual loading rate ( $177 \text{ g N m}^{-2} \text{ yr}^{-1}$ ). *Carex* TDN removal was significantly better than unplanted columns ( $p = 0.04$ ) and intermediate between the better performing *Phragmites* and *Schoenoplectus* treatments and the poorer performing *Deschampsia* and *Typha* treatments. Virtually zero TDN remained in *Carex* columns by day 6 at higher temperatures for all OC loads, whereas some TDN remained at low temperatures and low OC loads. Nitrogen removal in both the *Deschampsia* and *Typha* treatments was not significantly different than the unplanted columns. Higher between-replicate variance in

Deschampsia and Typha at warm temperatures (and in Carex at low temperatures) appeared to reflect differences in plant growth and health. Both Deschampsia and Typha grew well initially, but the overall health of some replicates declined during the data collection period. The observed between-replicate variation of TDN can be qualitatively related to the quantity of plant biomass as a surrogate for plant health which varied widely between replicates (Table 1). Due to the poor health of several of the replicates of these plants, the results for both species are likely not representative of potential plant capacity.

Table 1. Cumulative nitrogen removal over one annual cycle and above-ground plant biomass at the end of the study.

| Treatment                                                                                                                                                                                                                | Carbon load 0                           |                            | Carbon load 1                           |                            | Carbon load 2                           |                            |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------|----------------------------|-----------------------------------------|----------------------------|-----------------------------------------|----------------------------|
|                                                                                                                                                                                                                          | TDN removal rate                        | Total above ground biomass | TDN removal rate                        | Total above ground biomass | TDN removal rate                        | Total above ground biomass |
|                                                                                                                                                                                                                          | (N g m <sup>-2</sup> yr <sup>-1</sup> ) | (g)                        | (N g m <sup>-2</sup> yr <sup>-1</sup> ) | (g)                        | (N g m <sup>-2</sup> yr <sup>-1</sup> ) | (g)                        |
| Non-planted                                                                                                                                                                                                              | 56.7 (18.2)                             | -                          | 132.3 (32.5)                            | -                          | 168.7 (4.0)                             | -                          |
| Typha                                                                                                                                                                                                                    | 113.52 (49.6)                           | 111 (125)                  | 157.4 (33.0)                            | 151 (116)                  | 173.7 (8.0)                             | 104 (84)                   |
| Deschampsia                                                                                                                                                                                                              | 117.7 (44.6)                            | 66 (58)                    | 151.0 (33.8)                            | 68 (49)                    | 169.6 (9.0)                             | 68 (53)                    |
| Carex                                                                                                                                                                                                                    | 165.7 (24.7)                            | 243 (125)                  | 171.7 (18.3)                            | 247 (130)                  | 175.6 (3.69)                            | 178 (80)                   |
| Phragmites                                                                                                                                                                                                               | 177.1 (0.4)                             | 348 (160)                  | 177.1 (0.32)                            | 261 (101)                  | 177.1 (1.2)                             | 139 (92)                   |
| Schoenoplectus                                                                                                                                                                                                           | 177.1 (0.46)                            | 511 (100)                  | 175.6 (9.8)                             | 412 (148)                  | 177.0 (1.9)                             | 312 (79)                   |
| <b><i>All results as either mean areal total dissolved nitrogen removal rate for August 2012 through August 2013 or total experimental above ground biomass with standard error of six replicates in parenthesis</i></b> |                                         |                            |                                         |                            |                                         |                            |

According to the intra-species (i.e. within plant treatment) statistical model, nitrogen removal in unplanted columns was most strongly influenced by OC load (enhanced at higher loads,  $p < 0.0001$ ), with temperature also playing a significant role (enhanced at higher temperature,  $p = 0.04$ ). TDN removal in Carex was seasonal,

however OC load was most influential ( $p = 0.040$ ) with poorer winter TDN removal at OC0x and OC1x compared to OC2x. Neither OC load nor temperature appeared to influence TDN removal in Phragmites and Schoenoplectus treatments; however, in spite of consistently low TDN concentrations, a statistical temperature dependency ( $p < 0.001$ ) was found in the Phragmites treatments. The Deschampsia treatments were significantly influenced by temperature ( $p = 0.0006$ ), while Typha treatments were not significantly influenced by any of the measured factors. Both Deschampsia and Typha treatments were likely most significantly affected by reductions in plant health.

Table 2. Dissolved organic carbon concentration averaged over days 3 to 15 at 4°C and 24°C.

| <u>Treatment</u> | <u>OC0x</u>                       |                                   | <u>OC1x</u>                       |                                   | <u>OC2x</u>                       |                                   |
|------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|
|                  | Winter<br>(C mg l <sup>-1</sup> ) | Summer<br>(C mg l <sup>-1</sup> ) | Winter<br>(C mg l <sup>-1</sup> ) | Summer<br>(C mg l <sup>-1</sup> ) | Winter<br>(C mg l <sup>-1</sup> ) | Summer<br>(C mg l <sup>-1</sup> ) |
| Unplanted        | 4.0 (0.2)                         | 5.1 (0.5)                         | 6.5 (0.3)                         | 9.9 (1.2)                         | 8.7 (0.4)                         | 15.1 (2.3)                        |
| Typha            | 3.0 (0.2)                         | 11.4 (2.6)                        | 4.8 (0.4)                         | 13.5 (1.9)                        | 8.3 (0.6)                         | 12.3 (1.9)                        |
| Deschampsia      | 5.2 (0.4)                         | 17.6 (1.9)                        | 6.8 (0.5)                         | 19.7 (2.4)                        | 9.5 (0.6)                         | 16.1 (1.7)                        |
| Carex            | 9.7 (0.7)                         | 27.9 (7.3)                        | 11.8 (0.7)                        | 17.9 (3.3)                        | 13.1 (0.8)                        | 14.4 (2.3)                        |
| Phragmites       | 10.6 (0.6)                        | 19.8 (3.8)                        | 10.1 (0.5)                        | 14.9 (3.2)                        | 11.2 (0.6)                        | 14.5 (2.2)                        |
| Schoenoplectus   | 11.3 (0.8)                        | 13.6 (2.6)                        | 11.0 (0.6)                        | 12.2 (2.1)                        | 11.2 (0.9)                        | 9.9 (1.8)                         |

*Data are means of four replicates (standard error in parenthesis).*

The organic carbon added to the system was quickly removed from all plant treatments in both the winter and summer seasons. Within the first 24 hours approximately 90% or greater removal was observed in the OC2x load and approximately 80% at OC1x. Figure 6 illustrates the temporal variation of dissolved organic (DOC) and inorganic (DIC) carbon. A general pattern of rapid organic carbon removal within the first 24 hours followed by a moderate increase until day 6 or 9 and then a decrease again by

day 15 is evident in all treatments at the OC1x and OC2x loads. Average values after incubation day 3 are shown in Table 2. Carex values approached  $30 \text{ mg l}^{-1}$  in summer, more than five times greater than unplanted controls. A two-way ANOVA model was run on the DOC data from two incubations representing summer and winter respectively including sample days 3, 6, 9, and 15, to determine what factors influenced OC levels after the first 24 hours. The interaction between season and plant treatment was most significant ( $p = 0.007$ ); therefore, to explore the interactions within a species, the intra-species model was used. DOC levels past day 1 in the unplanted columns increased with both carbon load ( $p < 0.0001$ ) and season ( $p = 0.014$ ). Deschampsia and Typha treatments performed similarly to the unplanted columns, showing effects of both carbon load and season, but in these treatments season was more influential ( $p < 0.0001$  and  $p = 0.004$ , respectively) than carbon load ( $p = 0.055$  and  $p = 0.027$ , respectively). Variation among Carex treatments was not well explained by either temperature or carbon load. Variation in the Schoenoplectus DOC levels was influenced most by season ( $p = 0.009$ ), with higher values in summer. Lastly in the Phragmites treatments, carbon load was the most influential factor ( $p < 0.001$ ); however, the relationship was negative, there was less OC past day 1 with increasing carbon load.

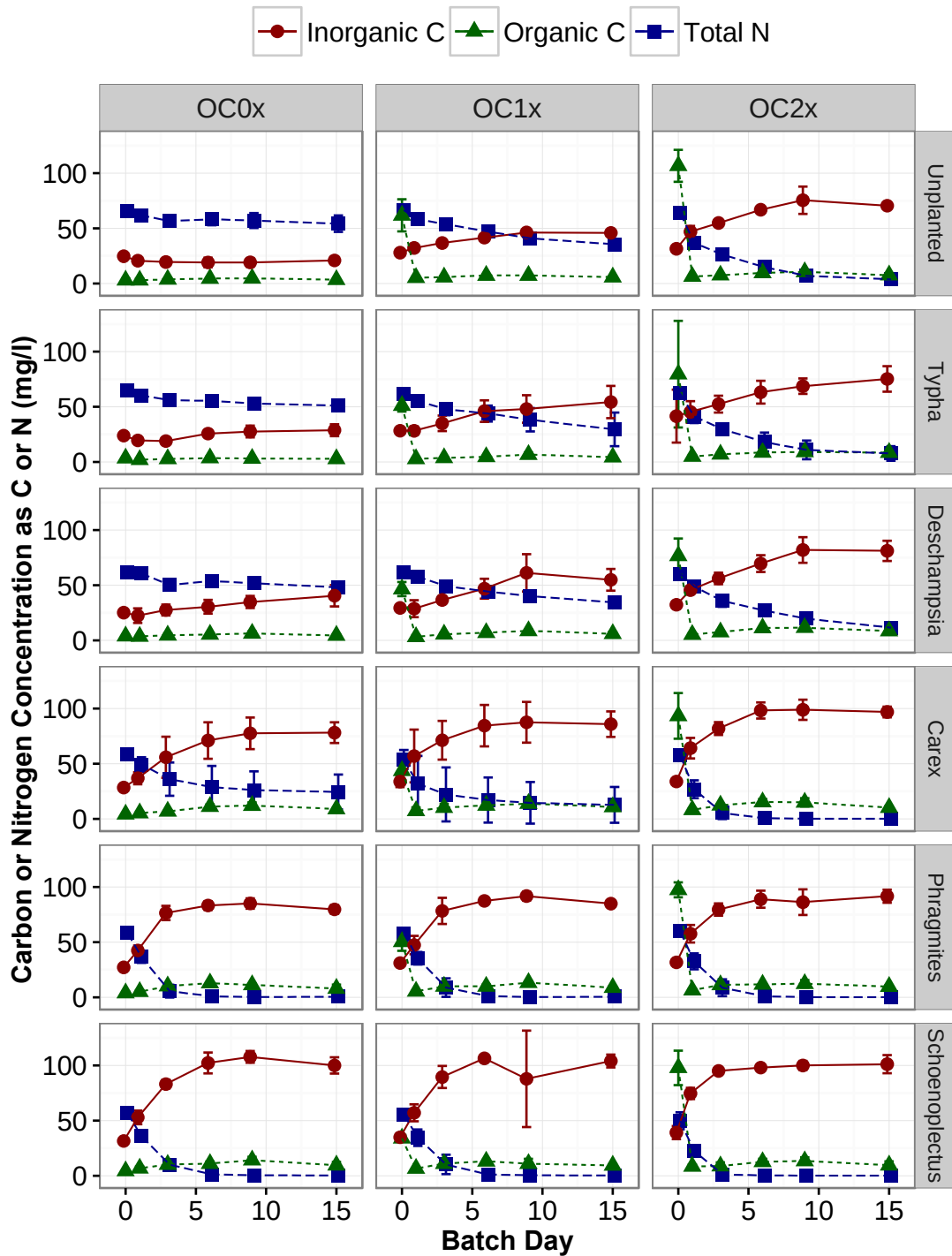


Figure 6. Mean organic (DOC) and inorganic (DIC) carbon concentrations across the 15-day incubation at 4 °C in the winter of 2013. The illustrated data represent the mean of four replicates with error bars representing the standard deviation.

### Plant Biomass and Nitrogen Uptake

Total above ground plant biomass measured at the end of the study (after 4 growing seasons) varied greatly between species and was significantly affected by organic carbon addition (Table 1). Above ground biomass for *Phragmites* and *Schoenoplectus*, the top two performing plant species in relation to nitrogen removal, was significantly greater at the OC0x load than at the OC2x load ( $p < 0.01$ ). *Carex* treatments also exhibited this trend but differences were not significant ( $p = 0.11$ ). Plant biomass differences between species were greatest at the OC0x load and as organic carbon load increased differences between species decreased. A general ranking of above-ground plant biomass roughly matches a ranking for TDN removal; better performing species had more biomass in general and species differences are most dramatic when no organic carbon is added. Adding OC enhanced TDN removal and had less influence on plant biomass in species with poor performance at OC0x.

The amount of applied TDN removed by plant uptake was estimated by comparing the nitrogen content measured in total plant biomass at the end of the study to the amount of N estimated to have been removed over the course of the experiment. The values varied by species and for all species more nitrogen was incorporated into the biomass of plants at the OC0x load compared to the OC2x load ( $p < 0.05$ ; Table 3). TDN removal attributed to plant uptake at the OC0x load was 65%, 60%, 38%, 20% and 28% for *Schoenoplectus*, *Carex*, *Phragmites*, *Deschampsia* and *Typha*, respectively, but at the OC2x load was only 41%, 35%, 20%, 20% and 16%, respectively. *Deschampsia* and *Typha* estimates may however be influenced by poor plant health.

Table 3. Mass of total dissolved nitrogen attributed to plant uptake and microbial removal over the study period.

| Treatment      | OC0x                  |                            |                        | OC2x                  |                            |                        |
|----------------|-----------------------|----------------------------|------------------------|-----------------------|----------------------------|------------------------|
|                | Plant uptake<br>(g N) | Microbial removal<br>(g N) | Total Removal<br>(g N) | Plant uptake<br>(g N) | Microbial removal<br>(g N) | Total Removal<br>(g N) |
| Typha          | 1.9                   | 4.7                        | 6.6                    | 1.5                   | 7.7                        | 9.2                    |
| Deschampsia    | 1.4                   | 5.5                        | 6.9                    | 1.8                   | 7.3                        | 9.1                    |
| Carex          | 5.2                   | 3.5                        | 8.7                    | 3.2                   | 6.0                        | 9.2                    |
| Phragmites     | 3.5                   | 5.7                        | 9.2                    | 2.4                   | 6.9                        | 9.3                    |
| Schoenoplectus | 6.0                   | 3.3                        | 9.3                    | 3.8                   | 5.5                        | 9.3                    |

Above ground biomass was used to determine annualized dry biomass accumulation rates assuming equal growth in each year. *Schoenoplectus* above ground biomass growth ranged between 4.4 and 7.2 kg m<sup>-2</sup> yr<sup>-1</sup>, far exceeding the 2.2 kg m<sup>-2</sup> yr<sup>-1</sup> measured by Tanner (1996) and the 2.3-3.2 kg m<sup>-2</sup> yr<sup>-1</sup> range found in natural stands by Miller and Fujii (2009). In contrast to those studies, *Schoenoplectus* produced more biomass than *Typha* and *Phragmites* in our study. *Phragmites* above ground biomass accumulation was between 2.0 and 4.9 kg m<sup>-2</sup> yr<sup>-1</sup>, a range similar to the maximum value reported by Vymazal and Kröpfelová (2005) and Borin and Salvato (2012). *Carex* above ground biomass accumulation ranged between 2.5 and 3.5 kg m<sup>-2</sup> yr<sup>-1</sup>, close to the first year growth of *Carex elata* documented by Borin and Salvato (2012). Average above ground biomass for *Deschampsia* and *Typha* was low and standard deviations between replicates were approximately equal to the mean, regardless of carbon load (Table 1). For comparison to other studies, average *Typha* annual production was (2.3 kg m<sup>-2</sup> yr<sup>-1</sup>), which was less than the first year growth found by Borin and Salvato (2012), less than

20% of their second year biomass accumulations, and less than 33% of the standing biomass value presented by Vymazal (2011).

### Discussion

Denitrification and plant uptake were the major TDN removal pathways in this study applying wastewater with  $72 \text{ mg N l}^{-1}$  at an application rate of approximately  $178 \text{ g N m}^{-2} \text{ yr}^{-1}$ . Other TDN removal pathways are possible (Kadlec and Wallace, 2009); however, consistently near neutral pH values minimized ammonia volatilization, removal of dissolved nitrogen by incorporation into particulate organic matter in the subsurface was deemed minimal (Borin and Salvato, 2012), and sorption of ammonium is always non-permanent and finite over the long term, leaving plant uptake and microbial transformation as the likely removal mechanisms. These data show a strong interplay between these two mechanisms. Higher OC loads significantly increased TDN removal except for *Phragmites* and *Schoenoplectus* (which displayed virtually complete TDN removal in all conditions) and in all cases increased the fraction of TDN removal attributable to denitrification. On the other hand, increased OC load decreased the biomass of the better TDN removing species and decreased the amount attributed to plant uptake. These results suggest that microorganisms outcompeted the plants for the available nitrogen at higher OC loading (Inselsbacher, Hinko-Najera Umana et al. 2010). The resulting nitrogen limiting conditions reducing above-ground biomass and thus plant nitrogen assimilation significantly.

The OC2x treatment (OC:N = 2.4:1 g g<sup>-1</sup>) was sufficient to facilitate removal of virtually all TDN by day six in all treatments at warmer temperatures but not in *Deschampsia*, *Typha* and unplanted columns at colder temperatures. This suggests that, previous work indicating an ideal OC:N ratio of 5:1 for effective nitrate removal (Baker 1998, Ingersol and Baker 1998) is too simplistic and generally higher than necessary, at least at our nitrogen loading rates. Increases in the OC:N ratio increased the importance of the microbial denitrification removal pathway and are believed to enhance the completeness of denitrification to N<sub>2</sub> gas (Mander, Dotro et al. 2014); however, higher OC:N ratios have also been correlated to increases in nitrous oxide production (Wu, Zhang et al. 2009). With many combinations of plant treatment and OC loading, the effect of temperature on TDN removal is evident, with decreased rates of removal at colder temperatures. The effect is dampened however with increasing OC load; at the OC2x load most plant treatments show reduced nitrogen removal at only 4°C and 8°C. In the OC2x treatments the winter removal mechanisms during plant senescence is likely dominated by microorganisms, with temperature dependent rates of growth, resulting in slower rates of microbial denitrification of the primarily nitrate based TDN applied.

The influent was comprised of 25% ammonium and at least 50% of the ammonium was removed from the water within the first 30 minutes, attributed primarily to sorption to gravel and below ground biomass. In the *Phragmites* and *Schoenoplectus* treatments, ammonium approached detection limits within 24 hours but in *Deschampsia* and *Typha* treatments, small measurable quantities persisted. More significant quantities remained in the unplanted columns. While ammonium contributed only 25% of the

incoming TDN, ammonium dynamics shed light on the influence of plants and OC loading on nitrogen transformations. An increase in nitrate often accompanied the ammonium decrease within the first 24 hours in the OC0x load, especially in the unplanted, *Deschampsia* and *Typha* treatments (Figures 2 and 3). At the OC2x load there was no accompanying increase in nitrate for these treatments, and unplanted columns showed no increase at the OC1x load. This suggests that there was sufficient oxygen to drive the nitrification rate in excess of the denitrification rate in these plant treatments when lightly loaded with organic carbon. At the higher carbon loadings and in the three high performing treatments according to N removal (*Carex*, *Phragmites* and *Schoenoplectus*), the removal of ammonium suggests nitrification was occurring but the rate was lower than the denitrification rate. Observed ammonium dynamics were consistent with behavior expected given bulk fluid redox values. The unplanted columns receiving OC0x had average measured Eh values above +200mV (data not shown), which is favorable for nitrification, but when OC was added, Eh levels were generally low enough (<+150 mV) to inhibit nitrification.

These results suggest that plants release dissolved organic carbon to the rhizosphere either as direct exudates or as a product of subsurface detritus decomposition, though no observation were made to ascertain the source or the bioavailability of the organic compounds. Organic carbon values for filtered samples, which represent the excess of OC remaining after consumption by all heterotrophic activity, increased with time in all planted treatments when no additional sucrose was supplied. At the OC0x load unplanted columns demonstrate a reduction in DIC and increase in DOC and very little

change in total dissolved carbon (Figure 4), suggesting active autotrophic metabolic activity consistent with some nitrification. In the planted treatments at OC0x, DOC gradually increases to an asymptotic value which varies by species but the increase in DIC is always much larger. This strongly suggest the plants were supplying organic carbon in quantities significant enough to drive heterotrophic metabolic activity. Similar temporal patterns were observed at 24°C as were similar subtle differences between plant treatments.

All *Phragmites* and *Schoenoplectus* treatments removed nitrate at the applied rate of  $133 \text{ g N m}^{-2} \text{ yr}^{-1}$  closely followed by *Carex* at  $121 \text{ g N m}^{-2} \text{ yr}^{-1}$  with respective TDN reductions of 177 and  $165 \text{ g N m}^{-2} \text{ yr}^{-1}$ . The application rate ( $177 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) and hence maximum removal rate is low compared to some treatment wetlands, but the removal is within the range of other experiments performed without addition of supplemental organic carbon. (Borin and Salvato 2012) reported that *C. elata* and *P. australis* could remove over  $200 \text{ g N m}^{-2} \text{ yr}^{-1}$  without the addition of organic carbon. Conservatively estimating that 30% ( $40 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) of the incoming nitrate (excluding additional nitrate created by nitrification) was removed via denitrification in OC0x treatments, plants must have supplied at least  $34 \text{ g C m}^{-2} \text{ yr}^{-1}$ . The value is likely greater since other heterotrophic activity undoubtedly consumed available organic carbon. Zhai et al. (2013) calculated that root exudation (excluding detritus decay) rates for *Phragmites*, *Iris* and *Juncus* species resulted in the potential to reduce 2.7 to  $9.4 \text{ g NO}_3^- - \text{N m}^{-2} \text{ yr}^{-1}$ . Iannelli et al. (2011) found rates of subsurface organic carbon supply during the growing season to range between 5.76 and  $7.02 \text{ g C m}^{-2} \text{ d}^{-1}$  for several wetland plant species. Total root

mass has been found to correlate to organic carbon rhizodeposition (Baptist, Aranjuelo et al. 2015) and this experiments in small containers with high root density may have enhanced plant effects; field scale studies are needed to verify results.

