

DENITRIFICATION AT THE MICROSCALE
IN TREATMENT WETLANDS

by

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TABLE OF CONTENTS

1. LITERATURE REVIEW.....	1
Treatment Wetlands	1
Wastewater Nutrient Removal	2
Types of Wetland	4
Greenhouse Gas Emissions	6
Denitrification and Associated Enzymes	7
Organisms Performing Denitrification.....	9
Methods for Measuring Denitrification	10
Denitrification in Wetlands.....	14
Wetlands Spatial Activity	15
Plant Species Activity	16
Experimental Design.....	17
Hypotheses	18
2. MATERIALS AND METHODS.....	20
Wetland Growth Conditions	20
Coring.....	21
Habitat Separation.....	22
Filling Incubation Vials	23
Incubation Conditions	24
Gas Sampling	26
Gas Chromatography Analysis	27
Gas Chromatography Standard Curves.....	28
DNA Extraction	29
qPCR Conditions.....	30
qPCR Optimization.....	31
qPCR Standard Curves.....	32
Normalizations and Data Imputation	34
Statistical Analysis	36
3. EXPERIMENTAL RESULTS.....	42
Results Overview	42
Data Set Grouping and Presentation	42
Quantification of 16S rDNA and Denitrification Genes.....	43
Quantification of Gas Denitrification Activity	46
Overview of Hypotheses	49
Carbon Load Influences Denitrification Primarily in Non-Planted Columns.....	50
Effects of Carbon Load and Plant Presence on Particulates	50
Effects of Carbon Load and Plant Presence on Gravel.....	53

TABLE OF CONTENTS – CONTINUED

Effects of Carbon Load and Plant Presence on Roots	55
Carbon Load Effects on Plant Species.....	57
Effects of Carbon Load and Plant Species on Particulates	57
Effects of Carbon Load and Plant Species on Gravel.....	59
Effects of Carbon Load and Plant Species on Roots	61
Effects of Carbon Load and Plant Species on 16S rDNA- Normalized Data	63
Summary of Carbon Load Effects	63
The Role of Habitat.....	64
Plant Species Effects Do Not Play a Large Role in Denitrification Activity.....	65
Effects of Plant Species on Particulates.....	65
Effects of Plant Species on Gravel.....	66
Summary of Plant Species Effects	68
Denitrification Gene Copy Numbers Correlate to Gas Assays	69
N ₂ O emission Correlates to Other Measurements	73
The Relative Completeness of Denitrification	77
Summary	80
 4. DISCUSSION	 81
Discussion Overview	81
Quantification of Denitrification Genes.....	83
Quantification of Denitrification Gas Emissions	87
Effects of Carbon Load	92
Comparing Between Habitats	94
Effects of Habitat	96
Effects of Species and Plant Presence.....	99
Genes to Gas Correlations.....	101
Minimizing N ₂ O Release	102
Design Parameters.....	104
Future Work	107
 REFERENCES CITED.....	 109
 APPENDICES	 120
APPENDIX A: Aggregated Data.....	122
APPENDIX B: Statistical Models	133
APPENDIX C: Gas Chromatography Additional Information	146
APPENDIX D: Particulate Total Organic Carbon Data	149

LIST OF TABLES

Table	Page
1. Synthetic wastewater recipe. All compounds were laboratory-grade, purchased from Fisher Scientific (New Jersey) except zinc sulfate from Ricca Chemical Company (Texas), manganese sulfate from MP Biomedicals (Ohio), and sucrose, which was food-grade. The zero-carbon load had no sucrose added but was otherwise identical.....	21
2. qPCR primer sets used in this study.	31
3. qPCR cycling conditions used in this study.....	31
4. qPCR standard curves for each primer set used in this study.	33
A1. All aggregated denitrification activity gas data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.....	123
A2. Aggregated 16S rDNA and <i>nosZ</i> gene abundance data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in copies g^{-1} sample or copies mL^{-1} sample.	126
A3. Aggregated <i>nirS</i> and <i>nirK</i> gene abundance data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in copies g^{-1} sample or copies mL^{-1} sample.....	129

LIST OF TABLES – CONTINUED

Table	Page
A4. Mixed-effects models of 16S rDNA-normalized data (16S = 16S rDNA). Carbon = carbon load. Habitat = particulates, gravel, or roots. Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. Interaction terms are noted by a $\times$ symbol. Replicate columns were included as random effects. One row is a single model. This collection of models was <i>p</i> -adjusted together. <i>P</i> -values ≤ 0.1 are bolded and were explored with post-hoc Tukey's tests. The planted data set excludes non-planted data, planted model effects refers to plant presence or absence.....	134
A5. Mixed-effects models of gas-to-gene and gas-to-gas correlations. 16S = 16S rDNA. All data was grouped for this analysis. Replicate columns were included as random effects. One row is a single model. This collection of models was <i>p</i> -adjusted together. <i>P</i> -values ≤ 0.1 in these cases imply correlation is significantly different from 0, and are bolded. They were explored with post-hoc Tukey's tests.....	135
A6. Fixed-effects models of separate habitat data. 16S = 16S rDNA. Carbon = carbon load. Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. DNE = Does not exist. Interaction terms are noted by a $\times$ symbol. One row is a single model. Each collection of habitat models was <i>p</i> -adjusted independently of the other habitats. <i>P</i> -values ≤ 0.1 are bolded and were explored with post-hoc Tukey's tests. Planted model effects refers to plant presence or absence.....	136
A7. Post-hoc Tukey's test for 16S rDNA-normalized data (16S = 16S rDNA). Carbon = carbon load ($-C$ and $+C$). Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. The “ $-$ ” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a $\times$ symbol. One column is a single model. <i>P</i> -values ≤ 0.05 are considered significant and are bolded. The planted data set excludes non-planted data. The $\neq$ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.	138

LIST OF TABLES – CONTINUED

Table	Page
A8. Post-hoc Tukey's test for particulate data. 16S = 16S rDNA. N ₂ O cons. = N ₂ O consumed. Carbon = carbon load (–C and +C). Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. <i>P</i> -values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.....	139
A9. Post-hoc Tukey's test for gravel data. 16S = 16S rDNA. N ₂ O cons. = N ₂ O consumed. Carbon = carbon load (–C and +C). Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. <i>P</i> -values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.....	141
A10. Post-hoc Tukey's test for particulate and gravel data. 16S = 16S rDNA. Carbon = carbon load (–C and +C). Species = <i>Carex</i> , <i>Schoenoplectus</i> , <i>Phragmites</i> , or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. <i>P</i> -values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.	143
A11. N ₂ O concentration potential standard curve fits. The selected fit is bolded.....	147

LIST OF TABLES – CONTINUED

Table	Page
A12. Total organic carbon (TOC) data for the particulate fraction. DNE = Does not exist. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Total carbon (TC), inorganic carbon (IC), and TOC are reported in mg L^{-1}	150

LIST OF FIGURES

Figure	Page
1. A horizontal subsurface flow treatment wetland. 1 inflow zone; 2 impermeable layer; 3 filtration material and roots; 4 plants; 5 water level; 6 outflow zone; 7 drainage pipe; 8 outflow structure. Figure from Vymazal, 2010.	5
2. The nitrogen cycle. Denitrification can lead to both N_2O and N_2 being released.....	7
3. Diagram of a TW column used in this study, designed to simulate horizontal subsurface flow.....	21
4. A diagram representing the sample processing. Sixteen columns total were cored near the top of the column and split into three habitats for assays. Example incubation vials for habitats are shown. Not to scale.	23
5. Treatment conditions for gas incubations. The first column lists what amendments were added to each treatment group to observe the reactions diagrammed on the right. Note the N_2O production was chemically inhibited, whereas N_2O consumption was restricted only by the incubation nutrient solution, and N_2O emission was uninhibited.	25
6. A color scheme representation of the main ways data was grouped. Note the non-planted root data does not exist. The horizontal axis represents the three plant species and non-planted columns. The vertical axis represents habitats within the column. The color of each category was constant through the results (<i>e.g.</i> , gravel is red throughout all analysis). Squares of species and habitat interaction are multicolored only in this figure, each figure in the results will show only one color scheme (<i>e.g.</i> , one graph will show either habitat or species).	39
7. Gene copy numbers split by habitat and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample or copies mL^{-1} sample.....	45
8. Gene copy numbers normalized by 16S rDNA and split by habitat and carbon load. This visualization of the data does not represent a statistical model. Units are in copies/16S rDNA copies $^{-1}$	46

LIST OF FIGURES – CONTINUED

Figure	Page
9. Gas assay data split by habitat and carbon load. Roots have the highest values, whereas particulate has the lowest. N ₂ O consumption has higher values than N ₂ O production. This visualization of the data does not represent a statistical model. Units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample.....	48
10. Gas assay data normalized by 16S rDNA and split by habitat and carbon load. Habitats are closer together than in the non-normalized data, with gravel exhibiting the highest gas values per 16S rDNA copy number. This visualization of the data does not represent a statistical model. Units are in mg hr ⁻¹ 16S rDNA copies ⁻¹	49
11. Particulate gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies mL ⁻¹ sample.	52
12. Particulate gas assay data split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in mg hr ⁻¹ mL ⁻¹ sample.....	53
13. Gravel gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g ⁻¹ sample.	54
14. Gravel gas assay data split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in mg hr ⁻¹ g ⁻¹ sample.	55
15. Root gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g ⁻¹ sample.	56
16. Root gas assay data split by carbon load. This visualization of the data does not represent a statistical model. Units are in mg hr ⁻¹ g ⁻¹ sample.	56
17. Particulate gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies mL ⁻¹ sample.	58

LIST OF FIGURES – CONTINUED

Figure	Page
18. Particulate gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.....	59
19. Gravel gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.	60
20. Gravel gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.	61
21. Root gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.	62
22. Root gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.	62
23. Correlation of N_2O produced to the <i>nir</i> genes, which contribute to N_2O production. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample; gene units are in copies g^{-1} sample or copies mL^{-1} sample.....	70
24. 16S rDNA-normalized correlation of N_2O produced to the <i>nir</i> genes, which contribute to N_2O production. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} 16\text{S rDNA copies}^{-1}$; gene units are in copies $16\text{S rDNA copies}^{-1}$	70

LIST OF FIGURES – CONTINUED

Figure	Page
25. Correlation of N ₂ O consumed to the <i>nosZ</i> gene, which contributes to N ₂ O consumption. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample; gene units are in copies g ⁻¹ sample or copies mL ⁻¹ sample.....	71
26. 16S rDNA-normalized correlation of N ₂ O consumed to the <i>nosZ</i> gene, which contributes to N ₂ O consumption. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ 16S rDNA copies ⁻¹ ; gene units are in copies 16S rDNA copies ⁻¹	72
27. Correlation of N ₂ O produced/consumed to <i>nir/nos</i> gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample; gene units are in copies g ⁻¹ sample or copies mL ⁻¹ sample.	73
28. Correlation of N ₂ O emitted to <i>nir/nos</i> gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample; gene units are in copies g ⁻¹ sample or copies mL ⁻¹ sample.....	75
29. Correlation of N ₂ O emitted to N ₂ O produced/consumed. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample.....	76
30. 16S rDNA-normalized correlation of N ₂ O emitted to <i>nir/nos</i> gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ 16S rDNA copies ⁻¹ ; gene units are in copies g ⁻¹ sample or copies mL ⁻¹ sample.....	76

LIST OF FIGURES – CONTINUED

Figure	Page
31. 16S rDNA-normalized correlation of N ₂ O emitted to N ₂ O produced/consumed. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Units are in mg hr ⁻¹ 16S rDNA copies ⁻¹ , mg hr ⁻¹ g ⁻¹ sample, or mg hr ⁻¹ mL ⁻¹ sample.	77
32. The N ₂ O emitted/produced ratio, split by habitat, plant presence, and carbon load. Units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample.....	79
33. Correlation of N ₂ O emitted/produced to <i>nir/nos</i> gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr ⁻¹ g ⁻¹ sample or mg hr ⁻¹ mL ⁻¹ sample; gene units are in copies g ⁻¹ sample or copies mL ⁻¹ sample.....	79

GLOSSARY

Abbreviations

BOD:	Biochemical oxygen demand, a measure of organic carbon in water
C _t value:	Cycle threshold value, used in qPCR
HSSF:	Horizontal sub-surface flow, describing a type of wetland
GC:	Gas chromatography
qPCR:	Quantitative PCR
ppm:	Parts-per-million
UHP:	Ultra-high purity, in this study referring to N ₂ gas
TW(s):	Treatment wetland(s)

Commonly used terms

Carbon load:	Either 0 or 0.391 g L ⁻¹ sucrose (0 or 2) in TW feed solution
Habitat:	Roots, gravel, or particulates
Species:	<i>Carex utriculata</i> , <i>Phragmites australis</i> , and <i>Schoenoplectus acutus</i>
N ₂ O produced:	Acetylene-inhibited reaction that blocks N ₂ formation
N ₂ O emitted:	Uninhibited denitrification, N ₂ O is measured
N ₂ O consumed:	The amount of N ₂ O converted into N ₂
16S rDNA:	A universal bacterial ribosomal gene
<i>nirS</i> or <i>nirK</i> :	Functionally equivalent genes which encode nitrite reductases
<i>nirS</i> + <i>K</i> :	The sum of <i>nirS</i> + <i>nirK</i>
<i>nosZ</i> :	A gene which encodes nitrous oxide reductase

ABSTRACT

Treatment wetlands (TWs) have been in use for over three decades for wastewater treatment, agricultural water treatment, and some industrial wastes. Thousands of TWs exist for treating wastewater globally, but the microbial processes and controls *in situ* primarily responsible for water treatment are poorly understood. In this study, 16 separate model TW columns consisting of three plant groups and one non-planted group were fed synthetic post-secondary wastewater with half receiving no added carbon and half receiving 0.391 g L^{-1} as sucrose. Core samples were taken from each of the TW columns and separated into three distinct habitats (roots, gravel, particulates). Each habitat was assayed for its ability to produce N_2O , consume N_2O , and emit N_2O , as well as for denitrification gene abundances (*nirS*, *nirK*, and *nosZ*) and bacterial gene abundance (16S rDNA). The addition of organic carbon to the wetland was found to increase denitrification activity and gene copy abundance in non-root fractions, but organic carbon addition did not affect the root fraction. Plant presence within the TW was found to increase gas assay and gene abundance values in non-root habitats. Differences between three plant species were minor compared to differences attributed to carbon addition and plant presence. Of all habitats, gravel was found to have the highest denitrification activity and denitrification gene copy abundance relative to the number of 16S rDNA copies, as well as the highest ratio of N_2O produced to N_2O emitted. Implications for this study suggest the gravel and root fractions should be studied in further detail for their ability to accommodate denitrifying microbes.

CHAPTER 1

LITERATURE REVIEW

Treatment Wetlands

Natural wetlands cover 2-6% of the world's land surface and were first used as wastewater discharge sites over 100 years ago (Kadlec and Wallace, 2009; Kayranli et al., 2010). A wetland is defined as having plants, water, and some type of media to harbor the plant roots (Kadlec and Wallace, 2009). Their often highly complex ecosystems include all three domains of life, with abiotic and biotic factors both playing large roles in the stability and overall health of the wetland; this unique wetland ecosystem provides a habitat to many species uncommon to other habitats, which increase their robustness for water treatment (Kadlec and Wallace, 2009).

Engineered wetlands are designed to optimize the efficiencies of natural wetland systems. Treatment wetlands (TWs) have been in use for over three decades for wastewater treatment, agricultural water treatment, nutrient removal, and the treatment of some industrial wastes (Vrhovsek et al., 1996; Brix and Arias, 2005; Chen et al., 2006; Kadlec and Wallace, 2009; Chon et al., 2011). Interest in TWs has been growing due to their low maintenance and construction costs compared to more traditional treatment alternatives (Vrhovsek et al., 1996; Chen et al., 2006; Kayranli et al., 2010). Many parameters affect the operation of a TW, including plant species, nutrient loading rates, type of flow, temperature, and wetland design (Vymazal, 2007; Kadlec and Wallace, 2009).

Wastewater Nutrient Removal

A traditional wastewater treatment plant performs primary and secondary treatment processes before discharging the water. These steps often remove large solids, total suspended solids (TSS), organic carbon (described as biochemical oxygen demand [BOD] or chemical oxygen demand [COD]), and nitrogen. The focus of this research remains on tertiary treatment, considered to be any additional treatment after secondary treatment, which encompasses a wide variety of processes. Wetlands are often considered for secondary and post-secondary treatment options, and are designed primarily to remove nitrogen and oxygen demand.

High levels of nitrogen in water can be harmful to the ecosystem as well as people, and lead to problems such as eutrophication, algal blooms, reduced biodiversity, and increased levels of bladder cancer, making the removal of nitrogen an important step for wastewater treatment (Sovik et al., 2006; Maltais-Landry et al., 2009; Kadlec and Wallace, 2009; Lu et al., 2009). Ammonium typically makes up over 50% of influent domestic wastewater nitrogen, but nitrate can be the primary species following a nitrification process (Kadlec and Wallace, 2009). Increasing regulatory pressures on wastewater treatment to remove total nitrogen instead of ammonia-nitrogen have led many to consider alternative options for denitrification, including recycling the nitrified waste to an earlier treatment step or using hybrid vertical and horizontal flow wetlands (Vymazal, 2005; Vymazal, 2010). Improving understanding of denitrification will make these processes more efficient.

TWs have been used to treat multiple types of wastewater and are proficient at removing organic material and other nutrients, often leaving nitrified water with low carbon concentrations (Arias et al., 2005; Brix and Arias, 2005; Vymazal, 2010; Canga et al., 2011). Nitrogen levels in wastewater TWs tend to be two to three orders of magnitude higher than those found in natural wetland systems (Canga et al., 2011).

Wetlands remove and/or transform nitrogen by many processes, including ammonification, nitrification, denitrification, anaerobic ammonia oxidation (anammox), adsorption, and biological assimilatory and dissimilatory pathways (Vymazal, 2007). Between 40-55% of total nitrogen is typically removed in TWs (Vymazal, 2007). Even though the majority of the total nitrogen removal is attributed to microbial processes, macrophytes are described as essential for efficient nitrate removal (Lin et al., 2002). Although anammox can compete with denitrification for nitrogen removal in a TW, most studies indicate denitrification removed more nitrogen than anammox processes (Zhang et al., 2008).

Carbon is a key component of cellular metabolism and many biological processes including denitrification. Carbon cycling inside a wetland is complex, involving biological and non-biological processes and multiple types of carbon (*e.g.*, inorganic and organic, dissolved and particulate), establishing a non-zero concentration of BOD (Kadlec and Wallace, 2009). In the context of wastewater treatment, carbon content is often referred to in terms of BOD, the amount of oxygen consumed by microorganisms to oxidize organic matter (Kadlec and Wallace, 2009). TWs for tertiary wastewater treatment typically receive an influent less than $40 \text{ mg L}^{-1} \text{ BOD}_5$, the amount of BOD in a

5-day incubation and remove BOD₅ with an efficiency of 61% (Vymazal and Kropfelova, 2009). Increased carbon loads have been shown to increase denitrification rates in wetlands (Lin et al., 2002; Kadlec and Wallace, 2009; Lu et al., 2009).

Types of Wetland

Wetlands for wastewater treatment can be described by the types of flow within the wetland: free-water surface, vertical subsurface flow, or horizontal subsurface flow (HSSF). Vertical flow wetlands are more efficient at performing nitrification and horizontal flow is more efficient for denitrification due to the aerobic operation of vertical flow systems and anoxic operation of horizontal flow wetlands (Arias et al., 2005; Vymazal, 2010; Canga et al., 2011).

Free water surface wetlands are the most similar to natural wetland systems and as such often attract local flora and fauna (Kadlec and Wallace, 2009). Typical use of these wetlands is as a last treatment step (tertiary or post-tertiary treatment) to remove carbon and nitrogen before discharging the wastewater (Kadlec and Wallace, 2009).

The system used in this research most closely resembles horizontal subsurface flow (Figure 1). The bottom of the wetland bed is lined with an impermeable layer, and filled with a medium such as gravel to provide support for plant and microbial growth (Vrhovsek et al., 1996). The design of these wetlands provides insulation in cold regions, as well as provides abundant rhizosphere habitat for associated microbes (Stein and Hook, 2005; Vymazal, 2010). Physical and chemical processes contribute to treatment, but much of the treatment is provided by microbial processes (Faulwetter et al., 2009; Vymazal, 2010).

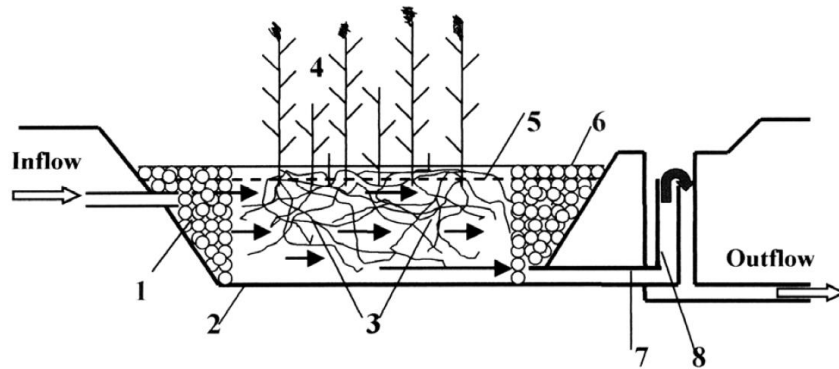


Figure 1: A horizontal subsurface flow treatment wetland. 1 inflow zone; 2 impermeable layer; 3 filtration material and roots; 4 plants; 5 water level; 6 outflow zone; 7 drainage pipe; 8 outflow structure. Figure from Vymazal, 2010.

Subsurface flow wetlands have a large degree of flexibility in their design and processing capabilities. Vertical flow wetlands are traditionally designed to have a high amount of oxygen available to the organisms in the wetland. Water is cycled through the wetland surface in a batch-loading scheme. Water is pumped over the surface, where it percolates through the wetland and into a drain pipe at the bottom (Vymazal, 2010). Between loads, the wetland is exposed to air to provide more oxygen during treatment (Vymazal, 2010). Variations of this design exist to optimize specific treatment processes (Kadlec and Wallace, 2009). HSSF wetlands exhibit a steep oxygen gradient on the water surface, making the wetland primarily anoxic, with some oxygen being tightly associated with the rhizosphere (Vymazal, 2010). Treatment designs combining a vertical flow and horizontal flow wetland are becoming more popular to combine nitrification and denitrification processes to remove a high amount of total nitrogen (Vymazal, 2005).

Greenhouse Gas Emissions

Denitrification leads to the release of gaseous nitrogen, typically in the form of dinitrogen gas (N_2) and the potent greenhouse gas nitrous oxide (N_2O). Nitrous oxide is a powerful greenhouse gas with a global warming potential 298 times that of carbon dioxide by mass. It is the third-most prevalent anthropogenic greenhouse gas (behind CO_2 and CH_4), responsible for roughly 6% of anthropogenic warming (IPCC, 2013). It can last 120 years in the atmosphere and is considered the dominant ozone-depleting substance in the 21st century (Ravishankara et al., 2009). The large levels of nitrogen found in wastewater systems can lead to high levels of N_2O being released, with reported values between 5-17 tonnes CO_2 equivalents per hectare per year in HSSF wetlands (Mander et al., 2005; Teiter and Mander, 2005; Sovik et al., 2006). Although TWs tend to have higher N_2O emissions than natural wetland systems due to higher nitrogen loading rates, they have lower emissions than traditional wastewater treatment practices, making wetlands an appealing alternative in terms of greenhouse gas emissions (Mander et al., 2005; Teiter and Mander, 2005; Sovik et al., 2006). Studies to reduce N_2O emissions are needed as anthropogenic N_2O levels are expected to rise from increased food production and as TWs become a more common solution for wastewater treatment (Mander et al., 2005; Teiter and Mander, 2005; Sovik et al., 2006; Liikanen et al., 2006; IPCC, 2013). One of the goals for this project is to provide additional understanding of the operation of microbial processes involved in TWs to provide support for the addition of a HSSF TW onto an existing treatment plant as a means to remove more total nitrogen through denitrification and to establish parameters to keep a low ratio of emitted nitrous oxide to

dinitrogen gas. The proposed research will lead to a TW that is a low-cost, efficient, and an environmentally-appropriate alternative to more traditional treatment practices.

Denitrification and Associated Enzymes

The nitrogen cycle describes the conversion of nitrogen species between their various states in the environment. Nitrification converts ammonium (NH_4^+) into nitrate (NO_3^-). Complete denitrification converts nitrate into the gaseous product dinitrogen (N_2), but incomplete denitrification can release gaseous nitrous oxide (N_2O) before it is converted to dinitrogen. Figure 2 outlines the nitrogen cycle.

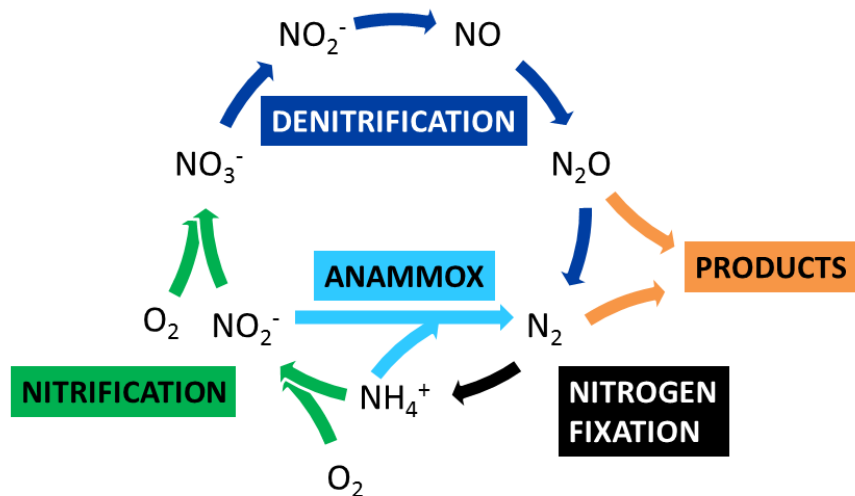


Figure 2: The nitrogen cycle. Denitrification can lead to both N_2O and N_2 being released.

Respiratory denitrification, the most prevalent denitrification process, occurs when denitrifiers use a nitrogen species as the terminal electron acceptor in their electron transport chain. Most denitrifiers are facultative anaerobes and will preferentially use oxygen over nitrogen species in the electron transport chain, as the reduction of nitrogen

species provides only about 60% of the energy of oxygen (Koike and Hattori, 1975a,b; Stouthamer et al., 1982). Denitrification is comprised of a series of four reductions under anoxic conditions, although in some cases small amounts of oxygen may be present (Torres et al., 2011). Nitrate (NO_3^-) is reduced to nitrite (NO_2^-), to nitric oxide (NO), to nitrous oxide (N_2O), and finally to dinitrogen gas (N_2) (Zumft, 1997). Each successive reduction provides roughly the same amount of energy to the cell on a per electron basis, making each intermediate in denitrification roughly equally favorable as an electron acceptor (Koike and Hattori, 1975b; Zumft and Cardenas, 1979; Stouthamer et al., 1982; Ferguson, 1994). Betlach and Tiedje (1981) reported that the accumulation of denitrification intermediates was due to different reduction rates rather than any specific inhibition. Many factors affect denitrification including concentrations of substrate, oxygen, carbon, as well as pH, temperature, moisture, and the denitrifying community (Vymazal, 2007; Saleh-Lakha et al., 2009; Lu et al., 2009).

Each step of microbial denitrification is mediated by an enzyme and its associated gene. Nitrate is converted to nitrite by a nitrate reductase, either Nar or Nap (genes *narG* or *napA*) (Bru et al., 2007). Nitrite is converted to nitric oxide by NirS or NirK (genes *nirS* or *nirK*) (Braker et al., 1998). Nitric oxide is converted to nitrous oxide by the nitric oxide reductase Nor (*nor* genes) (Arai et al., 1999). The final step, converting nitrous oxide to dinitrogen gas, is carried out by the nitrous oxide reductase NosZ (gene *nosZ*) (Zumft, 1997; Scala and Kerkof, 1998; Throback et al., 2004). Current denitrification research focuses predominantly on two sets of enzymes, Nir and Nos. Nitrite reductases are comprised of two functionally identical, mechanistically distinct enzymes NirS and

NirK (Zumft, 1997; Braker et al., 1998). NirK contains copper, while NirS contains cytochrome *cd*₁ (Zumft, 1997; Braker et al., 1998). Denitrification is controlled by a complex regulatory system involving multiple promoters to regulate the expression of genes based on environmental conditions (Zumft, 1997; Arai et al., 1999).

Organisms Performing Denitrification

Various steps of denitrification have been found in all three domains of life (Zumft, 1997; Kim et al., 2009; Rusch, 2013). The microorganisms with denitrifying capabilities determine to a large extent the amount of nitrous oxide released into the atmosphere during denitrification processes. These organisms consist of few eukaryotes and over 60 genera of Bacteria and Archaea, including over 25 species of Archaea which inhabit a wide range of habitats (predominantly extremophiles); most currently known denitrifiers are bacteria (Zumft, 1997; Kim et al., 2009; Rusch, 2013). The hallmark classification of denitrifiers is their ability to reduce nitrite to nitric oxide with Nir enzymes, as this is seen as a commitment step to the denitrification process (Zumft, 1997; Jones et al., 2008).

