

**Elucidating the Impact of Biosolids-Derived Antimicrobials on Denitrifying
Microbial Community Function and Structure in Agricultural Soil**

by

Ryan Michael Holzem

Department of Civil and Environmental Engineering
Duke University

Date: _____

Approved:

Claudia Gunsch, Supervisor

Heather Stapleton

Rytas Vilgalys

Lingchong You

Francis de los Reyes III

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Civil and Environmental Engineering in the Graduate School
of Duke University

2014

ABSTRACT

**Elucidating the Impact of Biosolids-Derived Antimicrobials on Denitrifying
Microbial Community Function and Structure in Agricultural Soil**

by

Ryan Michael Holzem

Department of Civil and Environmental Engineering
Duke University

Date: _____

Approved:

Claudia Gunsch, Supervisor

Heather Stapleton

Rytas Vilgalys

Lingchong You

Francis de los Reyes III

An abstract of a dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
Civil and Environmental Engineering in the Graduate School of
Duke University
2014

Copyright by
Ryan Michael Holzem
2014

Abstract

More than 50% of wastewater biosolids are applied to agricultural fields as fertilizer in the U.S. This technique has been used for decades as a widely accepted beneficial reclamation method for biosolids, which meet the established regulatory levels for nutrients, metals, and pathogens. A major drawback to land application is the potential environmental release of non-regulated organic contaminants, which accumulate in biosolids during the wastewater treatment process. Recent studies have been performed to identify and quantify the presence of emerging contaminants in biosolids, and others have investigated the effects of compounds already identified as ‘priority pollutants’ and whose use is waning. However, there is limited research on the effect of emerging organic contaminants on soil microbial ecology and nutrient cycling. Because many of the compounds found in biosolids are specifically designed to elicit biological modifications (e.g., antimicrobials), there is a risk that these compounds will disrupt microbial soil functions, decrease soil productivity, and ultimately affect the long term viability of these ecosystems, resulting in unforeseen economic and social costs. Therefore, there is a clear need to characterize the effects of novel contaminants on soil health.

This dissertation was divided into three distinct parts examining the impacts of emerging organic contaminants on soil microbial ecology with increasing complexity to

better reflect environmental conditions. To assess the ecological impacts, the functional endpoint of denitrification was selected because it provides a vital indication of soil health. Denitrifying bacteria play a critical role in this process, and thus, were used as indicator organisms for determining contaminant ecotoxicological potential.

Furthermore, antimicrobial agents (a.k.a., bactericides or biocides) were selected as model contaminants because they are designed specifically to deactivate microorganisms, are heavily used in the U.S with over \$1 billion in yearly sales, and have been measured in biosolids.

Overall, the objectives of this dissertation were to: 1) develop a rapid, high-throughput functional assay that measured denitrification inhibition for screening potential ecological impacts of biosolids-derived antimicrobial agents, 2) determine the potential effects of common and emerging biosolids-derived antimicrobial agents on denitrification by a model soil denitrifier, *Paracoccus denitrificans* PD1222, 3) examine the impacts of the most commonly used antimicrobial, triclosan (TCS), on wastewater treatment efficiency in bench scale sequencing batch reactors (SBRs) coupled with anaerobic digesters, 4) examine the impacts of biosolids aged and spiked with TCS on denitrification under simulated agricultural soil conditions, and 5) evaluate potential impacts of TCS in 'traditional' biosolids on denitrification in agricultural soil under field conditions.

The first phase of research pertaining to Objectives 1 and 2 examined the baseline interactions between biosolids-derived antimicrobial agents and soil microbial ecology. However, to isolate the effect of an individual contaminant from the myriad of contaminants found in biosolids, there was a need for developing a rapid, high-throughput method to evaluate general ecotoxicity. In the first part of this dissertation, we developed a novel assay that measured denitrification inhibition in a model soil denitrifier, *Paracoccus denitrificans* Pd1222. Two common (TCS and triclocarban) and four emerging (2,4,5 trichlorophenol, 2-benzyl-4-chlorophenol, 2-chloro-4-phenylphenol, and bis(5-chloro-2-hydroxyphenyl)methane) antimicrobial agents found in biosolids were analyzed as model contaminants. Overall, the assay was reproducible and measured impacts on denitrification over three orders of magnitude exposure. The lowest observable adverse effect concentrations (LOAECs) were 1.04 μM for TCS, 3.17 μM for triclocarban, 0.372 μM for bis-(5-chloro-2-hydroxyphenyl)methane, 4.89 μM for 2-chloro-4-phenyl phenol, 45.7 μM for 2-benzyl-4-chlorophenol, and 50.6 μM for 2,4,5-trichlorophenol. Compared with gene expression and cell viability based methods, the denitrification assay was more sensitive and resulted in lower LOAECs. Of the six compounds examined, four resulted in LOAECs that were below or within an order of magnitude of concentrations that were measured in the environment, indicating potential ecological impacts.

In the second part of the dissertation, the impacts of emerging contaminants were examined first under laboratory conditions mimicking wastewater treatment processes (Objective 3) and then agricultural fields (Objective 4). For this phase, TCS, which is the most widely used antimicrobial agent and identified in the first phase for potential ecological impacts, was used as the model contaminant. To mimic wastewater treatment processes, bench scale SBRs coupled with anaerobic digesters were set up and operated. The SBRs and digesters were seeded with activated and anaerobically digested sludge from the North Durham Water Reclamation Facility (NDWRF, Durham, NC). Reactors were fed synthetic wastewater with or without 0.73 μM of TCS. Samples were taken periodically to monitor chemical oxygen demand (COD), ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), total suspended solids (TSS), volatile suspended solids (VSS), dissolved oxygen (DO), and phosphate (PO_4^{3-}) and pH. In addition, biomass samples were collected for DNA extraction and microbial community analysis using terminal restriction fragment length polymorphism (T-RFLP) of 16S SSU rDNA. Methane production was also monitored for the anaerobic digesters. In addition, the final digested biosolids that were generated from the SBRs fed with and without TCS were analyzed for TCS concentration, TSS, VSS, TKN, phosphorus (as P_2O_5), potassium (as K_2O), and pH. Overall, biological processes associated with nitrogen removal (nitrification and denitrification), were impacted by TCS entering the SBRs regardless of the starting microbial community. Both of the SBRs that were not receiving TCS reached

steady-state at greater than 92% NH_4^+ removal within the first week of operation, whereas the SBRs receiving TCS took 42 and 63 days to reach steady-state removal at that level. However, while NH_4^+ removal was temporarily inhibited, elevated levels of NO_3^- and NO_2^- in the effluent of the TCS fed SBRs, suggested longer-term impacts on nitrite oxidizing bacteria (NOB) and denitrifiers. After Day 58, the NO_3^- effluent concentration for the SBRs receiving TCS was 3.9 ± 0.16 mg/L, which was 2.4 times greater than the NO_3^- effluent of the SBRs not receiving TCS (1.7 ± 0.08 mg/L). Similarly, after Day 58, the NO_2^- effluent of the SBRs receiving TCS reached a steady-state concentration of 8.7 ± 0.75 mg/L. The mean NO_2^- concentration in the controls after Day 58 was 7.7 times lower at 1.1 ± 0.78 mg/L, but was still trending towards 0 when the reactors were stopped. No inhibition was observed for COD and PO_4^{3-} removal. In addition, non-metric multidimensional scaling (NMS) ordination analysis showed that the microbial communities between SBRs fed with and without TCS were similar on Day 0, but increased in difference to Day 41, around when the major changes in nitrification were observed. After a slight increase in similarity between the control and TCS SBR microbial communities on Day 41, the communities increased in difference to Day 63.

To mimic agricultural field conditions, containers of soil were amended with the biosolids generated from the SBRs. The containers were maintained in a growth-chamber to simulate field lighting and watering conditions. Three biosolids treatments

were examined: 1) biosolids generated from the SBRs not fed TCS, but that still had low backgrounds of TCS (a.k.a., Control Biosolids); 2) biosolids generated from the SBRs fed with TCS (a.k.a., Aged TCS Biosolids); and 3) biosolids that were generated by the SBRs not fed TCS, but spiked with TCS 24 h before application (a.k.a., Spiked TCS Biosolids). Alfalfa was planted in half of the containers receiving the Control and Aged TCS Biosolids to assess differences due to vegetation. To assess the overall ecotoxicity of biosolids aged and spiked with TCS, the function, abundance, and diversity of the soil denitrifying communities were examined. The impacts on total bacteria abundance and diversity were also examined for comparison. Specifically, the denitrifying enzyme activity (DEA) assay was used to measure functional impacts, quantitative polymerase chain reaction (qPCR) was used to measure impacts on abundance, and T-RFLP was used to measure impacts on diversity. Correlations between these methods were also examined for possible interactions between denitrifier function and community structure and to provide insight into targets of inhibition. Lastly, a denitrification inhibition score was developed to quantify global impacts of TCS on denitrification. The containers with plants that received biosolids aged with and spiked with TCS showed potential long-term inhibition based on measurement of soil denitrification at 26.9 ± 4.6 $\mu\text{g}/\text{kg}$ and 68.6 ± 26.9 $\mu\text{g}/\text{kg}$ of TCS, respectively. Denitrifier abundance and diversity, however, were more sensitive to TCS in biosolids and inhibition was observed throughout the experiment, with maximum inhibition on Days 7 and 28. Inhibition of

denitrifier abundance and diversity was observed at TCS concentrations as low as $17.9 \pm 1.93 \mu\text{g/L}$, which was about 10 to 3000 times lower than concentrations reported by other studies that showed impacts on other functional endpoints (i.e., respiration, phosphatase activity, NO_3^- and NO_2^- production, and Cy17 stress biomarker abundance), even after taking pH into account. Five significant correlations were developed, three of which related qPCR and the DEA assay, or abundance and activity. However, the analyses that were correlated did not yield the same results as far as significant inhibition in the presence of TCS. Thus, while the results suggested some relatedness between activity, abundance, and diversity, the results generally support the use of multiple methods to determine the ecotoxicity of biosolids-derived organic contaminants. As a result, a denitrification inhibition score was developed that took into account all three methods to determine the overall ecotoxicity of TCS in biosolids. Overall, the denitrification inhibition score showed that denitrification was inhibited by both biosolids that were aged and spiked with TCS over the extent of the 84 day experiment, but maximum inhibition occurred after a week to about a month. While the denitrification inhibition score indicated that the TCS in the biosolids aged with TCS was less bioavailable than in the spiked biosolids, the impacts of the aged and spiked biosolids could have also been due to differences in TCS concentrations.

Objective 5 consisted of a long-term soil sampling campaign on four agricultural fields receiving Class B municipal biosolids. Soil samples were taken before and after

biosolids application and were analyzed to elucidate potential impacts of TCS in the biosolids on denitrification. Again, to assess the overall impacts of TCS on the soil denitrifying community, the DEA assay, qPCR, and T-RFLP were used to measure impacts on function, abundance, and diversity, respectively. Similar to Objective 4, the analysis included an examination of potential correlations between denitrifying community structure and function, and quantification of global impacts using the denitrification inhibition score. As expected, the results in this pilot-study reflected the complexity of the system that was analyzed and many more samples, which account for variables including, but not limited to soil characteristics, biosolids characteristics, biosolids application rates, and chemical composition and quantities, would be needed to show any statistically significant differences. Nevertheless, several key results were obtained. Again potential long-term inhibition of denitrification was observed using the DEA assay, however the effects of exhaustion of resources, such as NO_3^- , or significant changes in the local environment were suspected, but could not be verified. Inhibition was also observed for denitrifier abundance, but little to no inhibition was observed when examining the relative number of denitrifying species. Thus, while the abundance of denitrifiers was reduced, and denitrification was eventually depressed, the number of species in the soil remained constant. When looking at the denitrification inhibition score, which took all three measurements into account, increased inhibition over time was observed with the exception of the measurements on Days 30 and 103, which

indicated overall, but weak inhibition of denitrification by the application of biosolids. NMS ordinations showed no correlation between the shift in denitrifying microbial community and TCS. Because of the complexity of the soil and biosolids and because of the myriad of contaminants likely in the biosolids, the results may not be significant and a more in-depth study was recommended.

Overall, the results presented in this dissertation provide a systematic evaluation of the effects of biosolids-derived TCS on agricultural soil microbial ecology. First, it was demonstrated that statistically significant inhibition of denitrification could be used as a potential indicator of biosolids-derived emerging organic contaminant ecotoxicity. The denitrification assay that was developed was then used to analyze ecotoxicological potential of six emerging biosolids-derived antimicrobial agents, and found inhibition of denitrification at environmentally relevant concentrations. The most widely used antimicrobial agent, TCS, was further shown to inhibit wastewater treatment processes, as well as, denitrification in simulated agricultural conditions after being aged with and spiked into biosolids. In addition, evidence showing potential inhibition of denitrification by TCS in 'traditional' biosolids under field conditions was also obtained. Based on these results, this dissertation asserts that biosolids-derived emerging organic contaminants pose a potential risk to agricultural soil microbial ecology and overall soil health. Future studies, however, are needed to examine the impacts of other contaminants that might be flagged with the assay developed in this dissertation under

more complex conditions mimicking the environment. Furthermore, other research is needed to examine the role microbial communities play in the bioavailability of emerging contaminants, especially TCS, and a more extensive, in-depth study is needed to characterize the individual impacts of emerging contaminants on soil microbial communities under field conditions.

Dedication

For Anne and Avni. I give you my very best.

Contents

Abstract	iv
List of Tables	xxi
List of Figures	xxii
Acknowledgements	xxvi
1. Motivation and Scope of Research	1
1.1 Introduction and Problem Definition	1
1.2 Research Hypotheses and Approach.....	5
2. Background and Literature Review	8
2.1 Wastewater Treatment.....	8
2.2 Biosolids.....	9
2.3 Emerging Contaminants.....	10
2.4 Antimicrobial Agents.....	12
2.4.1 Triclosan.....	13
2.4.2 Triclocarban.....	14
2.4.3 2,4,5 Trichlorophenol	15
2.4.4 2-Benzyl-4-chlorophenol	16
2.4.5 2-Chloro-4-phenylphenol	17
2.4.6 Bis(5-chloro-2-hydroxyphenyl)methane	17
2.4.7 Inactivation Mechanisms.....	17
2.5 Denitrification	19

2.5.1 Biology of Denitrification.....	24
2.5.2 Ecology.....	26
2.5.3 Factors Driving Soil Denitrification.....	27
2.5.3.1 Proximate Factors.....	28
2.5.3.2 Distal Factors.....	31
3. Determining the Ecological Impacts of Organic Contaminants in Biosolids Using a High-Throughput Colorimetric Denitrification Assay: A Case Study with Antimicrobial Agents.....	36
3.1 Introduction.....	36
3.2 Materials and Methods.....	40
3.2.1 Cell Growth and Preparation.....	40
3.2.2 Batch Reactor Preparation.....	41
3.2.3 Experimental Procedure.....	42
3.2.4 Denitrification Assay.....	44
3.2.5 Gene Expression Analysis.....	45
3.2.6 Cell Viability Assay.....	46
3.2.7 Analytical Methods.....	47
3.2.8 Statistical Analysis.....	48
3.3 Results and Discussion.....	48
3.3.1 Assay Development with Antimicrobial Agents.....	48
3.3.2 Implications of Potential Ecological Impacts.....	52
3.3.3 Denitrification Assay Comparison.....	54
3.3.4 Gene Expression.....	55

3.3.5 Cell Viability.....	58
3.3.6 Potential Assay Applications.....	59
4. Evaluating the impacts of Triclosan on Biological Wastewater Treatment Processes using Bench-Scale Activated Sludge Sequencing Batch Reactors Coupled with Anaerobic Digesters.....	62
4.1 Introduction.....	62
4.2 Materials and Methods.....	64
4.2.1 SBR Design and Operation.....	64
4.2.2 TCS Addition.....	68
4.2.3 Anaerobic Digester Design and Operation.....	69
4.2.4 Analytical Methods.....	71
4.2.5 DNA Extraction and PCR Conditions.....	73
4.2.6 T-RFLP Analysis.....	74
4.2.7 Statistical Analysis.....	76
4.3 Results and Discussion.....	76
4.3.1 TCS Concentrations.....	76
4.3.2 Reactor Characteristics.....	78
4.3.3 Treatment Efficiencies.....	78
4.3.4 Digestion Characteristics and Performance.....	83
4.3.5 Impacts on Microbial Community Structure.....	84
4.3.6 Implications of TCS on Wastewater Treatment Plant Performance.....	87

5. Evaluating the Community Structure and Functional Impacts of Soil Denitrifying Bacteria in Containers Amended with Laboratory Generated Biosolids Aged with Triclosan	88
5.1 Introduction.....	88
5.2 Materials and Methods	93
5.2.1 Plant Container and Growth-Chamber Setup.....	94
5.2.2 Biosolids Development.....	96
5.2.3 Biosolids Application and Sampling Protocol	98
5.2.4 Analytical Methods	101
5.2.5 Functional Impact Analysis	103
5.2.6 DNA Extraction	104
5.2.7 Community Impact Analysis.....	104
5.2.8 Statistical Analysis.....	109
5.3 Results and Discussion	110
5.3.1 Simulated Biosolids Characteristics.....	110
5.3.2 Soil Characteristics	112
5.3.3 Plant Biomass.....	115
5.3.4 Impacts on denitrifiers.....	115
5.3.4.1 Function.....	116
5.3.4.2 Abundance.....	119
5.3.4.3 Diversity	123
5.3.5 Methods Correlation	128
5.3.6 General Implications of Biosolids Application	129

6. Elucidating the Impacts of Biosolids on Native Agricultural Soil Denitrifying Bacteria	140
6.1 Introduction.....	140
6.2 Materials and Methods.....	143
6.2.1 Sample Location and Collection.....	143
6.2.2 Analytical Methods.....	146
6.2.3 Functional Impact Analysis	146
6.2.4 DNA Extraction	146
6.2.5 Community Impact Analysis.....	146
6.2.6 Statistical Analysis.....	147
6.3 Results and Discussion	148
6.3.1 TCS Concentrations.....	148
6.3.2 Soil Characteristics	149
6.3.3 Impacts on denitrifiers.....	151
6.3.3.1 Function.....	151
6.3.3.2 Abundance.....	154
6.3.3.3 Diversity	157
6.3.4 Method Correlation.....	159
6.3.5 Implications of Biosolids Application	160
7. Conclusions and Engineering Significance	164
7.1 Key Findings	164
7.2 Recommendations and Future Work.....	170

Appendix A – Chapter 3 Supplemental Information	175
A.1 Nitrate and Ammonium Measurements.....	175
A.2 Emerging Contaminants.....	176
Appendix B – Chapter 5 Supplemental Information	183
B.1 qPCR Primer Optimization.....	183
B.2 NMS Ordination Plots	186
References	188
Biography	208

List of Tables

Table 1: Physical-chemical properties of target antimicrobial agents.	19
Table 2: SBR characteristics (mean values).....	78
Table 3: Physical-chemical properties of the Control and TCS Biosolids.	99
Table 4: Linear correlations between methods used to analyze denitrification.	129
Table 5: Calculated neutral TCS in this and previous studies.	138
Table 6: Soil physical-chemical properties.	151
Table 7: Linear correlation between methods used to analyze field denitrification.	160
Table 8: List of phenols and chlorinated phenols examined in preliminary experiments.	179

List of Figures

Figure 1: Simplified schematic of nitrogen cycle [83].	21
Figure 2: Denitrification pathway. Nar, NO ₃ ⁻ reductase; Nir, NO ₂ ⁻ reductase; Nor, NO reductase; Nos, N ₂ O reductase [94].	26
Figure 3: Relative denitrification rate in the presence of varying concentrations of six antimicrobial agents and measured by the denitrification assay. (*) indicates a statistical significant difference from the control (p ≤ 0.05). (@) indicates a statistical significant difference from 0 (p ≤ 0.05). Values < 1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error.....	51
Figure 4: Gene expression and cell viability assay results for triclosan and triclocarban. Gene expression is represented by the absolute gene transcript numbers of nirS and nosZ relative to 16s rRNA (left Y-axis) and absolute gene transcript numbers for 16S rRNA (right Y-axis). Cell viability is represented by percent viable relative to the control. (*) indicates a significant different from control (p-value ≤ 0.05). Cell viability values < 1 indicate decreased cell viability as compared to the control. Error bars represent one standard error.....	55
Figure 5: Schematic and photo of SBR and anaerobic setup. Note that the SBRs are not yet covered and have not been inoculated, and the SBR and the anaerobic digesters are not connected.....	67
Figure 6: COD, PO ₄ ³⁻ , and NH ₄ ⁺ , removal percentages. Error bars represent one standard error.....	81
Figure 7: Effluent nitrate and nitrite concentrations. Error bars represent one standard error.....	83
Figure 8: Relative abundance of species in SBRs over time. (@) indicates a significant difference from Day 0 (p-value ≤ 0.05). (#) indicates a significant difference from the control at the same sample day (p-value ≤ 0.05). (\$) indicates a marginal significant difference from the control at the same sample day (p-value ≤ 0.10). Values > 1 indicate an increase in the number of species compared to Day 0. Error bars represent one standard error.....	85

Figure 9: NMS ordination analysis of 16S in SBRs. (C) indicates control SBR samples. (T) indicates TCS SBR samples.....	86
Figure 10: Growth-Chamber with plant containers.	96
Figure 11: Day 0 TCS concentration in biosolids amended soil treatments. (*) indicates a significant difference (p-value ≤ 0.05) from the respective control with and without plants.....	112
Figure 12: Day 0 and 84 amended soil OC, TKN, and NO_3^- concentrations for each treatment. (@) indicates a significant difference (p-value ≤ 0.05) from Day 0. (%) indicates a marginal significant difference (p-value ≤ 0.10) from Day 0. Only Day 0 measurements were taken for the unamended soil. Error bars represent one standard error.....	114
Figure 13: Mean total alfalfa biomass for treatments with plants. No replicates were set up with plants that received biosolids spiked with TCS. Error bars represent one standard error.....	115
Figure 14: Relative denitrification rate of control, TCS aged, and TCS spiked biosolids-amended soil with and without plants. (@) indicates a significant difference from Day 0 (p-value ≤ 0.05). (%) indicates a marginal significant difference from Day 0 (p-value ≤ 0.10). (#) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.05). (\$) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.10). Values < 1 indicate inhibition of denitrification as compared to Day 0. Error bars represent one standard error.....	119
Figure 15: Relative total bacteria and denitrifier abundance. (@) indicates a significant difference from Day 0 (p-value ≤ 0.05). (%) indicates a marginal significant difference from Day 0 (p-value ≤ 0.10). (#) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.05). (\$) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.10). Values < 1 indicate a decrease in absolute copy number or ratio of target gene to 16S as compared to the Day 0. Error bars represent one standard error.	123
Figure 16: Relative total bacteria and denitrifier diversity. (@) indicates a significant difference from Day 0 (p-value ≤ 0.05). (%) indicates a marginal significant difference from Day 0 (p-value ≤ 0.10). (#) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.05). (\$) indicates a significant difference from the respective no plants or plants control on the same day (p-value \leq	

0.10). Values < 1 indicate a decrease in in the number of species compared to the Day 0 Error bars represent one standard error.	127
Figure 17: Denitrification inhibition score for TCS treatments.....	134
Figure 18: Pre and post-biosolids application TCS concentration in soil. Error bars represent one standard error.	149
Figure 19: Relative denitrification rates measured in examination fields before and after biosolids application. (*) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.05). Values < 1 indicate inhibition of denitrification as compared to the Day 0. Error bars represent one standard error.....	154
Figure 20: Relative total bacteria and denitrifier abundance. (*) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.05). (#) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.10). Values < 1 indicate a decrease in absolute copy number or ratio of target gene to 16S compared to the Day 0. Error bars represent one standard error.	156
Figure 21: Relative total bacteria and denitrifier diversity. (*) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.05). (#) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.10). Values < 1 indicate a decrease in the number of species compared to the Day 0. Error bars represent one standard error.	158
Figure 22: NMS ordination of T-RFLP data.	159
Figure 23: Denitrification inhibition score for field biosolids application.	163
Figure 24: The effects of various emerging contaminants on denitrification. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05). Values <1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error.	177
Figure 25: The effects of several QACs on denitrification by <i>P. denitrificans</i> PD1222. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05). Values <1 indicate inhibition of denitrification as compared to the control. Error bars represent one standard error.	178

Figure 26: Relative denitrification rate in the presence of triclocarban and selected phenols and chlorinated phenols. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05), (@ indicates a marginal statistical significant difference from the control (p-value ≤ 0.06), and (#) indicates a weak statistical significant difference from the control (p-value ≤ 0.15). Values <1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error..... 180

Figure 27: Relative denitrification for four species of denitrifiers exposed to Bis(5-chloro-2-hydroxyphenyl)methane. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05). Values <1 indicate inhibition of denitrification as compared to the control. Error bars represent one standard error..... 182

Figure 28: nosZ qPCR primer optimization. 185

Figure 29: nirS qPCR primer optimization. 185

Figure 30: nirK qPCR primer optimization. 186

Figure 31: NMS ordination of growth-chamber T-RFLP data. 187

Acknowledgements

I would first like to thank my wife, Anne, for always being my biggest supporter and emotional foundation. Anne, I honestly could have never have made it through my Ph.D. without you. You were there for me during the darkest times of exhaustion and frustration, and you always kept a smile when “just 15 minutes” turned into several hours. Thank you and I love you. I would also like to thank my daughter, Avni. Knowing that I would come home every night and see you helped me stay strong during the last year and a half of my research. I hope the completion of this document and all the work it entailed, shows you that you can accomplish your loftiest of goals.

I would like to thank my family and friends for their support and encouragement. I would especially like to thank my Grandpa and my parents for doing all they could to keep me going and showing that they were always there for me. All of the visits and care-packages made home not feel so far away. Thank you to my HLM A.J, as well as Nate and Eric for visiting me in North Carolina. I look forward to spending more time with you guys now that we will be in same state.

I would like to thank my adviser Dr. Claudia Gunsch for all of the time and support she committed to me throughout my dissertation research. Without her, I am not sure what I would be doing now. She believed in me in 2006, when she hired a kid from a small, agricultural state-school in Wisconsin as an REU student. Then, she encouraged me to return for my Ph.D. in 2010 even with limited microbiology

background. Her confidence in me and her ability to keep me motivated, especially when everything failed in the lab, was truly amazing and inspiring. I only hope that I can be as strong of a mentor to others, as she has been to me.

I would also like to thank Dr. Heather Stapleton, Dr. Rytas Vilgalys, Dr. Francis de los Reyes, and Dr. Lingchong You for serving on my Ph.D. committee and for their support and guidance on my research. It is truly my honor to have had your involvement in my Ph.D. career. I would also like to thank Dr. Stapleton again for letting me use her laboratory as I attempted to learn analytical chemistry.

Lastly, I would like to thank my fellow new and former colleagues at Duke, Kaoru, Christina, Thomas, Matt, Ashley, Grace, Lauren B., Carley, Courtney, Ross, Tony, Lauren C., Lauren R., for all of the help and interesting conversations over the years. I would also like to thank the undergraduate researches Bridget and Shanice, who helped me complete this research. A special thanks to Kaoru for teaching me how to work in a lab and for always taking the time to answer my research and life related questions.

The following sources of funding made this work possible: NIH's Center for Biomolecular and Tissue Engineering Training Program and the National Science Foundation Grant CBET-0854167. Any opinions, findings, and conclusions or recommendations expressed in this dissertation are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

1. Motivation and Scope of Research

The following section outlines the motivations and the scope of the research that was completed for my Ph.D.

1.1 Introduction and Problem Definition

Currently, land application of sewage sludge, also known as biosolids, accounts for approximately 50% of treated wastewater solid disposal in the United States (U.S.).

Biosolids have traditionally been applied to agricultural fields in order to recover their macronutrients, primarily nitrogen and phosphorus, and as an alternative to incineration or land filling [1-3]. This technique has been used for decades as a widely accepted beneficial reclamation method for biosolids, which meet the established regulatory levels for nutrients, metals, and pathogens established under 40 CFR Part 503 [4]. A major drawback to land application is the potential environmental release of non-regulated organic contaminants, which accumulate in biosolids during the wastewater treatment process [2, 5]. In fact, a recent review found that more than 500 different organic chemicals have been identified in biosolids around the U.S. [6]. Recent research has been performed to identify and quantify the presence of emerging contaminants in biosolids. However, there is limited research available on the effect of these organic contaminants on soil microbial ecology and nutrient cycling. Because many of the compounds found in biosolids are specifically designed to elicit biological modifications

(e.g., antimicrobials) there is a risk that these compounds will disrupt ecologically important microbial soil functions, decrease soil productivity, and ultimately affect the long term viability of these ecosystems, resulting in unforeseen economic and social costs. Therefore, there is a clear need to characterize the effects of novel contaminants on soil health.

The main objective of my Ph.D. was to determine the effects of biosolids-derived emerging organic contaminants on the microbial ecology and function of agricultural soil. To assess these impacts, the functional endpoint of denitrification was selected because it provides a vital indication of soil health. Denitrification consists of the stepwise reduction of the soluble nitrogen oxides, nitrate (NO_3^-) and nitrite (NO_2^-), to nitrous oxide (N_2O) and N_2 [7]. Denitrifying bacteria play a critical role in this process, and thus, are ideal indicator organisms for determining contaminant ecotoxicological potential. Specifically, denitrifiers: 1) play a major role in both the nitrogen and carbon cycles, which are essential for microbial, soil, and plant health [8]; 2) are known to degrade synthetic fertilizers added to agricultural fields and produce a major portion of the greenhouse gas nitrous oxide (N_2O) [9-13]; 3) are active in the amelioration of excess nitrogen fertilizer, which reduces the runoff load to streams after precipitation events [14]; and 4) are known to produce intermediates that may be toxic to the local ecosystem, such as NO_2^- and nitric oxide (NO) [7]. Because of the global implications of denitrification, a close examination of these organisms is essential in fully understanding

the impacts of the biosolids-derived emerging contaminants on soil microbial ecology. Any observable changes in denitrifying community activity and structure may reflect overall soil health and resilience.

Previous studies have focused on compounds already identified as ‘priority pollutants’ and whose use is waning as a result [15]. One important class of biosolids-derived emerging organic contaminants is antimicrobial agents (a.k.a., bactericides or biocides). These chemicals are of concern because they are designed to deactivate microorganisms, are heavily used in the U.S with over \$1 billion in yearly sales [16], and have been measured in biosolids. The most common antimicrobials found in biosolids are triclosan (TCS), triclocarban, and the quaternary ammonium compounds (QACs), which are cationic surfactants sometimes used for their biocidal characteristics [17]. TCS and triclocarban have received the most attention because they are the most used, and as a result, have been found up to part per million concentrations in biosolids [6, 18]. QACs, on the other hand, despite having been measured in biosolids up to 103 mg/kg dry wt., are expected to be degraded rapidly in the environment [17]. Other chlorinated phenols similar in structure to TCS have been found in biosolids at only parts per billion (ppb) concentrations, but are expected to increase in usage [6, 17]. Thus, a total of six contaminants were examined for this research; two commonly used antimicrobials, TCS and triclocarban, and four emerging antimicrobials; 2,4,5-trichlorophenol, 2-Chloro-4-phenylphenol (chlorinated phenol), bis(5-chloro-2-

hydroxyphenyl)methane and benzyl-4-chlorophenol [19, 20] [21-24]. Primary focus was given to TCS for the latter part of this dissertation because it is the most widely used. Overall, the six compounds cover a range of chemical properties, which indicated a range of potential environmental fates, such as the octanol-water coefficient ($\log K_{ow}$ between 2.5 and 4.8) and water solubility (0.16 to 4,984 μM).

Based on the research needs outlined, the specific research goals of my doctoral research were to:

- 1) Develop a rapid, high-throughput functional assay that measures denitrification inhibition for screening potential ecological impacts of biosolids-derived organic contaminants,
- 2) Determine the potential effects of common and emerging biosolids-derived antimicrobial agents on denitrification by a model soil denitrifier, *Paracoccus denitrificans* PD1222.
- 3) Examine the impacts of the most commonly used antimicrobial, TCS, on wastewater treatment efficiency in bench scale sequencing batch reactors (SBRs) coupled with anaerobic digesters,
- 4) Examine the impacts of biosolids aged and spiked with TCS on denitrification under simulated agricultural soil conditions, and

- 5) Evaluate potential impacts of TCS in biosolids on denitrification in agricultural soil under field conditions and assess whether a larger, more extensive study should be completed.

1.2 Research Hypotheses and Approach

The overarching hypothesis of this dissertation research was that biosolids-derived antimicrobial agents significantly affected agricultural soil microbial ecology. To test this hypothesis, the impacts of common and emerging antimicrobial agents on the community function and structure of soil denitrifiers were examined. Objective 1 and 2 were completed concurrently and were carried out using batch reactors with pure cultures of the model soil denitrifier, *Paracoccus denitrificans* PD1222. Within these batch reactors, the model organism was exposed to increasing concentrations of the target antimicrobial agents, and then denitrification was monitored using a colorimetric method that measured the reduction of nitrite (NO₂⁻) over time. Based on the observed inhibition, lowest observable effect concentrations (LOAECs) of the compounds were established. To validate the denitrification assay, two other methods (i.e., gene expression and cell viability assays) were tested on *P. denitrificans* PD1222.

Objective 3 consisted of operating bench scale SBRs coupled with anaerobic digesters. The SBRs and digesters were seeded with sludge from the aeration basin and anaerobic digesters from the North Durham Water Reclamation Facility (NDWRF,

Durham, NC). Reactors were either fed synthetic wastewater (SWW) or SWW spiked with 0.73 μM TCS. Samples were taken periodically to monitor chemical oxygen (COD), ammonium (NH_4^+), nitrate (NO_3^-), NO_2^- , total suspended solids (TSS), volatile suspended solids (VSS), dissolved oxygen (DO), phosphate (PO_4^{3-}) and pH. Biomass samples were also taken for DNA extraction and microbial community analysis using terminal restriction fragment length polymorphism (T-RFLP) of 16S SSU rDNA. Methane production was monitored for the anaerobic digesters. In addition, the digested biosolids generated from the SBRs fed SWW and SWW with TCS were analyzed for TCS concentration, TSS, VSS, Total Kjeldahl nitrogen (TKN), phosphorus (as P_2O_5), potassium (as K_2O), and pH.

Objective 4 was accomplished by amending containers of soil with the biosolids generated from Objective 3. The containers were maintained in a growth-chamber to mimic agricultural field conditions. Three biosolids treatments were examined: 1) biosolids generated from the SBRs fed with only SWW and as a result only had low background concentrations of TCS (a.k.a., Control Biosolids); 2) biosolids generated from the SBRs fed with SWW spiked with TCS (a.k.a., TCS Biosolids); and 3) biosolids that were generated by the SBRs fed with only SWW and spiked with TCS 24 h before application (a.k.a., Spiked TCS Biosolids). In addition, alfalfa was planted in half of the containers receiving the Control and TCS Biosolids to assess impacts of vegetation. To assess the overall ecotoxicity of biosolids aged and spiked with TCS, the function,

abundance, and diversity of the soil denitrifying communities were examined. The impacts on total bacteria abundance and diversity were also examined for comparison. Specifically, the denitrifying enzyme activity (DEA) assay was used to measure functional impacts, quantitative polymerase chain reaction (qPCR) was used to measure impacts on abundance, and T-RFLP was used to measure impacts on diversity. Correlations between these methods were also examined to examine any interactions between denitrifying function and community structure. Finally, a denitrification inhibition score was developed to quantify global impacts of TCS on denitrification.

Objective 5 consisted of a long-term soil sampling campaign on four agricultural fields receiving Class B municipal biosolids. Soil samples were taken before and after biosolids application and were analyzed to evaluate potential impacts of TCS in the 'traditional' biosolids on denitrification. Again, to assess the overall impacts of TCS on the soil denitrifying community, the DEA assay, qPCR, and T-RFLP were used to measure impacts on function, abundance, and diversity, respectively. Similar to objective 5, the analysis included an examination of potential correlations between denitrifying community structure and function, and quantification of global impacts using the denitrification inhibition score.

2. Background and Literature Review

The following chapter briefly summarizes the current scientific literature regarding the general topics addressed in this dissertation. More detailed reviews specific to each objective can be found in the corresponding research chapters (Chapter 3 – 6).

2.1 Wastewater Treatment

One of the main purposes of wastewater treatment processes is to reduce the loads of pathogens and excess nutrients, which if released back into the environment, could result in adverse effects to human and environmental health. Wastewater treatment does not however efficiently remove all types of chemicals. Thus, even treated effluent water can be responsible for introducing pollutants into the environment. A recent survey by the United States Geological Survey (U.S.G.S) identified 95 different pharmaceutical, hormone, and other organic wastewater contaminants in over 139 U.S. streams across 30 states. The prevalence of these compounds was further confirmed in a study conducted in 2002, when more than 80 compounds were detected in various aquatic environments around the country [25]. A majority of these were pharmaceuticals and anthropogenic chemicals that entered the environment through wastewater effluent discharge from residential homes, industries and medical facilities. Other compounds, however, particularly some of the more lipophilic, hydrophobic, and non-ionic compounds will not be discharged via the effluent, but instead will partition to the solids during the

wastewater treatment process. Then when these solids are applied as fertilizers to fields and forests the pollutants are introduced back into the environment through this route as well [17].

2.2 Biosolids

Biosolids are the residual, solid material remaining after secondary water treatment, which is often dewatered and then processed into a soil-like material. Historically, biosolids were disposed of in landfills, dumped into the ocean, or incinerated [1].

However, incineration has become an expensive and publically unfavorable approach, and ocean dumping was banned in 1991. Until the ban, about 5% of U.S. sewage was being disposed of by ocean dumping [26]. Additional research showed that the land application of biosolids was also a beneficial way to recycle nutrients, improve soil properties, and increase fertility [17, 27, 28]. Thus, land application of biosolids has become more popular. In 1980, it was estimated that approximately 25% of the total sludge generated in the U.S. was applied to land [4]. The rate has now increased to greater than 50% [1].

Because of the new trend towards land application, more focus has been given to biosolids, especially to the contaminants that may be contained therein. A recent literature review identified 516 different organic chemicals in biosolids measured around the U.S. [6]. Of these, 83% were not on the U.S. Environmental Protection

Agency (EPA) established list of priority pollutants and target compounds. Thus, the authors of this study concluded that current and future studies only targeting compounds on the EPA lists would only detect a small fraction of the organic chemicals in sludge. Furthermore, a study showed that antimicrobial pharmaceuticals (e.g., fluoroquinolone and tylosin) sorbed to the biosolids can accumulate in the soil over time (a.k.a., terraccumulation) [29]. These and similar compounds were then found to be mobilized in the soil and transported to ground- or surface water sources [30]. This highlights a need to understand the release of these organic chemicals into the surrounding soils through biosolids application that may affect human and environmental health both directly and indirectly. In fact, a recent study found that biosolids effluent (presumably from land runoff after mobilization) resulted in DNA damage to fathead minnows in an adjacent stream, which the authors attributed to the high levels of organic contaminants (i.e., PAHs and PBDEs) in the biosolids [31]. If effects of these compounds are being observed in fathead minnows, other effects on other biota, including microorganisms in the soil of land-applied biosolids may also be of concern.

2.3 Emerging Contaminants

Current regulations (i.e., 40 CFR Part 503) that require periodic monitoring of biosolids land application are in place to reduce the risk of pathogen and metal exposure and

release into soils and the surrounding environment. However, there are no regulations on organic chemicals in biosolids, despite studies that have shown that some of the emerging contaminants found in biosolids are at levels that harm micro- and macro-organisms in receiving soil [32]. Conversely, one study investigated organic contaminant risks from biosolids land applications in Canada and concluded that the mixing of biosolids with uncontaminated soils did not pose an environmental risk from organic substances such as chlorinated dioxins, furans, polychlorinated biphenyls (PCBs) and chlorinated pesticides [15]. However, this observation was most likely a result of regulations restricting the use of these chlorinated compounds, in which a decrease would be expected. Similar trends have been observed for other persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs) [33, 34]. However, there are new classes of emerging pollutants of concern that are increasing in concentration in biosolids and could have ecotoxicological impacts during land application. These compounds are commonly lipophilic, hydrophobic, and non-ionic, which make them particularly persistent and bioaccumulative, and pose a potential risk to the human food chain [6]. Specifically, these chemicals have the potential to affect food production by inhibiting crop growth or the processes important to crop growth or human health directly by bioaccumulating in crops that are consumed by humans and livestock [35-38]. These include chemicals that are primarily used as antimicrobial

agents. Each of these is briefly described below to show their environmental persistence and range of chemical characteristics.

2.4 Antimicrobial Agents

The use of antimicrobial agents has increased in recent years with 700 products already on the market back in 1992 and over \$1 billion in yearly sales by 2000 [16]. Many of these products contain TCS or triclocarban [39]. Other compound, such as 2-Benzyl-4-Chlorophenol, 2-Chloro-4-phenylphenol, and bis(5-chloro-2hydroxyphenyl)methane, which are similar in structural features to TCS, are also used for antimicrobial purposes. In addition to structure, these six compounds cover a range of log K_{ow} values (2.5-4.8) and solubility values (0.16 to 4,984 μM), suggesting the compounds are persistent in the environment and bioaccumulative. A summary of the physical and chemical properties of the six target compounds can be seen in Table 1. Of the six compounds targeted, 2,4,5-Trichlorophenol is the most soluble and least hydrophobic. Triclocarban and TCS, however, are the least soluble and most hydrophobic. Overall, several of these compounds have already been found in biosolids at concentrations that alone, or taken together, have negatively impacted micro and macro organisms in environments receiving the biosolids. The six target compounds are described in more detail below. TCS and triclocarban are described first, as there is substantial background literature on

these compounds. Little to no information for the remaining compounds could be found. Nevertheless, what was available is summarized in the following sections.

2.4.1 Triclosan

TCS also known as 5-chloro-2-(2,4-dichlorophenoxy) phenol is a broad range antibacterial and antifungal agent commonly used since 1972 in shampoos, soap, detergent and even in toothpaste to prevent gingivitis in the range of 0.1-0.3% (w/w) [40]. In addition, TCS can be further found in many other products including textiles, sportswear, bed clothes, shoes, and carpets at the same levels [41]. As a result, these domestic applications are likely the major sources of TCS, as well as, triclocarban, for wastewater treatment plants [42]. In fact, 95% of the TCS produced in the U.S. is used in personal care products (PCPs), which are disposed mainly by being washed down residential drains [43]. The Log K_{ow} of TCS is 4.8, and the acid dissociation constant (pKa) is 7.9 (Table 1), suggesting that it is fairly bioaccumulative (non-ionized) under ambient conditions and partitions to solids in wastewater treatment [44]. TCS has a half-life greater than 266 days in soil [45]. TCS also has a solubility of about 13.8 μM in water and phosphate buffer (pH 7.4, 30° C), respectively [46], which was verified in the lab. It has been measured at ppb levels in water samples [25, 47] and is of concern due to the potential to facilitate the evolution of antibiotic resistant strains of bacteria. Algae and snails in surface water have been shown to bioaccumulate TCS [48, 49]. TCS, and its

microbial metabolite methyl-TCS, have been identified in water and sediments adjacent to wastewater treatment plants (WWTPs) [22], and in biosolids collected from several different WWTPs with measured concentrations as high as 133 mg/kg dry weight [17, 21, 23, 32, 50-53]. This led to the discovery that TCS was bioaccumulating in the tissue of earthworms found in agricultural soils receiving biosolids [32]. Numerous studies have measured TCS in biosolids, however, few have actually examined the concentrations of TCS in the soil following biosolids application. The studies that were conducted, reported a large range of TCS concentrations between 4.1 ng/kg to 1,840 µg/kg and were dependent upon the source biosolids, the application rate of the biosolids, and soil mixing type (e.g., till vs. no till) and mixing depth [32, 54-56]. Another study determined that 79% of TCS entering a WWTP was biologically degraded, 15% sorbed to biosolids, and the remaining 6% was discharged directly to the receiving surface water [41]. Other studies reported higher partitioning to biosolids [51, 52]. Furthermore, TCS was found in 59% of the 139 streams tested by the U.S.G.S [25] and many other receiving streams for wastewater treatment [57]. Overall, these data suggest significant loadings of TCS to the environment may occur during land application of biosolids.

2.4.2 Triclocarban

Table 1 shows the physical and chemical properties for triclocarban. Triclocarban is a topical antiseptic that is added to soaps, cosmetics, detergents, cleansing lotions, wipes,

and other PCPs for its germicidal properties. Because of its high log K_{ow} , low solubility, and trichlorinated aromatic structure, triclocarban is persistent in the environment, with half-lives of 120 and 540 days in soil and sediment, respectively [57]. Triclocarban has been measured in water and wastewater up to 0.022 μM [57] and estimated in sediment and biosolids amended soil pore space up to 0.867 μM [51, 53, 54, 58, 59]. Triclocarban has been measured at levels of 2.17 to 51 mg kg^{-1} dry weight in digested municipal sludge [51, 54, 59]. Cha and Cupples (2009) found that triclocarban concentrations in soil increased over time after subsequent amendments of biosolids [54]. The EPA also found triclocarban in all of the 84 biosolids samples measured across the U.S. with a range of 0.187 to 441 mg kg^{-1} dry weight [53].