The TDN application rate in this experiment ( $177 \text{ g N m}^{-2} \text{ yr}^{-1}$ ) was less than the removal potential of all *Phragmites* and *Schoenoplectus* treatments but exceeded the maximum winter removal of *Carex* and the year round removal of *Deschampsia* and *Typha* without the addition of organic carbon. The maximum removal rates of these systems appears to be both species and season dependent, and different species may support different primary removal pathways. For example, *Carex rostrata* is more dependent on nutrient uptake as a nitrogen removal pathway, supporting less denitrification per dry root mass than other wetland species (Hallin, Hellman et al. 2015). This illustrates the potential for greater seasonal effects compared to *P australis* and *S acutus*. which may support more denitrification that mediates seasonal effects. The true nitrogen removal capacity of these best performing species is unknown since the combination of denitrification and plant assimilation exceeded the amount applied. Additionally, it is conceivable that for maximum TDN removal some plant species might display a maximum organic carbon load; additional loading would reduce plant biomass so much that the reduced plant uptake would exceed the increase in denitrification stimulation.

### Conclusions

Removal of ammonium, nitrite and nitrate when applied at concentrations of 18  $\text{mg NH}_4^+ \text{-N l}^{-1}$  and 54  $\text{mg NO}_3^- \text{-N l}^{-1}$  and at a rate of  $177 \text{ g N m}^{-2} \text{ yr}^{-1}$  was strongly

affected by plant species, the level of organic carbon addition and the combined effect of season and temperature. Equally strong interactions between these factors underscore the complexity of nitrogen dynamics in treatment wetlands. Unplanted columns responded as anticipated, displaying better removal as both organic carbon loading and temperature increased. Columns with plant species *P australis* and *S acutus* removed virtually all available nitrogen regardless of organic carbon addition or temperature while the two plant species with lower plant biomass and questionable health (*D cespitosa* and *T latifolia*) removed nitrogen at a level similar to the unplanted columns. *C utriculata* removed all available TDN at warmer temperatures at regardless of organic carbon addition; however, nitrogen removal decreased in the winter when the organic carbon was not added.

Reductions in plant biomass with the addition of organic carbon, and hence reductions in plant nitrogen uptake, were statistically significant, presumably because microbes more rapidly scavenged the available nitrogen. The competition between plant uptake and denitrification for available nitrogen has potentially important ramifications for nitrate nitrogen removal in TWs. Increasing available organic carbon increases nitrate removal, presumably through increasing denitrification rates. However, denitrification is enhanced at the expense of optimal plant growth. Equally apparent from the data is that plants do not remove a fixed quantity of nitrogen, rather that the uptake is influenced by how much organic carbon is available to drive the competitive microbial removal process including denitrification; the relative importance of this competition is species specific. Therefore it is likely that the amount of additional organic carbon required and the

appropriate influent C:N ratio for optimal TDN removal is plant species-specific. As nitrate loading rates increase, it is likely that the need to add organic carbon to drive denitrification increases as the maximal plant uptake occurs at the lowest organic carbon loading rates.

This research illustrates the potential to design nitrate-loading schemes that are plant specific to optimize plant growth and accumulation into biomass, as well as enhance microbially mediated removal with plant input as the sole carbon source. Optimizing the loading schemes of wetlands to transform nitrate without additional organic carbon will be beneficial to hybrid TW design as well as systems designed to reduce non-point source nitrate pollution.

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## CHAPTER THREE

### INFLUENCE OF PLANTS AND ORGANIC CARBON ADDITION ON GREENHOUSE GAS EMISSIONS FROM MODEL TREATMENT WETLANDS

#### Contribution of Authors and Co-Authors

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Contributions: Co-author on initial experiment proposal. Assisted with experimental set-up, data collection and sample analysis. Provided edits on the manuscript.

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Contributions: Assisted with experimental set-up, data collection and sample analysis. Provided edits on the manuscript.

Co-Author: Otto R. Stein

Contributions: Primary investigator on initial experiment proposal. Advised on experimental design and analysis. Provided substantial edits on the manuscript.

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INFLUENCE OF PLANTS AND ORGANIC CARBON ADDITION ON  
GREENHOUSE GAS EMISSIONS FROM MODEL  
TREATMENT WETLANDS

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**Keywords:** ammonium, carbon dioxide (CO<sub>2</sub>), Chemical oxygen demand, methane (CH<sub>4</sub>), nitrate, nitrous oxide (N<sub>2</sub>O)

Abstract

The emissions of greenhouse gasses carbon dioxide, methane and nitrous oxide were measured from 48 wetland microcosms treating synthetic wastewater with 72 mg l<sup>-1</sup> total dissolved nitrogen and planted in monocultures of five plant species or left unplanted. Organic carbon was added to half the microcosms to create a C:N ratio of 2.4 while half received no influent organic carbon. Emissions from a sealed headspace were measured over 24 hour periods five times within a 15-day incubation, once in the summer (24 °C) and again in the winter (4 °C). Carbon dioxide emissions ranged over two orders of magnitude, were significantly increased by plant presence and were significantly greater in the summer. CO<sub>2</sub> emissions increased in unplanted, *T. latifolia* and *D. cespitosa* planted microcosms when organic carbon was added but in *C. utriculata*, *P. australis* and *S. acutus*, more CO<sub>2</sub> was emitted without organic carbon addition. Methane emissions were virtually zero in the winter and always low in the summer, but plant presence significantly raised summer CH<sub>4</sub> emissions. N<sub>2</sub>O emissions ranged over three orders of magnitude, were universally and significantly higher when organic carbon was added and

significantly higher in the presence of plants, but seasonal effects were highly dependent on plant species and organic carbon load. Incubation-averaged and temporal variation of nitrous oxide emissions significantly correlated to removal of total dissolved nitrogen. The fraction of total dissolved nitrogen removed from water and released as  $\text{N}_2\text{O}$  increased significantly with organic carbon addition and plant presence.

### Introduction

Wastewater treatment systems and natural wetlands emit greenhouse gases (GHG) (Christensen, Panikov et al. 2003, Kampschreur, Temmink et al. 2009, Foley, de Haas et al. 2010, Mander, Dotro et al. 2014). Interest in GHG emissions from treatment wetlands (TWs) has increased in recognition that wastewater addition to artificial wetlands may create a significant source of GHG. TWs may emit more carbon dioxide, and less-emphasized but more potent GHGs such as methane and nitrous oxides compared to either conventional wastewater treatment systems or natural wetlands. On the other hand, TWs are the only wastewater systems employing vascular plants. The sequestration of  $\text{CO}_2$  into plant biomass (Brix, Sorrell et al. 2001, Mander, Lohmus et al. 2008) may allow TWs to act as a net sink for GHG. Thus, emissions from TW may be better or worse than alternative wastewater treatment systems, with a disproportionate influence on climate change relative to the quantity of water they treat on a global scale.

Research on GHG emissions from TWs is limited and has shown emissions to be highly variable; changes in constituent loading rates, temperature/season, plant species and time of day can have significant influence on emissions from an individual TW and

even larger differences between TWs have been noted depending on the specific treatment purpose, hydraulic design and overall climate (Teiter and Mander 2005, Mander, Dotro et al. 2014). The primary GHGs of concern, carbon dioxide, methane and nitrous oxide, are generated by the vascular plant and microbial processes that drive water quality improvements in TWs. Carbon dioxide (CO<sub>2</sub>) has a 100-year absolute global warming potential of  $2.49\text{e}^{-14} \text{ W m}^{-2} \text{ yr}^{-1} \text{ kg}^{-1}$  and is the reference gas for other GHGs. Methane (CH<sub>4</sub>) and nitrous oxide (N<sub>2</sub>O) have 100 year global warming potentials 28 and 265 times greater than carbon dioxide respectively, not considering climate-carbon feedbacks (Myhre, Shindell et al. 2013). Despite high potential emissions of GHG, many TW have been shown to act as a net GHG sink as plants sequester atmospheric CO<sub>2</sub> (Mander, Lohmus et al. 2008). The processes that generate and consume the GHG are complex and interrelated, and rates vary over space and time in the TW subsurface. Minimizing the potential release of GHG from TW without sacrificing their overall effectiveness in water quality amelioration requires an understanding of the TW processes that produce and consume the gasses.

### Carbon Dioxide

Photosynthesis couples atmospheric CO<sub>2</sub> and sunlight to produce organic carbon compounds that wetland plants incorporate into biomass, acting as a sink for atmospheric CO<sub>2</sub> (Brix, Sorrell et al. 2001). However, shoot and root respiration returns some CO<sub>2</sub> directly to the atmosphere on a diel cycle at rates that vary based on species, existing biomass and growth rate (Bloom, Sukrapanna et al. 1992, Pregitzer, Zak et al. 2007). In natural wetland systems and in some TW the root exudation of labile organic carbon

commonly referred to as chemical oxygen demand (COD) and plant detritus provide the major sources of COD to the wetland environment (Whiting and Chanton 2001, Pícek, Čížková et al. 2007). Microbial processes in the rhizosphere oxidize this COD, returning significant quantities of the plant-sequestered CO<sub>2</sub> to the atmosphere (Brix, Sorrell et al. 2001, Zhou, Zhou et al. 2009). Plants can also emit CO<sub>2</sub> directly during respiration, a process that can increase almost five fold when they take up nitrate instead of ammonium (Bloom, Sukrapanna et al. 1992). Microbial degradation of root exudates and plant detritus is influenced by external factors such as temperature, oxidation reduction potential and location in the rhizosphere (Weber and Legge 2013). Thus in temperate climates, there is a strong seasonal pattern to the subsurface interactions influencing CO<sub>2</sub> sequestration and release (Liikanen, Huttunen et al. 2006).

In TWs processing significant external COD inputs, GHG emission is affected by total COD input and total input of electron acceptors. In these cases, the availability of electron acceptors may limit the rate of mineralization and CO<sub>2</sub> release. Vertical flow (VF) and intensified TWs optimized for oxygen transfer are therefore able to degrade large quantities of COD and produce higher areal rates of CO<sub>2</sub>, which in a review by Jahangir et al. (2016) have been found to be as large as 916 mg C m<sup>-2</sup> hr<sup>-1</sup> and average 276 mg C m<sup>-2</sup> hr<sup>-1</sup>. In sub-surface horizontal flow (HF) and free water surface wetlands (FWS), CO<sub>2</sub> production is less than VF, with averages of 42 and 70 mg C m<sup>-2</sup> hr<sup>-1</sup> respectively. Mander et al. (2014) noted a negative relationship between COD loading and CO<sub>2</sub> production in HF wetlands indicating that CO<sub>2</sub> production may be limited by the input of electron acceptors. As COD loading increases, aerobic metabolisms give way to

the anaerobic digestion of COD which generally proceeds at slower rates and with more methane production. In natural wetlands and TW systems with medium to low annual loading rates of influent COD, plant derived COD may represent the primary organic carbon input and be the major driver for carbon emissions (Picek, Čížková et al. 2007, Iannelli, Bianchi et al. 2011).

### Methane

Methane production in wetlands is a result of fermentation and methanogenic decomposition of COD. Methanogenic microorganisms are highly redox dependent and are generally inhibited above -200 mV (Kadlec and Wallace 2008). Though they have been found to be active over a wide range of temperatures (Hanson and Hanson 1996), reductions in methane production during winter appear to be common in TW (Teiter and Mander 2005, Liikanen, Huttunen et al. 2006). High organic loading rates in free water surface (FWS) and horizontal flow (HF) wetlands can favor methane production over processes creating carbon dioxide, increasing the GHG warming potential per amount of COD removed. However, in these TW types, large portions of the methane produced in anaerobic zones may be consumed as the gas is transported through the water column into anoxic or aerobic zones where methane may be oxidized by sulfate or other ions, limiting the release of CH<sub>4</sub> to the atmosphere (Mander, Dotro et al. 2014). Conversely, methane and other gasses may be preferentially transferred from the subsurface by plant roots and shoots because convective transport through root aerenchyma provides less resistance than diffusion through water, thus providing a mechanism for direct release of generated methane to the atmosphere (Colmer 2003). Fractionation of mineralized

organic carbon into  $\text{CO}_2$  and  $\text{CH}_4$  released to the atmosphere is not currently predictable given the complexities surrounding methane production and release in TW, but the warming potential is likely enhanced when more methane is produced in situations such as high organic loading rates, low redox levels and higher temperatures.

### Nitrous Oxide

Of the three main GHG produced in TW, nitrous oxide is by far the most powerful, thus small differences in its release can have much greater impacts on climate change. Plant, fungal and microbial mediated redox reactions driving transformations vital to nitrogen removal in TW can produce  $\text{N}_2\text{O}$ . Plants can influence  $\text{N}_2\text{O}$  emissions by direct production of  $\text{N}_2\text{O}$  during nitrate assimilation, by affecting microbial processes in the subsurface, and by increasing gas transport from the subsurface via aerenchyma (Smart and Bloom 2001, Colmer 2003, Pregitzer, Zak et al. 2007). Direct production of  $\text{N}_2\text{O}$  has been shown to be very limited in wheat, accounting for 0.02 to 0.2% of the nitrate assimilated (Smart and Bloom 2001). Little information is available for direct production by wetland plant species and the potential release of  $\text{N}_2\text{O}$  by plant assimilation of nitrate cannot be dismissed in TW systems as many treat nitrate-rich wastewater. Fungal denitrifiers may produce the majority of  $\text{N}_2\text{O}$  emissions in grassland systems (Laughlin, Stevens et al. 2008). In TW, fungal activity is two to three orders of magnitude less than bacterial activity thus the relative importance of fungal denitrification in TWs has been thought to be minimal (Chang, Wu et al. 2015). The role of fungi in TW is however open to debate as (Lingua, Copetta et al. 2015) found

increased nitrate removal capacity in *Phragmites australis* planted mesocosms inoculated with arbuscular mycorrhizae.

Microbial processes likely dominate  $\text{N}_2\text{O}$  production in most TW. Nitrifying microorganisms may produce  $\text{N}_2\text{O}$  via two pathways: (1) through the stepwise oxidation of ammonium to nitrate, producing  $\text{N}_2\text{O}$  as an intermediary product between hydroxylamine and nitrite (classic nitrification) or, (2) through autotrophic ammonium oxidizing bacteria which internally utilize nitrite as an initial electron acceptor (nitrifier denitrification) (Wrage, Velthof et al. 2001). Though nitrification has been implicated as the greatest contributor to  $\text{N}_2\text{O}$  production in some TW (Wang, Inamori et al. 2008), most systems contain significant anoxic zones and produce the majority of the  $\text{N}_2\text{O}$  through incomplete denitrification (Faulwetter, Gagnon et al. 2009, Vilain, Garnier et al. 2014). Denitrification is the stepwise reduction of nitrate to di-nitrogen gas, the last step transitioning from  $\text{N}_2\text{O}$  to  $\text{N}_2$  via nitrous oxide reductase encoded by the *nosZ* gene. However not all denitrifying microorganisms contain the *nosZ* gene, creating the possibility of  $\text{N}_2\text{O}$  release before the last step is completed. In addition, the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$  has been shown to be inhibited by decreasing temperature, increasing ORP, low pH, and a lack of labile organic carbon (Cuhel, Simek et al. 2010).

These current research investigates the effects of plant species, temperature/season and the influent organic carbon to nitrogen ratio on total nitrogen removal and greenhouse gas emission in model TW microcosms. The specific application is treatment of partially nitrified effluent entering a horizontal sub-surface flow wetland with varying levels of prior COD removal, but is applicable to any TW treating influent

high in nitrate. To simulate such systems influent nitrogen was divided into 75% nitrate and 25% ammonium. Three levels of influent organic carbon (COD), including zero COD, allowed investigation into plants' capacity to add organic carbon to fuel nitrogen removal via denitrification. Five plant species representing varying levels of performance for COD removal in a previous experiment (Taylor, Hook et al., 2011) were chosen to represent a range of expected performance in this study. Water quality results for the five plant species and unplanted controls are reported in Allen et al. (2016a), and this paper reports the GHG emission results comparing the lowest (zero) and highest level of COD addition.

### Methods

The experiment was performed in a climate-controlled greenhouse at a latitude of 45.7° N. In October 2010, 18 replicates of five plant species (with family), *Carex utriculata*, and *Schoenoplectus acutus* (Cyperaceae), *Deschampsia cespitosa* and *Phragmites australis* (Poaceae), and *Typha latifolia* (Typhaceae), were planted into 15-cm diameter by 40-cm deep PVC columns. For simplicity all plant treatments will be referred to using the genus name, non-italicized to emphasize the naming convention and the potential variation between species in a genus. All columns, including 18 unplanted controls, were filled to a depth of 30 cm with gravel sieved between 2.4 and 4.8 mm. A period of warm temperature, supplemental lighting and nutrient addition aided plant establishment. Starting July 2011 temperature was changed on 45-day increments from 24°C, to 18°C, to 12°C, to 8°C, to 4°C and stepwise back to 24°C following the same

pattern. Starting in October 2011, synthetic wastewater containing  $18 \text{ mg NH}_4\text{-N l}^{-1}$  and  $54 \text{ mg NO}_3\text{-N l}^{-1}$  and one of three levels of COD supplied as sucrose was applied. Macronutrients P, Mg, K, Ca, and S as well as micro-nutrients were also added consistent with previous experiments (Taylor, Hook et al. 2011). A new batch incubation was initiated every 15 days in which the TW columns were drained and refilled with 1.8 liters of synthetic wastewater. There were three incubations per temperature step. The 18 replicates were subdivided into three treatment groups of six replicates. Each group received either zero COD (OC0x) or one of two levels of organic carbon representing exactly one (OC1x) or two times (OC2x) the stoichiometric equivalent required to remove all nitrogen by classic denitrification. These feed solutions represented COD:N of zero, 2.7 and 5.5 g COD per g N, respectively. Six groups of 18 columns, each containing one replicate of each combination of COD load and plant treatment were organized in a randomized block design.

Beginning in January 2012, 30 ml water samples were taken during the third incubation of each temperature and seasonal setting (8 per year) on incubation days 0, 1, 3, 6, 9 and 15. Incubation day zero samples were collected within 30 min of feeding the columns, with all subsequent samplings at the approximate same time of day as the initial feeding. Samples were passed through a  $0.2\mu\text{m}$  filter and analyzed for dissolved concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$  and  $\text{HPO}_4^{2-}$  using ion chromatography (Metrohm USA. Samples taken at both temperature extremes ( $4^\circ$  and  $24^\circ\text{C}$ ) were also filtered and analyzed for dissolved inorganic (DIC) and dissolved organic carbon (DOC) (Skalar Formacs). Total above ground plant biomass was collected from the columns at

the end of the experiment in September 2014. All sampled biomass was dried at 40°C, weighed, and ground for nutrient analysis. Additional details of the experimental design and water sampling and biomass protocols are presented in Allen et al. (2016).

To assess GHG emissions, custom-made forty liter bags made of a tube of 10-mil polypropylene were designed to completely enclose one column and the associated above-ground plant biomass. Each was sealed at the top and outfitted with a water sampling port (3 mm nylon hose barb fitting) approximately 10 cm from top of plant column, upper and lower gas sampling ports (6 mm nylon hose barb fitting) approximately 15 and 120 cm respectively from top of plant column, and electrical connections for the ORP probes (coax cable connectors), all gas-tight. The bag volume was held constant by a piece of 1 m tall by approximately 1 m wide nylon garden fencing rolled into a cylinder and inserted into the bag to keep the bag fully expanded and attached to a frame above the bags to keep them upright . Any biomass taller than approximately 80 cm and detritus easily disassociated from the plant was removed and saved prior to gas sampling.

Gas headspace sampling occurred during a summer incubation (24 °C; August/September, 2013) and a winter incubation (4 °C; March 2014). The bags were slipped over the plants and sealed to the bottom of the microcosm using rubber gaskets and large band clamps the day prior to the start of the 15-day incubation and were not removed until the incubation ended. Gasses from the headspace of all plant species from both OC0x and OC2x carbon treatments were sampled (four replicates of each species

and carbon load combination). Gas headspace measurements and concurrent water quality samples were taken from the same replicate.

Gas accumulation was measured on days 0-1, 3-4, 6-7, 9-10, and 14-15 over 24 hour periods to account for variation attributed to diurnal fluctuations. During this time the bags were completely sealed. At the end of the 24-hour measurement period, the headspace inside the bags was mixed by massaging the bag and agitating any freestanding biomass. Gas samples were collected from the lower gas sampling port using 60 ml syringes and transferred to 100ml FlexFoil gas sampling bags (SKC Inc PA. USA). Carbon dioxide measurements were taken immediately using a handheld CO<sub>2</sub> meter and measurements taken during winter 2014 were verified on a Varian GC with a thermal conductivity detector. Nitrous oxide analysis was performed using a HP 5890 (winter and summer 2013) or a Varian GC (winter 2014) with an electron capture detector, while methane was always measured on the HP 5890 using a flame ionization detector. To maintain atmospheric gas composition between measurement periods, the sample ports were opened and a low head (6 mm water) blower system (flow rate approximately 10 L min<sup>-1</sup> per column) flushed the bags with fresh air. Atmospheric CO<sub>2</sub> concentrations at the start of each sample day were verified by placing a handheld CO<sub>2</sub> meter at the exit sampling port prior to resealing the bags.