Genes encoding for denitrification enzymes are found in highly diverse bacterial species. *NirS* and *nirK* have not been found in the same strain of a species, but have both been found in different strains of the *Alcaligenes* and *Pseudomonas* genera (Coyne et al., 1989). *NirK* is found in a wider diversity of organisms, but often in fewer gene copy numbers than *nirS* (Coyne et al., 1989). Homologs of *nirK* have been found in a few nitrifiers, in contrast to previous beliefs that only denitrifiers encoded for Nir enzymes (Casciotti and Ward, 2001). Using *nir* genes is still common to estimate the total

denitrifier population (Braker et al., 1998; Jones et al., 2008; Hallin et al., 2009; Hamonts et al., 2013). Approximately one-third of denitrifiers incompletely reduce nitrate to nitrous oxide and do not have the *nosZ* gene (Zumft, 1997; Henry et al., 2006; Jones et al., 2008). Although uncommon, a few non-denitrifiers are able to reduce nitrous oxide to dinitrogen (Zumft, 1997; Sanford et al., 2012).

While it is thought that denitrification genes are more abundant than what is currently known, environmental qPCR primer sets to detect denitrification genes often select for primarily Alpha-, Beta-, and Gamma-proteobacteria (Jones et al., 2013; Rusch, 2013). Denitrification genes have also been detected in Delta- and Epsilon-proteobacteria, firmicutes, bacteroidetes, among other phyla (Jones et al., 2013). 16S rDNA genes encode for ribosomes and have some highly conserved regions across all bacteria. Phylogenetic trees based on 16S rRNA overlap poorly with phylogenetic trees based on denitrification genes, which suggests the denitrification genes evolved differentially from the 16S rRNA genes, likely due to horizontal gene transfer, duplication, and divergence (Jones et al., 2008). Microbial probes therefore have to be carefully designed to capture reliable and accurate information about the denitrification community.

Methods for Measuring Denitrification

Gas chromatography and qPCR are the two most commonly used analytical methods used to measure the denitrification process. This section will review the techniques.

A test of potential denitrification enzyme activity (measuring total N_2 and N_2O emissions as N_2O from NO_3^- conversion), established decades ago, is still one of the primary measurements in denitrification gas emission research (Balderston et al., 1976; Yoshinari and Knowles, 1976; Tiedje et al., 1989). Known as the acetylene-inhibition method, potential denitrification activity, and sometimes denitrification enzyme activity, acetylene is added to the headspace (typically 10%) of an airtight vial containing a sample, inhibiting NosZ. To keep gas terminology consistently focused on N_2O , this study uses the term N_2O produced or N_2O production to refer to this method. Thus, denitrification proceeds from NO_3^- to N_2O as the final product which is collected and quantified (Groffman et al., 2006). This method assumes that there is no significant increase in cell numbers or enzyme expression during the incubation period so the results are indicative of the potential denitrifying enzyme activity of the original sample. This method usually includes the addition of nutrients (carbon and nitrogen) to the incubation so that denitrification is not inhibited due to lack of substrate. Problems with this method have been well-established, and include: incompletely inhibiting N_2O reduction, inhibiting nitrification, and scavenging NO (Bollman and Conrad, 1997; Watts and Seitzinger, 2000; Groffman et al., 2006; Yu et al., 2010). All three of the problems listed above cause an underestimation of denitrification. However, the simplicity, low cost, and lack of a better high-throughput alternative have sustained the widespread use of this assay (Groffman et al., 2006; Butterbach-Bahl et al., 2013).

Less-commonly used methods include ^{15}N tracers and direct N_2 quantification methods. The former typically supply a sample with labeled $^{15}\text{NO}_3^-$ as the nitrogen source

and employ a mass spectrometer to determine the molecules with ^{15}N after a set incubation period. This is useful for measuring rates of denitrification respiration and could be used to locate regions of differential activity. The latter requires the replacement of any N_2 in the headspace gas with an inert gas, typically with helium or argon, so that all measured N_2 in the headspace is attributable to N_2 emission. This latter method is perhaps the most direct measurement of N_2 production of all the listed methods, but also the most costly. These techniques are not as often used as the others because of the challenges and cost associated with the methodology (Groffman et al., 2006).

Although the acetylene-inhibition method is widely used to measure denitrification, it fails to address the difference in production between N_2O and N_2 . Small variations to the assay can generate more information about the denitrification process and greenhouse gas emissions, rather than focusing solely on total potential denitrification. Adding nutrients without acetylene allows for the measurement of the total uninhibited N_2O released by the system over the incubation period, which is referred to in this study as N_2O emitted or N_2O emission; previous studies have referred to this assay as N_2O flux, N_2O emission, N_2O transient accumulation, N_2O evolution, and N_2O production (Watts and Seitzinger, 2000; Henry et al., 2008; Liu et al., 2010; Maltais-Landry et al., 2009; Mander et al., 2005; Philippot et al., 2009a; Khalil et al., 2005; Teiter and Mander, 2005). Another such modification measures potential N_2O reduction enzyme activity by adding N_2O to the headspace of a sample with carbon and no other nitrogen sources, to measure the remaining amount of N_2O after an incubation period. No

publications were found that included this assay to measure denitrification, it is referred to as N_2O consumed or N_2O consumption throughout this study.

Most of the genetic research in denitrification has focused on the genes *nirK*, *nirS*, and *nosZ*. Bacterial 16S rDNA genes are used to estimate the total number of bacteria. In contrast to nitrate reductases, which can often be part of ammonification and assimilatory nitrate reduction pathways in non-denitrifiers, Nir is found primarily in denitrifiers and is therefore used as an estimate of the number of denitrifiers present in a sample (Braker et al., 1998; Hendriks et al., 2000). The NosZ enzyme is often studied because of its direct relation to N_2O reduction. A few primer sets for these genes have dominated the literature because they are widely comparable between studies, have been optimized for qPCR, and capture a diverse range of bacteria. Using multiple primer sets for a single gene could lead to the problem of double-counting some gene copies. Advances in the genetic research are ongoing; a recent study designed an environmental *nosZ* primer to quantify a unique group of *nosZ*-harboring bacteria (Jones et al., 2013).

The ratio of $\text{N}_2\text{O}:\text{N}_2$ has often been overlooked in favor of reporting N_2O emissions directly. The ratio describes the amount of N_2O that is released in comparison to N_2 released, capturing some insight into the efficiency of the denitrification process to release low quantities of N_2O . This ratio has been found to be as high as 1:1 and as low as 1:1000 (Mander et al., 2005; Henry et al., 2008). Having large amounts of nitrate or nitrite present is thought to contribute to larger $\text{N}_2\text{O}:\text{N}_2$ ratios, as these substrates are expected to be used preferentially since they provide more energy due to an increased number of times they can accept electrons in comparison to N_2O (Khalil et al., 2005).

Reports combining $\text{N}_2\text{O}:\text{N}_2$ gas emission data with gene copy number are so far inconclusive, with one study finding a strong correlation to *nosZ* and one finding no correlation to *nirS* (Henry et al., 2008; Philippot et al., 2009a). Increased carbon load has been shown to increase denitrification activity, but its relationship to the $\text{N}_2\text{O}:\text{N}_2$ ratio is still not well understood (Lin et al., 2002; Picek et al., 2007; Philippot et al., 2009b).

Denitrification in Wetlands

Many studies have measured denitrification in terms of gas activity or gene abundance, but few have related the two (Correa-Galeote et al., 2013; Hamonts et al., 2013; Liu et al., 2010; Garcia-Lledo et al., 2011; Attard et al., 2011; Henry et al., 2008; Philippot et al., 2009a; Song et al., 2011). Comparing denitrification gas and genetic assays has been done with inconsistent methodology and only three used wetlands (Garcia-Lledo et al., 2011; Correa-Galeote et al., 2013; Song et al., 2011). Many more studies have focused on only one or the other of these. Publications have examined potential denitrification enzyme activities in various soils, natural wetland systems, and TWs (Mander et al., 2003; Mander et al., 2005; Teiter and Mander, 2005; Hunt et al., 2006). Similarly to denitrification gas emission studies, many qPCR studies have focused on environmental samples. Relatively few studies have examined the denitrification gene copy numbers in treatment wetlands (Chon et al., 2011; Song et al., 2011; Correa-Galeote et al., 2013; Ji et al., 2012). Understanding the microbial processes and how they influence these emissions is an important step towards reducing N_2O emissions, which could have broader implications for other denitrifying systems (Faulwetter et al., 2009).

Some correlations have been found between denitrification gene copy number and gas emissions, but these emissions may not be representative of the denitrification rates. The *nirS* gene copy number and community abundance has been correlated to potential denitrification enzyme activity; another study found a correlation between the relative abundance of *nosZ* (by 16S rDNA gene copy number) to potential denitrification enzyme activity (Philippot et al., 2009a; Song et al., 2011; Hamonts et al., 2013). Despite correlation with a potential denitrification enzyme activity, environmental variables such as carbon and nitrogen availability and hydrology have been found to be more closely correlated to the actual denitrification rates (Song et al., 2010, 2011, 2012; Attard et al., 2011). Using additional gas assays in combination with genetic assays will help elucidate these correlations more clearly.

Wetlands Spatial Activity

TWs encourage microbial growth by providing nutrients and attachment surfaces for microbes. A majority of treatment within a wetland is credited to microbial transformations rather than the plants within a wetland (Lin et al., 2002; Faulwetter et al., 2009). In particular, the plant rhizosphere is an important habitat for bacteria. Multiple studies have found large spatio-temporal gradients with regards to denitrification gas emission rates as well as gene copy numbers, and some have noted rhizosphere fractions are more active than non-rhizosphere fractions and have greater denitrification gene copy numbers than non-rhizosphere fractions (Teiter and Mander, 2005; Groffman et al., 2006; Hamonts et al., 2013; Faulwetter et al., 2013; Philippot et al., 2009b; Tanner, 2001). Plants are able to modify the micro-environments of the rhizosphere, which is the major

reason cited for higher denitrification activity in the rhizosphere (Kjellin et al., 2007; Hamonts et al., 2013). Even though roots provide a source of oxygen, spatial gradients make denitrification possible only micrometers away, and a coupling can exist between nitrification at the root surface and nearby “bulk soil” conducting denitrification (Kadlec and Wallace, 2009). One study found higher activity in the sediment fraction compared to subsurface plants and micro-algae, but this study did not examine the root fraction (Bastviken et al., 2003). Another study found large spatial gradients of denitrification gene copy numbers within a wetland, but only looked at sediment samples (Correa-Galeote et al., 2013). Few studies have explicitly separated habitats within a wetland to compare between them (Faulwetter et al., 2013). It has been recommended that further research examine the ability of micro-habitats for denitrification activity as well as examine the relationship between gene copy number and the ratio of $\text{N}_2\text{O}:\text{N}_2$ (Bastviken et al., 2003; Butterbach-Bahl et al., 2013; Faulwetter et al., 2009).

Plant Species Activity

The presence of plants can greatly impact the microbial diversity and abundance, typically greatly improving treatment compared to non-planted systems (Song et al., 2010, 2012; Faulwetter et al., 2013; Tanner, 2001; Brisson and Chazarenc, 2009; Stein and Hook, 2005; Allen et al., 2002; Taylor et al., 2011). The selection of different plant species can also lead to differences in microbial wetland communities, which influences denitrification activity (Lin et al., 2002; Brisson and Chazarenc, 2009; Maltais-Landry et al., 2009; Garcia-Lledo et al., 2011; Correa-Galeote et al., 2013). This is believed, in large part, to be caused by variations in root structure and exudates (Burgmann et al.,

2005; Faulwetter et al., 2013). However, due to variation in experimental conditions and lack of multiple studies for many species, more evidence is needed to further examine the effects of specific plant species in TWs (Brisson and Chazarenc, 2009).

Experimental Design

Our study considered the impact of two different water qualities (both at high nitrogen levels without added carbon or with added carbon) and three independent micro-habitats (particulates, roots, and gravel) on the denitrification activity of a treatment wetland, to help characterize the factors governing denitrification. Gravel was separated based on density and visual confirmation, roots were defined as the non-gravel portion not passing through a #35 (500 μm) sieve, and particulate was any material passing through a #200 (75 μm) sieve.

The simulated subsurface flow wetland column design used in this study has been used successfully in earlier studies at Montana State University (Allen et al., 2002; Stein and Hook, 2005; Stein et al., 2007; Taylor et al., 2011; Faulwetter et al., 2013; Allen et al., 2013). This system harbored an anoxic zone beneath the surface (typical of HSSF), a favorable environment for denitrification, as well as gave some modular capabilities such as allowing each plant column to be sampled independently of the others. This helped reduce the plant species effects in our samples to a single species. The focus of this thesis was to examine some of the factors which may correlate to N_2O production from a wastewater treatment wetland. This was explored through considering denitrification gas assay data as well as functional gene copy number data at the microscale level including the factors of carbon loading rate, habitat within the wetland, and plant species. Habitats

of roots, gravel, and particulate fractions from within the wetland were separated and three plant species and a non-planted group tested, along with two carbon loading rates. These samples were tested with three denitrification gas assays as well as gene copy number of three functional denitrification genes.

Hypotheses

Three main hypotheses for this study were generated: (1) Carbon load affects denitrification in particulate and gravel fractions more than in root fractions, (2) plant species affects denitrification activity, and (3) ratios between gas assays and gene copy numbers will be correlated. Within each hypothesis a series of testable predictions existed. For the first hypothesis, the following predictions were made: gravel and particulates have higher activity in non-planted carbon-positive systems than carbon-negative systems, planted gravel and particulates have higher activity in carbon-positive systems than carbon-negative systems, and root activity is mostly unaffected by carbon load. For the second hypothesis, the following predictions were tested: gravel and particulates have a higher activity in planted columns than non-planted columns, and root activities are species-specific. The third hypothesis tested a series of correlations between the *nirS* + *nirK* and N₂O produced, *nosZ* and N₂O consumed, as well as correlated N₂O emissions to other gas and genetic measurements.

Hypothesis 1: Carbon load affects denitrification in particulate and gravel fractions more than in root fractions. This was the main hypothesis of the study. It split the wetland column into multiple habitats to determine where denitrification was primarily occurring. It was expected the roots would provide some organic carbon in the

carbon-negative treatments. Some carbon from the roots was expected to be found in the particulate fraction, but very little was expected in the gravel fraction. Therefore, gravel carbon-negative fractions were expected to exhibit the lowest denitrification activity and gene abundances, and roots were expected to exhibit the highest denitrification activity and gene abundances, particularly in carbon-negative columns.

Hypothesis 2: Plant species affects denitrification activity. The species chosen for this experiment were all known to assist in the removal of nitrogen. Each species could create different micro-environments based on various factors, including root structure, carbon compound type and quantity emitted from the roots, and oxygen emitted from the roots, which could affect denitrification processes. This study addressed the ability of each species individually to affect the micro-scale denitrification processes.

Hypothesis 3: Ratios between gas assays and gene copy numbers are correlated. This hypothesis was more academic in nature than it was a practical design parameter. Examining the N_2O emission term was of primary importance. Regardless of the genetics or “laboratory-induced” gas assays, the N_2O emission term was the one most representing the greenhouse gas emission which this study addressed. Therefore, this measurement has been given special attention. All other measurements were seen as secondary to this one in their importance. If gas emissions could be correlated back to a gene copy number, it may be possible to design TWs to encourage favorable emission ratios while maintaining a high level of treatment.

CHAPTER 2

MATERIALS AND METHODS

Wetland Growth Conditions

The wetland system used in these experiments at Montana State University had been denitrifying for over 3 years. Plants were grown in a temperature-controlled greenhouse in separate PVC columns 30 cm tall, with 15 cm diameter, filled to 28 cm with pea gravel 0.3-1.3 cm in diameter. The column design can be seen in Figure 3. The three plant species *Carex utriculata*, *Phragmites australis*, and *Schoenoplectus acutus* (henceforth *Carex*, *Phragmites*, and *Schoenoplectus*) along with a non-planted group were fed synthetic wastewater every 15 days in batch. Components of the synthetic wastewater are listed in Table 1. *Phragmites* was chosen as a comparison to other wetland studies, *Carex* and *Schoenoplectus* were chosen based on previous experiments (Allen et al., 2002; Stein et al., 2007; Taylor et al., 2011; Allen et al., 2013). Temperature was cycled from 4 °C to 24 °C every year with the natural light cycle to match seasonal changes and induce annual growth and senescence patterns. Two columns of each plant treatment (8 total) had no carbon provided in the synthetic wastewater, and a corresponding set of columns (8 total) were fed two equivalents of carbon needed to stoichiometrically reduce NO_3^- to N_2 , with a C/N ratio of 1.47 (0.391 g L⁻¹ sucrose). Sixteen total columns were used in this research, divided into two carbon loads, four species, and two biological replicates of each type.

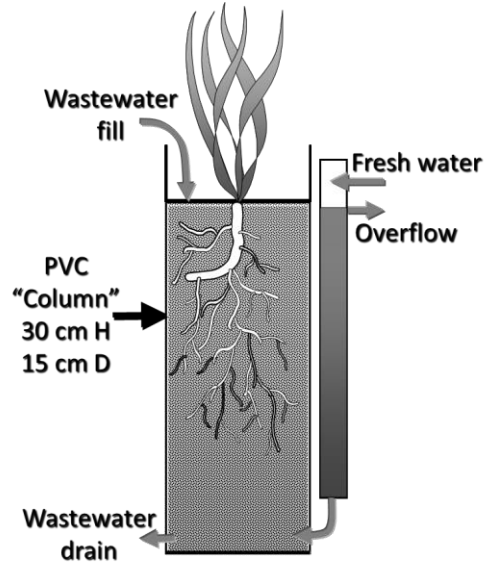


Figure 3: Diagram of a TW column used in this study, designed to simulate horizontal subsurface flow.

Table 1: Synthetic wastewater recipe. All compounds were laboratory-grade, purchased from Fisher Scientific (New Jersey) except zinc sulfate from Ricca Chemical Company (Texas), manganese sulfate from MP Biomedicals (Ohio), and sucrose, which was food-grade. The zero-carbon load had no sucrose added but was otherwise identical

Synthetic Wastewater	
Required Water Volume (L)	100
Reagent (g)	
$C_{12}H_{22}O_{11}$ (sucrose)	39.10
$FeCl_3$	0.04
$MgSO_4 \cdot 7H_2O$	6.20
K_2HPO_4	4.40
NH_4NO_3	11.43
KNO_3	28.87
H_3BO_3	1.00
$CuSO_4$	0.08
KI	0.19
$MnSO_4$	0.78
Na_2MoO_4	0.40
$ZnSO_4$	0.78
$CaCl_2$	0.19

Coring

Sample cores from the wetland columns were taken on day 3 of the column feeding cycle at 24 °C. Cores of the columns were extracted with a 1¼" hole saw. First, the column was drained and the water stored. The PVC side wall was drilled through at a height of 24.5 cm and the PVC disc was discarded. A core sample containing roots, gravel, and particulates (described below) was extracted with the hole saw and stored in a

nanopure-water-cleaned sealed jar for under twenty minutes before habitats were separated.

Habitat Separation

Sample cores were separated into particulates, roots, and gravel (Figure 4). “N₂ water” was made by sparging reverse-osmosis water with ultra-high purity (UHP) N₂ for 10-15 minutes to remove some of the oxygen. This N₂ water was poured into the core sample jar, stirred, and decanted onto a #35 (500 µm) sieve, stacked on a #200 (75 µm) sieve. Density of the gravel usually kept it from being decanted out of the sample jar, but any gravel observed visually on the sieves was returned to the sample jar. This mixing and decanting was continued until the N₂ water decanted from the sample jar was visibly free of most particulate matter and roots. Flowthrough from the sieves was collected and N₂ water was added until the final volume reached 500 mL to create the particulate fraction. The particulate fraction was therefore primarily composed of organic and inorganic particulates from the columns; roots were a major component of the particulate fraction in planted columns. The material between the two sieves was discarded, and the material not passing through the #35 sieve was labeled the root fraction. Larger roots were cut into small sections to make the root fraction more homogeneous across all incubation replicates. The rinsed gravel fraction was put through a final separation step by pouring it into a cylindrical tube and flowing pressurized N₂ water up from the bottom to fluidize and eject particles with a lower density than the gravel. The water and associated material from this final gravel purification step was discarded to produce the gravel habitat. Excess material from each habitat (not used in the gas incubations) was

stored at -20°C for DNA extraction. Typically all roots were used for incubation, so root material was recovered post-incubation and stored at -20°C for subsequent DNA extraction. DNA gene abundances were expected to change very little over a period of 3 hours.

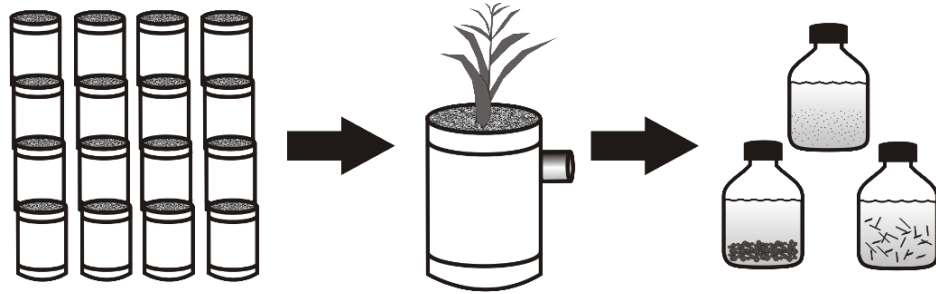


Figure 4: A diagram representing the sample processing. Sixteen columns total were cored near the top of the column and split into three habitats for assays. Example incubation vials for habitats are shown. Not to scale.

Filling Incubation Vials

Once all three habitats were separated, nine 10 mL glass incubation vials were prepared from each habitat, making 27 vials per core. These nine vials were for three different incubation conditions to be run in triplicate, as described below. Each habitat was kept submerged in N_2 water until it was divided into incubation vials, at which point the roots or gravel were blotted with a kimwipe (Kimtech Precision Wipes 05511, Kimberly-Clark Worldwide, Georgia) to remove excess water and give a more accurate (but still wet) mass. The amount of material added to each incubation vial varied across habitat: 2.0 ± 0.1 g gravel were added, roots were divided as homogeneously as possible for each sample core, and 5 mL of particulates were added. The mass of roots and gravel added to each incubation vial was recorded. N_2 water was added to each incubation vial

as follows: (1) particulates had no N_2 water added, (2) gravel had 4.25 mL added, and (3) roots had the difference between 5 mL and the average root volume added, assuming 1 g of roots was roughly equivalent to 1 mL of water. Concentrated nutrients (50 μ L) were then added to each vial so as to provide a negligible contribution to the total volume. For the potential N_2O production and total N_2O emission treatments, 1 mM of both glucose and potassium nitrate were added. For the N_2O consumption treatment, 1 mM glucose was added. Vials were then capped and crimped to seal them. Preliminary experiments confirmed all values of added nutrients were sufficient to maintain zero-order reaction kinetics for the N_2O production and consumption assays throughout the entire incubation period.

Incubation Conditions

The sparging station used for gas assays consisted of a manifold which provided twelve 25-gauge needles connected to gas flow. All N_2 used was UHP. Both N_2 and N_2O tanks were connected via 3 mm (OD) copper tubing to a shut-off valve. All other tubing connecting the shut-off valve to the manifold and connecting the manifold to needles was silicone (1.6 mm and 3.1 mm, Masterflex 96400-14 and 96400-16, Cole-Parmer, Illinois).

Each treatment measured a different section of the denitrification pathway, as seen in Figure 5. Three treatment groups per habitat were run in triplicate. All vials had the headspace and liquid sparged with UHP N_2 gas for 6-10 minutes to remove excess O_2 . Two needles pierced the septum of each vial; one was an outlet to the ambient air, the other was connected to the sparging station with gas tanks.

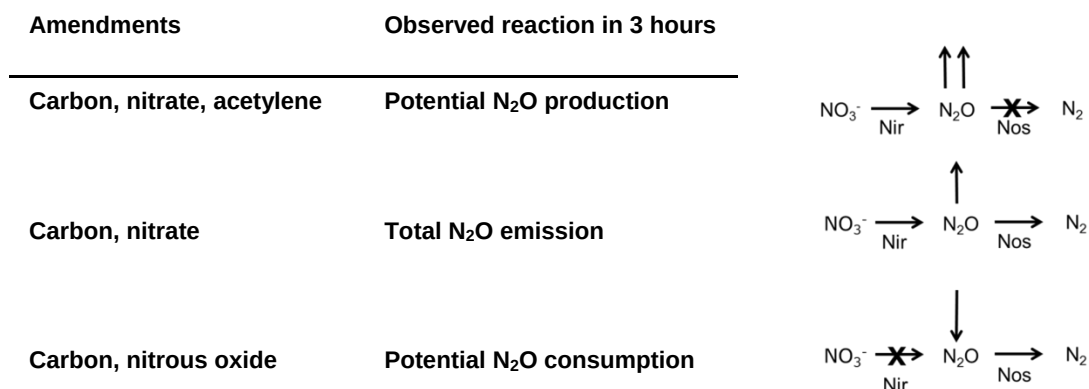


Figure 5: Treatment conditions for gas incubations. The first column lists what amendments were added to each treatment group to observe the reactions diagrammed on the right. Note the N₂O production was chemically inhibited, whereas N₂O consumption was restricted only by the incubation nutrient solution, and N₂O emission was uninhibited.

Modifications to the N₂O production and consumption treatment groups were made, and then all vials were turned upside down and incubated with shaking for 3 hours. The treatment group for N₂O consumption had nitrogen added as N₂O gas at 1200 ± 300 ppm mixed with the sparging UHP N₂ gas. For each set of vials sparged together (sparging group), three vials filled to 5 mL with N₂ water were used as controls. Variance within control vials for a sparging group was within an average of 5.0%, but variance between controls from different sparging groups was larger, causing the large concentration variance. Acetylene was added to the headspace (10% v/v) in the potential N₂O production treatment to block the conversion of N₂O into N₂.

Gas Sampling

At the end of 3 hours, the headspace of the vials were sampled for N_2O measurement. Using a two-syringe system, the vial septum was pierced with an empty 10-mL syringe and with a second 10-mL syringe full of nanopure water. 5.5-6 mL of nanopure water was injected from the syringe into the vial, which filled the empty syringe with 5.1-6 mL of headspace gas. Collecting over 5 mL of gas in the syringe was important to prevent atmospheric air from entering the syringe. The gas syringe was then removed from the incubation vial and depressed until only 5 mL remained. A short (~1 cm) silicone tube (1.6 mm, Masterflex 96400-14, Cole-Parmer, Illinois) connected the gas syringe to a gas collection bag (MS-252 FlexFoil bags, 3W $\times$ 7L, no eyelets, single PP fitting, standard label, SKC, Pennsylvania). The gas bag was opened for sample injection and closed before the syringe was removed. Three to four pumps of atmospheric air were used between samples to clean the gas syringe. Gas samples were diluted in the bag with N_2 to produce a detectable N_2O concentration on the gas chromatograph, as determined by trial and error. The potential N_2O production treatment was diluted 20-fold, total N_2O emission was diluted 5-fold for initial sample cores and diluted 20-fold in later sample cores, and the potential N_2O consumption was diluted 100-fold. In three cases only two technical replicates were performed for a given gas measurement due to experimental errors. No data were removed as outliers.

Gas bags were cleaned and reused according to an experimentally-derived protocol. Bags were flushed three times with N_2 and were partially filled with N_2 and left for 8-20 hours. Bags were flushed three more times with N_2 and were considered clean,

as confirmed by gas chromatography N_2O analysis, by being below ambient air concentrations.

Gas Chromatography Analysis

Gas bags were stored no longer than 72 hours before they were sampled on a HP 5890 Series II gas chromatograph, with a flame ionization detector (FID, for CH_4) and an electron capture detector (ECD, for N_2O). Ambient temperature was monitored with a NIST-traceable electronic thermometer and ambient pressure was monitored with a rooftop barometer. A 10-port custom injection valve used a 1 cm^3 sample loop to backflush into a $25\text{ cm} \times 0.32\text{ cm}$ (OD) precolumn with Porapak-T 80/100 mesh, to prevent water vapor from entering the analytical columns. Two $183\text{ cm} \times 0.32\text{ cm}$ (OD) analytical columns packed with Chromosorb 102 80/100 mesh and Porapak-Q 80/100 mesh, respectively, separated gas in series. Temperatures were $55\text{ }^\circ\text{C}$ for the oven, $240\text{ }^\circ\text{C}$ for the FID, and $360\text{ }^\circ\text{C}$ for the ECD. UHP N_2 was further purified through a Molecular Sieve 5A, activated charcoal, and an oxygen scrubber before being used as a carrier gas. High-purity CO_2 was introduced at 2% of the carrier flow rate just before the sample entered the ECD to encourage ionization. Equivalent flow was maintained to both detectors with a 4-port valve providing the ability to switch the sample from one detector to another. All flows were set at approximately 30 mL min^{-1} . At 2.5 minutes, flow was directed through the ECD to detect N_2O at 4 minutes.

Gas Chromatography Standard Curves

Standards of 0.1, 1, 5, 10, 25, and 50 parts-per-million (ppm) were created by diluting N₂O with N₂ inside gas bags. Ambient air samples from near the GC (henceforth ambient lab air samples or lab air) were run with every gas chromatography (GC) measurement batch, N₂O standards were run 9 times total throughout gas data collection periods. Peak areas from the chromatograms were converted into a concentration.