2.4.3 2,4,5 Trichlorophenol

2,4,5-trichlorophenol is an algaecide and microbiocide, is one of the more toxic chlorinated phenols, and is very persistent. It is often used as a fungicide in paper mills, as a herbicide, and as an intermediate in the manufacture of pesticides [60]. 2,4,5-trichlorophenol has been shown to be acutely and chronically toxic to humans and has a reference dose, or daily oral exposure that is likely to be without harmful effects during a life time, of 0.1 mg/kg of body weight per day [60]. In addition, 2,4,5-trichlorophenol was shown to reduce cell yield in simulated wastewater treatment by approximately

50% through continuous addition of between 6.9 and 8.6 μM [61]. Several key physical and chemical properties are reported in Table 1.

2.4.4 2-Benzyl-4-chlorophenol

2-benzyl-4-chlorophenol, also known as chlorophene, is a bactericide and fungicide used in the preservation of cosmetics and has been identified as an emerging contaminant in biosolids [62, 63]. Physical and chemical properties important to the ultimate fate and transport of this compound can be found in Table 1. 2-benzyl-4-chlorophenol is also used as a disinfectant in liquid and solid soaps [64, 65]. Wastewater influent concentrations of 2-benzyl-4-chlorophenol have been found up to 0.89 μM with as low as 10% remaining in the effluent [66]. Another study found much lower concentrations in the influent of wastewater treatment plant in Baltimore, MD of around 2.5E-3 μM . However, this was similar to the TCS concentrations at this plant and may represent an area with low loading of these compounds in general. Still nearly 73% of the influent compound in this study did not make it to the effluent, most likely due to biotransformation, although the authors did not take adsorption into account [24].

2.4.5 2-Chloro-4-phenylphenol

2-chloro-4-phenylphenol is a microbiocide [67]. Select physical and chemical properties can be found in Table 1. No information, however, could be found in the literature regarding the presence or abundance of this compound in the environment.

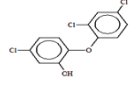
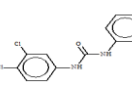
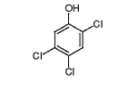
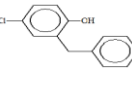
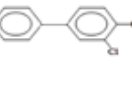
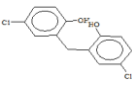
2.4.6 Bis(5-chloro-2-hydroxyphenyl)methane

Bis(5-chloro-2-hydroxyphenyl)methane, also called dichlorophene, is a microbiocide. Bis(5-chloro-2-hydroxyphenyl)methane was found in anaerobically digested sludge up to 0.52 μM [20]. In addition, bis(5-chloro-2-hydroxyphenyl)methane has also been shown to be bioaccumulative and has been found in the tissue of fish exposed to waste water effluents [19]. Physical and chemical properties of bis(5-chloro-2-hydroxyphenyl)methane can be found in Table 1.

2.4.7 Inactivation Mechanisms

The microbial inactivation mechanisms of these compounds are not well understood, with the exception of TCS, and to a lesser extent triclocarban. TCS inhibition has been thoroughly examined and at high concentrations is known to inhibit fatty-acid production, eventually leading to cell lysis [68, 69]. At low concentrations, TCS has been shown to have some impact on gene expression [70, 71]. Triclocarban, on the other hand, has not been fully characterized, but is suspected of adsorbing to the cell membrane and

compromising its permeability [72]. The inhibition mechanisms of the remaining compounds have not been analyzed to date, but are expected to be complex and consist of multiple targets [72].

Table 1: Physical-chemical properties of target antimicrobial agents.						
Compound Name	CAS	Solubility (μM)	log Kow	pKa	Molecular weight	Structure ^[73]
Triclosan	3380-34-5	13.8	4.8 ^[46]	7.9 ^[45]	289.54	
Triclocarban	101-20-2	0.16 ^[74] , 0.206 ^[75] , 6.3-14.6 ^[76] , 34.9 ^[77]	2.5- 4.2 ^[74]	12.7 ^[45]	315.59	
2,4,5-trichloro-phenol	95-95-4	4,984 ^[78]	3.6 ^[79]	6.97 ^[80]	197.45	
2-benzyl-4-chloro-phenol	120-32-1	681 ^[73]	3.6- 4.2 ^[73]	-	218.68	
2-chloro-4-phenyl-phenol	92-04-6	342-371 ^[73]	3.92 ^[73]	8.07 ^[81]	204.65	
Bis(5-chloro-2-hydroxy-phenyl) methane	97-23-4	111 ^[73]	4.3 ^[73]	9.92 ^[82]	269.12	

2.5 Denitrification

Denitrification, also known as dissimilatory nitrate reduction to ammonia (DNRA) or NO_3^- respiration, is the stepwise reduction of the soluble nitrogen oxides, NO_3^- and NO_2^- , to the dinitrogen gases, nitrous oxide (N_2O) and N_2 . Denitrification is coupled with electron transport phosphorylation, and as mentioned, is a dissimilatory process, which means that the reduced nitrogen is not used by the cell [7]. A simplified schematic of the nitrogen cycle can be seen in Figure 1 [83]. Overall, denitrification is believed to be the largest of at least six different processes that reduce NO_3^- ; others including nitrogen

assimilation, dissimilatory respiratory denitrification, dissimilatory NO_3^- reduction to NH_4^+ , non-respiratory denitrification, and chemodenitrification (not shown in Figure 1) [84]. Respiratory and non-respiratory denitrification make up biological denitrification, but being that respiratory denitrification is the dominant process, it is the only one discussed further. In general, the biological ability to denitrify is widely found in nature, including in soil, fresh and salt water, sediments, waste treatment systems, and animal gastrointestinal tracts, and appears to be ultimately only restricted by oxygen levels [7]. The focus of this research was on denitrification in agricultural settings. A previous research review examined typical denitrification rates in various types of soils and found that rates for agricultural soil have been reported between 0 and 239 kg N/ha-year, with the highest rates typically measured in irrigated, nitrogen-fertilized soils [85]. Barton *et al.*, [85] calculated a mean rate of 13 kg N/ha-year for agricultural soils from a thorough literature review. For agricultural soils in particular, the literature has cited six main reasons why denitrification is important.

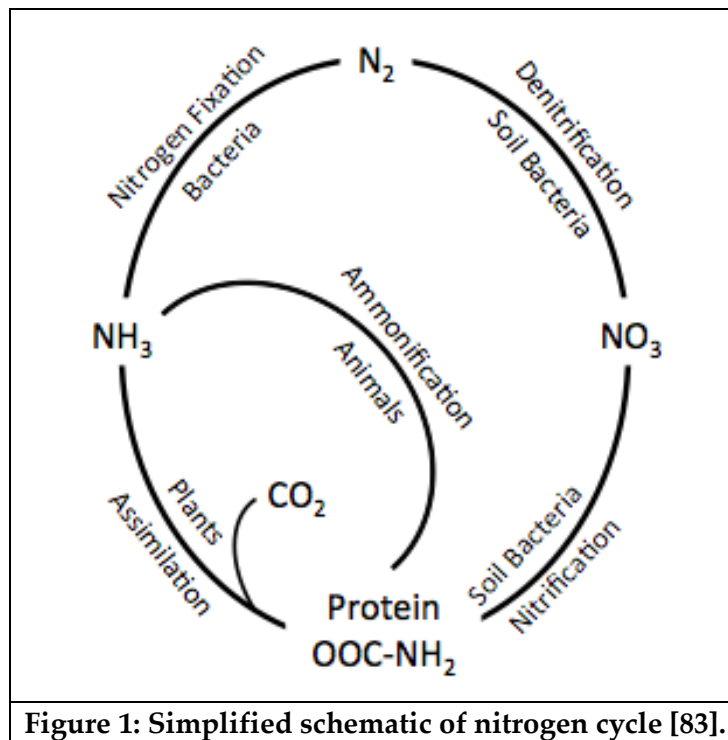


Figure 1: Simplified schematic of nitrogen cycle [83].

First, denitrification is incorporated into every domain of life and provides the preferred metabolic function in anaerobic environments, such as the conditions found just below the surface in agricultural soil. The denitrification process has been reported in more than 50 genera and more than 130 species. In addition, denitrifiers compose all three energy source classes, organotrophs (most popular), lithotrophs, and phototrophs. Within these classes, there are denitrifying representatives that are aerobic, oligocarbophilic, fermentative, halophilic, thermophilic, sporeformer, magnetotactic, N_2 fixing, pathogenic, hydrogen oxidizers, sulfur oxidizers, and ammonia reducers [7]. Several studies have shown that microorganisms capable of denitrification make up approximately 5% of the total soil microbial community, making it more abundant than

any of the other nitrogen cycle organisms [86, 87]. The robustness of this phylogeny is somewhat expected, however, because of the potentially high energy gained from denitrification. When paired with carbon oxidation, denitrification can yield a potential of about 1.2 V per reaction (exothermic) [88].

Denitrification also plays a major role in both the nitrogen and carbon cycles, which are essential for microbial, soil, and plant viability. Denitrification completes the global nitrogen cycle by supplying N_2 to the atmosphere to be used by the nitrogen fixers, which is then subsequently made available to crops. Furthermore, denitrification is a catabolic process - the use of organic carbon as an electron donor will result in less complex molecules – affecting the use (as in the fate and transport) of carbon in the soil.

Three of the reasons why denitrification is important in agricultural soil are tied together and are concerned with the advantages and disadvantages of converting NO_3^- and NO_2^- to the nitrogen gases. As an advantage, denitrification is active in the amelioration of excess nitrogen fertilizer, which reduces the runoff load to streams after precipitation events. If NO_3^- , and even NO_2^- , makes it into nearby streams, algal blooms may form, and eutrophication may occur. NO_3^- is of particular importance because it is a pollutant in groundwater and surface water, especially when the water is eventually used as drinking water. Levels above the EPA regulated 10 mg/L have been linked to “blue baby syndrome” [14].

As a disadvantage, denitrification is known to degrade nitrogen fertilizers added to agricultural fields, resulting in both productivity and economic losses. Nitrogen is a vital nutrient for plants, and in fact, for all living things, as it is a component of amino acids, the building blocks of proteins, purines, and pyrimidines [8]. For plants, however, N_2 gas in the atmosphere is much less available than ammonia (NH_3), or even NO_2^- and NO_3^- in the soil. As a result, nitrogen, mostly as NO_3^- and NO_2^- , is the most limiting nutrient in crop production and denitrification has been linked to between 0 and 70% loss of NO_3^- and NO_2^- in fertilizers to the atmosphere in the form of N_2O or N_2 gases (typically in the range of 0 to 25%) [9, 10]. Fertilizer NO_3^- and NO_2^- losses generally increase with fertilization, which subsequently results in lower crop production. Under normal conditions, this corresponds to a denitrification rate range of 0 to 200 kg N/ha-year [11]. To compound this further, the reduction of the nitrogen fertilizers produces a major portion (70%) of the total global greenhouse gas emissions of N_2O [10]. According to the Intergovernmental Panel on Climate Change (IPCC), N_2O is about 300 times more potent of a greenhouse gas than carbon dioxide and contributes around 6% of the anthropogenic greenhouse effect [12]. Making it worse, N_2O is also directly involved with the depletion of the stratospheric ozone layer [13].

Lastly, denitrification can produce intermediates that may be toxic to the local ecosystem. The potentially toxic intermediates, NO_2^- and nitric oxide (NO) have been shown to negatively impact local ecosystems at high concentrations [7]. For example,

NO_2^- has been shown to inhibit nodulation of rhizobia. Also, NO_2^- has been shown to react with secondary amines to produce the carcinogens, nitrosamines [7].

Overall, denitrification plays an important role in agricultural soils. The following sections will briefly cover the current scientific literature regarding the biology and ecology behind denitrification in agricultural soils. Also, because this research focused on denitrification in an agricultural environment, the following sections briefly cover the key factors that drive denitrification in these environments.

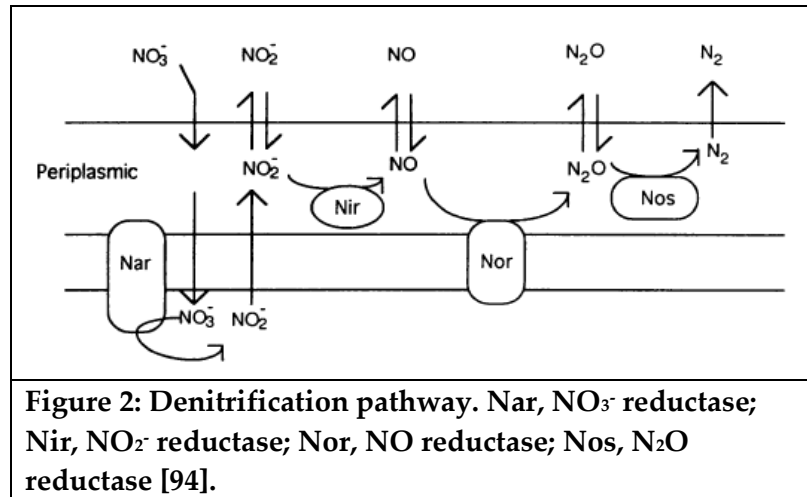
2.5.1 Biology of Denitrification

As alluded to earlier, denitrifying organisms are very diverse (i.e., Archaea, Bacteria, and Fungi), are typically heterotrophs, and are also facultative microorganisms, which under aerobic conditions use oxygen as an electron acceptor. Bacteria are generally believed to be the dominant denitrifying species in most environments, although with some controversy [89]. Fungi have been shown to be more abundant in grassland soils [90]. Nevertheless, this diversity, which makes it impossible to identify denitrifiers by taxonomy alone, is most likely due to horizontal gene transfer in evolution [91]. When oxygen levels decrease below about $10 \mu\text{mol O}_2$, denitrification enzymes begin to be synthesized and nitrogen becomes the preferred electron acceptor [92]. Under aerobic conditions, however, it appears that competition for carbon is the major determinant of denitrifier population density and composition. In addition to oxygen and carbon

availability, denitrification is further influenced by pH, natural growth limitations, temperature, electron acceptor availability, and predation, among others that will be discussed later. [89, 93]. Although denitrifiers are ubiquitous in most soils, these conditions determine which and when genes for denitrification are expressed.

The overall enzymatic denitrification pathway can be seen in Figure 2 [94]. NO_2^- and NO reductase are membrane proteins and NO_2^- and N_2O reductases are enzymes found in the periplasm [84]. NO_3^- reduction is associated with two enzymes *nar* (membrane-bound) and *nap* (periplasmic-bound), which have also been found in bacteria that do not denitrify. Thus, the genes *narG* and *napA*, which code for these enzymes, are not widely used to characterize denitrifying microorganisms [89]. NO_2^- reduction genes are the most widely used for denitrifier community studies because they catalyze the first step leading to gaseous nitrogen formation. Two NO_2^- reductases are known, a copper containing enzyme encoded by *nirK* and cytochrome *cd1* encoded by *nirS*, but have different evolutionary origins. NO reductase contains a cytochrome bc complex and the lack of this enzyme appears to be lethal to the cell from the toxic accumulation of NO. NO reduction is also completed by two enzymes; one that receives electrons from cytochrome *c* (pseudoazurin), *cnor*, and the other that receives electrons from a quinol pool, *qnor*. *Nor* and *qnor* are responsible for the formation of the N-N bond, and because of this, these genes have received much focus in the literature [89]. Lastly, the gene *nosZ* codes for N_2O reductase. Tiedje (1988) reported that most

denitrifiers isolated from nature have the entire pathway from NO_3^- to N_2O [7, 95]. For example, *Paracoccus denitrificans* can complete all of the denitrification steps [14]. However, some denitrifying organisms do not synthesize all of the enzymes required in complete denitrification. For example, *Wolinella succinogenes* has been shown to lack the nitrite reductase step [95]. Thus, denitrification may become a community process [91].



2.5.2 Ecology

To understand denitrification in agricultural soils, it is important to understand what organisms complete the process and how communities of these organisms form. Myrold and Tiedje (1985) showed that denitrifying community structure was dependent on the aerobic competition for carbon [96] and not based on the capacity to denitrify. Another study showed that denitrifier population density decreases with increasing depth in soil [97]. However, Kennedy and Lawless (1985) found a chemotaxis response to NO_3^- and

NO_2^- concentrations greater than 10^{-3} M [98], so NO_3^- may also play an important role in determining the community structure. NO_3^- , however, only appears to be a determining force in denitrifying community structure in fertilized fields, where enough NO_3^- is available [99]. Soil denitrifying communities are further structured by pH, moisture content, and temperature [89]. Nevertheless, in agricultural soils *Pseudomonas* was shown to be the dominant species at between 75 and 98%, and *Alcaligenes* making up most of the remaining population [7, 100, 101].

Significant differences have been shown between community function and structure [102, 103]. Thus, it is important to examine both when determining the impact of any variable on denitrification. In a review completed by Eichner (1990), N_2O emissions ranged from 0.2 to 42 kg $\text{N}_2\text{O-N}$ /ha-year [104]. Another research review examined typical denitrification rates in various types of soils and found that rates for agricultural soil have been reported between 0 and 239 kg N/ha-year with the highest rates typically measured in irrigated, nitrogen-fertilized soils [85]. Barton *et al.*, [85] calculated a mean rate of 13 kg N/ha-year for agricultural soils.

2.5.3 Factors Driving Soil Denitrification

There are many factors that have been found in the literature that have impacts on denitrification in agricultural soils. It is important to understand these factors, so that denitrification can be optimized for beneficial agricultural purposes. Inhibition of

denitrification can be broken up into two main groups, proximate and distal factors. Proximate factors include oxygen levels, nitrogen and carbon availability, enzymatic kinetics, and growth rate. The distal factors include moisture conditions (*i.e.*, rainfall, irrigation, and groundwater), seasonal changes (*i.e.*, temperature), seasonal cycles (*e.g.*, freezing/thawing, wetting/drying, etc.), soil texture, plants, organic matter, physical disruptions, and competition or excretions by other organisms [7, 10, 89]. The distal factors appear to guide the community structure over the long-term, whereas the proximate factors impact individual communities in real-time. Unfortunately, understanding the cause and effect of these variables on denitrification is not all that easy. A quick examination of the literature reveals an intricate, somewhat convoluted web of contradictory interactions that is in need of sorting out. This is especially true because many of the studies lack a complete characterization and accounting of all proximate and distal variables that may be impacting the results stated. The following paragraphs attempt to summarize the major trends of the findings.

2.5.3.1 Proximate Factors

The proximate factors seem to drive the real-time fluctuations in denitrifiers. The following sections will discuss the three major proximate factors that drive denitrification; oxygen levels, NO₃⁻ availability, and carbon availability.

2.5.3.1.1 Oxygen

The most important driving factor for denitrification is the level of available oxygen. For the most part oxygen inhibits denitrification, although there are two known species have been shown to be microaerophilic (tolerant to small levels of oxygen), *Aquaspirillum magnetotacticum* and *Nitrosomonas europaea* (also denitrifies), which are presumed to need the oxygen for oxygenase reactions [7]. In most cases, oxygen represses enzyme synthesis and activity. *NosZ* is especially sensitive to oxygen, and as a result, exposure to oxygen causes differential production ratios of N_2O and N_2 [102]. Typically lower N_2O/N_2 ratios reflect lower redox conditions with greater availability of carbon, whereas higher ratios correspond to lower pHs, and the presence of oxygen [105, 106]. A previous review found that the threshold value of oxygen below which denitrification rapidly increases was in the range between 0.2 and 21 μM as O_2 . Due to measurement limitations and difficulties converting temperature, salt, and pressure dependent units reported in this study, however, the authors recommended that a safe estimate O_2 threshold is about 10 μM (stated earlier), although a lower concentration is probable [7].

The distal factors that alter oxygen levels also have a potentially substantial effect on denitrification. Rainfall and other moisture sources not only can carry oxygen, which inhibits denitrification, but also can decrease oxygen diffusion, which encourages denitrification. As a result, factors like soil texture and the plants evapotranspiration rate also influence denitrification. Plants can further stimulate respiration, which removes oxygen, but also can use water, subsequently drying and aerating the soil [107].

Respiration, which is driven by carbon and sometimes water, is the major O_2 removal mechanism [7].

2.5.3.1.2 Nitrate

By definition, denitrification requires a nitrogen source (NO_3^- or NO_2^-) to proceed. The water content in soils, and the rate of production and use by other pathways determines the “native” NO_3^- supply. NO_3^- is primarily produced through nitrification and the NH_3 used in nitrification is produced through mineralization. In addition, NO_3^- assimilation, DNRA, and anaerobic NH_4^+ oxidation (anammox) use NO_3^- and could compete with denitrification. In agricultural soils, plant removal of NO_3^- has a significant impact on denitrification [7]. Typically, however, NO_3^- is usually supplemented in agricultural soils through organic and mineral fertilizers.

2.5.3.1.3 Carbon

Carbon is used as an electron donor in denitrification to reduce nitrate. Carbon, in its many forms, can play three roles in denitrification: 1) serves as an electron donor, 2) serves as a source of NO_3^- , 3) drives respiration (water helps), which reduces oxygen [10]. Thus, amount of available carbon is dependent on whether the environment is anaerobic or aerobic. In aerobic environments, the denitrifiers, which make up a small population of heterotrophs, proportionally consume a smaller percentage of the available carbon. Under anaerobic conditions, however, denitrifiers consume a greater

percentage of carbon because they will be more abundant. Denitrification still ceases under anaerobic conditions if the carbon resources become exhausted. Water, plants, physical disruptions, and competition are the distal factors that influence the amount of available carbon [7]. Water can stimulate metabolism in dry soil and serve as a transport media. Plants can release carbon exudates that stimulate respiration in the root zone. Physical disruptions like freezing and thawing and wetting and drying cycles, and cultivation and non-tilling free trapped carbon that was otherwise biologically unavailable. Lastly, competition with or excretion by other organisms also influences the amount of carbon available for denitrification [7].

2.5.3.2 Distal Factors

The distal factors guide the community structure of the denitrifiers. The distal factors include moisture conditions (*i.e.*, rainfall, irrigation, and groundwater), temperature (seasons), seasonal changes (freezing/thawing, wetting/drying, etc.), soil texture, plants, organic matter, physical disruptions, and competition or excretions by other organisms [7, 10, 89]. Of these, denitrification has been shown to have seasonal changes, with rates typically highest in the spring to fall months, especially during thawing periods [10]. Similarly, higher rates of denitrification were shown after drying-wetting cycles across several studies [10], and the longer the “wet” treatment, the higher the denitrification

[108]. However, the distal factors reported to have the most significant effects on denitrification are crop-type [11], fertilizer-type [11], and chemical application [10].

2.5.3.2.1 Crops

Plants have many impacts on denitrification, depending mostly on how they impact the distal factors. Increased water consumption by plants increases the soil gas exchange and oxygen content [10]. Plants consume NO_3^- and water, but nitrate can also be formed in the root zone when root exudates are mineralized. NO_3^- can also become bioavailable when the plant dies and decomposes. Plants further release carbon in the form of mucilage (high molecular weight polysaccharides), exudates (low molecular weight compounds), and root cap cells, among others, which have also been shown through various studies to be correlated with denitrification [10]. Overall, the addition of plants was shown to result in a rhizosphere community shift [109, 110] and an increase in denitrifier abundance [111, 112]. This has led to hypotheses that denitrification is actually a selective advantage for microorganisms in the rhizosphere [113]. According to a review completed by Philippot *et al.* [10] denitrification rates also varied by crop type, with higher denitrification rates typically reported for larger root masses and legumes. The common hypothesis, although somewhat contested, is again that denitrification by the rhizobia were the cause of the increased rates.

2.5.3.2.2 Fertilizers

As mentioned previously, denitrification serves as a sink for nitrogen fertilizers added to agricultural fields. This loss is substantial, making up between 0 and 70% of fertilizer loss, with the highest rates immediately after fertilizer addition [114]. In addition to supplying the source of nitrogen for denitrification, fertilization has been shown to affect denitrification in three main ways: 1) by changing the pH of the soil or local environment, 2) altering the amount and form of organic carbon (OC) that is available for microorganisms, and 3) altering the amount and form of nitrogen available for denitrification. Fertilization has also been shown to affect the ratio of N_2O and N_2 as the end product of denitrification and depends primarily on the type of fertilizer used; mineralized fertilizer or poultry waste (higher denitrification rates) versus organic, cattle, and pig waste (lower denitrification rates) [115].

Fertilizers can directly change the pH of the soil, which subsequently has an impact on denitrification. In general, denitrification tends to be higher in neutral conditions, and thus, is higher for alkaline versus acidic fertilizers. This impact also extends to community structure. Enwall *et al.* [116] showed that fertilizers with lower pH showed a shift in *narG* and *nosZ* community shifts, however no correlation between the pH and shift could be developed. Fertilizers also affect the carbon availability of the soil. Organic fertilizers tend to have higher denitrification rates because of the availability of more organic carbon [10]. However, this is sometimes offset by the availability of nitrogen posed by the fertilizer, as organic fertilizers also tend to sequester

nitrogen more than mineral fertilizers [10]. Several community analysis studies have obtained mixed results regarding the form of the nitrogen being applied. One study showed a shift in the denitrifying gene *nirK* based on increasing ammonium concentrations [117]. In contrast, another study completed by Deiglmayer *et al.* [118] found that the structure of the *narG* denitrifying community did not shift by varying the NO_3^- concentration. However, two different sources of nitrogen were used in these experiments and could account for the different community responses. Compounding the ability to elucidate the effects of fertilizer addition further, in a review, Philippot *et al.* [10] showed that denitrification was further affected by soil type, fertilizer type, fertilizer application rate, and sequence of applying different fertilizers.

2.5.3.2.3 Pollutants

Denitrifiers have been shown to be sensitive to heavy metals. Metals like arsenic, cadmium, chromium, copper, lead, silver, and zinc have all shown to inhibit denitrification activity and diversity [10]. Cadmium, copper, and zinc in particular, have been shown to affect nitrous oxide reduction, and thus, the levels of nitrous oxide present. Again, other factors such as pH, cation exchange capacity, and natural organic matter content can affect the availability of the heavy metal and ultimately the impact on denitrification [10].

As far as organic contaminants, many different classes of compounds have been shown to inhibit denitrification, of which include, polyaromatic hydrocarbons (PAHs),

pesticides, and heavy metals [10]. However, when denitrification is inhibited, not all of the enzymes are affected equally and *nosZ* seems to be the most sensitive to pollutants, as well as to the variables mentioned earlier, including oxygen levels, carbon to nitrogen (C:N) ratios, and pH [119-122]. In all, fungicides inhibit denitrification more than herbicides and pesticides, which have mixed results because they appear to be influenced more by environmental variables [123]. This effect is mostly due to experimental design variables, such as to soil type, concentration added, and the active ingredient. This uncertainty is further broadcasted by community analyses that show that some compounds lead to a shift in denitrifiers, while others do not. To date, no studies have examined the effect of the target compounds in this study on denitrifiers. Pell *et al.* [123] found that, the related antimicrobial 2,4-Dichlorophenol inhibited denitrification at levels of 100 $\mu\text{g/g}$ dry soil. However, because the compounds targeted in this study are designed to deactivate microorganisms, there is a potential that they will inhibit denitrification in soil once land applied via biosolids.

3. Determining the Ecological Impacts of Organic Contaminants in Biosolids Using a High-Throughput Colorimetric Denitrification Assay: A Case Study with Antimicrobial Agents

This chapter was published in Holzem, R.M., H.M. Stapleton, and C.K. Gunsch. (2014).

Environmental Science and Technology, 48(3), 1646-1655 and addresses Objectives 1 and 2.

3.1 Introduction

Dewatered solid residual materials remaining after secondary wastewater treatment (a.k.a., biosolids) have traditionally been applied to agricultural fields in order to recover their macronutrients, primarily nitrogen and phosphorus [1-3]. Current regulations pertaining to biosolids land application (i.e., 40 CFR Part 503) regulate both pathogen reduction and heavy metal exposure. However, these regulations do not cover any organic contaminants. In fact, a recent review found that more than 500 different organic chemicals have been identified in biosolids around the United States [6]. The compounds that are likely to accumulate in biosolids and could have ecotoxicological impacts during land application are commonly lipophilic, hydrophobic, and non-ionic. These characteristics make these chemicals particularly persistent and bioaccumulative, and pose a potential risk to the human food chain [6]. Specifically, these chemicals have the potential to affect food production by inhibiting crop growth or the processes important to crop growth or human health directly by bioaccumulating in crops that are

consumed by humans and livestock [35-38]. While current concentrations of emerging organic contaminants have been measured in biosolids and receiving soils at levels below what may harm macro-organisms, there is growing concern that these same compounds may impact ecologically important microorganisms [32].

Little is known about the effect of biosolids-derived organic compounds on soil microbial function and, in turn, soil health. Denitrification is one functional endpoint, which has received little attention, but provides a vital indication of soil health.

Denitrification consists of the stepwise reduction of the soluble nitrogen oxides, nitrate (NO_3^-) and nitrite (NO_2^-), to nitrous oxide (N_2O) and N_2 [7]. Denitrifying bacteria play a critical role in this process, and thus, are ideal indicator organisms for determining contaminant ecotoxicological potential. Specifically, denitrifiers: 1) play a major role in both the nitrogen and carbon cycles, which are essential for microbial, soil, and plant health [8]; 2) are known to degrade synthetic fertilizers added to agricultural fields and produce a major portion of the greenhouse gas N_2O [9-13]; 3) are active in the amelioration of excess nitrogen fertilizer, which reduces the runoff load to streams after precipitation events [14]; and 4) are known to produce intermediates that may be toxic to the local ecosystem, such as NO_2^- and nitric oxide (NO) [7].

One class of biosolids-derived emerging organic contaminants are antimicrobial agents (a.k.a., bactericides or biocides). These chemicals are of concern because they are designed to deactivate microorganisms, are heavily used in the U.S with over \$1 billion

in yearly sales [16], and have been measured in biosolids. The most common antimicrobials found in biosolids are TCS, triclocarban, and the quaternary ammonium compounds (QACs), which are cationic surfactants sometimes used for their biocidal characteristics [17]. TCS and triclocarban have received the most attention because they are the most used, and as a result, have been found up to part per million concentrations in biosolids [6, 18]. QACs, on the other hand, despite having been measured in biosolids up to 103 mg/kg dry wt., are expected to be degraded rapidly in the environment [17]. Other chlorinated phenols similar to TCS have been found in biosolids, but only at parts per billion (ppb) concentrations [6, 17]. Despite being ubiquitous in biosolids and found at high concentrations, very limited research has examined the impacts of TCS and triclocarban on microbial function and most are limited to aqueous environments. Many published studies examined the general toxicity of TCS and triclocarban and were not focused on microbial processes. These studies reported a large range of inhibitory concentrations for microorganisms, 0.863 to 276 μM for TCS and 31.7 μM to 127 M for triclocarban [124]. As far as studies targeting specific microbial processes, median effective concentrations (EC50) of 6.29, 69.1, and 825 μM have been reported based on BOD degradation, oxygen consumption, and glucose utilization, respectively [76].

Because there are so many different types of contaminants found in biosolids, a cost-effective, high-throughput method for determining their ecotoxicological potential is needed. In particular, an assay is needed which quantifies a functional endpoint

relevant to ecological impacts. In the present case-study, a colorimetric assay was developed and used to measure the impacts of several antimicrobial agents on denitrification. For comparison and validation purposes, the assay, experiments were also conducted with two commonly used methods, gene expression and cell viability. *Paracoccus denitrificans* Pd1222, a common soil denitrifier, was selected as the model bacterium for the present study because the microorganism is fully sequenced and, unlike *Pseudomonas spp.*, does not have a known resistance to contaminants [100, 125]. *P. denitrificans* Pd1222 is a facultative microorganism, meaning it relies metabolically on denitrification under anaerobic conditions and respiration under aerobic conditions, and contains the entire denitrification pathway. In total, six antimicrobials were selected as target compounds in this case-study: two “historical” antimicrobial agents, which have been extensively studied (TCS and triclocarban), and four emerging antimicrobials (2,4,5 trichlorophenol, 2-benzyl-4-chlorophenol, 2-chloro-4-phenylphenol, and bis(5-chloro-2-hydroxyphenyl)methane). A summary of the physical and chemical properties of the six target compounds is shown in Table 1. While these compounds are designed and used to deactivate microorganisms, the inactivation mechanisms are not well understood, with the exception of TCS, and to a lesser extent triclocarban. TCS inhibition has been thoroughly examined and at high concentrations is known to inhibit fatty-acid production, eventually leading to cell lysis [68, 69]. At low concentrations, TCS has been shown to have some impact on gene expression [70, 71]. Triclocarban, on the other hand,

has not been fully characterized, but is suspected of adsorbing to the cell membrane and compromising its permeability [72]. The inhibition mechanisms of the remaining compounds have not been analyzed to date, but are expected to be complex and consist of multiple targets [72].

3.2 Materials and Methods

The following section will describe the methods used in this chapter.

3.2.1 Cell Growth and Preparation

P. denitrificans PD1222 was grown on Luria-Bertani (LB) agar plates and single colonies were used to inoculate 100 mL of autoclaved LB broth. Cells were maintained by constant shaking at 125 rpm on a shaker table in an incubator at 37 °C. One mL of late log culture was transferred to a 250 mL Erlenmeyer flask containing 100 mL of LB broth (~21 °C) on a shaker table at 125 rpm. For use in subsequent experiments, 5 mL of late log cells were harvested by centrifugation at 5,500 rpm for 5 min using a SORVALL® Super T21 centrifuge (Thermo Scientific, Waltham, MA). Cells were washed with previously described minimal medium (MM) modified to contain 36.6 mg/L of (NH₄)₂SO₄, and repelletized [126]. All chemicals were obtained from Thermo Fisher Scientific (Waltham, MA) and VWR (West Chester, PA).

3.2.2 Batch Reactor Preparation

The experiments were carried out in batch reactors consisting of 200 mL clear Neutraglas serum bottles (Thomas Scientific, Waltham, MA). 100 mL of MM was added to each bottle and heat sterilized. Bottles were wrapped in aluminum foil to prevent photodegradation of the target contaminant and inoculated with 9×10^8 cells/L (72 ± 3.5 mg dry weight/L). Preliminary tests showed that this was the minimum concentration of cells that was needed to quantify significant changes in denitrification over a 24 h timeframe.

Target concentrations were selected based on solubility (Table 1), concentrations found in the environment, and concentrations that were found to impact denitrification in preliminary experiments. For TCS, the concentrations selected were 10% of the aqueous solubility, and ten- and hundred-fold dilutions of that concentration (10.4, 1.04, and 0.104 μM , respectively). The concentrations selected for triclocarban were 31.7, 3.17, and 0.032 μM based on the reported solubility range of 0.16 to 34.9 μM and that preliminary results showed no inhibition below 0.16 μM . The concentrations for the remaining compounds were similarly selected to be below solubility and cover the transition from not inhibiting denitrification to inhibiting denitrification. The concentrations selected were 50.6, 5.06, and 0.506 μM for 2,4-5-trichlorophenol, 48.9, 4.89, and 0.489 μM for 2-chloro-4-phenylphenol, 37.1, 3.72, and 0.372 μM for bis(5-chloro-2-hydroxyphenyl)methane, and 45.7, 4.57, and 0.457 μM for 2-benzyl-4-

chlorophenol. The target compounds were added using a Hamilton Syringe (Hamilton Company, Reno, NV). Following the compound introduction, 0.05 g/L of glucose and 25 mg/L of NO_2^- (KNO_2 stock in DI water) were added to each bottle [127]. Quadruplicates of each treatment condition were prepared. In addition, biological, solvent, NO_2^- and inactive controls were prepared. The biological control consisted of adding the cells, glucose, and NO_2^- , and the solvent control consisted of cells, glucose, NO_2^- , and 200 μL of acetone. The NO_2^- control consisted of adding only the glucose and the NO_2^- to account for any non-biological NO_2^- reduction. Finally, an inactive control was prepared that contained the cells, glucose, and NO_2^- , but was kept aerobic. The inactive control was used to account for any nitrogen assimilation during cell growth. All bottles were crimp capped with Neutraglas serum bottle seals and butyl rubber stoppers (Thomas Scientific, Waltham, MA). To ensure anaerobic conditions in all samples, the bottles were evacuated and acetylene was added [128, 129].

3.2.3 Experimental Procedure

Samples were collected from each bottle using a 1 mL Hamilton syringe (Hamilton Company, Reno, NV) for the beginning time point. Optical density (OD), pH, NO_2^- , ammonium (NH_4^+), NO_3^- and the target compound concentration were measured at the initial time point and after 24 h. In addition, NO_2^- and OD were further measured every 4 h after the initial time point until the experiment ended (24 h). The total sample

volume for pH (10 μ L), NO_2^- (50 μ L), OD (200 μ L), NH_4^+ (200 μ L), and NO_3^- (500 μ L) analyses was placed in a 1.5 mL centrifuge tube. OD_{620} was measured immediately after all samples were collected using a Multiskan MCC spectrophotometer (Fisher Scientific, Hampton, NH). The tubes were then microcentrifuged for 5 min after which NO_2^- , NH_4^+ , and NO_3^- were measured. The pH was measured last using pH strips (EMD Chemicals Inc., Gibbstown, NJ). Samples collected for chemical analysis were placed in 2 mL glass vials (Agilent Technologies, Santa Clara, CA) and immediately stored at $-20\text{ }^\circ\text{C}$.

A separate set of batch reactors was set up for gene expression measurements. Nitrite was measured at time point 0 and 6 h to verify that denitrification was occurring and remained consistent with other experiments. At those time points, 9.5 mL was removed from each reactor and centrifuged at 5,500 rpm for 5 min using a SORVALL® Super T21 centrifuge (Thermo Scientific, Waltham, MA). The cells were then washed in 1 mL of RNase and DNase free PBS buffer, mixed by pipetting, and transferred to a 1.5 mL centrifuge tube. The pellet was washed again with 1 mL of PBS. After the second PBS wash, the supernatant was removed and the pellet resuspended in 1 mL of RNALater (Life Technologies, Carlsbad, CA) and incubated at $4\text{ }^\circ\text{C}$.

Finally, a separate set of batch reactors was prepared for the cell viability assay. Again, nitrite was measured at time point 0 and 6 h. At 6 h, 1 mL of cells was removed from each reactor and processed immediately with the cell viability assay.

3.2.4 Denitrification Assay

Functional changes were quantified using a colorimetric denitrification assay in which NO_2^- reduction was measured instead of N_2O production. NO_2^- reduction was measured instead of NO_3^- to avoid competing pathways that use or produce NO_3^- and because NO_2^- reductase has been shown to be the rate limiting step of denitrification [130-132]. NO_2^- was measured colorimetrically using a modified version of the Griess reagent method [133]. Briefly, 50 μL of sample were added to a 96 well plate. A standard curve ranging from 0 to 25 mg/L as NO_2^- was developed by serially diluting a stock of KNO_2 made in the modified MM described earlier. To each well, 50 μL of sulfanilamide reagent (1 g sulfanilic acid dissolved in 70 mL of water and 30 mL of acetic acid) was dispensed and the plate was shaken for 10 s at 1,500 rpm, covered with aluminum foil, and incubated for 5 min. Then, 50 μL of N-1-naphthylethylenediamine dihydrochloride (NED) reagent (100 mg of NED dissolved in 60% acetic acid) was added to each well, shaken, covered, and incubated again. NO_2^- was then measured at a wavelength (λ) of 540. The relative denitrification rate was calculated as the ratio of the reduction of NO_2^- per cell (determined from OD_{620} measurements) per h of the treatment and the reduction of NO_2^- per cell per h of the biological control containing no antimicrobial contaminant. The NO_2^- reduction rate was calculated over which ever time was shorter, the time until NO_2^- concentration decreased to less than 10% of the starting concentration before then

or 24 h. To close the nitrogen mass balance, NO_3^- and NH_4^+ were also measured as described in Appendix A

3.2.5 Gene Expression Analysis

The absolute quantification of NO_2^- reductase (*nirS*), and N_2O reductase (*nosZ*) as well as the reference gene (*16S*) was performed using qRT-PCR to verify denitrification inhibition. Total RNA was extracted from each quadruplicate sample within 48 h using the Qiagen RNeasy mini kit (Chatsworth, CA) following the manufacturer's instruction. Complementary DNA (cDNA) was synthesized within 24 h following RNA extraction using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Foster City, CA).

Absolute quantification by qPCR targeting *nirS*, *nosZ*, and *16S rDNA* was performed on a Stratagene Mx3000p real-time PCR apparatus (Cedar Creek, TX) using iTaq SYBR Green Super Mix with ROX binding dye (Bio-Rad, Hercules, CA). Primer sequences for target genes were designed specifically for *P. denitrificans* PD1222 using the open source program Primer3 (v.0.4.0) [134]. Several primer sets were designed and tested and the pairs that resulted in the presence of a unique band of the expected size with the most intensity in a 2% agarose gel stained with ethidium bromide were selected for each of the target genes. For *nirS* the forward primer was 5'-ATCGAGACCTCGAAGATGGA-3' and the reverse primer was 5'-

CTGCTCGTCGTAGATCATGC-3'. For *nosZ* the forward and reverse primers were 5'-GCCTGTCCAAGTTCTCGAAG-3' and 5'-GTCCCAGACCGACTTGATGT-3', respectively. Primers U519F and E685R used to quantify *16S* were previously described elsewhere [135, 136]. Amplicons from *P. denitrificans* PD1222 genomic DNA were used as the standards for each target gene. All samples were run at qPCR efficiencies between 90 and 110% and R^2 value ≥ 0.985 . Results are reported as the absolute transcript ratios of the denitrifying genes *nirs* and *nosZ* to the reference gene *16S*. The absolute transcript values for *16S* are reported without normalization. RT-qPCR results are reported following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [137].

3.2.6 Cell Viability Assay

Membrane integrity was measured using the Invitrogen LIVE/DEAD® fluorescent plate assay (Grand Island, NY) per manufacturer's instructions. Briefly, cells were washed, pelletized, and then resuspended in PBS to reach an $OD_{670} = 0.06$. Then, 100 μ L of the cells were aliquoted into 96 well plates in triplicate and a propidium iodide/SYTO 9 mix was added for staining. After 20 min of incubation at room temperature in the dark, the fluorescence intensity at λ of 530 nm (green) and 630 nm (red) was measured using a Spectramax M5 plate reader (Sunnyvale, California). The excitation λ was 485 nm. Standard curves for both "live" and "dead" curves were run on every plate. "Dead"

cells were treated with isopropyl alcohol. Autofluorescence background was subtracted from the final live/dead cell ratios [138].

3.2.7 Analytical Methods

TCS and triclocarban concentrations were measured at 0 and 24 h to ensure no degradation occurred over the span of the experiment. The samples were first diluted to have a final total mass of 10-100 ng. The diluted samples were then spiked with 100 μ L of the internal standard (i.e., ^{13}C labeled TCS (at 1.0 $\mu\text{g}/\text{mL}$) or triclocarban (at 0.6 $\mu\text{g}/\text{mL}$) and filtered through a methanol-rinsed 0.2 μm polytetrafluoroethylene (PTFE) filter. The samples were then analyzed using liquid chromatography/tandem mass spectrometry (LC/MS-MS) as previously described[139]. A five point standard curve was prepared that covered the range of expected values in the samples. The remaining four compounds were only analyzed using the denitrification inhibition assay and no analytical analyses were completed. For the denitrification, gene expression, and cell viability assays, no significant degradation of TCS and triclocarban was observed over the extent of the experiments and up to 97% of the spiked stock compound was recovered.

3.2.8 Statistical Analysis

Experimental values are reported as the mean \pm standard error. To analyze statistical differences between treatments, one-way analysis of variance (ANOVA) coupled with Tukey's post-hoc analysis using the open source statistical software R (v.2.15.1) was used. Differences were considered significant for p-values ≤ 0.05 [140].

3.3 Results and Discussion

The following section describes the development and demonstration of a denitrification assay that can be used to determine the ecological impacts of biosolids-derived emerging organic contaminants. The assay is also validated using two traditional methods for determining ecotoxicity, gene expression and cell viability. In addition, the environmental implications of the assay results and the potential applications of the assay are discussed.

3.3.1 Assay Development with Antimicrobial Agents

Initial assay testing demonstrated that the denitrification process was successfully isolated and that no competing pathways were used by *P. denitrificans* PD1222. The NH_4^+ concentration remained constant over the duration of the experiment, no NO_3^- was produced and NO_2^- remained steady in the inactive control. The ability to rely on NO_2^-

measurements as opposed to a suite of nitrogenous compounds removes the need for specialized and potentially costly equipment.