#### Data and Statistical Analysis

Gas emission over the incubation period was calculated by linearly interpolating measured concentrations between measurement days and integrating to estimate the total quantity produced. Total and daily production rates were normalized to the units of m<sup>-2</sup>

hr<sup>-1</sup> by dividing each production value by the surface area and 24-hour measurement duration. Linear mixed effects models (function lme() in package nlme) were used in the program R version 3.1.2 to assess factors influencing the emission of each GHG. The three main effects tested were: plant treatment (either 2 levels: planted and unplanted or 6 levels: unplanted, Carex, Deschampsia, Phragmites, Schoenoplectus, and Typha), COD load (2 levels: OC0x, OC2x), and season (winter (temp = 4 °C) and summer (temp = 24 °C)). The model evaluated fixed effects for plant treatment, OC load and temperature, and the three two-way interactions between fixed effects. In some cases additional linear models for a specific plant treatment were used to assess the effect of sampling season (winter/summer) or COD load as main effects.

### Results and Discussion

Plant species, the addition of COD, and season all significantly affected GHG emission. Carbon dioxide (CO<sub>2</sub>) emissions were significantly affected by plant presence versus absence ( $p < 0.001$ ), and the two-way interactions between plant species and carbon load, plant species and temperature, as well as, carbon load and temperature were also all significant ( $p < 0.001$ ). Methane (CH<sub>4</sub>) emissions were only measured in the summer and were not affected by either plant presence or COD load. Nitrous oxide (N<sub>2</sub>O) emissions were significantly affected by the interaction between COD addition and season ( $p = 0.02$ ). Emission rates of CO<sub>2</sub>, CH<sub>4</sub> and N<sub>2</sub>O averaged over the 15-day incubation are found in Table 4 which also includes values for the quantity of total dissolved nitrogen (TDN) removed, annualized rate of total dissolved nitrogen removal,

and total above ground plant biomass measured at the end of the experiment. Time series values for net emissions of CO<sub>2</sub>, and N<sub>2</sub>O are presented in Figures 7 and 8, respectively. For better interpretation, results and discussion are separated into sections for each GHG measured followed by an integrated discussion.

### Carbon Dioxide Emissions

Unplanted microcosm CO<sub>2</sub> emissions were significantly higher when COD was applied in both summer and winter ( $p = 0.002$  and  $0.02$ , respectively) and was marginally higher ( $p = 0.06$ ) in winter than in summer at both COD loadings. The CO<sub>2</sub> in the headspace of the unplanted microcosms accounted for 33% and 66% of the total dissolved organic carbon (DOC) added (OC2x) in summer and winter respectively. Assuming the dissolved inorganic carbon (DIC) was consistent with measured values in other incubations for the same conditions, the sum of measured CO<sub>2</sub> and assumed DIC concentrations accounts for 75% and 100% of the added DOC in the summer and winter, respectively. As expected, indicators of respiration (CO<sub>2</sub> + CH<sub>4</sub> emission) for the unplanted microcosms in the absence of applied COD (OC0x) was the lowest recorded, but some CO<sub>2</sub> was emitted, presumably by surface algae or other autotrophic microbial creation of organic carbon followed by heterotrophic degradation activity.

Table 4. Mean measured greenhouse gas emissions rates and total dissolved N ion removal over a 15-day incubation and above-ground biomass at the end of the experiment as influenced by season and plant species treatment (standard error is given in parenthesis)

|                                                             |                                                              | Unplanted      |                 | Carex             |                   | Deschampsia       |                   | Phragmites        |                  | Schoenoplectus     |                   | Typha            |                  |
|-------------------------------------------------------------|--------------------------------------------------------------|----------------|-----------------|-------------------|-------------------|-------------------|-------------------|-------------------|------------------|--------------------|-------------------|------------------|------------------|
|                                                             |                                                              | OC0x           | OC2x            | OC0x              | OC2x              | OC0x              | OC2x              | OC0x              | OC2x             | OC0x               | OC2x              | OC0x             | OC2x             |
| Summer                                                      | CO <sub>2</sub><br>(mg-C m <sup>-2</sup> hr <sup>-1</sup> )  | 7.64<br>(1.36) | 12.87<br>(2.07) | 297.59<br>(54.40) | 226.54<br>(47.52) | 917.04<br>(79.89) | 451.25<br>(54.31) | 204.13<br>(85.97) | 89.40<br>(16.32) | 444.08<br>(101.82) | 137.93<br>(31.26) | 46.49<br>(18.12) | 67.95<br>(17.73) |
|                                                             | CH <sub>4</sub><br>(mg-C m <sup>-2</sup> hr <sup>-1</sup> )  | 0.01<br>(.01)  | 0.03<br>(.01)   | 0.24<br>(.13)     | 0.22<br>(.08)     | 0.27<br>(.07)     | 1.09<br>(.46)     | 1.41<br>(.55)     | 0.17<br>(.05)    | 0.45<br>(.18)      | 0.35<br>(.17)     | 0.20<br>(.05)    | 0.27<br>(.03)    |
|                                                             | N <sub>2</sub> O<br>(mg-N m <sup>-2</sup> hr <sup>-1</sup> ) | 0.28<br>(.09)  | 0.42<br>(.11)   | 0.30<br>(.04)     | 0.69<br>(.10)     | 0.12<br>(.04)     | 0.69<br>(.11)     | 0.03<br>(.01)     | 0.68<br>(.10)    | 0.05<br>(.01)      | 0.70<br>(.11)     | 0.31<br>(.08)    | 0.82<br>(.05)    |
| Winter                                                      | CO <sub>2</sub><br>(mg-C m <sup>-2</sup> hr <sup>-1</sup> )  | 9.74<br>(.46)  | 23.94<br>(1.51) | 47.93<br>(12.66)  | 65.92<br>(15.49)  | 100.02<br>(10.52) | 82.46<br>(10.77)  | 81.60<br>(15.41)  | 49.53<br>(8.31)  | 60.81<br>(14.25)   | 45.82<br>(13.35)  | 27.07<br>(6.12)  | 33.83<br>(6.48)  |
|                                                             | CH <sub>4</sub><br>(mg-C m <sup>-2</sup> hr <sup>-1</sup> )  | 0.01<br>(.01)  | 0.01<br>(.00)   | 0.01<br>(.00)     | 0.01<br>(.00)     | 0.01<br>(.00)     | 0.01<br>(.00)     | -0.02<br>(.00)    | -0.02<br>(.01)   | -0.02<br>(.00)     | -0.02<br>(.00)    | -0.02<br>(.00)   | -0.02<br>(.00)   |
|                                                             | N <sub>2</sub> O<br>(mg-N m <sup>-2</sup> hr <sup>-1</sup> ) | 0.02<br>(.01)  | 0.29<br>(.05)   | 0.33<br>(.26)     | 1.53<br>(.44)     | 0.11<br>(.02)     | 1.33<br>(.48)     | 0.01<br>(.01)     | 0.40<br>(.15)    | 0.03<br>(.01)      | 0.59<br>(.13)     | 0.07<br>(.02)    | 1.13<br>(.27)    |
| Total Above Biomass (g)                                     |                                                              | -              | -               | 243<br>(125)      | 178<br>(80)       | 66 (58)           | 68 (53)           | 348<br>(160)      | 139<br>(92)      | 511<br>(100)       | 312<br>(79)       | 111<br>(125)     | 104<br>(84)      |
| TDN Removal rate<br>(N g m <sup>-2</sup> yr <sup>-1</sup> ) |                                                              | 56.7<br>(18.2) | 168.7<br>(4.0)  | 165.7<br>(24.7)   | 175.6<br>(3.69)   | 117.7<br>(44.6)   | 169.6<br>(9.0)    | 177.1<br>(0.4)    | 177.1<br>(1.2)   | 177.1<br>(0.46)    | 177.0<br>(1.9)    | 113.52<br>(49.6) | 173.7<br>(8.0)   |

The presence of a plant universally resulted in an increase in CO<sub>2</sub> ( $p < 0.01$ ); CO<sub>2</sub> production rates rose to as much as 100 times that of the unplanted treatments depending on plant species, season and COD load. Within planted treatments, the warm, summer measurement period resulted in significant increases in CO<sub>2</sub> emission ( $p < 0.01$ ); CO<sub>2</sub> emissions were between 2 and 9 times greater in summer for a given combination of species and COD load. Incubation-averaged rates of summer CO<sub>2</sub> emission in the planted treatments ranged between 46 (Typha, OC0x) and 917 mg C m<sup>-2</sup> hr<sup>-1</sup> (Carex, OC0x). The low value is within the low range of values for HF wetlands reported by Mander et al. (2008), however, the high values exceed their highest reported values by a factor of almost two. Counterintuitively, more CO<sub>2</sub> was emitted when no COD was added to the planted treatments, especially for the Carex, Phragmites and Schoenoplectus treatments ( $p = 0.05$ ). In only 3 cases (two in winter, one in summer) was more CO<sub>2</sub> emitted when COD was added and the increase was not statistically significant. The increase of CO<sub>2</sub> emission at the OC0x loading between the unplanted and planted treatments is much greater in the summer with planted treatments emitting 2.3 times more CO<sub>2</sub> averaged over all planted treatments but only 1.1 times more in the winter. Differences between species in CO<sub>2</sub> emission due to COD loading are closely tied to species differences in above ground plant biomass due to COD loading; Carex, Phragmites and Schoenoplectus treatments had large emission differences and ranged between 60% and 250% more biomass when COD was absent, while both CO<sub>2</sub> emissions and plant biomass differences due to COD load were minor for Deschampsia and Typha.

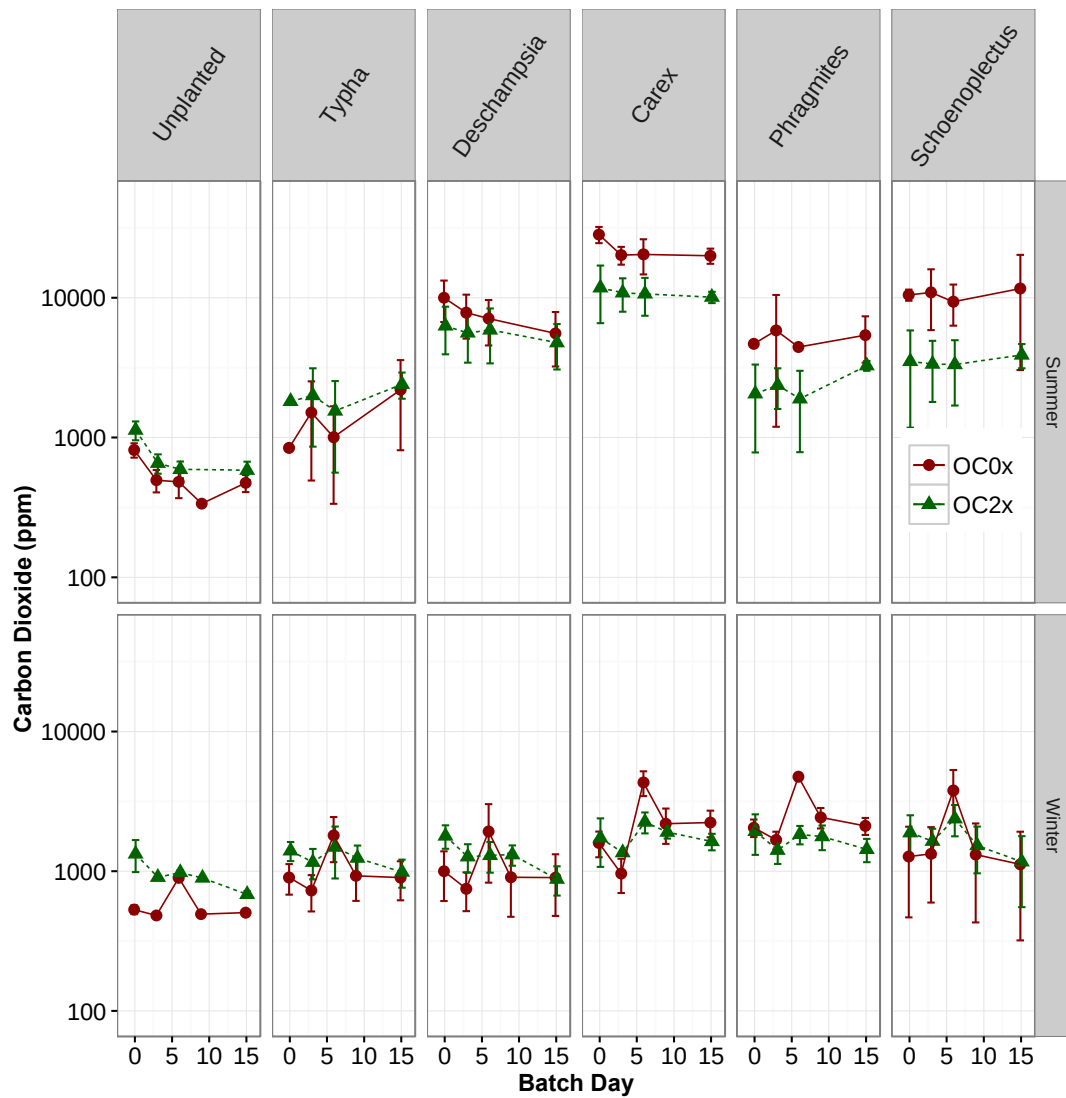


Figure 7. CO<sub>2</sub> emissions from four replicate columns with and without COD addition separated by plant species and season, winter (4°C) and summer (24°C). Headspace sampling occurred on incubation days 0-1, 2-3, 5-6, 9-10, and 14-15. The line represents sample mean and error bars represent 95% confidence intervals

As illustrated in Figure 7, macrophytes clearly stimulated CO<sub>2</sub> emissions in both winter and summer regardless of species. Several possible plant-driven mechanisms include CO<sub>2</sub> release via uptake of nitrate, root and shoot respiration, and stimulation of heterotrophic microbial activity by supplying significant quantities of organic carbon via

direct exudation and/or decomposition of dead plant biomass. Estimating the quantity of nitrate versus ammonium plant assimilation and separating the effects of plant respiration, root carbon exudation and detritus degradation is not possible given the experimental design. However, the observed plant species differences, seasonal patterns and the influence of COD addition offer important clues to their combined effects. A simple measure of above-ground plant biomass regardless of species did not correlate with CO<sub>2</sub> emissions, however a species-specific growth response to COD addition did correlate with CO<sub>2</sub> emissions. Species with little growth variation when COD was supplied (*Deschampsia* and *Typha*) emitted the same amount of CO<sub>2</sub> whether COD was supplied or not, suggesting that the added organic carbon had no influence on heterotrophic microbial activity. The three species that grew better in the absence of COD addition (*Carex*, *Phragmites* and *Schoenoplectus*) emitted more CO<sub>2</sub> when plant biomass was high even though no COD was added, suggesting that the plant-generated organic carbon more than compensated for the lack of added COD. In all cases more CO<sub>2</sub> was emitted in the summer. Importantly, the rate of CO<sub>2</sub> production in all planted treatments greatly exceeded that which would have been produced by the removal of all the supplied nitrogen by classic denitrification and/or plant uptake of nitrate. In many cases, all the nitrogen was removed even when no COD was supplied (Allen, Burr et al. 2016a). Greater CO<sub>2</sub> emissions in the summer are consistent with greater heterotrophic utilization of available organic carbon in the summer (Brix, Sorrell et al. 2001, Mander, Dotro et al. 2014, Groh, Gentry et al. 2015). In combination, these results strongly suggest that plants supplied significant quantities of labile organic carbon and some species generated more

when none was externally supplied as sucrose. The available organic carbon stimulated a suite of heterotrophic metabolic pathways including, but not limited to, denitrification.

Allen et al. (2016a) postulated that the externally supplied COD stimulated denitrification to the point that plant growth suffered from a lack of available nitrogen. The CO<sub>2</sub> emissions suggest that the resulting smaller plant biomass decreased the amount of organic carbon available for all heterotrophic microbial activity with minimal effect on the denitrification pathway. Plants release oxygen in addition to supplying organic carbon and the interplay between available oxygen and organic carbon influences the oxidation-reduction potential (ORP) of the system (Stein and Hook 2005) which in turn offers clues as to potential electron acceptors besides nitrate that may have been utilized.

All plants were net emitters of carbon dioxide regardless of COD loading. However, the net production of CO<sub>2</sub> could be biased by several experimental effects. The 24-hour measurement period was designed to remove diurnal variation. Bags were sealed and measurements taken in the middle of the day during the period of maximum daily photosynthesis (Brix, Sorrell et al. 2001), but after accruing the result of previous night's respiration. This sampling bias could have overestimated total CO<sub>2</sub> production. The increased concentrations of gasses inside the sealed headspace likely influenced reaction rates for some processes. Plants have been shown to increase photosynthesis rates and increase rhizodeposition (Carney, Hungate et al. 2007, Fenner, Ostle et al. 2007, Blagodatskaya, Blagodatsky et al. 2010) during short-term exposure to increased CO<sub>2</sub>. Elevated CO<sub>2</sub> could affect methanogenesis rates, however, most heterotrophic processes would be more likely to be altered by changes in oxygen concentration than CO<sub>2</sub>.

Changes in oxygen were monitored and found to not change within the sensitivity of the technique. Gas headspace measurements were taken near the end of the annual growth period and in mid-winter. Winter measurements were taken at the lowest temperature step during a period of visible senescence, corresponding with a time period with minimal photosynthesis while summer measurements were taken when plant growth rates were beginning to decline for the year and subsurface respiration was likely increasing. Both measurement periods missed the early growth period when plants would have been adding biomass, and most likely sequestering CO<sub>2</sub>, thus our values cannot be considered indicative of an entire annual cycle.

#### Methane Emissions

In all cases, CH<sub>4</sub> emissions were at least 200 times less than CO<sub>2</sub> emissions (Table 4). In the summer, unplanted columns continued to statistically emit no methane, but the presence of a plant universally resulted in an increase in CH<sub>4</sub> emissions ( $p = 0.04$ ). Emissions were higher for the three species with greater biomass (*Carex*, *Phragmites* and *Schoenoplectus*), but within species CH<sub>4</sub> emissions did not correlate to either biomass or COD load. Even when considering the increased warming potential of methane over carbon dioxide (24 times), the impact of methane emissions was minimal compared to the emissions of CO<sub>2</sub>.

#### Nitrous Oxide Emissions

Nitrous oxide emission rates averaged over the 15-day incubation are shown in Table 4 with the time series illustrated in Figure 8. Generalizations about N<sub>2</sub>O emissions

are confounded by the significant interaction between the influence of COD load and season ( $p = 0.02$ ).  $N_2O$  emissions were universally greater when COD was added (OC2x); the influence was significant ( $p < 0.001$ ), and differences were greater in the winter than the summer. In general, more  $N_2O$  was emitted in the presence of plants especially when COD was added (OC2x) and in winter. Differences between seasons depended on the plant treatment and carbon load; significantly more  $N_2O$  was emitted in the summer for *Schoenoplectus* at both COD loadings ( $p = 0.01$ ) with similar trends visible for the unplanted and *Phragmites* treatments as well as the OC0x loading for *Carex* and *Typha*. The highest  $N_2O$  emissions occurred at the OC2x loading in winter for *Carex*, *Deschampsia* and *Typha*. As expected,  $N_2O$  emissions are closely tied the removal of total dissolved nitrogen (TDN), defined as the summation of ammonium, nitrate and nitrite. For purposes of assessing  $N_2O$  emissions, TDN can be considered to be predominantly nitrate, ammonium comprised a maximum 23% of the measured TDN within 30 minutes of filling the columns. TDN removal rates over the entire experiment are shown in Table 4. Changes in both  $N_2O$  headspace concentrations and TDN water concentrations with time within an incubation are shown in Figure 8.