The N₂O standard curve was a linear plot using values which had been adjusted with a log transformation. The standard curve for N₂O concentrations was tightened by accounting for the ambient lab air samples of N₂O at the beginning and ending of running samples on the gas chromatograph. All N₂O standards were converted into the number of moles in the GC sample loop using $PV = nRT$. This accounted for small fluctuations in temperature and pressure that occurred in the GC room between sampling days. The peak area was log-transformed, as this tracked well with standards separated by GC sample batch. Each individual sample was assigned a lab air value that was the linear interpolation between the initial N₂O lab air and the concluding N₂O lab air in the GC sample batch. In log-transformed space, the lab air values linearly adjusted the peak area of each sample based on its relation to the average lab air value, and then samples were transformed back to a linear space using the equation $adjusted\ peak\ area = e^{(\ln(peak\ area) - (\ln(lab\ air) - \ln(lab\ air\ average)))}$, where lab air average was the average value of all lab air samples. The standard curve of moles in the sample loop against adjusted peak area was plotted. A linear model fit using an `lm` function in R (statistical package v. 3.1.2 and the `nlme` package, described in more detail

below) of linear axes and a forced y-intercept of zero was chosen ($slope = 2.176 \times 10^{-16}$, $adjusted R^2 = 0.9897$) (R core team, 2014; Pinheiro et al., 2015). From this standard curve, all adjusted peak areas were converted into moles of N₂O in the sample loop, which were converted back into a concentration (mg N₂O g⁻¹ sample or mg N₂O mL⁻¹ sample). Final adjustments were then made, including accounting for dilution into gas bags and subtracting the N₂O consumption term from controls. Additional information has been provided in Appendix C.

DNA Extraction

Previously stored samples were removed from -20 °C and thawed on ice. A single DNA extraction of each sample provided the DNA for all qPCR measurements of that sample type (*e.g.*, one extraction was performed on each replicate of an added-carbon non-planted column). Extractions were performed using the FastDNA SPIN Kit for Soil (MP Biomedicals, Ohio) and DNA was eluted with 100 µL DEPC-treated water (AM9906, Life Technologies Corporation, California). To prepare the particulate fraction, 33 mL were filtered through a 0.2 µm polycarbonate filter paper (Poretics Corporation, California). The filter paper was added directly to a DNA extraction tube. Gravel was extracted without the use of the DNA extraction beads as gravel naturally provides a rough surface during the homogenization step. 1.6 g (wet mass) of gravel and between 0.12 and 0.77 g (wet mass) of roots were added to each DNA extraction tube. After extraction, each sample was checked with a NanoDrop ND-1000 (Thermo Scientific, Massachusetts) to measure DNA concentration and purity (data not shown).

DNA samples were also run through a OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, California) to remove excess inhibitors.

qPCR Conditions

Table 2 lists the primer sets used in this study. Base pair length was used in the calculation for gene copy number. Each reaction contained 25 μL total volume with 12.5 μL SYBR Green PCR Master Mix (Life Technologies Corporation, California), 5 μL DEPC-treated water, 5 μL DNA template, and 1.25 μL of each primer at 10 μM . Cycling conditions for qPCR were modified from literature values, shown in Table 3. A melting-curve analysis was performed on all samples as quality control. Two independent qPCR replicates were performed for all samples analyzed, with an average C_t difference of less than 0.2 between duplicates. Duplicates that were more than 1 C_t apart were run in duplicate a second time, and the average of all four C_t values was used for analysis. No data were removed as outliers. Samples were diluted 10-fold for the 16S rDNA primer set, and 100-fold for *nirS*, *nirK*, and *nosZ*. In three cases 16S rDNA samples were diluted 100-fold when the 10-fold dilution was found to have an inhibitory effect, as inspected visually in the C_t curve. Each qPCR run contained standards of 10^{-2} and 10^{-7} $\text{ng } \mu\text{L}^{-1}$ genes corresponding to the primer set to check for consistency with standard curves. All threshold C_t values were obtained using the Rotor Gene Series software package v. 1.7 (QIAGEN, Maryland) and were exported for further analysis.

Table 2: qPCR primer sets used in this study.

Gene	Primer Set	Base pairs (bp)	Reference
16S rDNA	341F/534R	174	Smits et al., 2004; Muyzer et al., 1993
<i>nirS</i>	Cd3aF/R3cd	425	Throback et al., 2004
<i>nirK</i>	876F/1040R	164	Henry et al., 2004
<i>nosZ</i>	nosZ2F/nosZ2R	267	Henry et al., 2006

Table 3: qPCR cycling conditions used in this study.

Gene	Initial Activation	Touchdown	Melting	Annealing	Extension	Cycles
16S rDNA	95 °C, 15 min	N/A	95 °C, 15s	60 °C, 30 s	72 °C, 30 s	35
<i>nirS</i>	95 °C, 15 min	N/A	95 °C, 30 s	57 °C, 60 s	72 °C, 60 s	35
<i>nirK</i>	95 °C, 15 min	6 cycles, anneal at 63 °C, -1 °C cycle ⁻¹	95 °C, 15 s	58 °C, 30 s	72 °C, 30 s	40
<i>nosZ</i>	95 °C, 15 min	6 cycles, anneal at 65 °C, -1 °C cycle ⁻¹	95 °C, 15 s	60 °C, 15 s	72 °C, 30 s	30

qPCR Optimization

The annealing temperature was optimized for each primer set by testing annealing temperatures ± 2 °C from published values on a Bio-Rad iCycler (Bio-Rad Laboratories, California) with a gradient block, using MyiQ software v. 3.1. The DNA template used for optimization was a combination of original DNA extract from six samples (two from each habitat) to account for potentially heterogeneous distributions of gene sequences and copy numbers among the samples (“multi-DNA template”). Other optimizations,

including determining the appropriate dilution value for DNA template samples, were performed on a Rotor-Gene 6000 (QIAGEN, Maryland), which was the instrument used for all other qPCR data collection.

Inhibition was determined by checking qPCR efficiency between each dilution in a dilution series with and without the PCR inhibitor removal kit. The *nirK* gene copy dilution series was matched to a dilution series where 2.7×10^8 copies were spiked into each sample in the dilution series. Sample inhibition would cause a higher C_t value than expected and show as an outlier on a standard curve. The lowest consistently uninhibited dilution factor was used for each gene.

qPCR Standard Curves

Genes from each primer set using the multi-DNA template (diluted 10-fold) were amplified by PCR following a modified version of the conditions outlined above with a GoTaq Green Master Mix (Promega Corporation, Wisconsin). All PCR cycling conditions were held at 95 °C for 2 minutes as an initial step (instead of 15 minutes), and were held at 72 °C for 5 minutes as a final step. Products were confirmed on a 1% agarose gel and purified with a QIAquick PCR Purification Kit (QIAGEN, Maryland). The purity and concentration was tested on a NanoDrop ND-1000 (data not shown), then samples were further purified using the OneStep PCR Inhibitor Removal Kit to match the general DNA extractions, since the multi-DNA template was generated before the purification kit was an established part of the protocol. Another purity and concentration test was performed on a NanoDrop ND-1000 (data not shown), and standard dilutions were made by first diluting to $1 \text{ ng } \mu\text{L}^{-1}$ and following with serial dilutions.

Standard curves were created with triplicate runs of all dilutions. Standard curves for all primers ranged from 10^{-2} through 10^{-7} ng μL^{-1} of the gene target, in 10-fold dilutions and were generated by plotting the C_t value versus the log of the sample concentration. The amplification efficiency was calculated using the formula $E = 10^{\frac{-1}{\text{slope}}} - 1$ with the best-fit line through the standard curve. Standard curves for qPCR are shown in Table 4.

Table 4: qPCR standard curves for each primer set used in this study.

Primer	Slope	Efficiency (%)	R^2
16S rDNA	-3.193	105.7	0.9971
<i>nirK</i>	-3.440	95.3	0.9986
<i>nirS</i>	-3.441	95.3	0.9996
<i>nosZ</i>	-3.710	86.0	0.9993

The standard curve allowed the conversion of raw C_t values into a concentration (ng μL^{-1}) using the calculation $\text{sample DNA } (\frac{\text{ng}}{\mu\text{L}}) = 10^{\frac{C_t - \text{intercept}}{\text{slope}}}$. By assuming 1 mole of base pairs was 650 g (Integrated DNA Technologies, 2011) and assuming all gene copies have exactly the same number of base pairs in their length for a single primer set, the number of gene copies can be determined by the equation $\text{copies} = \frac{\text{DNA amount (ng)} * 6.022 * 10^{23} (\frac{\text{copies}}{\text{mole}})}{(\text{amplicon bp length}) * 650 (\frac{\text{g}}{\text{mole}}) * 10^9 (\frac{\text{ng}}{\text{g}})}$. This value is reported as total gene copies per g of sample or total gene copies per mL of sample.

Normalizations and Data Imputation

Two distinct types of normalization were applied to the data. The first type, standardizing units, was applied universally. The data were unit-normalized before all analysis. The second type, normalizing to 16S rDNA gene copy number, was used to make a second data set that was analyzed separately from the original data. From here on out, the terms “normalized” and “non-normalized” refer only to the data sets in relation to 16S rDNA normalization unless specifically stated otherwise.

Roots and gravel gas samples were standardized by mass of sample (g) and time (hr). Particulate fraction gas samples were standardized by volume of sample (mL) and time (hr). DNA measurements were standardized to a copy number per gram (roots and gravel) or copy number per mL (particulates) material extracted. For comparisons within one habitat, these per gram and per mL values were used. For comparisons across habitats, these values were further normalized by the copy number of 16S rDNA per sample. DNA values are typically normalized to ng DNA and/or to g of sample; g of sample was chosen to relate to the gas assays. Technical replicates for all measurements were averaged together before being processed. Data were log-transformed during analysis to ensure normal distribution. Log transformations were applied once to any ratio of data sets rather than separately for each data set. For example, a ratio of $\frac{nosZ}{16S\ rDNA}$ would be $\log(\frac{nosZ}{16S\ rDNA})$.

A small number of gas samples registered above the 50 ppm standard curve. These values were all capped at the standard-curve-extrapolated value of 60 ppm. The capping affected two N₂O production measurements and one N₂O consumption

measurement. An error estimation calculated from the expression $\frac{(extrapolated - capped)}{average(extrapolated + capped)} * 100$ suggested selecting the 50 ppm value would introduce an approximate error of 28%, whereas capping at 60 ppm instead would result in only a 13% error. Although the 60 ppm cap was likely not entirely accurate due to instrument limitations, it was expected to be more accurate than a 50 ppm cap. These data should have been run again after further dilution, but the gas bags had already been cleaned and sample discarded before the data points were analyzed.

Approximately 16% of gas values were extrapolated below the standard curve in the region between 0 ppm and 0.1 ppm. To account for the samples below the standards, the forced intercept was included in the standard curve such that 0 moles in the GC would result in 0 peak area. This extrapolation affected 11 (of 44) N₂O emission samples, 10 (of 44) N₂O production samples, and 1 (of 44) N₂O consumption sample.

The *nirS*, *nirK*, and *nosZ* values all had correction factors applied to data below the detection limit. Undetectable *nirS*, *nirK*, and *nosZ* values (2, 16, and 7 samples, respectively) were substituted for the detection limit divided by 2 and adjusted for the habitat of the sample. The detection limit was set at 10^{-7} ng μL^{-1} of the gene target, which was the last sample in the standard curve. As a result of substituting 36% *nirK* values (explained below), *nirK* was only included in analysis when it was being summed with the *nirS* to represent the amount of nitrite reductase.

Statistical Analysis

Great effort was taken to carefully design the statistical tests used in this experimental design to address the hypotheses. Statistical models (henceforth models) were designed to incorporate as many effects as possible to reduce the total number of models needed, as well as to provide more conservative *p*-values to reduce type I errors.

Statistical analysis was performed with R statistical package v. 3.1.2 and the *nlme*, *lme4*, and *multcomp* packages (R core team, 2014; Pinheiro et al., 2015; Bates et al., 2014; Torsten et al., 2008). Linear mixed-effects models were generated to assess the relationships between denitrification activity, gas assays, and gene copy numbers. *P*-values were obtained by performing a marginal-type ANOVA on each model, and model assumptions were checked by performing a Q-Q plot and residuals plot analysis, and additionally performing a Levene's test for equality of variance for models when appropriate. When models failed these tests, a model weighted by $\frac{1}{\text{variance}}$ of the sample groups was used in its place. If the variance of a sample group was 0, the variance for that group was taken to be the average variance from all other groups. It is worth noting, however, that in cases where a weighted model was used, the equality of variance assumption was forced at the cost of potentially violating the normal distribution assumption. A full list of the models run can be found in Appendix B.

Linear mixed-effects models are a type of regression analysis in which the variance among some factors is considered to be random (*e.g.*, a technical triplicate) and the variance among other factors is considered to be fixed (*e.g.*, multiple plant species). Multiple factors may be tested within a single model, but it is important to recognize the

differences between two similar models. By including more than one factor in a model (hypothetically, *factor A* and *factor B*), the question being asked is: Does factor A have an effect after the effects of factor B are accounted for (and vice versa)? The math testing a model which includes only factor A is distinct from the math testing factor A in a model which includes both factors A and B.

In this study, the only random effect considered was the column replicate number. Such labeling accounts for non-independence of measurements coming from each of the three habitats from the same column. When the data was split by each habitat there was no random effect listed, because multiple samples within a model were no longer coming from the same column. Interaction terms (which may be written as $\text{factor1} \times \text{factor2}$) between the main effects were often included in the models used for this study. An interaction term between two effects is best described as one factor influencing the correlation of the other factor. For example, one plant species may perform well as carbon-positive and poorly as carbon-negative, while another plant species may perform equally well at both carbon loads. This interaction term may or may not trigger significance for the individual factors, but the interaction term operates within the model as its own factor independent of the main effects.

Data was split into three main groupings for analysis: all data excluding non-planted samples (“planted”), all data excluding roots (“non-root”), and all data split by habitat (generally as “habitat,”; specifically as “gravel,” “particulate,” and “root”). Data had to be broken up in this way due to the included interaction terms between tested factors—the non-planted root data does not exist, therefore interactions between species

and habitat factors cannot be modeled and the data set must be split to remove all roots or remove the non-planted (Figure 6). The whole data set was used for comparing ratios, as this did not examine the habitat or species as factors. Two ways of examining the effects of plant presence in TW columns were explored. The gravel and particulate data sets include non-planted samples as a fourth “species,” whereas the root data set does not include these samples. One additional category exists which groups all plant species together as “planted” to compare against the “non-planted” samples. Planted can therefore refer to a data set or to a factor; if a factor it will be planted column samples in comparison to non-planted column samples, and often referred to as plant presence. For all data sets, one model using the lme function was generated for each unique gas assay along with each of *nirS*, *nirS+nirK* (henceforth *nirS+K*), and *nosZ* gene copy numbers. Statistical models were never run independently on *nirK*, so throughout this study “all genes” or “all gene abundances” in the context of statistical models refer to *nirS*, *nirS+K*, *nosZ*, and 16S rDNA.

For habitat data sets, 16S rDNA gene copy number was used in its own model. For the planted and non-root data sets, each of the measurements was normalized by 16S rDNA gene copy number. The habitat data sets were not normalized to 16S rDNA gene copy number.

Fixed main effects of carbon load, habitat, and species were included with random effects of column replicate number. When data was grouped by habitat a fixed-effect lm model was used, and habitat was not included as a fixed effect. All possible fixed-effects interaction terms were included in the model, but the three-way interaction was dropped

when it was found to be statistically insignificant. An example model would be: in the planted data set, a response of *nosZ* copy number was tested by the factors of habitat, species, carbon load, habitat interacting with species, habitat interacting with carbon load, and species interacting with carbon load. The same models were also run with main effects of carbon load, habitat, and plant presence to account for differences that may only be obvious when grouping all plant species together. The factor of plant presence could not be run on the root or the planted data sets, as these were non-existent in non-planted samples by definition; therefore the corresponding root models used only one main effect of carbon load.

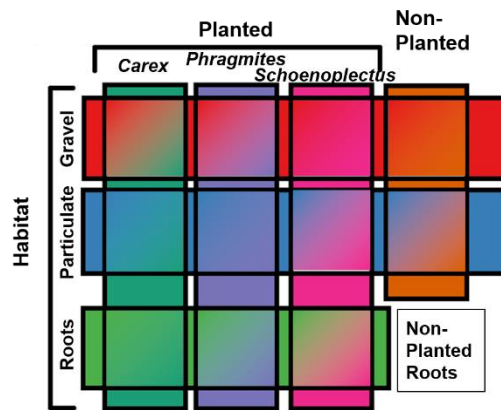


Figure 6: A color scheme representation of the main ways data was grouped. Note the non-planted root data does not exist. The horizontal axis represents the three plant species and non-planted columns. The vertical axis represents habitats within the column. The color of each category was constant through the results (e.g., gravel is red throughout all analysis). Squares of species and habitat interaction are multicolored only in this figure, each figure in the results will show only one color scheme (e.g., one graph will show either habitat or species).

Multiple additional mixed-effects models were generated for the whole data set to correlate gas assays with gene copy numbers, and to examine the N_2O emission term. Two different model types were generated; the first correlated gene data to their

corresponding gas assays and the second examined the N₂O emission values and how they correlate with other measurements. The first type includes 5 models: the potential amount of N₂O produced with *nir* gene copy numbers as a fixed effect, the potential amount of N₂O consumed with *nosZ* gene copy number as a fixed effect, the normalized-data versions of the previous two models, and the ratio of N₂O produced/consumed as a response with a ratio of *nir/nos* as the effect. The second type of model includes 14 models: the N₂O emission term was the response for 5 models which have each measurement separately as the only effect, 2 models which had N₂O emission as the response with the ratio of *nir/nos* or N₂O produced/consumed as effects, 6 models were the normalized version of these (*e.g.*, normalized N₂O emitted by normalized *nosZ*), and one model was N₂O emitted/produced as the response by *nir/nos* as the effect. These models did include column replicate as a random effect, but did not account for carbon load, habitat, species, or plant presence differences as fixed effects, as the data was left ungrouped.

Because multiple models were used, a multiple comparison correction was performed using Benjamini-Hochberg false discovery rate set to $\alpha = 0.05$ (Benjamini and Hochberg, 1995). A multiple comparison correction adjusts the 95% confidence for a single model and applies (in this case because $\alpha = 0.05$) the 95% confidence that all models adjusted in the same group were correct. This correction factor was applied separately to subsets of the data grouped by the data set models were performed with, which closely corresponded to the hypotheses being tested: planted; non-root models with species and with plant presence effects; particulate fraction with species and with

plant presence effects; gravel with species and with plant presence effects; roots with species (testing carbon load only) effects; and all gas-to-gene and gas-to-gas correlations were grouped together.

Significant effects as determined by an ANOVA of the models $p \leq 0.1$ (after p -value adjustment) were further explored. The lmer function generated a new model which was analyzed by the multcomp package to provide post-hoc Tukey's tests between the fixed-effects groups (Bates et al., 2014; Torsten et al., 2008). When performing post-hoc analysis on an interaction term for the planted and non-root data sets, all non-significant interaction terms and the main effects of the interacting factors were removed from the model (*e.g.*, if a species and habitat interaction term was being tested, the model included the interaction term and carbon load as effects). Significance was set at $p \leq 0.05$ for post-hoc tests. P -values are reported as either F -test (ANOVA) or as Tukey's (post-hoc).

All results, including figures, were generated after all value substitutions and imputations were made, unless specifically noted otherwise. All technical replicates were averaged. Biological replicates were not averaged, but were accounted for as random effects within all models that permit the use of random effects (*i.e.*, all models not based on a habitat-specific data set).

CHAPTER 3

EXPERIMENTAL RESULTS

Results Overview

In this study, 16 simulated HSSF TW columns consisting of two carbon loads (0, 39.1 g L⁻¹) and four plant treatments (three species: *Carex*, *Phragmites*, *Schoenoplectus*; one non-planted) were divided into three distinct habitats (particulate, gravel, root). Each of these samples was analyzed for denitrification activity using three gas assays (N₂O production, N₂O emission, N₂O consumption) and 4 qPCR assays for genes associated with microbial abundance (16S rDNA) or denitrification activity (*nirS*, *nirK*, *nosZ*). All measurements were aggregated into 44 total samples, split into: 22 of each carbon load; 16 particulates, 16 gravel, 12 roots; 12 of each species, 8 of non-planted. Denitrification activity was assessed according to carbon load, habitat, and plant species, and analyzed to determine any correlations with minimizing N₂O emission, as well as correlations between gas assays and gene copy number. The following three sections are structured to provide a brief introduction to the data collected before addressing the main hypotheses.

Data Set Grouping and Presentation

Due to the complex nature of the data, many of the statistical models overlap in certain areas, testing different aspects of the same data. Multiple data sets (all data, planted, non-root, particulates, gravel, root) were generated to completely address all hypotheses. This has provided a more thorough, although perhaps more nuanced,

understanding of the results. The full set of models and post-hoc tests can be found in Appendix B. To ease the complicated nature of the data sets, figures have been presented in color. Colors for most data sets are unique (Figure 6, Habitat: gravel is red, particulate is blue, roots are green; Carbon Load and Plant Presence: carbon-negative non-planted columns are light blue, carbon-negative planted columns are light green, carbon-positive non-planted columns are blue, carbon-positive planted columns are green; Species: *Carex* is teal, non-planted is orange, *Phragmites* is purple, *Schoenoplectus* is pink). The data figures do not represent the statistical models. For the scatterplots, an R^2 value is not representative of a mixed-effects model so none have been provided. Regression lines are provided based on the data in the figure, which do not account for the random effects in a mixed-effects model.

Distinguishing between denitrification activity and gene abundance in each habitat was a major focus of this study; as such, it was important to generate a system by which to compare between habitats. All data collected were analyzed as normalized and non-normalized. The normalized data was relative to the 16S rDNA gene copy number, and was comprised of the planted and non-root data sets. The non-normalized data was not relative to 16S rDNA copy number, and was comprised of the three habitat data sets (particulates, roots, gravel). Comparisons across habitat were primarily made in the normalized data.

Quantification of 16S rDNA and Denitrification Genes

In each habitat and condition, the quantity of genes associated with the denitrification process was assessed. These genes include *nirS*, *nirK* (both for nitrite

reductase), and *nosZ* (for nitrous oxide reductase). 16S rDNA copies were measured as an indication of overall bacterial abundance. Non-normalized data are presented first, followed by the 16S rDNA-copy-normalized data. Across all qPCR data, 14% of samples were below the detectable limit. Approximately 34% of all *nirK* samples were below the limit of detection (and were substituted), followed by 7 samples of *nosZ* and 3 samples of *nirS* (16 and 7%, respectively, both of which were substituted). Due to the high percentage of *nirK* data points that were substituted, *nirK* copies were included in analysis only when summed with *nirS*, to represent the total nitrite reductase activity (See Appendix A for aggregated data).

In the non-normalized qPCR data, gene copy numbers showed consistent patterns with respect to habitat (Figure 7). Roots universally exhibited the highest copies for each gene tested, with little difference in copy number between carbon loads. The particulate and gravel fractions exhibited lower copy numbers than roots, and showed an increase in copy number at the carbon-positive load compared to the carbon-negative load. When a group of the same habitat and carbon load was compared across two genes (*e.g.*, carbon-positive roots 16S rDNA copy number versus carbon-positive roots *nirS* copy number), gene copy numbers for functional genes were an order of magnitude lower than for 16S rDNA, whereas *nosZ* and *nirS+K* were of the same magnitude. *NirS* was found on average to be five times more abundant than *nirK* in the samples tested, explaining the similarity between the *nirS* and *nirS+K* boxplots. No statistical models compared these data across habitat or carbon load in the complete, non-normalized data set presented in Figure 7.

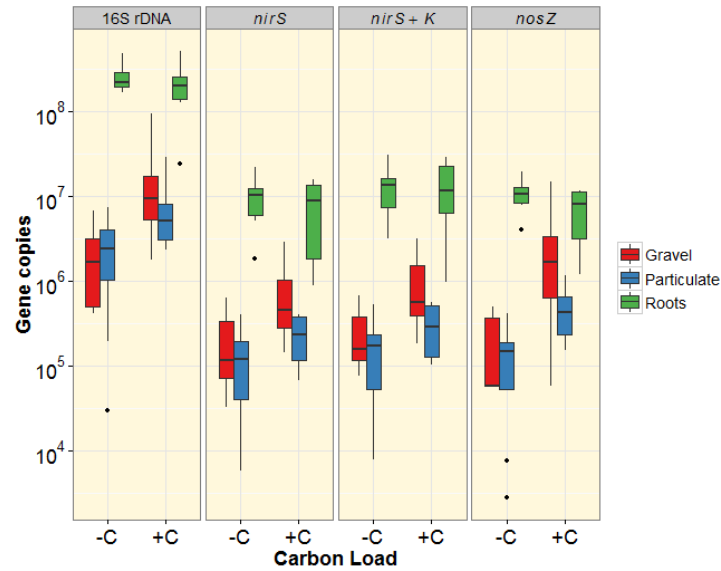


Figure 7: Gene copy numbers split by habitat and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample or copies mL^{-1} sample.

Normalized copy numbers presented a different perspective on the trends just described (Figure 8). The relative gene abundance among denitrification genes was highest among gravel samples and lowest among root samples. The trend of comparable quantity in the non-normalized data for *nirS+K* and *nosZ* was extended through the normalized data set as well. Gene abundance ranged from 1.5 to 30.9% *nirS+K* and 1.2 to 35.5% *nosZ* (Figure 8). There was much less difference between habitats and between carbon loads when 16S rDNA-normalized gene copy numbers were compared to non-normalized gene copy numbers. Relative to the number of bacteria (using 16S rDNA copy number as a surrogate measurement), habitat and carbon load did not have a large effect on the number of denitrifier gene copies. Statistical models and further analysis of these trends are presented with their respective hypotheses.

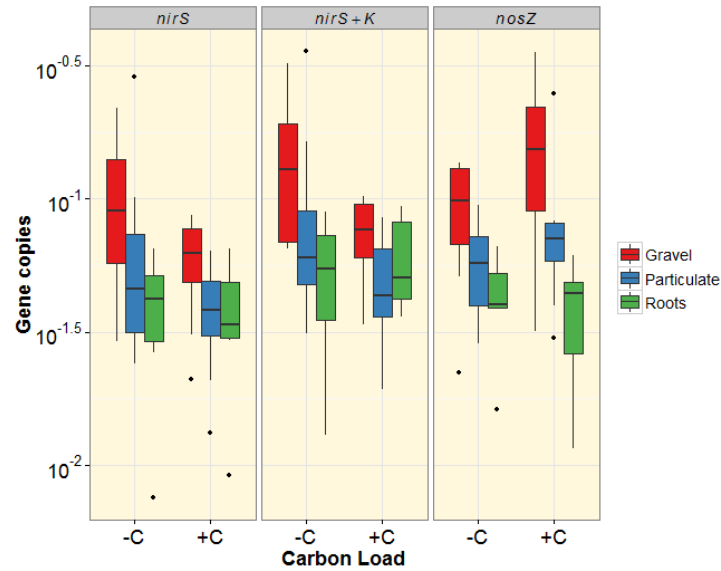


Figure 8: Gene copy numbers normalized by 16S rDNA and split by habitat and carbon load. This visualization of the data does not represent a statistical model. Units are in copies/16S rDNA copies⁻¹.

Quantification of Gas Denitrification Activity

The quantity of denitrification capable by the TW columns was assessed via gas chromatography of three independent incubation assays (N_2O production, N_2O consumption, N_2O emission). Approximately 17% of all gas values were extrapolated below the standard curve, including 10 of N_2O produced, 11 of N_2O emitted, and 1 of N_2O consumed (each of 44 total measurements). A slight extrapolation was made for samples above the standard curve, where 1 N_2O consumed sample and 2 N_2O produced samples were capped. Non-normalized data will be presented first, followed by the 16S rDNA-copy-normalized data.

Non-normalized gas data showed similar trends to the gene data across habitat (Figure 9). Roots exhibited the highest values for every assay, and remained unaffected

by differences in carbon load. Particulate and gravel samples had higher values in the carbon-positive systems than carbon-negative systems and their values remained below those of the root fraction (Figure 9). For a given gas assay sample group of the same habitat and carbon load, the uninhibited N_2O emission exhibited the lowest values and was greatly exceeded by N_2O consumption values, with the total potential N_2O production values in between the other two. N_2O emission was expected to be the lowest-valued term, as it measured a byproduct of the uninhibited denitrification process, and therefore included both N_2O production and consumption terms. The amount of N_2O consumed was on average an order of magnitude greater than the amount of total potential N_2O produced, with only 3 samples producing more N_2O than consuming it and 2 samples going above a 90:1 ratio of consumption to production. The N_2O emission terms were larger than N_2O production subtracted from N_2O production due mainly to concentration-dependent kinetics in the N_2O emission assay, a low estimate of N_2O production from the acetylene-inhibition assay, and a potential over-estimate of the N_2O consumed measurement because of the high amount of N_2O added to that assay. No statistical models were used to compare across the entire data set as presented in Figure 9.

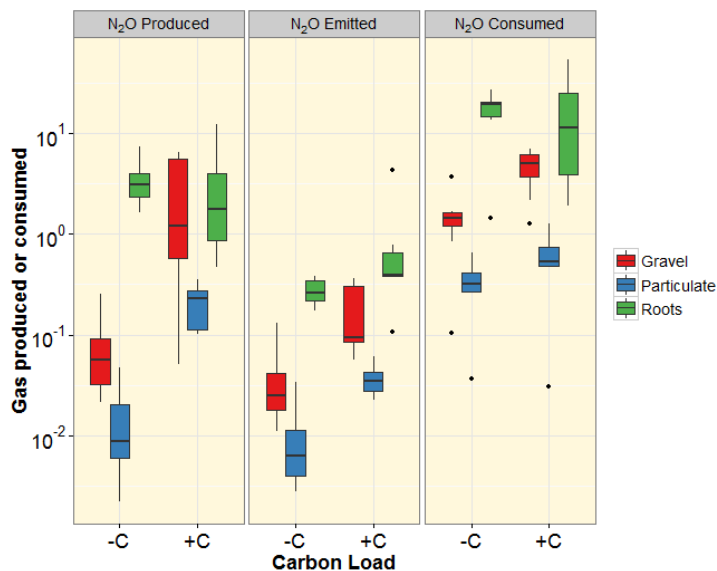


Figure 9: Gas assay data split by habitat and carbon load. Roots have the highest values, whereas particulate has the lowest. N_2O consumption has higher values than N_2O production. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1} \text{sample}$ or $\text{mg hr}^{-1} \text{mL}^{-1} \text{sample}$.