The assay was effective at reproducibly measuring changes in denitrification rates for antimicrobials at concentrations ranging over three orders of magnitude with an average standard error of $\pm 8.77\%$ (n=24, Figure 3). While this standard error is quite large, it compares favorably to the 10 to 20% analytical variability commonly reported for the most widely used method for measuring potential denitrification (i.e., the Denitrification Enzyme Activity (DEA) assay) [84, 127]. Overall, the assay results show that the antimicrobial agents exhibit a dose-response relationship where an increase in antimicrobial concentration corresponds to a decrease in denitrification.

The assay's sensitivity was evaluated by measuring the lowest observable adverse effect concentrations (LOAECs) for each antimicrobial. The LOAEC was defined as the contaminant concentration at which the functional endpoint (i.e., nitrite concentration) was significantly different from the biological control. LOAECs of 1.04 μM were obtained for TCS, 3.17 μM for triclocarban, 3.71 μM for bis-(5-chloro-2-hydroxyphenyl)methane, 4.89 μM for 2-chloro-4-phenyl phenol, 45.7 μM for 2-benzyl-4-chlorophenol, and 50.6 μM for 2,4,5-trichlorophenol. The LOAECs reported in this study were consistent with other studies reporting toxicity and microbial process inhibition concentrations ranges of 0.863 to 276 μM for TCS and 31.7 μM to 127 M for triclocarban [124]. The LOAEC reported herein for 2,4,5-trichlorophenol was higher than, although

consistent with a previous study showing 50% reduction in cell yield in simulated wastewater between 10.1 and 12.6 μM . LOAEC values, or similar inhibition values for the remaining compounds could not be found in the literature for comparison. Alone, these LOAECs provide insight into what antimicrobial agents are the most effective biocides. TCS and triclocarban are found in most cleaning products and were shown in this study to be the most effective antimicrobials against *P. denitrificans* Pd1222. The remaining compounds in order of their effectiveness were bis-(5-chloro-2-hydroxyphenyl)methane, 2-chloro-4-phenyl phenol, 2-benzyl-4-chlorophenol, and lastly 2,4,5-trichlorophenol.

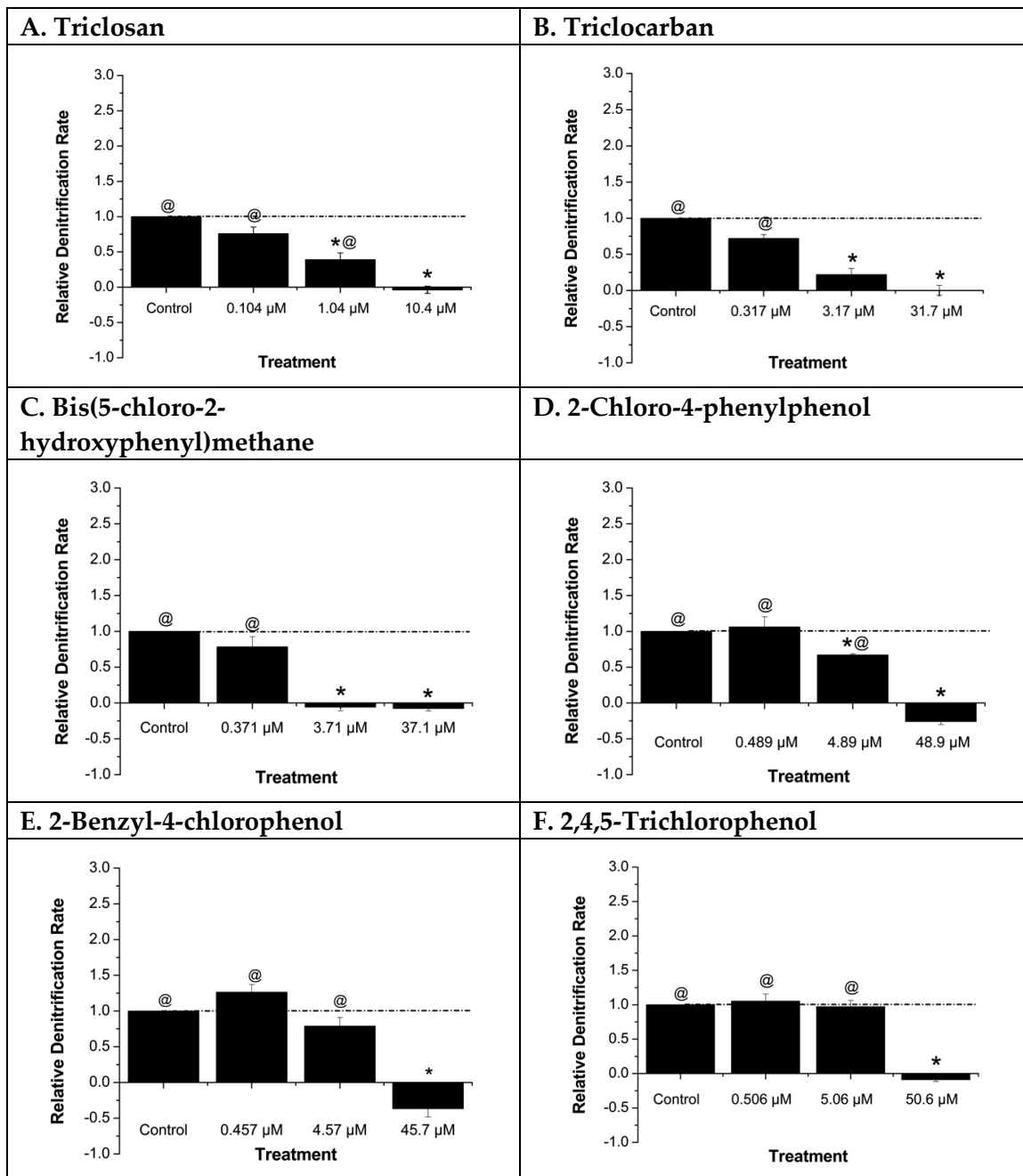


Figure 3: Relative denitrification rate in the presence of varying concentrations of six antimicrobial agents and measured by the denitrification assay. (*) indicates a statistical significant difference from the control ($p \leq 0.05$). (@) indicates a statistical significant difference from 0 ($p \leq 0.05$). Values < 1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error.

3.3.2 Implications of Potential Ecological Impacts

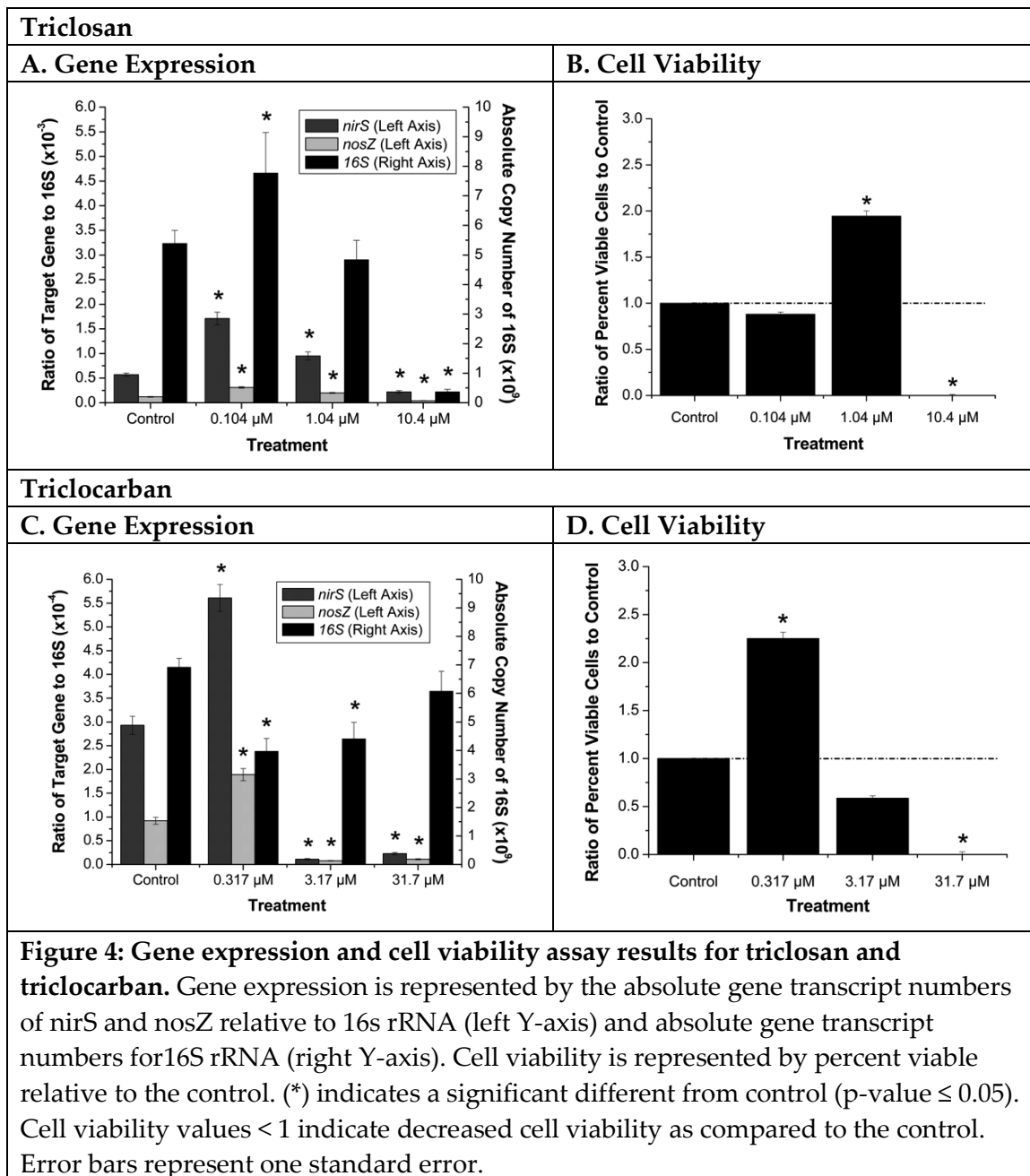
Five of these six antimicrobial agents have been measured or estimated in the environment at concentrations below or near this study's LOAEC values, and one has been measured at concentrations higher than this study's LOAEC values. TCS, has been measured in water and wastewater up to 1.94 μM [22, 141], estimated in pore water of sediment and biosolids amended soil up to 1.32 μM [58], and in measured in biosolids collected from several different WWTPs at concentrations as high as 113 mg/kg dry weight [17, 21, 23, 32, 50-53]. While the LOAEC for TCS in the study is slightly below the aqueous concentrations found in the environment, the LOAEC calculated on a dry weight basis, 4.19 g/kg, was much greater than the concentrations found in biosolids. This is likely a reflection of differences in sample solids content. For instance, whereas the solids concentration herein was low (72 ± 3.5 mg dry weight/L), the average mixed liquor suspended solids concentration in activated sludge samples average 2,000-2,500 mg/L [142]. It should also be noted that the LOAEC in the present study was normalized to the mass of active cells, whereas the values reported in the literature for biosolids are normalized to total solids, which include inactive cells as well as other non-cellular materials. Triclocarban has been measured in water and wastewater up to 0.022 μM [57], estimated in sediment and biosolids amended soil pore space up to 0.867 μM [58], and measured in biosolids up to 441 mg/kg dry [51, 53, 54, 59]. The LOAEC for triclocarban is within one order of magnitude of environmental aqueous concentrations. However,

the solids concentration should be considered when comparing the values in our study to other biosolids studies. On a dry weight basis, the LOAEC (14.0 g/kg) is much greater than the concentrations found in biosolids. Bis(5-chloro-2-hydroxyphenyl)methane was found in anaerobically digested sludge at concentrations up to 0.52 μM , which is within an order of magnitude of the LOAEC for *P. denitrificans* PD1222 [20]. Similarly, 2-Benzyl-4-chlorophenol was found in wastewater influent at 0.12 μM , which is also within an order of magnitude of the LOAEC for *P. denitrificans* PD1222. While the occurrence of concentrations at levels below or near the LOAECs reported in this study may indicate potential ecological impacts, further analysis is warranted as the denitrification assay developed herein uses a liquid medium and the actual bioavailability of these compounds may be substantially different when sorbed to biosolids. In addition, bioavailability is greatly impacted by whether the compound is in the neutral or ionic form. Neutral compounds are generally more bioavailable, but also sorb more readily to natural organic matter (NOM). Based on the pKa of the compounds and assuming a pH of 7, the fraction of the compound in the neutral form was calculated to be 89% for TCS, 100% for triclocarban, 100% for bis-(5-chloro-2-hydroxyphenyl)methane, 92% for 2-Chloro-4-phenyl phenol, and 48% for 2,4,5-trichlorophenol. The pKa of 2-benzyl-4-chlorophenol could not be estimated, and therefore, the fraction in the neutral form was not calculated. Under environmental conditions, sorption would be further impacted by the sorbent material present and the concentrations of ions and other chemical

constituents in the soil or pore-space water. However, these results demonstrate the capabilities of the denitrification assay to provide an initial screening of potentially harmful contaminants.

3.3.3 Denitrification Assay Comparison

To validate the denitrification assay, two other methods (i.e., gene expression analysis and cell viability assays) were tested on *P. denitrificans* PD1222 (Figure 4). These methods were selected for comparison purposes as they are commonly used to establish potential ecotoxicological impacts of contaminants in lieu of proteomic based approaches, which require very specialized and costly equipment [138, 143, 144]. Only TCS and triclocarban were used for this part of the study because the genetic targets are known for these two antimicrobial agents. Due to their widespread use in consumer applications, there is a substantial amount of literature available on these contaminants as compared to the other four antimicrobials.



3.3.4 Gene Expression

Based on gene expression analysis of the functional genes *nirS* and *nosZ*, LOAECs of 10.4 μM and 3.17 μM were obtained for TCS and triclocarban, respectively (Figure 4 A and

C). Strictly examining the LOAECs, the denitrification assay was more or as sensitive, yielding lower values for TCS (1.04 μM) but equal levels for triclocarban (3.17 μM).

Following an initial increase in expression at low concentrations, transcription was increasingly repressed as the concentration of either TCS or triclocarban increased. The increase in transcript numbers for *nirS* and *nosZ* at the low and medium concentrations of TCS found in this study are consistent with the previous studies showing an up-regulation in membrane associated gene transcript numbers [71]. This increase in transcript numbers may be counterintuitive at first since TCS inhibition is known to consist primarily of post-translational inhibition of the enoyl-acyl carrier enzyme (FabI) which blocks fatty acid biosynthesis, and eventually leads to a compromised cell membrane [68, 69]. However, Escalada *et al.* [70] showed that TCS binding with FabI could not explain the bactericidal nature alone and that other mechanisms existed.

Another study supported this finding when they measured an increase in gene transcripts, especially for several proteins associated with the membrane, including the nitrate reductase genes, *narH* and *narJ* in the presence of non-lethal doses of TCS [71].

Transcript numbers for both *nirS* and *nosZ* also increased at the lowest concentration of triclocarban. The explanation for this upregulation is unclear, however, as the mode of action of triclocarban has not been fully characterized. McDonnell and Russell suggested that triclocarban adsorbs to the cell membrane and ultimately compromises its permeability [72]. Similarly to TCS, the inhibition by triclocarban is thought to be

complex and consist of multiple, yet unknown targets [72]. The upregulation observed herein suggests that triclocarban elicited a cell response similar to that of TCS at the lowest concentration. At the highest concentrations for both compounds, the denitrifying genes were suppressed, which could be due to either down regulation of the genes or degradation of the transcripts. Lysing of the cells and the release of cellular components, followed by mRNA and rRNA degradation could explain the decrease in the transcript numbers at this concentration. This explanation is consistent with the cell viability results discussed later in this manuscript.

An advantage of gene expression over the denitrification assay method is that gene expression data may provide some indication as to the mechanisms of inhibition imparted by the contaminant of interest. While this method does not provide a direct functional end point measurement, it may still provide useful information if the mode of inhibition is at the transcription level and the correct gene targets are known. However, for many contaminants, impacts are post-transcriptional and no effect may be observed in gene expression studies unless the cells are severely stressed and in the process of dying. For example, although TCS has been shown to have some impact on gene transcription, relegated mostly to membrane-associated transcripts, this did not directly correlate to activity, as shown by the denitrification assay. A proteomic-based approach could provide more targeted mechanistic and functional endpoint information (as compared to transcriptomic-based approaches), however, these methods are very cost-

prohibitive and time intensive. For these reasons, proteomic approaches have not been widely implemented [145].

3.3.5 Cell Viability

The second method that was compared to the denitrification assay was cell viability.

Using the cell viability assay, LOAECs of 10.4 μM and 31.7 μM were obtained for TCS and triclocarban, respectively (Figure 4 B and D). Both LOAECs are higher than those obtained with the denitrification assay suggesting the lower sensitivity of the cell viability assay. This result is not unexpected as cell viability only provides a measure of ultimate cell death, which is the final result of more complex cellular interactions.

Similarly to the gene expression measurements, cell viability can provide some indication of inhibitory mechanisms, albeit limited and less targeted than gene expression. Using the cell viability assay, a dose response relationship was not observed.

At the highest concentration for both compounds, near complete cell lysis occurred.

However, at the medium TCS and low triclocarban concentrations, a statistically significant increase in cell viability was observed as compared to the negative control, suggesting that at these concentrations, TCS and triclocarban may have helped sustain the cell integrity for a longer duration. We are not the first to observed this increase, which was previously attributed to intrinsic uptake mechanisms such as efflux pump extrusion of the compound from the cell or a change in the cell permeability [146-150].

These contradictory results illustrate some of the difficulties in utilizing cell viability to assess ecotoxicological impacts of contaminants. Cells with compromised membranes may still be partially active, and inversely, intact cells may not be fully active. Cell viability data only provide surrogate information as opposed to direct functional data. Finally, cell viability is limited to compounds that lead to the cell membrane being compromised, and therefore, may obscure more complex interactions between contaminants and microorganisms.

3.3.6 Potential Assay Applications

We have demonstrated that the denitrification assay developed herein can be used effectively as an initial indicator for potential adverse ecological impacts by antimicrobial contaminants. This assay is particularly attractive for biosolids where it is difficult to measure the ecotoxicological impacts. The strengths of this assay are: 1) its ability to measure a functional endpoint; 2) its adaptability to high-throughput screening and, 3) its reliance on non-specialized equipment thereby decreasing its implementation cost. However, one of the general drawbacks of this method is that it does not provide any mechanistic details regarding potential inhibitory mechanisms. However, as an initial test to determine which of the myriad emerging contaminants found in biosolids require further examination, the denitrification assay provides a viable alternative that coincides with the initial toxicological testing outlined in the US Environmental

Protection Agency (USEPA) guidelines regarding the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) and recommended for the Toxic Substances and Control Act (TSCA).

Much of the current toxicological research is focused on the direct impacts of emerging contaminants on human health and often requires some form of animal testing. Research on potential indirect impacts, such as the impacts on microorganisms beneficial to humans, has been limited. The fact that *P. denitrificans* Pd1222 is a facultative aerobic microorganism allowed for the isolation of the denitrification pathway and measurement of denitrification. Still, the same method could be expanded to other ecologically important organisms or groups of organisms. For example, other model denitrifiers could be used in lieu of *P. denitrificans* Pd1222, if they can complete the entire denitrification pathway. Alternatively, multiple denitrifiers that together have the complete denitrification pathway could be used instead, as long as any differences in growing conditions were appropriately addressed. In addition, other assays could be developed that provide measurements of other functional endpoints that can also indicate soil health, including those relevant to carbon cycling and decomposition.

An important advantage of the method outlined in this study is its short experimental length (24 h) and its reliance on low cost instrumentation, including basic culturing equipment and a spectrophotometer. Denitrification was quantified colorimetrically, which is significantly cheaper than measuring the denitrification end

products (N_2O or N_2) or measuring a stable nitrogen isotope throughout the denitrification process, which both require specialized equipment such as a gas chromatographer with mass spectrometry capabilities. One limitation, however, is that measuring NO_2^- reduction may not directly reflect the amount of end product (N_2O or N_2) formed [84]. However, the use of acetylene in combination with nitrite instead of nitrate and a pure culture should avoid many of the limitations associated with the traditional methods [151].

Finally, it may be advisable to expand this method to more complex media that better simulate biosolids and soil. The soil microbial ecology test protocols outlined for FIFRA and recommended for TSCA require the addition of standard soil to the assays. However, there are no suitable biosolids standards that can be used to isolate the impacts of individual contaminants. This issue is further complicated as Alexander as well as Pannu *et al.* [152] demonstrated that contaminant bioavailability drastically changes when a contaminant is spiked into biosolids as opposed to gradually introduced during wastewater and aged through digestion [153]. Therefore, obtaining solid media for these tests could prove challenging. For these reasons, it may be preferable to perform initial screens in simple systems such as that described herein and later extrapolate to a more complex and realistic conditions if initial screenings indicate ecotoxicological potential.

4. Evaluating the impacts of Triclosan on Biological Wastewater Treatment Processes using Bench-Scale Activated Sludge Sequencing Batch Reactors Coupled with Anaerobic Digesters.

The following chapter describes the portion of the work completed for Objective 3.

4.1 Introduction

Triclosan (TCS), also known as 5-chloro-2-(2,4-dichlorophenoxy) phenol, is a broad range antimicrobial agent commonly used in personal care products (PCPs), such as shampoos, soap, detergent ,and toothpaste, among many others [40, 41], and is used heavily in the U.S. with over \$1 billion in yearly sales [16]. As a result of their extensive use, these products are likely the major contributors of TCS to wastewater treatment plants [6, 25, 42]. TCS has been measured in wastewater up to 1.94 μM [22, 141]. TCS also has a solubility of approximately 6.9 to 13.8 μM [46]. The Log K_{ow} of TCS is 4.8, and the acid dissociation constant (pKa) is 7.9, suggesting that it is fairly bioaccumulative (non-ionized) under ambient conditions and partitions to solids (biological material) in wastewater treatment, as evidenced by the high concentrations in biosolids (113 mg/kg) [6, 17, 44]. Because TCS is deliberately used to deactivate microorganisms, partitioning to solids, or flocs, where much of a wastewater treatment plant's biological process occur, may lead to a greater potential to negatively impact beneficial microorganisms.

The mechanism of TCS microbial inhibition has been thoroughly examined. At high concentrations TCS inhibition primarily consists of post-translational inhibition of the enoyl-acyl carrier enzyme (FabI), which blocks fatty acid biosynthesis, and eventually leads to a compromised membrane [68, 69]. At low concentrations, TCS has been shown to have some impact on membrane-associated gene expression [70, 71].

Because TCS is antimicrobial and the inhibition mechanisms are relatively well characterized, many studies have examined the general toxicity of TCS, with a few focused on biological wastewater processes and microbial processes in general. These studies reported a large range of inhibitory concentrations of TCS for microorganisms, 0.863 to 276 μM [124]. As far as studies targeting specific microbial processes, our previous research showed that triclosan inhibited denitrification, a major component of the nitrogen cycle, at concentrations as low as 1.04 μM [154]. Other studies reported median effective concentrations (EC₅₀) on various pure and mixed cultures of microorganisms of 6.29 μM , based on biochemical oxygen demand (BOD) degradation, 69.1 μM based on oxygen consumption, and 825 μM based on glucose utilization [76, 155]. Examining a continuous-flow activated sludge system, Stasinakis *et al.* [156] showed that nitrification was temporarily inhibited at a TCS concentration of 1.73 μM of TCS, and the EC₅₀ of ammonium (NH_4^+) uptake was 34.4 μM . In a follow-up study also on a continuous flow activated sludge system by the authors, NH_4^+ removal was inhibited at an even lower concentration (3.45 μM) [157]. However, in both instances,

TCS was not fed to the reactors, but rather, TCS inhibition was measured on samples taken from the reactors. Thus, any impacts may have been artifacts of solids not acclimated with TCS. Federle *et al.* [158] incrementally increased TCS feed from 0.14 to 6.90 μM to sequencing batch reactors (SBRs) inoculated with activated sludge and found no inhibition of chemical oxygen demand (COD), BOD, or NH_4^+ removal. However, the primary focus of this study was on TCS degradation and the authors specifically selected for TCS resistant microorganisms by feeding the reactors continuously with domestic wastewater dosed with TCS at slowly increasing concentrations. On the other hand, the study presented herein examined the impact of simulated wastewater dosed with triclosan on startup and steady-state wastewater treatment processes (COD, nitrogen, and phosphorus removal) and microbial community structure using sequencing batch reactors (SBRs) coupled with anaerobic digesters.

4.2 Materials and Methods

The following section describes the methods used for the research presented in this chapter.

4.2.1 SBR Design and Operation

Four reactors, consisting of 11.5 L Mr. Aqua rectangular glass fish-tanks (30.0 cm x 17.8 cm x 20.3 cm, 0.64 cm thick) sealed with silicon (Taiwan), were set up in parallel in a

chemical containment hood and were maintained at room temperature (~20 °C) (Figure 5). The walls of the reactors were covered in aluminum foil to prevent photodegradation of TCS. The top of the reactor was covered with an aluminum foil-lined plastic container. The peak volume in each reactor was 9 L and the volume after decanting was 3.2 L. The SBRs were operated on a 6 h cycle, with 30 min of synthetic influent feeding without aeration, 3 h of aeration and complete mixing, 1 h of settling, and 30 min of decanting, and 1 h idle. The SBRs were kept idle (the mixers were not turned on) during influent feeding to encourage phosphorus removal [159]. A solids retention time (SRT) of 10 days was selected to promote nitrification [160]. The operating hydraulic retention time (HRT) was 5.6 h, which was in the range of contact stabilization [159]. Once per day, during the last 5 min of idle time, the mixers were turned on and 250 mL of waste was decanted and wasted or collected in the anaerobic digester (after 30 days of operation when steady-state conditions were reached). All four of the reactors were fed synthetic wastewater (SWW) using a previously published recipe [161]. The SWW had an average of 450 mg/L COD and 40 mg/L of ammonium. Two of the reactors were fed only SWW (Control A and B), while two others were fed SWW with TCS (TCS A and B).

Ambient air was fed to the reactors via two PetSmart Top Fin Air 8000 aerators (Phoenix, AZ). Each aerator had four separate tubes attached to PetSmart Top Fin Fine bubble air stone diffusers (Phoenix, AZ) that were placed evenly throughout the reactors in order to maintain even aeration. The dissolved oxygen was maintained above 4.2

mg/L. Multi-head peristaltic pumps were used to feed the influent and the effluent (Cole Parmer, Masterflex L/STM, Vernon Hills, IL). Three days of influent SWW were stored for each reactor in a PetSmart Great Choice 76 L rectangular glass tank (76.2 x 30.5 cm x 30.5 cm) lined with aluminum foil and covered to prevent photodegradation (Phoenix, AZ). Influent tubing was weighed down with stainless steel nuts. Effluent from each reactor was collected in a separate unlined, but covered 76 L glass tank. Reactors were mixed with large stir bars on stir plates at 500 RPM. All cycling components were controlled with GE Digital SunSmart digital (Fairfield, CT) and Intermatic TN311C (Grove, IL) timer-controllers. All tubing entering the reactors was secured to the wall with suction cups PetSmart Great Choice Airline Holders (Phoenix, AZ) and zip-ties at the appropriate heights. The SBRs were initially spiked with 9 L of activated sludge taken from the North Durham Water Reclamation Facility (NDWRF, Durham, NC), which currently treats 20 MGD completes biological nutrient removal of BOD, NH_4^+ , and phosphorus. Also, to determine the impact of variations between inoculum microbial communities, reactors Control A and TCS A were inoculated with a different composite activated sludge sample than Control B and TCS B. However, in both cases the inoculum was added to the SBRs within 2 h of collection.

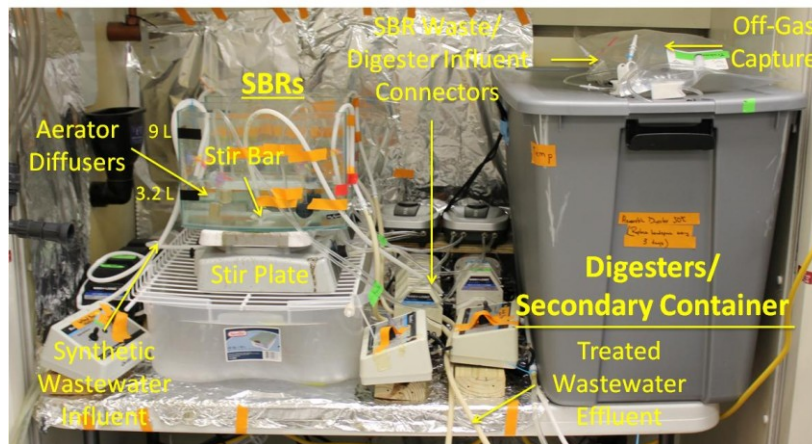
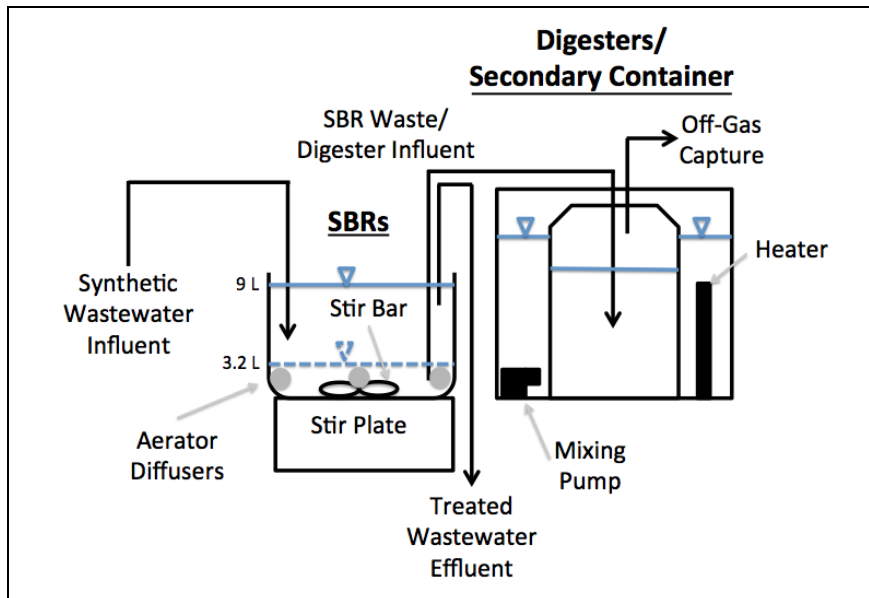


Figure 5: Schematic and photo of SBR and anaerobic setup. Note that the SBRs are not yet covered and have not been inoculated, and the SBR and the anaerobic digesters are not connected.

SBR analysis began immediately after inoculation and was repeated approximately every 7 days thereafter until operation ceased. COD, NH_4^+ , nitrate (NO_3^-),

nitrite (NO_2^-), total suspended solids (TSS), volatile suspended solids (VSS), dissolved oxygen (DO), and pH were completed on each sampling day. Phosphate (PO_4^{3-}) and microbial community analyses, on the other hand, were completed every 2 to 3 sampling days. For each reactor and in triplicate, the total sample volume for NH_4^+ (200 μL), NO_2^- (50 μL), and PO_4^{3-} (250 μL) analyses was obtained from the influent and effluent and placed in a 1.5 mL centrifuge tube. The samples were then microcentrifuged for 5 min at 13K rpm on an Eppendorf 5424 centrifuge (Hamburg, Germany) after which NO_2^- , NH_4^+ , and PO_4^{3-} (when appropriate) were measured. Triplicate 10 mL samples were obtained from the influent and effluent for NO_3^- and COD analyses. In addition, triplicate 25 mL samples were obtained from the influent, reactor, and effluent for pH. Lastly, triplicate 25 mL samples were obtained from each reactor for TSS and VSS analyses. Once every 10 to 20 days, triplicate 1 mL samples were obtained only from the Control and TCS replicate B reactors, centrifuged for 5 min at 13K rpm, the supernatant removed via pipetting, and the pellet stored at -20°C for further processing for microbial community analysis. Samples for microbial community analysis were not collected from Replicate A reactors by mistake.

4.2.2 TCS Addition

The target concentration of TCS was selected to reflect a wastewater treatment plant that generated biosolids with high levels of TCS and was calculated based on available

published adsorption characteristics of TCS (albeit for soil and biosolids) and published range of concentrations measured in biosolids [23, 42, 59, 162] The concentration selected was 0.73 μM of TCS, which was calculated from a theoretical biosolids concentration of 30 to 50 mg/kg dry weight (dependent upon the adsorption isotherm used). Stock TCS was made in acetone and spiked into the influent tank using a Hamilton Syringe (Hamilton Company, Reno, NV) and mixed using a stainless-steel spoon. Fresh influent with TCS was made every two or three days.

4.2.3 Anaerobic Digester Design and Operation

One anaerobic digester was set up for each SBR treatment: Control B and TCS B SBRs. The digester consisted of 19 L solvent-rinsed, glass carboys by Learn to Brew, LLC (Moore, OK). Each digester was sealed with a VWR rubber stopper (Radnor, PA) and silicon. The respective waste decanting tube from the SBR was fed into the digester through the stopper. Another tube was placed into the digester through the stopper and was connected to a 5 L SKC Tedlar Sample Bag (Eighty Four, PA). Once the SBRs reached steady-state (Day 30), the digesters were inoculated with 100 mL of anaerobically digested sludge from the NDWRF (Durham, NC). After inoculation, the tubing and the stoppers were sealed and the headspace of the carboys was evacuated and replaced with nitrogen gas at atmospheric pressure. The glass carboys were then placed in a 113 L plastic container and weighed down using sand bags. The container

was filled with water. A 200 Watt Aqueon aquarium heater (Franklin, WI) was used to maintain the water temperature at 27 °C and a PetSmart 950 Aqueon circulation pump (Phoenix, AZ) was used to continuously mix the water. The container with the digesters was covered in order to minimize photodegradation. Two hundred fifty mL was wasted from each SBR to the anaerobic digesters per day for 47 days and when the SBRs were shutdown, all remaining solids from the SBR were pumped to the digesters. The digesters were then isolated from the SBRs and digestion commenced for 20 days. The digesters were mixed manually every 3 days throughout the entire operation to ensure complete digestion.

Completely mixed samples were obtained from the digester after digestion was complete and were analyzed for TCS concentration, TSS, VSS, Total Kjeldahl nitrogen (TKN), phosphorus, potassium, and pH. Triplicate 10 mL samples were obtained from each digester for TSS and VSS analyses. Triplicate 50 mL composite sample were obtained for TKN, phosphorus, potassium, and pH analyses and was sent to Waters Agricultural Laboratories, Inc. (Georgia USA) for processing. Forty mL samples were obtained for TCS analysis and were placed in volatile organic analysis certified Thermo Scientific 50 mL amber vials with silicone/polytetrafluoroethylene (PFTE) septum in polypropylene caps (Waltham, MA), stored at 4 °C and processed the within 24 h.

4.2.4 Analytical Methods

COD (mercuric digestion method) and nitrate (cadmium reduction method) were measured using HACH (Loveland, CO) reagents. NO_2^- was measured colorimetrically using a modified version of the Griess reagent method and published previously [133, 154]. A modified version of the phenate method (Section 4500 of the Standard Methods for the Examination of Water and Wastewater) was used to measure NH_4^+ and described previously [154, 163]. PO_4^{3-} was also measured according to a previously published method [133]. TSS and VSS analyses were completed according to Section 2450 of the Standard Methods for the Examination of Water and Wastewater [163]. Finally, pH was measured using a Beckman Coulter, Inc. pH/temperature/millivolt meter and probe (Fullerton, CA).

Gas production by the digesters was also monitored to evaluate digester performance. Briefly, triplicate 10 mL gas samples were taken from each gas sample bag using a Poulten & Graf Ltd Fortuna 10 mL gas-tight syringe (Barking, Essex, UK) and Becton Dickinson and Company 25 gauge needles (Franklin Lakes, NJ). The samples were then injected into 9 mL gas vials crimp capped with butyl stoppers that were flushed with nitrogen gas (N_2) and evacuated as previously described [127]. Vials, butyl stoppers, and aluminum crimp caps were obtained from Grace Davison Discovery Science (Deerfield, IL). The methane of the gas samples was measured with a modified Shimadzu gas chromatographer (GC) 17A version 3 (Kyoto, Japan) and Tekmar

headspace autosampler (Vernon, BC, Canada) within 7 days. The GC was retrofitted in 2007 with a methanizer, ECD, flame ionization detector (FID), and Valco Instruments Co. Inc. valves (Houston, TX) to approximate the GC-2014 Greenhouse Gas Analyzer. The remaining gas volume was measured using a Singer (Elster) American Meter Co. flow meter (Nebraska City, NE).

To verify the TCS dosage, stock concentrations were measured. TCS stock concentrations were first diluted to have a final total mass of 10 to 100 ng. The diluted samples were then spiked with 100 μ L of the internal standard (i.e., ^{13}C labeled triclosan (at 1.0 $\mu\text{g}/\text{mL}$) and filtered through a methanol-rinsed 0.2 μm polytetrafluoroethylene (PTFE) filter. The samples were then analyzed using liquid chromatography/tandem mass spectrometry (LC/MS-MS) as previously described [139].

To measure the TCS concentration in the simulated digested biosolids, the liquid and solids fractions were first separated via centrifugation by an Eppendorf 5810R centrifuge (Hamburg, Germany). The aqueous portion was then transferred to a 50 mL Kinman glass vial (Des Moines, IA) centrifuged tube and extracted with 50:50 dichloromethane (DCM):hexane three times. Briefly, 10 mL of 50:50 DCM:hexane was added to the sample and vortexed for 10 s. The samples were then centrifuged at 2,500 rpm for 5 min. The supernatant was then collected in a VWR 15 mL glass test tube (Radnor, PA). The extraction was repeated for a total of three times. The samples were further processed and analyzed using LC/MS-MS as previously described for biosolids

samples starting with the sulfuric acid purification step [139]. The solid portion of the samples were analyzed according to the same method, but starting with homogenization with sodium sulfate and pressurized fluid extraction [139]. All solvents were high pressure liquid chromatography (HPLC) grade and were obtained from Sigma Aldrich (Milwaukee, WI).

4.2.5 DNA Extraction and PCR Conditions

Total DNA was extracted from all replicates on all days using the MO BIO PowerLyzer PowerSoil DNA Extraction Kit by (Carlsbad, CA). All extractions were performed following a modified version of the manufacturer's protocol [164]. Briefly, after the addition of reagent C1, 400-500 μ L of 25:24:1 phenol/chloroform/IAA was added to each tube and vortexed. Following, instead of homogenizing the samples, the samples were placed on a horizontal shaker at max speed for 5 min. Also, 25 μ L of eluent buffer (C6) was added to the white filter membrane, the samples were incubated for 5 min and centrifuged for 30 s at 13K rpm. This step was then repeated. After DNA extraction was completed a spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to verify the concentration and purity of the DNA. PCR of the bacterial 16S SSU rRNA gene region was completed based on previously described method by Lukow *et al.* [165] and modified by Alito and Gunsch [166]. 6-FAM labeled 27F was used as the forward primer and unlabeled 1392R was used as the reverse primer [165]. For each PCR

reaction, 1 μ L of purified template DNA was used. Following amplification, the presence of the correct length PCR amplicons was confirmed via visualization on a 1 % agarose gel containing 0.1% ethidium bromide. PCR amplicons were then purified using a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). The final PCR product concentrations and purity were again verified using the NanoDrop.

4.2.6 T-RFLP Analysis

The PCR amplicons were digested with restriction enzymes as described in Lukow *et al.* [165]. However, no bovine serum albumin (BSA) was used in the reactions as preliminary experiments showed interference. One hundred ng of purified PCR product and 10 U of the restriction enzyme, *MspI*, (New England Bio-labs Inc., Beverly, MA) were used for each reaction. The reactions were then incubated for 2 h at 37 °C. Following incubation, samples were stored at -20 °C until further processing by the Duke University DNA Analysis Facility. The Duke University DNA Analysis Facility desalted the samples through a spin column filtration and completed fragment analysis using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (BioVentures, Inc., Murfreesboro, TN).

T-RFLP profiles were evaluated using Applied Biosystem's Genescan v3.7.1 analysis software (Foster City, CA). Manual inspection of each profile was completed to

ensure only true peaks were chosen for statistical analyses. The raw data was then imported into T-REX in the following format; Dye/Peak, Sample File Name, Size, Height, Area in Point, Area in BP [167]. All peaks less than 50 basepairs (bp) were excluded from the analysis because they were possible primer dimer fragments. Only T-RFs with a minimum peak height threshold of 50 relative fluorescent units were used. T-REX was used to align the profiles based on the replicates using the “Environments” function in the software. Presence/absence data files were imported into PAST (Paleontological Statistics software) for data analysis [168]. Peak area is bias towards the abundance of a specific species (each fragment), whereas, presence/absence gives all species (each fragment) the same weight during analysis. Thus, presence/absence is especially important when species of low abundance are significantly impacted more by the treatments. Also, because of inherent biases with PCR, peak area measurements are only semi-quantitative. After the data were analyzed, the relative species abundance was calculated based on the number of peaks present in each sample. The relative abundance values were then compared between the control and TCS SBRs over time. The data were then analyzed using principal nonmetric multidimensional scaling (NMS) coupled with Jaccard similarity distance for group clustering. The Jaccard similarity distance was used because it works well for binary data, such as the presence/absence data compared herein. Measured variables such as, NH_4^+ reduction, and effluent NO_3^- and NO_2^- were then compared to the NMS ordination results and by using linear regression models.

The correlation coefficients between each environmental variable and the NMS axis scores are presented on the ordination plots as vectors from the origin. The vectors relative length and direction indicate the extent of the correlation.

4.2.7 Statistical Analysis

Experimental values are reported as the mean \pm standard error. To analyze statistical differences between treatments, one-way and two-way analysis of variance (ANOVA) coupled with Tukey's post-hoc analysis using the open source statistical software R (v.2.15.1) was used. Differences were considered significant for p-values ≤ 0.05 [140]. Marginal significance was considered for p-values ≤ 0.10 . In addition, linear regression analysis was also completed using R and significance was again determined using ANOVA.

4.3 Results and Discussion

The results obtained from the SBRs coupled with anaerobic digesters are provided below.

4.3.1 TCS Concentrations

TCS stock concentrations were quantified to verify the correct dosage was being applied to the SBRs. For the SBRs, 93.6% ($\pm 1.0\%$) of the spiked stock compound was recovered.

Concentrations were not measured within the reactors because they were being fed fresh SWW with TCS 6 times each day and new SWW with TCS was made every 3 days. As stated earlier, the TCS concentration was selected to achieve a final biosolids concentration of 30 to 50 mg/kg. The concentrations measured in the digested control and TCS biosolids were significantly different from each other and were 2.85 ± 0.69 mg/kg for the control SBR and 82.7 ± 11.1 mg/kg from the TCS dosed SBR. The aqueous portions comprised $1.48 \pm 0.43\%$ and $4.06 \pm 0.35\%$ for the control and TCS biosolids, respectively. Published TCS concentrations measured in biosolids range from 0.09 mg/kg, all the way up to 133 mg/kg [23, 42, 44, 53, 54, 59, 157, 169-172]. Of this data set, the average concentration was calculated to be about 4.9 mg/kg (note that this average was based on published data where in many instances, only ranges were provided). Thus, while the concentration in the control was higher than expected, the control and the TCS biosolids fall on the lower and higher ends of the published range of values, respectively. The higher than expected concentration for the control biosolids was likely due to background concentrations in the anaerobically digested sludge used for inoculum, from inoculum activated sludge that remained in the SBR after steady state was reached, or from external contamination due to high use of TCS containing domestic products (dishwashing detergent, etc.). The biosolids aged with TCS were noticeably higher than what was estimated, but this was likely due to differences

between the conditions within the SBR and the soil and biosolids used to develop the adsorption isotherms, such as pH.

4.3.2 Reactor Characteristics

Throughout the operation of the SBRs, TSS, pH and DO were monitored and can be seen in Table 2. For all of the characteristics, there was no significant difference between treatments within a replicate (Control A vs. TCS A and Control B vs. TCS B). However, the DO, control influent and reactor pH, and TCS reactor pH were significantly different between A and B (p-value = 0.002).