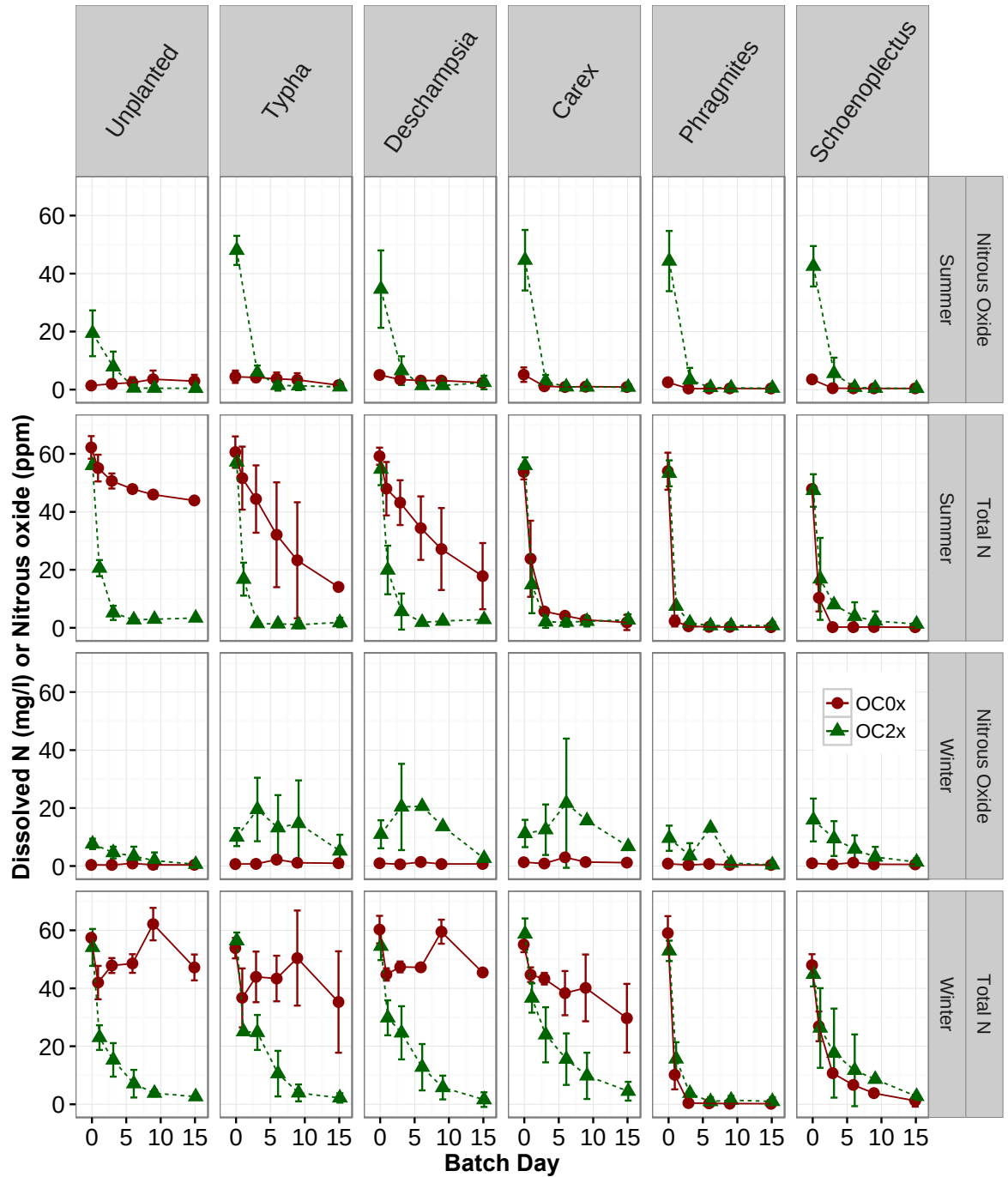


Figure 8.  $N_2O$  emissions (top panel) and total dissolved nitrogen values (bottom panel) from four replicate columns with and without COD addition separated by plant species and season, winter (4°C) and summer (24°C). Headspace sampling occurred on incubation days 0-1, 2-3, 5-6, 9-10, and 14-15. Dissolved nitrogen water quality sampling occurred on days 0, 1, 3, 6, 9, and 15. The line represents sample mean with error bars represent 95% confidence intervals.

In the unplanted columns both  $\text{N}_2\text{O}$  emissions and TDN removal were significantly greater when COD was added at twice the stoichiometric equivalent required for complete denitrification ( $\text{OC}_{2x}$ ;  $p = 0.03$  and  $p < 0.01$ , respectively). In both seasons when COD was applied to unplanted columns, virtually all TDN was removed by the end of the 15-day incubation. The rate of removal and the amount of  $\text{N}_2\text{O}$  emitted was greatest early in the incubation and steadily decreased with time (Figure 8). There is a strong correlation between the time trends of TDN removal and  $\text{N}_2\text{O}$  emitted in both the summer and winter (correlation coefficient = 0.73 and 0.48, respectively,  $p < 0.001$ ). Without COD addition to unplanted columns,  $\text{N}_2\text{O}$  emission and TDN removal were minimal in the summer, and not statistically different than zero in the winter.

Without the confounding effects of plants, nitrogen removal in unplanted columns was likely microbially driven and denitrification likely the dominant heterotrophic activity given that nitrate dominated the TDN measurements, the addition of COD positively influenced TDN removal and electron acceptors other than oxygen and nitrate are lacking in the system. The time trend in  $\text{CO}_2$  emissions (Fig. 7) supports this conclusion. Production rates of all parameters were greatest early in the incubation period and the summation of  $\text{CO}_2$  emitted and estimated DIC in the water column at the end of the incubation accounts for the majority of DOC applied. Interestingly, approximately 90% of the added organic carbon was removed within the first 24 hours with only small changes thereafter (Allen, Burr et al. 2016a), but the time trends for summation of  $\text{CO}_2$  emitted and DIC produced as well as the TDN removal and  $\text{N}_2\text{O}$  emission were more gradual as would be expected from microbial growth kinetic models. This suggests that

dissolved organic carbon is somehow sorbed rapidly from the water column but is utilized by microbial activity at a rate different from apparent DOC removal. Regardless, the carbon and nitrogen mass balances over the 15-day incubation supports denitrification as the dominant nitrogen removal pathway and primary microbial activity in the unplanted columns.

The presence of plants universally and significantly ( $p = 0.03$ ) increased  $N_2O$  emissions compared to unplanted columns when COD was added (OC2x; Table 4). The difference was most dramatic in the winter when incubation-averaged emission rates at the OC2x loading were 2 to 5 times greater, depending on species. The presence of plants had no significant influence on  $N_2O$  emissions at the OC0x loading in either season. In summer,  $N_2O$  emissions tended to follow an exponential decay with the majority of release occurring within the first 24 hours (Fig. 8). At the OC2x load the decay was strongly exponential; a minimum of 53% of the total emissions occurred within the first 72 hours regardless of species and there was no significant difference in emissions between COD loadings in measurements taken on day 3 or later. There was no obvious temporal trend in  $N_2O$  emission rate in winter, values were consistently low at OC0x load and higher with a general downward trend with time at OC2x, but high replicate variance, especially in *Carex*, *Deschampsia* and *Typha* treatments, masked any conclusive winter time trend at the OC2x load. Summer  $N_2O$  emission rates strongly correlated to changes in TDN, which were always greatest within the first 24 hours. The summer OC2x correlation coefficient between  $N_2O$  emitted and TDN removed was 0.80 ( $p < 0.001$ ), and ranged from 0.69 to 0.88 when considering specific plant species.

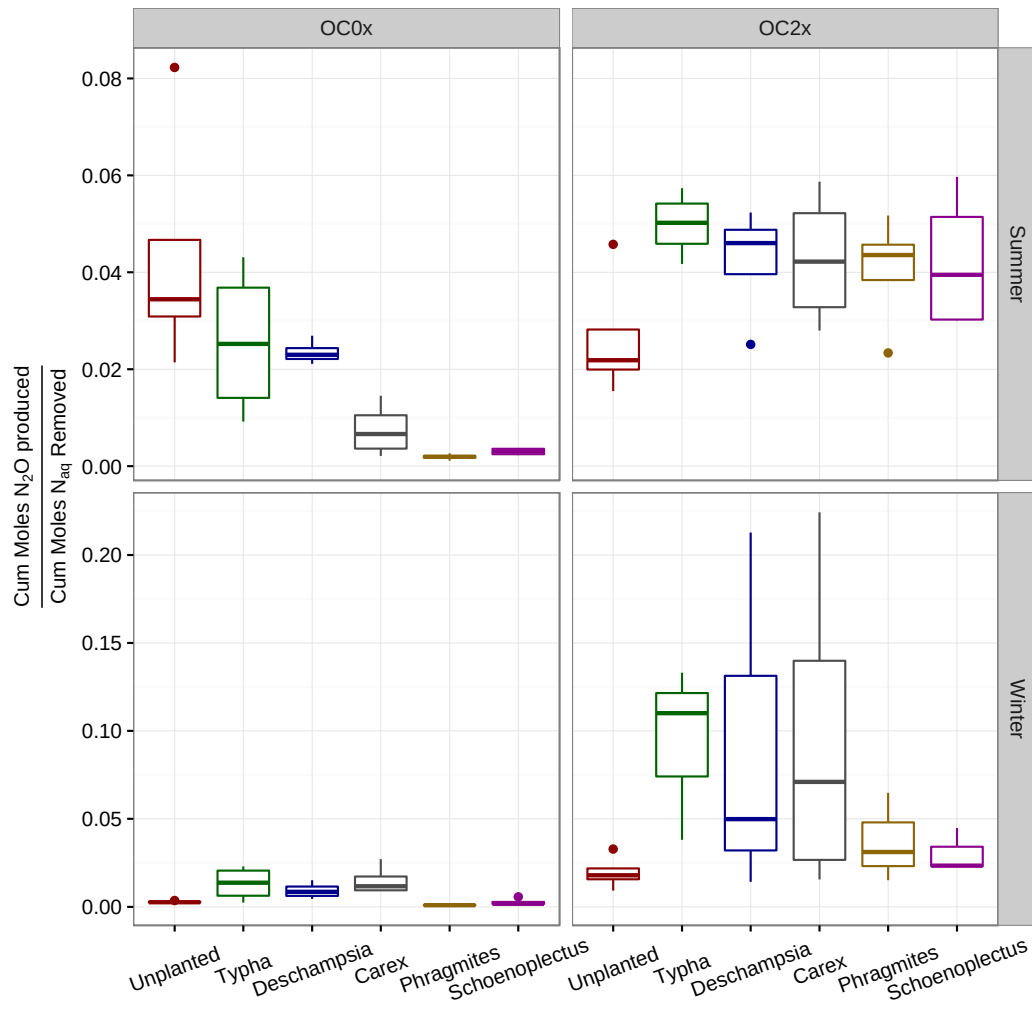


Figure 9. The emission ratio of the quantity of  $N_2O$  emitted to the total amount of dissolved nitrogen removed by the system over the duration of the 15-day incubation. Please note difference in scale between summer and winter.

The amount of  $\text{N}_2\text{O}$  emitted relative to the amount of TDN removed is an important indicator of the completeness of the denitrification process. An “emission ratio” (the ratio of  $\text{N}_2\text{O}$  emitted to the TDN removed (mole N basis)) ranged from 0.05% to 10.0%, depending on COD loading, plant treatment and season (Fig. 9). In the unplanted columns at the OC2x loading, the ratio was 2.6% and 1.8% in the summer and the winter, respectively. At the OC0x loading the corresponding unplanted values are 4.0% and 0.5%. Adding COD always increased the  $\text{N}_2\text{O}$  emission ratio in planted treatments ( $p < 0.001$  in both summer and winter). Focusing on the OC2x treatments, which universally resulted in near complete TDN removal by day 15, the presence of plants in summer always increased the relative production of  $\text{N}_2\text{O}$  from 2.6% (unplanted) to 4.3% (planted average), with  $p \leq 0.05$  for specific species except *Phragmites* for which  $p < 0.10$ . There were no significant differences between planted treatments. The emission ratio was always higher in the presence of plants in winter at OC2x as well; increases in *Carex*, *Deschampsia* and *Typha* treatments were large (emission ratio averaging 9.4%) but only moderately significant ( $p < 0.10$ ), due to considerable  $\text{N}_2\text{O}$  emission variability. The values for *Schoenoplectus* and *Phragmites* were intermediate at 3.2 and 3.8%, respectively, and not different than unplanted or other plant treatments. Species effects on the emission ratio were different at the OC0x loading. In summer without COD, emission ratios were not significantly greater than zero for the three plant treatments that facilitated complete TDN removal—*Carex*, *Phragmites*, and *Schoenoplectus*. Their means were significantly lower than the unplanted treatments ( $p < 0.005$ ) and the *Deschampsia* and *Typha* treatments ( $p \leq 0.10$ ). *Deschampsia* and *Typha* emitted more  $\text{N}_2\text{O}$  than these

species despite removing less TDN. In winter at the OC0x load, emission ratios were generally the lowest measured; the *Phragmites* and *Schoenoplectus* means were not significantly different than zero despite being the only plant treatments to remove virtually all TDN in winter without COD.

Mander et al. (2014) used an “emission factor”, defined as the percentage of incoming total nitrogen emitted as N<sub>2</sub>O to compare N<sub>2</sub>O emissions from different TW systems. Emission factors from nine HF systems analyzed by Mander et al. (2014) averaged 0.79% with a range of 0.04-3.01% while Jahangir et al. (2016) performed a similar analysis and found N<sub>2</sub>O emission factors averaged  $0.6 \pm 0.38\%$ . Though an emission factor is a valuable comparator across a variety of systems, it does not take into account potential differences in the amount of total nitrogen removed. Total nitrogen removal efficacy averaged only 31% in the Jahangir et al. (2016) study, thus the emission ratio would be about 3 times higher than the emission factor. Taking this into account, their emission ratio is about 3.5%, lower than our planted treatment average at OC2x (5.5%) and greater than our average (1.5%) at the OC0x loading.

The presence of macrophytes increased N<sub>2</sub>O emissions and the emission ratio when COD was added regardless of species or season. At the OC0x loading, plant presence had no effect on emission rates and mixed effects on the emission ratio. Several possible plant-driven mechanisms influencing N<sub>2</sub>O emissions include supporting a larger denitrifying microbial population and/or providing additional labile organic carbon to increase denitrification, or inhibiting complete denitrification through the introduction of oxygen into the subsurface. The CO<sub>2</sub> emission data suggests that plant presence increased

overall heterotrophic microbial activity, but the influence on denitrification activity is not clear since all planted treatments produced more  $\text{CO}_2$  than required to remove all TDN by denitrification whether or not carbon was added. The fact that total  $\text{N}_2\text{O}$  emissions and the emission ratio increased in presence of plants at the OC2x load, when all the TDN was removed even in the unplanted treatments, implicates incomplete denitrification as a main mechanism increasing  $\text{N}_2\text{O}$  emissions in planted treatments. Planted treatment emissions and emission ratios were generally higher in the winter when oxygenation of the subsurface could be expected to be greater (Allen, Hook et al. 2002; Taylor, Hook et al. 2011). Those studies had higher levels of applied COD, and it is possible that more oxygen was available during the summer as well as in the more lightly loaded columns of this study.

A second line of reasoning also implicates incomplete denitrification as a contributing factor to  $\text{N}_2\text{O}$  emissions. Virtually all TDN was removed in Phragmites and Schoenoplectus treatments regardless of season and COD load, yet the emission ratio increased and  $\text{CO}_2$  emissions decreased when COD was supplied. The system may have been sufficiently carbon limited to affect the completeness of denitrification without affecting the overall denitrification rate. The winter  $\text{N}_2\text{O}$  emission at the OC2x loading was correlated to TDN removal in the three treatments that produced the lowest fraction of  $\text{N}_2\text{O}$  (Unplanted, Phragmites and Schoenoplectus), and were not correlated in the three treatments that produced largest quantities of  $\text{N}_2\text{O}$  (Carex, Deschampsia, and Typha). The bulk of the  $\text{N}_2\text{O}$  produced in these treatments occurred in the middle of the incubation, after added organic carbon was removed. It is possible that the subsurface microbial

communities in these planted treatments were more diverse or there was greater relative oxygen input creating a carbon limited environment that resulted in partial denitrification.

### Integration

These interpretations should be considered in light of a few confounding factors. First, the influent was 25% ammonium and 75% nitrate. Ammonium removal was very rapid at the beginning of each incubation and is attributed to sorption to gravel and below-ground biomass with gradual nitrification rejuvenating sorption sites for the next incubation (Allen, Burr et al. 2016a). Therefore, some of the  $N_2O$  emitted may have been produced by the nitrification process and the separation the nitrification from denitrification emission pathways is not possible. In the simplified case of the unplanted columns, oxygen input must have been low especially when COD was supplied and unplanted columns maintained measureable ammonium throughout the incubation. In unplanted columns it appears that, potential  $N_2O$  emission by nitrification was very low. Regardless, most of the nitrified ammonium would have had to be eventually denitrified to justify the observed TDN removal, suggesting that a minimum of 80% of the occurring nitrogen transformation processes was denitrification.

Some of the observed TDN removal is attributable to plant uptake, though the strong temporal correlations between TDN removal and  $N_2O$  emissions suggest denitrification dominated. Nitrogen removed by plant assimilation would not have been available for microbial processes. Presumably plant uptake was greater in summer and in species with higher biomass, thus plant uptake may be a contributing factor to the lower emission ratio values at the OC0x load and extremely low emission ratios for *Phragmites*

and *Schoenoplectus* which had the highest biomass. Nevertheless, plants can emit  $\text{N}_2\text{O}$  when taking up nitrate as the nitrogen source, so that the effect of plant uptake on nitrous oxide emissions is unknown.

### Conclusions

Emissions of carbon dioxide, methane and nitrous oxide were significantly influenced by influent organic carbon concentrations, presence and species of macrophytes and by season. Equally strong interactions between these factors underscore the complexity of GHG emissions from treatment wetlands. Methane emissions were generally low. Relatively low organic carbon loading rates likely limited methanogenic processes.  $\text{CO}_2$  emissions were at least two orders of magnitude larger than  $\text{CH}_4$  emissions (mass C basis). The presence of plants universally increased  $\text{CO}_2$  emissions;  $\text{CO}_2$  emissions were also larger in the summer than the winter in the planted treatments. The unplanted treatment and planted treatments with limited plant biomass had more  $\text{CO}_2$  emissions when organic carbon was added, but plants with significantly more plant biomass emitted more  $\text{CO}_2$  in the absence of organic carbon addition. This is attributed to the plants' ability to supply organic carbon for heterotrophic processes by exudation and detritus decay or by increased respiration. We believe these plants had less biomass when organic carbon was added because the organic carbon stimulated denitrification to the point that plant growth was inhibited by a lack of nitrogen, thus tying  $\text{CO}_2$  release to the nitrogen cycle.

Nitrous oxide emissions are closely tied to the removal of total dissolved nitrogen, both in magnitude over the incubation and temporally within the incubation. Adding organic carbon universally increased  $\text{N}_2\text{O}$  emissions and almost always increased the ratio of  $\text{N}_2\text{O}$  emitted to TDN removed with emissions ratios that increased from approximately 1.6% to 4.75% with organic carbon addition. The presence of plants generally increased the emission ratio, especially at higher organic carbon loading. Increased overall denitrification rates contribute to the increased  $\text{N}_2\text{O}$  emissions. However, in several cases all of the TDN was removed regardless of organic carbon addition but the emission ratio increased when organic carbon was applied, suggesting that the carbon addition influenced the completeness of the denitrification process. With the addition of organic carbon approximately 4% of the nitrogen removed may be emitted as  $\text{N}_2\text{O}$  in the summer which increased to approximately 6% in the winter with some variation due to plant species.

Conclusions must be placed in the context of the study which was designed to assess the nitrogen removal capacity of a horizontal flow TW receiving water high in nitrate but with remaining ammonium and limited organic carbon, e.g. a typical tertiary system or one designed primarily for runoff. Treatment wetlands designed primarily for COD removal would have much higher C:N ratios and the majority of the nitrogen would be in the form of ammonium. In our study influent total nitrogen averaged  $72 \text{ mg N l}^{-1}$  with 75% as nitrate and 25% as ammonium. Organic carbon was added to the influent at twice that required for complete nitrogen removal by classic denitrification or not at all. The total nitrogen loading rate was  $177 \text{ g N m}^{-2} \text{ yr}^{-1}$  which, due to the long incubation

time of 15 days, is low by many operational TW standards. Nitrogen removal in tertiary systems with higher nitrogen loading rates would likely be more influenced by denitrification as opposed to plant uptake and require more organic carbon hence a higher C:N to effectively remove nitrogen with unknown effects on GHG emissions.

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## CHAPTER FOUR

### EMPIRICAL MODELING OF PLANT INFLUENCE AND ORGANIC CARBON ADDITION ON NITROGEN REMOVAL IN TREATMENT WETLANDS

#### Contribution of Authors and Co-Authors

Manuscript in Chapter 4:

Author: Christopher R. Allen

Contributions: Generated physical experimental design. Performed experiment set-up, data collection, analysis and generated the initial draft of the manuscript.

Co-Author: Otto R. Stein

Contributions: Primary investigator on initial experiment proposal. Advised on experimental design and analysis. Provided edits on the manuscript.

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EMPIRICAL MODELING OF PLANT INFLUENCE AND ORGANIC CARBON  
ADDITION ON NITROGEN REMOVAL IN TREATMENT WETLANDS

Abstract

Time series nitrogen data from batch-operated wetland microcosms were modeled to investigate the effects of carbon addition, season and plant species on removal kinetics. The feed solution consisted of 72 mg N l<sup>-1</sup> (75% nitrate, 25% ammonium) plus one of three C:N ratios: zero (OC0x), 1.2 (OC1x) and 2.4 (OC2x), with organic carbon added as sucrose. Plants treatments included: unplanted, *C. utriculata*, *D. cespitosa*, *P. acutus*, *S. acutus*, and *T. latifolia*. Linear models approximating zero through second order kinetics, and a non-linear regression representing the k-C\* model were fit to 180 unique combinations of temperature, plant species and organic carbon treatments. The model for each of the unique combinations was created using the data from four replicates. R<sup>2</sup> comparisons between the kinetic models, illustrated a general shift from zero to higher order removal kinetics as carbon addition and plant influence increased. The k-C\* model better characterized the dynamics observed in the system, but the application of the k-C\* model was limited at low removal rates. An Arrhenius relationship was applied to determine the k<sub>20</sub> and the temperature dependency  $\theta_k$  within the k-C\* model for nitrogen removal. The k<sub>20</sub> estimates were found to increase with carbon addition for all plant treatments (OC0x mean k<sub>20</sub> = 1.13d<sup>-1</sup> and OC2x mean k<sub>20</sub> = 2.07d<sup>-1</sup>). The k<sub>20</sub> values determined were likely exaggerated compared to field systems, however, the effects of temperature on the k<sub>20</sub> should apply and  $\theta_k$  was approximately 1.07, averaged across all planted treatments. Processes involved in nitrogen removal were investigated by

comparing seasonal estimates of plant N uptake with observed N removal, illustrating a seasonal pattern of plant uptake dominated N removal during the growing season and microbe dominated winter N removal.