After normalizing by 16S rDNA copies, gas trends were similar to normalized gene trends (Figure 10). The gravel fraction exhibited higher values in each gas assay than roots and particulates relative to the microbial abundance, suggesting the gravel fraction was slightly enriched for denitrifiers. Roots appeared to have the least 16S rDNA-normalized denitrification enzyme activity as measured by each gas assay (excepting the carbon-negative particulate fraction in N_2O production). In contrast to the normalized gene copy number data, the normalized gas data maintained more heterogeneity between habitats and assay type. However, differences between the two carbon loads were mostly eliminated. Results of the statistical analysis of these trends are presented with their respective hypotheses.

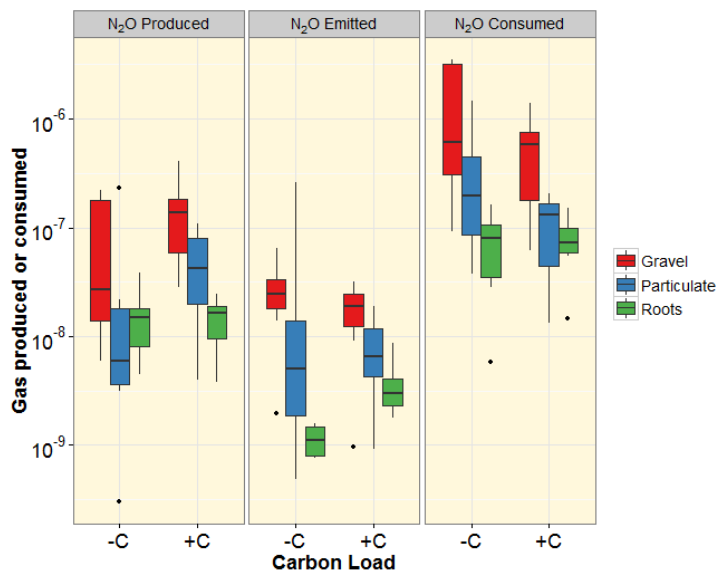


Figure 10: Gas assay data normalized by 16S rDNA and split by habitat and carbon load. Habitats are closer together than in the non-normalized data, with gravel exhibiting the highest gas values per 16S rDNA copy number. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{ 16S rDNA copies}^{-1}$.

Overview of Hypotheses

Three main hypotheses drove this study: (1) Carbon load affects denitrification in particulate and gravel fractions more than in root fractions, (2) plant species affects denitrification activity, and (3) ratios between gas assays and gene copy numbers are correlated. That is not to imply these hypotheses were the only relevant aspects of the system, but that they were the main focal points of the study. Further exploration of the statistical modeling also revealed aspects of the system that were not explicitly or entirely covered under the hypotheses. These are also presented.

Carbon Load Influences Denitrification Primarily in Non-Planted Columns

The effects of carbon load were explored through the three habitats and three plant species plus the non-planted columns used in this study. Denitrification activity and gene abundance in root samples were minimally affected by carbon load under all circumstances, whereas the impact of carbon load on denitrification activity and gene abundance of gravel and particulates varied from minimal to large, depending on other system parameters. Carbon load heavily affected samples from non-planted columns, as seen in data not normalized to microbial abundance (Figure 11 through Figure 14). Carbon load had little effect on data normalized to microbial abundance. Planted systems did not see the large differences in denitrification activity due to carbon load, so there were relatively few cases where denitrification in a plant species was affected by carbon load. Figure 11 through Figure 16 demonstrate the influence of carbon load on planted and non-planted columns. Although there were no non-planted columns with roots, Figure 15 and Figure 16 demonstrate the lack of effect carbon load had on the root habitat across the planted samples with and without added carbon. Each habitat is presented in succession in the following sections, as separated first by plant presence and carbon load, and later as separated by plant species and carbon load.

Effects of Carbon Load and Plant Presence on Particulates

In particulate samples, gene copy number trends were consistent, but gas assay trends were not (Figure 11 and Figure 12). The samples with the lowest gene copy numbers were consistently carbon-negative non-planted, whereas all other samples were

grouped together. The 16S rDNA gene copies were an order of magnitude larger than the functional gene copies. Gas assays showed two different trends, where the matching carbon load terms were clustered together in N₂O produced and N₂O emitted, but the planted and non-planted samples cluster together in the N₂O consumed. N₂O production and N₂O emission were therefore shown to be more affected by carbon load, whereas N₂O consumption was more affected by plant presence. Visual inspection of Figure 11 and Figure 12 suggest planted carbon-negative samples had a much higher genetic capacity of N₂O production (*nirS*+*K* copy number), but lacked the higher values of N₂O production or emission which would have shown a correlation to higher denitrification gas activity. Statistical models support these observations, as every measured value except N₂O consumed was deemed significantly larger in the carbon-positive than carbon-negative loads for particulate measurements ($p \leq 0.018$, Tukey's).

Further analysis of the system examined the carbon load and plant presence interaction term, revealing carbon-positive non-planted samples had significantly higher gene copies than carbon-negative non-planted samples for all genes tested ($p \leq 0.004$, Tukey's). The carbon-negative planted samples have significantly higher gene copies than the carbon-negative non-planted samples for all genes tested ($p \leq 0.001$, Tukey's). This suggests carbon-negative non-planted columns have significantly fewer denitrifiers than their carbon-positive non-planted column counterparts. Even though *nosZ* copy abundance was deemed in higher statistical abundance in carbon-positive planted samples compared to carbon-negative planted samples ($p = 0.039$, Tukey's), this was not deemed practically significant as the differences between carbon load were similar to differences

seen in the *nir* and 16S rDNA gene copies, which were not statistically significant ($p \geq 0.353$, Tukey's). No model showed samples from carbon-positive non-planted columns were statistically different from carbon-positive planted samples ($p \geq 0.200$), suggesting non-planted systems have the ability to denitrify at a similar level as planted systems when extra carbon was provided.

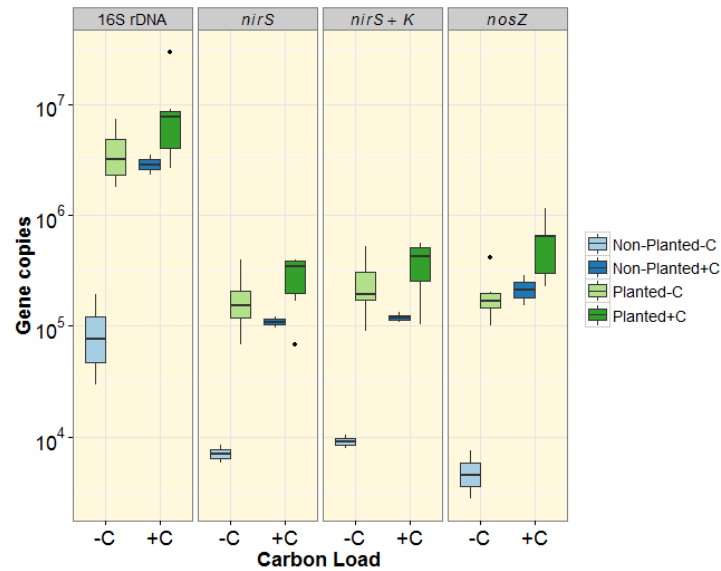


Figure 11: Particulate gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies mL^{-1} sample.

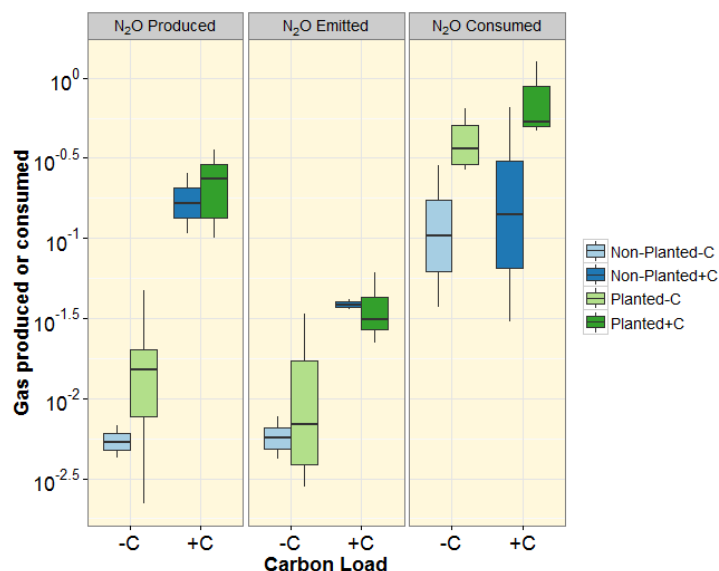


Figure 12: Particulate gas assay data split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.

Effects of Carbon Load and Plant Presence on Gravel

Gravel samples showed inconsistent patterns between the gene copy and gas assay data (Figure 13 and Figure 14). To some extent, carbon load does affect each tested gravel measurement in both gas and gene assays. Across the all measurements, values of gravel from planted columns seem to cluster in the middle of gravel values from non-planted columns, where the carbon-negative non-planted gravel was at the lowest value and the carbon-positive non-planted gravel was at the highest. Models confirm that the activity from all measurements in carbon-positive samples was significantly higher than activity in carbon-negative samples ($p \leq 0.016$, Tukey's). Consistently, the carbon-positive non-planted column gravel outperforms carbon-positive planted and carbon-negative non-planted column gravel for all gas and gene measurements obtained. Models

have confirmed this as significant only for the gene copies ($p \leq 0.003$, Tukey's), and not any gas assays.

Where the particulate samples were shown to differ among carbon-negative non-planted and planted samples, gravel samples were only shown to differ in the carbon-negative non-planted and planted samples for *nirS* copies. There was no case where carbon-positive planted gravel samples were statistically different from carbon-negative planted gravel samples ($p \geq 0.180$). Gravel non-planted samples were particularly strongly affected by changes in carbon load. Gravel carbon-positive non-planted samples were suggested by the data to have the highest number of denitrifiers, as well as the highest denitrification gas activity. Gravel in the carbon-negative non-planted columns seemed to have limited ability to harbor denitrifiers and to perform the denitrification process.

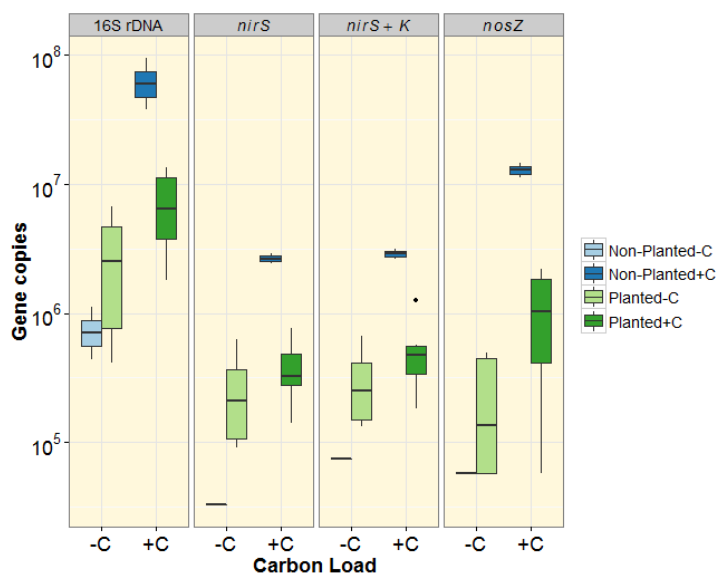


Figure 13: Gravel gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.

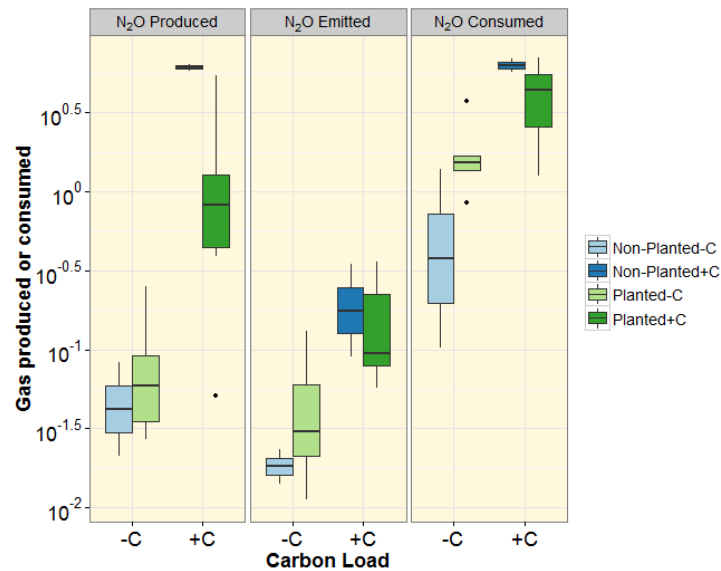


Figure 14: Gravel gas assay data split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.

Effects of Carbon Load and Plant Presence on Roots

Root models did not show any statistically-significant differences between carbon loads (Figure 15 and Figure 16, $p \geq 0.690$, F -test). By definition, the roots were from a planted system, particularly from the area most capable of dynamic interaction with microbes *in vivo*. The roots were therefore not expected to differ in denitrification activity due to carbon load, because they could provide carbon in the rhizosphere. Contrast this result to the particulate and the gravel analysis, where non-planted systems were most affected by changes in carbon load.

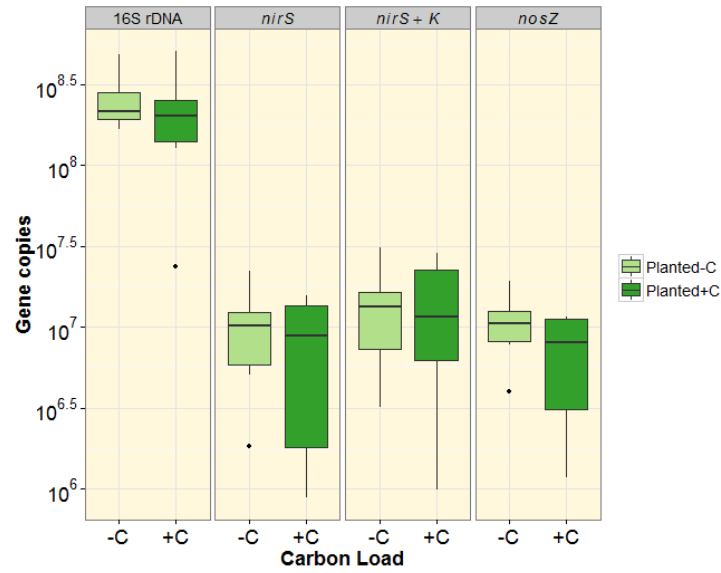


Figure 15: Root gene copy numbers split by plant presence and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.

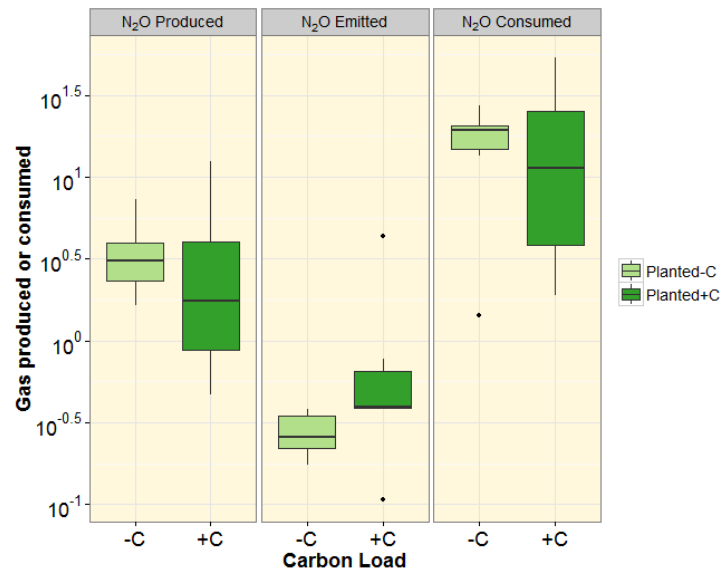


Figure 16: Root gas assay data split by carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.

Carbon Load Effects on Plant Species

In the interest of being thorough in analysis, and to further develop the picture of carbon load's influence, the data will be briefly readdressed with each plant species (*Carex*, *Phragmites*, *Schoenoplectus*, plus non-planted) examined individually. Figure 17 through Figure 22 show the same data as Figure 11 through Figure 16, but the planted group was split into its respective species for visualization and statistical analysis. Many of the patterns remained the same as seen above, with non-planted columns exhibiting the most sensitivity to carbon load, although in some cases a species did differ in its denitrification activity due to carbon load. Hypothesis 1 tested the effect of carbon load on planted and non-planted samples, which included comparisons across some individual plant species.

Effects of Carbon Load and Plant Species on Particulates

Carbon load differences in denitrification genes for TW particulates were largely based on plant presence (Figure 17 and Figure 18). Carbon load effects on particulate from columns were seen primarily in the gene copy number data. Carbon load as a single factor was only significant in two measurements (N₂O produced and 16S rDNA copy number) ($p \leq 0.026$, Tukey's). However, it was the carbon load and species interaction term that showed significance in all functional genes as well as N₂O produced and consumed ($p \leq 0.043$, *F*-test), where the carbon-positive non-planted samples have higher copies than carbon-negative non-planted samples for each of the terms listed ($p \leq 0.028$, Tukey's). As for carbon effects on species, measurements of N₂O produced and N₂O emitted were significantly affected by carbon load in *Phragmites* (N₂O produced and

N_2O emitted) and *Schoenoplectus* (N_2O produced) ($p \leq 0.022$, Tukey's). These trends suggest the denitrifying community in TW columns planted with *Phragmites* was more sensitive to carbon load than the other plant species tested. For carbon-negative systems, gene copies in the particulate habitat were greatly affected by the presence of plants, but for carbon-positive systems, plant presence was less important in terms of denitrification activity.

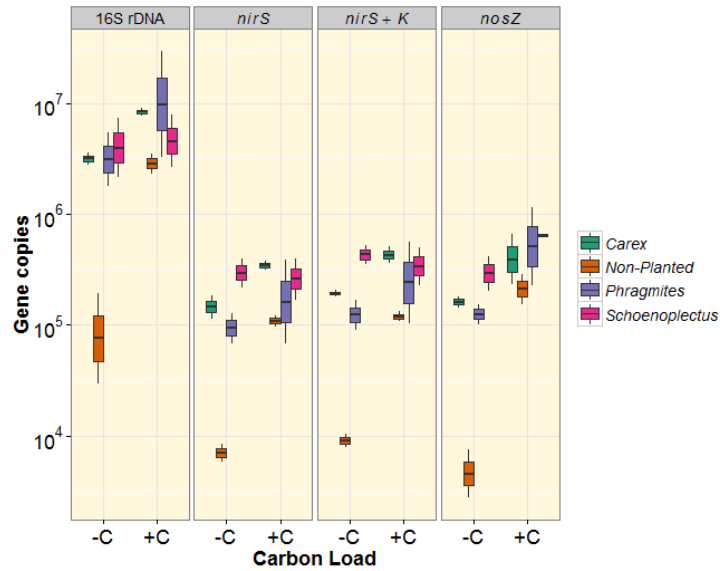


Figure 17: Particulate gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies mL^{-1} sample.

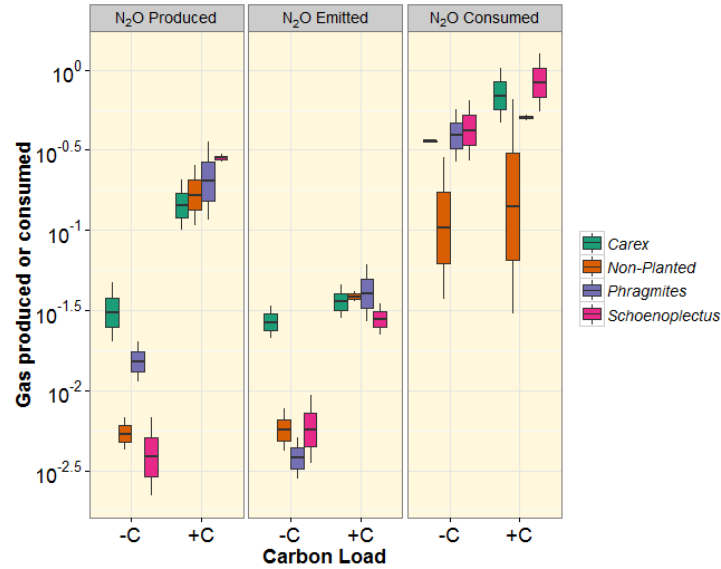


Figure 18: Particulate gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in mg hr⁻¹ mL⁻¹ sample.

Effects of Carbon Load and Plant Species on Gravel

Although carbon load was important for gravel samples when comparing non-planted to planted samples, carbon load had little effect on gas and gene assays when comparing between gravel in various plant species systems (Figure 19 and Figure 20). Due partially to the large amount of heterogeneity in gravel samples between plant species, statistical models showed fewer significant differences between non-planted and planted columns, particularly in the gas measurements, than was previously seen when plant species were grouped together. Across all genes and the N₂O emitted term, carbon-positive non-planted samples were statistically more active than carbon-negative non-planted samples ($p \leq 0.027$, Tukey's). In two cases, N₂O emitted and 16S rDNA copies, microbes in the *Phragmites* column were significantly more active with added carbon than without ($p \leq 0.011$, Tukey's). Although this trend was only shown to be statistically

significant in two measurements, it suggests denitrification activity in *Phragmites* was more sensitive to carbon load effects than for the other plant species, which was also observed in the particulates fraction.

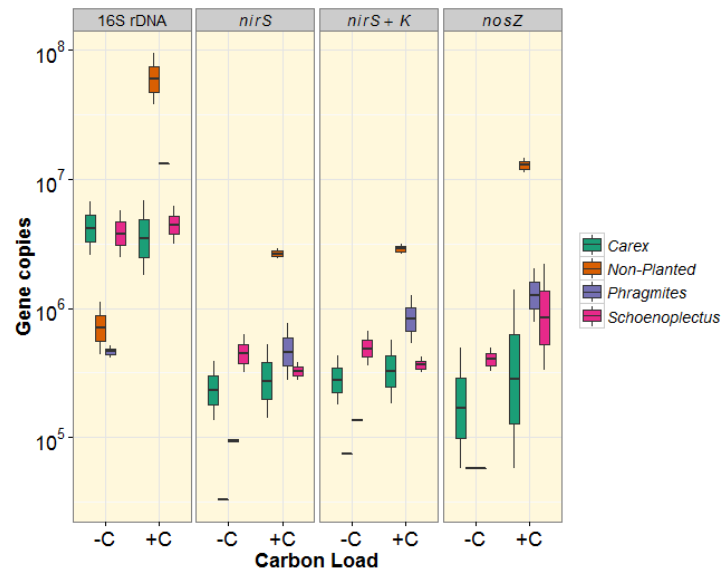


Figure 19: Gravel gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.

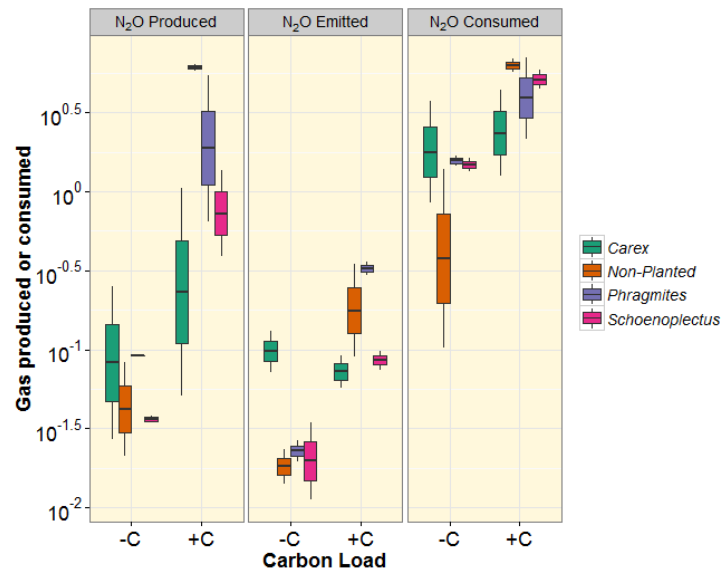


Figure 20: Gravel gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.

Effects of Carbon Load and Plant Species on Roots

The root fraction did not show large differences between carbon loads, even when separated into individual species, which was confirmed by models showing no significance between gas values or gene copy numbers for any of the factors tested (Figure 21 and Figure 22, $p \geq 0.690$, F -test). Variance between species in the root fraction was much greater for carbon-positive samples, implying that roots showed the most heterogeneity in denitrification activity when there was more carbon available. The dynamic interaction between roots and microbes provided the ability to remain statistically unaffected by carbon load.

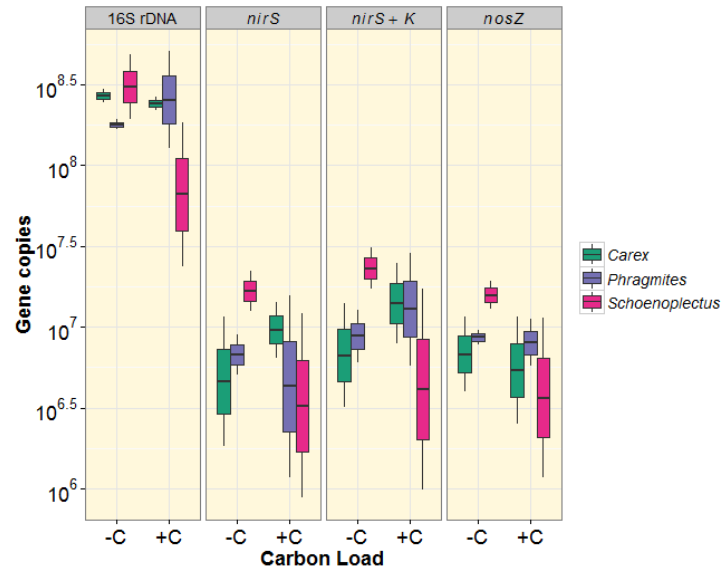


Figure 21: Root gene copy numbers split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in copies g^{-1} sample.

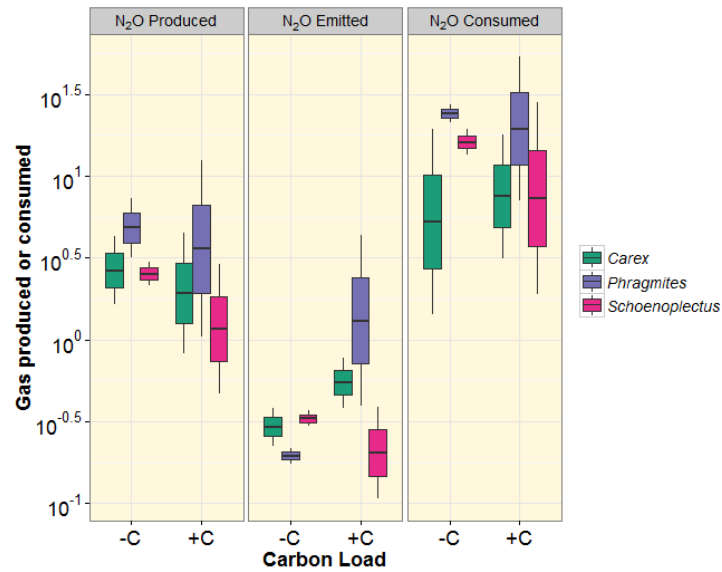


Figure 22: Root gas assay data split by species and carbon load. This visualization of the data does not represent a statistical model. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample.

Effects of Carbon Load and Plant Species on 16S rDNA-Normalized Data

Differences in denitrification activity between carbon loads among samples normalized to 16S rDNA microbial abundance were practically negligible (Figure 8, Figure 10). There was one instance across the planted data set where, in the normalized N₂O produced measurement, carbon-positive gravel was shown to be more active than the other carbon-positive habitats, and particulate carbon-negative was shown to be less active than the other carbon-negative fractions ($p \leq 0.025$, Tukey's). This result was not found for any other measurements in the non-root or the planted data sets, and signifies that the normalized data sets showed a different aspect of analysis from the non-normalized data sets. Denitrification activity was similar across carbon loads when normalized to the relative number of bacteria present, suggesting denitrification activity was limited by available carbon.

Summary of Carbon Load Effects

This section has shown how carbon load affects denitrification activity. Non-planted fractions in both gravel and particulate were affected particularly strongly, whereas planted columns were less affected and there were very few differences among carbon loads for samples that came from different plant species. Coupled with the results that root fractions never varied by carbon load, this suggests the roots of a system had a stabilizing effect for denitrification activity, even on samples from other habitats. The parameter of carbon load most heavily impacted the particulate and gravel fractions, accounting for heavy support of hypothesis 1.