Characteristic	A Replicates (n=10)		B Replicates (n=11)	
	Control	TCS	Control	TCS
TSS (mg/L)	2,370 ± 222	2,248 ± 217	3,084 ± 202	3,327 ± 275
DO (mg/L)	7.461 ± 0.15		4.21 ± 0.66	
Influent pH	6.74 ± 0.15	6.7 ± 0.13	6.38 ± 0.05	6.42 ± 0.14
Reactor pH	7.73 ± 0.12	7.78 ± 0.09	7.37 ± 0.07	7.33 ± 0.06
Effluent pH	7.85 ± 0.05	7.95 ± 0.06	7.74 ± 0.13	7.74 ± 0.12

4.3.3 Treatment Efficiencies

Removal of COD, PO₄³⁻, and NH₄⁺ were examined to determine if the addition of TCS had any impact on treatment efficiency (Figure 6). COD removal was maintained between 86 and 100% throughout the entire operation of both Control and TCS SBRs and there was no significant difference between the two treatments. COD removal was not impacted by the addition of TCS, which was consistent with a previous study

showing only a minor effect on organic substrate removal at much higher concentrations (6.9 μM) [156]. Similarly, PO_4^{3-} , was not impacted by the addition of TCS. There was a significant difference between SBRs receiving the same inoculum. Both Control A and TCS A reach nearly completed PO_4^{3-} removal, whereas Control B and TCS B reached a maximum of about 10 to 20% removal. The difference between the replicates for A and B, was most likely due to differences in the inoculum activated sludge or in the measured DO. Recall that Control A and TCS A received activated sludge that was collected from the same wastewater treatment plant, but at a different time than Control B and TCS B. In addition, the DO was significantly different between replicates A and B. Because biological phosphorus removal only occurs under anaerobic conditions, the higher DO in the B replicates during aeration could have led to higher DO during the anoxic/anaerobic cycles of the SBRs and decreased removal of NO_3^- and therefore, decreased phosphorus removal. While a static influent feed was used to promote phosphorus removal in this study, simultaneous nitrogen and phosphorus removal can be difficult to achieve in an SBR [159]. Regardless, there was no difference in PO_4^{3-} removal at steady-state conditions between the treatments when SBRs that received the same inoculum were compared.

NH_4^+ was greatly impacted by the addition of TCS. Again, there was a significant difference between replicates A and B at startup; Control A and TCS A had lower initial NH_4^+ removal than Control B and TCS B. Again, this was most likely due to differences

in the inoculum. However, both of the Controls reached steady-state at greater than 92% NH_4^+ removal within the first week of operation, whereas TCS A and TCS B took 42 and 63 days to reach steady-state removal at that level, respectively. Inhibition of NH_4^+ removal could be attributed to the bacterial communities slowly acclimating to TCS, however, inhibition continued past 30 days (3x SRTs) when the solids from the inoculum should have already been removed. In addition, temporary inhibition of nitrification was also observed by Stasinakis *et al.* [156] at albeit at a slightly higher TCS concentration of 1.73 μM . The increased sensitivity to external changes and slower growth rate of autotrophic nitrifiers compared to the heterotrophic bacteria responsible for COD removal, likely explains why TCS impacted NH_4^+ removal, but not COD removal in this study [142, 173]. NH_4^+ is removed from wastewater treatment plants by ammonia oxidizing bacteria (AOB) and inhibition of these organisms, such as that shown in this study could result in the WWTP exceeding effluent nutrient permit levels.

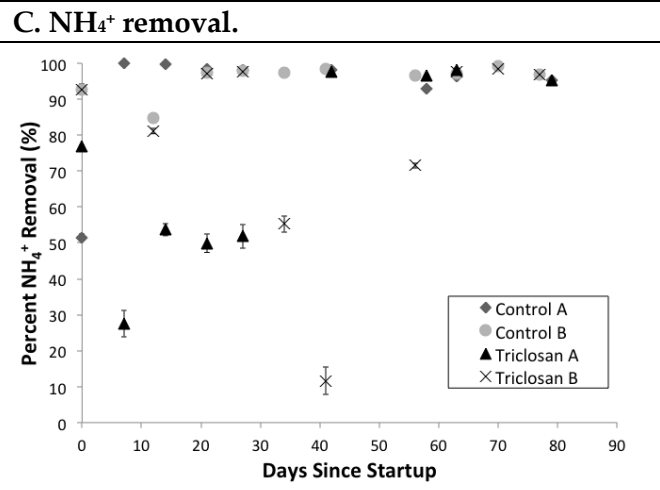
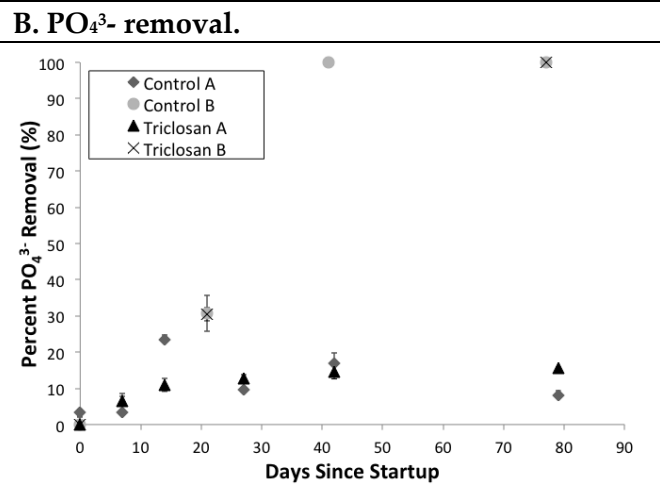
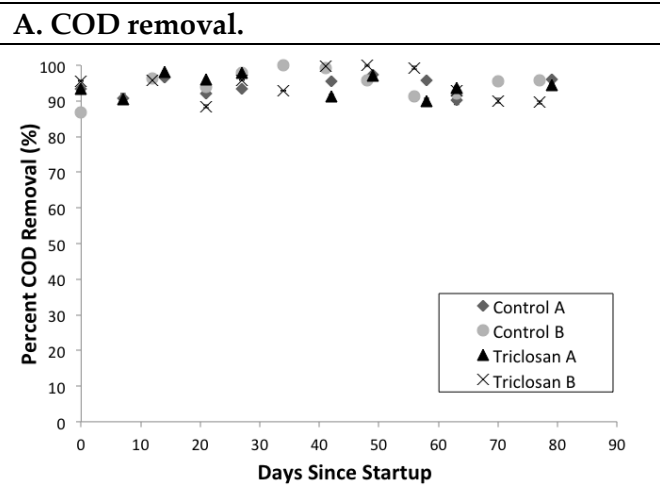
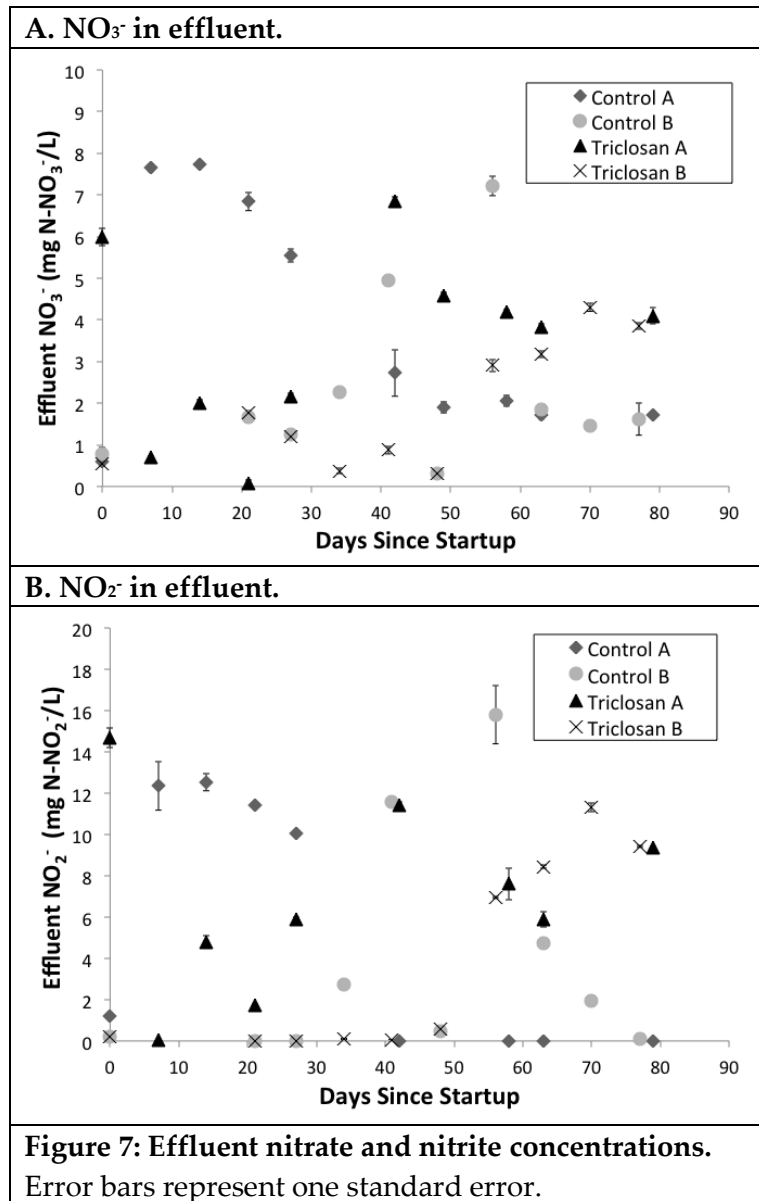


Figure 6: COD, PO₄³⁻, and NH₄⁺ removal percentages. Error bars represent one standard error.

Effluent NO_3^- and NO_2^- concentrations were also measured (Figure 7). For both the Controls and the TCS SBRs, the effluent NO_3^- and NO_2^- concentrations were much more variable than COD and PO_4^{3-} removal, and unlike NH_4^+ removal, were more variable for both the control and TCS SBRs until around Day 58. After Day 58, the NO_3^- effluent concentrations for the TCS replicates was 3.9 ± 0.16 mg/L, which was 2.4 times greater than the NO_3^- effluent of the controls (1.7 ± 0.08 mg/L) and was significant.. Similarly, after Day 58, the NO_2^- effluent of the TCS replicates reached a steady-state concentration of 8.7 ± 0.75 mg/L. The mean NO_2^- concentration of the controls after Day 58 was 7.7 times lower at 1.1 ± 0.78 mg/L, but was still trending towards 0 when the reactors were stopped. The difference between control and TCS NO_2^- after Day 58 was significant. These data suggest that while AOBs were inhibited temporarily, the nitrite oxidizing bacteria (NOB), which complete the second step of nitrification (i.e., oxidation of NO_2^- to NO_3^-) and denitrifiers were inhibited by the addition of TCS on a more long-term basis. In addition, the concentration used in this study ($0.73 \mu\text{M}$) was lower than the concentration shown to significantly inhibit denitrification by the common soil denitrifier *Paracoccus denitrificans* PD1222 ($1.04 \mu\text{M}$) (Chapter 3), and lower than concentrations shown to inhibit denitrification in other studies [154, 156, 157].



4.3.4 Digestion Characteristics and Performance

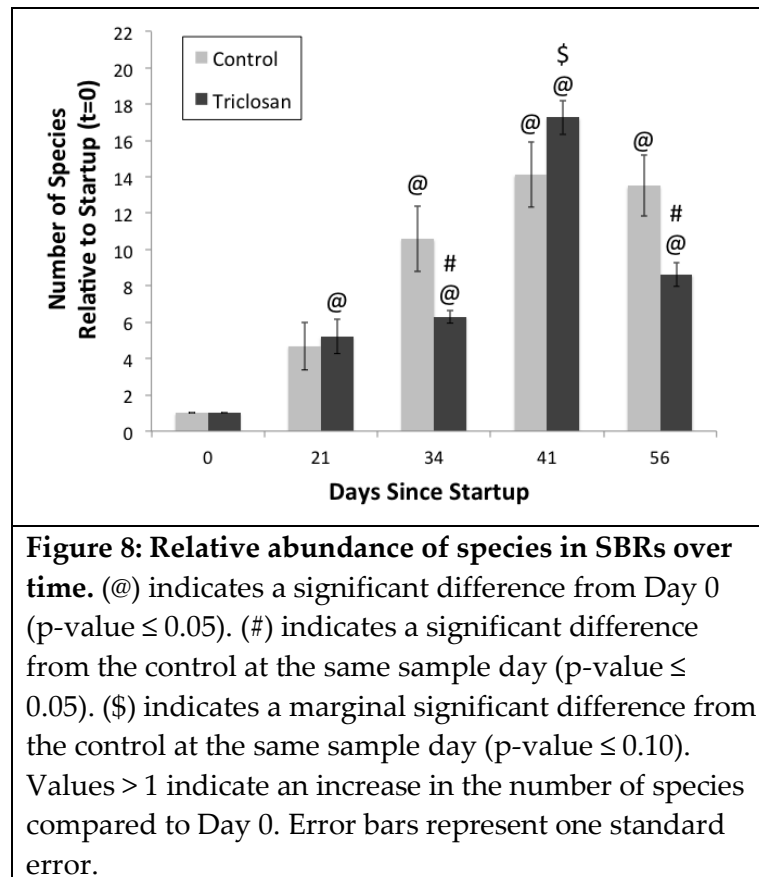
Solids were wasted to the anaerobic digesters for 47 days from Control B and TCS B SBRs. On the final day of SBR operation (Day 77), the entire idle-volume of the SBRs were emptied into the corresponding digesters. The TKN, phosphorus (as P₂O₅), and

potassium (K_2O) concentrations, and pH measured in the control biosolids were $4,667 \pm 273$, $1,028 \pm 22$, and 182 ± 3.1 mg/kg, and 6.68 ± 0.04 , respectively. In the TCS biosolids, the values were $3,777 \pm 82$, 781 ± 7.9 , and 153 ± 0.64 mg/kg, and 6.71 ± 0.02 , respectively. The TSS of the control and TCS biosolids was $5,877 \pm 46.4$ and $6,223 \pm 132$ mg/L, respectively. TKN, P_2O_5 , K_2O , and OC values of the biosolids generated in this study were approximately an order of magnitude lower than those reported for typical biosolids [174]. However, when the TKN, P_2O_5 , and K_2O concentrations were normalized to TSS, there were no significant differences between the two biosolids treatments (calculated in Chapter 5). The total gas produced by the control and TCS digesters were 19.3 and 14 L, respectively. The concentration of methane within the digester gas was 15.1 ± 1.5 and 13.9 ± 1.9 g/m³, respectively. Neither the volume nor methane concentration was significantly different. Still, any difference between the values was most likely due to the difference in TSS.

4.3.5 Impacts on Microbial Community Structure

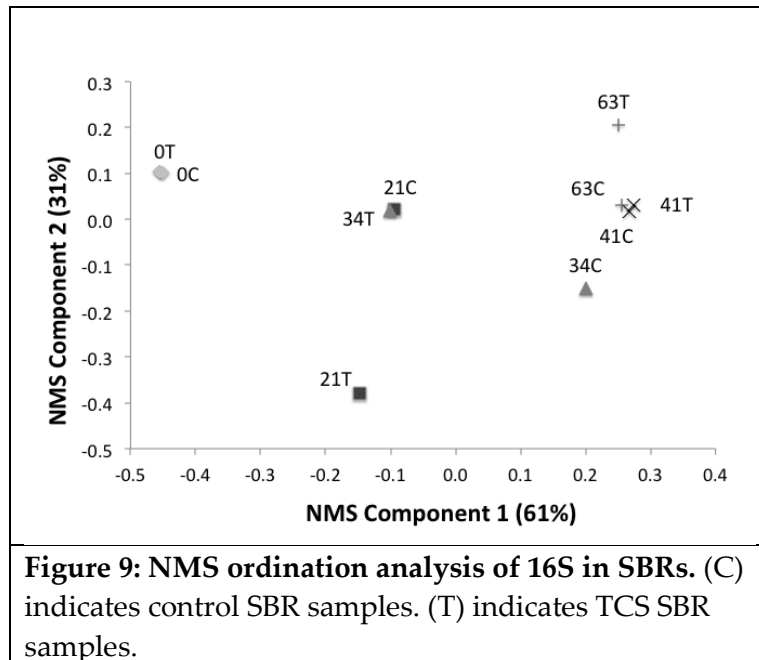
T-RFLP of the housekeeping gene, 16S rDNA was completed on Control B and Triclosan B SBRs to examine the impact of TCS on microbial communities. The relative abundance of fragments compared to the control is shown in Figure 8. For both the control and TCS fed SBRs, the number of species increased over time. Whereas the control and TCS SBRs were not significantly different at day 0, on day 34 and 56 the TCS fed SBRs had

significantly lower number of species. However, looking at the species abundance alone does not provide information regarding any differences in composition of the microbial communities – samples with the same number of species (i.e., fragments) may not necessarily have the same species. To examine the differences between the microbial communities a NMS ordination analysis was completed.



The convergent, final NMS ordination solution for 16S is in Figure 9. The stress of the NMS plot solution was 0.082, which indicates a strong correspondence between the

distances among points. R values for the first and second axes were 0.61 and 0.31, respectively (total R = 0.92). Figure 9 shows that at Day 0, the control and TCS communities are similar, but become less similar until Day 41. On Day 41, the communities within the control and TCS SBRs appear to be similar again and then increase in difference by Day 63. This suggests that the microbial communities, predominated by the nitrifiers and specifically the AOBs, may have assimilated to TCS around Day 41, which was supported by the NH_4^+ , NO_3^- , and NO_2^- data. On Day 63, the differences in microbial communities could be due to differences in the NOBs.



4.3.6 Implications of TCS on Wastewater Treatment Plant Performance

This study indicates that biological processes associated with nitrogen removal (nitrification and denitrification), may be impacted by TCS entering the wastewater treatment plant regardless of the starting microbial community. However, while NH_4^+ removal was temporarily inhibited, elevated levels of NO_3^- and NO_2^- in the effluent of the TCS fed SBRs, suggest longer-term impacts on NOB and denitrifiers. No inhibition was observed for COD removal, which is completed by heterotrophic microorganisms that grow more rapidly and are less sensitive than the autotrophs that carry out nitrification [142, 173]. PO_4^{3-} also was not impacted by TCS. However, due to less frequent sampling, and as a result, limited available data, a complete statistical analysis of PO_4^{3-} on the microbial communities could not be completed. Because phosphorus removal is tied to nitrification (phosphorus removal requires anaerobic conditions), the impacts of phosphorus removal by TCS should be examined in more depth. The results in this study indicate that wastewater treatment could be negatively impacted by TCS concentrations as low as $0.73 \mu\text{M}$ and that in the short-term, existing plants receiving influent with TCS at these concentrations could be operating at less than optimal performance. However, it is expected that the continued addition of TCS in the influent would select for resistant bacteria, and eventually microbial community would acclimate and wastewater treatment processes, and efficiencies, will likely be restored.

5. Evaluating the Community Structure and Functional Impacts of Soil Denitrifying Bacteria in Containers Amended with Laboratory Generated Biosolids Aged with Triclosan

The following chapter describes the research completed to address Objective 4.

5.1 Introduction

Processing and disposal of solids generated during the secondary treatment make up a significant portion of wastewater treatment plants' cost and operations [142]. Land application is the most cost effective approach for disposing solids (a.k.a., biosolids) and accounts for approximately 50% of wastewater solids disposal in the U.S. [1]. Biosolids are land applied to recover their macronutrients, primarily nitrogen and phosphorus, and as an alternative to incineration or landfilling. In fact, until the practice was banned in 1991, ocean dumping accounted for nearly 5% of disposal [26]. Land application of biosolids has been used for decades as a widely accepted beneficial reclamation method, and the biosolids must meet the established regulatory levels for nutrients, heavy metals, and pathogens established under 40 CFR Part 503.

A major drawback to land application, however, is the potential environmental release of non-regulated organic contaminants, which accumulate in biosolids during the wastewater treatment process. Much recent research has been performed to identify and quantify the presence of emerging contaminants in biosolids. A recent literature

review of these studies identified 516 different organic chemicals in biosolids measured around the U.S. [6]. Of these, 83% were not on the U.S. Environmental Protection Agency (EPA) established list of priority pollutants and target compounds. Still, research to determine the ecotoxicity of these organic contaminants once land applied, is limited. Determining ecotoxicity of these compounds is of particular concern because the compounds that are likely to accumulate in biosolids are commonly lipophilic, hydrophobic, and non-ionic, making these chemicals particularly persistent and bioaccumulative [6]. In addition, because many of the compounds found in biosolids are specifically designed to elicit biological modifications (e.g., kill microorganisms), there is a risk that these compounds will disrupt ecologically important microbial soil functions (i.e., carbon, nitrogen, and phosphorus cycling), decrease soil productivity, and ultimately affect the long term viability of these ecosystems, resulting in unforeseen economic and social costs [35-38]. Therefore, there is a clear need to characterize the effects of novel contaminants on soil ecological health.

Many of the over 500 contaminants already identified in biosolids are associated with domestic products, including flame retardants, pharmaceuticals and personal care products (PPCPs), pesticides, surfactants, and antimicrobial agents [6]. One common class of contaminants found in biosolids, the antimicrobial agents, are of particular concern because they are designed to deactivate microorganisms and are heavily used in the U.S., with over an estimated \$1 billion in yearly sales [16]. Triclosan (TCS) is one of

the most commonly found antimicrobial agents in biosolids, and has been measured on average at concentrations of 4.9 mg/kg dry weight (note that this average was based on published data where in some instances, only ranges were provided) up to 113 mg/kg dry weight [53]. Such high concentrations are a result of the widespread use, easy disposal, and chemical characteristics of TCS and products that contain TCS. Greater than 95% of the TCS produced is used in personal care products (PCPs) such as, shampoos, soaps, detergents, and toothpaste, among many others, which are mainly washed down residential drains [43]. In addition, TCS has an octanol-water partitioning coefficient ($\text{Log } K_{ow}$) of 4.8, an acid dissociation constant (pKa) of 7.9, a solubility of approximately 6.9 to 13.8 μM , and a soil half-life greater than 266 days [44-46]. These characteristics allow TCS to partition readily to solids during wastewater treatment and be persistent and bioaccumulative (non-ionic) under ambient conditions. Furthermore, once applied, TCS has been shown to accumulate in the soil and plants grown in biosolids-amended soil [29, 35-38, 152, 175, 176].

The general toxicity of TCS on microorganisms has been well characterized. At high concentrations, TCS' mode of action consists of post-translational inhibition of the enoyl-acyl carrier enzyme (FabI), which blocks fatty acid biosynthesis, and eventually leads to a compromised membrane [68, 69]. At low concentrations, TCS has been shown to have some impact on membrane-associated gene expression [70, 71]. In addition, the range of inhibitory concentrations of TCS for microorganisms have been reported to be

0.863 to 276 μM [124]. Many of these studies comprise general toxicity studies where the levels of mortality of a given pure-culture microorganism were determined at various concentrations of TCS. Few studies have examined the impact of TCS on other microbial process endpoints in pure culture or under environmental conditions. Of those, most of the studies have focused on biological processes in wastewater treatment. For example, median effective concentrations (EC₅₀) of 6.29, 34.4, 69.1, and 825 μM were reported based on biochemical oxygen demand (BOD) degradation, ammonium uptake, oxygen consumption, and glucose utilization [76, 155]. Other studies reported impacts on nitrification in activated sludge and denitrification in liquid media by a model soil microorganism at 1.73 and 1.04 μM , respectively [154, 156]. The impact of TCS on soil microorganisms is also limited. Several studies showed impacts of TCS on soil microbial processes, but were confined to spiking TCS directly into the soil. Because of the important role organic carbon (OC) can play in adsorption, values for each study are shown in parentheses. For one study that spiked TCS, negative impacts on microbial soil activity were observed at 5 mg/kg (OC = 8.5 mg/kg), 50 (OC = 8.5 to 18.5 mg/kg), based on soil respiration and nitrate (NO_3^-) and nitrite (NO_2^-) production (nitrification) and enzyme activity, respectively [177]. Two other studies showed negative, but temporary impacts on soil microbial communities. Liu *et al.* [35] and Butler *et al.* [178], showed temporary inhibition of soil respiration at 10 mg/kg of TCS (OC = 17 to 28 g/kg) for 4 and 7 days, respectively. Liu *et al.*, [35] also reported temporary inhibition of phosphatase

activity (2 days) at 0.1 to 50 mg/kg (no OC data provided). One study, reported no inhibition of nitrification at < 2 mg/kg (no OC data provided) [179]. Because TCS partitions readily to solids, which could potentially reduce the bioavailability of TCS, and thus its toxicity, several other studies spiked TCS into biosolids before mixing them with soil. Pannu *et al.* [152] measured no significant difference in CO₂ evolution and NH₄⁺-N uptake and NO₃⁻-N+NO₂⁻-N release at TCS concentrations ≤ 10 mg/kg (soil OC= 11 to 48 g/kg, biosolids OC = 250 mg/kg) [152]. Park *et al.* [180], however, found a significant decrease in biomass at 10 to 50 mg/kg after 7 to 30 days, but did not see a significant effect on microbial diversity compared to the control, as measured by phospholipid fatty acids (PLFA) (soil OC = 10.8 mg/kg and biosolids OC = 350 g/kg). The study did find increased indicators of stress in TCS dosed biosolids [180]. Lastly, Harrow *et al.* [181] showed a decrease in heterotrophic community diversity after 10 weeks in soil amended with TCS-dosed greywater (6.91 μM). Not only were the amended soil concentrations in all of the studies very high, in the parts per million range, these studies do not properly account for aging as TCS was directly spiked into biosolids before applying to soil. In a critical review, Alexander [153] showed that bioavailability, and therefore the toxicity of a chemical is greatly impacted by the time a chemical was partitioned to solids (i.e., biosolids or soil). Presumably then, the bioavailability of TCS in wastewater treatment plant biosolids, which can take months to

produce, would be greatly different than the bioavailability of TCS spiked into biosolids and mixed with soil within a few min to h [152, 180].

Thus, to address the TCS bioavailability limitations in the previous study, the scope of the present study was to assess the impact of biosolids aged with TCS on soil microbial function and community structure after land application. To this end, laboratory biosolids were generated using sequencing batch reactors (SBRs) coupled with anaerobic digesters. The SBRs were fed synthetic wastewater (SWW) dosed with TCS at a concentration that produced biosolids that reflected those reported in the literature. To our knowledge, this is the first time that biosolids aged with a specific organic contaminant were generated in the laboratory with the intent of determining ecological impacts. Soil denitrifiers were targeted to assess the impacts of the biosolids on soil microbial communities based on our previous research that identified these organisms as potential models for ecotoxicological testing [154].

5.2 Materials and Methods

The following section describes the methods that were used for the research that contributed to this chapter.

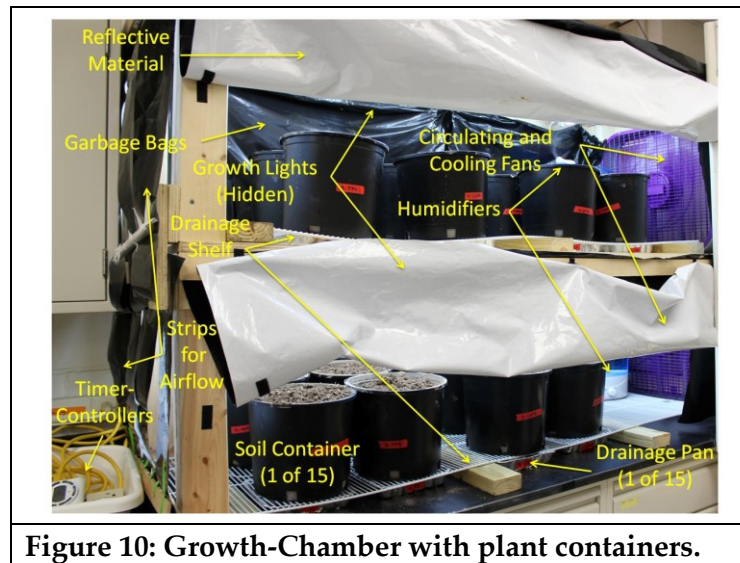
5.2.1 Plant Container and Growth-Chamber Setup

Fifteen 11.3 L Nursery Supplies, Inc. high-density polyethylene (HDPE) containers (diameter = 26.7 cm, height = 24.1 cm) obtained from Fifth Season Gardening (Carrboro, NC) were used to contain the soil. Each container had pre-cut holes in the bottom for drainage. New York Wire Aluminum screen wire was placed at the bottom of the containers to prevent sediment washout (Hanover, PA). Local soil was obtained from Sands and Soils (Durham, NC), which was harvested from wooded area that had never been amended with biosolids. Native North Carolina (NC) soil was used instead of manufactured soil to encourage the propagation of native soil denitrifying communities. The soil was placed on and covered by a plastic tarp, and manually mixed once per day for 4 days to homogenize. Approximately 15 kg of soil was added to each container. The containers with soil were then placed on supported wire drainage shelves in a two-story, constructed growth-chamber (Figure 10). A 20.3 x 20.3 cm Handi-Foil aluminum rectangular container (Wheeling, IL) was placed under each plant container to collect any drainage, however, no substantial drainage was observed throughout the experiment.

The frame of the growth-chamber (2 m x 0.69 m x 1.2 m) was constructed from untreated lumber. Each of the two levels was 0.6 m in height, leaving 35.6 cm between the top of the chamber level and the top of the containers. The front and sides of the growth-chamber were covered with black and white polyethylene reflective material

from Fifth Season Gardening (Asheville, NC) and secured with Velcro. The back of the growth-chamber was covered with Walmart Great Value black garbage bags (Bentonville, AR). The Sun System Sun Blaze T5 Strip Light fixtures, each with 2,500 °K, blue, high output fluorescent lights were specifically selected to encourage indoor plant growth and to reflect field conditions by covering the entire ultra-violet (UV) spectrum. Five lights, 30.5 cm apart were placed on each level 26.8 cm above the top of the plant containers and were turned on for 12 h per day. A 50.8 cm Holmes in box fan (Milford, MA) was set up on each level in order to provide air circulation. Also, each level had a Sunpentown 7.6 L ultrasonic humidifier (Industry, CA) to maintain morning and afternoon relative humidities between 60 and 80%, which were typical values reported for Raleigh, NC [182]. Ultrasonic humidifiers were selected to allow for more control over temperature (as opposed to warm mist humidifiers) and to reduce biofilm buildup within the machine and possible cross-contamination. The temperature was maintained between 18 and 27 °C, which was consistent with the average daily minimum and maximum air temperatures recorded by the weather station at the North Durham Water Reclamation Facility for January 1 to December 31, 2013 (Durham, NC) [183]. Both temperature and relative humidity were monitored with a Mondri Mini Greenhouse Thermo-Hygrometer (Vancouver, B.C., Canada). The growth lights and thermo-hygrometer were obtained from Fifth Season Gardening (Carrboro, NC). All cycling components (lights, humidifier, and fans) were controlled with GE Digital SunSmart

timer-controllers (Fairfield, CT). All plant container and growth-chamber parts were rinsed prior to assembly with high-pressure liquid chromatography (HPLC) grade acetone, hexane, and methanol obtained from Sigma Aldrich (Milwaukee, WI).



5.2.2 Biosolids Development

A description of the setup and operation of the SBRs coupled with anaerobic digesters has been reported elsewhere (Chapter 4) [154]. Briefly, two glass SBRs with silicone seals were set up in parallel that were capable of treating up to 23 L/day of SWW [161]. One SBR was fed SWW (a.k.a., Control SBR) and one was fed SWW dosed with 0.73 μM of TCS (a.k.a., TCS SBR). The SBRs were operated on 6 h cycles that designed to promote nitrification and phosphorus removal. The tanks were also completely covered with aluminum foil to limit photodegradation of TCS. Further, each SBR was attached to an

anaerobic digester made from 19 L solvent-rinsed, glass carboys. The digesters were kept in a container to block light and in a water bath heated to 27 °C. Every 3 days throughout the study, the digesters were mixed manually to ensure complete digestion.

The SBRs were initially spiked with 9 L of activated sludge taken from the North Durham Water Reclamation Facility (NDWRF, Durham, NC), which currently treats 20 MGD and completes biological nutrient removal of BOD, NH_4^+ , and phosphorus. After 30 days of operation, when steady-state conditions were reached, 250 mL of solids were decanted daily to the anaerobic digesters. Immediately prior to sending solids to the digesters, the digesters were inoculated with 100 mL of anaerobically digested sludge from the NDWRF (Durham, NC) and sealed. The headspace in the digesters was then evacuated and flushed with nitrogen gas at atmospheric conditions. Anaerobic conditions were maintained throughout the operation of the digesters. Two hundred and fifty mL of waste was then pumped to each of the reactors per day for 47 days, and when the SBRs were shutdown, all remaining solids from the SBR were pumped to the digesters. The digesters were then isolated from the SBRs and digestion commenced for 20 days. When digestion was complete, the biosolids from the Control SBR (a.k.a., Control biosolids) and the TCS SBR (a.k.a., Aged TCS biosolids) were analyzed for TCS concentration, TSS, VSS, Total Kjeldahl nitrogen (TKN), phosphorus, potassium, and pH.

5.2.3 Biosolids Application and Sampling Protocol

Twenty four h prior to biosolids application, 3 L of mixed biosolids from the Control SBR were removed and placed in a 4 L Erlenmeyer flask covered in aluminum foil. Using a Hamilton Syringe (Hamilton Company, Reno, NV), stock TCS in HPLC-grade acetone was added to the flask to yield a concentration of 1.76 μM or an estimated concentration based on the control biosolids total suspended solids (TSS) (7,346 mg/L) of 70 mg/kg. The biosolids spiked with TCS (a.k.a., Spiked TCS biosolids) were then mixed with a solvent rinsed stir-bar on a stirplate for 24 h at 500 rpm. The biosolids spiked with TCS were made to serve as a comparison to the aged biosolids (Control and Aged TCS biosolids).

On the start day of the experiment, the control biosolids, biosolids aged with TCS, and biosolids spiked with TCS were added to the plant containers and were mixed in the top 4 in of soil using a scupula to mimic tilling conditions. First, the carboy or flask was mixed by hand and then poured into a solvent rinsed 1 L graduated cylinder, which was then slowly poured onto the soil in each container. In order to add the same mass of solids to each treatment, 0.84 L of the control biosolids and biosolids spiked with TCS and 1 L of the biosolids aged with TCS were added to the containers. Six containers received control biosolids and biosolids aged with TCS and 3 containers received biosolids spiked with TCS. Raw TSS, TKN, phosphorus (as P_2O_5), potassium (as K_2O), pH and TCS values were reported in Chapter 4. Values normalized to TSS are shown in

Table 3. The TKN added to each container corresponded to an agronomic rate of 98 kg/m² which falls in the range of typical alfalfa values for NC [184]. As stated in Chapter 4, the TCS concentrations measured in the digested control and TCS biosolids were significantly different from each other and were 2.85 ± 0.69 mg/kg for the control SBR and 82.7 ± 11.1 mg/kg from the TCS dosed SBR. The majority of the TCS was partitioned to the solids in the biosolids, and the aqueous phase comprised 1.48 ± 0.43% and 4.06 ± 0.35% for the control and TCS biosolids, respectively. The TCS concentrations in the control and biosolids aged with TCS were consistent with low and high ranges of the published biosolids concentrations [23, 42, 44, 53, 54, 59, 157, 169-172]. TCS concentrations were measured approximately 2 weeks prior to application in order to develop application rates and were not measured again prior to application.

Physiochemical Property (mg/L)	Control Biosolids	TCS Biosolids
TSS (mg/L)	6220 ± 48.2	6220 ± 131
TKN (mg/L)	3950 ± 232	3780 ± 81.7
P ₂ O ₅ (mg/L)	817 ± 18.2	781 ± 7.91
K ₂ O (mg/L)	154 ± 2.58	153 ± 0.636
pH	6.68 ± 0.037	6.71 ± 0.015
TCS (mg/kg)	2.85 ± 0.069	82.7 ± 0.015

Following biosolids application, soil samples were taken from each container. Specifically, 4 to 5 cores were taken from the entire depth of each container using a modified aluminum soil corer. The cores were then placed on aluminum pie-tins,

homogenized with a scupula, and distributed to storage containers for further downstream processing. Separate scupulas and aluminum food containers (diameter = 24.1 cm, height = 3.81 cm) were used for each plant container and were solvent rinsed prior to sampling. For the Denitrifying Enzyme Activity (DEA) assay, duplicate 5 g samples were distributed directly to the 125 mL Kimble Chase Kimax glass Erlenmeyer flasks with ground glass tops (Vineland, NJ). Triplicate 5 g samples were placed in volatile organic analysis certified Thermo Scientific 50 mL amber vials with silicone/polytetrafluoroethylene (PFTE) septum in polypropylene caps (Waltham, MA), stored at -80 °C for TCS analysis. Duplicate 1.5 g samples were placed in 1.5 mL microcentrifuge tubes and stored at 20 °C for DNA extraction and microbial community analyses. Forty g samples were placed in VWR 50 mL polypropylene centrifuge tubes for TKN, OC, and pH analyses and stored at -80 °C (Radnor, PA). Lastly, triplicate 2 g samples were placed in 50 mL centrifuge tubes and stored at 4 °C for dry weight, ash content, and moisture content analyses. Soil sampling was repeated 1, 7, 28, and 84 days after biosolids application. Again, all sampling utensils were solvent rinsed prior to each use.

Alfalfa seeds were planted in the top 1.27 cm in triplicate containers that received the control biosolids and the biosolids aged with TCS 24 h after biosolids application. No replicates with plants were set up for the containers that received biosolids spiked with TCS. Alfalfa was selected because of its low nitrogen agronomic rate, which required

less biosolids to be generated. HybriForce – 2400 Alforex Alfalfa Seeds from the Dairyland Seed Company (Clinton, WI) were selected because they grow well indoors, are surface sterilized, and do not require inoculation with N fixing microorganisms for germination. Seeds were applied to the containers at a rate of 25 pounds per acre, which was within the recommended range for broadcasting alfalfa seeds [185]. On day 84, after soil samples were collected, all of the available alfalfa biomass was collected, rinsed with DI water, and weighed. The samples were then wrapped in aluminum foil and stored at -80 °C for future analysis (not completed in this study).

Throughout the extent of the experiment, the containers were watered with DI water every other day to keep the soil moist. Specifically, the containers were watered until the soil at the bottom of the container was observed to be moist, but was stopped before the water drained from the soil. While there was no substantial flow-through, any water that did drain was dumped back on the soil. Water was distributed using a Chapin 7.6 L plastic tank sprayer (Batavia, NY).

5.2.4 Analytical Methods

Methods used to measure TSS, TKN, P₂O₅, K₂O, pH and TCS values in the laboratory-generated biosolids were reported in Chapter 4. The TCS concentration in the biosolids spiked with TCS was not measured because the entire volume was used during application. TKN, OC, and pH of the biosolids-amended soil samples in this study were

measured by Waters Agricultural Laboratories, Inc. for days 0 and 84 only (Georgia USA). Samples for TKN, OC, and pH were taken, however, analyses were only completed on the initial and ending time point samples to determine any overall changes throughout the experiment. Dry weight and moisture and ash content were determined by first aliquoting triplicate 2 g samples to 57 mm VWR aluminum dishes (Radnor, PA). Then the samples were covered with aluminum foil and dried at 105 °C for 24 h. After 24 h, the samples were weighed, covered, baked at 550 °C for 3 h, and weighed again. Moisture content was calculated by dividing the difference between the initial weight and weight after baking at 105 °C, by the initial weight. The dry weight was calculated as 1 minus the product of the moisture content and the initial weight. Ash content was calculated by dividing the difference between the initial weight and the weight after baking at 550 °C, by the initial weight.

Because the focus of this study was to examine the impacts of applying biosolids aged with TCS on microbial communities in agricultural soil and not the fate of TCS, TCS was only measured on day 0. For day 0 samples, TCS was measured in triplicate following methods previously described for biosolids [139]. Samples for other sampling time points were not analyzed and changes in TCS were considered to be consistent between all of the containers. One of the limitations of the analytical methods used herein was that the methods require solvents to extract the compound from the soil and thus it is impossible to differentiate between the bioavailable and non-bioavailable

portion of the contaminant available to the microorganisms. Thus, while additional measurements could have provided information regarding any changes in the overall TCS concentration in the soil over time (e.g., through degradation), limited information on the amount of TCS resulting in toxicity would be obtained [153].

5.2.5 Functional Impact Analysis

The DEA assay was used to measure the functional activity of the denitrifiers in the soil samples. Specifically, the DEA assay measures potential denitrification, which is the amount of NO_3^- that can be denitrified when carbon and NO_3^- are in excess under anaerobic conditions [186]. Duplicate, 5 g homogenized soil samples from each container were measured using the method published previously [127] and modified [187]. Gas samples were collected in 9 mL gas vials crimp capped with butyl stoppers that were flushed with nitrogen gas (N_2) and evacuated as previously described [127]. Vials, butyl stoppers, and aluminum crimp caps were obtained from Grace Davison Discovery Science (Deerfield, IL). Nitrous Oxide (N_2O) in the gas samples was measured on a modified Shimadzu gas chromatographer (GC) 17A version 3 (Kyoto, Japan) and Tekmar headspace autosampler (Vernon, BC, Canada) within 7 days. The GC was retrofitted in 2007 with a methanizer, ECD, flame ionization detector (FID), and Valco Instruments Co. Inc. valves (Houston, TX) to approximate the GC-2014 Greenhouse Gas

Analyzer. Relative denitrification rates were then calculated by normalizing the production of N₂O in a treatment by the Day 0 measurement of each treatment.

5.2.6 DNA Extraction

Total DNA was extracted from all replicates on all days using the MO BIO PowerLyzer PowerSoil DNA Extraction Kit (Carlsbad, CA). All extractions were performed following a modified version of the manufacturer's protocol [164]. Briefly, after the addition of reagent C1, 400-500 µL of 25:24:1 phenol/chloroform/IAA obtained from Sigma-Aldrich was added to each tube and vortexed (Milwaukee, WI). Following, instead of homogenizing the samples, the samples were placed on a horizontal shaker at maximum speed for 5 min. Also, 25 µL of eluent buffer (C6) was added to the glass microfiber white filter membrane, the samples were incubated for 5 min and centrifuged for 30 s at 13,000 rpm. This step was then repeated. The samples were then stored at -20 °C for further microbial community impact analysis.

5.2.7 Community Impact Analysis

Changes in the abundance and diversity of total bacteria and denitrifying populations were used to measure impacts of biosolids aged and spiked with TCS on community structure. Specifically, the absolute abundance of the reference gene (16S rRNA) as well as the functional denitrifier genes, NO₂⁻ reductases (*nirS* and *nirK*) and N₂O reductase

(*nosZ*), was quantified by quantitative polymerase chain reaction (qPCR). Terminal Restriction Fragment Length Polymorphisms (T-RFLP) of the same denitrifier genes and reference gene was completed to determine changes in diversity.

To measure abundance, following DNA extraction, the Zymo DNA Clean & Concentrator-5 kit was used to purify the samples. A spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was then used to verify the concentration and purity of the DNA. The DNA was then diluted with RNase and DNase free autoclaved water to 1.67 ng/ μ L. Absolute quantification of *nirS*, *nirK*, *nosZ*, and 16S *rDNA* was performed on a Stratagene Mx3000p real-time PCR apparatus (Cedar Creek, TX) using iTaq SYBR Green Super Mix with ROX binding dye (Bio-Rad, Hercules, California). Previously published primers were used for *nirS* [188], *nirK* [86], *nosZ* (NosZ1) [189], and 16S rRNA (U519F and E685R) [135, 136]. The qPCR reaction for *nirS*, *nirK*, and *nosZ* consisted of 4.31 μ L RNase free, DNase free, autoclaved water, 12.5 μ L of iTaq SYBR Green Super Mix with ROX binding dye, 1.28 μ L of the forward and reverse primers (3.125 μ M stock), 0.63 μ L bovine serum albumin (BSA), and 5 μ L each of diluted template DNA. BSA was obtained from New England Bio-labs Inc. (Beverly, MA). The reaction for 16S rRNA was the same as for the denitrifying genes except BSA was replaced with additional water of the same volume. Preliminary tests were performed to optimize the annealing temperature of the primers and showed that touchdown PCR conditions were not needed (shown in Appendix B). The annealing temperature for all primers was

determined to be 56°C, with the exception of 16S rRNA primers, which was 54 °C. Conditions for qPCR were 95 °C for 10 min, followed by 45 cycles at 95 °C for 30 s, 1 min at the respective annealing temperatures, 30 s at 72 °C, and dissociation curve to assess the quality of the primers. All samples were run in duplicate, and qPCR efficiencies between 90 and 110% and R² value ≥ 0.985 were obtained. Purity of the amplified product was further verified by the observation of a single melting peak and the presence of a unique band of the expected size in a 2% agarose gel stained with ethidium bromide. In addition, no PCR inhibition was observed through serial dilutions of sample DNA. Results are reported as the absolute quantity ratios of the denitrifying genes *nirS*, *nirK*, and *nosZ* to the reference gene *16S* relative to Day 0. The absolute quantity for *16S* was reported relative to Day 0. To date, only single copies per genome have been found for the denitrifying genes. However, 16S rRNA can range from 1 to 15 copies per genome [190]. However, due to the diversity of denitrifiers, research with other reference genes is limited [91]. Thus, a major assumption in this study was that the average copies of 16S rRNA on the genome were the same for all treatments and that all prokaryotic microorganisms with 16S rRNA were impacted equally.