### Introduction

The enhancement of nitrate removal in treatment wetlands (TW) and the amelioration of agricultural runoff and tile drainage is currently of great concern, as nitrates cause eutrophication of waterways and, at high levels, become an acute public health risk. In the effluent of many Vertical Flow TWs (VFTWs) as well as in agriculturally-polluted water, high levels of nitrate occur without significant quantities of organic carbon, limiting the effectiveness of the denitrification microbial removal pathway. Denitrification is a primarily heterotrophic, stepwise process that couples oxidized N species like nitrate with organic carbon to eventually produce dinitrogen gas. It is the presumed primary permanent removal pathway for oxidized N in wetlands (Faulwetter, Gagnon et al. 2009). Without incoming organic carbon (OC), plant primary production may represent the main source for OC in the wetland, and as a result be the main driver of nitrogen removal (Miller and Fujii 2009, Zhai, Piwpuan et al. 2013). Wetland systems provide nitrogen removal in carbon limited waters without the addition of OC, however, the effectiveness is influenced by design parameters such as the presence of a free water surface and the amount of contact between water and wetland sediments (Kadlec and Wallace 2008, Iannelli, Bianchi et al. 2011, Collins and Gillies 2014). Because of the confounding effects in many field scale applications, the potential influence of plants is not well characterized. Often the pollutant mass loading of TWs

designed to treat storm and stream water is less than wetlands receiving domestic waste, and as a result the impacts of plant growth may be larger.

Nitrogen can be removed from influent water by a variety of pathways, some of which are practically permanent and others only temporary. The primary nitrogen removal pathways include sorption, plant uptake, and microbial respiration and uptake. Gaseous removal of ammonium is also worth noting, though generally insignificant in wetland systems with neutral pH values (García, Rousseau et al. 2010). While microbial respiration of nitrogen via denitrification permanently removes aqueous nitrogen from the system by forming nitrogen gas, other forms of removal from the water column may move the nitrogen from one pool in the system to another.

Plant uptake removes both nitrate and ammonium from the water column. The plant then assimilates the nitrogen into biomass. While the biomass is alive this nitrogen is bound, however, as the dead above and below ground biomass decays it represents a slowly biodegradable source. The nitrogen bound in plant matter varies in its level of recalcitrance, with the majority of the N potentially becoming available over the time period of one year (Hume, Fleming et al. 2002).

Sorption represents both a permanent and temporary pool for nitrogen. The primarily negatively charged soils / planting medium have an affinity for positively charged ammonium ions. Depending on the wetland media, some ammonium may be very tightly bound to sorption sites and for all practical purposes be permanently removed from the water column. The majority of the N sorbed, however, may still be highly bioavailable as both plants and microorganisms can access the loosely sorbed

nitrogen for growth processes, either incorporating the N into biomass or releasing the N as an oxidized nitrogen compound as a result of nitrification (García, Rousseau et al. 2010). The total sorption and amount of practically permanent sorption is a function of the planting media.

Microbial processes including assimilation and denitrification can represent both temporary and permanent removal mechanisms. Microbial assimilation also places the N into biomass. Portions of the microbial biomass may recycle, becoming bioavailable as cells die, while a smaller fraction will become more recalcitrant and bound into particulate organic matter. Depending on the subsurface redox conditions and the level of recalcitrance of the N containing compounds, N may be bound in particulate matter on a timescale that for all practical purposes represents permanent removal (García, Rousseau et al. 2010). Denitrification is the stepwise reduction of nitrate to (ultimately) di-nitrogen gas and is performed primarily by heterotrophic microorganisms that couple organic carbon and oxidized nitrogen species for respiration. Denitrification is dependent on the availability of electron donors like organic carbon, oxidized nitrogen species, and is inhibited by oxygen (Kadlec and Wallace 2008).

Primary production of plants may facilitate the removal of nitrogen in two ways, the seasonal uptake of nitrogen and the production of carbon compounds to fuel denitrification. Plant species vary in the amount of N per gram of plant biomass, and produce different amounts of biomass. The biomass of various plant species also decays at different rates. Plants can contribute organic carbon via direct exudation of excess photosynthate and the degradation of plant detritus and plants also input oxygen via the

transport of atmospheric oxygen into the subsurface of the wetland system. The ability to provide both carbon and oxygen allows plants to provide both electron donors and acceptors facilitating the growth of microorganisms that can uptake nitrogen into their biomass and when organic carbon is in excess, perform denitrification. All of these processes work in concert in a wetland system, removing nitrogen from the incoming water.

Modeling the removal of N from the system can improve our ability to scale up experimental results into practical designs as well as help test hypotheses surrounding the mechanics of N removal. Models for constituent removal vary considerably in their complexity, ranging from empirical mass removal rate models to sophisticated biokinetic models that incorporate and predict the concentrations of multiple constituents simultaneously (Langergraber, Rousseau et al. 2009, Samsó and Garcia 2013, Pálffy and Langergraber 2014, Meyer, Chazarenc et al. 2015). Empirical rate models provide an easy-to-apply tool for analysis of gross process rates and offer the potential for laboratory results to be scaled up and used in design (Kadlec and Wallace 2008). However, the ability to test process-based-hypotheses is limited. Rate models vary in form, with statistically estimated coefficients that depend on the form of the model. The most common kinetic models are zero, first, second, and modified first order models (Clark 1996).

Rate Models:

$$\frac{dC}{dt} = k \xrightarrow{\text{Integration yields}} C_N = C_0 - kt \quad \text{Zero order}$$

$$\frac{dC}{dt} = k[C_N] \xrightarrow{\text{Integration yields}} C_N = C_0 e^{-kt} \quad \text{First order}$$

$$\frac{dC}{dt} = k[C_N - C^*] \xrightarrow{\text{Integration yields}} C_N = C^* + (C_0 - C^*)e^{-kt} \quad \text{Modified first order, } kC^*$$

$$\frac{dC}{dt} = k[C_N]^2 \xrightarrow{\text{Integration yields}} C_N = \frac{1}{\left(kt + \frac{1}{C_0}\right)} \quad \text{Second order}$$

In all presented models,  $C_N$  is the concentration of total dissolved nitrogen (TDN) at time  $t$ ,  $t$  is time in days,  $k$  is an empirically calibrated constant with units that vary based on the kinetic model ( $\text{mg N day}^{-1}$ ,  $\text{day}^{-1}$ , and  $\text{mg}^{-1} \text{ N day}^{-1}$ , for zero, first and second order rate constants, respectively),  $C_0$  is the concentration of TDN at  $t=0$ , and  $C^*$  is the concentration of TDN as  $t$  approaches infinity. Each rate model provides limited information about the kinetics occurring in the system with respect to the measured or modeled parameter. Zero order kinetics are characterized by linear removal rates with time, and occur when removal is limited by factors other than the availability of the substance. For instance, nitrate removal may be linear with time if the system is limited by the availability of organic carbon, or when low temperatures reduce reaction rates.

First order and modified first order removal kinetics indicate that the rate of removal is a function of the concentration of the constituent. Many microbial consumption processes are modeled using first order kinetics, and more complex biokinetic models may be well approximated by a series of sequential and interrelated first order kinetic models. A single first order model often accounts for several simultaneously-occurring first order reactions, as the rate constant for each is

incorporated into a global rate constant for the system and is determined empirically. Modified first order kinetics are similar in mathematical form, however, the modified version accounts for a non-zero asymptote. This model is often referred to as the  $k$ - $C^*$  model where  $C^*$  is a non-zero asymptote residual concentration. The  $k$ - $C^*$  model has been used to provide both mass and areal based rates of removal in treatment wetlands and is one the most widely applied models in horizontal flow wetland design (Stein, Biederman et al. 2006, Kadlec and Wallace 2008).

The residual concentration of various constituents in wetland systems may reflect physical realities such as non-biodegradable COD or OC production, or may be indicative of poor treatment when there is no physical reasoning behind the presence of a concentration residual, as when modeling nitrate in horizontal flow wetlands. For some constituents such as nitrate, which should theoretically reach an asymptote close to zero in wetland systems (Kadlec and Wallace 2008),  $C^*$  may simply reflect artifacts of calibration experiments that are short in duration compared to the actual removal rates, which could lead to false conclusions about the involved physical processes.

Lastly, second order removal rates are valid when the rate is proportional to the concentration of two constituents, or in the special case when the reaction rate is influenced by two reactants with the same initial concentration and coupled with 1:1 stoichiometry, the rate is proportional to the concentration of one of the constituents squared (Clark 1996).

The first order kinetic constant can be adjusted for temperature using the Arrhenius equation.

$$k_T = k_R \theta^{T-T_R}$$

*Arrhenius equation*

For this equation,  $k_R$  is the rate constant at 20 °C,  $k_T$  is the rate constant at temperature T, and  $\theta$  is an empirically determined constant. The Arrhenius equation physically represents the decrease in available energy in a system as temperature decreases. In the simplified form shown above, the equation can be considered an empirical expression incorporating the effect of temperature and can therefore be applied to any model parameter expected to vary with temperature (Kadlec and Wallace 2008). Thus the effect of temperature on both  $k$  and  $C^*$  can be empirically fit by applying an Arrhenius equation to values of either determined at various temperatures. In wetland systems the empirical nature of this approach allows the equation to account for temperature effects of processes such as seasonal plant growth and senescence (Stein, Biederman et al. 2006).

In wetlands where treatment processes may be tied to plant inputs and exports, seasonal plant biomass production and senescence likely play a large role in the efficacy of the treatment. At low nitrogen loading rates (total nitrogen loadings  $120 \text{ g N m}^{-2} \text{ yr}^{-1}$  or below), plant uptake dominates nitrogen removal processes (Kadlec and Wallace 2008) and the seasonal sequestering of nitrogen into plant biomass is important because plant uptake may represent a temporary removal mechanism with net nitrogen inputs from the plant occurring during plant senescence.

Senescence is a period of very low plant activity, where the plants shut down photosynthesis for the winter with effects both above and below ground. Above ground biomass often desiccates and decays, potentially acting as an input of both OC and nitrogen during decomposition. An annual cycle of root turnover is also common. Plants

store OC and nutrients in some roots during senescence, while other roots decay, providing another potential OC and nitrogen source, with an annual rate of root turnover in wetland species of approximately 58% in temperate climates (Gill and Jackson 2000). Plant activity such as the uptake of nutrients is generally limited, thus TW nitrogen removal should be primarily microbial in nature. The OC required for microbial removal of nitrates likely originates from decaying organic matter as the diel inputs of excess photosynthate seasonally cease. The rate of decay of plant derived organic matter which makes OC labile is affected by temperature, the composition of the organic matter, and how much oxygen is available. Assuming other inputs of OC are limited, the total capacity of nitrate-specific microbial removal depends on the total mass of carbon input into the subsurface on an annual cycle.

Modeling plant growth patterns can assist in determining when plant uptake vs microbial processes may dominate nitrogen removal in TWs. During plant growth, N uptake rates are as high as  $0.04 \text{ mg N d}^{-1} \text{ gDW}_{\text{root}}^{-1}$  (Raman, Spanswick et al. 1995), but vary based on N stress of the plant (Šimůnek and Hopmans 2009). Microbial uptake varies based on quantities of required nutrients, inhibitors, and the amount of microbial biomass (Langergraber, Rousseau et al. 2009). The competition between microbes and plants for nitrogen is a topic of current debate. Microorganisms can outcompete plants for nitrogen in grassland systems, retarding plant growth (Inselsbacher et al. 2010). Over longer periods however, as microbial populations stabilize, plants often take up N remaining from the decay of the microbial biomass. Thus the availability of nitrogen for plants may be delayed, and reduced by the amount of nitrogen lost to denitrification and

into recalcitrant particulate organic matter. The net result of competition between plant uptake and microbial processes is likely system dependent. In highly aerobic soils the fraction of N lost to denitrification may be low, while in wetland systems Allen et al. (2016) found that the total biomass of plants was negatively correlated to organic carbon loading and was attributed to nitrogen limitation due to denitrification stimulated by the OC addition.

Investigating the seasonal relationship between plant growth and N removal is important to elucidate the total removal capacity of the system, determine if removal capacity varies seasonally, and help to formulate hypotheses that may be addressed by further experimentation or biokinetic modeling. Previous chapters have outlined the general water quality and greenhouse gas emission results from this experiment. This chapter uses a combination of primarily empirical modeling techniques and an annualized nitrogen mass balance to further investigate potential mechanisms and seasonal trends.

### Methods

Time series water quality data from the experiments presented by Allen et al. (2016a) were used in this analysis and detailed information on the sampling and experimental design may be found there. In brief, the climate-controlled greenhouse experiment was performed in Bozeman, Montana (45.7° N, 111.0° W) between November 2010 and September 2014. In October 2010, 18 replicates of five plant species (with family), *Carex utriculata*, and *Schoenoplectus acutus* (Cyperaceae); *Deschampsia cespitosa* and *Phragmites australis* (Poaceae), and *Typha latifolia* (Typhaceae), were planted into 15-cm diameter by 40-cm deep PVC columns filled to a depth of 30 cm with

gravel having a sorption capacity estimated to be around 190 meq kg<sup>-1</sup>. Eighteen additional replicates contained unplanted gravel. For simplicity all plant treatments will be referred to using the genus name, non-italicized to emphasize the naming convention and the potential variation between species in a genus. After a nine-month plant establishment period characterized by elevated temperatures (20 °C) and artificial lighting, the temperature was changed on 45-day increments from 24 °C, to 18 °C, to 12 °C, to 8 °C, to 4 °C and back to 24 °C following the same pattern.

Every 15 days the TW columns were gravity drained and approximately 1.8 liters of synthetic wastewater was added to each column to initiate an incubation. The synthetic wastewater contained 18 mg NH<sub>4</sub><sup>+</sup>-N l<sup>-1</sup> and 54 mg NO<sub>3</sub><sup>-</sup>-N l<sup>-1</sup> and one of three levels of OC supplied as sucrose. Micro-nutrients consistent with previous experiments (Taylor, Hook et al. 2011) were also added. The 18 replicates of each plant treatment were subdivided into three groups of six replicates. Each group received either zero OC (OC0x) or one of two levels of OC representing exactly once (OC1x) or twice (OC2x) the stoichiometric equivalent required to remove all nitrogen by classic denitrification. These feed solutions represented OC:N mass ratios of zero, 1.2 and 2.4 and COD:N ratios of zero, 2.7 and 5.5 g COD per g N, respectively.

Beginning in January 2012, 30 ml water samples were taken at the geometric center of the gravel bed via a permanently installed sample tube. Collection occurred during the third incubation of each temperature and seasonal setting (8 per year) on incubation days 0, 1, 3, 6, 9 and 15. Incubation day zero samples were collected within 30 min of feeding the columns, with all subsequent samplings at the approximate same

time of day as the initial feeding. The samples were filtered at the time of the collection through a 0.2µm filter and were analyzed for dissolved concentrations of  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$  and  $\text{HPO}_4^{2-}$  using ion chromatography (Metrohm USA.). For this modeling analysis total dissolved nitrogen data (the summation of the nitrogen containing ions nitrite, nitrate and ammonium) from 10 incubations (two at each temperature over an annual cycle) were included, comprising all the previously published TDN data presented in this experiment. At the terminus of the experiment all plants in OC0x and OC2x treatments were destructively sampled, to determine the overall mass of N incorporated into total plant biomass.

#### Statistical Analysis:

Regression models were run using the time series data from each unique combination of six plant species, three OC loadings and ten seasonal incubations. Each regression used the conglomerated data from four replicates of each combination resulting in a total of 180 unique incubation combinations. Four models were run for each unique combination, three linear models using the function `lm()` and one non-linear regression using the function `nlme()` in the program R build 3.2.4 (Pinheiro, Bates, 2016, R Core Team 2016). Inputs to the linear models depend on the model; the raw non-transformed data over time, the log transformed data over time and one over the data with time, for zero through second order kinetics respectively (Clark 1996). The final model was a non-linear regression based on the k-C\* model following the form presented in Equation 1.

$$C_n = C^* + (C_0 - C^*) * e^{-kt} \quad \text{Equation 1}$$

Goodness of fit metrics were summarized and printed for each model. The model goodness of fit metrics included: a plot of the data with the model, residual and a quantile quantile (QQ) plots for each model,  $R^2$ , and all model coefficients. All models were compared using the  $R^2$  metric in conjunction with the visual assessment of the residuals and QQ plots.

The k-C\* model results were further analyzed to determine the temperature dependency of the decay rate (k), initial concentration ( $C_0$ ), and residual concentration ( $C^*$ ). Log transformed coefficient values were modeled using the linear model presented in equation 2 to determine  $\Theta$  and  $k_{20}$  values.

$$\ln(k_T) = \ln(k_{20}) + \ln(\Theta_k) (T - 20) \quad \text{Equation 2}$$

### Results and Discussion

Previous analysis indicated that the mass removal of TDN is significantly affected by plant species, temperature, and carbon loading (Allen, Burr et al. 2016a). Questions remain regarding the kinetic effects of these factors on TDN removal from the nitrate dominated influent. Visual analysis of the total dissolved nitrate (TDN) time series data indicated all three experimental factors (temperature, plant species and carbon addition) influence the shape of the degradation curve. It was hypothesized that if the system was limited by organic carbon removal should be zero order with respect to TDN and, at higher OC levels the removal would be better represented by a higher order model. To

test this general hypothesis, the zero, first and second order kinetic models were compared using the  $R^2$  value. The model with the highest  $R^2$  was then chosen to best represent the removal of the four replicates in that incubation for that plant and organic carbon treatment combination. The number of of times a given model provided the highest  $R^2$  value can be found in Table 5. The level of organic carbon addition increases from left to right, and the plant treatments are ranked from lowest to highest in their ability to increase the modeled kinetic removal order.

Table 5. Frequency of either a zero, first or second order model best representing the time series data for a unique treatment combination based on the  $R^2$  value separated by plant treatment and OC load. Each block of three model results sums to 10, representing the number of incubations analyzed, with the exception of the Phragmites OC0x and OC1x, and the Schoenoplectus OC2x, each of which had one incubation analysis fail.

|                | OC0x |       |        | OC1x |       |        | OC2x |       |        |
|----------------|------|-------|--------|------|-------|--------|------|-------|--------|
|                | Zero | First | Second | Zero | First | Second | Zero | First | Second |
| Unplanted      | 9    | 0     | 1      | 3    | 5     | 2      | 0    | 7     | 3      |
| Typha          | 8    | 1     | 1      | 5    | 5     | 0      | 2    | 6     | 2      |
| Deschampsia    | 6    | 4     | 0      | 3    | 7     | 0      | 2    | 8     | 0      |
| Carex          | 2    | 6     | 2      | 1    | 1     | 8      | 2    | 3     | 5      |
| Schoenoplectus | 0    | 7     | 3      | 0    | 6     | 4      | 1    | 1     | 7      |
| Phragmites     | 0    | 2     | 7      | 0    | 1     | 8      | 1    | 3     | 6      |
| Total          | 25   | 20    | 14     | 12   | 25    | 22     | 8    | 28    | 23     |

As seen in Table 5, the zero order kinetic model decreases in frequency of best fit as carbon load increases. The zero order kinetics were expected to be most heavily represented on the OC0x treatments as organic carbon, not nitrogen, was limiting the denitrification reaction. This relationship is especially evident in the unplanted columns where the zero order kinetics model fits the data best 9 out of 10 times at OC0x and 0 out of 10 times in the OC2x treatments. This trend can also be seen in the Typha and

Deschampsia plant treatments which were characterized by relative poor plant health, low biomass values, and limited plant effect on water quality changes (Allen, Burr et al. 2016a).

In the OC0x treatments, plant presence facilitated a transition from a zero order model to a higher order kinetic model. The level of this effect is correlated to the total nitrogen removal efficacy measured by Allen et al. (2016a), with the removal from higher performing species (Carex, Schoenoplectus and Phragmites) being better modeled using first or second order removal kinetics. Though second order kinetics would be conceptually appropriate in the OC1x treatments as carbon and nitrogen were added at equal concentrations and are removed via approximately 1:1 stoichiometry, second order models appear to best represent the data more often when removal rates were high, specifically in the OC2x, high performing plant species Carex, Schoenoplectus and Phragmites.

Some of the fitted models did not capture the intricacy of the data. This was especially evident when TDN concentrations dropped significantly within the first two days of the incubation length. As an example, Figure 10 shows (in this case for OC2x) that the first and second order models often under-estimated the rates of reduction, and miss the highly curvilinear shape within the first 48 hours. The resulting intercepts were often significantly lower than the measured initial concentrations, sometimes by an order of magnitude. Despite these obvious limitations, using simple one parameter kinetic models have merit, illustrating the general change in kinetics as organic carbon becomes less limiting.