The Role of Habitat

Although the entirety of habitat (roots, gravel, particulates) effects was not explicitly covered by hypothesis 1, it is worth noting the effects habitat played in denitrification activity and gene abundance (Figure 8, Figure 10). Habitat was the only statistically significant effect in the normalized data set (4 of 6 measurements, $p \leq 0.073$, F -test), except one case where carbon load interacts with habitat, making it the most important factor for normalized data. In N_2O emitted and N_2O consumed normalized measurements, each habitat was shown by models to be a statistically distinct group ($p \leq 0.021$, Tukey's). In measurements of N_2O emitted, N_2O consumed, *nirS*, and *nirS+K* gene copy number, roots and gravel were found to be significantly different from each other, with gravel having the most denitrification gene abundance and activity relative to 16S rDNA microbial abundance in comparison to roots and particulates ($p \leq 0.001$, Tukey's). The N_2O produced measurement tied the habitat significance to carbon load, because the particulate carbon-negative and the gravel carbon-positive showed higher and lower activity for N_2O produced, respectively, than the other habitats within the same carbon load. As confirmed by statistical models (described above), in some cases gravel samples have a significantly higher ratio of denitrifier relative abundance compared to the root and particulate fractions. Although habitat was a large factor in non-normalized data as well (Figure 7, Figure 9), some of those effects were due to differences in separation methods between habitats, so comparisons between habitats in non-normalized data have to be made with care.

Plant Species Effects Do Not Play a Large Role in Denitrification Activity

Plant species did not have a large impact on denitrification activity. The effects of species were found to be less important in comparison to plant presence or absence. There was a minimal, although inconsistent, species effect on denitrification activity of particulates and gravel, and no effect on roots (Figure 17 through Figure 22). The activity of the particulate fraction was more clearly divided between non-planted and planted samples. Normalized data showed no significant effects by differences in plant species. To fully address this hypothesis, some carbon load effects are necessarily considered as they relate to species effects, although the majority of them were covered in the previous section. Furthermore, some species effects that have been covered in terms of carbon load are re-addressed in terms of species, including an analysis of the non-planted columns.

Effects of Plant Species on Particulates

The effects of plant species on the particulate habitat are considered. Non-planted carbon-negative columns were clearly less abundant in gene copies from each species in carbon-negative gene copy data (Figure 17). All species and the non-planted group were of comparable gene abundance in the carbon-positive copy data. In the gas data, *Carex* exhibited a comparable amount of N₂O emission at both carbon loads (Figure 18). All other plant species showed lower emission in carbon-negative systems, which led to *Carex* showing statistically higher N₂O emission compared to all other species ($p \leq 0.031$, Tukey's). The only other statistically significant species effect in particulates from planted samples was that N₂O produced measurements in *Schoenoplectus* samples were

more affected by carbon load than in *Carex* samples ($p = 0.026$, Tukey's). As this trend was not seen elsewhere, it was deemed practically insignificant.

All other significance was related to non-planted columns. Each carbon-negative planted species particulate fraction had significantly higher gene copy abundance than all particulate carbon-negative non-planted samples, but no statistical differences were seen between the carbon-positive non-planted samples and any carbon-positive plant species samples ($p \geq 0.323$, Tukey's). This suggests that carbon-negative particulates had a different profile of activity dependent on plant presence, but had similar activity among the species present in their respective TW system. The exception to this was *Carex*, which was shown in some gas assays to exhibit similar N_2O emission and N_2O production across carbon loads, whereas other species exhibited more distinction between carbon loads. As previously described, when plant species were grouped together, all gene copies were shown to differ significantly in carbon-negative planted compared to carbon-negative non-planted samples, but the difference was not seen in any carbon-positive planted samples compared to non-planted. Again, the trends suggest particulates for carbon-negative systems were distinct between plant presence and absence, but in carbon-positive systems, the effects of plant presence on denitrification activity were minimized. In context, because particulates in planted systems were primarily plant material, these results are reasonable.

Effects of Plant Species on Gravel

The activity of gravel exhibited slightly different statistical results from the particulates in that it was also affected greatly by plant presence, but the distinction

between individual plant species was not as clear (Figure 19 and Figure 20). Among the species-specific models, only 16S rDNA and *nosZ* copy number, as well as N₂O produced measurements were shown to have differences between planted species ($p \leq 0.031$, Tukey's). Gene copy numbers in *Phragmites* samples were more affected by carbon load than *Carex* and *Schoenoplectus*, in that the *Phragmites* carbon-negative samples were lower and its carbon-positive samples were higher than the other two species (Figure 19). However, the N₂O produced in *Phragmites* differs minimally in carbon load compared to other species, so this statistical distinction was viewed as more of an artifact of heterogeneous variance across species samples than it was about producing more or less N₂O.

Every other statistically significant term involves the non-planted column gravel, so the differences in non-planted gravel compared to species-specific column gravel will now be considered. Similarly to the previous models with plant presence, gene copy number data suggest the carbon-negative non-planted gravel had lower copy numbers than carbon-negative planted gravel (for *nirS*), and the carbon-positive non-planted samples have higher copy numbers than carbon-positive planted gravel ($p \leq 0.049$, Tukey's). However, the models did not support these carbon load differences in gene copy number as strongly as when comparing between planted and non-planted column gravel, and the non-planted was only shown to differ in some cases of *nirS*, *nirS*+*K*, and 16S rDNA, but not all cases, from *Schoenoplectus* or *Carex*. The gas assay measurements had variances much too high to distinguish between species effects. Gravel non-planted samples, particularly the gene copy number data, were greatly affected by carbon load,

but these large changes in activity were not always shown to be statistically distinct from gravel samples taken from wetland systems including plant species. For the grouped species models, *nirS* gene copies differed from carbon-negative planted to carbon-negative non-planted samples, and all gene copies differed from carbon-positive planted to carbon-positive non-planted (described above). The trends of gravel denitrification activity relative to species expand upon the trends described previously with gravel, namely that carbon load interacted with the gravel non-planted samples strongly, but this did not always correspond to a statistical significance between planted and non-planted denitrification activity.

Summary of Plant Species Effects

Plant presence and absence was a much more important factor affecting the denitrification activity than was the specific species of plant among planted samples. The N_2O consumption term was least affected by plant species. The data have shown that non-planted particulate gene copy numbers had more distinct plant species profiles than non-planted gravel, even though gravel seemed to be affected more strongly by plant presence or absence in terms of carbon load effects. This was partially due to the high amount of variance among gravel replicate samples, which lowered the ability of statistics to differentiate two groups as significantly distinct, and partially due to the particulate samples including fine roots when they were from planted samples, expanding the distinction between planted and non-planted samples. Microbes in *Carex* columns were shown to emit N_2O at a particularly high value in the carbon-negative particulate samples. Microbes in *Phragmites* were shown to differ from those in *Schoenoplectus* and

Carex in some cases, which was partially attributable to the low variance among *Phragmites* samples, and partially attributable to the denitrification capability of *Phragmites* being more strongly affected by carbon load than the denitrification capability of other species.

Denitrification Gene Copy Numbers Correlate to Gas Assays

Generally, gene copy numbers correlated to gas assays. As the tested interactions become more complex, the gene-to-gas correlations were not necessarily upheld. In a basic sense, N₂O production should be related to *nir* genes, and N₂O consumption to *nos* genes. Each model presented was first considered independently before general assertions were made.

Models testing the one-term factors were significantly correlated. The values of N₂O produced were tested by the effect of *nirS+K* copy number. One normalized and one non-normalized model were run, both showing similar positive correlations ($p \leq 0.001$, *F*-test). The same was true of N₂O consumed, which was tested against *nosZ* copy number (again, $p \leq 0.001$, *F*-test). These four models are represented by Figure 23 through Figure 26. These models confirmed that a positive correlation existed between the gene copy number and its expected production or consumption of N₂O in both normalized and non-normalized data.

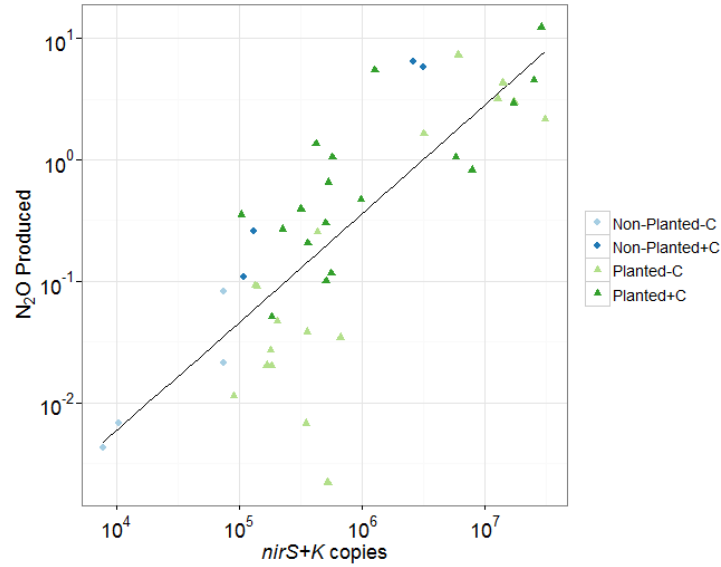


Figure 23: Correlation of N_2O produced to the *nir* genes, which contribute to N_2O production. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample; gene units are in copies g^{-1} sample or copies mL^{-1} sample.

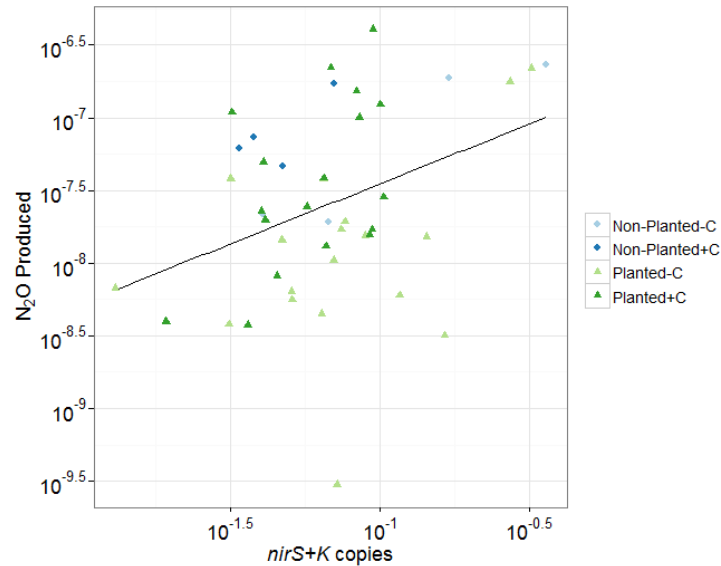


Figure 24: 16S rDNA-normalized correlation of N_2O produced to the *nir* genes, which contribute to N_2O production. This visualization of the data does not represent a

statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} 16\text{S rDNA copies}^{-1}$; gene units are in $\text{copies } 16\text{S rDNA copies}^{-1}$.

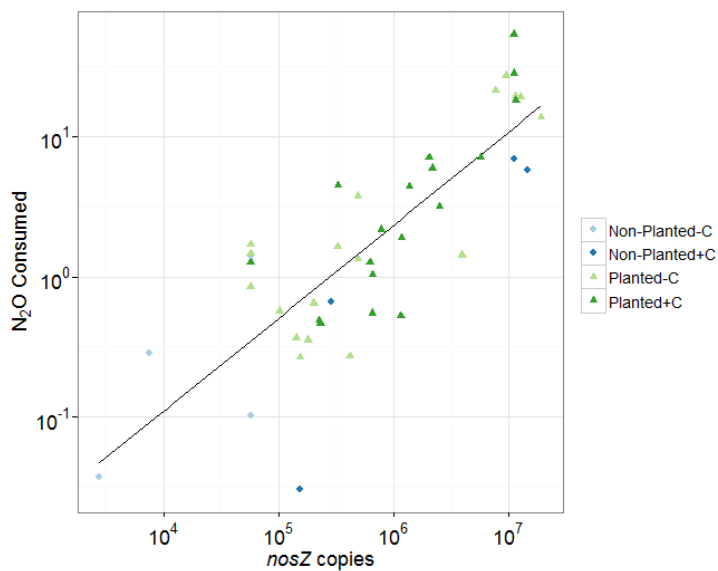


Figure 25: Correlation of N₂O consumed to the *nosZ* gene, which contributes to N₂O consumption. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1} \text{sample}$ or $\text{mg hr}^{-1} \text{mL}^{-1} \text{sample}$; gene units are in $\text{copies g}^{-1} \text{sample}$ or $\text{copies mL}^{-1} \text{sample}$.

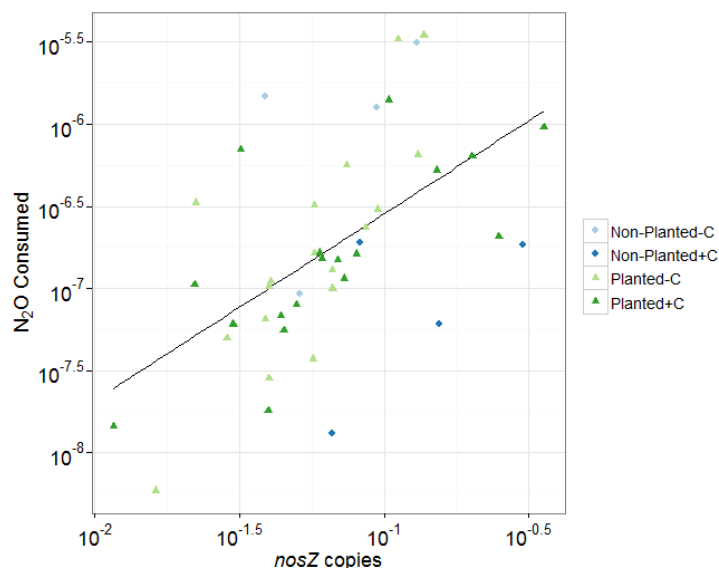


Figure 26: 16S rDNA-normalized correlation of N_2O consumed to the *nosZ* gene, which contributes to N_2O consumption. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in mg hr^{-1} 16S rDNA copies $^{-1}$; gene units are in copies 16S rDNA copies $^{-1}$.

Combining the previous models, the correlation between the ratios of N_2O production/consumption to *nir/nos* was weaker, but still showed significance ($p \leq 0.020$, *F*-test, Figure 27). Keep in mind that a ratio of two measurements was equivalent mathematically to both ratios being normalized, since the 16S rDNA gene copy number was the same for both measurements. This combined correlation exhibited a negative relationship, suggesting a high *nirS+K* copy number was correlated to more N_2O consumption. This result does not fit with the expected hypothesis. All single-effect correlations were positive, suggesting that a higher number of gene copies was associated with a higher value of its corresponding N_2O production or consumption, but the combined correlation was negative suggesting a higher concentration of *nirS+K* was associated with a lower value of N_2O produced. Conceptually, this was not an intuitive

relationship. The N_2O consumption term dominated the y-axis ratio, and the *nir/nos* did not have a dominant term since they were of the same order of magnitude, which could contribute to the unintuitive negative correlation. This suggests that at a fundamental level, a one-factor gas and gene copy number correlation was strong, but an increase in the complexity of the correlation was linked with an increase in complexity of the included variables to other factors within the system.

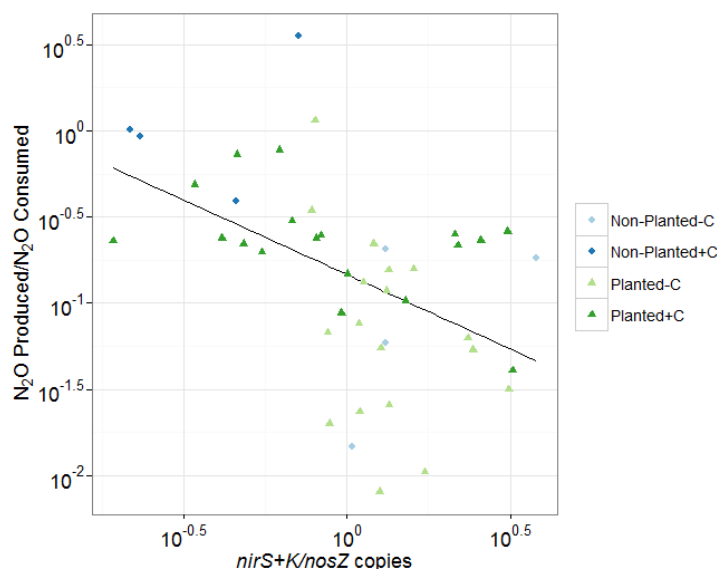


Figure 27: Correlation of N_2O produced/consumed to *nir/nos* gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample; gene units are in copies g^{-1} sample or copies mL^{-1} sample.

N_2O emission Correlates to Other Measurements

After supporting the claim that *nir* gene copies correlated to N_2O produced, and *nos* gene copies correlated to N_2O consumed, it stands to check what measurements could be correlated with N_2O emitted. This emission term was important for the purposes of minimizing greenhouse gas emissions from the wetland system. Figure 28 through Figure

31 represent models which correlated the non-normalized and normalized N₂O emission term to the other measurements obtained. Models confirmed N₂O emission was positively correlated with all single measurement factors (16S rDNA, N₂O produced, N₂O consumed, *nirS*+*K*, *nosZ*), whether using normalized or non-normalized data ($p \leq 0.010$, *F*-test). Higher values of all other terms, including *nosZ* and N₂O consumed, correlated to N₂O emission. When the single measurements were combined, such as N₂O emitted by *nir/nos* (Figure 28), the combined error due to the ratio was greater than the error of single factors, and the ratios were not correlated ($p \geq 0.366$, *F*-test) except in the case of non-normalized N₂O emitted by N₂O produced/consumed ($p = 0.001$, *F*-test, Figure 29). Figure 28 and Figure 30 show the poor correlation between *nir/nos* gene copies and N₂O emission ($p = 0.835$, *F*-test). Figure 29 and Figure 31 correlated N₂O production/consumption to N₂O emission. Only the non-normalized N₂O emission was significant ($p = 0.001$, *F*-test). Each measurement associated with N₂O emission had some associated error. The error associated with correlating a ratio (such as *nir/nos*) to N₂O emission was greater than the error of correlating just one factor (such as only *nir*) to N₂O emission. This was again believed to be due to more complex interactions in the system; simple correlations were much stronger than complex correlations.

The ratio correlations make sense mathematically, despite the unintuitive result. Because both *nirS*+*K* and *nosZ* were positively correlated with N₂O emission (with similar slopes, ~ 0.6), and the genes were of the same order of magnitude in abundance, correlating the ratio between them with N₂O emission removes the positive correlation.

The same can be said of the normalized N_2O emission correlation to ratios of $\text{nirS}+\text{K}/\text{nosZ}$ and N_2O production/consumption.

Gas and genetic components of the system did correlate positively with one another as single factors, but the ratios between them were not well-understood by data reported herein. N_2O emission was positively correlated with all single-factor measurements (16S rDNA, N_2O produced, N_2O consumed, $\text{nirS}+\text{K}$, nosZ), associating more N_2O production and genetic production potential as well as more N_2O consumption and genetic consumption potential with higher releases of N_2O . Hypothesis 3 has been supported in a basic sense, but not to the level originally expected.

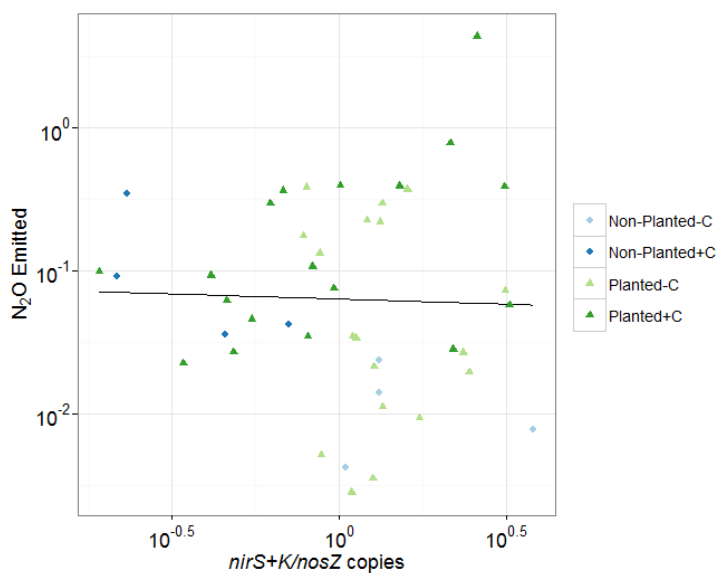


Figure 28: Correlation of N_2O emitted to nir/nos gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample; gene units are in copies g^{-1} sample or copies mL^{-1} sample.

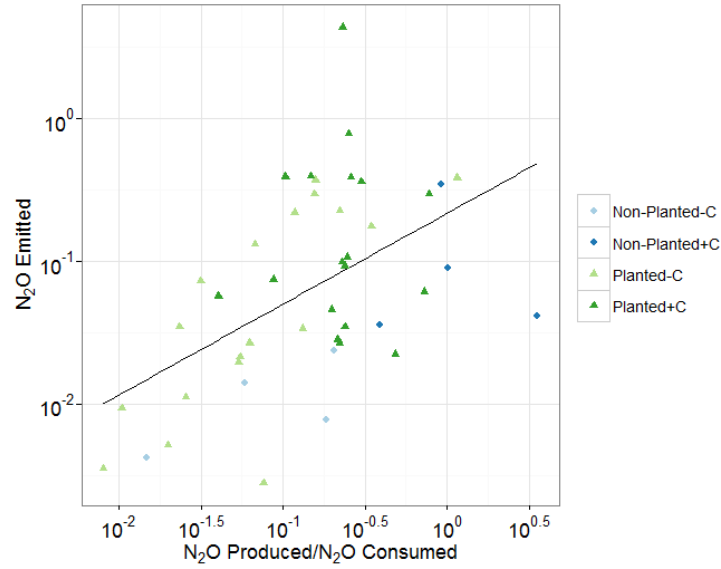


Figure 29: Correlation of N_2O emitted to N_2O produced/consumed. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.

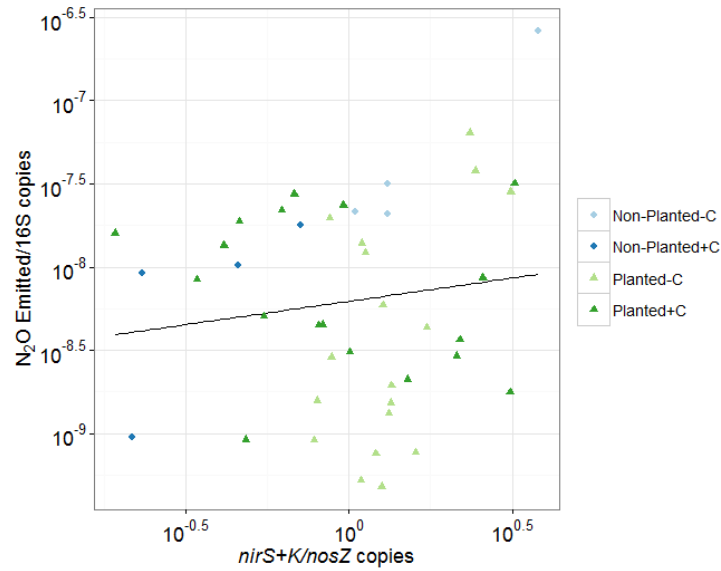


Figure 30: 16S rDNA-normalized correlation of N_2O emitted to *nir/nos* gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} 16\text{S rDNA copies}^{-1}$; gene units are in copies g^{-1} sample or copies mL^{-1} sample.

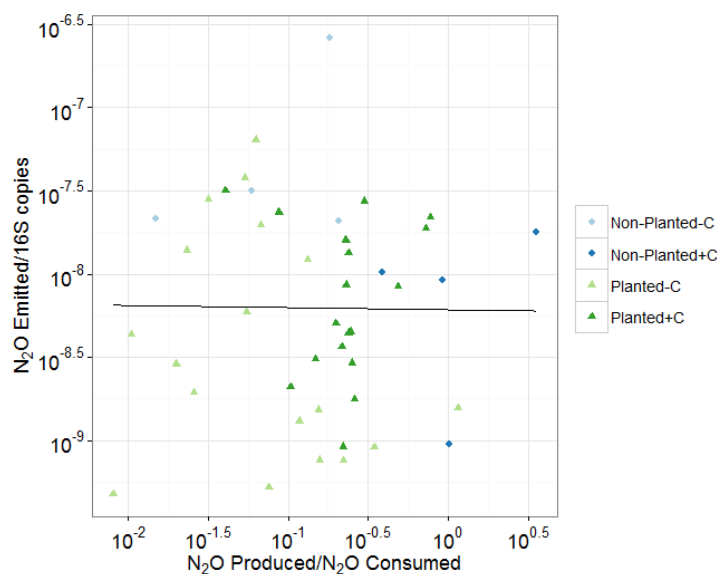


Figure 31: 16S rDNA-normalized correlation of N_2O emitted to N_2O produced/consumed. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Units are in $\text{mg hr}^{-1} 16\text{S rDNA copies}^{-1}$, $\text{mg hr}^{-1} \text{g}^{-1}$ sample, or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.

The Relative Completeness of Denitrification

Improving TW operation and design through reducing the amount of N_2O emitted while maintaining a high level of denitrification activity was a primary focus of this study. The final product in denitrification is N_2 , although N_2O can also be released before it is reduced to N_2 . As such, the ratio of N_2O emitted to N_2O produced was considered. From the measurements used in this study, the N_2O emission/production terms were used to represent the completeness of denitrification, such that the measurement represents the fraction of denitrification completed that was released as N_2O .

Gravel samples from the carbon-positive non-planted systems exhibited the lowest N_2O emitted to N_2O produced ratio. Figure 32 visualizes the ratio of N_2O emitted to N_2O produced a wetland would release by plant presence and habitat. Both the total

amount of denitrification and the quantity of N_2O emissions are important parameters for denitrifying treatment wetlands. Examining the ratio of these quantities gave more valuable insight; a low N_2O emission term on its own could have been due to a sample with very poor denitrification activity. The carbon-positive non-planted gravel samples had the lowest ratio, followed by planted carbon-negative root samples. Except for the carbon-negative root fractions, the remaining carbon-negative particulate and gravel had the highest ratios, suggesting the carbon-positive wetland samples were able to denitrify more material while emitting less N_2O . This topic will be revisited in more detail in the discussion.

An estimate of how well the ratio of total N_2O emissions correlate to genetic production/consumption capacity was created by correlating N_2O emitted/produced to *nir/nos* copy number (Figure 33, $p = 0.001$, F -test). Carbon-negative samples appeared to be just above a 1:1 ratio of *nir* to *nos* gene copies, whereas the carbon-positive samples appeared to have a wide range of ratio values, extending from below 1:1 to above 1:1 *nir:nos*. Although a correlation was visible, there was a large amount of heterogeneity among the samples, which suggests this correlation was not capturing all of the factors involved.

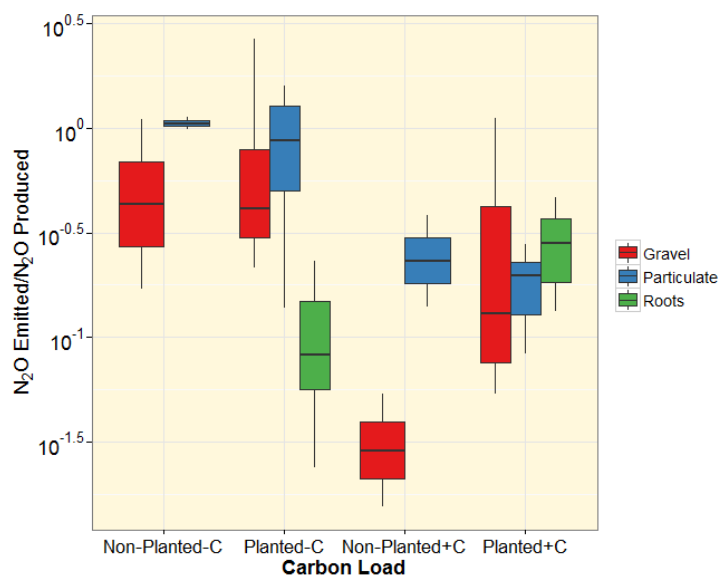


Figure 32: The N_2O emitted/produced ratio, split by habitat, plant presence, and carbon load. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.

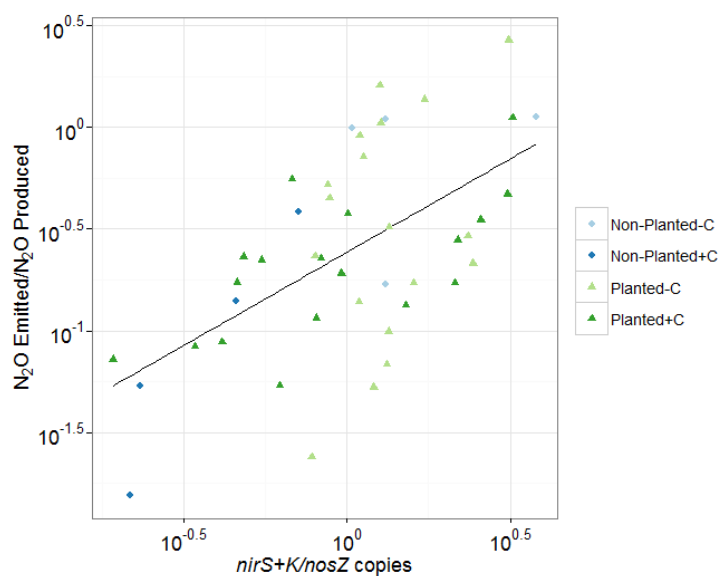


Figure 33: Correlation of N_2O emitted/produced to *nir/nos* gene copies. This visualization of the data does not represent a statistical model. The best-fit line is a regression line based on the data in the figure. Gas units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample; gene units are in copies g^{-1} sample or copies mL^{-1} sample.