To measure changes in diversity, following DNA extraction, a spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to verify the concentration and purity of the DNA. Replicates were kept separate before and after DNA extraction. PCR amplification on a GeneAmp 9600 thermal cycler (Applied Biosystems, Foster City, CA)

of bacterial 16S SSU rDNA gene fragments, *nirS*, *nirK*, and *nosZ* were performed as described previously [165, 191-193]. The primers, used for amplification were published elsewhere: *16S* [165] (16S27F and 1392R), *nirS* [193] (nirS1F and nirS6R), *nirK* [194] (nirK1F and nirK5R), *nosZ* [192] (nos661F and bis1773R). The forward primer for *16S*, *nirS* and *nosZ* gene fragments and the reverse primer for *nirK* were fluorescently labeled with 6-carboxyfluorescein (FAM).

BSA was used in some of the reactions to limit primer dimer formation and humic acid interference, and MgCl₂ was added as a cofactor for *Taq* polymerase and to stabilize the two strands of DNA. The *16S* thermocycle profile given by Lukow *et al.* [165] was followed with the exception that 10 cycles were added to ensure total template DNA amplification. The *nirS*, *nirK*, and *nosZ* thermocycle profiles were also previously published by Braker *et al.* [193], Wolsing and Prieme [191], and Scala and Kerkhof [192], respectively. Again, 10 cycles were added to the profiles to ensure total template DNA amplification. Following amplification, the presence of the correct length PCR amplicons was confirmed via visualization on a 1% agarose gel containing 0.1% ethidium bromide. PCR amplicons were then purified using a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). The final PCR product concentrations and purity were again verified using the NanoDrop.

The PCR amplicons were digested with restriction enzymes as described in Lukow *et al.* [165], Braker *et al.* [193], Wolsing and Prieme [191], and Scala and Kerkhof

[192], and for *16S*, *nirS*, *nirK*, and *nosZ*, respectively. However, no BSA was used in the reactions as preliminary experiments showed interference. One hundred ng of purified PCR product and 10 U of restriction enzyme (New England Bio-labs Inc., Beverly, MA) were used for each reaction. Following incubation samples were stored at -20 °C until further processing by the Duke University DNA Analysis Facility. The Duke University DNA Analysis Facility desalted the samples through a spin column filtration and completed fragment analysis using an Applied Biosystems 3100 capillary sequencer (Foster City, CA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (BioVentures, Inc., Murfreesboro, TN).

T-RFLP profiles were evaluated using Applied Biosystem's Genescan v3.7.1 analysis software (Foster City, CA). Manual inspection of each profile was completed to ensure only true peaks were chosen for statistical analyses. The raw data was then imported into T-REX in the following format; Dye/Peak, Sample File Name, Size, Height, Area in Point, Area in BP [167]. All peaks less than 50 basepairs (bp) were excluded from the analysis because they were possible primer dimer fragments. Also, only T-RFs with a minimum peak height threshold of 50 relative fluorescent units were used. T-REX was used to align the profiles based on the replicates using the "Environments" function in the software. Presence/absence data files were imported into PAST (Paleontological Statistics software) for data analysis [168]. Presence/absence was selected because presence/absence gives all species (each fragment) the same weight during analysis,

whereas, peak area is biased towards the abundance of a specific species (each fragment). Thus, presence/absence is especially important when species of low abundance are significantly impacted more by the treatments. Also, because of inherent biases with PCR, peak area measurements are only semi-quantitative. After the data were analyzed, the relative species abundances on each sampling day was calculated, which was the ratio of the number of peaks present in each treatment to Day 0. The data were then analyzed using principal nonmetric multidimensional scaling (NMS) coupled with Jaccard similarity distance for group clustering (Appendix B). The Jaccard similarity distance, which ignores abundance and measures the similarity between binary sample sets, was used because presence/absence data are binary. However, there were no noticeable groupings of the samples on the NMS ordination plots, and thus, were not presented in the results section.

5.2.8 Statistical Analysis

Experimental values are reported as the mean \pm standard error. To analyze statistical differences between treatments, one-way and two-way analysis of variance (ANOVA) coupled with Tukey's post-hoc analysis using the open source statistical software R (v.2.15.1) was used. Differences were considered significant for p-values ≤ 0.05 [140]. Marginal significance was considered for p-values ≤ 0.10 . In addition, linear regression

analysis was also completed using R and significance was again determined using ANOVA.

5.3 Results and Discussion

To determine the impacts of TCS containing aged and spiked biosolids on denitrifying activity, five treatment conditions were examined; Control – No Plants, Control – Plants, Aged TCS – No Plants, Aged TCS – Plants, and Spiked TCS – No Plants. Following biosolids amendment, denitrifying activity and community structure were monitored for 84 days. The results from these experiments are presented below.

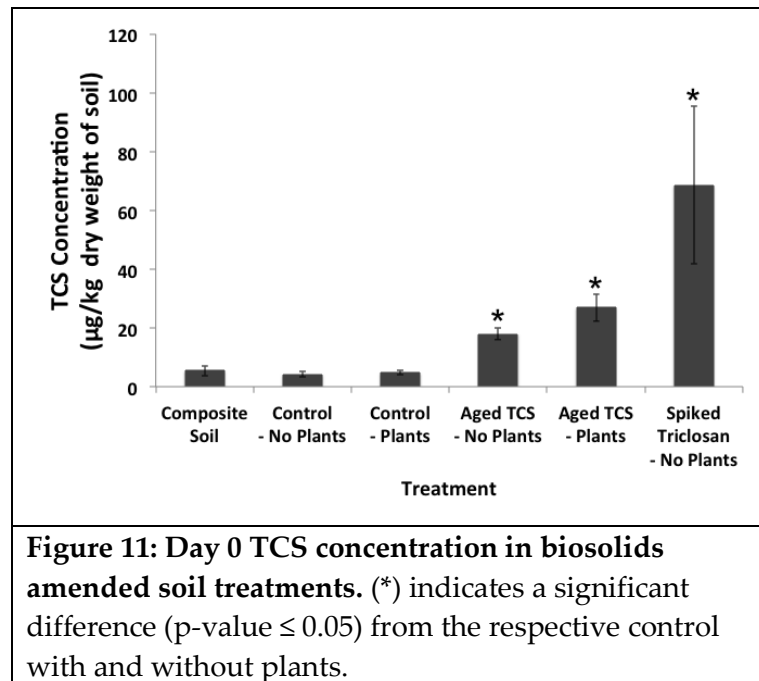
5.3.1 Simulated Biosolids Characteristics

Following biosolids application on Day 0, soil samples were collected and measured for TCS concentration (Figure 11). The plants and no plants treatments receiving the control biosolids had TCS concentrations of 4.2 ± 0.95 and 4.7 ± 0.79 $\mu\text{g}/\text{kg}$, respectively and were not significantly different from the unamended soil used in the study (5.4 ± 1.8 $\mu\text{g}/\text{kg}$). Both the plants and no plants treatments that received the biosolids aged with TCS had TCS concentrations of 17.9 ± 1.93 and 26.9 ± 4.6 $\mu\text{g}/\text{kg}$, respectively. These concentrations were significantly different from 0 and from each other (not shown in Figure 11), which was most likely due to incomplete mixing before aliquoting and applying the biosolids. Incomplete mixing that did not completely resuspend the solids in the digester carboys would result in the first few applications receiving fewer solids,

and therefore less TCS than later applications. The containers that did not eventually have plants received the biosolids aged with TCS prior to those that eventually had plants, and therefore a lower concentration of TCS. The TCS concentration in the containers receiving biosolids spiked with TCS was $68.6 \pm 26.9 \mu\text{g}/\text{kg}$, which was significantly higher than the containers with and without plants that received the biosolids aged with TCS (not shown in Figure 11). The difference between the TCS concentrations in the soil receiving the spiked TCS versus the aged TCS biosolids may have been caused by degradation during the 2 weeks between when the TCS in the biosolids aged with TCS was measured and when the biosolids were applied. Overall, the average percent detection of the internal standard (^{13}C TCS) for all samples was $67.7 \pm 2.4\%$ (standard deviation = $\pm 14.1\%$), which was consistent with the publication where the method was obtained [172]. In addition, TCS was present in the blanks at low levels (average = $5.4 \pm 2.1 \text{ ng}$).

The concentrations measured in the biosolids used for the present study were representative of concentrations reported for soil. Numerous studies have measured TCS in biosolids, however, few have actually examined the concentrations of TCS in the soil following biosolids application. Published studies report a large variability of TCS concentrations ranging between $4.1 \text{ ng}/\text{kg}$ to $1,840 \mu\text{g}/\text{kg}$. These concentrations were dependent upon the source biosolids, the application rate of the biosolids, and soil mixing type (e.g., till vs. no till) and mixing depth [32, 54-56]. In addition, both the

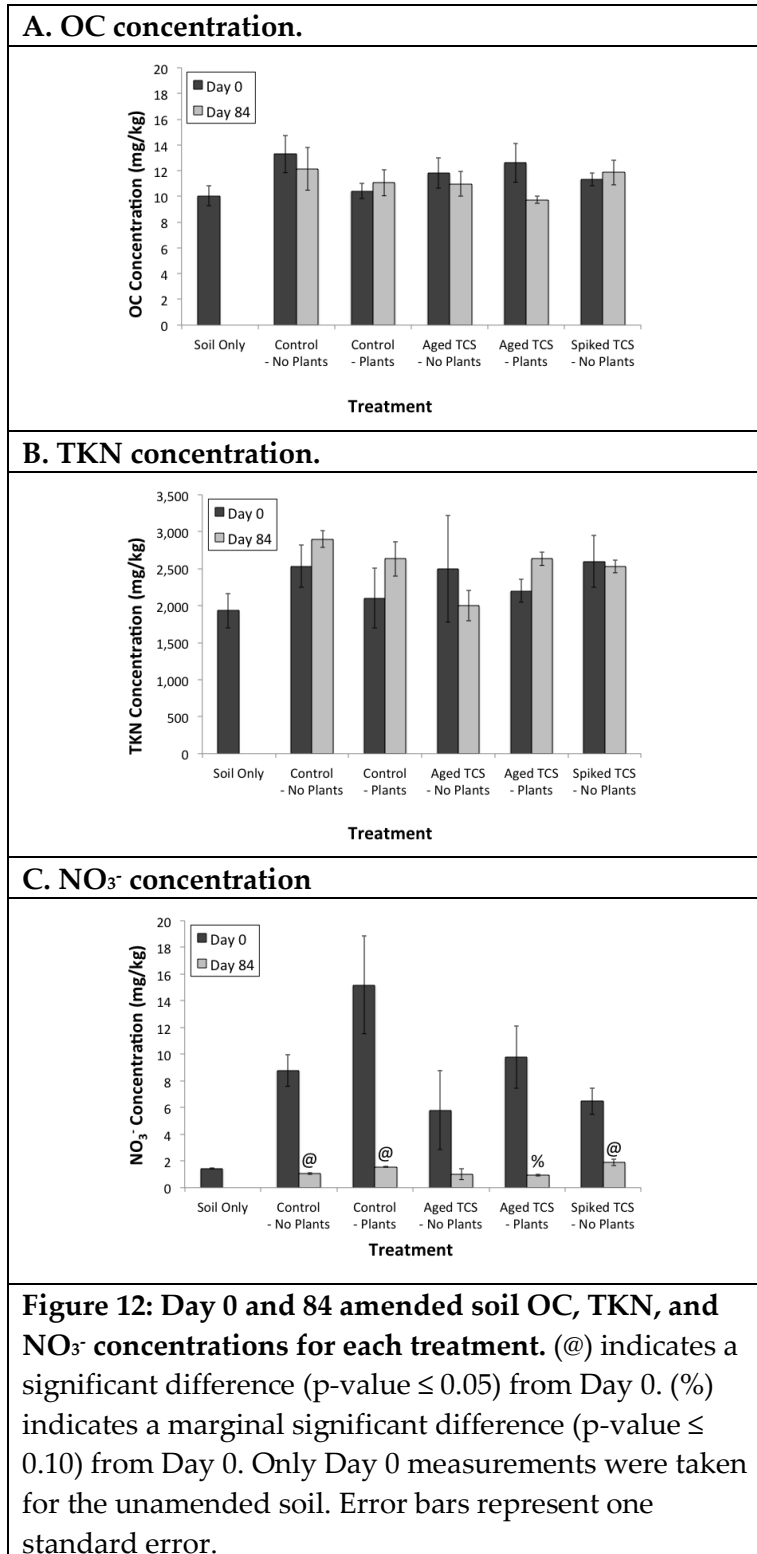
amended soil concentrations in this study and the concentrations reported in the literature, were much lower than those used in previous studies examining the impact of TCS on soil microbial processes. Previous studies that spiked TCS directly into soil or into biosolids before mixing with soil had final soil concentrations in the parts per million (ppm) range [35, 152, 177-181].



5.3.2 Soil Characteristics

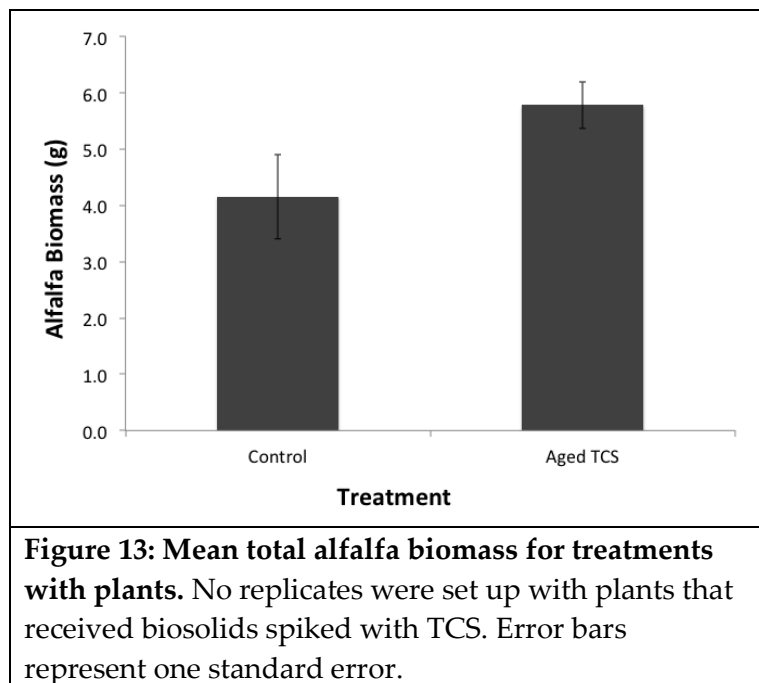
The initial (Day 0) and ending (Day 84) OC, TKN, and NO_3^- average values for each treatment are shown in Figure 12. No significant differences were measured for TKN and OC between Days 0 and 84 for all treatments. In addition, there were also no significant differences between treatments that received the biosolids spiked or aged

with TCS and the respective no plants and plants treatments that received the control biosolids. The OC and TKN concentrations reported in this study were consistent with previous studies [35, 152, 177-181]. NO_3^- , on the other hand, significantly decreased between Day 0 and 84 for containers with and without plants that received the control biosolids and containers that received biosolids spiked with TCS. There was a marginally significant decrease of NO_3^- (p-value = 0.061) in the containers with plants that received biosolids aged with TCS. Containers without plants that received biosolids aged with TCS were not significant because there was not enough soil to complete one of the replicates. However, there was no significant difference in NO_3^- between the containers that received biosolids spiked and aged with TCS and the respective containers with and without plants that received the control biosolids. pH values were also not significantly different between treatments for Day 0 or 84 and over the extent of the experiment for each treatment, with the exception of the containers with plants that received biosolids aged with TCS, which had a significant decrease in pH (p-value = 0.006). The average Day 0 and 84 soil pH for all treatments was 5.6 ± 0.031 and 5.4 ± 0.029 , respectively.



5.3.3 Plant Biomass

The alfalfa seedlings germinated around Day 4 (3 days after sowing) and continued to grow until Day 84. The total plant biomass harvested on Day 84 for the containers that received the control biosolids and biosolids aged with TCS is shown in Figure 13. Plant biomass was not significantly different (p -value > 0.05).



5.3.4 Impacts on denitrifiers

To assess the overall ecotoxicity of biosolids aged and spiked with TCS, the function, abundance, and diversity of the soil denitrifying communities were examined. The impacts on abundance and diversity of total prokaryotic microorganisms were also examined for comparison. The DEA assay was used to measure functional impacts,

qPCR was used to measure impacts on abundance, and T-RFLP was used to measure impacts on diversity.

5.3.4.1 Function

The relative denitrification rate for all treatments on all sample days is presented in Figure 14. In general, the denitrification rate decreased after Day 1 and remained lower throughout the remainder of the experiment. The initial promotion of denitrification by organic fertilizers, such as manure and sewage sludge has been shown previously, and has been attributed to an increase in OC [116, 191, 195]. Typically, the maximum denitrification occurs within 24 to 48 h of application of organic amendments [196]. However, the NO_3^- used by denitrifiers is often at lower concentrations or less bioavailable in organic fertilizers such as biosolids than in synthetic, mineral fertilizers, which can result in a decrease in denitrification [10]. Thus, one possible explanation for the observed decrease in denitrification potential of all treatments, was that NO_3^- available to the denitrifiers in the soil was exhausted. The reduction in NO_3^- over the extent of the experiment (Figure 12) supports this conclusion. A decrease in TKN was not observed likely because TKN is a broad measurement of nitrogen in a soil that is not bioavailable to denitrifiers (i.e., the sum of organic nitrogen, ammonia (NH_3) and NH_4 [7]).

On Days 1 and 7, there were no significant differences between the containers that received biosolids that were spiked or aged with TCS and the respective controls with and without plants. Denitrification was significantly lower in the containers that received biosolids spiked with TCS on Day 28 and marginally significantly lower (p-value = 0.090) in the containers with plants that received biosolids aged with TCS on Day 84, indicating potential long-term inhibition. The corresponding concentrations were $68.6 \pm 26.9 \mu\text{g/kg}$ and $26.9 \pm 4.6 \mu\text{g/kg}$, respectively. However, the inhibitory effects in the containers that received biosolids spiked with TCS disappeared by Day 84. No additional measurements were taken after Day 84 to verify a rebound in the denitrification in the containers with plants that received the aged biosolids. By contrast, denitrification in the containers that received biosolids aged with TCS and without plants was marginally significantly greater on Day 84 (p-value = 0.052). The rebounding, inhibition, and stimulation of denitrification in the containers that received the highest, middle, and lowest TCS concentration, respectively, could be due to complex acclimation responses of the denitrifiers to TCS. The two highest TCS concentrations resulted in significant and marginally significant inhibition on Day 28 and 84, respectively, but by Day 84 denitrification had rebounded in the presence of the highest concentration of TCS. At the lowest concentration, no inhibition occurred, but marginally significant stimulation of denitrification was observed on Day 84. Similar non-dose related responses were observed by Liu *et al.* [35] and Butler *et al.* [178] on soil

respiration, but at much higher TCS concentrations than in this study. Briefly, no inhibition was observed at concentrations < 1 mg/kg, inhibition was observed within the first 2 days ≥ 1 mg/kg, and at concentrations ≥ 30 mg/kg inhibition was observed from 2 to 4 days [35]. In addition, between 4 and 12 days, soil respiration was actually promoted for treatments that received ≥ 10 mg/kg TCS, but the magnitude of the promotion was not directly correlated to TCS concentration [35]. Butler *et al.* [178] also observed an initial inhibition of soil-induced respiration (< 7 days), which was generally greater as the concentration increased from 10 to 1000 mg/kg TCS, but not always, followed by a recovery, which was not correlated with TCS concentration. Butler [178] further showed that subsequent dosing of TCS resulted in higher soil respiration than the control that received no TCS. Still, only Butler *et al.* [178] examined long-term impacts of TCS on soil microorganisms, albeit only after respiking TCS into the soil on Day 14, and showed sustained inhibition of basal respiration at concentrations as low as 10 mg/kg.

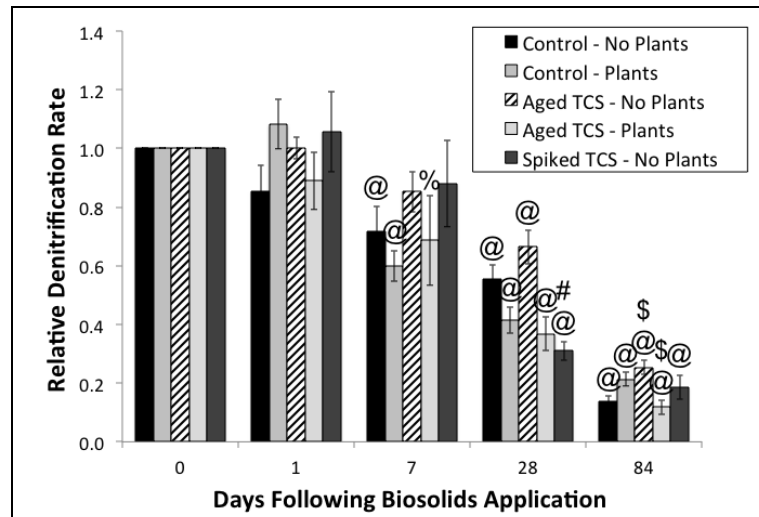


Figure 14: Relative denitrification rate of control, TCS aged, and TCS spiked biosolids-amended soil with and without plants. (@) indicates a significant difference from Day 0 (p-value ≤ 0.05). (%) indicates a marginal significant difference from Day 0 (p-value ≤ 0.10). (#) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.05). (\$) indicates a significant difference from the respective no plants or plants control on the same day (p-value ≤ 0.10). Values < 1 indicate inhibition of denitrification as compared to Day 0. Error bars represent one standard error.

5.3.4.2 Abundance

The total prokaryotes (16S rRNA) and populations with the common denitrifying genes (*nosZ*, *nirS*, and *nirK*) were quantified for each treatment over the extent of the experiment (Figure 15) [86, 189]. The range of absolute copy numbers of 16S, *nirS*, *nirK*, and *nosZ* per ng of DNA were 2.08×10^4 to 2.17×10^5 , 1.10×10^3 to 2.98×10^4 , 2.50×10^1 to 1.68×10^4 , and 2.11×10^3 to 6.31×10^4 , respectively. These values were consistent with a previously published study [188].

Examination of the 16S relative copy numbers showed a general increase in the prokaryotes over the course of the experiment. Abundance in the containers without plants that received biosolids aged with TCS was not significantly lower than the control throughout the experiment. The containers with plants that received biosolids aged with TCS showed significantly lower abundance compared to the control on Days 1 and 28 and marginally significantly lower abundance on Day 84 (p-value = 0.051). Abundance in the containers that received biosolids spiked with TCS was significantly lower on Day 7 only.

For *nosZ*, containers with plants that received biosolids aged with TCS were significantly lower on Day 7 and 28, containers without plants that received the biosolids aged with TCS were marginally significantly lower on Days 1 and 7 (p-values = 0.074 and 0.057, respectively), and containers that received the spiked biosolids were significantly lower on Days 1 and 28 and marginally significantly lower on Day 7 (p-value = 0.092). The overall *nosZ* containing population increased slightly until Day 28 before decreasing to or marginally greater than starting levels by Day 84. Denitrifiers carrying the *nosZ* gene have been shown to be the most sensitive to pollutants and other changes in environmental conditions (e.g., oxygen levels, carbon to nitrogen (C:N) ratios, and pH) [119-122].

The quantity of *nirS*, similarly to the DEA assay results, decreased after Day 1 to Day 7 and remained low until the end of the experiment. The only significant inhibition

observed for *nirS* was for the containers with plants that received biosolids aged with TCS and the containers that received spiked biosolids on Day 7. Similar to Day 84 results for the containers without plants that received biosolids aged with TCS measured by the DEA assay that showed a marginally significant stimulation, on Day 28 *nirS* abundance was significantly higher than in the control. The overall similarity of the DEA assay and *nirS* abundance results may indicate potential correlations between the methods.

The population with the *nirK* gene increased about 4 times the starting abundance by Day 28. By Day 84, the *nirK* abundance had stabilized at approximately 2 times the starting levels. There were no significant differences in abundance observed for the containers that received biosolids spiked or aged with TCS and the corresponding no plants and plants controls.

Overall, the qPCR data presented herein suggested that inhibition of denitrifiers occurred between Days 1 and 84 for containers that received spiked biosolids (68.6 ± 26.9 $\mu\text{g}/\text{kg}$ TCS) and those with (26.9 ± 4.6 $\mu\text{g}/\text{kg}$ TCS) and without (17.9 ± 1.93 $\mu\text{g}/\text{kg}$ TCS) plants that received aged biosolids. Maximum inhibition was observed around Day 7. In addition, examining gene abundance was more sensitive to impacts of TCS than measuring activity, at least in the short-term, particularly shown for *nosZ* (< 84 days). No studies could be found that examined the impact of TCS in biosolids on total bacteria and denitrifying gene abundance or any other functional gene, in order to put the results of this study in perspective. However, two other studies were found that examined the

impact of biosolids on microbial abundance. One study examined the impacts of biosolids application on soil microorganisms and found that the number of heterotrophs increased following application, as measured by plate count[197]. TCS concentrations were not measured in that study. Park *et al.* [180], however, found a delayed decrease in biomass after 7 to 10 days in soil mixed with TCS-spiked biosolids and biomass remained low until the end of the experiment (Day 30).

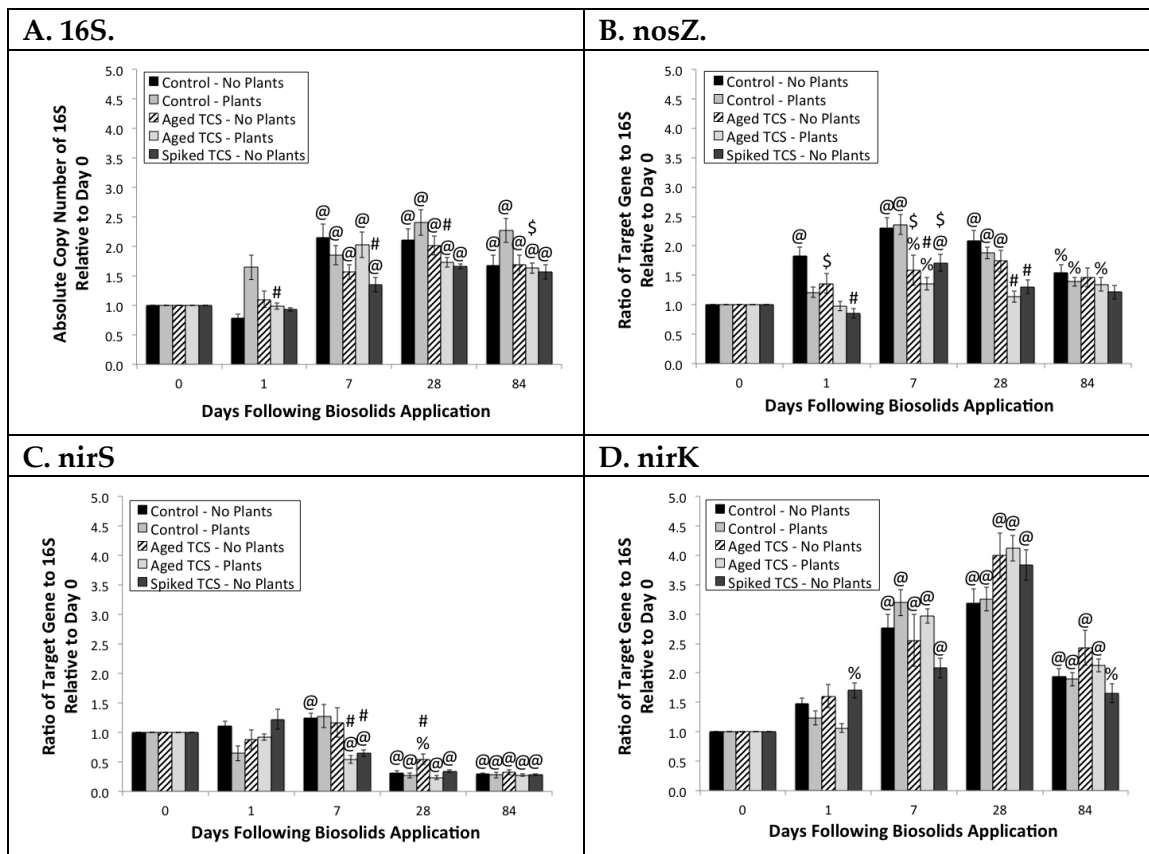


Figure 15: Relative total bacteria and denitrifier abundance. (@) indicates a significant difference from Day 0 (p -value ≤ 0.05). (%) indicates a marginal significant difference from Day 0 (p -value ≤ 0.10). (#) indicates a significant difference from the respective no plants or plants control on the same day (p -value ≤ 0.05). (\$) indicates a significant difference from the respective no plants or plants control on the same day (p -value ≤ 0.10). Values < 1 indicate a decrease in absolute copy number or ratio of target gene to 16S as compared to the Day 0. Error bars represent one standard error.

5.3.4.3 Diversity

The diversity of total bacteria and denitrifier populations were also examined (Figure 16). The number of fragments measured for 16S, *nirS*, *nirK*, and *nosZ* were 1 to 84, 0 to 116, 0 to 1, and 0 to 23, respectively. Much lower diversity was observed for all denitrifying genes in this study compared with field biosolids amended soil samples

measured in Chapter 6, especially for *nirK*. This is likely due to there either being a low number of species that actually contain the *nirK* gene or the primers were not able to account for the different species that were present and will be discussed further, later in this section.

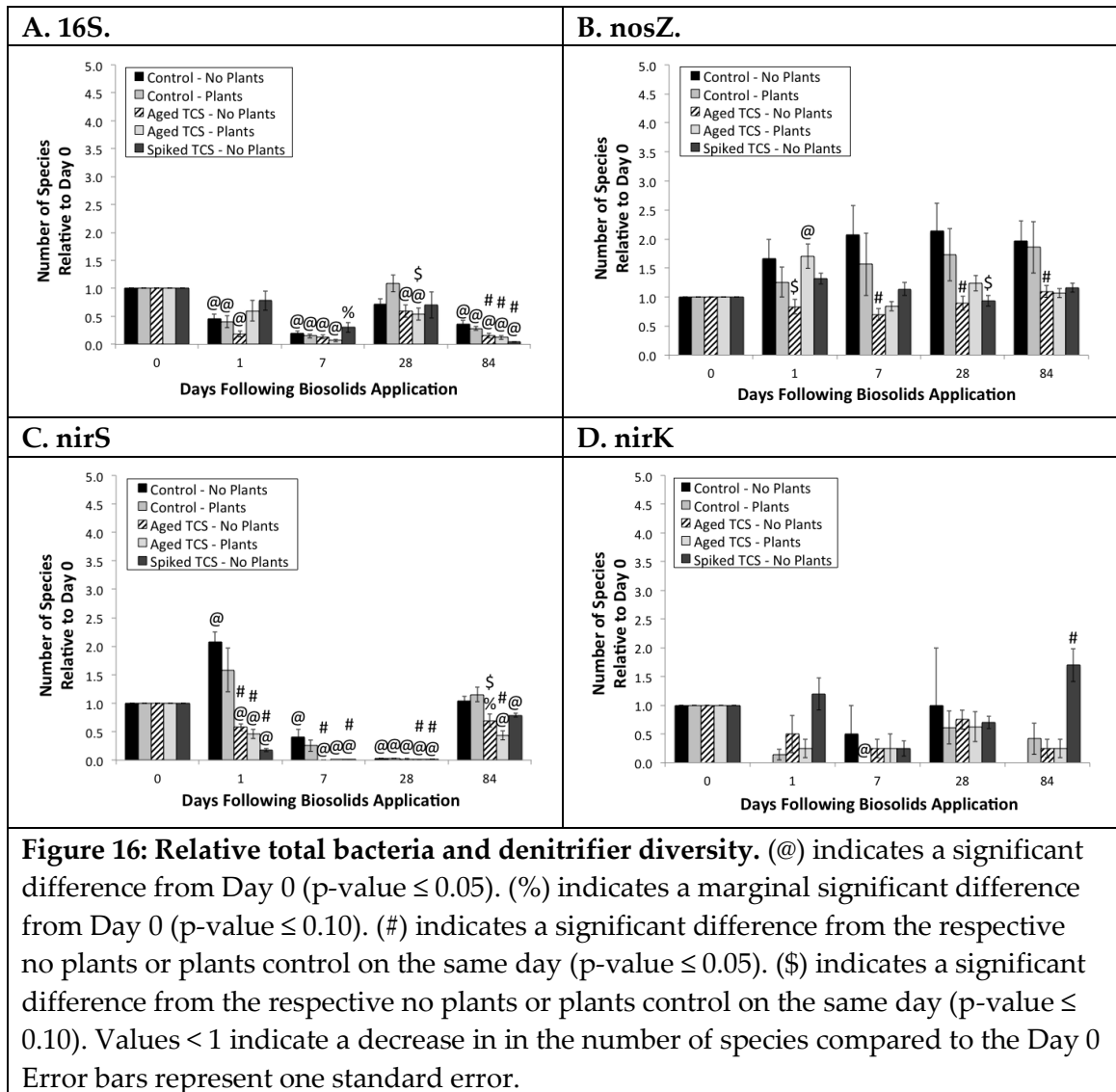
The relative diversity of the general bacteria population, as measured by 16S rRNA decreased until Day 7, then slightly increased at Day 28, but was not as high as the initial diversity, and then decreased again on Day 84. Diversity was marginally significantly lower than in the control for the containers with plants that received biosolids aged with TCS on Day 28 (p-value = 0.060) and significantly lower for all three TCS treatments on Day 84. The diversity of the denitrifier population with *nosZ* remained the same for the entirety of the experiment for both the controls and the TCS treatments. Marginal inhibition was observed for the containers without plants that received biosolids aged with TCS on Day 1 and significant inhibition on Days 7, 28, and 84. Marginal inhibition was observed for the containers that received biosolids spiked with TCS on Day 28. Diversity for the containers with plants that received biosolids aged with TCS was not significantly different from the control on any day, but was likely due to the high variability of the corresponding control with plants. For the most part, the diversity of *nirS*, similarly to the abundance of *nirS*, and activity, as measured by the DEA assay, decreased throughout the experiment. However, unlike abundance of *nirS* and activity, *nirS* diversity increased on Day 84. The diversity for all three TCS

treatments was significantly lower than the controls on Day 1. On Day 7, the containers without plants that received the biosolids aged with TCS and spiked with TCS were significantly inhibited. On Day 28, the containers with plants that received biosolids aged with TCS and the containers that received the spiked biosolids were significantly inhibited. On Day 84, the containers without plants that received the aged TCS biosolids were marginally significantly inhibited, whereas the containers with plants that received the TCS aged biosolids was significantly inhibited (p-value = 0.094). These data clearly did not indicate a dose-response, but as discussed earlier, such a response may not be expected. In addition, although the sampling protocol was developed to maintain as much homogeneity as possible, slight changes in the heterogeneity of the samples could account for the varying responses observed for the three TCS treatments. Low numbers of *nirK* fragments (0-1) were obtained for all of the samples analyzed, and resulted in the high errors shown in Figure 16D. Because of the larger errors, none of the TCS treatments were significantly different from the control, with the exception of the containers that received the spiked biosolids on Day 84, which was significantly higher. One explanation for the low number of fragments for *nirK* was that it is just a reflection of low number of species that contain the *nirK* gene in the samples [191]. Another explanation was that the *nirK* denitrifiers in this study were more distantly related to those used to develop the primers and thus were not digested the same [191]. This has

been shown to occur for the *nirS* primers and would also explain why there was amplification by the *nirK* qPCR primers, but not the T-RFLP primers.

Overall, similar to the denitrifier abundance, inhibition of denitrifier diversity occurred from Day 1 to 28 and then decreased thereafter to Day 84 for all three TCS treatments. Unfortunately again, there were very few studies that have examined the impacts of biosolids application on microbial community distribution, let alone TCS specifically, in order to directly compare the results. Enwall *et al.* [116] showed a shift in *nosZ* communities using denaturing gradient gel electrophoresis (DGGE) in soil treated with sewage sludge compared to no treatment. The shift in *nosZ* was suspected of being related to pH, as plots treated with sewage sludge had a lower pH than in the unamended control in that study, which was attributed to the long-term acidification of the soil by the fertilizers. The study showed no significant correlation between soil pH and microbial activity (i.e., soil respiration), but did not quantify the impacts of pH on microbial diversity. It should be noted that no emerging contaminants were measured in the study. Several studies have looked at microbial community structure changes in soils or biosolids spiked with TCS. Liu *et al.* [35] showed a shift in the microbial communities for low (0, 0.1, and 1 mg/kg) and high TCS concentrations (10, 30, 50 mg/kg) that was related to carbon utilization. Park *et al.* [180] correlated a shift in microbial communities based on PLFA using canonical correspondence analysis (CCA) and TCS spiked with TCS directly into the soil at concentrations of 0, 10 and 50 mg/kg and soil that received

biosolids spiked with the same concentrations of TCS. Finally, a long-term decrease in heterotroph diversity was observed by Harrow *et al.* [181] in soil inoculated with TCS spiked greywater (6.19 μM).



5.3.5 Methods Correlation

One of the major difficulties with determining ecotoxicity on microorganisms under representative environmental conditions is that different indicators (e.g., specific endpoint functions or community structure measurements) yield different results in terms of impact magnitudes and concentrations. In addition, no studies have really even attempted to determine the most effective indicator for determining impacts under any environmental conditions. [198]. While many of the previous studies examining the impacts of TCS on soil microorganisms focused on microbial activity alone, the present study independently examined three targets, activity, abundance, and diversity. Using linear regression analysis, we sought to correlate these three targets in order determine and evaluate any relatedness between the measurements (Table 4). Five significant correlations were developed, three of which related qPCR and the DEA assay, or abundance and activity. Of these, the strongest correlation ($R = 0.77$) was a positive correlation between qPCR of *nirS* and the DEA assay. Graham *et al.* [199] also found a strong correlation ($R = 0.89$ p-value < 0.01) between absolute *nirS* abundance and denitrification rate, however denitrification was measured using stable isotopes. They also found a high correlation with *nirK*, which was not found in this study. The strong correlation between the abundance of *nirS* and the denitrification rate could be because the NO_2^- reductase gene is often the rate-limiting step for denitrification and *nirS* is the most abundant of the NO_2^- reductases [14, 130, 188]. Four other correlations were also

significant: qPCR of *nirK* and the DEA assay, qPCR of 16S and the DEA assay, the diversity of 16S communities and DEA Assay, and the diversity and abundance of *nosZ*. However, the analyses that were correlated did not yield the same results as far as significant inhibition in the presence of TCS. Thus, while the results suggested some relatedness between activity, abundance, and diversity, the results generally support the use of multiple methods to determine the ecotoxicity of biosolids-derived organic contaminants.

Table 4: Linear correlations between methods used to analyze denitrification.

Method A (X)	Method B (Y)	F-Statistic	R	p-value	Intercept	Slope
qPCR 16S	DEA	15.3	0.611	0.0007*	1.34	-0.436
qPCR nirS	DEA	36.1	0.771	0.000004*	0.181	0.690
qPCR nirK	DEA	8.56	0.490	0.008*	1.04	-0.170
qPCR nosZ	DEA	2.10	0.210	0.161	0.997	-0.226
T-RFLP 16S	DEA	4.87	0.373	0.038*	0.472	0.392
T-RFLP nirS	DEA	1.17	0.084	0.290	0.597	0.130
T-RFLP nirK	DEA	0.468	-0.164	0.501	0.611	0.107
T-RFLP nosZ	DEA	2.33	0.228	0.141	0.981	-0.239
qPCR 16S	T-RFLP 16S	3.31	0.297	0.082	0.913	-0.261
qPCR nirS	T-RFLP nirS	0.973	-0.032	0.334	0.374	0.306
qPCR nirK	T-RFLP nirK	0.778	-0.095	0.387	0.755	-0.078
qPCR nosZ	T-RFLP nosZ	8.41	0.486	0.008*	0.553	0.514

(*) indicates a significant difference from 0 (p-value \leq 0.05)

5.3.6 General Implications of Biosolids Application

In this study, biosolids aged with and spiked with TCS showed potential long-term inhibition based on measurement of activity (denitrification). However, inhibition was

limited and because the DEA assay historically has low sensitivity smaller impacts may have been overlooked [84, 127]. Denitrifier abundance and diversity were slightly more sensitive to TCS and inhibition was observed throughout the experiment, with maximum inhibition occurring from Day 1 to 28. This suggests that both the total number of denitrifiers and species of denitrifiers were influenced more heavily by TCS addition and that those that were not impacted by TCS addition, continued to denitrify with minimal potential long-term impacts. In addition, there was an overall lack of correlation between the abundance, diversity, and function of the denitrifiers. However, due to the complexity of the environment examined (i.e., soil and biosolids) the results were also complex, and all three methods were needed to determine the overall ecotoxicity of TCS in biosolids.

In total, 7 analyses were completed directly relating to denitrifiers; qPCR and T-RFLP were used to examine 3 denitrifying genes for abundance and diversity and the DEA assay examined the overall denitrification activity. In order to quantify the net impacts shown by all the assays, a denitrification inhibition score was developed and shown in Figure 8. The denitrification inhibition score was developed by first assigning values of 1, 0.5, 0, -0.5, or -1 to each treatment receiving TCS or Spiked TCS biosolids at each time point for the 7 analyses. A value of 1 was assigned when the treatment was significantly lower than the control at that sample-day indicating inhibition, 0.5 was assigned when the treatment was marginally significantly lower than the control,

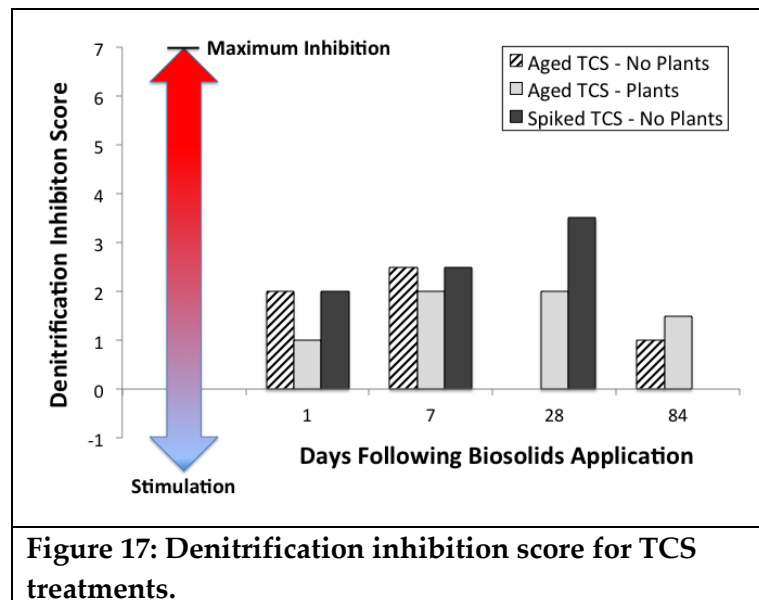
indicating marginal inhibition, 0 was given if the treatment was not significantly different than the control, -0.5 was assigned when the treatment was marginally significantly greater than the control, indicating marginal stimulation, and -1 was assigned if the treatment was greater than the control, indicating stimulation. The values for each treatment at each sample-day were then summed and represented the overall denitrification inhibition score. Because 7 analyses were completed, maximum inhibition would correspond to a score of 7, and maximum stimulation would correspond to the minimum score of -7. Overall, the denitrification score assumed that there were no differences in magnitude for the target of each analysis and that each target was equal in importance. This limitation may have resulted in effects being masked, especially if targets of more importance were less sensitive to inhibition than less important targets. Further research would be needed to elucidate the magnitude of each target and corresponding analysis in relation to overall impacts on denitrifiers. In addition, the denitrification inhibition score assumed that marginal significance represented 50% of the weight of significance. Further work would also be needed to determine if this assumption was appropriate.