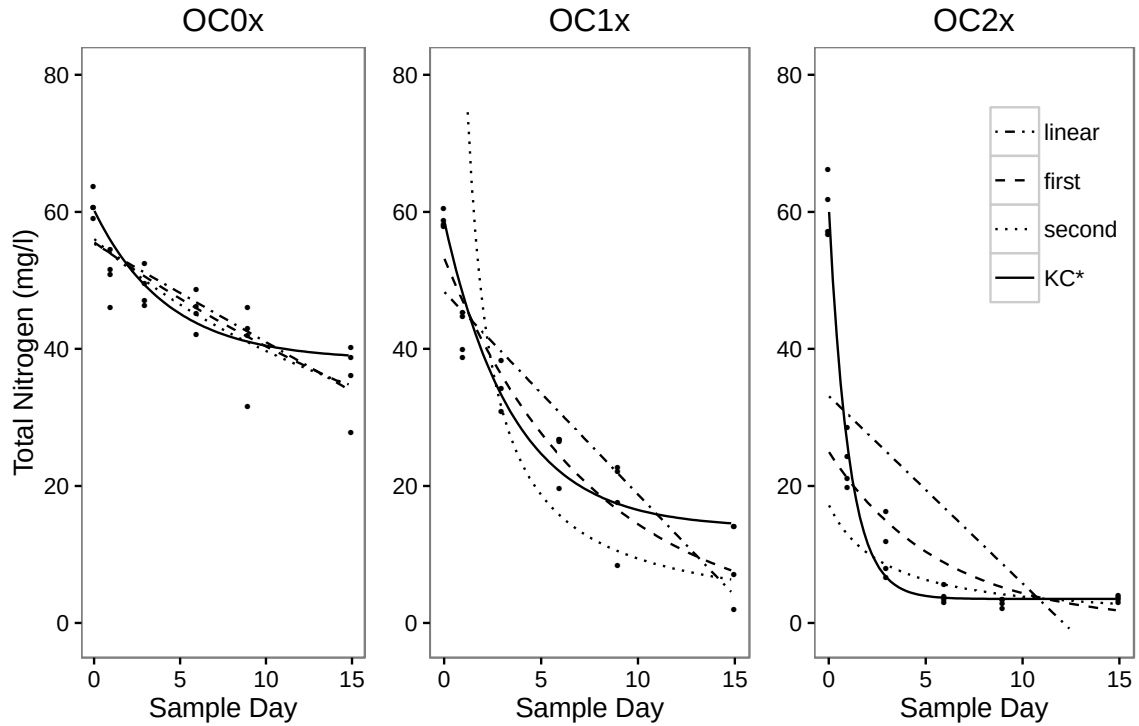


Figure 10. Total dissolved nitrogen removal across an incubation with model approximations for the unplanted OC0x, OC1x, and OC2x treatments in the fall 18 °C temperature step.

The k-C\* model offers a potential improvement to one parameter models by allowing for an asymptotic value greater than zero, and is often used as the industry standard for design (Kadlec and Wallace 2008). The k-C\* model is fit through non-linear regression and adds another parameter which should inherently improve fit, but may not necessarily provide a better model. To assess the goodness of fit, the mean  $R^2$  values from the 10 incubations are presented in Table 6 for both the best fit single parameter model and the k-C\* model. Though the  $R^2$  value is not an ideal metric for comparing non-linear models, in this application the  $R^2$  value adequately assesses which model accounts for more data variability, without addressing model superiority. In all cases the

$R^2$  metric for goodness of fit is higher for the k-C\* model than the best fitting one parameter model suggesting that it was better able to represent the TDN removal time series data. As OC addition and TDN removal due to the effects of specific plant species increase, moving from OC0x to OC2x, the k-C\* model increases in goodness of fit compared to the single parameter model. The difference between goodness of fit between model types is low without additional organic carbon and when the effects of specific plant species results in limited TDN removal. This reinforces the transition from lower order models to higher order models as removal increases and highlights the k-C\* model's ability to represent the non-linear removal of TDN.

Table 6. Comparison of  $R^2$  values between the single factor model and the k-C\* model aggregated by organic carbon load and plant treatment.

|                | OC0x                   |      | OC1x                   |      | OC2x                   |      |
|----------------|------------------------|------|------------------------|------|------------------------|------|
|                | Best fit single factor | K-C* | Best fit single factor | K-C* | Best fit single factor | K-C* |
| Unplanted      | 0.52                   | 0.58 | 0.83                   | 0.85 | 0.69                   | 0.95 |
| Typha          | 0.60                   | 0.67 | 0.71                   | 0.83 | 0.66                   | 0.93 |
| Deschampsia    | 0.65                   | 0.75 | 0.73                   | 0.89 | 0.58                   | 0.94 |
| Carex          | 0.60                   | 0.81 | 0.53                   | 0.77 | 0.56                   | 0.97 |
| Schoenoplectus | 0.63                   | 0.96 | 0.63                   | 0.96 | 0.57                   | 0.95 |
| Phragmites     | 0.68                   | 0.97 | 0.68                   | 0.97 | 0.55                   | 0.97 |

The k-C\* model was not without pitfalls. When the reduction of TDN was slow, the k-C\* model often exaggerated the rate of initial removal and fit an asymptotic value of TDN ( $C^*$ ) while the concentration of TDN appeared to still be declining. This effect is well illustrated in the model coefficient results from the data presented in Figure 10. The first order rate constants ( $k$ ) were 0.03, 0.13 and 0.17  $d^{-1}$  for OC0x, OC1x, and OC2x

respectively for the single parameter first order model while the k-C\* model resulted in k coefficients that were 0.23, 0.28 and 0.97 d<sup>-1</sup> respectively with corresponding C\* values of 38.5, 13.8 and 3.5 mg l<sup>-1</sup>. Focusing on the rate constants from the OC0x treatments (0.03 and 0.23 d<sup>-1</sup> for the first order single parameter and k-C\* model respectively), the k-C\* model k value was an order of magnitude greater than the single parameter model and fit a non-linear curve to what visually appears to be a linear dataset. The general linearity of this dataset is reinforced by the zero order single factor model providing the best single parameter model fit ( $R^2 = 0.714$ ) followed closely by the first order model with a coefficient close to zero ( $R^2 = 0.711$ ). The first order model did not perform significantly worse than the zero order model and likely provided an approximate first order decay rate that physically represents the system better than the k-C\* model. Focusing on the OC2x treatment in Figure 10, the k-C\* model appears to provide a superior fit to the data. This is reinforced by the k-C\*  $R^2$  of 0.97 compared to the best fit single parameter model (first order)  $R^2$  of 0.42. The k value of 0.17 d<sup>-1</sup> from the first order single parameter model underestimated the rate of removal and resulted in an intercept (24.5 mg N l<sup>-1</sup>) which is much less than the measured (72 mg N l<sup>-1</sup>) or estimated from the k-C\* model (60.8 mg N l<sup>-1</sup>). The ability of the k-C\* model to better represent the data as OC load and plant effect increased was ubiquitous across incubations.

Table 7. k-C\* model results summary with the rate constant at 20 °C ( $k_{20}$ ) on the left followed by the temperature coefficient  $\Theta_k$  followed by the incubation residual  $C^*$  and the initial concentration  $C_0$  with their respective temperature coefficient.

| Plant Treatment | Carbon Treatment | $k_{20}$ | $\Theta_k$ | $C^*_{20}$ | $\Theta_{C^*}$ | $C_0$ | $\Theta_{C_0}$ |
|-----------------|------------------|----------|------------|------------|----------------|-------|----------------|
| Unplanted       | OC0x             | 0.19     | 0.97       | 44.5       | 0.99           | 62.4  | 1.00           |
|                 | OC1X             | 0.23     | 1.03       | 8.6        | 0.92           | 59.4  | 1.00           |
|                 | OC2x             | 1.22     | 1.07       | 3.8        | 1.02           | 60.5  | 1.01           |
| Carex           | OC0x             | 1.00     | 1.04       | 0.4        | 0.83           | 56.3  | 1.00           |
|                 | OC1X             | 1.81     | 1.09       | 0.2        | 0.84           | 52.1  | 1.00           |
|                 | OC2x             | 2.46     | 1.07       | 0.5        | 1.02           | 59.4  | 1.00           |
| Deschampsia     | OC0x             | -        | -          | -          | -              | -     | -              |
|                 | OC1X             | 0.57     | 1.12       | 1.3        | 0.93           | 55.5  | 1.00           |
|                 | OC2x             | 1.38     | 1.09       | 1.4        | 0.95           | 54.6  | 1.00           |
| Phragmites      | OC0x             | 2.46     | 1.08       | 0.2        | 0.99           | 58.8  | 1.00           |
|                 | OC1X             | 2.24     | 1.07       | 0.2        | 0.97           | 57.6  | 1.00           |
|                 | OC2x             | 2.73     | 1.06       | 0.2        | 1.01           | 56.9  | 1.00           |
| Schoenoplectus  | OC0x             | 1.85     | 1.07       | 0.2        | 0.97           | 55.0  | 1.00           |
|                 | OC1X             | 2.62     | 1.08       | 0.2        | 0.99           | 56.7  | 1.00           |
|                 | OC2x             | 2.28     | 1.02       | 0.3        | 1.06           | 51.1  | 1.00           |
| Typha           | OC0x             | 0.17     | 0.98       | 10.4       | 0.90           | 61.0  | 1.00           |
|                 | OC1X             | 0.60     | 1.07       | 0.2        | 0.74           | 57.3  | 1.00           |
|                 | OC2x             | 1.68     | 1.08       | 0.3        | 0.90           | 58.7  | 1.00           |

Carbon load, plant species, and the inseparable temperature/season factor all contributed to the rate of removal and fitted residual concentration. To model seasonal changes, the  $\Theta$  and  $K_{20}$  values within the Arrhenius relationship were fit to the three model parameters  $k$ ,  $C^*$  and  $C_0$ . The results of the modeling effort for TDN are summarized in Table 7.

As previously discussed, when removal rates were low, the models occasionally failed, as was the case with the OC0x Deschampsia treatment where too few models ran without failure thus an Arrhenius relationship could not be determined. In general, the

rate constant or  $k_{20}$  increased with carbon load, though the effect was dampened in the Phragmites and Schoenoplectus treatments with high removal rates at all OC loads. Both Phragmites and Schoenoplectus treatments had  $k_{20}$  values that increased less than  $0.5 \text{ d}^{-1}$  from OC0x to OC2x, while all other treatments had an average increase of  $1.33 \text{ d}^{-1}$ . The Phragmites and Schoenoplectus treatments also had higher  $\Theta_k$  values at OC0x than OC2x suggesting that warmer temperatures had a more positive effect at lower OC loading. Most other treatments had a higher  $\Theta_k$  values at the OC2x loading demonstrating the empirical model's capacity to describe the interactions between season, plant species and OC loading consistent with the statistical analysis of the raw data (Allen, Burr et al. 2016a).

The k-C\* model accounted for low removal rates in the effective incubation residual,  $C^*$ , which varied greatly between the poor performing plant treatments and little between the high performing plant treatments. For the high performing species, including Carex, the  $C^*_{20}$  values were less than  $1 \text{ mg N l}^{-1}$  for all carbon loads. In the unplanted as well as the Deschampsia and Typha treatments, the  $C^*$  values ranged from as high as  $44 \text{ mg N l}^{-1}$  in the unplanted OC0x treatments to  $0.3 \text{ mg N l}^{-1}$  in the Typha OC2x treatments and  $C^*$  consistently decreased as OC addition increased from OC0x to OC2x. As illustrated in Table 7, the residual concentration values were found to vary in their relationship to temperature and carbon load. For the high performing species Phragmites and Schoenoplectus, the seasonal dependency was minimal with the exception of the Schoenoplectus OC2x treatments which were shown to increase with temperature. With the exception of Deschampsia and Typha all OC2x treatments had a  $\Theta_{C^*}$  that was

positive, indicating that the residual concentration increased with higher temperature. This result was curious based on the elevated rates of reaction during the warmer months and was attributed to residual levels of ammonium (less than  $5 \text{ mg l}^{-1}$ ) that could not be nitrified due to low ORP and was not incorporated into plant biomass. In the OC0x and OC1x treatments the  $\Theta_{C^*}$  was universally less than one indicating that the  $C^*$  increased at lower temperatures. The most extreme examples of winter  $C^*$  increase were in the Typha OC1x and Carex OC0x treatments in which the  $\Theta_{C^*}$  values were 0.73 and 0.83, respectively. The inverse relationship with temperature was expected, as lower rates of removal were associated with cooler temperatures and accounted for in the  $\Theta_{C^*}$  value.

For all models, the  $C_0$  or initial TDN values did not vary significantly or systematically with carbon addition, plant treatment or temperature. The  $C_0$  values were modeled statistically in this analysis instead of using the average values measured in the feed solutions for these reasons: 1) potential hysteresis effects between incubations for some species, 2) potential changes in dilution of feed water as different species may have changed the water retention of the columns, and 3) the fast removal of ammonium attributed to sorption within the first 30 minutes of feeding prior to the first sample. By allowing the model to fit the  $C_0$ , general systematic changes in the data could be observed. Most models estimated the  $C_0$  to be between 50 and 60  $\text{mg N l}^{-1}$ , approximately 10 to 20  $\text{mg N l}^{-1}$  lower than measured feed concentrations. These values are consistent but still lower than the approximately 63  $\text{mg N l}^{-1}$  expected in the feed if approximately 50% of the 17  $\text{mg l}^{-1}$  of ammonium added with the feed solution was removed via sorption prior to the first measurement, as reported by Allen et al. (2016a). Increasing the

confidence in the modeled  $C_0$  values is the almost complete lack of systematic change with temperature, with all  $\Theta_{C_0}$  values equaling approximately 1.0.

The areal rate constants for N removal modeled in this experiment were quite high, especially compared to the measured total annual mass removal rate. The overall mass removal of N for the top performing species exceeded  $170 \text{ g N m}^{-2} \text{ yr}^{-1}$ , resulting in an equivalent annual removal areal rate constant of approximately  $170 \text{ yr}^{-1}$  or  $0.46 \text{ day}^{-1}$ . As reported by Kadlec and Wallace (2009), the mean areal removal rate constant for nitrate dominated systems is  $42 \text{ m yr}^{-1}$  or  $0.11 \text{ day}^{-1}$ . The annual rate constant determined by annual mass loading from this experiment fell within the 90<sup>th</sup> percentile of the presented removal rates. The rates observed in this study quantified on a daily basis were in general much larger, as high as  $2.73 \text{ day}^{-1}$  or  $1000 \text{ yr}^{-1}$  which is likely highly exaggerated and could result from several factors including: 1) small experimental container sizes with a high concentration of roots and 2) nutrient starved initial conditions for each incubation. The small experimental units may have exaggerated plant effect by increasing root density which may allow the plants to uptake more nitrogen and input more carbon on an areal basis. The nutrient starved initial conditions also inflate the initial rates of plant uptake. Nitrogen uptake rates in plants can be very fast, and in this root bound system could account for 100% of the nitrogen removal in as little as 30 minutes. Plant uptake is not limitless and the rates of uptake can vary based on the level of nitrogen stress the plant may be under (Šimůnek and Hopmans 2009). From a process perspective, this results in N uptake proceeding at a much higher rate if the plants are

coming from a state of N limitation and a rate proportional to growth when nitrogen is not limiting.

Though the daily removal rates for the wetland systems were significantly higher than the annual removal rates presented in other studies, the variation of the rates with temperature and season should be applicable to other systems. In this system the  $\Theta_k$  values were lower than the mean and median presented by Kaldec and Wallace (2009) with a mean of approximately 1.07 opposed to the presented 1.11, indicating less seasonal dependence.

Modifying the rates and  $C^*$  values of the model using the Arrhenius equation allows the empirical lumping of multiple parameters to address process changes in the wetland using temperature as a surrogate for season. In the spring and fall there may be discrepancies between the temperature and growth rates of the plants. In spring, active growth begins while in fall full senescence may have already been reached for the same temperature. Prior to performing a more detailed process based model to elucidate potential carbon contributions of plants, it was deemed important to investigate the potential seasonality of nitrogen removal pathways, distinguishing plant uptake and denitrification. An attempt was made to model plant growth and superimpose this growth on the seasonal N removal measured. For this analysis, detailed plant biomass data was required and limited to the OC0x and OC2x treatments. Only end of experiment nitrogen incorporation into plant matter was available and due to the significant decline in plant health for multiple replicates of the *Deschampsia* and *Typha* treatments, modeling was

only performed for the *Carex*, *Phragmites*, and *Schoenoplectus* OC0x and OC2x treatments.

To model the potential amount of nitrogen removed per incubation by either plant uptake or denitrification, a plant growth model was superimposed on the total nitrogen removed for each incubation. For each plant species, the duration of growth was first considered and correlated with qualitative laboratory assessments of plant growth. Seasonal growth models from Brix et. al (2001) were used for *P. australis*. Konings et al. (1992) estimated that growth for several *Carex* species started after six days of temperatures above 6 °C, and senescence begins in September. Limited information on the growth patterns of *S. acutus* were acquired, and *S. acutus* was the only species to not totally senesce in the winter greenhouse environment, retaining some green year round. The seasonal upticks and downshifts in growth determined for *Carex* were therefore applied to *Schoenoplectus* to account for the lengthy apparent growing season. For *Schoenoplectus*, the winter uptake of some N was allowed to represent the lack of full senescence.

Seasonal growth patterns for each species were transformed into unit growth curves by plotting the relative growth rate over an annual time period and normalizing the area under the curve to a value of 1. Each unit curve was scaled by the total nitrogen incorporated into biomass averaged over three growing seasons. These data were overlaid with the total nitrogen removal measured from each incubation providing an estimate of seasonal plant uptake vs microbial removal (Figure 11).

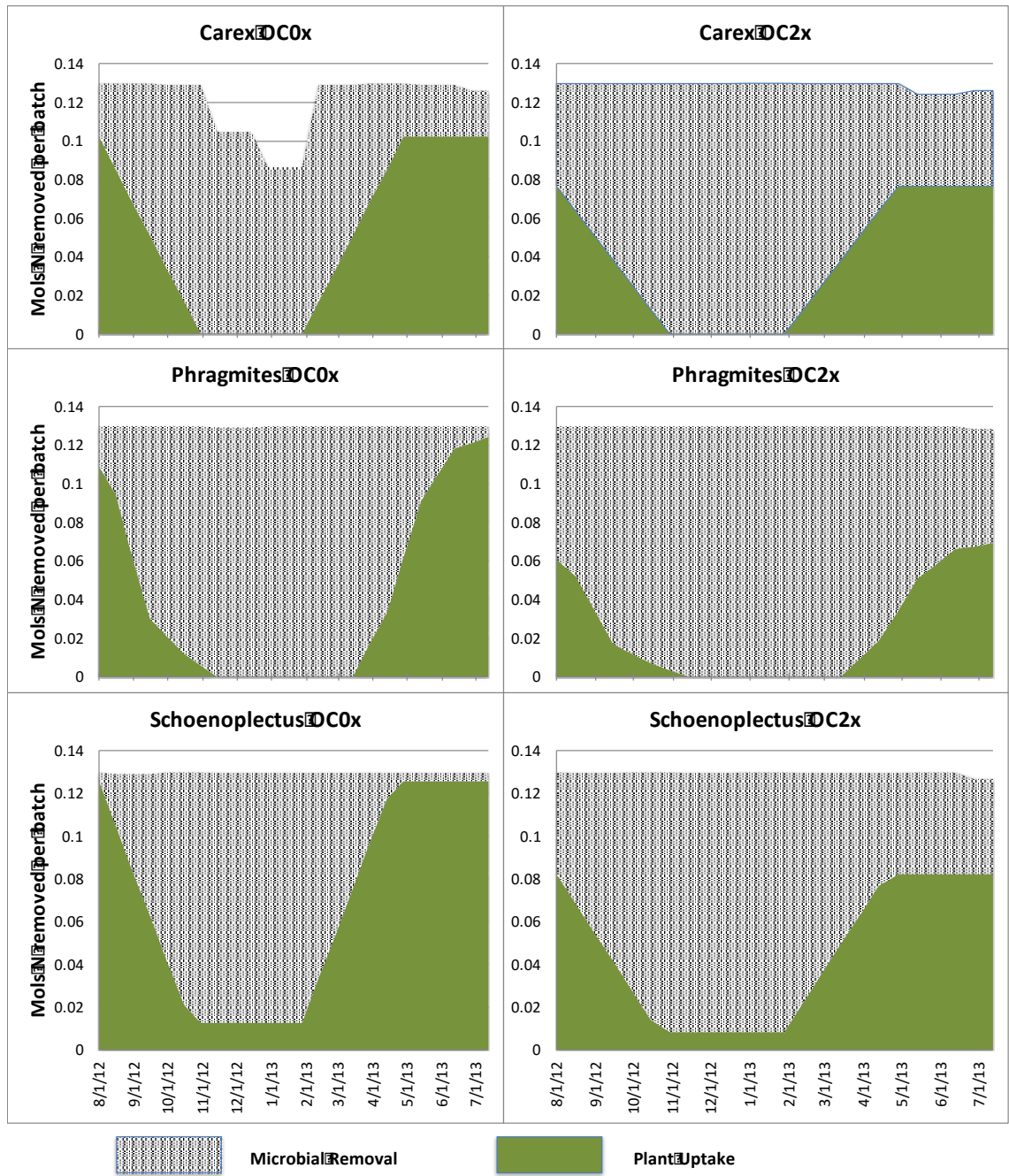


Figure 11. Seasonal model of microbial removal vs. plant uptake for Carex, Phragmites, and Schoenoplectus. with OC0x on the left and OC2x on the right.

As illustrated in Figure 11, plant uptake played a large role in N removal for all three species, especially without additional carbon. In the OC0x treatments, the removal of TDN was primarily attributable to plant uptake. It is likely that the growing season application of N could have been increased for these treatments, as microbial removal represented such a small fraction of the TDN removed and the summer growth of wetland species facilitates nitrate removal via denitrification from carbon inputs at a rate of 5.6 to 7.02 g C m<sup>-2</sup> d<sup>-1</sup> (Iannelli, Bianchi et al. 2011). Winter N removal is likely related to total carbon banked in the growing season and therefore potentially negatively impacted by the addition of additional N during the growing season if that addition results decreases in the amount of carbon stored in the subsurface during the growing season.