Summary

All of the original hypotheses stated for this study have been addressed herein. Carbon load was found to have an insignificant impact on denitrification activity for planted columns. Carbon load was a significant factor for non-planted columns, with carbon-positive samples providing more denitrification activity than carbon-negative samples. Habitat was found to be a significant factor in normalized denitrification activity, with gravel exhibiting the most denitrification activity relative to the amount of microbial abundance. *Phragmites* was shown to be the most different plant species from the others, typically due to having more sensitivity in denitrification processes to carbon loads, although it was still within a comparable range to the others. Models comparing gas to gene data were partially supported. Comparing a single gas to single gene (N_2O production to *nir* and N_2O consumption to *nos*) was entirely supported, but once the ratio of gas values or gene copy numbers were used, results were mixed, suggesting that more simplistic single-factor correlations were stronger than complex correlations to what was likely a more complicated relationship between ratios of gene copies and gas assays. Non-planted carbon-positive gravel was the most efficient at denitrifying with a low quantity of N_2O emitted, but planted columns were the most productive for denitrification.

CHAPTER 4

DISCUSSION

Discussion Overview

In this study, the fundamental processes surrounding nitrogen removal in TWs were assessed by characterizing the effects of plant species, carbon loading rate, and spatial orientation (assessed as habitat) on the microbial community dynamics within a TW. This research will continue to improve TW design and operation by expanding upon knowledge of microbial denitrification dynamics within a wastewater TW for tertiary treatment, which should ultimately lead to an increase in the use of TWs as a means of tertiary wastewater treatment. Use of TWs as a tertiary treatment option for municipal wastewater treatment typically consumes fewer resources than the traditional wastewater treatment corollary (Chen et al., 2006). Understanding the dynamics between wetland nitrogen removal, denitrification, and nitrous oxide emission will help mitigate the greenhouse gas effects caused by nitrous oxide release into the atmosphere. These described areas of focus were selected for analysis in this study because they have been classified as high priority research targets by previous studies (Brisson and Chazarenc, 2009; Faulwetter et al., 2009; Ravishankara et al., 2009; IPCC, 2013).

A model HSSF TW system was used in a controlled environment to isolate the effects of the desired variables and diminish the potential effects from confounding environmental variables that affect field-scale TWs. Research was conducted during the summer at ~24 °C, although the entire wetland system was cycled seasonally. Synthetic

wastewater was batch-fed to sixteen single-plant wetland columns consisting of 4 plant treatments (*Carex*, *Phragmites*, *Schoenoplectus*, non-planted) and 2 carbon loading rates (0 g L^{-1} sucrose, 0.391 g L^{-1} sucrose). Core samples, representing a wetland microcosm, were taken from each TW column; core samples were separated into three distinct habitats (particulates, roots, gravel); each selected habitat representing a unique environment within the wetland: roots possess microbial attachment sites as well as a provide dynamic interactions including supplying root exudates and oxygen; gravel provides microbial attachment sites, but does not supply nutrients; the particulates fraction was a collection comprised primarily of loosely-associated biofilm and dissolved and particulate organics from the other two habitats and was collected as a potentially important location for microbial activity. This experimental design provided the best division between the desired variables of spatial distribution, carbon load, and plant species effects.

Previous TW research at MSU has used model TW systems to address species effects, temperature effects, and microbial effects on diverse contaminant removal. *Schoenoplectus* and *Carex* were selected for this study because of their involvement in reducing chemical oxygen demand (COD) in previous TW studies at MSU, particularly in low temperatures (Allen et al., 2002; Taylor et al., 2011). *Phragmites* planted columns performed only moderately well, but this plant has become somewhat of a model species for wetland studies (Brisson and Chazarenc, 2009). At the heart of plant species effects research is the ability of the roots to interact with microbial communities. Rhizosphere interactions, particularly relating to redox conditions, with microbial communities are

thought to be a major influence on treatment capabilities (Stein and Hook, 2005; Faulwetter et al., 2009; Faulwetter et al., 2013). Fundamental research into microbial communities, their location within a wetland, and their interactions with plants in a TW will provide a better understanding of wetland treatment processes.

Denitrification activity was measured by gas assays (N_2O produced, N_2O emitted, N_2O consumed) and denitrification genetic potential was measured by gene copy numbers (16S rDNA, *nirS*, *nirK*, *nosZ*). Three major hypotheses were tested: (1) Carbon load affects denitrification in particulate and gravel fractions more than in root fractions, (2) plant species affects denitrification activity, and (3) ratios between gas assays and gene copy numbers are correlated. Results demonstrated that carbon load had a large effect on the denitrification activity of gravel and particulates habitats measured by gas emissions and gene copy number, but no effect on the root habitat. Plant species effects were minor in comparison to the larger effects caused by plant presence in a TW; plant presence primarily affected copy number in the root and gravel habitats, and affected gas denitrification activity to a lesser extent. Simple ratios comparing a specific gene abundance with its corresponding gas measurement assay were positively correlated, but more complicated gene and gas relationships were not always correlated intuitively.

Quantification of Denitrification Genes

Four genes were assayed using qPCR to examine the microbial community by assessing abundance of total microbes (16S rDNA) and denitrification genes (*nirS*, *nirK*, *nosZ*). Studies measuring denitrification genes have been done in a variety of environments, including garden systems, marshes, glacier forelands, and many types of

agricultural soils (Hallin et al., 2009; Hammonts et al., 2013; Henry et al., 2008; Henry et al., 2006; Kandeler et al., 2006; Liu et al., 2010; Lopez-Gutierrez et al., 2004; Philippot et al., 2009a), but relatively few are comparable to tertiary wastewater TWs (Garcia-Lledo et al., 2011; Chon et al., 2011; Ji et al., 2012). The following paragraphs will address each gene function independently, and *nirS* and *nirK* will be presented together.

The 16S rDNA gene is commonly used in papers that assess denitrification gene copy numbers as a surrogate measurement for total microbial abundance to obtain an estimate of the relative abundance of denitrifiers to the total population. Because 16S rDNA copy numbers are known to vary from 1 to 13 copies per cell, the value of 16S rDNA cannot be taken as a direct cell count (Fogel et al., 1999). The range of 16S rDNA copies for this study (10^4 - 10^8 copies g^{-1}) was 4 orders of magnitude below that found in a TW treating tertiary wastewater, which was between 10^8 - 10^{12} copies g^{-1} (Garcia-Lledo et al., 2011; Chon et al., 2011). Studies that tested other environments were also in the range of 10^8 - 10^{12} copies g^{-1} (Hallin et al., 2009; Hammonts et al., 2013; Henry et al., 2006; Kandeler et al., 2006; Lopez-Gutierrez et al., 2004). The exact reasons for this difference remain unclear, but multiple explanations address low copies in this system. The sample masses were taken with a wet weight rather than a dry weight, artificially lowering the observed number of microbes per mass. Furthermore, the PCR inhibitor removal kit was expected to recover 50-90% of the original DNA. It was also possible the DNA extraction efficiency was low. All of these apply to the DNA extraction and quantitation methods and therefore apply to *nirS*, *nirK*, and *nosZ* as well. Because these errors were

systemic across all qPCR data in this study, the trends among data were expected to remain consistent.

Measurements of gene copies encoding the nitrite reductase functionality (*nirS* and *nirK*) are often taken as an estimate of the total denitrifier population within a sample site. *NirS* and *nirK* are functionally equivalent, but have been found to differ in abundance depending on environmental conditions (Hallin et al., 2009; Attard et al., 2011). Genomes encoding one or the other of these genes are almost exclusive to denitrifiers, meaning that they contain genes to process nitrate to nitrous oxide or dinitrogen gas (Casciotti and Ward, 2001; Zumft, 1997). Therefore, studies focusing on microbial aspects of denitrification often use these genes as an estimate of the total number of denitrifiers within a system. In this study, *nirK* had 15 (out of 44, approximately 34%) samples below the limit of detection, while *nirS* had 3 samples (out of 44) below the limit of detection. It was estimated the gene copy numbers for *nirS* and *nirK* in this study (which range from 10^3 - 10^7 and 10^2 - 10^6 copies g^{-1} , respectively) were up to 2-5 orders of magnitude below expected values of 10^6 - 10^9 copies g^{-1} for *nirS* and 10^5 - 10^{11} copies g^{-1} for *nirK*, for studies that used the same primer sets (Attard et al., 2011; Chen et al., 2010; Chon et al., 2011; Garcia-Lledo et al., 2011; Hallin et al., 2009; Hammonts et al., 2013; Henry et al., 2006; Kandeler et al., 2006). A strong correlation between *nirS* and *nirK* was found in TWs from this study, and *nirS* was found to be about 4.5 times as abundant as *nirK*. In other systems, these two genes were not always correlated strongly, and were present in various ratios, thought to be a byproduct of the environmental conditions (Hallin et al., 2009; Attard et al., 2011). The copy numbers

reported herein are only an estimate of the number of denitrifiers because the primer sets used were designed to be environmental primer sets, and therefore represent certain types of bacteria more than other types. However, both *nirS* and *nirK* are single-copy genes, with few observed cases of having 2-3 copies per genome, so they can be seen as a cell count estimate, representing a minimum number of denitrifying microbes in a given sample (Kandeler et al., 2006; Jones et al., 2008; Sanford et al., 2012). Values of 16S rDNA-relative gene abundance ranges from 1.5 to 30.9% *nirS+K* in this study, which was much higher than the more commonly observed maximum of 3.6% (Chon et al., 2011; Garcia-Lledo et al., 2011; Hallin et al., 2009; Hammons et al., 2013); although one study in a microbial fuel cell did report *nirS* values as high as 90% (Vilar-Sanz et al., 2013).

NosZ, the nitrous oxide reductase encoded by *nosZ*, is of particular interest in denitrification studies because of its direct interaction with the emission of the potent greenhouse gas, nitrous oxide. In the TW system from this study, N₂O cannot be readily converted into N₂ without NosZ, increasing the potential for releasing N₂O into the atmosphere. Seven (out of 44) *nosZ* gene assays were below the limit of detection in this study. Copy numbers measured in this study (ranging from 10³-10⁷ copies g⁻¹) were up to 2 orders of magnitude below observed ranges of 10⁵-10⁹ copies g⁻¹ (Chon et al., 2011; Garcia-Lledo et al., 2011; Henry et al., 2006; Hallin et al., 2009; Hammons et al., 2013; Ji et al., 2012; Kandeler et al., 2006). *Nir* and *nos* gene copies have sometimes been observed to be of the same order of magnitude, but often *nos* gene copies have been observed as an order of magnitude lower than *nir* gene copies (Garcia-Lledo et al., 2011).

As with the *nir* primer sets, the *nosZ* primer set used in this study captured *nosZ* copies more efficiently in certain types of bacteria, particularly in those bacteria with a traditional *nosZ* gene (defined by its DNA sequence). Approximately one-third of denitrifiers lack the traditional *nosZ* gene (Zumft, 1997; Henry et al., 2006; Jones et al., 2008). Recent literature has suggested a second *nosZ* primer set that captures a non-overlapping segment of *nosZ* from other microbes in equal magnitude to the gene copies captured with the primer set used in this study (Jones et al., 2013). As such, it was likely the *nosZ* gene copy number abundance was underestimated in the current study and quantification of this second set of organisms could account for a more accurate correlation between *nosZ* copy number and N₂O consumption (Orellana et al., 2014). *NosZ* is a single-copy gene, except for a few observed cases of having 2-3 copies per genome, so it can be seen as a cell count estimate (Kandeler et al., 2006; Jones et al., 2008; Sanford et al., 2012). Relative to 16S rDNA gene abundance for *nosZ* was found to range from 1.2 to 35.5%, whereas most studies report a 3% maximum (Chon et al., 2011; Garcia-Lledo et al., 2011; Hallin et al., 2009; Hammons et al., 2013). The large relative abundance values are thought to be more of a reflection on the low 16S rDNA copies in this study rather than a high relative abundance of denitrifiers.

Quantification of Denitrification Gas Emissions

To assay the enzymatic denitrification process within the experimental system, three gas assays were used. The first (N₂O produced) measures total denitrification potential by preventing the conversion of N₂O to N₂. The second (N₂O emitted) measures the amount of N₂O emitted during uninhibited denitrification. The third (N₂O consumed)

measures the total amount of N_2O the TW sample can convert to N_2 . All three gas assays were performed using gas chromatography on column sample incubations. Gas assays represent the amount of denitrification a sample was capable of at the time the sample was taken from the wetland column, which is dependent upon the conditions provided to the TW column. No values have been previously reported for the N_2O consumption term. The following paragraphs will address some of the methodology common to all measurements before considering each measurement independently of the others.

The incubations used to test gas values were designed to meet certain equipment limitations and provide comparison to previous literature values. For gas incubation samples, excess carbon and nitrogen were supplied to maintain non-limiting conditions for the reaction kinetics. In doing so, the measurements from the wetland samples were providing potentials for denitrification activity based on the wetland's community of microbes and denitrification enzymes at the time samples were taken from the TW column, rather than providing denitrification values that would be observed in the wetland directly. Normalizing the gas assay values by 16S rDNA copy number was done specifically to make direct comparisons across 3 fundamentally distinct habitats for gas and functional gene copy values. Using this normalization for gas measurements was a new procedure allowing for all measurements to be viewed on a per microbe basis, and helps bridge some of the differences between the various habitats. This normalization was further supported by standardizing all gas assay and gene assay units to the mass of the sample instead of using the ratio of gene assays to mass of DNA extracted, as is sometimes the case. As mentioned previously, even though the reported 16S rDNA

numbers did not match literature values, valuable information was contained in the trends. N₂ was used as a headspace gas in this study, and N₂O was measured for all gas assays, due to the high expense and equipment required for some other measurements such as isotope labeling or gas-flow core incubations (Mander et al., 2003; Mander et al., 2005; Teiter and Mander, 2005).

One concern with all gas assays was microbial growth during the incubation period. Some studies have used chloramphenicol to inhibit new enzyme synthesis. However, no chloramphenicol was used in the gas assays due to some undesirable interactions with the denitrification process (Brooks et al., 1992; Dendooven et al., 1994; Pell et al., 1996; Murray and Knowles, 1999). Instead, the length of incubation time was set to 3 hours, which was sufficiently short so that enzyme production would contribute minimally compared to other factors, as preliminary data confirmed (data not shown) (Pell et al., 1996).

N₂O production is the most commonly used method to assess the denitrification potential of a system. Values of N₂O production presented in this study (0.002-12.4 mg N₂O g⁻¹ hr⁻¹) range up to 3 times published values, which have been reported as high as 4.1 mg N₂O g⁻¹ hr⁻¹ in TWs (Bastviken et al., 2003; Correa-Galeote et al., 2013; Hunt et al., 2006; Kjellin et al., 2007). However, when the total N₂O production values from this study from each pair of replicate TW columns were averaged (*e.g.*, all carbon-negative *Phragmites* samples), the column-averaged values all fell within the upper bounds for reported ranges. Therefore, it is proposed that splitting the columns into habitats was the primary reason for some of the variation in measurements. Acetylene was used as an

inhibitor in these assays to prevent the nitrous oxide reductase from converting N_2O into N_2 . Historically, the acetylene-inhibition method has been used as the N_2O production assay; current studies use this method or an alternative method such as gas-flow core incubations, which are gaining more popularity. Groffman et al. (2006) and Butterbach-Bahl et al. (2013) discuss some of the issues with the acetylene-inhibition method: nitrification inhibition, slow diffusion of acetylene, contamination of acetylene, conversion of NO to NO_2^- , and decomposition of acetylene.

Despite its shortcomings, the acetylene-inhibition method is still widely used, in large part because it is simple, cheap, and there is a wide range of literature to compare values. This study has attempted to avoid many of the pitfalls of the technique. Groffman et al. (2006) suggest one major check for the method to help validate the results: linear accumulation of N_2O . Preliminary results for this study validated a linear accumulation of N_2O (data not shown). Furthermore, to address concerns over the conversion of NO to NO_2^- and the inhibition of nitrification, 1 mM each of carbon and NO_3^- were provided to the incubation vials, to minimize limiting nutrient conditions (Christensen et al., 1989; Saleh-Lahka et al., 2009; Yu et al., 2010). Though certain corrections were made to the method used, this value was still expected to be an underestimate of the actual N_2O production value.

The N_2O emission measurement represents the amount of N_2O that would be released into the atmosphere if the denitrification process within the TW columns was carried out uninhibited with its enzyme capacity for denitrification at the time of sampling. Amendments of nitrate and carbon provide nutrients for all denitrification

processes to occur at maximum potential. Published gas assay values for N₂O emission (assayed similarly to this study) in TWs range from 0 to 0.001 mg N₂O hour⁻¹ g⁻¹ soil (Hunt et al., 2006; Correa-Galeote et al., 2013). All values for this study fell 1-2 orders of magnitude above that range, with an outlier at 4.4 mg N₂O g⁻¹ hour⁻¹ (the next-highest value was 0.78 mg N₂O g⁻¹ hour⁻¹). When the values from paired replicate columns were averaged (including the outlier), all values fell under 1 mg N₂O g⁻¹ hour⁻¹. This suggests that the N₂O emission values from this study were higher than previously reported values. The reasons for this are still unclear.

Although no publications were found that employ the N₂O consumption approach, this study linked the *nosZ* gene with a direct measurement of NosZ activity. Typically, N₂O production and sometimes the N₂O emission values are used as a measure of denitrification gas activity. The only papers that were found to use a similar measurement technique have done so for the purposes of kinetic characterization, which was beyond the scope of this study, therefore no literature values were readily comparable. N₂O consumption was consistently the largest gas measurement value obtained, and was typically an order of magnitude more than N₂O production values for the same sample group. This was thought to be a result of the low estimate of N₂O production due to acetylene-inhibition method, a high estimate of N₂O consumption due to the high initial concentration of N₂O used in the assay, as well as kinetics. A one-step conversion of N₂O to N₂ was quantified as the N₂O consumption term, whereas a three-step conversion of NO₃⁻ to N₂O was required to measure the N₂O production term, which likely means the N₂O emission term has N₂O-concentration-dependent kinetics.

Effects of Carbon Load

The effects of carbon load were important in TW denitrification, as the data strongly supported Hypothesis 1: carbon load affects denitrification in particulate and gravel fractions more than in root fractions. Even though the models did not always support the statistical differences between carbon loads (0 and 0.391 g L⁻¹ sucrose), it is clear from Figure 7 and Figure 9 that gravel and particulate carbon-positive samples exhibited higher values for every gas and gene assay compared to carbon-negative samples. In terms of the non-normalized data, the root fraction showed very little difference between the two carbon loads. In some cases the root carbon-negative samples exhibited higher activity than root carbon-positive activity, but non-root fractions were statistically different between carbon loads. Gene assays were more affected than gas assays by carbon loads. This is because gene assays had tighter distributions (are more consistent) than gas assays within sample groups (*e.g.*, carbon-negative *Phragmites*), which led to smaller confidence intervals and, simultaneously, more confidence that significant differences exist between two groups. This trend makes some sense as genes indicated potential denitrification capability, whereas the gas measurements indicated enzyme activity, which were more immediately affected by external conditions.

Carbon load affected non-planted columns more significantly than planted columns (Figure 11 through Figure 16). Of the 7 gas and gene assays tested, carbon-positive non-planted samples exhibited significantly higher values than carbon-negative non-planted samples in a combined 12 of 14 measurements (7 measurements, 2 habitats, 1 plant “species”) in the particulate and gravel samples. But in particulate and gravel data

for species-specific carbon load effects, the same was true for only 5 out of 42 measurements (7 measurements, 2 habitats, 3 species). Of note, the activity of the microbial community within *Phragmites* TW columns accounts for 4 of those 5 significant results, suggesting that of all species tested by this study, the denitrification activity of the microbial community within *Phragmites* was moderately affected by carbon load. The proposed reason for fewer significant differences in denitrification activity within planted columns is that the roots in a TW provided some dampening effect on the system due to their ability to release and uptake nutrients (Jones et al., 2009; Tanner et al., 2001). This effect extended through the wetland even after the habitat fractions were separated from one another.

Carbon load had no significant effect on denitrification activity or gene abundance normalized to 16S rDNA copy number (Figure 8, Figure 10). Although in the normalized data some denitrification activity for carbon-positive samples was seen to be higher than carbon-negative samples, those differences were not statistically significant. This trend can best be explained through metabolic capabilities of a system. With high levels of nitrate, and an anoxic environment, denitrification was a favorable process in the wetland columns. Carbon is a key nutrient to this process. In carbon-negative systems, fewer microbes were present. The microbes that were present were more likely to possess denitrification capabilities, because the other nutrients for denitrification were still present. In the case of carbon-positive wetland columns, conditions were still favorable for denitrification and the carbon existed to support the process, so the absolute amount of denitrification increased. The extra carbon supplied allowed more microbial

metabolic pathway options that would otherwise have been out-competed by denitrification and the relative amount of denitrification activity decreased. Thus, normalizing denitrification activity by microbial 16S rDNA abundance shrunk the differences between carbon loads.

Comparing Between Habitats

Because the factor of habitat ties in so closely with differences in carbon load, the habitat fractions will be considered before moving to address the other hypotheses. The collection of each habitat was designed to capture the majority of material coming from the TW sample while keeping the habitats as distinct as possible. Separation of the habitats in a timely fashion was important to keep assays representative of the microbial communities as seen in the TW column. Gravel and roots were selected as distinct environments (abiotic and biotic, respectively) that collectively made up the main locations for microbial and biofilm growth. The particulates fraction was designed to capture microbial activity not represented by the first two fractions, which effectively compares the loosely-associated microbes to the main habitats. Determining how to compare across these habitats was therefore an important aspect of this study.

The root and gravel fractions were expected to be most different from each other in denitrification activity, with the particulate fraction somewhere in between. A way to compare habitats was needed. Gravel had the most rigorous separation method of all tested habitats, meaning that only strongly-associated microbes remained. The root fraction had some unseparated particulate fraction trapped within the root structure. Due to the washing steps, loosely-associated particles and microbes from the gravel and roots

likely ended up in the particulate fraction. Differences among habitats were specifically examined to look for any large effects which could be attributed to separation techniques. The similarity of trends between gas assays and qPCR assays for all factors indicated that the differences caused by separation techniques, such as any discrepancy in the amount of DNA extracted from gravel compared to roots, were likely of the same order of magnitude, so the focus could remain on the differences between factors rather than separation techniques.

Multiple attempts were made to develop a method that allowed for direct comparison of gas and gene copy assays across all habitats. Gravel and roots had mass and surface area, but measuring the mass of the particulates was challenging, and measuring the surface area of the particulates was incompatible with the definition of that habitat. Gravel also had a much higher density than roots, such that 1 gram of roots had a much higher surface area (more root abundance) than 1 gram of gravel. However, mass and volume were easier to accurately measure and were more compatible with the literature, which typically reports activity based on mass. A total organic carbon (TOC) measurement was considered for each particulate sample, but using a volume was ultimately chosen for this habitat fraction to provide consistency across the planted and non-planted samples, due to pieces of root being exclusively part of the planted particulate samples. TOC data for particulate samples have been provided in Appendix D. Gravel and roots were standardized to a mass basis (g) and particulates were standardized to a volume basis (mL). Comparing between habitats statistically was performed only on the 16S rDNA-normalized data set because this justification did not seem strong enough

for quantitative comparisons, but some comparisons were made qualitatively between the habitats.

Effects of Habitat

The differences in denitrification activity between habitats showed habitat as perhaps the most important factor in this study. Roots were shown to have the highest gene abundance and gas activity; fluctuations in gene abundance and gas activity paired with fluctuations in carbon load suggest the root-associated denitrifying microbial community was least affected by carbon load. This provides further evidence to previous studies that claim that certain regions (namely roots) of the TW are extremely active in the denitrification process and account for the majority of the denitrification, and that other regions within the TW contribute little to the overall sum of denitrification activity (Faulwetter et al., 2013; Hamonts et al., 2013; Philippot et al., 2009b; Tanner, 2001).

Column gravel was the habitat most affected by carbon load, because pathways for gravel to obtain external carbon were much more restricted compared to root and particulate habitats. Tiny root fragments were observed in the particulate fraction (because this was only separated by size), which undoubtedly contributed to carbon availability to the microbes in the TW column. Carbon availability was a key factor in explaining why gravel was also impacted more than particulate fractions by plant presence and absence in TW systems. In non-planted systems, the number of sites to harbor microbial growth was limited. Roots were the favored site when they were present, which was seen by the large amount of 16S rDNA compared to other habitats (Figure 7). When fine roots were absent in non-planted systems, the column was

relatively free of particulates, so non-planted particulate fractions had less material present than planted particulate fractions. Gravel becomes the main site for bacteria to attach and grow. To increase the complexity by incorporating carbon load effects, when carbon was not added to the system, roots could act as or provide a source of carbon. In the carbon-negative non-planted systems, there were very little nutrients to support heterotrophic microbial growth. When carbon was present in the non-planted systems, the particulate fraction was still lacking and gravel was the primary site for microbial growth supported by the added carbon.

Few 16S rDNA microbial analyses have explicitly compared roots to non-root fractions. Faulwetter et al. (2013) report gravel from planted columns can have more 16S rDNA copies than roots, but other studies report higher 16S rDNA activity in the roots (Hamonts et al., 2013; Garcia-Lledo et al., 2011). Studies have also used other methods to show roots have higher microbial abundance than “bulk” areas (Henry et al., 2008; Brisson and Chazarenc, 2009; Burgmann et al., 2005). Faulwetter et al. (2013) completely separated roots from gravel instead of separating the same material that was either classified as root-associated or “bulk”. As a result of discrete habitat separation procedures, Faulwetter et al. (2013) suggested that the DNA extraction efficiencies can vary for the different habitats, causing part of the discrepancy between habitats in this study. As previously mentioned, that was also a consideration for this study and likely contributes to the low value of gravel gene abundances. DNA extraction concentrations as measured by a NanoDrop ND-1000 (Thermo Scientific, Massachusetts) (data not shown) clustered below $40 \text{ ng } \mu\text{L}^{-1}$ for gravel, were split evenly between 0-40 and 40-80

ng μL^{-1} for roots, and fell mostly between 40-80 ng μL^{-1} for particulates with a few outliers above and below.

Comparing results for non-normalized (for 16S rDNA) data across habitat was therefore difficult; however, the roots were the most active fraction on a per gram or per mL basis. Roots have been cited previously as a site of higher denitrification activity (Lin et al., 2002; Tanner, 2001; Brisson and Chazarenc, 2009). In this study, each gas and gene measurement showed higher microbial denitrification activity in the root fraction compared to that in gravel and particulates. However, when the data were normalized to 16S rDNA gene copy number, the microbial community in the roots was not the most active fraction, and in fact were in most cases the lowest fraction. Microbes on the gravel had significantly higher normalized denitrification activity and gene abundance in 4 of 6 models (N_2O emission, N_2O consumption, *nirS*, and *nirS+K*), and carbon-positive gravel had higher activity than carbon-positive roots for N_2O production (Figure 8, Figure 10). The particulate fraction microbial denitrification activity falls in between that of the gravel and root fraction microbial denitrification activity.

In all cases, gravel samples showed the highest denitrification activity and abundance relative to microbial abundance (Figure 8, Figure 10). The exact reasons for this are unclear, but one possibility is presented after considering the effects of plant presence. It is possible that the roots were able to harbor more bacteria due to the dynamic interactions between the rhizosphere and microbes, but the presence of oxygen, which the roots provide *in vivo*, could have inhibited some denitrification processes at the root surface and therefore encouraged non-denitrifiers to compete with denitrifiers on the

root surface, decreasing the relative abundance of denitrification genes (Kadlec and Wallace, 2009; Hamonts et al., 2013; Garcia-Lledo et al., 2011). Since many denitrifiers are facultative anaerobes, for this idea to be supported, it would follow that the difference in gas assays between roots and gravel would be larger than the difference in gene abundance between roots and gravel, because the gene copy numbers could be present in roots with lower enzyme expression or otherwise inhibited enzymes present in the root fractions. Such was the case, where in the normalized data, the difference in gas assays between roots and gravel fractions was close to an order of magnitude, whereas the gene copy numbers differed by less than half of that.

Effects of Species and Plant Presence

The hypothesis that practical differences exist between treatment wetland plant species of *Carex*, *Schoenoplectus*, and *Phragmites* was not supported by this research (Figure 17 through Figure 22). Of all factors tested in this study, plant species was least important. Plant species in this study were shown in some models to be a statistically significant factor in denitrification activity, although no consistent or strong patterns among plant species effects were found across the entire data set. No significant species effects were found in the root fractions. At first glance, this appears to contrast previous studies at MSU, which have found significant effects in COD removal due to plant species in a TW (Allen et al., 2002; Taylor et al., 2011; Stein and Hook, 2005). However, those studies all compared each plant species to the non-planted system which acted as the control TW. This study also found a large difference between planted and non-planted systems, but little difference was found between the individual species tested in those

studies. Brisson and Chazarenc (2009) provided an analysis of studies exploring species effects, and concluded more information is needed to be confident that certain species perform better than others for a given context, but that planted systems were shown in almost all cases to perform better than non-planted systems.

Of particular note is that all species chosen for this study were known to perform relatively well in TWs designed for denitrification (Allen et al., 2002; Taylor et al., 2011; Stein and Hook, 2005). Had this not been the case, it is possible that more differences would have been observed between plant species. For example, microbial and denitrification gene abundance in *Phragmites* TWs were found in both particulate and gravel samples to be statistically different between the two carbon loads, although it was clear from the data (Figure 17 through Figure 27) that carbon-negative microbial and denitrification gene abundance in *Phragmites* TWs were typically lower than other species and carbon-positive values higher than other species. It was also the case that *Carex*-associated microbes were least affected by carbon load, and the values for carbon-positive *Carex* samples were often very close to the corresponding values of carbon-negative *Carex* samples. The ability of *Carex* to dampen carbon load differences was not explicitly tested for by the statistical models, as they were testing for differences between groups rather than similarity.