The denitrification inhibition scores for both aged and spiked TCS treatments are shown in Figure 17. Containers with and without plants that received biosolids aged with TCS had an increase in the score from Day 1 to 7 that then decreased to Day 84. The containers without plants had a score of 0 on Day 28 and a score of 1 on Day 84. The

containers with plants decreased from a score of 2 on Days 7 and 28 to a score of 1.5 on Day 84. These results could reflect the difference in concentrations between the two treatments or the addition of plants in one of the treatments. Based on TCS concentrations, this result was somewhat expected because the no plant treatment had a lower TCS concentration than the plant treatment. However, as was discussed earlier, acclimation of TCS by the denitrifiers reported by Butler *et al.* [178] and Liu *et al.* [35], showed that we may not necessarily expect a dose-response to TCS. These results may need to be replicated to confirm the observed responses. If the difference between the two treatments was due to the presence of plants, the results were somewhat unexpected. Opposite of what was observed in this study, less impact was expected in the presence of plants because the plants could uptake TCS, reducing the exposure to the denitrifiers, or the plants could provide protection for the denitrifiers. Previous work examining the effects of plants on TCS and denitrification in soil is conflicting to results presented herein. Several studies have shown uptake of TCS by soybeans, lettuce, radish, carrots, barley, meadow fescue and Bahia grass [152, 175, 200]. Wu *et al.* [175] and Macherius *et al.* [200] spiked TCS directly into the soil the plants were growing in, but did not take biosolids adsorption into account, which may have resulted in a higher bioavailability of TCS than measured. However, plant uptake of TCS originating in biosolids applied under field conditions was much more limited [152, 201]. Pannu *et al.* [152] found that the plants in soil receiving biosolids spiked with TCS accumulated more

TCS than those in unspiked biosolids .Further complicating matters is the complex interaction between plants and denitrification. Smith and Tiedje [202] found that at low NO_3^- concentrations (2 mg/kg dry soil) denitrification was decreased when plants were present. By day 84, NO_3^- concentrations were below 2 mg/kg dry soil in this study, which could have contributed to inhibition of denitrification when the plants were present. In addition, microbial community composition has been shown to be different when plants are present, which in turn could have accounted for the increased inhibition [109, 110]. Nevertheless, the higher impacts on Day 7 and 28 of both TCS-aged treatments indicate TCS in biosolids negatively impacted soil denitrifiers in the short-term.

The Spiked TCS treatment, which had an initial soil TCS concentration of $68.6 \pm 26.9 \mu\text{g/kg}$, resulted in inhibition throughout the experiment, and 2.5 and 3.5 of the 7 analyses showed inhibition on Days 7 and 28. These results are consistent with previous studies where TCS was spiked directly into the soil or into biosolids. The higher, more sustained inhibition score observed for the Spiked TCS treatment, was consistent with the expected higher bioavailability for the Spiked TCS treatment [153]. However, the higher, more sustained inhibition could just be a result of a higher TCS concentration than the biosolids aged with TCS.



Overall, the results in this study showed potential negative impacts of TCS on soil microbial community structure, and potential long-term impacts on function. These data were consistent with previous studies which showed negative impacts, but at significantly higher concentrations. In the present study, the inhibition was observed by biosolids aged with TCS at concentrations as low as $17.9 \pm 1.93 \mu\text{g}/\text{kg}$, which was still nearly 10 to 3,000 times lower than the previous studies [35, 177, 178, 180]. This result was unexpected as the aging process used in this study was expected to decrease the bioavailability of TCS. However, there are several possible explanations for the observed inhibition at low TCS concentration. First, the functional endpoint (i.e., denitrification) targeted in this study may have been more sensitive to TCS than other endpoints examined in other studies (e.g., dehydrogenase, soil respiration and nitrification) [35, 178, 203] suggesting this function may provide good indication of potential ecotoxicity

of organic compounds. Second, other studies focused on the activity of soil microorganisms, whereas in our study, community structure (i.e., abundance and diversity) was more sensitive to TCS. Finally, toxicity is highly variable and based on soil characteristics and microbial community characteristics, which may differ between this and previous studies. Specifically, differences in soil characteristics, such as in pH, OC, and pore space could explain the difference between this and previous studies [153].

The low pH range in this study likely resulted in an increased fraction of neutral TCS and subsequently increased the bioavailability of TCS. Neutral compounds are generally more bioavailable, but also sorb more readily to natural organic matter (NOM). Based on the pK_a of TCS and the pH range measured in this study, the fraction of the compound in the neutral form was calculated to be 96.1 to 97.5%, which was high and may only be encountered in more acidic soils. Similar calculations were completed on previous studies that examined the impact of TCS on soil microbial activity based on the reported pH and TCS concentrations that resulted in inhibition (Table 5). Still, the TCS concentrations that resulted in inhibition and adjusted for the percent in the neutral form in this study were nearly 10 times lower than the next closest. It should be noted that differences in OC were not taken into account in in this calculation, but are presented in Table 5 for comparison purposes as OC, in addition to the composition of the soil, plays an important role in adsorption prediction.

While low, the pH range reported in the present study was similar to that used by Enwall *et al.* who showed no correlation between pH and the microbial shift in *nosZ* after biosolids application [116]. At the lower pH, more TCS should have been adsorbed to the soil, which should not have resulted in the transport of the compound to the bottom of the soil container. Still, this was accounted herein by controlling watering so that no significant flow-through occurred and by taking cores throughout the depth of the container. Nevertheless, the bioavailability of TCS in the soil used in this study would represent a worst-case-scenario for agricultural fields.

Nevertheless, the much lower TCS concentration that resulted in microbial inhibition obtained in this study indicated a need to better understand the bioavailability dynamics for TCS. While other organic compounds have been shown to be made more bioavailable by microorganisms in the soil, which could explain the inhibition of denitrification at such low concentrations, no such studies have been completed on TCS [153]. In addition, research is currently lacking that examines how environmental stressors (e.g., nutrient limited conditions) play a role in the toxicity of organic contaminants on microorganisms. Limited work has been completed on nutrient availability and heavy metal toxicity and organic contaminant biodegradation [204, 205]. This would be of particular interest for persistent, bioaccumulative compounds, such as TCS, which would remain in the soil after microbial nutrient sources were exhausted and stress induced. In the present study, NO_3^- concentrations decreased over time,

which could have resulted in stress on the denitrifying community and thus, an increase susceptibility to toxicity. NO_3^- was not measured on day 7 when maximum inhibition was observed, but the bioavailable fraction could have already been exhausted by then.

Table 5: Calculated neutral TCS in this and previous studies.

Study	TCS Addition to Soil	pH	TCS Inhibition Concentration (mg/kg)	Inhibition Target	Calculated Percent Neutral	Calculated Neutral TCS (mg/kg)	Reported OC (mg/kg)
Present Study	Aged with Biosolids	5.4 - 5.6	0.018	Denitrification	99.5- 99.7	0.01781 – 0.01784	8.6 – 15.6
	Spiked	5.68	10	Respiration	99.4	9.54	18.2 ^a
Liu <i>et al.</i> [35]	Spiked	5.68	0.1 - 50	Phosphatase Activity	99.4	0.099 – 49.7	18.2 ^a
	Spiked	5.4	5	NO ₃ ⁻ and NO ₂ ⁻ Production	99.7	4.99	8.5 – 18.5
Waller and Kookana [177]	Spiked	7.4	50	Respiration and NO ₃ ⁻ and NO ₂ ⁻ Production	76.0	38.0	8.5 – 18.5
	Spiked in Biosolids	6.3 - 7.5	50	Cy17 Stress Biomarker	71.5-97.5	35.8–48.8	10.8 ^b
Butler <i>et al.</i> [178]	Spiked in Biosolids	6.9 - 7.5	10	Respiration	71.5-90.9	7.15-9.09	17.1 – 27.8 ^a

^a reported as total carbon.
^b reported as carbon.

Overall, this study showed that soil quality, as indicated by the abundance, diversity, and function of denitrifying bacteria, was negatively impacted at environmentally relevant concentrations by the addition of TCS in biosolids, and ageing may have reduced the magnitude of the impact. However, the pH range in this study was consistent with more acidic soils, when the bioavailability of TCS would be increased. While the results of this study indicate potential negative impacts on denitrifying community structure and function, the background concentration of other contaminants could have contributed to the impacts observed. As indicated by the high TCS concentration in the “control” biosolids, the volume of inoculum likely contributed loadings of TCS and other compounds. While, future studies examining the impact of biosolids-derived contaminants should generate the biosolids under laboratory settings, the inoculation volume to the digesters should be minimized as the presence of other organic contaminants could bias the data.

6. Elucidating the Impacts of Biosolids on Native Agricultural Soil Denitrifying Bacteria

The following chapter describes the work completed to address Objective 5.

6.1 Introduction

The use of antimicrobials has grown significantly over the past 10 years in the U.S., with over an estimated \$1 billion in yearly sales [16]. One of the main antimicrobial agents used in personal care products (PCPs), such as shampoos, soap, detergent, and toothpaste, among many others, is triclosan (TCS) [40, 41]. In fact, 95% of the TCS produced in the U.S. is used in PCPs, which are disposed mainly by being washed down residential drains [43]. Because TCS has an octanol-water partitioning coefficient ($\text{Log } K_{ow}$) of 4.8, an acid dissociation constant (pK_a) of 7.9, and a solubility of approximately 6.9 to 13.8 μM , the compound partitions readily to solids during wastewater treatment and is persistent and bioaccumulative (non-ionic) under ambient conditions [44-46]. Eventually, the solids generated during secondary wastewater treatment (a.k.a., biosolids) and the TCS contained therein, are disposed of, and in the U.S. over 50% of these solids are disposed via land application. TCS has been measured in biosolids up to 113 mg/kg dry weight [53]. Current regulations (i.e., 40 CFR Part 503) established levels for nutrients, heavy metals, and pathogens for land application of biosolids, but not for organic contaminants. Much of the recent research has been performed to identify and

quantify the presence of emerging contaminants in biosolids, however, research to determine the ecotoxicity of organic contaminants likely to accumulate in biosolids once land applied, is limited. Of particular concern are compounds found in biosolids that are specifically designed to deactivate microorganisms, there is a risk that these compounds will disrupt ecologically important microbial soil functions. TCS, which at high concentrations blocks fatty acid biosynthesis in microorganisms eventually leading to compromised membrane [68, 69] and at low concentrations depresses membrane-associated gene expression [70, 71], is one such compound. Thus, there is a need to determine the impacts of TCS on soil ecological health.

A limited number of studies have attempted to examine the ecotoxicity of TCS on soil microbial processes by mimicking field application of biosolids. Several studies spiked TCS directly into soil. One study observed inhibition of soil respiration, nitrate (NO_3^-) and nitrite (NO_2^-) production, and enzyme activity at 5 to 50 mg/kg [177]. Temporary impacts on soil respiration and phosphatase activity were observed for TCS concentrations of 0.1 to 50 mg/kg in two other studies that used the 'spiked' approach [35, 178]. Yet another study showed no inhibition at 2 mg/kg [179]. Other studies attempted to address the underestimate of TCS bioavailability from spiking TCS directly into the soil, by spiking TCS into biosolids prior to mixing with soil [153]. As expected, these studies found much more limited impacts on microbial processes. Pannu *et al.* [152] measured no impacts on CO_2 evolution and $\text{NH}_4^+\text{-N}$ uptake and $\text{NO}_3^-\text{-N}+\text{NO}_2^-\text{-N}$

release at TCS concentrations ≤ 10 mg/kg. However, Park *et al.* [180] observed significant decrease in biomass and increased indicators of microbial stress, but no effects on microbial diversity between 10 and 50 mg/kg TCS. Not only were the concentrations in these studies much greater than those measured in the field, but also aging of the TCS with the soil was not taken into account. Alexander showed that bioavailability, and therefore the toxicity of a chemical is greatly impacted by the time a chemical was partitioned to a solid (i.e., soil) [153]. The bioavailability of TCS in biosolids applied under field conditions would likely be less than the TCS in the spiked studies completed to date. In Chapter 5, we addressed this shortcoming by examining the impact of biosolids aged with TCS on microbial community function and structure under laboratory conditions. However, no studies have examined the impacts of TCS on microorganisms in the field. A major drawback to field studies, is that individual impacts of compounds on microorganisms are difficult to elucidate due to the complex, heterogeneity of biosolids and soil and because there are numerous organic compounds in biosolids which may inhibit various microbial processes making it difficult to reach conclusions with respect to potential ecotoxicity [6].

One method to address the complexity of field conditions would be to conduct an experimental survey examining fields that covered a wide range of all of the key variables related to the ecotoxicity of TCS in land applied biosolids to soil microorganisms. Once these variables, including, but not limited to soil characteristics,

biosolids characteristics, biosolids application rates, and chemical composition and quantities, were compiled, statistical methods could be used to determine correlations between TCS and microbial impacts. However, this type of approach would undoubtedly require extensive time and effort. The scope of this pilot-study was to evaluate the impacts of 'traditional' biosolids containing TCS on soil microbial community and structure under field conditions on a much smaller scale, and provide some initial input to determine if a much larger, more extensive study is warranted. Again, as in Chapters 3 and 5, soil denitrifiers were targeted to assess the impacts of the biosolids on soil microbial communities [154].

6.2 Materials and Methods

The methods that were used for the research that contributed to this chapter are described in the following section.

6.2.1 Sample Location and Collection

Four fields receiving Class B municipal biosolids from a North Carolina community were examined for this study. The biosolids were digested anaerobically under mixed conditions. Soil samples were obtained for each field prior to biosolids application.

Fields were arbitrarily labeled as Fields A, B, C, and D. Biosolids were only applied to each field once during the study period. Following application, Fields A, B, C, and D

were tracked for 147, 126, 112, and 21 days respectively, from April to September 2013. During this time frame, soil samples were collected based on access to the fields. Specifically, fields could only be accessed when biosolids were being applied during weekdays and not within 24 h of a precipitation event. Fields were previously seeded with grass, which once grown, was harvested and prepared for livestock. During the study the grass in Field A grew from 1 to 4 ft, and was harvested prior to the last sampling day (Day 147). The grass in Field B grew from 3 to 5 ft over the extent of the study and was not harvested. Sampling on Field C started after the grass was harvested and grew less than 1 ft by the end of the study. Finally, over the 21 days Field D was monitored, the grass remained approximately 1 ft in height.

The initial sampling location within each field was selected randomly. The initial sampling location relative to each field was different between fields because of differences in size and shape of each field. Samples were then collected immediately prior to and immediately following biosolids applications. Twelve, 15.2 to 30.5 cm soil cores were collected randomly within a 9.1 m diameter with an AMS 2.54 cm x 61.0 cm stainless steel soil (American Falls, ID). The number of soil cores was based on obtaining a soil volume large enough for all of the biological and chemical analyses. The soil depth was based on how far the probe could reasonably driven into the soil using a rubber mallet. The cores were then placed in a Handi-Foil aluminum-roasting pan (Wheeling, IL) and homogenized with a stainless-steel gardening shovel. The pan, corer, and shovel

were solvent rinsed with high-pressure liquid chromatography (HPLC) grade acetone, hexane, and methanol prior to each sampling event. Solvents were obtained from Sigma-Aldrich (Milwaukee, WI). The soil was then distributed to storage containers for downstream processing. Triplicate 5 g samples were distributed VWR 50 mL polypropylene centrifuge tubes for the Denitrifying Enzyme Activity (DEA) assay. Likewise, 40 g samples were placed in another set of VWR 50 mL tubes for TKN, organic carbon (OC), and pH analyses. Two g samples were placed in 50 mL centrifuge tubes for dry weight, ash content, and moisture content analyses. Five g samples were placed in volatile organic analysis certified ThermoScientific 50 mL amber vials with silicone/polytetrafluoroethylene (PFTE) septum in polypropylene caps (Waltham, MA), stored at -80 °C for TCS analysis. Finally, 1.5 g samples were placed in 1.5 mL microcentrifuge tubes for DNA extraction and microbial community analyses. All samples were taken in triplicate and temporarily stored in a cooler with ice packs while in the field. Back in the laboratory, the DEA assay and dry weight, ash content, and moisture content samples were stored at 4 °C and processed within 24 h. The TKN, OC, and pH samples and the samples for TCS analysis were stored at -80 °C. Samples for DNA and extraction and microbial community analysis were stored at -20 °C. Follow-up samples were obtained randomly from the same 9.1 m diameter location, which was determined using a Garmin handheld global position system (GPS, Wichita, KS).

6.2.2 Analytical Methods

TKN, OC, and pH of the soil samples before and after biosolids applications were measured by Waters Agricultural Laboratories, Inc. for all days tested (Georgia, USA). Dry weight and moisture and ash content were determined, as described in Chapter 5. The TCS concentration was measured in triplicate for the samples prior to and immediately after biosolids application using previously described methods for biosolids [139].

6.2.3 Functional Impact Analysis

As in Chapter 5, the DEA assay was used to measure the functional activity of the denitrifiers in the soil samples using the method published previously [127] and modified [187].

6.2.4 DNA Extraction

Total DNA was extracted using a modified version of the MO BIO PowerLyzer PowerSoil DNA Extraction Kit (Carlsbad, CA) described in Chapter 5 [164].

6.2.5 Community Impact Analysis

Changes in the abundance and diversity of total prokaryotic microorganisms and denitrifying populations were again used to measure impacts of TCS biosolids on

community structure. The absolute abundance of the reference gene (16S rRNA) as well as the functional denitrifying genes, NO₂⁻ reductases (*nirS* and *nirK*) and N₂O reductase (*nosZ*), were quantified by quantitative polymerase chain reaction (qPCR). Terminal Restriction Fragment Length Polymorphisms (T-RFLP) of the same denitrifying genes and reference gene was completed to determine changes in diversity.

Following DNA extraction, samples for abundance analysis were purified and processed as in Chapter 5. Samples for diversity analysis were also processed according to Chapter 5. However, in addition to calculating the relative species abundances on each sampling day, nonmetric multidimensional scaling (NMS) coupled with Jaccard similarity distance for group clustering was used to examine the T-RFLP data. In addition, while presence/absence data were used for the analyses completed in this Chapter, T-RFLP peak area data was examined to determine whether the model organism used in Chapter 3 was present in the field samples. Based on *nosZ* fragments, *Paracoccus denitrificans* PD1222 was present in 59% of the field samples examined. *nirS* was also examined for the model organisms, but the results were inconclusive.

6.2.6 Statistical Analysis

Experimental values are reported as the mean ± standard error. To analyze statistical differences between treatments, one-way and two-way analysis of variance (ANOVA) coupled with Tukey's post-hoc analysis using the open source statistical software R

(v.2.15.1) was used. Differences were considered significant for p-values ≤ 0.05 [140].

Marginal significance was considered for p-values ≤ 0.10 . In addition, linear regression analysis was also completed using R and significance was again determined using ANOVA.

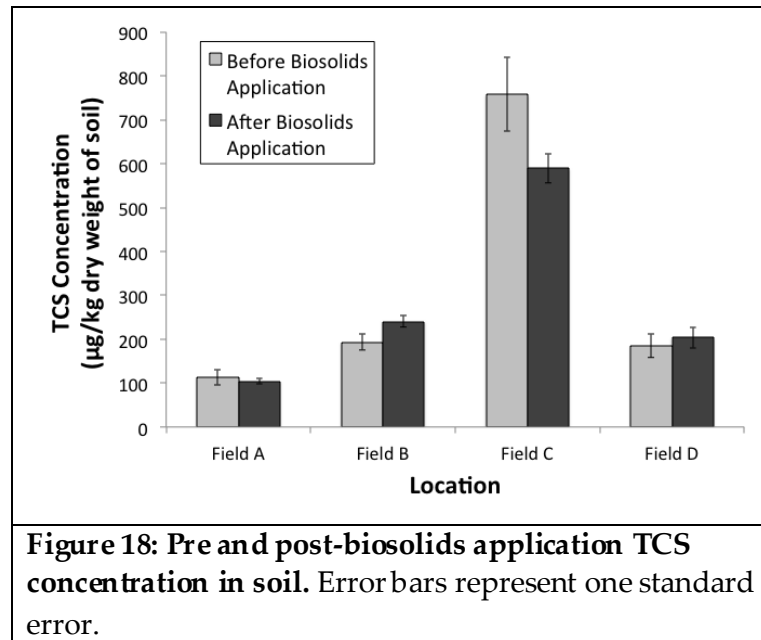
6.3 Results and Discussion

The results obtained for the pilot field study are provided below. Note that Day 0 corresponds to pre-application of biosolids and Day 1 corresponds to immediately following biosolids application.

6.3.1 TCS Concentrations

The average TCS concentrations before and after biosolids application are shown in Figure 18. There was no significant difference between the before and after samples, which indicates that either the TCS concentration in the biosolids for this application were low or that the TCS concentration in the soil was built up over years of application. Significant differences in TCS concentration prior to biosolids application were observed between Field 1 and 3 (p-value = 0.0002), Field 2 and 3 (p-value = 0.0011) and Field 3 and 4 (p-value = 0.0004). A marginal significant difference was observed between Field 1 and 2 (p-value = 0.059). After biosolids application, Field 1 and 3 (p-value = 0.0002), Field 2 and 3 (p-value = 0.0007), and Field 3 and 4 (p-value = 0.0004) were still significantly

different. Field 1 and 2 were no longer marginally significantly different following biosolids application. Overall, the average percent detection of the internal standard (^{13}C TCS) for all samples was $57 \pm 2.8\%$ (standard deviation = $\pm 12.8\%$), which was consistent with the paper where the method was obtained [172]. In addition, TCS was present in the blanks at low levels (average = 2.5 ± 0.83 ng).



6.3.2 Soil Characteristics

Several soil characteristics were measured for each location throughout the study period and are shown in Table 6. TKN and pH were measured on each sampling day for all four locations. Data for total Carbon (TC), humic matter (HM), phosphorus (as P_2O_5) and potassium (as K_2O) were somewhat limited due to errors in the analyses. Nevertheless,

TKN and pH did not appear to consistently change with the application of biosolids or change over the extent of the experiment. However, TKN does not account for NO_3^- , which was discussed in Chapter 5 to be more readily bioavailable to denitrifiers [10]. NO_3^- was not measured in this study, but should be considered for a more extensive study. Similarly, for the data available, phosphorus and potassium were not consistently impacted by biosolids application or change over time. Humic substances have been shown to reduce the bioavailability, and subsequently, toxicity of the compound through humification [206]. However, HM was also not significantly different between the sample locations. TC in the soil decreased over the first 103 days at Field A and 68 days of Field C. TC in Field B remained the same over the first 82 days. However, because TC is a measurement of both organic carbon (OC) and inorganic carbon (IC), it does not necessarily reflect soil microbial activity.

Location	Day	pH	TC (g/kg)	HM (%)	TKN (mg/kg)	P (mg/kg)	K (mg/kg)
Field A	0	7.7	61.9	0.56	6,200	178	3.14
Field A	1	7.6	63.5	0.27	6,500	195	1.38
Field A	30	7.6	56.5	0.32	5,600	167	1.91
Field A	103	7.5	49.5	0.36	6,500	166	2.17
Field A	147	7.9			5,500		
Field B	0	7.2	60.2	0.36	6,500	582	0.62
Field B	1	7.0	56.9	0.27	5,600	755	0.36
Field B	82	7.2	62.6	0.41	5,600	535	0.77
Field B	105	7.3			8,800		
Field B	126	7.4			7,300		
Field C	0	7.2	147.3	0.27	12,300	500	0.54
Field C	1	7.4	119.3	0.66	9,900	595	1.11
Field C	68	7.2	81.7	0.27	7,000	1185	0.23
Field C	112	7.6			10,400		
Field D	0	7.6			8,500		
Field D	1	7.5			8,100		
Field D	8	7.6			7,900		
Field D	21	7.6			8,600		

6.3.3 Impacts on denitrifiers

The function, abundance, and diversity of the soil denitrifiers were examined to determine potential impacts from the application of ‘traditional’ biosolids containing TCS.

6.3.3.1 Function

Figure 19 shows the relative denitrification rate results from the DEA assay. For three of the four fields examined, denitrification increased following biosolids application and

then decreased below pre-application levels around 100 days. Specifically, denitrification in Field A increased for the first 40 days and then returned to pre-application denitrification levels by Day 103. In Field B, increased denitrification was observed immediately following application, but was significantly lower by Day 82. Denitrification could have continued to increase in Field B until Day 82, but was not measured due to rain. Field C denitrification increased up until at least Day 68, but by Day 112 was depressed. The initial promotion of denitrification by organic fertilizers, such as manure and sewage sludge has been shown previously, and has been attributed to an increase in OC [116, 191, 195, 207]. Others have shown that maximum denitrification typically occurs within 24 to 48 h of application of organic amendments [196]. In the present study, Field D had little change immediately after application and a significant decrease in denitrification after 7 days. However, by Day 21, denitrification was at pre-application levels. Still, by Day 105 in Fields A, B, and C, denitrification was significantly reduced. This could be indicating longer-term impacts of biosolids on denitrification, similar to some of the results in Chapter 5, or could be an indication that field conditions changed significantly between pre-application and Day 105, and as a result, denitrification rates changed.

Very few other studies have examined the impacts of biosolids-derived organic contaminants on denitrification. Johansson *et al.* [208] showed no correlation between several anthropogenic compounds measured in biosolids on denitrification capacity.

However TCS was not included in the group of compounds examined. Other studies that spiked TCS directly into soil or into biosolids that were mixed with soil reported inhibition of other microbial function endpoints (i.e., respiration, phosphatase activity, and NO_3^- and NO_2^- production) at much higher concentrations [35, 177, 178]. The significant decreases after Day 105 could also be related to NO_3^- concentration, as discussed in Chapter 5.

Linear regression analysis was completed to examine any possible links between the denitrification profiles of each of the fields and TCS concentration. There were no significant correlations between the magnitude of the increase in denitrification rate following application or the average decrease in denitrification rate over time and the pre, post, and difference between pre and post-biosolids application TCS concentrations. There was also no significant correlation between the TCS concentrations and the denitrification rates measured immediately after application (Day 1), when all fields could be compared directly.

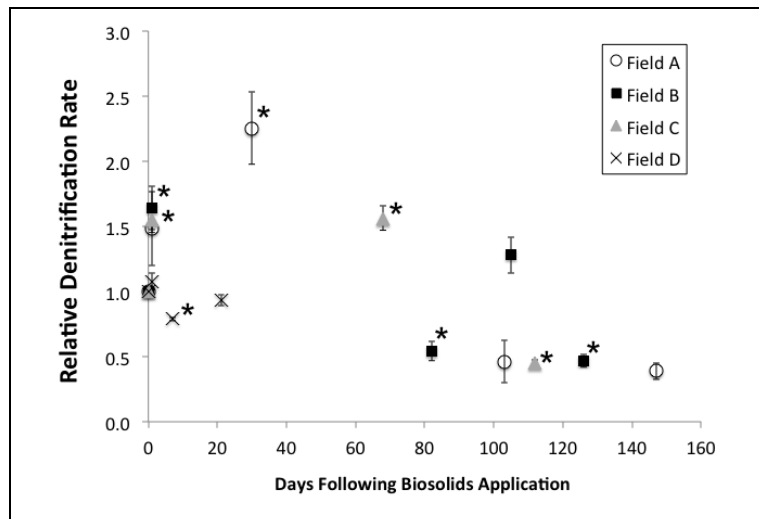


Figure 19: Relative denitrification rates measured in examination fields before and after biosolids application. (*) indicates a significant difference from Day 0 (pre-application of biosolids) (p-value ≤ 0.05). Values < 1 indicate inhibition of denitrification as compared to the Day 0. Error bars represent one standard error.

6.3.3.2 Abundance

The total (16S) and denitrifying (*nosZ*, *nirS*, and *nirK*) bacteria abundances were quantified for each field over the extent of the experiment (Figure 20). The range of absolute copy numbers of 16S, *nirS*, *nirK*, and *nosZ* per ng of DNA were 2.45×10^2 to 2.64×10^5 , 4.15×10^{-1} to 4.98×10^3 , 5.52×10^0 to 5.40×10^3 , and 5.94×10^{-1} to 7.91×10^4 , respectively. These values were consistent with Chapter 5 and with a previously published study [188].

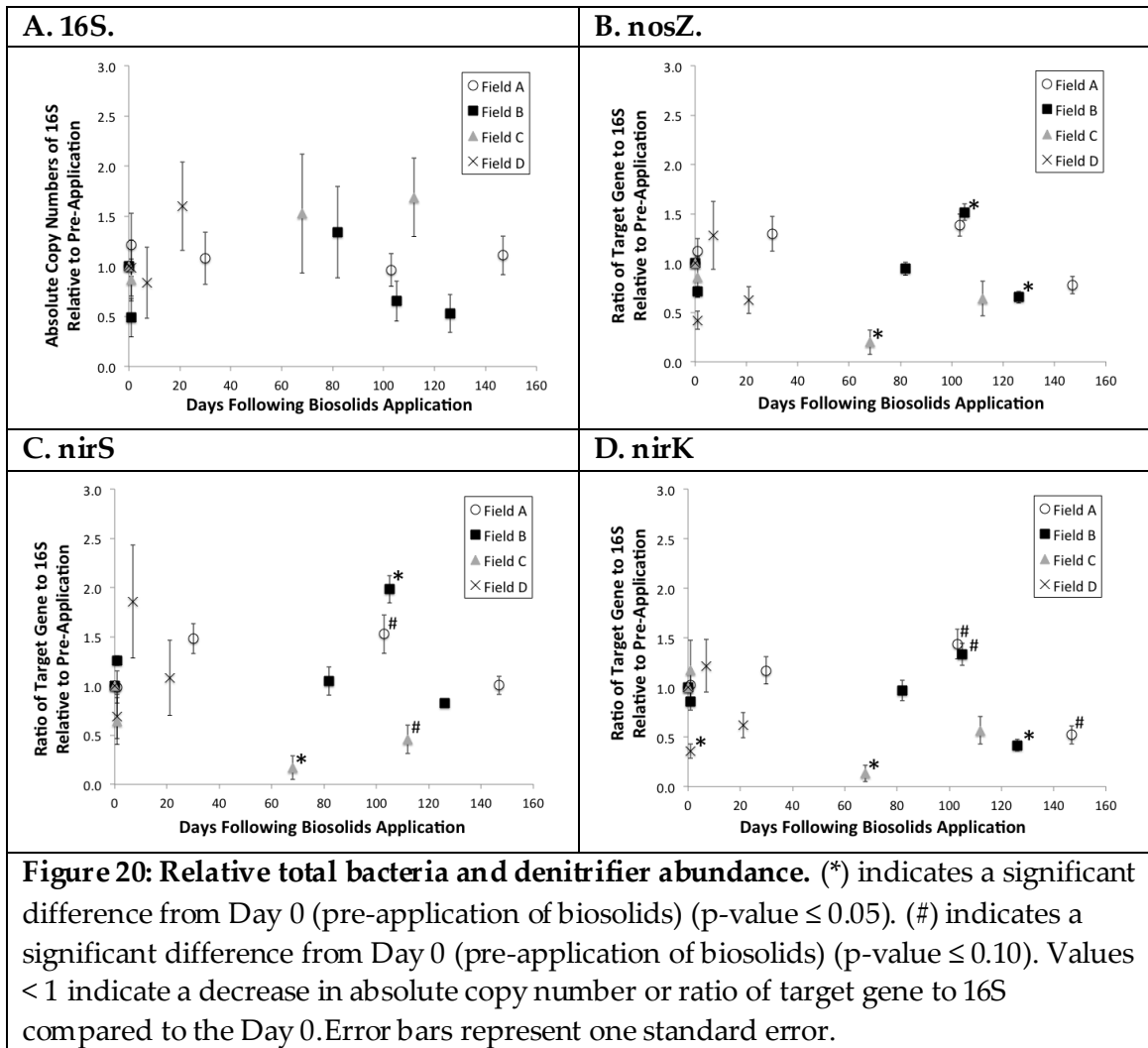
In general, total prokaryote abundance remained the same throughout the experiment. This was somewhat consistent with previous studies that showed an

increase in heterotrophs via plate count after the application of biosolids , but not consistent with another that showed a delayed decrease in biomass due to the addition of TCS-spiked biosolids [180, 197].

The denitrifying populations followed the general trend of an increase in abundance following application, followed by a decrease to significantly lower abundances after about Day 100. For all three genes, abundances for Field A remained at or above pre-application levels until Day 147, when *nosZ* was significantly lower. Abundances of all three denitrifying genes in Field B were not significantly different from the control up to Day 82, but were significantly greater on Day 105 for *nosZ* and *nirS* and marginally significantly greater for *nirK* (p-value = 0.056). Abundance of *nosZ* and *nirK* was then significantly lower on Day 126. Abundances in Field C were significantly less than pre-application on Day 68 for all three denitrifying genes. However by Day 112 only *nirS* was marginally significantly lower (p-value = 0.073). Lastly, *nirK* was significantly lower in Field D immediately after biosolids application and then rebounded to pre-application levels. Field D *nirS* and *nosZ* did not significantly change over the extent of the experiment.

Overall, *nirK* seemed to be the most impacted by the biosolids application with the most relative abundance values significantly or marginally significantly below pre-application levels (4). Only Days 103 and 105 for Field A and B had values significantly greater than pre-application. The increase for Field B at Day 105 was consistent for all

three denitrifying genes. Denitrifying genes *nosZ* and *nirS* were impacted the second most (3 sample days). Only two other studies were found examining microbial abundance in response to biosolids application and were described in more detail in Chapter 5; one reporting an increase in heterotrophs and one reporting a decrease in biomass following biosolids application [180, 197].

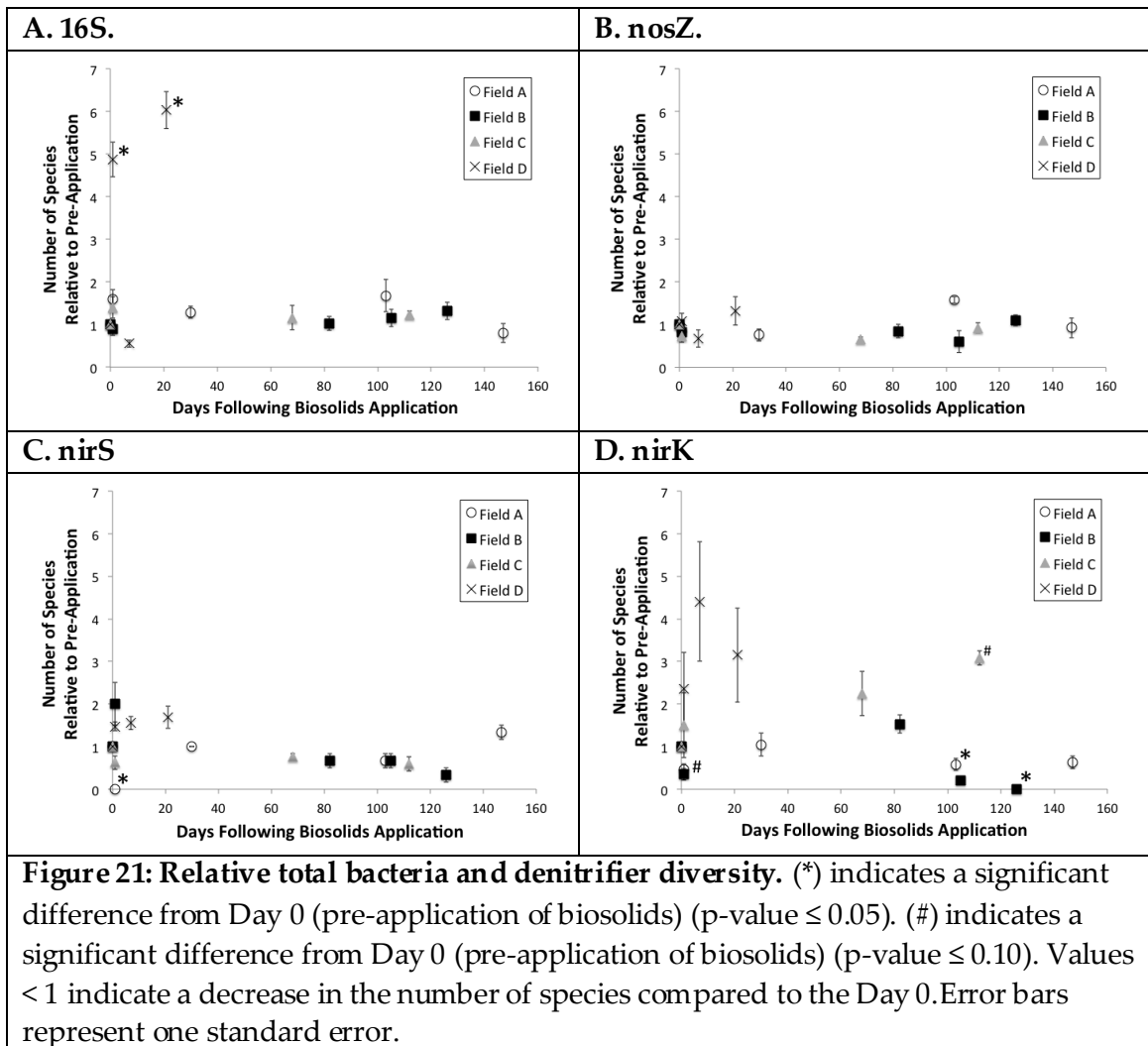


6.3.3.3 Diversity

T-RFLP was used to examine the impacts of TCS in biosolids on total bacteria and the denitrifier populations (Figure 21). The number of fragments measured for 16S, *nirS*, *nirK*, and *nosZ* ranged from 0 to 154, 0 to 116, 0 to 13, and 3 to 58, respectively.

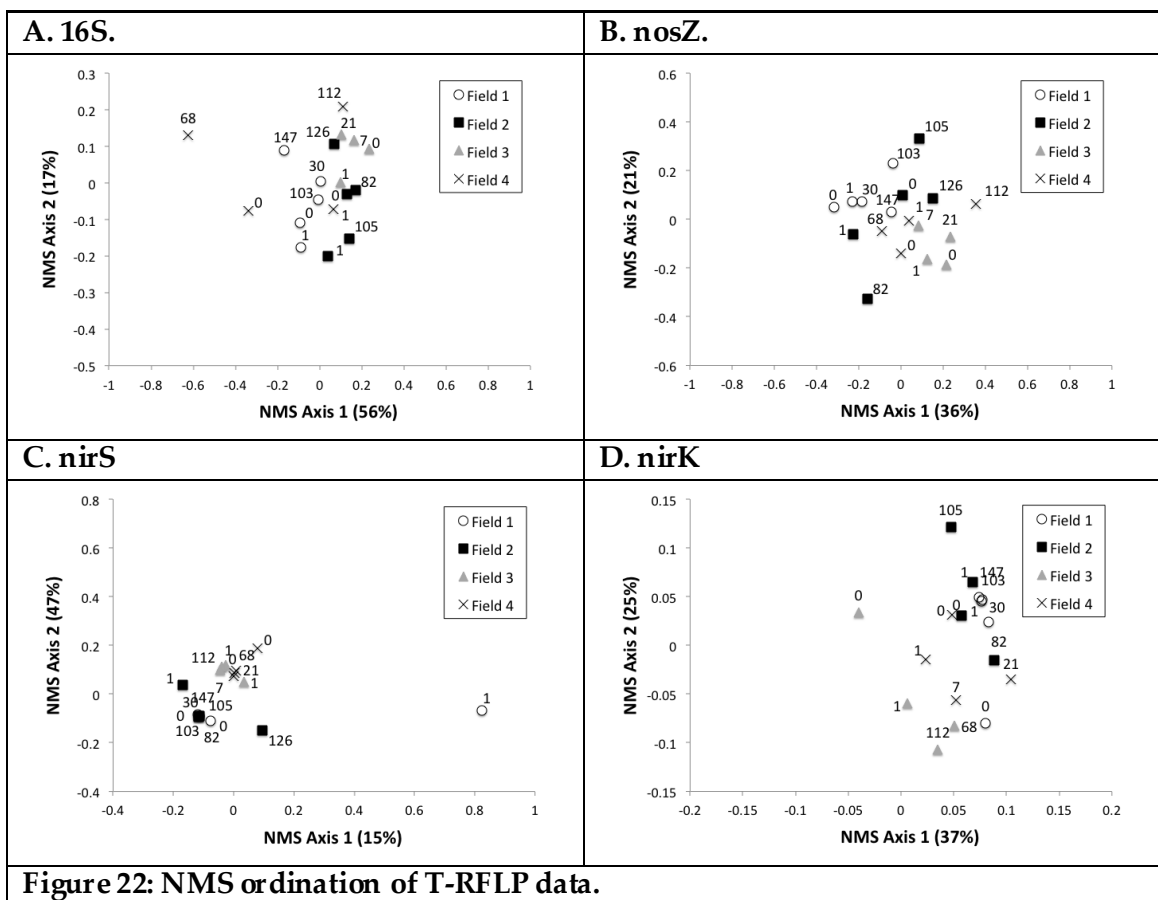
For the most part, both the total bacteria and denitrifying population diversity remained the same throughout the study period for all four fields. Only the diversity in Field A and B for *nirK* were inhibited. *nirK* in Field A was significantly inhibited on Day 103 and in Field B was marginally significantly inhibited on Day 1 and significantly inhibited on Day 126. Overall, it appears that diversity of both the total bacteria and denitrifiers was not measurably impacted by the addition of biosolids containing TCS. This could mean that while the abundance, and even activity, of the denitrifiers were inhibited by the application of biosolids, the diversity of these populations remained consistent. While data regarding the impacts of biosolids application on soil microbial community structure were limited, a few have shown impacts from biosolids application. Enwall *et al.* [116] showed a shift in *nosZ* communities using denaturing gradient gel electrophoresis (DGGE) in soil treated with sewage sludge compared to no treatment. TCS, nor any other emerging contaminant, were measured during the experiment, and thus were not analyzed for potential impacts. Several studies that mimicked biosolids application and spiked TCS directly into the soil or into biosolids that were mixed with soil showed a correlation between community shifts and TCS

concentration based on carbon utilization and phospholipid fatty acids characteristics [35].



The convergent, final NMS ordination solution for all four genes is shown in Figure 22. The stress of the NMS plot solution for 16S, nosZ, nirS, and nirK were 0.1864, 0.2693, 0.1591, and 0.1893, respectively, which indicated a relatively strong

correspondence between the distances among the points. R values for each axis are displayed in parentheses in the axis description of each plot. For all four genes, there was not a consistent pattern for the grouping of the field samples. Thus, the impact of TCS concentration in biosolids on the denitrifying communities was not apparent.



6.3.4 Method Correlation

The activity, abundance, and diversity yielded varying results in regard to application of 'traditional' biosolids containing TCS. As in Chapter 5, correlations between activity,

abundance, and diversity were developed using linear regression analysis (Table 8). Unlike in Chapter 5, no significant correlations were found between the DEA assay and qPCR. The only significant correlation was between *nosZ* diversity and activity (DEA assay). In addition, linear regression analysis was used to determine if TKN had any impacts on activity, diversity, or abundance. However, no significant correlations were found. This was somewhat expected because, as shown in Chapter 5, NO_3^- concentrations play a more significant role in denitrification.

Table 7: Linear correlation between methods used to analyze field denitrification.

Method A (X)	Method B (Y)	F-Statistic	R	p-value	Intercept	Slope
qPCR 16S	DEA	0.288	-0.209	0.599	1.26	-0.202
qPCR nirS	DEA	0.039	-0.245	0.845	0.990	0.056
qPCR nirK	DEA	0.407	-0.190	0.533	0.855	0.221
qPCR nosZ	DEA	0.070	-0.241	0.795	0.959	0.098
T-RFLP 16S	DEA	0.001	-0.248	0.972	1.05	-0.003
T-RFLP nirS	DEA	0.099	-0.237	0.758	0.972	0.080
T-RFLP nirK	DEA	0.305	-0.207	0.588	1.13	-0.058
T-RFLP nosZ	DEA	5.43	0.455	0.033*	2.04	-1.06
qPCR 16S	T-RFLP 16S	1.88	0.221	0.189	0.149	1.39
qPCR nirS	T-RFLP nirS	0.796	-0.110	0.386	0.711	0.241
qPCR nirK	T-RFLP nirK	0.751	-0.122	0.399	2.03	-0.695
qPCR nosZ	T-RFLP nosZ	0.019	-0.247	0.891	0.959	-0.025

(*) indicates a significant difference from 0 (p-value ≤ 0.05).

6.3.5 Implications of Biosolids Application

As expected, the results presented in this pilot field study reflected the complexity of the system that was analyzed and many more samples, which account for variables

including, but not limited to soil characteristics, biosolids characteristics, biosolids application rates, and chemical composition and quantities, would be needed to show any statistically significant differences. However, the main objective of this study was to determine if a larger, more extensive study should be completed. With that in mind, several key results were obtained. The long-term inhibition of denitrification was observed using the DEA assay. However, the inhibition observed after about Day 100 could be due to exhaustion of resources, such as NO_3^- , or significant changes in the local environment. Unfortunately, NO_3^- and NO_2^- were not measured, and as expected TKN showed no correlation with activity, or abundance and diversity. Increased inhibition over the extent of the study period was observed for denitrifier abundance, suggesting that impacts measured by activity may be delayed compared to the density of the denitrifying populations. Little to no inhibition was observed when examining the relative number of denitrifying species. Thus, while the abundance of denitrifiers was reduced, and denitrification was eventually depressed, the number of species in the soil remained constant.

As described in Chapter 5, a denitrification inhibition score was calculated for each time point and presented in Figure 23. The score for Day 1 was not included in Figure 23, but had a denitrification inhibition score of 0 out of 28 analyses, indicating no inhibition. Each field was measured on Day 1, which resulted 4 times the total analyses than the other sample days. Each of the days presented in Figure 23 have a maximum

score of 7, which corresponds to the 7 analyses completed on each day (DEA assay and 3 genes each for qPCR and T-RFLP; one field per day) and to maximum inhibition.