In all cases, the winter removal of N is dominated by microbial processes. In the OC0x treatments, the microbial removal of N over the winter can occur only if sufficient OC from the growing season is banked in the subsurface and made available for microbial consumption. The effective TDN removal witnessed in the OC0x Carex treatments was limited throughout the winter until the spring growing period initiates. This could indicate a depletion of OC availability over the winter. Overall biomass accumulation in the Carex treatments was lower than both Phragmites and Schoenoplectus. If the amount of OC banked into the subsurface is a function of overall plant biomass accumulation, it is possible that there exists an inflection point between Carex and Phragmites at which point the plants are unable to provide sufficient OC storage for year round nitrogen removal at this annual loading rate. It is possible that with

higher annual nitrogen loading rates, similar patterns would be illustrated in all plant treatments as nitrate availability outstrips net OC production.

### Conclusions

Single parameter kinetic approximation models indicated a transition from zero to higher order kinetics as OC addition and plant effect increased. The k-C\* model provided the most robust tool for modeling the general trends associated with N removal in this nitrate dominated system. The  $k_{20}$  values estimated from the modeling results generally increased with organic carbon addition. In the unplanted, Carex, Deschampsia and the Typha treatments the mean increases in  $k_{20}$  from OC0x to OC2x was 1.33 while the plant treatments Phragmites and Schoenoplectus mediated the  $k_{20}$  increase to less than 0.5. The  $k_{20}$  values from this experiment were significantly higher than in other reported values likely due to the small experimental container size and nutrient limiting conditions prior to each incubation. Despite the exaggerated removal rates, temperature dependency values are likely applicable to other nitrate dominated systems, with the average  $\theta_k$  found to be 1.07.

There were significant limitations to the application of the k-C\* model to this dataset when nitrogen removal was slow due to lack of carbon or plant effect. Specifically, the associated rate constant would be inflated and a high C\* value fit, resulting in a good mathematical approximation with little physical interpretation. In addition, the model would occasionally fail to converge, especially if N concentrations increased after the beginning of the incubation. This limitation was illustrated in the OC0x treatments of Deschampsia where nitrification of initially sorbed ammonium

increased measured TDN resulting in the k-C\* model failing enough times to not be able to generate an Arrhenius relationship model. Due to these limitations, future analysis should involve a more process-based model that can account for constituent speciation, a variety of microbial and physical processes, and allow for hypothesis testing on plant effect and carbon input.

Initial investigations into the likely processes driving N removal in the system illustrated large seasonal trends of removal dominated by plant uptake in the growing season, and by denitrification in the winter months. During the growing season the plant treatments *Carex*, *Phragmites*, and *Schoenoplectus* could have likely removed more nitrogen than was supplied in this experiment. In the OC0x treatments, winter removal was facilitated by carbon that had been banked in the subsurface during the growing season. It is unknown how increases in the N application rate during the growing season may affect carbon banking and therefore winter nitrate removal.

## CHAPTER 5

## FUTURE DIRECTIONS

This research both addressed and generated important questions regarding the maximum N removal capacity of treatment wetlands receiving nitrate rich waters and the processes at play in wetlands. Annual nitrogen removal potential for two species, *Phragmites australis*, and *Schoenoplectus acutus*, exceeded the annualized experimental loading rate applied even when no organic carbon (OC) was added (Allen, Burr et al. 2016a). Removal from *Carex utriculata* treatments also exceeded the application load during seasons of maximum plant growth but not during the winter season. Total greenhouse gas emissions were dominated by CO<sub>2</sub>, the production of which was influenced by plant species and season. The addition of OC surprisingly increased the emission of N<sub>2</sub>O (Allen, Burr et al. 2016b). This counter-intuitive result is one of the primary sources for new directions for future experimentation. These include new hypotheses regarding rates and mass accrual of species-specific OC rhizodeposition, field scalability of experimental values, the time series of plant uptake under various nitrogen loading schemes, and microbial community composition and structure. Addressing these questions can directly impact the ability to apply results to future designs and use the collected data to improve our understanding of the mechanics and biology behind treatment processes in wetlands.

Future work can be divided into three primary foci. 1) The first involves investigating the maximum nitrate removal capacity of the three high performing species at a field scale. This work is critical for design applications and includes, but is not

limited to, development of scaling factors for the greenhouse research to field sites as well as increasing the rates of application to determine theoretical maxima. 2) Biokinetic modeling of the existing data would allow hypotheses to be tested on relative plant inputs and microbial interactions elucidating carbon dynamics and likely microbial removal pathways. 3) Investigations into the composition and structure of rhizosphere microbial communities in this experiment would elucidate plant effect on microbial communities and comparing data from this experiment to data measured from model root bioreactors would allow further hypothesis testing on how microorganism community and structure changes can affect water quality results.

#### Plant Mediated Nitrate Removal

The current work illustrated that two plant species *Phragmites australis*, and *Schoenoplectus acutus*, are able to remove N at a rate above our loading rate of  $177 \text{ g m}^{-2} \text{ yr}^{-1}$ , indicating that the full potential for removal is currently underestimated. Similarly, *Carex utriculata* treatments illustrate a potential to remove more N during the growing season, while the winter ceiling for treatments was exceeded in this experiment. Mass removal values and rates may not be directly applicable to field applications due to enhanced plant effects caused by the small dimensions of the experimental unit. The experimental units used were shallow compared to the potential rooting depths for all plants (USDA 2016), and narrow in radius compared to the normal root distribution for these plants in more natural settings. An illustration of the root bound nature of a *Schoenoplectus* column can be found in Figure 12.



Figure 12. Schoenoplectus OC2x treatment removed from column during destructive sampling. The root bound nature of the subsurface can be seen by both the mat of roots against the column wall, and the ability of the roots to self-support the gravel.

The root bound nature of the columns likely enhanced a plant effect, as root density may be larger compared to field systems. Increases in root density may increase areal removal rates by incorporating more root biomass per area for nitrogen uptake and increase relative root surface area, providing more habitable surfaces for microorganisms. The increase in surface area for microorganisms facilitates the potential growth of more microbial biomass which in turn processes nutrients more quickly. Lastly, areal removal rates may be higher than field settings as the effective areal primary plant production may be higher as plants do not compete with neighboring plants for sunlight. In the greenhouse, all columns were separated by at least 15 cm, allowing the aboveground biomass more area to capture energy and concentrate that energy into the smaller areal footprint of the experimental column. With these confounding factors, it is very likely that the plant effect in these experiments is systematically exaggerated, however the degree of the potential exaggeration is unknown. Applying similar experimental loading rates in the field would facilitate creation of a scaling factor that would make the lab results applicable to future designs.

For testing nitrate removal rates across seasons at the field scale, experiments can be run at one or both of the MSU treatment wetland field sites at either Bridger Bowl or the Ennis National Fish Hatchery. Both of these TW systems are designed to operate as VFTW, however, water level control structures are in place allowing the water level to rise and, for an entire wetland cell to be operated in a batch mode. High nitrate, low organic carbon wastewater can be applied to the wetland cell with a small recirculation pump to provide mixing. At both sites two or more species are planted in each cell,

confounding plant species effects, however, approximate correlations of treatment in the field to lab results could be obtained.

In the greenhouse setting performing similar experiments with higher loading rates may also provide an interesting addendum to this experimental dataset. To increase the loading rate, reductions in incubation length would be preferable to increases in N concentration. Increasing N concentration could have the combined effect of using nitrogen concentrations that may not be field relevant and could be phytotoxic. Running the system in a continuous flow mode would also potentially provide better steady state data, as plant uptake rates can be highly variable as the plant transitions from a state of nitrogen abundance to limitation over the course of an incubation (Šimůnek and Hopmans 2009). It is likely that all high performing plants in this experiment helped to increase initial removal rates as the plants were experiencing nitrogen limitations at the end of each experimental incubation. Continuing these experiments over the course of an annual cycle would also help to address hypotheses surrounding annual rates of rhizodeposition and the winter consumption of banked carbon.

### Biokinetic Modeling

Biokinetic models generally increase significantly in complexity compared to the k-C\* model and similar bulk performance models. Biokinetic models apply numerical methods and assumed mathematical relationships between constituents to estimate concentrations over time, given specific starting conditions. The more physical processes and constituents added the greater the complexity.

Most biokinetic models in use for the modeling of treatment wetlands are based on the microbial kinetics assumed in activated sludge models with fitting parameters such as microbial biomass estimates and maximum process rates that are used to better represent the results seen in the wetlands (Meyer, Chazarenc et al. 2015). In modeling vertical flow and often in SSHF wetlands, the majority of error associated with the models has been derived from changes or variation in the model hydraulics. As wetland modeling improves and the effects of loading and flow regime become more calculable, the selection of macrophyte species may become the next most important optimization step in wetland design (Brisson and Chazarenc 2009), because plant selection can impact the efficacy of microbial processes and quantity of nutrient removal to standing biomass. In wetlands designed to ameliorate nitrate polluted waters with few incoming carbon sources, the rate and total mass of carbon inputs from any plant, let alone specific plant species, is currently unknown. This dataset provides a potential tool to calibrate models to include plant carbon input. By operating our systems as no-flow batch experiments, we eliminated the flow component, allowing the bio-kinetics to take center stage and facilitate the calibration of biokinetic model parameters. The data from similar systems at MSU has proved to be invaluable to the wetland modeling community as the biokinetic components of both primary models have been calibrated using past batch experiments (Mburu, Sanchez-Ramos et al. 2012, Mburu, Rousseau et al. 2014, Pálffy and Langergraber 2014). These modeling efforts have focused on the addition of aerobic capacity facilitated by plants and modeled the species-specific effects of plants on the subsurface microbial densities, attributing greater treatment of COD rich wastewater to

more oxygen and a greater number of microbes. With the dataset provided by this recently performed experiment, we can investigate plants on a spectrum of their capacity to act as net producers of electron donors or acceptors.

The two wetland models primarily in use are the BioPore module in Comsol and the Constructed Wetland Model1 (CWM1 and 2D) in Hydrus (Langergraber, Rousseau et al. 2009, Meyer, Chazarenc et al. 2015). Both models use matrices of reactants and reactions based on the Activated Sludge Models developed by Gujer and Henze (1991). Microbial growth is described using Monod kinetics with aerobic, anoxic and anaerobic processes that include the cycling and removal carbon, oxygen, and phosphorus (Gujer and Henze 1991, Gujer, Henze et al. 1999).

Other physical processes can be accounted for, such as plant uptake of nutrients and water. Plant nutrient uptake can be modeled seasonally and has been incorporated to follow seasonal growth patterns, often proportional to plant water uptake. Our current research illustrates that greater complexity can be incorporated into the plant uptake models, and Šimůnek and Hopmans (2009) propose that the rate of nitrogen uptake can be modeled with two maxima: 1) A greater maximum when the plant is N stressed facilitating active plant uptake; and 2) a lower rate when plants are not N stressed and plant uptake can be modeled passively with water uptake. The input of oxygen by plants can already be simply modeled in both Comsol and Hydrus as a constant input that decreases with root density into the subsurface, extending the existing model functionality to OC inputs is therefore not an issue of model capacity but of adequate data to estimate and test plant derived organic carbon input parameters.

In general, both the Hydrus and Comsol models handle saturated flow dynamics well, however each has been developed to fit two types of flow scenarios: The Hydrus model excels in vertical non-saturated flow dynamics (Langergraber, Rousseau et al. 2009), while the Comsol model has been modified to account for the production of particulate matter and the resulting loss of hydraulic conductivity, simulating SSHFTW clogging (Samsó and Garcia 2013). Neither model currently considers degradation of plant material or the production and release of labile carbon compounds by plants. Proceeding with modeling efforts in Comsol and the BioPore kinetic tool box is likely the best path forward due to its current use in horizontal flow wetlands, the type most likely to be employed for denitrification processes.

Despite the only direct measurement of plant based carbon output in the system being CO<sub>2</sub>, biokinetic models can allow us to test hypothesis surrounding the measured removal of nitrogen and the consumption / production of other constituents to estimate plant carbon input. Work on this experiment up to this point has only been able to attribute bulk quantities of nitrogen removal to two pools, either plant uptake or microbial removal. The addition of carbon into the subsurface and the seasonal transition between plant dominated and microbial dominated treatment is a rich area of modeling research. Correlating CO<sub>2</sub> dynamics to the processes in the system can help elucidate total carbon input into the system and potentially the total carbon required to outpace the addition of oxygen by plants, therefore allowing denitrification to occur. Similarly, the increase in N<sub>2</sub>O production with the addition of organic carbon was counter-intuitive and warrants further investigation, especially into the microbial processes and communities in the

subsurface. Modeling the microbial processes simultaneously with plant effects could help provide context on why the addition of labile carbon, which should enhance complete denitrification, resulted in incomplete denitrification and N<sub>2</sub>O production.

Using the extensive time series data set acquired from this experiment to test process based models will allow for superior estimates of plant inputs into carbon limited systems. Having better quantification and estimation of plant inputs in carbon limited systems holds the potential to drastically change the ease of applicability of wetlands for nitrate amelioration.

### Rhizosphere Analysis

The intricacies of plant root and microbial symbiosis are extremely complicated. Plant roots change both temporally and spatially. As the roots change so do the microbial communities associated with them. In many natural systems, the rhizosphere is the most active part of the subsurface environment, with plant inputs driving complete food webs of micro and macro-organisms (Hawkes, DeAngelis et al. 2007). Understanding how these communities are structured, both spatially and in their composition will aid in more accurate biokinetic models and potentially shed light on some of the experimental results.

In this experiment the interaction between N<sub>2</sub>O production and carbon load is intriguing. Plant uptake does not fully account for the differences in N<sub>2</sub>O production between the two tested carbon loads, especially in the winter months when plant uptake should have been minimal. One hypothesis is that the organic carbon addition alters the structure and composition of the subsurface microbial communities. Aspects of this hypothesis are supported by enzymatic investigations performed on sample cores taken

from microcosms running in parallel to this experiment (Spengler 2015), however the mechanics behind how those communities are supported is unknown. Active enzyme assays from samples shows a spatial shift in activity between the OC0x and OC2x loadings. In the OC0x treatments, activity was highly associated with the roots with little activity associated with the gravel. In the OC2x treatments, high activity was still associated with the roots, but an increase in activity as defined by increased 16sDNA qPCR copy numbers associated with the gravel was noted (Spengler 2015). This spatial shift in activity is important and also suggests a potential for a shift in microbial community composition. Performing microbial community analysis on the samples taken as a part of this experiment could shed light on community population shifts with the addition of organic carbon. The inherent spatial and temporal heterogeneity of rhizosphere communities complicates the elucidation of trends in TW rhizosphere analysis. To overcome some of the challenges associated with working in the dynamic rhizosphere, a physical model root analogue has been proposed that would reduce the inherent variability associated with growing plant roots.

A proposal has been submitted to investigate the microbial communities along the root using micro profiles of roots and a model root zone bioreactor. The model root zone bioreactor is based on a hollow fiber membrane to create a physical root analogue. Two fluids can be used with the hollow fiber membrane to create a physical system similar in structure to a submerged plant root system. Fluids can be moved through the hollow fiber membrane lumen to simulate plant exudates, which can diffuse through the membrane to the microbial communities growing on the membrane surface. Constituents in the water

can include oxygen, carbon compounds, and various plant produced acids and compounds proven to effect microbial communities. Outside the hollow fiber membrane, consistent synthetic wastewater or other fluids may be circulated to model the bulk fluid in a TW. Microbial populations that grow on the surface of the membrane can then be accessible to micro-electrodes capable of generating profiles of activity in the biofilm. The system also allows testing of hypotheses over time as the system can be run at a steady state, without needing to account for temporal changes in the root over time. Results from this model system can then be directly compared to the large datasets accrued at MSU for both oxygen and carbon limited treatment dynamics.

### Conclusions

This experiment was large in scale and duration. Over 5,200 individual water quality samples plus over 800 gas samples were analyzed, generating a pool of over 37,000 data points not including automated data acquisition such as redox and temperature. The experiment followed the growth of the plants for almost four years with data spanning the growth of all and the death of several of the plant treatments. Despite this massive dataset more questions were raised than addressed. The five wetland species tested illustrated their potential capacity to thrive in nitrate dominated wetlands, and for three species, *Carex utriculata*, *Phragmites australis* and *Schoenoplectus acutus*, the plants demonstrated potential to remove N exceeded the loading. A great need exists for the amelioration of nitrate polluted waters and this experiment illustrates the potential of wetlands to accomplish that goal. Several experimental factors exist that limit that direct application of these results to full scale systems. The limitations on this dataset can be

overcome with field scale application testing, which is within the capacity of the MSU wetland group with their current access to TW infrastructure.

The potential understanding to be gleaned from this dataset is also very large. This body of work used primarily statistical methods to examine the overarching conclusions from this experiment. Detailed analysis of the data using a process-based bio kinetic model will likely shed light on some of the interesting hypotheses generated, while providing valuable quantification of plant carbon input. Coupling this already robust dataset with additional information surrounding microbial community analyses would also likely increase our general understanding of the processes at play in the wetland system. Building on this large data set as well as the datasets generated by other wetland researchers at MSU can allow the robust testing of multiple parameters in a lab scale model root reactor.

By performing subsequent field scale trials, biokinetic modeling, microbial community analysis and root modeling this experiment will greatly add to the immediate and practical application of wetlands for nitrate removal, and shed light on the fundamental processes at play in the TW environment.

APPENDIX A

TEMPERATURE, PLANT SPECIES AND RESIDENCE TIME EFFECTS ON  
NITROGEN REMOVAL IN MODEL TREATMENT WETLANDS

# TEMPERATURE, PLANT SPECIES AND RESIDENCE TIME EFFECTS ON NITROGEN REMOVAL IN MODEL TREATMENT WETLANDS

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## Abstract

Removal of total nitrogen (TN) in treatment wetlands (TWs) is challenged by the complexity of the nitrogen cycle and the variety of influent nitrogen types. Plant species selection, season/temperature, and hydraulic loading rate likely influence root oxygenation and hence the magnitude and mechanisms of nitrogen removal, especially for ammonium-rich wastewater. Nitrogen data was collected from two previous experiments utilizing batch-loaded (lengths of 3, 6, 9 and 20 days), sub-surface TWs monitored for at least a year during which temperature was seasonally varied between 4°C and 24°C. Synthetic wastewater containing approximately 17 mg N L<sup>-1</sup> as NH<sub>4</sub> and 27 mg N L<sup>-1</sup>, 450 mg L<sup>-1</sup> COD, and 13 mg L<sup>-1</sup> SO<sub>4</sub>-S was applied to four replicates the plant species *Carex utriculata*, *Schoenoplectus acutus* and *Typha latifolia* and unplanted controls. Presence and species of plants had a more dramatic effect on TN removal than temperature or residence time. Planted columns achieved approximately twice the nitrogen removal of unplanted controls (40-95% versus 20-50% removal) regardless of season and temperature. *Carex* outperformed both *Typha* and *Schoenoplectus* and demonstrated less temperature dependency. TN removal in *Carex* was excellent at all temperatures and loading rates; in *Schoenoplectus* and *Typha* TN removal improved at lower loading rates. In all cases reductions in TN were not accompanied by increases in NO<sub>3</sub> which were consistently below 1 mg N L<sup>-1</sup>.

**Keywords:** Ammonium, COD, Loading rate, Season, Sulfate

### Introduction

Nutrient removal in treatment wetlands (TWs) occurs through a complex series of biogeochemical mechanisms, the rate of which has been shown to be influenced by temperature, plant species, system design, and the loading characteristics (Vymazal 2007, Kadlec and Wallace 2009). Total nitrogen (TN) removal is an important aspect in the success of many TWs designed for wastewater treatment. Nitrogen removal in wetland systems can occur through both abiotic and biotic pathways including the loss of nitrogen through ammonia volatilization, sorption, accumulation into recalcitrant particulate matter, plant uptake, and through microbially mediated processes (Vymazal 2007). The metabolism of nitrogen compounds by microbes in the rhizosphere has been shown to account for the majority of TN removal. In most instances sequential nitrification and denitrification are thought to represent the primary removal pathway (Faulwetter, Gagnon et al. 2009, Kadlec and Wallace 2009, Langergraber, Leroch et al. 2009), however, annamox and other ammonium oxidation removal pathways have been found in TW systems and may play an important role in nitrogen removal depending on system design and influent water conditions (Dong and Sun 2007, Paredes, Kuschik et al. 2007, Paredes, Kuschik et al. 2007, Vymazal 2007, Erler 2008, Wallace and Austin 2008).

Root zone aeration plays a large role in treatment wetland efficacy when systems are loaded with large quantities of organic carbon (typically measured as biochemical oxygen demand, BOD or chemical oxygen demand, COD) and reduced nitrogen. In subsurface-flow TWs, physical means have been used to increase substrate aeration by exposing the wetland media to air through forced aeration, by actively varying the water

level, and in vertical flow wetlands, by pulse loading the treatment bed (McBride and Tanner 2000, Wallace 2001, Brix and Arias 2005, Vymazal 2005, Vymazal 2007, Kadlec and Wallace 2009, Langergraber, Leroch et al. 2009, Langergraber, Pressl et al. 2010). Plants also play a role in subsurface aeration by transporting oxygen into the rhizosphere; however, planted-mediated oxygen transfer rates are difficult to measure and estimates vary widely (Bezbaruah and Zhang 2005, Dong, Zhu et al. 2011). The amount of oxygen and other exudates beneficial to microorganisms in the rhizosphere has been found to vary by plant species (Laskov, Horn et al. 2006) and species selection may significantly affect the overall treatment efficacy of a TW system; certainly the presence of plants significantly increases TW performance (Brisson and Chazarenc 2009). Plants actively and passively exude or release a variety of substances that impact microbial growth around the roots including carbon compounds, acids, vitamins and most notably in wetland systems oxygen (Bertin, Yang et al. 2003, Mainiero and Kazda 2005, Cardon 2007). The amount of microbial biomass found in soil and wetland systems increases proximal to roots, and increased microbial activity and diversity can be correlated to the presence of plants (Cardon 2007, Faulwetter, Gagnon et al. 2009). The greater abundance and potential diversity of microbes supported by plants is the likely means by which increased treatment efficacy is achieved in planted systems.