The effect of roots in planted columns does not stop at the root fraction. Root material provides some additional resource to the particulate and gravel fraction. The idea that roots have a positive effect on denitrification activity has been reported elsewhere (Lin et al., 2002; Tanner, 2001; Brisson and Chazarenc, 2009), but these studies primarily

compare samples of the same material from the sampling site. This study takes the research one step further by separating three distinct habitats of different material from the wetland system. What was seen with these results (Figure 17 through Figure 27), but not in other studies, was that that non-planted gravel could, in the presence of carbon, perform better than the sum of planted root and gravel fractions. The sum of activity from planted systems exceeded the activity in non-planted systems, but the amount of activity occurring in each habitat differed between the two types of systems.

Genes to Gas Correlations

Both in 16S rDNA-normalized and non-normalized data, the correlations between *nirS+K* and N₂O production were strong, as was the correlation between *nosZ* and N₂O consumption. The non-normalized data had less variance in the correlation than normalized data (Figure 23 through Figure 26). The negative correlation between the ratios of these values (Figure 27) is still not well-understood, but was possibly a result of the gene abundance terms being of the same order of magnitude and the gas activity N₂O consumption being a dominant term over N₂O production. Previous studies have reported correlations between *nir* genes and potential N₂O production (as the term was defined in this study) (Attard et al., 2011; Correa-Galeote et al., 2013; Hammonts et al., 2013). *NosZ* has not been found to correlate to N₂O production (this relationship was not expected based on the mechanistic process) (Correa-Galeote et al., 2013; Hammonts et al., 2013). A correlation between N₂O consumption and *nosZ* presence has not been previously reported.

Ratios of gene copies to gas assays did not correlate in a predictable pattern beyond the single correlations of gene abundance to its function in denitrification, as measured by gas assay. The ratio of *nir/nos* copies correlated negatively to the ratio of N₂O produced/N₂O consumed. The particular reasons for this negative correlation are not well understood, but it is thought that the N₂O consumption term dominated the gas assay, while the *nir* and *nos* genes were of the same magnitude and therefore had a higher ratio of signal to noise.

Minimizing N₂O Release

A major goal for this project was to investigate N₂O emission and suggest how to reduce it in treatment wetlands. Ideally, a high level of denitrification could be paired with a low release of N₂O. This idea has been previously examined, where it was determined that planted systems provided better global performance with a high amount of nutrient removal paired to the low amounts of greenhouse gas production, accounting for N₂O, CO₂, and CH₄ (Maltais-Landry et al., 2009). The authors suggested more research is needed, with a specific focus on the mechanism of performance. This study addressed aspects of species selection, habitat, and nutrient loading on TW denitrification performance. As the idea of minimizing emissions is highly related to performance in TWs, the amount of N₂O emission and the amount of N₂O production (as the N₂O emitted to N₂O produced ratio) are considered in this section.

Some of the correlations associating N₂O emission with other measurements were intuitive, while others were counter-intuitive. Every measurement (N₂O production, N₂O consumption, 16S rDNA copies, *nir* copies, *nos* copies) in this study was positively

correlated with N₂O emission. While this could be intuitive at first glance, it means that higher values of N₂O consumption and *nosZ* abundance were correlated with higher levels of N₂O emission. This was likely due to all values increasing together; a sample that had higher microbial activity was likely to have more copy numbers of all genes and higher values of all gas assays (Garcia-Lledo et al, 2011; Hamonts et al., 2013). Two correlations in particular elucidate a more complete account of this relationship. 16S rDNA copy number was the least correlated with N₂O emission, suggesting that the number of microbes present in a sample had the least impact on the amount of N₂O being released. As 16S rDNA copies did not directly deal with denitrification activity while all other measurements did, this was a reassuring result that any measurement of denitrification activity was a better indicator for N₂O release than was the 16S rDNA microbial abundance. A higher ratio of N₂O production to N₂O consumption was positively correlated with N₂O emission. In the previous section, the negative correlation between the ratio of *nir/nos* to N₂O production/N₂O consumption was attributed to too much noise in the gene copy ratio. By replacing the gene ratio with N₂O emission, the resulting positive correlation supports the expected interaction between these terms.

Carbon-positive TW systems not only had higher values of N₂O production, but also had lower amounts of N₂O emitted in total denitrification, making carbon-positive TWs more desirable for nitrogen removal by denitrification. Gravel-associated microbes in non-planted TW columns provided the second-highest N₂O production values with the highest ratio of N₂: N₂O (Figure 32). Therefore, gravel non-planted systems presented the best treatment efficiency in terms of total denitrification capacity coupled with the lowest

greenhouse gas (as N_2O) emissions. Other gas emissions (*e.g.*, CO_2) should be considered as well, but were beyond the scope of this study. Expanding this idea to TW design will be explored further in the following section.

Design Parameters

For a denitrification treatment wetland, the most important measurement is the amount of dissolved nitrogen removal the wetland can achieve. For this study, the closest measurement to determine nitrate removal was N_2O production, which tracks the conversion of NO_3^- into N_2O , and inhibits the conversion of N_2O into N_2 , providing a measure of potential for denitrification. This research aimed to provide insight into how a low N_2O emission could be achieved with high nitrogen removal (estimated through N_2O production).

Traditional wastewater treatment plants typically treat denitrification in a similar way as wetlands—with microbes. The most important factors for wetland design are the characteristics of the influent water. This study has focused on tertiary treatment of municipal wastewater. Typical values for post-secondary treatment are 20 mg L^{-1} BOD_5 , 75 mg L^{-1} COD, 20 mg L^{-1} total nitrogen (Kadlec and Wallace, 2009). Heterotrophic biological denitrification treatment design focuses on effluent parameters such as BOD and COD removal and nitrate removal. Treatment goals are designed for by considering factors of carbon availability, aeration, temperature, and pH (Metcalf and Eddy, 1978). Often, it is necessary to add a carbon source to provide energy for the denitrification process. In a TW, the design focus to meet effluent treatment goals is on climate, wetland type, plant species, carbon availability, pH, temperature, and media (Faulwetter et al.,

2009; Kadlec and Wallace, 2009). Effects of some factors, such as the plant species and climate, are still not well characterized (Brisson and Chazarenc, 2009).

The results from this study have shown that planted columns typically produced more N_2O from denitrification than non-planted columns, but non-planted columns could produce more N_2O from denitrification if extra carbon was added to the column. This is particularly relevant for wetland system design, where nitrate removal is an important factor. The planted and non-planted distinction did not play a large role in N_2O emission. However, despite the non-planted columns seeming to perform better than carbon-positive planted columns, plants provided a substantial benefit to TWs that was not accounted for simply by those measurements, including providing buffering capacity of the system to shock events and (non-nitrate) nutrient and pollutant removal (Tanner, 2001; Brisson and Chazarenc, 2009; Kadlec and Wallace, 2006). The benefit of these extra factors supports a design for denitrification TWs in which plants should be encouraged. A design should encourage plants with a known reasonable ability to reduce nitrogen and most literature suggests the wetland performance will be enhanced from the case where no plants are present (Brisson and Chazarenc, 2009; Tanner, 2001). There is currently not enough information to understand under what conditions non-planted systems perform better than planted systems. While multiple studies have considered the effects of plant species, the variability between sampling sites and procedures is large, and most tested species have relatively few studies performed with them (Brisson and Chazarenc, 2009). Data from this study suggests that under the studied conditions, wetlands with *Carex* and *Schoenoplectus* released less N_2O during denitrification than

Phragmites by more than an order of magnitude. Although they also had slightly lower N₂O production measurements, the lower N₂O production values were on the same order of magnitude and therefore exhibited a comparative advantage; they performed comparable denitrification with reduced N₂O release into the atmosphere. Similar results were found in previous studies at MSU, suggesting higher performance from *Carex* and *Schoenoplectus* with lower performance by *Phragmites* (Allen et al., 2002; Stein et al., 2007; Taylor et al., 2011; Allen et al., 2013).

Results from this study also suggest having more carbon available yields more denitrification activity and denitrification gene abundance. This result has been confirmed in wetlands, and it is common to add carbon in traditional wastewater denitrification processes (Lu et al., 2009; Lin et al., 2002; Kadlec and Wallace, 2009; Philippot et al., 2009a; Metcalf and Eddy, 1978). When measurements were normalized to 16S rDNA copies, the relative values of gas measurements and gene copy number were similar between carbon loads, suggesting a larger carbon load leads to a higher microbial population but not necessarily a more relatively abundant denitrification population. This result appears to be supported by Henry et al. (2008), although the authors did not perform a statistical analysis on the relative abundances of denitrifying genes as they were influenced by carbon. It appears that both denitrifiers as well as non-denitrifiers increase in a somewhat fixed proportion to each other with the addition of carbon. Therefore, adding carbon in the wetland system will increase denitrification by providing nutrients for more microbial growth and by removing O₂, allowing denitrification to proceed.

Future Work

This study targeted areas of high importance in TW denitrification as set forth in previous publications. This project analyzed denitrification activity by gas and genetic quantification within a model TW system designed to simulate wastewater tertiary treatment. Research had previously characterized some effects of temperature and plant species on treatment within a similar model wetland system (Stein and Hook, 2005; Taylor et al., 2010; Allen et al., 2002; Allen et al., 2013). Microbial characterization of the wetland system focused on sulfate removal, nitrification, and 16S rDNA profiles (Faulwetter et al., 2008; Faulwetter et al., 2013). Comprehensive work linking enzymatic (gas) and microbial (gene copy) data was presented in this thesis to attempt to explain some of the plant species, carbon load, and spatial variances within TWs at a more fundamental level. This work provides a foundation for elucidating the mechanisms of microbial nutrient removal and transformations within TWs.

Elucidating the microbial mechanisms by which nutrient cycling within a TW occurs was beyond the scope of study. More targeted research is needed to understand the interactions between the TW environment and microbial dynamics. Further genetic work could be to perform a microbial community analysis with 16S rDNA sequencing, as well as perform qPCR targeting RNA for denitrification genes. This would provide a more complete characterization of the denitrifying community showing not only the microbial denitrification population that is present, but also which genes are being actively transcribed. A full comparison between these data would be useful in understanding conditions that encourage denitrifier growth as well as trigger denitrification genes to be

transcribed. Understanding environmental conditions that regulate transcription would be immensely helpful in creating favorable environments for denitrification, and potentially for encouraging the production of certain enzymes, such as NosZ, to lower TW N₂O emissions.

One of the major goals of this research, encouraging better design and performance of TWs, will be more easily achievable when microscale systems can be predictive of macroscale systems. This study supports that goal by addressing microbial processes and abundance and linking them with denitrification performance from gas data on the microscale. Research should attempt to combine microscale studies with mesoscale and macroscale studies to link the microbial processes with trends in scaled-up TWs. It would be useful to identify trends that do and that potentially do not hold true from the microscale studies to larger systems so that appropriate measures can be taken to address gaps in methodological or theoretical assumptions. In combining these areas of research, TW systems will be more easily optimized for nutrient removal to meet treatment goals.

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APPENDICES

APPENDIX A

AGGREGATED DATA

APPENDIX A: Aggregated Data

Data used in this study will be presented in an aggregated form. Technical replicates for each measurement are shown as averages with the standard deviations. Column replicates (biological replicates, reported as either a “1” or a “6”) are presented. This is the data format used for all statistical modeling.

Table A1: All aggregated denitrification activity gas data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in $\text{mg hr}^{-1} \text{g}^{-1}$ sample or $\text{mg hr}^{-1} \text{mL}^{-1}$ sample.

CL	CR	Species	Habitat	N ₂ O produced	standard deviation		N ₂ O emitted	standard deviation		N ₂ O consumed	standard deviation	
–	1	Non-Planted	Particulate	6.81E-03	9.1E-04	**	7.70E-03	5.9E-04	**	3.70E-02	6.4E-02	**
–	1	Non-Planted	Gravel	2.13E-02	5.9E-03	**	2.34E-02	7.4E-03	**	1.03E-01	9.6E-02	**
–	1	<i>Carex</i>	Particulate	4.69E-02	7.4E-03	**	3.36E-02	2.3E-03	**	3.55E-01	1.1E-01	**
–	1	<i>Carex</i>	Gravel	2.68E-02	3.1E-03	**	7.21E-02	6.7E-02	**	8.50E-01	2.1E-01	**
–	1	<i>Carex</i>	Roots	4.29E+00	5.8E-01	**	2.25E-01	3.6E-02	**	1.94E+01	6.3E+00	**
–	1	<i>Phragmites</i>	Particulate	2.03E-02	7.2E-03	**	2.80E-03	3.6E-04	**	2.67E-01	9.6E-02	**
–	1	<i>Phragmites</i>	Gravel	9.06E-02	5.3E-03	**	1.94E-02	6.7E-03	**	1.69E+00	2.1E-01	**
–	1	<i>Phragmites</i>	Roots	7.31E+00	8.1E+00	** †	1.75E-01	1.6E-02	**	2.11E+01	5.5E+00	**
–	1	<i>Schoenoplectus</i>	Particulate	6.77E-03	6.8E-04	**	9.28E-03	1.8E-03	**	6.49E-01	4.4E-02	**
–	1	<i>Schoenoplectus</i>	Gravel	3.81E-02	7.0E-04	*	3.47E-02	4.6E-03	**	1.63E+00	2.0E-01	**
–	1	<i>Schoenoplectus</i>	Roots	2.99E+00	8.3E-01	**	2.96E-01	1.4E-01	**	1.92E+01	4.9E+00	**
–	6	Non-Planted	Particulate	4.23E-03	2.5E-04	**	4.18E-03	3.9E-04	**	2.86E-01	4.5E-02	**
–	6	Non-Planted	Gravel	8.25E-02	2.7E-02	**	1.40E-02	2.8E-03	**	1.40E+00	1.4E-01	**
–	6	<i>Carex</i>	Particulate	2.01E-02	1.5E-03	**	2.12E-02	1.1E-03	**	3.66E-01	1.3E-01	**
–	6	<i>Carex</i>	Gravel	2.53E-01	1.9E-02	**	1.32E-01	3.3E-02	**	3.75E+00	5.0E-01	**
–	6	<i>Carex</i>	Roots	1.65E+00	1.8E-01	**	3.83E-01	1.5E-01	**	1.43E+00	6.4E-01	**
–	6	<i>Phragmites</i>	Particulate	1.14E-02	3.8E-03	**	5.11E-03	1.4E-03	**	5.70E-01	4.3E-01	**

Table A1 (cont'd)

CL	CR	Species	Habitat	N ₂ O produced	standard deviation		N ₂ O emitted	standard deviation		N ₂ O consumed	standard deviation	
—	6	<i>Phragmites</i>	Gravel	9.16E-02	3.4E-02	**	2.67E-02	2.0E-03	**	1.46E+00	3.0E-01	**
—	6	<i>Phragmites</i>	Roots	3.20E+00	1.2E+00	*	2.19E-01	4.8E-02	**	2.72E+01	2.8E+00	**
—	6	<i>Schoenoplectus</i>	Particulate	2.19E-03	2.4E-04	**	3.51E-03	1.9E-03	**	2.72E-01	1.6E-01	**
—	6	<i>Schoenoplectus</i>	Gravel	3.44E-02	1.4E-02	**	1.11E-02	6.1E-03	**	1.34E+00	4.6E-01	**
—	6	<i>Schoenoplectus</i>	Roots	2.15E+00	3.2E-01	**	3.69E-01	5.4E-02	**	1.36E+01	2.7E+00	**
+	1	Non-Planted	Particulate	1.08E-01	2.6E-02	**	4.15E-02	1.8E-02	**	3.04E-02	3.1E-02	**
+	1	Non-Planted	Gravel	6.42E+00	2.7E+00	**	3.45E-01	8.2E-03	**	6.93E+00	1.4E-01	**
+	1	<i>Carex</i>	Particulate	2.05E-01	2.6E-02	**	4.56E-02	4.7E-03	**	1.03E+00	1.6E-02	**
+	1	<i>Carex</i>	Gravel	1.05E+00	1.4E-01	**	9.23E-02	1.3E-02	**	4.40E+00	8.7E-01	**
+	1	<i>Carex</i>	Roots	4.51E+00	6.3E-01	**	7.77E-01	9.8E-02	**	1.80E+01	3.8E+00	**
+	1	<i>Phragmites</i>	Particulate	3.54E-01	8.1E-02	**	6.12E-02	2.7E-02	**	4.87E-01	1.9E-01	**
+	1	<i>Phragmites</i>	Gravel	5.46E+00	3.7E-01	**	2.93E-01	4.0E-02	**	7.04E+00	4.0E-01	**
+	1	<i>Phragmites</i>	Roots	1.24E+01	2.0E+00	**	4.35E+00	1.5E+00	**	5.36E+01	1.1E+01	**
+	1	<i>Schoenoplectus</i>	Particulate	3.00E-01	2.0E-02	**	3.46E-02	1.5E-03	**	1.26E+00	2.2E-02	**
+	1	<i>Schoenoplectus</i>	Gravel	3.91E-01	2.5E-01	**	7.46E-02	1.8E-03	**	4.45E+00	8.2E-01	**
+	1	<i>Schoenoplectus</i>	Roots	2.92E+00	2.3E-01	**	3.90E-01	3.0E-02	**	2.82E+01	3.0E+00	**
+	6	Non-Planted	Particulate	2.56E-01	5.4E-02	**	3.58E-02	1.4E-02	**	6.59E-01	0.0E+00	**
+	6	Non-Planted	Gravel	5.84E+00	4.0E-01	**	9.01E-02	2.9E-03	**	5.75E+00	4.1E-01	**
+	6	<i>Carex</i>	Particulate	1.00E-01	9.5E-03	**	2.81E-02	3.4E-04	**	4.65E-01	2.0E-01	**
+	6	<i>Carex</i>	Gravel	5.11E-02	4.0E-03	**	5.69E-02	8.0E-03	**	1.26E+00	1.5E-01	**
+	6	<i>Carex</i>	Roots	8.21E-01	6.1E-02	**	3.85E-01	5.8E-02	**	3.16E+00	5.0E-01	**

Table A1 (cont'd)

CL	CR	Species	Habitat	N ₂ O produced	standard deviation		N ₂ O emitted	standard deviation		N ₂ O consumed	standard deviation	
+	6	<i>Phragmites</i>	Particulate	1.16E-01	4.8E-03	**	2.67E-02	1.5E-03	**	5.22E-01	6.0E-02	**
+	6	<i>Phragmites</i>	Gravel	6.51E-01	2.1E-02	**	3.61E-01	1.9E-01	**	2.17E+00	3.8E-01	**
+	6	<i>Phragmites</i>	Roots	1.05E+00	1.1E-01	**	3.94E-01	6.2E-02	**	7.08E+00	4.1E+00	**
+	6	<i>Schoenoplectus</i>	Particulate	2.67E-01	9.6E-03	**	2.23E-02	3.1E-03	*	5.49E-01	1.5E-01	**
+	6	<i>Schoenoplectus</i>	Gravel	1.36E+00	8.3E-02	**	9.82E-02	4.0E-02	**	5.92E+00	6.9E-01	**
+	6	<i>Schoenoplectus</i>	Roots	4.69E-01	1.1E-01	**	1.07E-01	1.7E-03	**	1.89E+00	1.6E+00	**

Table A2: Aggregated 16S rDNA and *nosZ* gene abundance data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in copies g⁻¹ sample or copies mL⁻¹ sample.

CL	CR	Species	Habitat	16S rDNA copies	standard deviation		<i>nosZ</i> copies	standard deviation		
–	1	Non-Planted	Particulate	2.94E+04	0.0E+00	*	2.77E+03	0.0E+00	***	†
–	1	Non-Planted	Gravel	1.12E+06	0.0E+00	*	5.72E+04	0.0E+00	***	†
–	1	<i>Carex</i>	Particulate	2.76E+06	1.6E+05	*	1.82E+05	5.5E+05	*	
–	1	<i>Carex</i>	Gravel	2.56E+06	0.0E+00	*	5.72E+04	5.8E+25	***	†
–	1	<i>Carex</i>	Roots	2.98E+08	3.0E+04	*	1.16E+07	2.0E+05	*	
–	1	<i>Phragmites</i>	Particulate	5.37E+06	7.9E+04	*	1.54E+05	2.2E+04	*	
–	1	<i>Phragmites</i>	Gravel	5.13E+05	0.0E+00	*	5.72E+04	0.0E+00	***	†
–	1	<i>Phragmites</i>	Roots	1.92E+08	7.5E+04	*	7.77E+06	1.8E+05	*	
–	1	<i>Schoenoplectus</i>	Particulate	2.14E+06	4.4E+05	*	2.03E+05	1.5E+05	*	
–	1	<i>Schoenoplectus</i>	Gravel	2.50E+06	0.0E+00	*	3.28E+05	2.6E+05	***	
–	1	<i>Schoenoplectus</i>	Roots	1.94E+08	3.1E+04	*	1.28E+07	7.5E+05	*	
–	6	Non-Planted	Particulate	1.94E+05	0.0E+00	*	7.54E+03	5.1E+04	***	
–	6	Non-Planted	Gravel	4.42E+05	0.0E+00	*	5.72E+04	0.0E+00	*	†
–	6	<i>Carex</i>	Particulate	3.58E+06	2.3E+05	*	1.43E+05	8.3E+05	*	
–	6	<i>Carex</i>	Gravel	6.67E+06	0.0E+00	*	4.95E+05	6.9E+04	*	
–	6	<i>Carex</i>	Roots	2.44E+08	8.7E+04	*	3.97E+06	2.2E+05	*	
–	6	<i>Phragmites</i>	Particulate	1.78E+06	8.0E+04	*	1.02E+05	6.8E+05	*	

Table A2 (cont'd)

CL	CR	Species	Habitat	16S rDNA copies	standard deviation		<i>nosZ</i> copies	standard deviation		
–	6	<i>Phragmites</i>	Gravel	4.17E+05	0.0E+00	*	5.72E+04	0.0E+00	*	†
–	6	<i>Phragmites</i>	Roots	1.67E+08	1.1E+05	*	9.57E+06	2.9E+05	*	
–	6	<i>Schoenoplectus</i>	Particulate	7.34E+06	2.0E+05	*	4.18E+05	1.0E+06	*	
–	6	<i>Schoenoplectus</i>	Gravel	5.74E+06	0.0E+00	*	4.95E+05	3.3E+05	****	
–	6	<i>Schoenoplectus</i>	Roots	4.83E+08	9.0E+04	*	1.93E+07	4.7E+04	*	
+	1	Non-Planted	Particulate	2.31E+06	3.5E+03	*	1.53E+05	1.3E+05	*	
+	1	Non-Planted	Gravel	3.77E+07	1.2E+04	*	1.13E+07	2.4E+05	*	
+	1	<i>Carex</i>	Particulate	9.02E+06	1.2E+05	*	6.58E+05	1.9E+05	*	
+	1	<i>Carex</i>	Gravel	6.87E+06	0.0E+00	*	1.38E+06	1.1E+05	*	
+	1	<i>Carex</i>	Roots	2.65E+08	1.8E+06	*	1.16E+07	1.7E+05	*	
+	1	<i>Phragmites</i>	Particulate	3.26E+06	1.1E+05	*	2.25E+05	6.5E+04	*	
+	1	<i>Phragmites</i>	Gravel	1.34E+07	3.8E+04	*	2.04E+06	3.4E+05	*	
+	1	<i>Phragmites</i>	Roots	5.07E+08	2.8E+05	*	1.12E+07	1.8E+04	*	
+	1	<i>Schoenoplectus</i>	Particulate	7.79E+06	3.1E+05	*	6.25E+05	9.0E+04	*	
+	1	<i>Schoenoplectus</i>	Gravel	3.17E+06	0.0E+00	*	3.30E+05	7.9E+04	****	
+	1	<i>Schoenoplectus</i>	Roots	1.85E+08	7.0E+05	*	1.13E+07	9.7E+04	*	
+	6	Non-Planted	Particulate	3.49E+06	9.8E+03	*	2.89E+05	4.2E+05	*	
+	6	Non-Planted	Gravel	9.45E+07	6.8E+04	*	1.46E+07	1.3E+06	*	
+	6	<i>Carex</i>	Particulate	7.69E+06	2.5E+05	*	2.31E+05	8.0E+05	*	
+	6	<i>Carex</i>	Gravel	1.79E+06	0.0E+00	*	5.72E+04	0.0E+00	**	†
+	6	<i>Carex</i>	Roots	2.18E+08	4.3E+03	*	2.53E+06	6.4E+04	*	

Table A2 (cont'd)

CL	CR	Species	Habitat	16S rDNA copies	standard deviation		<i>nosZ</i> copies	standard deviation	
+	6	<i>Phragmites</i>	Particulate	2.92E+07	1.4E+05	*	1.16E+06	3.4E+05	*
+	6	<i>Phragmites</i>	Gravel	1.32E+07	6.1E+04	*	7.87E+05	6.2E+04	***
+	6	<i>Phragmites</i>	Roots	1.28E+08	1.5E+04	*	5.75E+06	7.0E+04	*
+	6	<i>Schoenoplectus</i>	Particulate	2.66E+06	6.2E+04	*	6.61E+05	4.8E+05	*
+	6	<i>Schoenoplectus</i>	Gravel	6.16E+06	0.0E+00	*	2.19E+06	1.5E+05	*
+	6	<i>Schoenoplectus</i>	Roots	2.37E+07	0.0E+00	*	1.18E+06	3.7E+05	***

Table A3: Aggregated *nirS* and *nirK* gene abundance data used in this study. CL = carbon load. CR = column replicate. * = 2 replicates, ** = 3 replicates, *** = 4 replicates, † = data substituted. Technical replicates have been averaged and are shown with their standard deviations. No data were removed as outliers. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Units are in copies g⁻¹ sample or copies mL⁻¹ sample.

CL	CR	Species	Habitat	<i>nirS</i> copies	standard deviation		<i>nirK</i> copies	standard deviation		
–	1	Non-Planted	Particulate	8.47E+03	1.9E+04	*	2.05E+03	0.0E+00	*	†
–	1	Non-Planted	Gravel	3.28E+04	7.1E+25	* †	4.22E+04	0.0E+00	*	†
–	1	<i>Carex</i>	Particulate	1.84E+05	4.9E+05	*	2.10E+04	8.0E+04	*	
–	1	<i>Carex</i>	Gravel	1.37E+05	5.2E+03	*	4.22E+04	0.0E+00	*	†
–	1	<i>Carex</i>	Roots	1.16E+07	0.0E+00	*	2.43E+06	1.1E+05	*	
–	1	<i>Phragmites</i>	Particulate	1.29E+05	6.0E+04	*	3.88E+04	1.1E+05	*	
–	1	<i>Phragmites</i>	Gravel	9.72E+04	3.7E+03	*	4.22E+04	0.0E+00	*	†
–	1	<i>Phragmites</i>	Roots	5.10E+06	4.2E+04	*	9.75E+05	3.4E+04	*	
–	1	<i>Schoenoplectus</i>	Particulate	2.17E+05	2.4E+05	*	1.35E+05	3.5E+05	*	
–	1	<i>Schoenoplectus</i>	Gravel	3.17E+05	1.4E+04	*	4.22E+04	0.0E+00	*	†
–	1	<i>Schoenoplectus</i>	Roots	1.25E+07	7.5E+05	*	4.71E+06	1.5E+05	*	
–	6	Non-Planted	Particulate	5.82E+03	0.0E+00	*	2.05E+03	0.0E+00	*	†
–	6	Non-Planted	Gravel	3.28E+04	0.0E+00	* †	4.22E+04	0.0E+00	*	†
–	6	<i>Carex</i>	Particulate	1.14E+05	1.1E+05	*	6.75E+04	2.7E+05	*	
–	6	<i>Carex</i>	Gravel	3.90E+05	5.9E+03	*	4.22E+04	0.0E+00	*	†
–	6	<i>Carex</i>	Roots	1.84E+06	9.6E+04	*	1.33E+06	2.7E+05	*	
–	6	<i>Phragmites</i>	Particulate	6.88E+04	6.4E+04	*	2.15E+04	1.7E+04	*	

Table A3 (cont'd)

CL	CR	Species	Habitat	<i>nirS</i> copies	standard deviation		<i>nirK</i> copies	standard deviation		
–	6	<i>Phragmites</i>	Gravel	9.19E+04	7.0E+03	*	4.22E+04	0.0E+00	*	†
–	6	<i>Phragmites</i>	Roots	8.97E+06	4.9E+04	*	3.72E+06	1.1E+04	*	
–	6	<i>Schoenoplectus</i>	Particulate	3.97E+05	6.2E+05	*	1.30E+05	1.5E+05	*	
–	6	<i>Schoenoplectus</i>	Gravel	6.25E+05	4.7E+03	*	4.22E+04	0.0E+00	*	†
–	6	<i>Schoenoplectus</i>	Roots	2.22E+07	1.2E+06	*	8.66E+06	1.7E+05	*	
+	1	Non-Planted	Particulate	9.74E+04	0.0E+00	*	1.13E+04	5.7E+04	*	
+	1	Non-Planted	Gravel	2.41E+06	9.1E+04	*	2.24E+05	2.1E+04	*	
+	1	<i>Carex</i>	Particulate	3.15E+05	5.4E+05	*	4.66E+04	1.3E+05	*	
+	1	<i>Carex</i>	Gravel	5.30E+05	0.0E+00	*	4.22E+04	0.0E+00	*	†
+	1	<i>Carex</i>	Roots	1.42E+07	5.3E+05	*	1.07E+07	5.8E+05	*	
+	1	<i>Phragmites</i>	Particulate	6.78E+04	2.4E+05	*	3.62E+04	2.8E+04	*	
+	1	<i>Phragmites</i>	Gravel	7.64E+05	2.9E+04	*	5.05E+05	7.1E+25	*	
+	1	<i>Phragmites</i>	Roots	1.56E+07	9.8E+04	*	1.32E+07	7.1E+25	*	
+	1	<i>Schoenoplectus</i>	Particulate	3.99E+05	3.1E+06	*	1.05E+05	2.3E+04	*	
+	1	<i>Schoenoplectus</i>	Gravel	2.76E+05	6.0E+04	*	4.22E+04	0.0E+00	*	†
+	1	<i>Schoenoplectus</i>	Roots	1.20E+07	3.0E+05	*	5.08E+06	3.4E+05	*	
+	6	Non-Planted	Particulate	1.21E+05	4.0E+05	*	1.05E+04	7.1E+25	*	
+	6	Non-Planted	Gravel	2.91E+06	2.2E+04	*	2.66E+05	1.1E+04	*	
+	6	<i>Carex</i>	Particulate	3.74E+05	2.0E+06	*	1.32E+05	1.9E+05	*	
+	6	<i>Carex</i>	Gravel	1.42E+05	2.0E+04	*	4.22E+04	0.0E+00	*	†
+	6	<i>Carex</i>	Roots	6.45E+06	1.4E+05	*	1.41E+06	4.1E+05	*	

Table A3 (cont'd)

CL	CR	Species	Habitat	<i>nirS</i> copies	standard deviation		<i>nirK</i> copies	standard deviation	
+	6	<i>Phragmites</i>	Particulate	3.86E+05	0.0E+00	*	1.74E+05	2.4E+05	*
+	6	<i>Phragmites</i>	Gravel	2.76E+05	1.0E+04	*	2.59E+05	7.1E+25	*
+	6	<i>Phragmites</i>	Roots	1.18E+06	1.0E+05	*	4.60E+06	9.1E+04	*
+	6	<i>Schoenoplectus</i>	Particulate	1.70E+05	1.1E+05	*	5.67E+04	3.6E+04	*
+	6	<i>Schoenoplectus</i>	Gravel	3.79E+05	1.1E+04	*	4.22E+04	0.0E+00	* †
+	6	<i>Schoenoplectus</i>	Roots	8.77E+05	3.0E+04	*	1.04E+05	0.0E+00	* †

APPENDIX B

STATISTICAL MODELS

APPENDIX B: Statistical Models

A full list of the statistical models will be given here. All models tested are shown first, followed by all post-hoc tests on models with a p -value ≤ 0.1 . Bolded values were statistically significant. Two terms with a “×” separating them (*e.g.*, Habitat×Species) represent the interaction term between the effects. Models were separated into three groups for analysis and p -value adjustment: the first group includes all normalized data models, the second group includes all gene-to-gas and N₂O emission models, and the third group includes all data models split by habitat. The “planted” factor refers to plant presence, so a sample was either classified as planted or non-planted. Note that for the models including species as a factor, the non-planted samples were included as a fourth “species”.