Therefore, -7 is the minimum score representing maximum stimulation. Overall, the denitrification inhibition scores showed slight inhibition over time, with the exception of Days 30 and 103, which correspond to samples taken from Fields C and A, respectively. Overall, these data indicate that the application of biosolids resulted in slight inhibition of denitrification that was sustained up to 150 days following application. However, these data do not indicate whether the TCS in the biosolids was leading to the inhibition. Also, because the samples were not taken on the same days following biosolids application, a more robust analysis of TCS concentration and denitrifying function and community structure could not be completed. In addition, any of the impacts observed in this study could be a result of another, or group of other compounds in the biosolids that had similar chemical characteristics to TCS. For example, the longer-term inhibition indicates that if a compound was resulting in the inhibition observed, the compound was likely not soluble. Nevertheless, the results in this study show potential negative impacts of applying biosolids containing TCS to soil denitrifying bacteria community structure and function. Because denitrification plays an integral part in overall quality and health, a more extensive study examining these impacts is recommended.

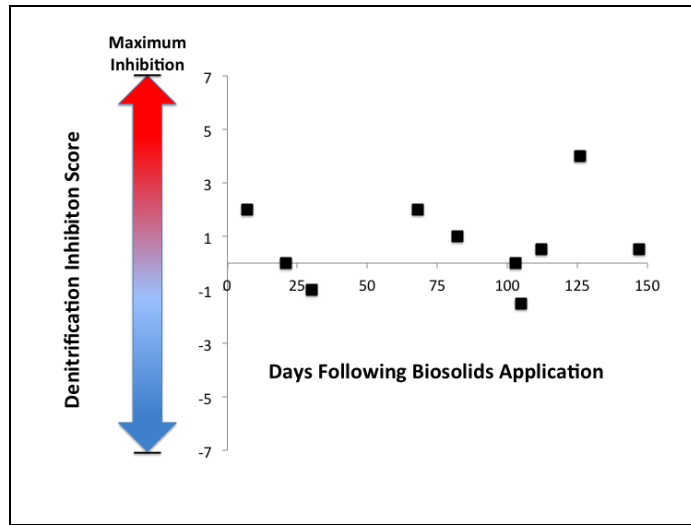


Figure 23: Denitrification inhibition score for field biosolids application.

7. Conclusions and Engineering Significance

Overall, the results presented in this dissertation provide a systematic evaluation of the effects of biosolids-derived antimicrobial agents on agricultural soil microbial ecology. First, it was demonstrated that denitrification could be used as an indicator of biosolids-derived antimicrobial agent ecotoxicity. The denitrification assay that was developed was then used to analyze ecotoxicological potential of six emerging biosolids-derived antimicrobial agents, and found inhibition of denitrification at environmentally relevant concentrations. The most widely used antimicrobial agent, TCS, was further shown to inhibit wastewater treatment processes, as well as denitrification in simulated agricultural conditions after being aged with and directly spiked into biosolids. In addition, evidence showing potential inhibition of denitrification under field conditions by biosolids addition was also obtained. Based on these results, this dissertation asserts that biosolids-derived TCS poses a potential risk to wastewater treatment processes and agricultural soil microbial denitrifiers, which could be an indication of broader impacts on overall microbial and soil health.

7.1 Key Findings

There were five main conclusions drawn from this dissertation, which correspond to the main objectives.

Conclusion 1: Denitrification is a potential indicator of ecotoxicity for biosolids-derived emerging organic contaminants.

As shown in Chapter 3, rapid, high-throughput method to evaluate the ecotoxicity of biosolids-derived antimicrobial agents was developed. The assay developed measured denitrification inhibition in a model denitrifier, *Paracoccus denitrificans* Pd1222. Two common (TCS and triclocarban) and four emerging (2,4,5 trichlorophenol, 2-Benzyl-4-chlorophenol, 2-chloro-4-phenylphenol, and Bis(5-chloro-2-hydroxyphenyl)methane) antimicrobial agents found in biosolids were then analyzed using the assay. Overall, the assay was reproducible and measured impacts on denitrification over three orders of magnitude exposure. Compared with gene expression and cell viability based methods, the denitrification assay was more sensitive and resulted in lower LOAECs. The increased sensitivity, low cost and high-throughput adaptability made this method an attractive alternative for meeting the initial testing regulatory framework for the Federal Insecticide, Fungicide, and Rodenticide Act, and recommended for the Toxic Substances Control Act, in determining the ecotoxicity of biosolids-derived emerging contaminants. In addition, this assay has the potential to be expanded to more complex media that better simulate biosolids and soil, other functional endpoints that can indicate soil health, and other ecologically important organisms or groups of organisms.

Conclusion 2: Four of the six antimicrobial agents examined were found to inhibit denitrification at environmentally relevant concentrations.

Also shown in Chapter 3, the lowest observable adverse effect concentrations (LOAECs) measured on the target compounds using the developed assay were 1.04 μM for TCS, 3.17 μM for triclocarban, 0.372 μM for bis-(5-chloro-2-hydroxyphenyl)methane, 4.89 μM for 2-Chloro-4-phenyl phenol, 45.7 μM for 2-benzyl-4-chlorophenol, and 50.6 μM for 2,4,5-trichlorophenol. Five out of the six compounds have been found at or near the LOAECs in the environment. TCS, has been measured in water and wastewater up to 1.94 μM [22, 141], estimated in pore water of sediment and biosolids amended soil up to 1.32 μM [58], and measured in biosolids collected from several different WWTPs at concentrations as high as 113 mg/kg dry weight [17, 21, 23, 32, 50-53]. While the LOAEC for TCS in the study is slightly below the aqueous concentrations found in the environment, the LOAEC calculated on a dry weight basis, 4.19 g/kg, was much greater than the concentrations found in biosolids, which was likely a reflection of differences in sample solids content. Triclocarban has been measured in water and wastewater up to 0.022 μM [57], estimated in sediment and biosolids amended soil pore space up to 0.867 μM [58], and measured in biosolids up to 441 mg/kg dry [51, 53, 54, 59]. The LOAEC for triclocarban is within one order of magnitude of environmental aqueous concentrations. On a dry weight basis, the LOAEC (14.0 g/kg) was much greater than the concentrations found in biosolids, but again was likely due to differences in sample solids content.

Bis(5-chloro-2-hydroxyphenyl)methane was found in anaerobically digested sludge at concentrations up to 0.52 μM , which was within an order of magnitude of the LOAEC for *P. denitrificans* PD1222 [20]. Similarly, 2-benzyl-4-chlorophenol was found in wastewater influent at 0.89 μM , which is also within an order of magnitude of the LOAEC for *P. denitrificans* PD1222.

Conclusion 3: Biological nitrogen removal and microbial communities were impacted in SBRs coupled with anaerobic digesters by the addition of TCS in the influent.

The work completed in Chapter 4 showed that biological processes associated with nitrogen removal (nitrification and denitrification), were impacted by TCS entering the SBRs regardless of the starting microbial community. Both of the SBRs that were not receiving TCS reached steady-state at greater than 92% NH_4^+ removal within the first week of operation, whereas the SBRs receiving TCS took 42 and 63 days to reach steady-state removal at that level. However, while NH_4^+ removal was temporarily inhibited, elevated levels of NO_3^- and NO_2^- in the effluent of the TCS fed SBRs, suggest longer-term impacts on NOB and denitrifiers. After Day 58, the NO_3^- effluent concentration for the SBRs receiving TCS was 3.9 ± 0.16 mg/L, which was 2.4 times greater than the NO_3^- effluent of the controls (1.7 ± 0.08 mg/L). Similarly, after Day 58, the NO_2^- effluent of the SBRs receiving TCS reached a steady-state concentration of 8.7 ± 0.75 mg/L. The mean NO_2^- concentration of the controls after Day 58 was 7.7 times lower at 1.1 ± 0.78 mg/L,

but was still trending towards 0 when the reactors were stopped. No inhibition was observed for COD and PO₄³⁻ removal.

Conclusion 4: TCS in aged and spiked biosolids negatively impacts denitrifying bacteria community structure and function in soil.

The containers with plants that received biosolids aged with and spiked with TCS showed potential long-term inhibition based on measurement of soil denitrifying activity at 26.9 ± 4.6 $\mu\text{g}/\text{kg}$ and 68.6 ± 26.9 $\mu\text{g}/\text{kg}$ of TCS, respectively. Denitrifier abundance and diversity, however, were more sensitive to TCS in biosolids and inhibition was observed throughout the experiment, with maximum inhibition from Day 1 to 28. Inhibition of denitrifier abundance and diversity was observed at TCS concentrations as low as 17.9 ± 1.93 $\mu\text{g}/\text{L}$, which was approximately 10 to 3,000 times lower than concentrations reported by other studies that showed impacts on other functional endpoints (i.e., respiration, phosphatase activity, NO₃⁻ and NO₂⁻ production, and Cy17 stress biomarker abundance), even after taking pH into account. Five significant correlations were developed, three of which related qPCR and the DEA assay, or abundance and activity. However, the analyses that were correlated did not yield the same results as far as significant inhibition in the presence of TCS. Thus, while the results suggested some relatedness between activity, abundance, and diversity, the results generally support the use of multiple methods to determine the ecotoxicity of

biosolids-derived organic contaminants. As a result, a denitrification inhibition score was developed that took into account all three methods to estimate the overall potential ecotoxicity of TCS in biosolids. Overall, the denitrification inhibition score showed that denitrification was inhibited by both biosolids that were aged and spiked with TCS over the extent of the 84 day experiment, but maximum inhibition occurred after one week and up to approximately one month. While the denitrification inhibition score indicated that the TCS in the biosolids aged with TCS was less bioavailable than in the spiked biosolids, the impacts of the aged and spiked biosolids could have also been due to differences in TCS concentrations.

Conclusion 5: Field-applied biosolids may have potential negative impacts on denitrifying bacterial communities.

As expected, the results presented in the pilot field study (Chapter 6) were more complex than those obtained under laboratory conditions (Chapter 5). First, because the TCS concentrations prior to and immediately after biosolids application were not significantly different, any inhibition in denitrification could not be correlated to the addition of TCS through biosolids application. However, potential long-term inhibition of denitrification by the addition of biosolids was still observed using the DEA assay, however the effects of exhaustion of resources, such as NO_3^- , or significant changes in the local environment could not be verified. Inhibition over the extent of the study

period was further observed for denitrifier abundance, but little to no inhibition was observed when examining the relative number of denitrifying species. Thus, while the abundance of denitrifiers was reduced, and denitrification was eventually depressed, the number of species in the soil remained relatively constant. The denitrification inhibition score, which took all three measurements into account, showed inhibition over time, with the exception of the measurements on Days 30 and 103. NMS ordinations showed no obvious groupings of the field samples, and thus, no association between the microbial communities and TCS concentration accumulated in the soil. Overall, any of the impacts observed in this study could be a result of characteristics of the biosolids, or if it was from a chemical within the biosolids, could be another, or group of other compounds in the biosolids that had similar chemical characteristics to TCS. For example, the longer-term inhibition indicates that if a compound was resulting in the inhibition observed, the compound was likely not soluble. Because of the complexity of the soil and biosolids and because of the myriad of contaminants likely in the biosolids, the results may not be significant and a more in-depth study was recommended.

7.2 Recommendations and Future Work

Broadly speaking, the results in this dissertation provide evidence for improved regulations and best management practices regarding; 1) environmental safety testing of

new compounds before marketing, 2) the use of similar, currently available compounds, 3) wastewater treatment to remove novel organic compounds from biosolids, and 4) general application rates of biosolids to avoid soil impairment. Improving regulations and practices in these four areas would provide a cradle to grave approach for dealing with new, potentially harmful compounds, especially those used in consumer products, which may accumulate in biosolids. While increased restrictions would undoubtedly result in increased costs for research and development of new compounds, as well as treatment and application strategies, continued investment in improving the quality of biosolids could be offset by the reduced use of synthetic fertilizers and the avoidance of alternative, often expensive reclamation means. Furthermore, human and environmental health costs associated with the production and use of synthetic fertilizers, and even manure from livestock, would also be saved [209-213]. In the end, maintaining biosolids as a low-cost alternative to synthetic fertilizers would benefit all farmers in the form of upfront costs and improved product quality.

In addition, several more specific recommendations were generated. The research recommendations listed in this section were not necessarily placed in order of highest priority to lowest priority. However, attempts should be made to address these recommendations carefully and expeditiously due the potential implications of their conclusions.

- Potential standards or removal processes should be examined to avoid inhibition of ecologically sensitive microbial communities by TCS. Based on the results presented herein, wastewater influent concentrations would need to be maintained below 0.73 μM and soil concentrations below 16 $\mu\text{g}/\text{kg}$. However, because an effect on treatment efficiencies has not been observed at actual wastewater treatment plants, the continued addition of TCS in the influent to a wastewater treatment plant may be selecting for resistant bacteria. This in itself is cause for concern and should be examined further. Further work should also be completed to determine the acceptable level of inhibition in regard to wastewater treatment processes and soil microbial processes (i.e., denitrification) in the presence of TCS and other emerging contaminants.
- The remaining 500 + contaminants already found in biosolids should be analyzed using the denitrification assay developed in this dissertation. Chemicals that result in significant inhibition of denitrification at concentrations lower than or near environmental concentrations should be flagged as having potential ecological impacts. In addition, manufacturers and researchers should use the denitrification assay to determine the potential ecological impacts of new chemicals, especially those not yet used by consumers. Modifications of the assay, so that it better reflects environmental conditions (e.g., the addition of standard soil), could be used to determine the environmental relevancy when no

environmental data exists. Contaminants flagged using the DEA assay should then be examined further under using laboratory-generated biosolids aged with the target compound and conditions mimicking agricultural field conditions.

- The denitrification assay developed in this study should be adapted to other ecologically important end-functions, as well as with other model organisms and groups of organisms. This and previous studies have shown that ecological impacts vary by the functional endpoint examined and analytical method used.
- Future studies examining the impact of contaminants on baseline wastewater processes should add the contaminant with the influent. Many of the emerging contaminants are already present in the influent and spiked studies do not adequately represent this loading scheme.
- This study can be used as an approach guideline for generating laboratory biosolids aged with a target compound. Future studies examining the impact of biosolids-derived contaminants should consider using laboratory-generated biosolids aged with the target compound. However, further chemical analyses should be completed to determine the persistence of the chemical in the SBRs. In addition, the volume of inoculum used for the anaerobic digesters should be minimized. It is also recommended that future studies attempt to quantify the bioavailability differences between the generated biosolids and when the compound is spiked directly into the biosolids.

- The role the microbial community plays in the bioavailability of TCS should be examined. This and previous studies have shown inhibition of microbial activity when bioavailability should be reduced due to adsorption.
- A more in-depth, extensive study should be completed to determine the impacts TCS on soil microbial communities under field conditions. The results presented in this dissertation indicate potential inhibition of denitrifying bacteria. However, more data regarding soil characteristics and microbial community characteristics tracked over time is needed to verify the significance of the correlation. In addition, several fields receiving varying amounts of TCS in the biosolids needs to be examined to evaluate a dose-response. Lastly, measurements for each field should be made over the same time increments, so differences in denitrification can be compared.
- Improved primers or alternative methods (e.g., genomic sequencing) should be used to quantify the diversity of denitrifiers containing the *nirK* gene.

Appendix A – Chapter 3 Supplemental Information

The following appendix contains the supplemental information for Chapter 3.

A.1 Nitrate and Ammonium Measurements

To close the nitrogen mass balance, NO_3^- and NH_4^+ were also measured. A modified version of the phenate method (Section 4500 of the Standard Methods for the Examination of Water and Wastewater) was used to measure NH_4^+ [163]. Briefly, 200 μL of sample was added to a 96 well plate. Again, a standard curve ranging from 0 to 10 mg/L as NH_4^+ was developed by serially diluting a stock of $(\text{NH}_4)_2\text{SO}_4$ in modified MM. To each well, 10 μL of phenate solution, 10 μL of sodium nitroprusside, and 20 μL of oxidizing solution were added. After each solution was added, the plate was shaken for 10 s at 1,500 rpm. The plate was then covered with aluminum foil and incubated at room temperature ($\sim 21^\circ\text{C}$) for 30 min. The plate was then shaken again and incubated for an additional 30 min. After the second incubation, the plate was shaken one last time and OD_{620} was measured. NO_3^- was quantified using a Dionex DX120 Ion Chromatographer (IC) with an Ionex IonPac[®] AS22 column and NG2 guard, and an ASRS[®] suppressor (Dionex Global, Sunnyvale, CA). The eluent used consisted of 0.45 M carbonate and 0.13 M bicarbonate in DI water. Five hundred μL samples were collected and transferred to new, sterile 1.5 mL centrifuge tubes and stored at 4°C for up to 48 h prior to analysis. Prior to IC analysis, each sample was diluted (1/5) in autoclaved DI water in 0.75 mL IC

vials (Sun SCI, Rockwood, TN). The vials were capped and vortexed for 5 s. To determine concentrations, a standard curve was prepared (0 to 25 mg/L as NO_3^-) by serially diluting stock KNO_3 made in modified MM. Analysis was carried out on the IC with an eluent flowrate of 1.0 mL/min and a residence time of 20 min.

A.2 Emerging Contaminants

Initially, the focus of the research in Chapter 3 was on a broader range of emerging contaminants, including pharmaceuticals, surfactants, and disinfectants. Denitrification by *P. denitrificans* PD1222 was measured in the presence of Ibuprofen (pharmaceutically active compound) [214, 215], naproxen (pharmaceutically active compounds, a.k.a., Aleve) [216-221] and 4-nonylphenol (surfactant) [6]. These compounds were originally selected because they had been measured in biosolids and covered a wide range of chemical properties important to their environmental fate. Figure 24 shows that of the three compounds, inhibition of denitrification was only observed for the highest concentrations of ibuprofen and 4-nonylphenol tested.

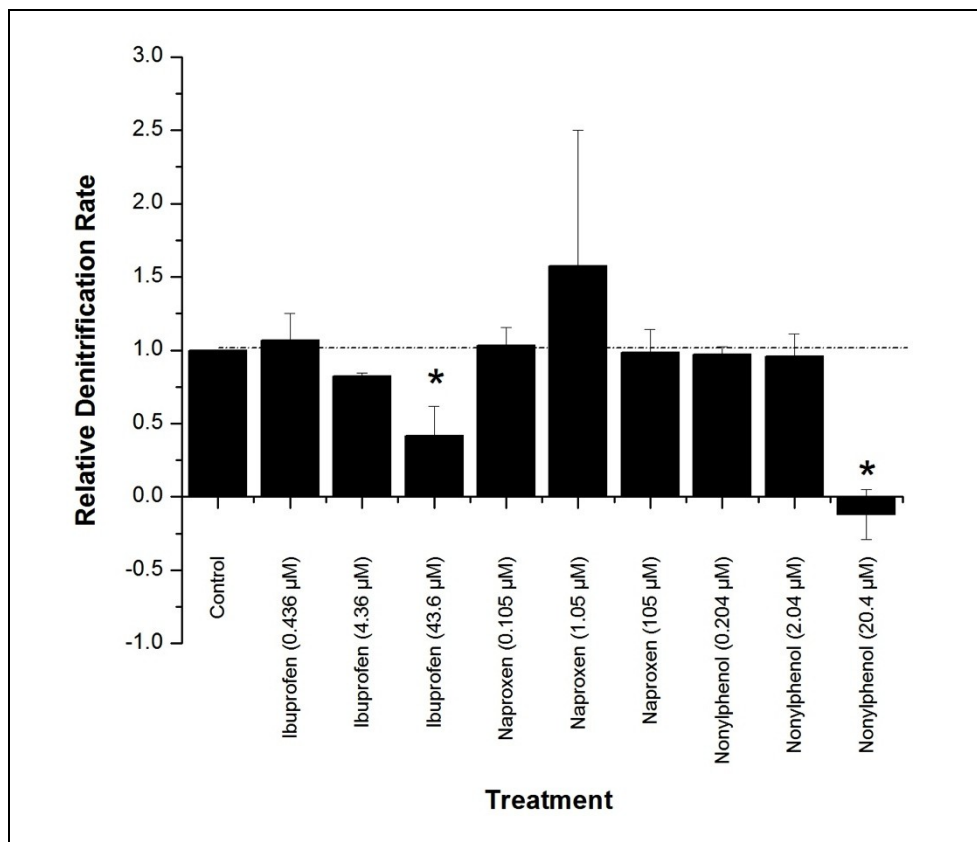


Figure 24: The effects of various emerging contaminants on denitrification. (*) indicates a statistical significant difference from the control (p -value ≤ 0.05). Values <1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error.

In an early attempt to narrow the target scope, the impacts on denitrification of two sets of common household disinfectant classes were tested, quaternary ammonium compounds (QACs), which are cationic surfactants used as disinfectant, several phenols, and chlorinated phenols, and triclocarban. The QACs examined were didecyltrimethylammonium bromide (DDAB, CAS 2390-68-3), tetradecyltrimethylammonium chloride (TTAC, CAS 4574-04-3),

benzyltrimethyltetradecylammonium chloride (BDAC, CAS 139-08-2), and cetylpyridinium (Cet, CAS 6004-24-6). The concentrations of DDAB, TTAC, BDAC, and Cet were 245, 343, 368, and 279 μM , respectively. As shown in Figure 25, all four of the QACs tested, resulted in complete inhibition of denitrification. Unfortunately, because of analytical limitations, the QACs were not pursued further.

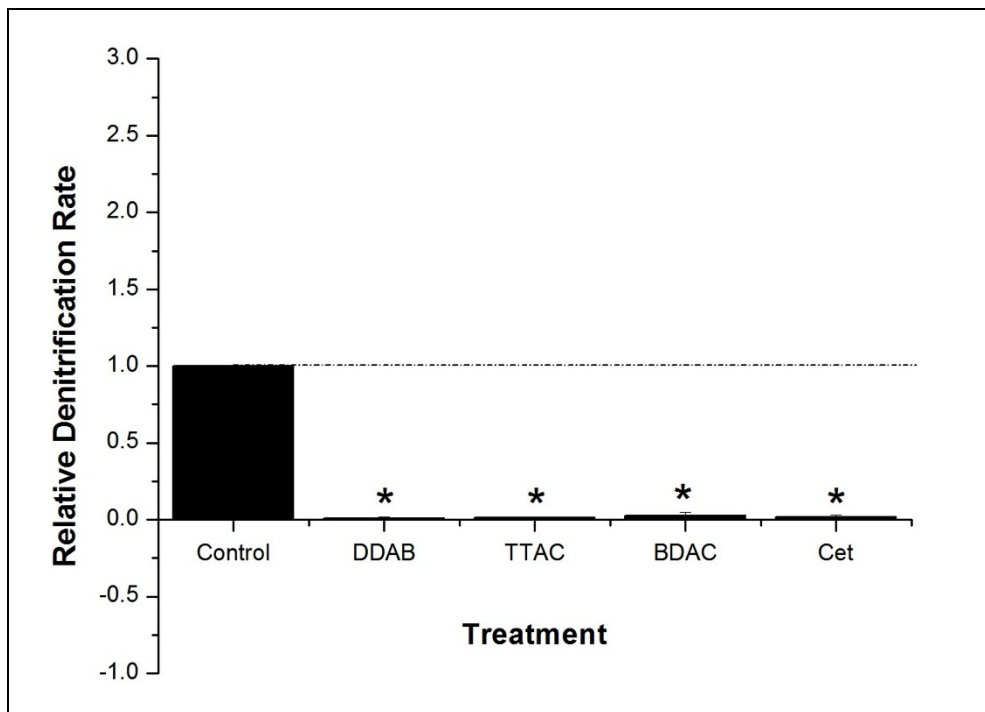


Figure 25: The effects of several QACs on denitrification by *P. denitrificans* PD1222. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05). Values <1 indicate inhibition of denitrification as compared to the control. Error bars represent one standard error.

All of the phenols and chlorinated phenols and triclocarban that were tested can be seen in Table 9. Figure 26 shows the results of the inhibition experiments. Figure 26

suggests that inhibition of denitrification occurred at lower concentrations as the number of benzene rings increased, as well as, the number of chlorine functional groups increased. Six of the compounds in Figure 26 significantly inhibited denitrification at concentrations around 50 μM or less. These included TCS, triclocarban, 2,4,5-trichlorophenol, 2-chloro-4-phenylphenol, bis(5-chloro-2-hydroxyphenyl)methane, and 2-benzyl-4-chlorophenol, and therefore, were selected as the target compounds in Chapter 3.

Abbreviation	Name	CAS
2C4PP	2-chloro-4-phenylphenol	92-04-6
TCP	2,4,5-trichlorophenol	95-95-4
24DCP	2,4-dichlorophenol	120-83-2
B5C2HM	Bis(5-chloro-2-hydroxyphenyl)methane	97-23-4
2B4C	2-benzyl-4-chlorophenol	120-32-1
2P	2-phenylphenol	90-43-7
Thymol	(2-isopropyl-5-methylphenol)	89-83-8
CHX	Chloroxylenol (4-chloro-3,5-dimethylphenol)	88-04-0
TCC	Triclocarban (3,4,'-trichloro-carbanilide)	101-20-2
TCS	Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol)	3380-34-5

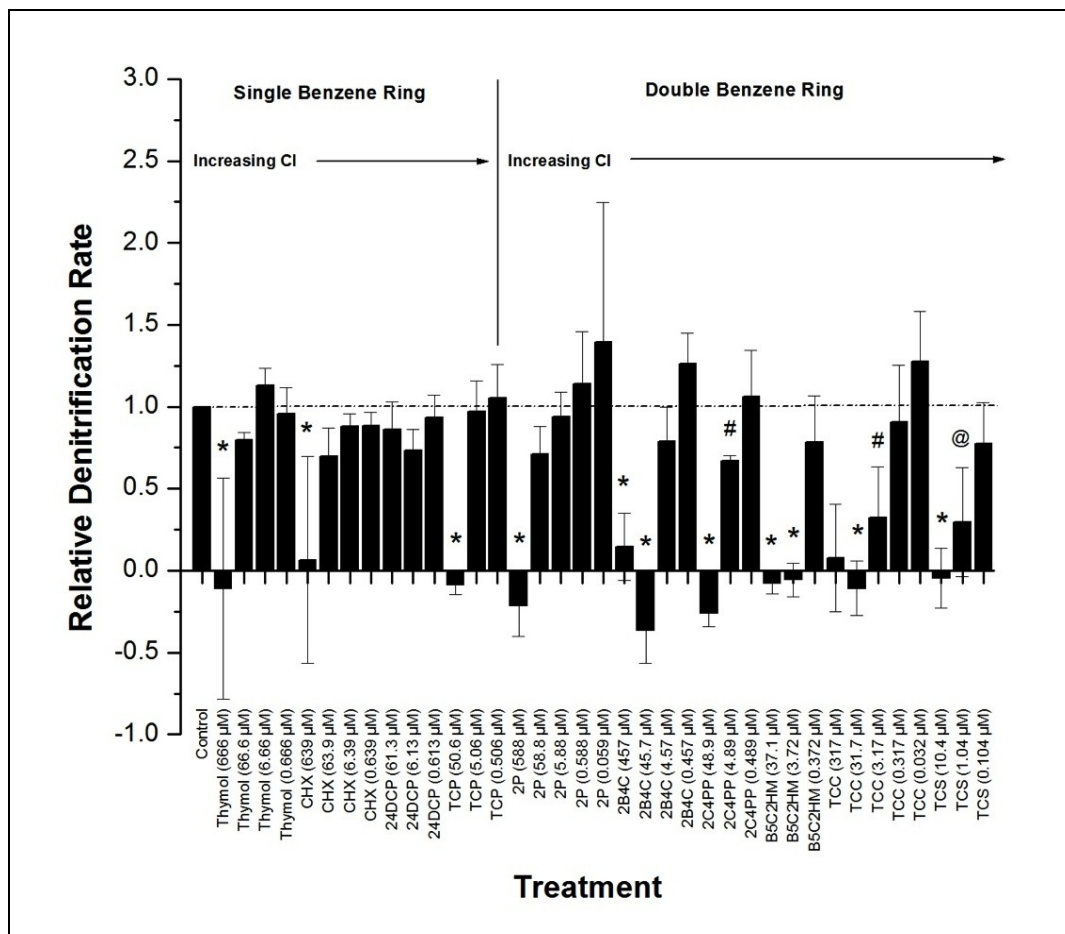


Figure 26: Relative denitrification rate in the presence of triclocarban and selected phenols and chlorinated phenols. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05), (@ indicates a marginal statistical significant difference from the control (p-value ≤ 0.06), and (#) indicates a weak statistical significant difference from the control (p-value ≤ 0.15). Values < 1 indicate inhibition of denitrification as compared to the control. Negative values correspond to a decrease in cell number when denitrification was completely inhibited. Error bars represent one standard error.

To determine if there were any differences in the efficacy of denitrification inhibition, four strains of model denitrifiers, *Paracoccus denitrificans* PD122 and *Pseudomonas stutzeri* JM300, Zobell, and DSM 50238 were exposed to 1.86 μM of Bis(5-chloro-2-hydroxyphenyl)methane. These cultures were obtained from Dr. Andrzej J.

Paszczynski of the University of Idaho (Moscow, Idaho). Figure 27 shows that the denitrification rate was only significantly inhibited for *P. denitrificans* PD1222 and not for the others. This may be due to natural resistance to bis(5-chloro-2-hydroxyphenyl)methane. Escalade *et al.* [70] demonstrated that *Pseudomonas* sp. Was resistant to TCS, a compound, which is chemically and physically similar to bis(5-chloro-2-hydroxyphenyl)methane. This supported the selection of this species as an indicator species of denitrification that was most vulnerable to contaminant exposure.

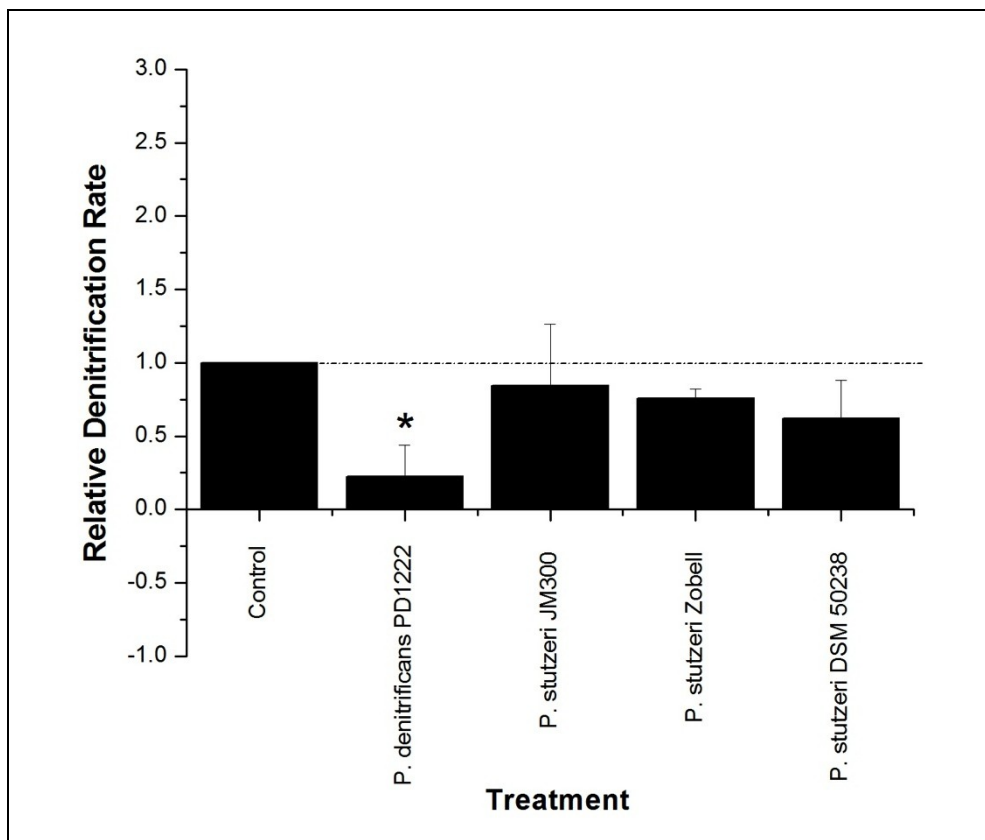


Figure 27: Relative denitrification for four species of denitrifiers exposed to Bis(5-chloro-2-hydroxyphenyl)methane. (*) indicates a statistical significant difference from the control (p-value ≤ 0.05 . Values < 1 indicate inhibition of denitrification as compared to the control. Error bars represent one standard error.

Appendix B – Chapter 5 Supplemental Information

The following appendix contains the supplemental information for Chapter 5.

B.1 qPCR Primer Optimization

Preliminary tests were completed to optimize the annealing temperature of the qPCR primers and to verify that touchdown PCR conditions were not needed. Conditions for qPCR were 95 °C for 10 min, followed by 45 cycles at 95 °C for 30 s, 1 min at the respective annealing temperatures, 30 s at 72 °C, and dissociation curve to assess the quality of the primers. Touchdown PCR conditions were previously published for *nirS* [188], *nirK* [86], *nosZ* (NosZ1) [189]. Annealing temperatures from 55 to 63 °C were examined. For initial assessment, 5 µL of PCR product from each annealing temperature and touchdown reaction were run on a 1.5% agarose gel also stained with ethidium bromide. The most intense single bands at the expected size with the least streaking were further analyzed on a 2% agarose gel, also stained with ethidium bromide. The expected size bands for *nirS*, *nirK*, and *nosZ* were 425, 164, and 259, respectively. The primers corresponding to the most intense single band without streaking from the second gel were selected for qPCR.

Figure 28 shows the amplification products of the *nosZ* primers for the various conditions. All bands, where present, were at the correct size. The second gel was run for annealing temperatures 55, 56, 57, and 63 °C and touchdown PCR, but the picture

was not saved properly. Nevertheless, the annealing temperature of 55°C resulted in the most intense band with no streaking, and was selected for qPCR analysis. Interestingly, no amplicon was detected using the previously published touchdown conditions.

Similar analyses were carried out for the *nirS* and *nirK* primers and shown in Figures 29 and 30. In both cases, the annealing temperature of 55°C also resulted in the most intense bands with the least streaking, and were selected for qPCR analysis. Again, no band was obtained with the *nirS* primers using the published touchdown PCR conditions. Touchdown conditions for the *nirK* primers yielded a band, however, the band was less intense than obtained with 55°C annealing temperature. In addition, a 55°C annealing temperature was also selected for *nirK* in order to keep the procedures for all three denitrifying genes consistent.

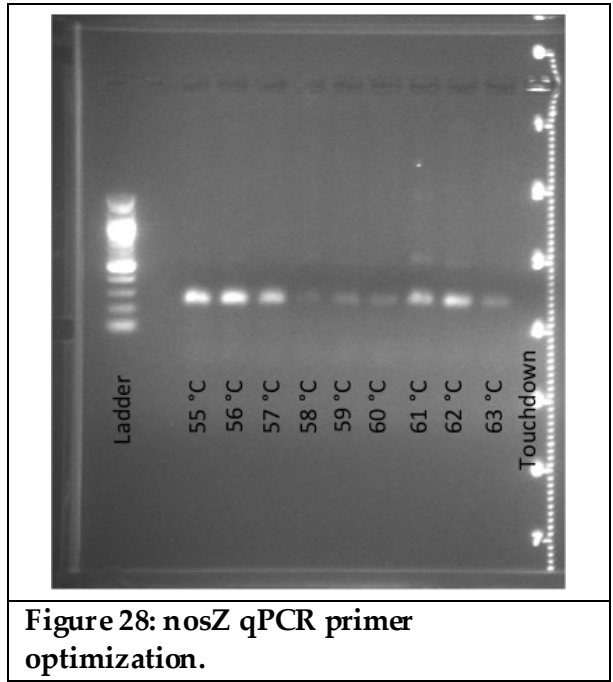
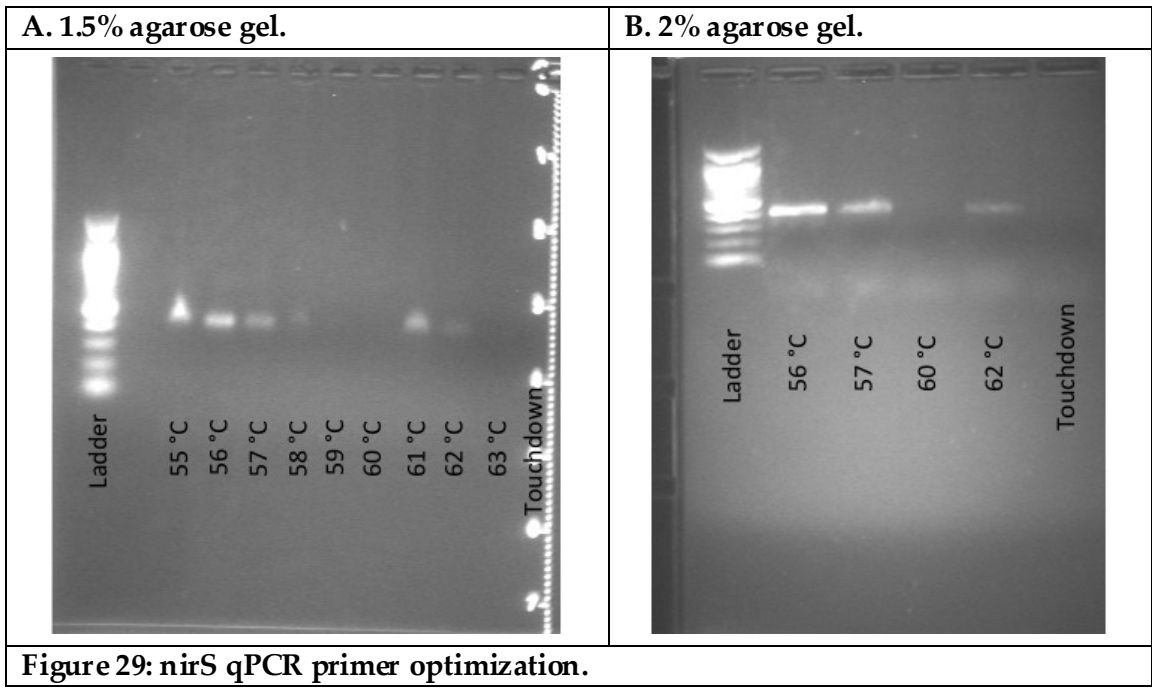
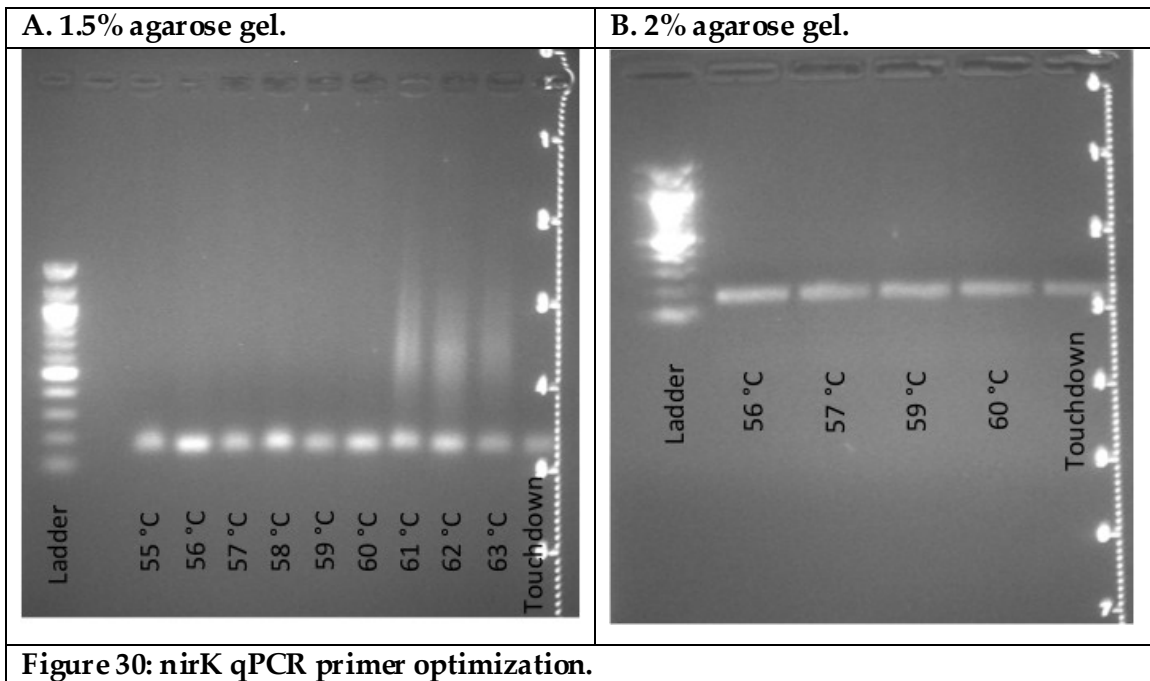


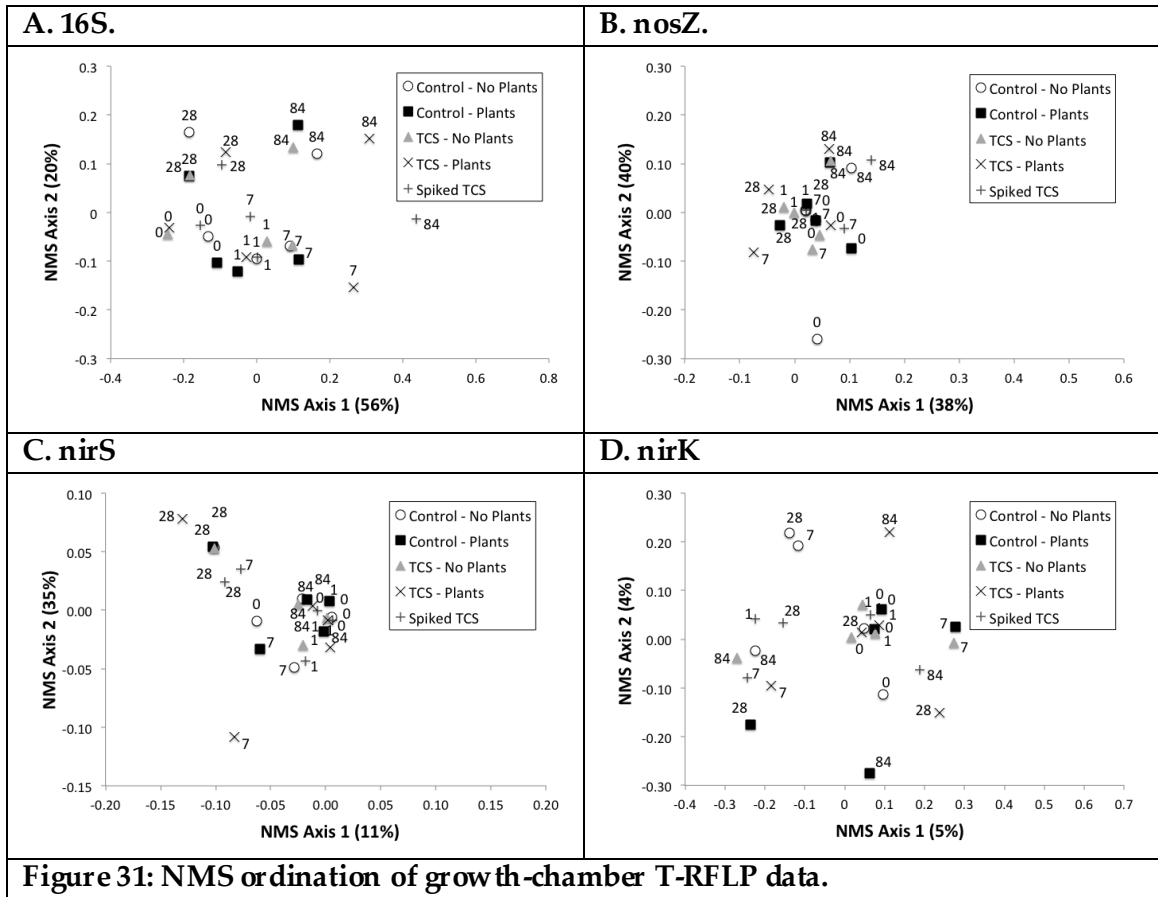
Figure 28: *nosZ* qPCR primer optimization.





B.2 NMS Ordination Plots

The NMS ordination plots completed in Chapter 5 are presented in Figure 31.