Oxygen, whether released by plants or otherwise introduced into the subsurface, acts as a high-energy electron acceptor for both heterotrophic and autotrophic bacteria which compete to oxidize either carbon compounds or ammonium. In planted subsurface-flow wetlands where the water level is not varied, plant roots represent the

primary input for oxygen into the subsurface. Redox gradients vary radially from the root (Bezbaruah and Zhang 2004) and longitudinally along the root (Faulwetter et al. 2012). Microbial biomass and diversity likely varies in response to these redox gradients providing a multitude of niches enabling the aerobic oxidation of carbon compounds and nitrification and anoxic processes such as denitrification in close proximity (Bezbaruah and Zhang 2004). Bulk water conditions do not represent the complexity of the interrelations occurring in the microbial communities along the roots surface, therefore researchers must use multiple tools, measurements, and methods to better investigate the black box of the rhizosphere.

In treatment wetlands designed to be loaded with raw or primary-treated wastewater, influent containing significant quantities of COD and reduced ammonium, root zone aeration capacity may dictate the treatment efficacy of the system. Oxygenation can be assumed to be the rate-limiting factor, effecting the removal of COD and the initial nitrification process required for both sequential nitrification and denitrification and annamox processes. Past research by our group has focused on correlating contaminant removal to plant oxygen input rates across seasons (Stein and Hook 2005, Stein, Borden-Stewart et al. 2007, Shultz, Stein et al. 2008, Taylor, Hook et al. 2011). COD removal efficacy has been correlated to temperature, loading-rate and plant species in batch fed subsurface TW microcosms. Levels of relative root zone oxygenation were determined by comparing COD removal rates, how amenable the system was to sulfate reduction, and bulk fluid oxidation reduction potential (ORP) measurements. These experiments suggest plants increase the oxygenation potential of

wetland systems with plant effects greatest in winter months which offsets any reductions in removal efficiency that would otherwise be attributed to temperature-induced decreases in reaction rates. To gain a better perspective on the interrelated mechanics at work in the black box of the rhizosphere, we reinvestigate these past experiments, incorporating before under-utilized nitrogen data to begin to better assess the interrelated processes of nutrient cycling in treatment wetlands.

### Methods

The goal of the current analysis is to utilize existing nitrogen data from two long-term controlled-temperature greenhouse experiments (Allen, Hook et al. 2002, Shultz, Stein et al. 2008) to further elucidate the seasonal effects of specific plant species. The first experiment investigated the removal of chemical oxygen demand (COD) over 20-day batch cycles, while the second built upon the results of first, utilizing a similar experimental design and identical synthetic wastewater recipe to look at the effects of increased loading rate on COD removal efficacy (by using 3, 6, and 9-day batch cycles). The two experiments utilized subsurface-flow wetland microcosms (“columns”) constructed from polyvinyl chloride (PVC) pipe (60 cm in height x 20 cm in diameter) and filled to a depth of 50 cm with washed pea-gravel (0.3–1.3 cm in diameter). The same locally sourced alluvial gravel was used in both experiments. The gravel was derived from noncalcareous rock of igneous and metamorphic origin consisting of approximately 80% quartz, 10% aluminum silicates and 10% mica. The mica and aluminum silicates in the gravel result in a high sorption capacity with a cation exchange capacity estimated to be approximately 19 meg per 100 grams of gravel.

The two experiments were conducted at Montana State University in Bozeman, Montana (46°N, 111°W; 1490 m elev.) from April 1997 to July 1999 and February 2006 to August 2007 respectively. Greenhouse temperatures sequenced annually from 4 to 24 to 4°C and used in conjunction with natural daily light cycles to mimic the seasons and induce annual plant growth and senescence patterns. Four replicates of three locally collected plant species, *Carex utriculata*, *Schoenoplectus acutus*, and *Typha latifolia* were compared to replicated unplanted control columns. All treatments were allowed to establish in the columns for at least 8 months prior to data collection. Though batch length varied among the experiments, the feed solution recipes were identical, with influent concentrations averaging 450 mg L<sup>-1</sup> COD, 44 mg L<sup>-1</sup> nitrogen, 8 mg L<sup>-1</sup> PO<sub>4</sub>-P, and 14 mg L<sup>-1</sup> SO<sub>4</sub>-S. The nitrogen was introduced into the columns in two forms, 27 mg L<sup>-1</sup> as amino-N (peptone) and 17 mg L<sup>-1</sup> as NH<sub>4</sub>-N. In a feeding cycle (batch), the columns were gravity drained and fed fresh synthetic wastewater from the top of the column. Samples were collected from a vertically-oriented sampling tube terminating in the geometric center of the column at days, 0.04 (20 min.), 1, 3, 6, 9 and 20, as truncated by batch length. Further details for each experiment can be found in the previous publications (Allen, Hook et al. 2002, Shultz, Stein et al. 2008).

Ammonium and COD were measured from all (filtered) samples using HACH colorimetric methods 10031 and 8000 respectively as were the common anions, nitrite, nitrate, sulfate, and phosphate using ion chromatography. In both experiments nitrate and nitrite were continually found to be below the detection limit (<1 mg N L<sup>-1</sup>). TN measurements (via HACH method 10072) were recorded in addition to the above

mentioned analyses at 4 and 24°C in the first experiment and at 8°C in the second. Previous nitrogen investigations (Riley, Stein et al. 2005), performed using the same experimental system and  $\text{NH}_4^+\text{-N}$  as the sole source of nitrogen, determined that organic-bound N in unfiltered samples remained comparatively low (around  $2 \text{ mg L}^{-1}$  which varied randomly across treatments and sample days), thus filtered aqueous samples were a good indicator of TN concentrations. Regardless, ammonium values cannot be directly used to indicate TN removal as the concentration of aqueous  $\text{NH}_4^+$  was found to be related to the sorption capacity of the gravel matrix, the biotic removal rate of TN and the hydrolysis of the amino-N to  $\text{NH}_4^+$  (Riley, Stein et al. 2005). However, comparing TN concentrations as opposed to  $\text{NH}_4^+$  allows for the discussion of nitrogen removal in the context of both COD and sulfate, normalizing all data to percent removal. To generate a relationship between TN and  $\text{NH}_4^+$  over time, a statistical model was created using the datasets from experiments for which TN and  $\text{NH}_4^+$  measurements were recorded.

Means across treatments ( $n= 4$ ) for collection events containing both TN and  $\text{NH}_4^+$  were calculated and used to construct a statistically-based comparison model assuming hydrolysis of the amino-N to  $\text{NH}_4^+$  follows a first order pattern. The difference between TN and  $\text{NH}_4^+$  magnitudes appear to follow an exponential decay with time corroborating the assumption of a first order transition from amino-N to  $\text{NH}_4^+$  (Fig. 1). Initial differences between TN and  $\text{NH}_4$  ( $\sim 27 \text{ mg N L}^{-1}$ ) decreased rapidly and were  $< 5 \text{ mg N L}^{-1}$  75% of the time at day 3 and 100% of the time by day 9 regardless of season, plant species or loading rate. To predict the difference between TN and  $\text{NH}_4^+$  as a function of time, a non-linear, mixed-effects model was fit to the data using R build

2.15.1 (Team 2012), and package nlme (Jose Pinheiro 2012) assuming an exponential decay of the difference between  $\text{NH}_4^+$  and TN. In the model, plant treatment was used as fixed effect, resulting in slightly different regression constants for each plant species. The model predicted measured TN values with a variance that decreased over time, with the largest magnitudes of error at 0.04 and 1 day after filling. By day 3 the model estimated TN within  $5 \text{ mg N L}^{-1}$  of the measured value 90% of the time with median absolute and relative errors of less than  $1.5 \text{ mg/l}$  and 14% respectively. From day 6 on, the model estimated TN within  $5 \text{ mg N L}^{-1}$  of the measured value 100% of the time with a median absolute error of approximately  $1\text{-mg N L}^{-1}$  and a relative error that was less than thirty percent, 80% of the time.

The two experiments followed slightly different annual temperature pattern but had the following temperature steps in common: 4, 8, 16, and  $24^\circ\text{C}$ . Only the data from common temperature steps are included in the results but contain approximately 1000 collection events for COD,  $\text{NH}_4^+$ , and sulfate. Utilizing the statistical model, an estimate for TN was calculated for each collection event by summing the mean measured  $\text{NH}_4^+$  concentration and the model-predicted difference between TN and  $\text{NH}_4^+$  at the given time. The initial concentration of TN for an individual feeding was assumed to be in the same proportion to the target  $44 \text{ mg L}^{-1}$  as the average measured proportions of all other measured constituents to their target concentrations.

### Results and Discussion

Nitrogen removal values were compared in conjunction with COD and sulfate removal values across seasons at multiple batch days. Direct comparisons between the

two experiments by season are difficult because the initiation of the annual temperature cycle is offset by approximately six months (one started and ended in winter the other started and ended in summer). In both experiments, the data followed seasonal trends that upon visual inspection correlated more heavily to temperature than plant growth and senescence patterns (results from intermediate temperatures in fall and spring were similar with species and batch length). Therefore, we have combined data collected from different seasons but at the same temperature. In correlation with past analyses, we address two major questions; to what extent does plant species and temperature influence removal rates, and how does loading rate affect the results?

Previously reported results from both experiments demonstrated that temperature/seasonal and plant species effects on COD and sulfate concentrations were pronounced and consistent by day 6 in all experiments with a minimum 6-day batch length. Schultz et al. (2008) demonstrated that there was no statistical difference in measured COD and sulfate concentrations at day 6 from experiments with batch lengths of 9 and 20 days. Additionally, model-estimated TN values had minimal variance by day 6. Figure 2 compares COD, sulfate and estimated TN removal at day 6 as a function of plant species and temperature aggregated across 9 and 20-day batch lengths. The number of data points per plant species varies between 12 and 16 depending on the temperature. A wide range of COD and sulfate removal values were measured at most temperature steps with the range generally increasing at lower temperatures, but differences in COD and sulfate with temperature and species were typically statistically significant within experiments (Stein and Hook 2005; Schultz et al. 2008). *Carex* and *Schoenoplectus*

demonstrated significantly greater sulfate removal at warmer temperatures while *Typha* and unplanted treatments demonstrated significantly greater COD removal at warmer temperatures. COD removal in *Carex* and *Schoenoplectus* and sulfate removal in *Typha* and unplanted treatments were insensitive to temperature. Variation in sulfate removal correlated with measured redox potential (ORP) with more removal associated with low ORP, suggesting that that lack of temperature dependency in *Carex* and *Schoenoplectus* COD removal is related to increased oxygenation of the rhizosphere in winter.

Interestingly, nitrogen removal has the smallest range of values and is generally the most insensitive to temperature. Clearly, the presence of plants increases nitrogen removal regardless of temperature and when plants are present there is a slight to modest increase in removal with increased temperature. Conversely, nitrogen removal decreases with increasing temperature in the unplanted gravel systems. Because plant uptake is an insignificant nitrogen removal mechanism (Paredes, Kuschik et al. 2007, Vymazal 2007, Langergraber, Lerach et al. 2009) all differences are likely due to differences in microbial metabolism influences on the nitrogen cycle. In the unplanted gravel, we hypothesize that TN removal decreases as temperature increases because heterotrophic bacteria out compete the autotrophic nitrifiers at higher temperatures for the very limited oxygen in the system; heterotrophic reaction rates may be sufficiently retarded at lower temperatures to increase slightly the oxygen availability for the nitrifying communities. However, the overall lack of available oxygen inhibits both heterotrophic and autotrophic aerobes at virtually all temperatures. In the planted systems improvement in nitrogen removal correlates more closely with plant species than temperature. *Typha* shows the

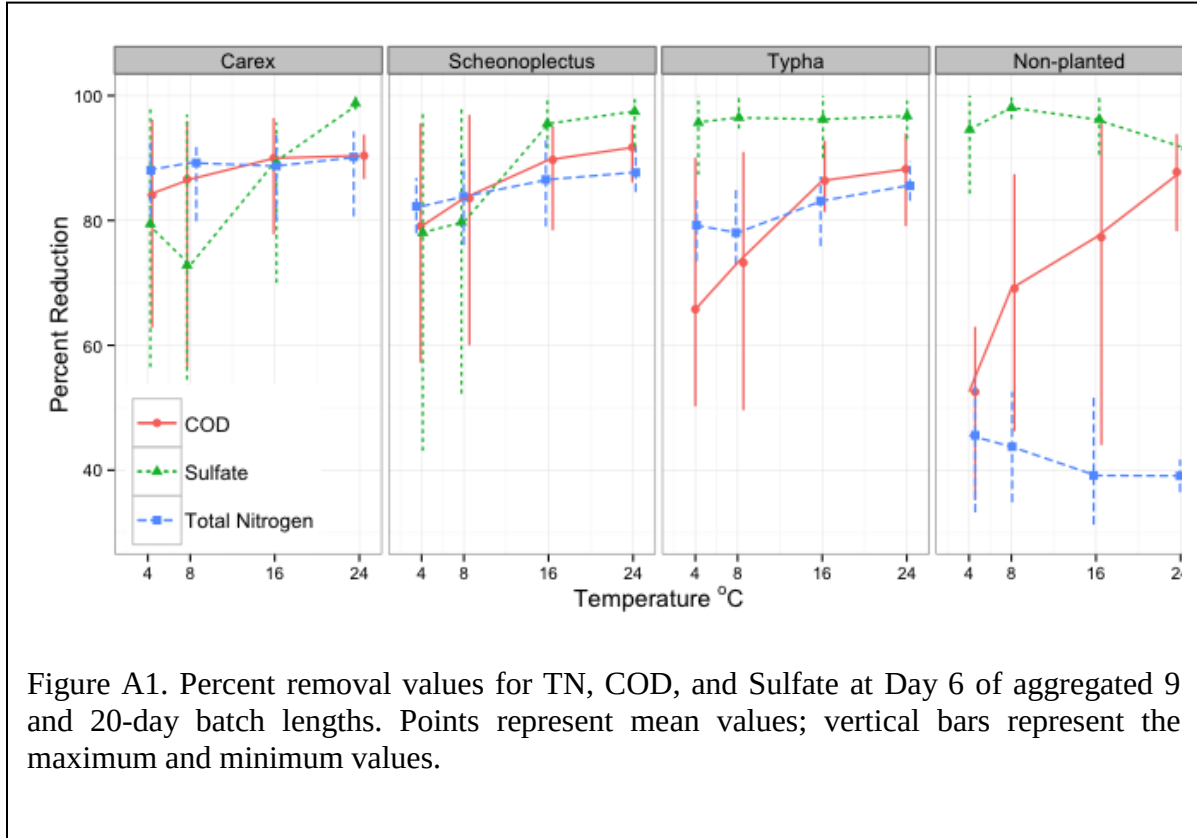


Figure A1. Percent removal values for TN, COD, and Sulfate at Day 6 of aggregated 9 and 20-day batch lengths. Points represent mean values; vertical bars represent the maximum and minimum values.

largest amount of temperature dependence and *Carex* the least. The *Carex* columns removed the most COD and TN regardless of temperature and given the lower sulfate removal values, especially at low temperatures, appear to aerate the subsurface to a high degree, data which has been corroborated with ORP in other publications Stein and Hook 2005; Stein et al. 2007). The *Schoenoplectus* columns more closely resemble the *Carex* columns but show slightly less removal and slightly more temperature dependence. These data suggest that aerobic nitrification is the factor limiting TN removal, but both temperature and species selection influence nitrification.

To address the effects of loading rate on removal efficacy, endpoint values (at day 3, 6, 9 and 20 for equivalent batch lengths) for COD, sulfate and estimated TN are compared in Figure 12. Batch length endpoint data have an inversely linear relationship

with loading rate. For example, a 3-day batch length has 2 times the loading rate of a 6-day batch and 3 times the loading rate of a 9-day batch for any given parameter, thus the data represent a nearly a sevenfold variation in loading rate.

In general terms, species selection appears to have a greater influence on efficacy than temperature or loading rate, but interactions are apparent. For example, *Carex* and *Schoenoplectus* are better for COD and especially TN removal (TN global average 88% and 76% respectively) than unplanted systems (global average = 34%) regardless of loading rate while *Typha* (global average = 69%) is intermediate but more similar to planted systems for TN. Within species, there is a general decrease in COD and TN removal and an increase in sulfate removal with increasing loading rate, but differences are generally smaller (or non-existent for unplanted systems) than between species at any given loading rate. For example, *Typha* and *Schoenoplectus* display the greatest variation in TN removal; temperature-averaged removal decreased by approximately 20 percentage points between the 9-day and 3-day batch lengths. Equivalent changes were only 3 percentage points in *Carex* and 10 percentage points in unplanted systems. Temperature effects on all parameters are fairly consistent across loading rates. Within species, parameters which showed poorer removal with increasing temperature tended to do so at all loading rates as did parameters demonstrating better performance with temperature, with the exception of sulfate removal in *Carex* and *Schoenoplectus*. For those species poorer sulfate removal at colder temperatures is expressed more at lower loading rates (longer batch lengths). This suggests that the increased aeration observed at lower

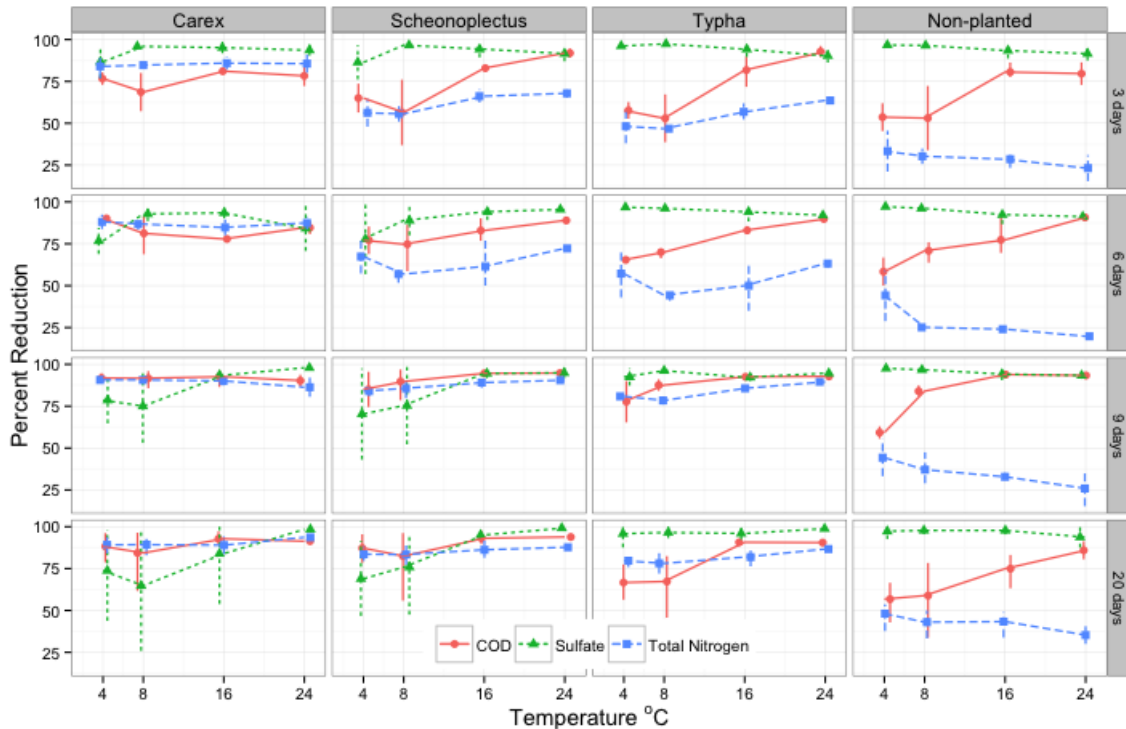


Figure A2. Percent removal of TN, COD, and sulfate at the final batch day for batch lengths of 3, 6, 9, and 20 days. Points represent mean values, vertical bars represent maximum and minimum values.

loading rates can be overwhelmed by high organic loading rates. This trend was also observed in Stein et al. (2007).

### Conclusion

While more detailed statistical analysis is continuing, general trends in the observed changes in concentration of COD, sulfate and TN (estimated by regression of  $\text{NH}_4^+$  measurements) suggest that variation in the aeration potential of the rhizosphere is the driving mechanism for variation in COD and TN removal efficacy. Plant species selection, temperature/season and loading rate influenced removal efficacy. Previously

reported negative correlations between measured ORP and sulfate removed and positive correlations between ORP and COD removal confirm that variation in COD removal is influenced by changes in aerobic versus anaerobic heterotrophic microbial activity. Variation in rhizosphere aeration also produces measureable differences in TN removal, presumably by influencing the efficacy of autotrophic nitrifiers. Species selection appears to have the greatest influence on rhizosphere aeration which follows a pattern of *C. utriculata* > *S. acutus* > *T. latifolia* > unplanted systems with greater aeration expressed in cold, winter conditions, especially for the two more aerating species. A sevenfold variation in loading rate had a relatively smaller effect on removal of COD and TN but higher loading rates appear to limit the effects of increased aeration of *Carex* and *Schoenoplectus*.

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