The response for a model is the term being tested by the effects. Only 1 response is allowed to be tested per model, but multiple effects can be tested for a given response. Tables presented below represent the linear mixed-effects or linear fixed-effects models.

Table A4: Mixed-effects models of 16S rDNA-normalized data (16S = 16S rDNA). Carbon = carbon load. Habitat = particulates, gravel, or roots. Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. Interaction terms are noted by a × symbol. Replicate columns were included as random effects. One row is a single model. This collection of models was *p*-adjusted together. *P*-values ≤ 0.1 are bolded and were explored with post-hoc Tukey's tests. The planted data set excludes non-planted data, planted model effects refers to plant presence or absence.

Model effects	Response	Data set	P-values					
			Habitat	Carbon	Species	Habitat× Carbon	Habitat× Species	Carbon× Species
Habitat×Carbon×Species	N ₂ O produced/16S	planted	0.285	0.633	0.302	0.046	0.312	0.321
Habitat×Carbon×Species	N ₂ O emitted/16S	planted	0.005	0.639	0.312	0.438	0.312	0.568
Habitat×Carbon×Species	N ₂ O consumption/16S	planted	0.004	0.997	0.321	0.676	0.321	0.312
Habitat×Carbon×Species	<i>nirS</i> /16S	planted	0.037	0.968	0.312	0.312	0.302	0.302
Habitat×Carbon×Species	<i>nirS</i> + <i>K</i> /16S	planted	0.073	0.997	0.302	0.302	0.194	0.312
Habitat×Carbon×Species	<i>nosZ</i> /16S	planted	0.361	0.647	0.470	0.524	0.670	0.776
Habitat×Carbon×Species	N ₂ O produced/16S	non-roots	0.503	0.781	0.399	0.832	0.448	0.399
Habitat×Carbon×Species	N ₂ O emitted/16S	non-roots	0.399	0.781	0.582	0.781	0.180	0.435
Habitat×Carbon×Species	N ₂ O consumption/16S	non-roots	0.399	0.928	0.691	0.842	0.448	0.399
Habitat×Carbon×Species	<i>nirS</i> /16S	non-roots	0.691	0.928	0.562	0.991	0.399	0.562
Habitat×Carbon×Species	<i>nirS</i> + <i>K</i> /16S	non-roots	0.691	0.991	0.562	0.928	0.399	0.562
Habitat×Carbon×Species	<i>nosZ</i> /16S	non-roots	0.991	0.781	0.781	0.781	0.781	0.928
Model effects	Response	Data set	Habitat	Carbon	Planted	Habitat× Carbon	Habitat× Planted	Carbon× Planted
Habitat×Carbon×Planted	N ₂ O produced/16S	non-roots	0.845	0.991	0.691	0.832	0.399	0.579
Habitat×Carbon×Planted	N ₂ O emitted/16S	non-roots	0.556	0.372	0.854	0.781	0.101	0.372
Habitat×Carbon×Planted	N ₂ O consumption/16S	non-roots	0.907	0.372	0.991	0.842	0.372	0.399
Habitat×Carbon×Planted	<i>nirS</i> /16S	non-roots	0.842	0.691	0.562	0.991	0.399	0.991
Habitat×Carbon×Planted	<i>nirS</i> + <i>K</i> /16S	non-roots	0.991	0.399	0.928	0.928	0.562	0.691
Habitat×Carbon×Planted	<i>nosZ</i> /16S	non-roots	0.556	0.582	0.907	0.781	0.832	0.862

Table A5: Mixed-effects models of gas-to-gene and gas-to-gas correlations. 16S = 16S rDNA. All data was grouped for this analysis. Replicate columns were included as random effects. One row is a single model. This collection of models was *p*-adjusted together. *P*-values ≤ 0.1 in these cases imply correlation is significantly different from 0, and are bolded. They were explored with post-hoc Tukey's tests.

Model effects	Response	Data set	<i>P</i> -values	Slope
<i>nirS</i> + <i>K</i>	N ₂ O produced	all data	0.001	0.946
<i>nosZ</i>	N ₂ O consumed	all data	0.001	0.674
<i>nirS</i> + <i>K</i> /16S	N ₂ O produced/16S	all data	0.001	1.160
<i>nosZ</i> /16S	N ₂ O consumed/16S	all data	0.001	1.394
<i>nirS</i> + <i>K</i> / <i>nosZ</i>	N ₂ O produced/consumed	all data	0.009	-0.736
16S	N ₂ O emitted	all data	0.001	0.542
N ₂ O produced	N ₂ O emitted	all data	0.001	0.623
N ₂ O consumed	N ₂ O emitted	all data	0.001	0.771
<i>nirS</i> + <i>K</i>	N ₂ O emitted	all data	0.001	0.646
<i>nosZ</i>	N ₂ O emitted	all data	0.001	0.586
<i>nirS</i> + <i>K</i> / <i>nosZ</i>	N ₂ O emitted	all data	0.835	-0.072
N ₂ O produced/consumed	N ₂ O emitted	all data	0.001	0.637
N ₂ O produced/16S	N ₂ O emitted/16S	all data	0.001	0.675
N ₂ O consumed/16S	N ₂ O emitted/16S	all data	0.001	0.779
<i>nirS</i> + <i>K</i> /16S	N ₂ O emitted/16S	all data	0.001	1.416
<i>nosZ</i> /16S	N ₂ O emitted/16S	all data	0.010	0.819
<i>nirS</i> + <i>K</i> / <i>nosZ</i>	N ₂ O emitted/16S	all data	0.366	0.312
N ₂ O produced/consumed	N ₂ O emitted/16S	all data	0.835	-0.038
<i>nirS</i> + <i>K</i> / <i>nosZ</i>	N ₂ O emitted/produced	all data	0.001	0.92

Table A6: Fixed-effects models of separate habitat data. 16S = 16S rDNA. Carbon = carbon load. Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. DNE = Does not exist. Interaction terms are noted by a $\times$ symbol. One row is a single model. Each collection of habitat models was *p*-adjusted independently of the other habitats. *P*-values ≤ 0.1 are bolded and were explored with post-hoc Tukey's tests. Planted model effects refers to plant presence or absence.

Model effects	Response	Data set	<i>P</i> -values		
			Carbon	Species	Carbon $\times$ Species
Carbon $\times$ Species	N ₂ O produced	particulate	0.049	0.043	0.068
Carbon $\times$ Species	N ₂ O emitted	particulate	0.574	0.020	0.072
Carbon $\times$ Species	N ₂ O consumed	particulate	0.198	0.709	0.903
Carbon $\times$ Species	<i>nirS</i>	particulate	0.210	0.002	0.058
Carbon $\times$ Species	<i>nirS+K</i>	particulate	0.210	0.002	0.059
Carbon $\times$ Species	<i>nosZ</i>	particulate	0.198	0.002	0.028
Carbon $\times$ Species	16S	particulate	0.001	0.051	0.158
Carbon $\times$ Species	N ₂ O produced	gravel	0.717	0.001	0.223
Carbon $\times$ Species	N ₂ O emitted	gravel	0.714	0.060	0.030
Carbon $\times$ Species	N ₂ O consumed	gravel	0.843	0.804	0.610
Carbon $\times$ Species	<i>nirS</i>	gravel	0.867	0.001	0.001
Carbon $\times$ Species	<i>nirS+K</i>	gravel	0.867	0.001	0.001
Carbon $\times$ Species	<i>nosZ</i>	gravel	0.843	0.001	0.002
Carbon $\times$ Species	16S	gravel	0.867	0.003	0.002
Carbon $\times$ Species	N ₂ O produced	root	0.890	0.690	0.931
Carbon $\times$ Species	N ₂ O emitted	root	0.690	0.890	0.690
Carbon $\times$ Species	N ₂ O consumed	root	0.890	0.690	0.890
Carbon $\times$ Species	<i>nirS</i>	root	0.690	0.690	0.690
Carbon $\times$ Species	<i>nirS+K</i>	root	0.690	0.690	0.690
Carbon $\times$ Species	<i>nosZ</i>	root	0.890	0.690	0.729
Carbon $\times$ Species	16S	root	0.690	0.690	0.690

Table A6 (cont'd)

Model effects	Response	Data set	Carbon	Planted	Carbon× Planted
Carbon×Planted	N ₂ O produced	particulate	0.003	0.282	0.574
Carbon×Planted	N ₂ O emitted	particulate	0.001	0.544	0.443
Carbon×Planted	N ₂ O consumed	particulate	0.903	0.282	0.911
Carbon×Planted	<i>nirS</i>	particulate	0.002	0.001	0.015
Carbon×Planted	<i>nirS+K</i>	particulate	0.002	0.001	0.014
Carbon×Planted	<i>nosZ</i>	particulate	0.001	0.001	0.003
Carbon×Planted	16S	particulate	0.002	0.001	0.016
Carbon×Planted	N ₂ O produced	gravel	0.003	0.767	0.128
Carbon×Planted	N ₂ O emitted	gravel	0.031	0.442	0.419
Carbon×Planted	N ₂ O consumed	gravel	0.092	0.413	0.246
Carbon×Planted	<i>nirS</i>	gravel	0.001	0.008	0.001
Carbon×Planted	<i>nirS+K</i>	gravel	0.001	0.001	0.001
Carbon×Planted	<i>nosZ</i>	gravel	0.001	0.171	0.001
Carbon×Planted	16S	gravel	0.002	0.318	0.023
Carbon	N ₂ O produced	root	0.690	DNE	DNE
Carbon	N ₂ O emitted	root	0.690	DNE	DNE
Carbon	N ₂ O consumed	root	0.890	DNE	DNE
Carbon	<i>nirS</i>	root	0.690	DNE	DNE
Carbon	<i>nirS+K</i>	root	0.690	DNE	DNE
Carbon	<i>nosZ</i>	root	0.690	DNE	DNE
Carbon	16S	root	0.690	DNE	DNE

Table A7: Post-hoc Tukey's test for 16S rDNA-normalized data (16S = 16S rDNA). Carbon = carbon load (−C and +C). Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. *P*-values ≤ 0.05 are considered significant and are bolded. The planted data set excludes non-planted data. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.

Significant Effect			Response, <i>P</i> -values					
Habitat	Model Effects	Data set	N ₂ O produced /16S	N ₂ O emitted /16S	N ₂ O consumed /16S	<i>nirS</i> /16S	<i>nirS</i> + <i>K</i> /16S	<i>nosZ</i> /16S
Particulate ≠ Gravel	Habitat×Carbon×Species	planted	-	0.021	0.004	0.137	0.345	-
Roots ≠ Gravel	Habitat×Carbon×Species	planted	-	0.001	0.001	0.001	0.001	-
Roots ≠ Particulate	Habitat×Carbon×Species	planted	-	0.019	0.010	0.069	0.089	-
Habitat×Carbon	Model Effects	Data set	N ₂ O produced /16S	N ₂ O emitted /16S	N ₂ O consumed /16S	<i>nirS</i> /16S	<i>nirS</i> + <i>K</i> /16S	<i>nosZ</i> /16S
Particulate−C ≠ Gravel−C	Habitat×Carbon×Species	planted	0.001	-	-	-	-	-
Roots−C ≠ Gravel−C	Habitat×Carbon×Species	planted	0.205	-	-	-	-	-
Roots−C ≠ Particulate−C	Habitat×Carbon×Species	planted	0.025	-	-	-	-	-
Particulate+C ≠ Gravel+C	Habitat×Carbon×Species	planted	0.010	-	-	-	-	-
Roots+C ≠ Gravel+C	Habitat×Carbon×Species	planted	0.001	-	-	-	-	-
Roots+C ≠ Particulate+C	Habitat×Carbon×Species	planted	0.376	-	-	-	-	-
Particulate+C ≠ Particulate−C	Habitat×Carbon×Species	planted	0.023	-	-	-	-	-
Roots+C ≠ Roots−C	Habitat×Carbon×Species	planted	1.000	-	-	-	-	-
Gravel+C ≠ Gravel−C	Habitat×Carbon×Species	planted	0.405	-	-	-	-	-

Table A8: Post-hoc Tukey's test for particulate data. 16S = 16S rDNA. N₂O cons. = N₂O consumed. Carbon = carbon load (–C and +C). Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. *P*-values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.

Significant Effect			Response, <i>P</i> -values						
Carbon Load	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
+C ≠ –C	Carbon×Species	particulate	0.026	-	-	-	-	-	0.001
Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
Non-Planted ≠ <i>Carex</i>	Carbon×Species	particulate	0.058	0.030	-	0.003	0.002	0.002	0.012
<i>Phragmites</i> ≠ <i>Carex</i>	Carbon×Species	particulate	0.615	0.009	-	0.863	0.819	0.974	1.000
<i>Schoenoplectus</i> ≠ <i>Carex</i>	Carbon×Species	particulate	0.026	0.031	-	0.606	0.452	0.770	0.993
<i>Phragmites</i> ≠ Non-Planted	Carbon×Species	particulate	0.320	0.790	-	0.007	0.004	0.003	0.013
<i>Schoenoplectus</i> ≠ Non-Planted	Carbon×Species	particulate	0.932	1.000	-	0.001	0.001	0.001	0.009
<i>Schoenoplectus</i> ≠ <i>Phragmites</i>	Carbon×Species	particulate	0.147	0.782	-	0.250	0.149	0.540	0.992
Carbon×Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
<i>Carex</i> +C ≠ 0. <i>Carex</i> –C	Carbon×Species	particulate	0.234	0.996	-	0.766	0.769	0.814	-
Non-Planted+C ≠ Non-Planted–C	Carbon×Species	particulate	0.004	0.028	-	0.015	0.014	0.003	-
<i>Phragmites</i> +C ≠ <i>Phragmites</i> –C	Carbon×Species	particulate	0.022	0.008	-	0.966	0.874	0.387	-
<i>Schoenoplectus</i> +C ≠ <i>Schoenoplectus</i> –C	Carbon×Species	particulate	0.001	0.075	-	1.000	1.000	0.877	-
Non-Planted–C ≠ <i>Carex</i> –C	Carbon×Species	particulate	0.150	0.083	-	0.008	0.005	0.005	-
<i>Phragmites</i> –C ≠ <i>Carex</i> –C	Carbon×Species	particulate	0.894	0.026	-	0.990	0.981	1.000	-
<i>Schoenoplectus</i> –C ≠ <i>Carex</i> –C	Carbon×Species	particulate	0.070	0.084	-	0.888	0.764	0.967	-
<i>Phragmites</i> –C ≠ Non-Planted–C	Carbon×Species	particulate	0.612	0.973	-	0.021	0.013	0.008	-

Table A8 (cont'd)

Carbon×Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS+K</i>	<i>nosZ</i>	16S
<i>Schoenoplectus</i> –C ≠ Non-Planted–C	Carbon×Species	particulate	0.998	1.000	-	0.002	0.001	0.002	-
<i>Schoenoplectus</i> –C ≠ <i>Phragmites</i> –C	Carbon×Species	particulate	0.335	0.971	-	0.510	0.339	0.841	-
Non-Planted+C ≠ <i>Carex</i> +C	Carbon×Species	particulate	1.000	1.000	-	0.500	0.323	0.959	-
<i>Phragmites</i> +C ≠ <i>Carex</i> +C	Carbon×Species	particulate	0.998	1.000	-	0.857	0.937	1.000	-
<i>Schoenoplectus</i> +C ≠ <i>Carex</i> +C	Carbon×Species	particulate	0.909	0.998	-	0.999	1.000	0.987	-
<i>Phragmites</i> +C ≠ Non-Planted+C	Carbon×Species	particulate	1.000	1.000	-	0.994	0.853	0.810	-
<i>Schoenoplectus</i> +C ≠ Non-Planted+C	Carbon×Species	particulate	0.971	0.992	-	0.757	0.528	0.618	-
<i>Schoenoplectus</i> +C ≠ <i>Phragmites</i> +C	Carbon×Species	particulate	0.998	0.982	-	0.983	0.996	1.000	-

Table A9: Post-hoc Tukey's test for gravel data. 16S = 16S rDNA. N₂O cons. = N₂O consumed. Carbon = carbon load (–C and +C). Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. The “–” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. *P*-values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.

Significant Effect			Response, <i>P</i> -values						
Carbon Load	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
+C ≠ –C	Carbon×Species	gravel	-	-	-	-	-	-	-
Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
Non-Planted ≠ <i>Carex</i>	Carbon×Species	gravel	0.942	0.044	-	0.026	0.079	0.665	0.068
<i>Phragmites</i> ≠ <i>Carex</i>	Carbon×Species	gravel	1.000	0.083	-	0.388	0.449	0.665	0.025
<i>Schoenoplectus</i> ≠ <i>Carex</i>	Carbon×Species	gravel	0.857	0.055	-	0.621	0.620	0.780	0.999
<i>Phragmites</i> ≠ Non-Planted	Carbon×Species	gravel	0.627	0.969	-	0.264	0.577	1.000	0.891
<i>Schoenoplectus</i> ≠ Non-Planted	Carbon×Species	gravel	0.995	0.998	-	0.005	0.014	0.001	0.083
<i>Schoenoplectus</i> ≠ <i>Phragmites</i>	Carbon×Species	gravel	0.001	0.991	-	0.074	0.088	0.001	0.031
Carbon×Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
<i>Carex</i> +C ≠ 0. <i>Carex</i> –C	Carbon×Species	gravel	-	0.998	-	1.000	1.000	1.000	1.000
Non-Planted+C ≠ Non-Planted–C	Carbon×Species	gravel	-	0.027	-	0.001	0.001	0.016	0.001
<i>Phragmites</i> +C ≠ <i>Phragmites</i> –C	Carbon×Species	gravel	-	0.011	-	0.172	0.050	0.217	0.006
<i>Schoenoplectus</i> +C ≠ <i>Schoenoplectus</i> –C	Carbon×Species	gravel	-	0.197	-	0.998	0.997	0.995	1.000
Non-Planted–C ≠ <i>Carex</i> –C	Carbon×Species	gravel	-	0.116	-	0.070	0.197	0.966	0.171
<i>Phragmites</i> –C ≠ <i>Carex</i> –C	Carbon×Species	gravel	-	0.205	-	0.695	0.761	0.966	0.069
<i>Schoenoplectus</i> –C ≠ <i>Carex</i> –C	Carbon×Species	gravel	-	0.143	-	0.898	0.897	0.989	1.000
<i>Phragmites</i> –C ≠ Non-Planted–C	Carbon×Species	gravel	-	1.000	-	0.532	0.869	1.000	0.994

Table A9 (cont'd)

Carbon×Species	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O cons.	<i>nirS</i>	<i>nirS+K</i>	<i>nosZ</i>	16S
<i>Schoenoplectus</i> –C ≠ Non-Planted–C	Carbon×Species	gravel	-	1.000	-	0.015	0.041	0.654	0.206
<i>Schoenoplectus</i> –C ≠ <i>Phragmites</i> –C	Carbon×Species	gravel	-	1.000	-	0.185	0.217	0.654	0.084
Non-Planted+C ≠ <i>Carex</i> +C	Carbon×Species	gravel	-	0.668	-	0.033	0.017	0.094	0.018
<i>Phragmites</i> +C ≠ <i>Carex</i> +C	Carbon×Species	gravel	-	0.183	-	0.966	0.507	0.851	0.413
<i>Schoenoplectus</i> +C ≠ <i>Carex</i> +C	Carbon×Species	gravel	-	1.000	-	1.000	1.000	0.961	1.000
<i>Phragmites</i> +C ≠ Non-Planted+C	Carbon×Species	gravel	-	0.912	-	0.114	0.228	0.483	0.298
<i>Schoenoplectus</i> +C ≠ Non-Planted+C	Carbon×Species	gravel	-	0.829	-	0.049	0.024	0.327	0.029
<i>Schoenoplectus</i> +C ≠ <i>Phragmites</i> +C	Carbon×Species	gravel	-	0.273	-	0.996	0.645	1.000	0.606

Table A10: Post-hoc Tukey's test for particulate and gravel data. 16S = 16S rDNA. Carbon = carbon load (−C and +C). Species = *Carex*, *Schoenoplectus*, *Phragmites*, or non-planted. The “−” symbol means the original model showed no significance for that significant effect. Interaction terms are noted by a × symbol. One column is a single model. *P*-values ≤ 0.05 are considered significant and are bolded. Planted model effects refers to plant presence or absence. The ≠ symbol is an abbreviation for the statistical test, which tests whether the difference between the two groups is significantly different from 0.

Significant Effect			Response, <i>P</i> -values						
Carbon Load	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
+C ≠ −C	Carbon×Planted	particulate	0.001	0.018	-	0.001	0.002	0.001	0.001
Planted	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
Planted ≠ Non-Planted	Carbon×Planted	particulate	-	-	-	0.001	0.001	0.001	0.001
Carbon×Planted	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
Non-Planted+C ≠ Non-Planted−C	Carbon×Planted	particulate	-	-	-	0.003	0.004	0.001	0.002
Planted−C ≠ Non-Planted−C	Carbon×Planted	particulate	-	-	-	0.001	0.001	0.001	0.001
Planted+C ≠ Non-Planted+C	Carbon×Planted	particulate	-	-	-	0.389	0.200	0.287	0.467
Planted+C ≠ Planted−C	Carbon×Planted	particulate	-	-	-	0.614	0.621	0.039	0.353
Carbon Load	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
+C ≠ −C	Carbon×Planted	gravel	0.001	0.016	0.003	0.001	0.001	0.001	0.001
Planted	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS</i> + <i>K</i>	<i>nosZ</i>	16S
Planted ≠ Non-Planted	Carbon×Planted	gravel	-	-	-	0.004	0.024	-	-

Table A10 (cont'd)

Carbon×Planted	Model Effects	Data set	N ₂ O produced	N ₂ O emitted	N ₂ O consumed	<i>nirS</i>	<i>nirS+K</i>	<i>nosZ</i>	16S
Non-Planted+C ≠ Non-Planted−C	Carbon×Planted	gravel	-	-	-	0.001	0.001	0.002	0.003
Planted−C ≠ Non-Planted−C	Carbon×Planted	gravel	-	-	-	0.017	0.094	0.707	0.576
Planted+C ≠ Non-Planted+C	Carbon×Planted	gravel	-	-	-	0.009	0.012	0.037	0.049
Planted+C ≠ Planted−C	Carbon×Planted	gravel	-	-	-	0.578	0.408	0.180	0.228

APPENDIX C

GAS CHROMATOGRAPHY ADDITIONAL INFORMATION

APPENDIX C: Gas Chromatography Additional Information

The N₂O standard curve from initial peak area and ppm data was not well-represented by a polynomial fit (which was the expected fit), so adjustments were needed to better fit the data. The standard curve for N₂O concentrations was tightened by converting all N₂O standards into the number of moles in the GC sample loop using $PV = nRT$. That conversion accounted for small fluctuations in temperature and pressure which affected the number of moles in the GC sample loop at any given time. Accounting for small fluctuations in the machine sensitivity through the use of ambient air samples also tightened the data. Ambient air samples were collected at the beginning and ending of every batch of samples that were run on the GC. Each sample was assigned an adjustment factor based on the linear interpolation of the lab airs from the GC sample batch. For example, if the lab air GC peak area was 10 counts in the beginning of sampling and 5 counts at the end of sampling and 4 samples were run in that batch, the samples would be given values of 9, 8, 7, and 6, in that order. Adjusting sample peak areas by the difference in their assigned adjustment factor and the average ambient lab N₂O peak area further tightened the standard curve. That adjustment was performed in log-transformed space with the equation $adjusted\ peak\ area = e^{(\ln(peak\ area) - (\ln(lab\ air) - \ln(lab\ air\ average)))}$, where lab air average was the average value of all lab air samples.

After the peak area adjustment, data points for respective concentrations along both log and linear standard curves more closely grouped resulting in tighter standard curves. Multiple fits and transformations were investigated using linear models. A

standard curve was fit to both the log-log plot of peak area versus moles of N₂O in the sample loop, as well as to the linear plot of the same values. The log-log plot fit an order of magnitude better at the 0.1 ppm standard, but was equal or worse at all other standard values. Applying two distinct standard curves for low and high N₂O concentrations was also considered for this. The three main curves considered are shown in Table A11. The linear plot was chosen as a better fit of the majority of data, which was above 1 ppm.

Table A11: N₂O concentration potential standard curve fits. The selected fit is bolded.

Standard curve (peak area vs moles N ₂ O)	Slope	Intercept	Adjusted R^2
Log-log (forced intercept)	-1.672	0	0.9602
Log-log	1.222	-39.35	0.9901
Linear-linear (forced intercept)	2.176×10^{-16}	0	0.9897

Some N₂O data points extend beyond the lower boundary of the standard curve. This was accounted for by making the standard curve pass through the point (0, 0).

One set of standards was excluded from the standard curve. Ambient lab air samples correlating to these standards were consistent with other samples, but the standards were clearly distinct from other standards in a plot. It was thought these standards were made incorrectly, and were removed as outliers.

APPENDIX D

PARTICULATE TOTAL ORGANIC CARBON DATA

APPENDIX D: Particulate Total Organic Carbon Data

Total organic carbon (TOC) data was collected for particulate samples but was not used in the analysis. It is presented here as supplemental information in Table A12. Samples were prepared with a homogenizer (T25S1, IKA, Germany). Samples were homogenized using 40 mL of stored biomass at 20500 rotations per minute for 30 seconds, separated by a 15 second homogenization of 70% ethanol followed by 15 second homogenization of nanopure water. Analysis was performed on a Skalar Formacs TOC/TN Analyzer (Skalar, Inc, GA), using a 950 °C total carbon temperature, and a 90 °C inorganic carbon temperature. Three triplicates were measured from the same sample solution. The averages are reported here. Three samples with high TOC values were analyzed a second time; the two triplicate averages were averaged and reported.

Table A12: Total organic carbon (TOC) data for the particulate fraction. DNE = Does not exist. Two biological column replicates were sampled, as represented by column replicate numbers 1 and 6. Total carbon (TC), inorganic carbon (IC), and TOC are reported in mg L^{-1} .

Carbon Load	Column Replicate	Species	TC	IC	TOC
–	1	Non-Planted	21.0	17.9	3.1
–	1	<i>Carex</i>	43.2	19.8	23.4
–	1	<i>Phragmites</i>	54.6	26.9	27.7
–	1	<i>Schoenoplectus</i>	63.8	18.1	45.7
–	6	Non-Planted	32.9	15.2	17.8
–	6	<i>Carex</i>	53.2	18.8	34.4
–	6	<i>Phragmites</i>	103.5	18.1	85.4
–	6	<i>Schoenoplectus</i>	75.3	26.5	48.8
+	1	Non-Planted	28.1	16.4	11.7
+	1	<i>Carex</i>	68.6	24.9	43.7
+	1	<i>Phragmites</i>	60.0	23.9	36.1
+	1	<i>Schoenoplectus</i>	98.7	30.4	68.3
+	6	Non-Planted	DNE	DNE	DNE
+	6	<i>Carex</i>	110.6	21.7	88.9
+	6	<i>Phragmites</i>	104.3	35.3	69.0
+	6	<i>Schoenoplectus</i>	162.0	26.7	167.5