References

1. USEPA, *Biosolids generation, use and disposal in the United States*. 1999 (EPA530-R-99-009): p. 74.
2. USEPA, *Process Design Manual: Land Application of Sewage Sludge and Domestic Septage*. Vol. EPA/625/R-95/001. 1995, Cincinnati: USEPA.
3. Bulluck Iii, L.R., et al., *Organic and synthetic fertility amendments influence soil microbial, physical and chemical properties on organic and conventional farms*. *Applied Soil Ecology*, 2002. 19(2): p. 147-160.
4. USEPA, *Land Application of Municipal Sludge*, in *Process Design Manual* 1983: Cincinnati, OH.
5. Wu, C.X., et al., *Uptake of Pharmaceutical and Personal Care Products by Soybean Plants from Soils Applied with Biosolids and Irrigated with Contaminated Water*. *Environmental Science & Technology*, 2010. 44(16): p. 6157-6161.
6. Harrison, E.Z., et al., *Organic chemicals in sewage sludges*. *Science of the Total Environment*, 2006. 367(2-3): p. 481-497.
7. Tiedje, J., *Ecology of denitrification and dissimilatory nitrate reduction to ammonia*, in *Biology of Anaerobic Organisms*. 1988.
8. Payne, W.J., *Denitrification*. 1981: John Wiley & Sons, Inc.
9. Firestone, M.K., *Biological Denitrification*. *Agronomy Monograph*, 1982. F.J. Stevenson Edition (No. 22): p. 289-326.
10. Philippot, L., S. Hallin, and M. Schloter, *Ecology of denitrifying prokaryotes in agricultural soil*. *Advances in Agronomy*, Vol 96, 2007. 96: p. 249-305.
11. Hofstra, N. and A. Bouwman, *Denitrification in Agricultural Soils: Summarizing Published Data and Estimating Global Annual Rates*. *Nutrient Cycling in Agroecosystems*, 2005. 72(3): p. 267-278.
12. Cicerone, R.J., *Analysis of Sources and Sinks of Atmospheric Nitrous-Oxide (N₂O)*. *Journal of Geophysical Research-Atmospheres*, 1989. 94(D15): p. 18265-18271.
13. Mosier, A.R., et al., *Assessing and Mitigating N₂O Emissions from Agricultural Soils*. *Climatic Change*, 1998. 40(1): p. 7-38.

14. Zumft, W.G., *Cell biology and molecular basis of denitrification*. Microbiology and Molecular Biology Reviews, 1997. 61(4): p. 533.
15. Bright, D.A. and N. Healey, *Contaminant risks from biosolids land application: Contemporary organic contaminant levels in digested sewage sludge from five treatment plants in Greater Vancouver, British Columbia*. Environmental Pollution, 2003. 126(1): p. 39-49.
16. Rosenberg S., *Consumer and market use of antibacterials at home*. The Pediatric Infectious Disease Journal, 2000. 19(10): p. S114-S116.
17. Clarke, B.O. and S.R. Smith, *Review of 'emerging' organic contaminants in biosolids and an assessment of international research priorities for the agricultural use of biosolids*. Environment International, 2011. 37(1): p. 226-247.
18. McClellan, K. and R.U. Halden, *Pharmaceuticals and personal care products in archived U.S. biosolids from the 2001 EPA national sewage sludge survey*. Water Research, 2010. 44(2): p. 658-668.
19. Hill, E.M., et al., *Profiles and Some Initial Identifications of (Anti)Androgenic Compounds in Fish Exposed to Wastewater Treatment Works Effluents*. Environmental Science & Technology, 2010. 44(3): p. 1137-1143.
20. Heidler, J.a.R.U.H., *Fate of organohalogenes in US wastewater treatment plants and estimated chemical releases to soils nationwide from biosolids recycling*. Journal of Environmental Monitoring, 2009(11): p. 2207-2215.
21. Chu, S.G. and C.D. Metcalfe, *Simultaneous determination of triclocarban and triclosan in municipal biosolids by liquid chromatography tandem mass spectrometry*. Journal of Chromatography A, 2007. 1164(1-2): p. 212-218.
22. Lindstrom, A., et al., *Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater*. Environmental Science & Technology, 2002. 36(11): p. 2322-2329.
23. McAvoy, D.C., et al., *Measurement of triclosan in wastewater treatment systems*. Environmental Toxicology and Chemistry, 2002. 21(7): p. 1323-1329.
24. Yu, J.T., E.J. Bouwer, and M. Coelhan, *Occurrence and biodegradability studies of selected pharmaceuticals and personal care products in sewage effluent*. Agricultural Water Management, 2006. 86(1-2): p. 72-80.

25. Kolpin, D.W., et al., *Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance*. Environmental Science & Technology, 2002. 36(6): p. 1202-1211.
26. Parr, J.F., and Sharon B. Hornick, *Utilization of Municipal Wastes*. Soil Microbiology Ecology, ed. J. F. Blaine Metting 1992, Richland, Washington: Marcel Dekker, Inc.
27. CEC, *Council Directive of 21 May 1991 Concerning Urban Waste Water Treatment (91/271/EEC)*. , in No. L 135/40-52, O.J.o.t.E. Communities, Editor, 1991: Brussels.
28. CEC, *Council Directive of 12 June 1986 on the Protection of the Environment, and in Particular of the Soil, when Sewage Sludge is Used in Agriculture (86/278/EEC)*. Official Journal of the European Communities, 1986. No. L 181/6-12 (Brussels).
29. Rooklidge, S.J., *Environmental antimicrobial contamination from terraccumulation and diffuse pollution pathways*. Science of the Total Environment, 2004. 325(1-3): p. 1-13.
30. Pedersen, J.A., M.A. Yeager, and I.H. Suffet, *Xenobiotic Organic Compounds in Runoff from Fields Irrigated with Treated Wastewater*. Journal of Agricultural and Food Chemistry, 2003. 51(5): p. 1360-1372.
31. Sullivan, C., C. L. Mitchelmore, R. Hale, and P. Van Veld, *Induction of CYP1A and DNA Damage in the Fathead Minnow (Pimephaes promelas) Following Exposure to Biosolids*. Science of the Total Environment 2007. Submitted.
32. Kinney, C.A., et al., *Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended With Biosolid or Swine Manure*. Environmental Science & Technology, 2008. 42(6): p. 1863-1870.
33. Clarke, B., et al., *Polybrominated diphenyl ethers and polybrominated biphenyls in Australian sewage sludge*. Chemosphere, 2008. 73(6): p. 980-989.
34. Wild, S.R., et al., *Organic contaminants in an agricultural soil with a known history of sewage sludge amendments: polynuclear aromatic hydrocarbons*. Environmental Science & Technology, 1990. 24(11): p. 1706-1711.
35. Liu, F., et al., *Terrestrial ecotoxicological effects of the antimicrobial agent triclosan*. Ecotoxicology and Environmental Safety, 2009. 72(1): p. 86-92.

36. Ki Chang Ahn, B.Z., Jiangang Chen, Gennady Cherednichenko, Enio Sanmarti, Michael S. Denison, Bill Lasley, Isaac N. Pessah, Dietmar Kültz, Daniel P.Y. Chang, Shirley J. Gee, Bruce D. Hammock, *In Vitro Biologic Activities of the Antimicrobials Triclocarban, Its Analogs, and Triclosan in Bioassay Screens: Receptor-Based Bioassay Screens*. *Environ Health Perspect.*, 2008. 116(9): p. 1203-1210.
37. Chen, J., et al., *Triclocarban Enhances Testosterone Action: A New Type of Endocrine Disruptor?* *Endocrinology*, 2008. 149(3): p. 1173-1179.
38. Aryal, N. and D.M. Reinhold, *Phytoaccumulation of antimicrobials from biosolids: Impacts on environmental fate and relevance to human exposure*. *Water Research*, 2011. 45(17): p. 5545-5552.
39. Bhargava, H.N. and P.A. Leonard, *Triclosan: Applications and safety*. *American Journal of Infection Control*, 1996. 24(3): p. 209-218.
40. Sabaliunas, D., et al., *Environmental fate of Triclosan in the River Aire Basin, UK*. *Water Research*, 2003. 37(13): p. 3145-3154.
41. Singer, H., Stephan Muller, Celine Tixier, and Laurent Pillonel, *Triclosan: Occurrence and Fate of a Widely Used Biocide in the Aquatic Environment: Field Measurements in Wastewater Treatment Plants, Surface Waters, and Lake Sediments*. *Environmental Science & Technology*, 2002. 36: p. 4998-5004.
42. Bester, K., *Triclosan in a sewage treatment process—balances and monitoring data*. *Water Research*, 2003. 37(16): p. 3891-3896.
43. Reiss, R., et al., *An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States*. *Environmental Toxicology and Chemistry*, 2002. 21(11): p. 2483-2492.
44. Ying, G.-G. and R.S. Kookana, *Triclosan in wastewaters and biosolids from Australian wastewater treatment plants*. *Environment International*, 2007. 33(2): p. 199-205.
45. Topp, E., et al., *Runoff of pharmaceuticals and personal care products following application of biosolids to an agricultural field*. *Science of the Total Environment*, 2008. 396(1): p. 52-59.
46. Grove, C., et al., *Improving the aqueous solubility of triclosan by solubilization, complexation, and in situ salt formation*. *Journal of Cosmetic Science*, 2003. 54(6): p. 537-550.

47. Hua, W.Y., E.R. Bennett, and R.J. Letcher, *Triclosan in waste and surface waters from the upper Detroit River by liquid chromatography-electrospray-tandem quadrupole mass spectrometry*. *Environment International*, 2005. 31(5): p. 621-630.
48. Coogan, M.A., et al., *Algal bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas wastewater treatment plant receiving stream*. *Chemosphere*, 2007. 67(10): p. 1911-1918.
49. Coogan, M.a.T.L.P., *Snail bioaccumulation of triclocarban, triclosan, and methyltriclosan in a North Texas, USA, stream affected by wastewater treatment plant runoff*. *Environmental Toxicology and Chemistry*, 2008. 27: p. 1788-1793.
50. Ying, G.G. and R.S. Kookana, *Triclosan in wastewaters and biosolids from Australian wastewater treatment plants*. *Environment International*, 2007. 33(2): p. 199-205.
51. Heidler, J., A. Sapkota, and R.U. Halden, *Partitioning, persistence, and accumulation in digested sludge of the topical antiseptic triclocarban during wastewater treatment*. *Environmental Science & Technology*, 2006. 40(11): p. 3634-3639.
52. Sapkota, A., J. Heldler, and R.U. Halden, *Detection of triclocarban and two co-contaminating chlorocarbanilides in US aquatic environments using isotope dilution liquid chromatography tandem mass spectrometry*. *Environmental Research*, 2007. 103(1): p. 21-29.
53. USEPA, *Targeted national sewage sludge survey sampling and analysis technical report*, U.E.P.A.O.o. Water, Editor 2009: Washington DC.
54. Cha, J. and A.M. Cupples, *Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids*. *Water Research*, 2009. 43(9): p. 2522-2530.
55. Lozano, N., et al., *Fate of triclosan in agricultural soils after biosolid applications*. *Chemosphere*, 2010. 78(6): p. 760-766.
56. Xia, K., et al., *Triclocarban, triclosan, polybrominated diphenyl ethers, and 4-nonylphenol in biosolids and in soil receiving 33-year biosolids application*. *Environmental Toxicology and Chemistry*, 2010. 29(3): p. 597-605.
57. Halden, R.U. and D.H. Paull, *Co-Occurrence of Triclocarban and Triclosan in U.S. Water Resources*. *Environmental Science & Technology*, 2005. 39(6): p. 1420-1426.

58. Chalew TE, H.R., *Environmental Exposure of Aquatic and Terrestrial Biota to Triclosan and Triclocarban*. J Am Water Works Assoc, 2009. 45(1): p. 4-13.
59. Chu, S. and C.D. Metcalfe, *Simultaneous determination of triclocarban and triclosan in municipal biosolids by liquid chromatography tandem mass spectrometry*. Journal of Chromatography A, 2007. 1164(1-2): p. 212-218.
60. Agency, U.S.E.P. *Technology Transfer Network Air Toxics Web Site. 2,4,5-Trichlorophenol 2012*; Available from: <http://www.epa.gov/ttnatw01/hlthef/trich1-p.html>.
61. Wei, Y., et al., *Minimization of excess sludge production for biological wastewater treatment*. Water Research, 2003. 37(18): p. 4453-4467.
62. Yamarik, T., *Safety Assessment of Dichlorophene and Chlorophene*. International Journal of Toxicology, 2004. 23(1): p. 1-27.
63. Ellis, J.B., *Pharmaceutical and personal care products (PPCPs) in urban receiving waters*. Environmental Pollution, 2006. 144(1): p. 184-189.
64. Services, N.L.o.M.S.I. *TOXNET*. 2004; Available from: <http://toxnet.nlm.nih.gov/>.
65. Xia, K., et al., *Occurrence and Fate of Pharmaceuticals and Personal Care Products (PPCPs) in Biosolids*. Journal of Environmental Quality, 2005. 34(1): p. 91-104.
66. Kasprzyk-Hordern, B., R.M. Dinsdale, and A.J. Guwy, *The removal of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs during wastewater treatment and its impact on the quality of receiving waters*. Water Research, 2009. 43(2): p. 363-380.
67. Kegley, S.E., Hill, B.R., Orme S., Choi A.H. *PAN Pesticide Database*. 2011; Version 10; [Available from: <http://www.pesticideinfo.org>].
68. McMurry, L.M., M. Oethinger, and S.B. Levy, *Triclosan targets lipid synthesis*. Nature, 1998. 394(6693): p. 531-532.
69. Levy, C.W., et al., *Molecular basis of triclosan activity*. Nature, 1999. 398(6726): p. 383-384.
70. Escalada, M.G., et al., *Triclosan inhibition of fatty acid synthesis and its effect on growth of Escherichia coli and Pseudomonas aeruginosa*. Journal of Antimicrobial Chemotherapy, 2005. 55(6): p. 879-882.

71. Bailey, A.M., et al., *Exposure of Escherichia coli and Salmonella enterica serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification*. Journal of Antimicrobial Chemotherapy, 2009. 64(5): p. 973-985.
72. McDonnell, G. and A.D. Russell, *Antiseptics and Disinfectants: Activity, Action, and Resistance*. Clinical Microbiology Reviews, 1999. 12(1): p. 147-179.
73. Agency, U.S.E.P., *EPI Suite v4.10 Structuring Drawing Program*, 2011.
74. Snyder, E.H., G.A. O'Connor, and D.C. McAvoy, *Toxicity and bioaccumulation of biosolids-borne triclocarban (TCC) in terrestrial organisms*. Chemosphere, 2011. 82(3): p. 460-467.
75. Halden, R.U.a.P., D. H., *Co-occurrence of triclocarban and triclosan in US water resources*. Environmental Science & Technology, 2005. 39(6): p. 1420-1426.
76. Consortium, T., *High Production Volume (HPV) Chemical Challenge Program Data Availability and Screening Level Assessment for Triclocarban: CAS #: 101-20-2, U.E.P. Agency, Editor 2002*.
77. Loftsson, T., et al., *Cyclodextrin Solubilization of the Antibacterial Agents Triclosan and Triclocarban: Effect of Ionization and Polymers*. Journal of Inclusion Phenomena and Macrocyclic Chemistry, 2005. 52(1): p. 109-117.
78. Blackman et al., *Archives of Biochemistry*, 1955. 54: p. 55-61.
79. Xie, Y.J.L., H.; Liu, H. X.; Zhai, Z. C.; Wang, Z. Y., *Determination of Solubilities and n-Octanol/Water Partition Coefficients and QSPR Study for Substituted Phenols* Bulletin of Environmental Contamination and Toxicology, 2008. 80(4): p. 319-323.
80. Simon, A.B., Csaba; Lente, Gabor; Fabian, Istvan *Structure-reactivity relationships and substituent effect additivity in the aqueous oxidation of chlorophenols by cerium(IV)* New Journal of Chemistry, 2011. 35(1): p. 235-241.
81. Rived, F., M. Rosés, and E. Bosch, *Dissociation constants of neutral and charged acids in methyl alcohol. The acid strength resolution*. Analytica Chimica Acta, 1998. 374(2-3): p. 309-324.
82. Broderius, S.J., M.D. Kahl, and M.D. Hoglund, *Use of joint toxic response to define the primary mode of toxic action for diverse industrial organic chemicals*. Environmental Toxicology and Chemistry, 1995. 14(9): p. 1591-1605.

83. Lawrence, E. *Henderson's Dictionary of Biological Terms*. 2010; 10th:[Available from: [http://www.starsandseas.com/SAS Ecology/SAS chemcycles/cycle nitrogen.htm](http://www.starsandseas.com/SAS_Ecology/SAS_chemcycles/cycle_nitrogen.htm)].
84. Tiedje, J.M., *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*. Soil Science Society of America Book Series, no. 5, ed. S.A. R. W. Weaver, Peter Bottomley, David Bezdicek, Scott Smith, Alit Tabatabai, Art Wollum 1994, Madison, WI.
85. Barton, L., et al., *Annual denitrification rates in agricultural and forest soils: a review*. Australian Journal of Soil Research, 1999. 37(6): p. 1073-1093.
86. Henry, S., et al., *Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR*. Journal of Microbiological Methods, 2004. 59(3): p. 327-335.
87. Henry, S., et al., *Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR (vol 59, pg 327, 2004)*. Journal of Microbiological Methods, 2005. 61(2): p. 289-290.
88. Madigan, M.T., John M. Martinko, Paul V. Dunlap, and David P. Clark, *Brock Biology of Microorganisms*. 12th ed 2009: Pearson Education Inc.
89. Wallenstein, M.D., David D. Myrold, Mary Firestone, and Mary Voytek, *Environmental Controls on Denitrifying Communities and Denitrification Rates: Insights from Molecular Methods*. Ecological Applications, 2006. 16(6): p. 2143-2152.
90. Laughlin, R.J. and R.J. Stevens, *Evidence for Fungal Dominance of Denitrification and Codenitrification in a Grassland Soil*. Soil Sci. Soc. Am. J., 2002. 66(5): p. 1540-1548.
91. Zumft, W., *The denitrifying prokaryotes*, in *The prokaryotes: an evolving electronic resource for the microbiological community*, S.f. M. Dworkin, E. Rosenberg, K.-H. Schelider, and E. Stackebrandt, Editor 1999.
92. Tiedje, J.M., Stephen Simkins, and Peter M. Groffman, *Perspectives on measurement of denitrification in the field including recommended protocols for a acetylene based methods*. Plant and Soil, 1989. 115: p. 261-284.
93. Delwiche, C.C. and B.A. Bryan, *Denitrification*. Annual Review of Microbiology, 1976. 30: p. 241-262.

94. Ye, R.W., Bruce A. Averill, and James M. Tiedje, *Denitrification: Production and Consumption of Nitric Oxide*. Applied and Environmental Microbiology, 1994. 60(4): p. 1053-1058.
95. Yoshinari, T., *N₂O reduction by Vibrio succinogenes*. Applied and Environmental Microbiology, 1980. 39(1): p. 81-84.
96. Myrold, D.D. and J.M. Tiedje, *Establishment of denitrification capacity in soil: Effects of carbon, nitrate and moisture*. Soil Biology and Biochemistry, 1985. 17(6): p. 819-822.
97. Vinther, F., *Total denitrification and the ratio between N₂O and N₂ during the growth of spring barley*. Plant and Soil, 1984. 76(1): p. 227-232.
98. Kennedy, M.J. and J.G. Lawless, *Role of Chemotaxis in the Ecology of Denitrifiers*. Applied and Environmental Microbiology, 1985. 49(1): p. 109-114.
99. Jacobson, S.N. and M. Alexander, *Nitrate loss from soil in relation to temperature, carbon source and denitrifier populations*. Soil Biology and Biochemistry, 1980. 12(5): p. 501-505.
100. Gamble, T.N., M.R. Betlach, and J.M. Tiedje, *Numerically Dominant Denitrifying Bacteria from World Soils*. Applied and Environmental Microbiology, 1977. 33(4): p. 926-939.
101. Sugahara, I., K. Hayashi, and T. Kimura, *Studies on Microbial Population in Coastal Waters - 2. Distribution and Generic Composition of Denitrifying Bacteria in Coastal and Oceanic Waters*. Bulletin of the Japanese Society of Scientific Fisheries, 1986. 52(3): p. 497-503.
102. Rich, J.J. and D.D. Myrold, *Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA*. Soil Biology & Biochemistry, 2004. 36(9): p. 1431-1441.
103. Boyle, S.A., et al., *Reciprocal transfer effects on denitrifying community composition and activity at forest and meadow sites in the Cascade Mountains of Oregon*. Soil Biology & Biochemistry, 2006. 38(5): p. 870-878.
104. Eichner, M.J., *Nitrous-Oxide Emissions from Fertilized Soils - Summary of Available Data*. Journal of Environmental Quality, 1990. 19(2): p. 272-280.

105. Firestone, M.K., R.B. Firestone, and J.M. Tiedje, *Nitrous-Oxide from Soil Denitrification - Factors Controlling its Biological Production*. Science, 1980. 208(4445): p. 749-751.
106. Weier, K.L., et al., *Denitrification and the Dinitrogen Nitrous-Oxide Ratio as Affected by Soil Water, Available Carbon, and Nitrate*. Soil Science Society of America Journal, 1993. 57(1): p. 66-72.
107. Smith, M.S. and L.L. Parsons, *Persistence of Denitrifying Enzyme Activity in Dried Soils*. Applied and Environmental Microbiology, 1985. 49(2): p. 316-320.
108. Bergsma, T.T., G.P. Robertson, and N.E. Ostrom, *Influence of soil moisture and land use history on denitrification end-products*. Journal of Environmental Quality, 2002. 31(3): p. 711-717.
109. Philippot, L., et al., *Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils*. Applied and Environmental Microbiology, 2002. 68(12): p. 6121-6128.
110. Cheneby, D., et al., *Genetic characterization of the nitrate reducing community based on narG nucleotide sequence analysis*. Microbial Ecology, 2003. 46(1): p. 113-121.
111. Vonberg, K.H.L. and H. Bothe, *The Distribution of Denitrifying Bacteria in Soils Monitored by DNA-Probing*. Fems Microbiology Ecology, 1992. 86(4): p. 331-340.
112. Claysjosseland, A., et al., *Influence of 2 Plant-Species (Flax and Tomato) on the Distribution of Nitrogen Dissimilative Abilities within Fluorescent Pseudomonas SPP*. Applied and Environmental Microbiology, 1995. 61(5): p. 1745-1749.
113. Ghiglione, J.F., et al., *Role of respiratory nitrate reductase in ability of Pseudomonas fluorescens YT101 to colonize the rhizosphere of maize*. Applied and Environmental Microbiology, 2000. 66(9): p. 4012-4016.
114. Ellis, S., et al., *Denitrification and N₂O emissions from a UK pasture soil following the early spring application of cattle slurry and mineral fertiliser*. Plant and Soil, 1998. 202(1): p. 15-25.
115. Skiba, U. and K.A. Smith, *The control of nitrous oxide emissions from agricultural and natural soils*. Chemosphere - Global Change Science, 2000. 2(3-4): p. 379-386.

116. Enwall, K., L. Philippot, and S. Hallin, *Activity and composition of the denitrifying bacterial community respond differently to long-term fertilization*. *Applied and Environmental Microbiology*, 2005. 71(12): p. 8335-8343.
117. Avrahami, S., R. Conrad, and G. Braker, *Effect of soil ammonium concentration on N₂O release and on the community structure of ammonia oxidizers and denitrifiers*. *Applied and Environmental Microbiology*, 2002. 68(11): p. 5685-5692.
118. Deiglmayr, K., L. Philippot, and E. Kandeler, *Functional stability of the nitrate-reducing community in grassland soils towards high nitrate supply*. *Soil Biology & Biochemistry*, 2006. 38(9): p. 2980-2984.
119. Bonin, P., M. Gilewicz, and J.C. Bertrand, *Effects of Oxygen on Each Step of Denitrification on Pseudomonas-Nautica*. *Canadian Journal of Microbiology*, 1989. 35(11): p. 1061-1064.
120. Holtan-Hartwig, L., et al., *Heavy metals tolerance of soil denitrifying communities: N₂O dynamics*. *Soil Biology & Biochemistry*, 2002. 34(8): p. 1181-1190.
121. Siciliano, S.D., R. Roy, and C.W. Greer, *Reduction in denitrification activity in field soils exposed to long term contamination by 2,4,6-trinitrotoluene (TNT)*. *Fems Microbiology Ecology*, 2000. 32(1): p. 61-68.
122. Cavigelli, M.A. and G.P. Robertson, *Role of denitrifier diversity in rates of nitrous oxide consumption in a terrestrial ecosystem*. *Soil Biology & Biochemistry*, 2001. 33(3): p. 297-310.
123. Pell, M., B. Stenberg, and L. Torstensson, *Potential Denitrification and Nitrification Tests for Evaluation of Pesticide Effects in Soil*. *Ambio*, 1998. 27(1): p. 24-28.
124. Chalew, T.E.A. and R.U. Halden, *Environmental Exposure of Aquatic and Terrestrial Biota to Triclosan and Triclocarban*. *JAWRA Journal of the American Water Resources Association*, 2009. 45(1): p. 4-13.
125. Schweizer, H.P., *Triclosan: a widely used biocide and its link to antibiotics*. *Fems Microbiology Letters*, 2001. 202(1): p. 1-7.
126. Gunsch, C.K., et al., *Identification of a homogentisate-1,2-dioxygenase gene in the fungus *Exophiala lecanii-corni*: a analysis and implications*. *Applied Microbiology and Biotechnology*, 2005. 68(3): p. 405-411.

127. Groffman, P., E. Holland, D. Myrold, G. Robertson, and X. Zou, *Standard soil methods for long-term ecological research*, ed. D.C.C. G. P. Robertson, C. S. Bledsoe, and P. Sollins 1999, Oxford: Oxford University Press.
128. Jensen, M.M., B. Thamdrup, and T. Dalsgaard, *Effects of Specific Inhibitors on Anammox and Denitrification in Marine Sediments*. *Applied and Environmental Microbiology*, 2007. 73(10): p. 3151-3158.
129. Walter, H.M., D.R. Keeney, and I.R. Fillery, *Inhibition of Nitrification by Acetylene*. *Soil Sci. Soc. Am. J.*, 1979. 43(1): p. 195-196.
130. Wilderer, P.A., W.L. Jones, and U. Dau, *Competition in denitrification systems affecting reduction rate and accumulation of nitrite*. *Water Research*, 1987. 21(2): p. 239-245.
131. Betlach, M.R. and J.M. Tiedje, *Kinetic Explanation for Accumulation of Nitrite, Nitric Oxide, and Nitrous Oxide During Bacterial Denitrification*. *Applied and Environmental Microbiology*, 1981. 42(6): p. 1074-1084.
132. Almeida, J.S., M.A.M. Reis, and M.J.T. Carrondo, *Competition between nitrate and nitrite reduction in denitrification by Pseudomonas fluorescens*. *Biotechnology and Bioengineering*, 1995. 46(5): p. 476-484.
133. Hernández-López, J. and F. Vargas-Albores, *A microplate technique to quantify nutrients (NO_2^- , NO_3^- , NH_4^+ and PO_4^{3-}) in seawater*. *Aquaculture Research*, 2003. 34(13): p. 1201-1204.
134. Rozen, S., and H. Skaletsky (ed.). *Primer3 on the WWW for general users and for biologist programmers*. . 2000.
135. Stults, J.R., et al., *Application of the 5' Fluorogenic Exonuclease Assay (TaqMan) for Quantitative Ribosomal DNA and rRNA Analysis in Sediments*. *Applied and Environmental Microbiology*, 2001. 67(6): p. 2781-2789.
136. Baker, G.C., J.J. Smith, and D.A. Cowan, *Review and re-analysis of domain-specific 16S primers*. *Journal of Microbiological Methods*, 2003. 55(3): p. 541-555.
137. Bustin, S.A., et al., *The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments*. *Clinical Chemistry*, 2009. 55(4): p. 611-622.

138. Arnaout, C.L. and C.K. Gunsch, *Impacts of Silver Nanoparticle Coating on the Nitrification Potential of Nitrosomonas europaea*. Environmental Science & Technology, 2012. 46(10): p. 5387-5395.
139. Davis, E.F., S.L. Klosterhaus, and H.M. Stapleton, *Measurement of flame retardants and triclosan in municipal sewage sludge and biosolids*. Environment International, 2012. 40: p. 1-7.
140. Team, R.D.C., *R: A language and environment for statistical computing.*, in *R Foundation for Statistical Computing* 2008: Vienna, Austria.
141. Thompson, A., et al., *The Fate and Removal of Triclosan during Wastewater Treatment*. Water Environment Research, 2005. 77(1): p. 63-67.
142. Eddy, M., *Wastewater Engineering: Treatment and Reuse*. 4th ed. ed, ed. G. Tchobanoglous, Burton, F.L., and Stensel, H.D. 2003, Boston: McGraw-Hill.
143. Hill, G.T., et al., *Methods for assessing the composition and diversity of soil microbial communities*. Applied Soil Ecology, 2000. 15(1): p. 25-36.
144. Saleh-Lakha, S., et al., *Microbial gene expression in soil: methods, applications and challenges*. Journal of Microbiological Methods, 2005. 63(1): p. 1-19.
145. VerBerkmoes, N.C., et al., *Systems Biology: Functional analysis of natural microbial consortia using community proteomics*. Nat Rev Micro, 2009. 7(3): p. 196-205.
146. Chuanchuen, R., et al., *Cross-Resistance between Triclosan and Antibiotics in Pseudomonas aeruginosa Is Mediated by Multidrug Efflux Pumps: Exposure of a Susceptible Mutant Strain to Triclosan Selects nfxB Mutants Overexpressing MexCD-OprJ*. Antimicrobial Agents and Chemotherapy, 2001. 45(2): p. 428-432.
147. Chuanchuen, R., R.R. Karkhoff-Schweizer, and H.P. Schweizer, *High-level triclosan resistance in Pseudomonas aeruginosa is solely a result of efflux*. American Journal of Infection Control, 2003. 31(2): p. 124-127.
148. Sekiya, H., et al., *Functional Cloning and Characterization of a Multidrug Efflux Pump, MexHI-OpmD, from a Pseudomonas aeruginosa Mutant*. Antimicrobial Agents and Chemotherapy, 2003. 47(9): p. 2990-2992.
149. Champlin, F.R., et al., *Effect of outer membrane permeabilisation on intrinsic resistance to low triclosan levels in Pseudomonas aeruginosa*. International Journal of Antimicrobial Agents, 2005. 26(2): p. 159-164.

150. Russell, A.D., *Bacterial resistance to disinfectants: present knowledge and future problems*. Journal of Hospital Infection, 1999. 43, Supplement 1(0): p. S57-S68.
151. Groffman, P.M., et al., *Methods for Measuring Denitrification: Diverse Approaches to a Difficult Problem*. . Ecological Applications, 2006. 16(6): p. 2091-2122.
152. Pannu, M.W., G.A. O'Connor, and G.S. Toor, *Toxicity and bioaccumulation of biosolids-borne triclosan in terrestrial organisms*. Environmental Toxicology and Chemistry, 2012. 31(3): p. 646-653.
153. Alexander, M., *Aging, Bioavailability, and Overestimation of Risk from Environmental Pollutants*. Environmental Science & Technology, 2000. 34(20): p. 4259-4265.
154. Holzem, R.M., H.M. Stapleton, and C.K. Gunsch, *Determining the Ecological Impacts of Organic Contaminants in Biosolids Using a High-Throughput Colorimetric Denitrification Assay: A Case Study with Antimicrobial Agents*. Environmental Science & Technology, 2014.
155. Neumegen, R.A., A.R. Fernandez-Alba, and Y. Chisti, *Toxicities of triclosan, phenol, and copper sulfate in activated sludge*. Environmental Toxicology, 2005. 20(2): p. 160-164.
156. Stasinakis, A.S., et al., *Investigation of triclosan fate and toxicity in continuous-flow activated sludge systems*. Chemosphere, 2007. 68(2): p. 375-381.
157. Stasinakis, A.S., et al., *Inhibitory effect of triclosan and nonylphenol on respiration rates and ammonia removal in activated sludge systems*. Ecotoxicology and Environmental Safety, 2008. 70(2): p. 199-206.
158. Federle, T.W., S.K. Kaiser, and B.A. Nuck, *Fate and effects of triclosan in activated sludge*. Environmental Toxicology and Chemistry, 2002. 21(7): p. 1330-1337.
159. *Wastewater Technology Fact Sheet: Sequencing Batch Reactors.*, in Office of Water, E.P. Agency, Editor 1999, EPA 832-F-99-073: Washington, D.C. .
160. Kos, P., *Short SRT (solids retention time) nitrification process/flowsheet*. Water Science and Technology, 1998. 38(1): p. 23-29.
161. Zeng, R.J., et al., *Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor*. Biotechnology and Bioengineering, 2003. 84(2): p. 170-178.

162. Wu, C., A.L. Spongberg, and J.D. Witter, *Adsorption and Degradation of Triclosan and Triclocarban in Soils and Biosolids-Amended Soils*. Journal of Agricultural and Food Chemistry, 2009. 57(11): p. 4900-4905.
163. *Standard Methods for the Examination of Water and Wastewater*. 21st Edition ed2005: Meircan Public Health Association, American Water Works Association, and Water Environment Federation.
164. Kucharzyk, K.H., Hsu-Kim, H., Deshusses, M.A., *Modifications of DNA extraction methods for sediments of various origins*. 2014. In progress.
165. Lukow, T., P.F. Dunfield, and W. Liesack, *Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants*. Fems Microbiology Ecology, 2000. 32(3): p. 241-247.
166. Alito, C.L. and C.K. Gunsch, *Assessing the Effects of Silver Nanoparticles on Biological Nutrient Removal in Bench-Scale Activated Sludge Sequencing Batch Reactors*. Environmental Science & Technology, 2013. 48(2): p. 970-976.
167. Culman, S.B., R.; Gauch, H.; Cadillo-Quiroz, H.; Buckley, D., *T-REX: software for the processing and analysis of T-RFLP data.*, 2009: BMC Informatics 10, (1), 171.
168. Hammer, O., Harper, D.A.T., and Ryan, P.D., *PAST: Paleontological Statistics software package for education and data analysis*, 2001: Paleontol Electronica 4: 9.
169. Morales, S., et al., *Microwave assisted extraction followed by gas chromatography with tandem mass spectrometry for the determination of triclosan and two related chlorophenols in sludge and sediments*. Journal of Chromatography A, 2005. 1082(2): p. 128-135.
170. Gatidou, G., et al., *Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry*. Journal of Chromatography A, 2007. 1138(1–2): p. 32-41.
171. Pothitou, P. and D. Voutsas, *Endocrine disrupting compounds in municipal and industrial wastewater treatment plants in Northern Greece*. Chemosphere, 2008. 73(11): p. 1716-1723.

172. Davis, E.F., S.L. Klosterhaus, and H.M. Stapleton, *Measurement of flame retardants and triclosan in municipal sewage sludge and biosolids*. *Environment International*, 2012. 40(0): p. 1-7.
173. E., D.J.W.B.a.R., *The Toxicity of Organic Chemicals to Treatment Processes*. *Water Science and Technology*, 1992. 25(3): p. 23-31.
174. Lu, Q., Z.L. He, and P.J. Stoffella, *Land Application of Biosolids in the USA: A Review*. *Applied and Environmental Soil Science*, 2012. 2012: p. 11.
175. Wu, C., et al., *Uptake of Pharmaceutical and Personal Care Products by Soybean Plants from Soils Applied with Biosolids and Irrigated with Contaminated Water*. *Environmental Science & Technology*, 2010. 44(16): p. 6157-6161.
176. Prosser, R.S., et al., *Bioaccumulation of triclosan and triclocarban in plants grown in soils amended with municipal dewatered biosolids*. *Environmental Toxicology and Chemistry*, 2013.
177. Waller, N.J. and R.S. Kookana, *Effect of Triclosan on microbial activity in Australian soils*. *Environmental Toxicology and Chemistry*, 2009. 28(1): p. 65-70.
178. Butler, E., et al., *Effects of triclosan on soil microbial respiration*. *Environmental Toxicology and Chemistry*, 2011. 30(2): p. 360-366.
179. Reiss, R., G. Lewis, and J. Griffin, *An Ecological Risk Assessment for Triclosan in the Terrestrial Environment*. *Environmental Toxicology and Chemistry*, 2009. 28(7): p. 1546-1556.
180. Park, I., et al., *Effects of Triclosan and Biosolids on Microbial Community Composition in an Agricultural Soil*. *Water Environment Research*, 2013. 85(12): p. 2237-2242.
181. Harrow, D.I., J.M. Felker, and K.H. Baker, *Impacts of Triclosan in Greywater on Soil Microorganisms*. *Applied and Environmental Soil Science*, 2011. 2011.
182. (SERCC), T.S.R.C.C. *Relative Humidity (%) for Selected Cities in the Southeast*. 2014 [cited 2014 February 4]; Available from: <http://www.sercc.com/climateinfo/historical/avgrh.html> - NC.
183. Carolina, S.C.O.o.N. *CRONOS Database - North Durham Water Reclamation Facility (DURH)*. 2014 [cited 2014 February 4]; Available from: <http://www.nc-climate.ncsu.edu/cronos/?station=DURH&temporal=monthly>.

184. Hardy DH, T.M., Stokes CE, *Crop fertilization based on North Carolina soil tests*, A.D. Department of Agriculture and Consumer Services, Editor 2014: North Caroliner.
185. Green, T.G.J., J. Paul Mueller, and Douglas S. Chamblee, *Planting Guide for Forage Crops in NC*, North Carolina State University: Raleigh, North Carolina.
186. Drury, C.F., D.D. Myrold, E.G. Beauchamp, and W.D. Reynolds, *Denitrification Techniques for Soil*, in *Soil Sampling and Methods of Analysis*, M.R.C.a.E.G. Gregorich, Editor 2008, CRC Press: Boca Raton, FL. p. 471-493.
187. Sutton-Grier, A., M. Ho, and C. Richardson, *Organic amendments improve soil conditions and denitrification in a restored riparian wetland*. *Wetlands*, 2009. 29(1): p. 343-352.
188. Kandeler, E., et al., *Abundance of narG, nirS, nirK, and nosZ Genes of Denitrifying Bacteria during Primary Successions of a Glacier Foreland*. *Applied and Environmental Microbiology*, 2006. 72(9): p. 5957-5962.
189. Henry, S., et al., *Quantitative Detection of the nosZ Gene, Encoding Nitrous Oxide Reductase, and Comparison of the Abundances of 16S rRNA, narG, nirK, and nosZ Genes in Soils*. *Applied and Environmental Microbiology*, 2006. 72(8): p. 5181-5189.
190. Větrovský, T. and P. Baldrian, *The Variability of the 16S rRNA Gene in Bacterial Genomes and Its Consequences for Bacterial Community Analyses*. *Plos One*, 2013. 8(2): p. e57923.
191. Wolsing, M. and A. Priemé, *Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of nir gene fragments*. *Fems Microbiology Ecology*, 2004. 48(2): p. 261-271.
192. Scala, D.J. and L.J. Kerkhof, *Horizontal Heterogeneity of Denitrifying Bacterial Communities in Marine Sediments by Terminal Restriction Fragment Length Polymorphism Analysis*. *Applied and Environmental Microbiology*, 2000. 66(5): p. 1980-1986.
193. Braker, G., et al., *Community structure of denitrifiers, Bacteria, and Archaea along redox gradients in pacific northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes*. *Applied and Environmental Microbiology*, 2001. 67(4): p. 1893-1901.

194. Bremer, C., et al., *Impact of Plant Functional Group, Plant Species, and Sampling Time on the Composition of nirK-Type Denitrifier Communities in Soil*. Applied and Environmental Microbiology, 2007. 73(21): p. 6876-6884.
195. Christensen, S. and B.T. Christensen, *Organic-Matter Available for Denitrification in Different Soil Fractions - Effect of Freeze Thaw Cycles and Straw Disposal*. Journal of Soil Science, 1991. 42(4): p. 637-647.
196. Dambreville, C., et al., *Compared effects of long-term pig slurry applications and mineral fertilization on soil denitrification and its end products (N₂O, N₂)*. Biology and Fertility of Soils, 2006. 42(6): p. 490-500.
197. Brendecke, J.W., R.D. Axelson, and I.L. Pepper, *Soil microbial activity as an indicator of soil fertility: Long-term effects of municipal sewage sludge on an arid soil*. Soil Biology and Biochemistry, 1993. 25(6): p. 751-758.
198. Torslov, J., *Comparison of Bacterial Toxicity Tests Based on Growth, Dehydrogenase Activity, and Esterase Activity of Pseudomonas fluorescens*. Ecotoxicology and Environmental Safety, 1993. 25(1): p. 33-40.
199. Graham, D.W., et al., *Correlations between in situ denitrification activity and nir-gene abundances in pristine and impacted prairie streams*. Environmental Pollution, 2010. 158(10): p. 3225-3229.
200. Macherius, A., et al., *Uptake of Galaxolide, Tonalide, and Triclosan by Carrot, Barley, and Meadow Fescue Plants*. Journal of Agricultural and Food Chemistry, 2012. 60(32): p. 7785-7791.
201. Gottschall, N., et al., *Pharmaceutical and personal care products in groundwater, subsurface drainage, soil, and wheat grain, following a high single application of municipal biosolids to a field*. Chemosphere, 2012. 87(2): p. 194-203.
202. Tiedje, J.M., et al., *Methods for the Production and Use of Nitrogen-13 In Studies of Denitrification*. Soil Sci. Soc. Am. J., 1979. 43(4): p. 709-716.
203. Kookana, R.S., G.G. Ying, and N.J. Waller, *Triclosan: its occurrence, fate and effects in the Australian environment*. Water Science and Technology, 2011. 63(4): p. 598-604.
204. Hutchins, S.R., *Optimizing btex biodegradation under denitrifying conditions*. Environmental Toxicology and Chemistry, 1991. 10(11): p. 1437-1448.

205. Brynhildsen, L., et al., *Effects of Glucose Concentrations on Cadmium, Copper, Mercury, and Zinc Toxicity to a Klebsiella sp.* Applied and Environmental Microbiology, 1988. 54(7): p. 1689-1693.
206. Bollag, J.-M., C.J. Myers, and R.D. Minard, *Biological and chemical interactions of pesticides with soil organic matter.* Science of the Total Environment, 1992. 123–124(0): p. 205-217.
207. Dambreville, C., et al., *Compared effects of long-term pig slurry applications and mineral fertilization on soil denitrification and its end products (N₂O, N₂).* Biology and Fertility of Soils, 2006. 42(6): p. 490-500.
208. Johansson, M., B. Stenberg, and L. Torstensson, *Microbiological and chemical changes in two arable soils after long-term sludge amendments.* Biology and Fertility of Soils, 1999. 30(1-2): p. 160-167.
209. Pimentel, D., et al., *Environmental, energetic, and economic comparisons of organic and conventional farming systems.* Bioscience, 2005. 55(7): p. 573-582.
210. Kramer, S.B., et al., *Reduced nitrate leaching and enhanced denitrifier activity and efficiency in organically fertilized soils.* Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(12): p. 4522-4527.
211. Hole, D.G., et al., *Does organic farming benefit biodiversity?* Biological Conservation, 2005. 122(1): p. 113-130.
212. Hanselman, T.A., D.A. Graetz, and A.C. Wilkie, *Manure-Borne Estrogens as Potential Environmental Contaminants: A Review.* Environmental Science & Technology, 2003. 37(24): p. 5471-5478.
213. Bicudo, J.R. and S.M. Goyal, *Pathogens and manure management systems: A review.* Environmental Technology, 2003. 24(1): p. 115-130.
214. Sabourin, L., et al., *Runoff of pharmaceuticals and personal care products following application of dewatered municipal biosolids to an agricultural field.* Science of the Total Environment, 2009. 407(16): p. 4596-4604.
215. Topp, E. and J.C. Germon, *Acetylene Metabolism and Stimulation of Denitrification in an Agricultural Soil.* Applied and Environmental Microbiology, 1986. 52(4): p. 802-806.

216. Boyd, G.R., et al., *Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada*. *Science of the Total Environment*, 2003. 311(1-3): p. 135-149.
217. Carballa, M., et al., *Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant*. *Water Research*, 2004. 38(12): p. 2918-2926.
218. Hernando, M.D., et al., *Trace-level determination of pharmaceutical residues by LC-MS/MS in natural and treated waters. A pilot-survey study*. *Analytical and Bioanalytical Chemistry*, 2006. 385(6): p. 985-991.
219. Rice, S.L. and S. Mitra, *Microwave-assisted solvent extraction of solid matrices and subsequent detection of pharmaceuticals and personal care products (PPCPs) using gas chromatography-mass spectrometry*. *Analytica Chimica Acta*, 2007. 589(1): p. 125-132.
220. Scheytt, T.J., P. Mersmann, and T. Heberer, *Mobility of pharmaceuticals carbamazepine, diclofenac, ibuprofen, and propyphenazone in miscible-displacement experiments*. *Journal of Contaminant Hydrology*, 2006. 83(1-2): p. 53-69.
221. Weigel, S., et al., *Determination of selected pharmaceuticals and caffeine in sewage and seawater from Tromsø/Norway with emphasis on ibuprofen and its metabolites*. *Chemosphere*, 2004. 56(6): p. 583-592.

Biography

Ryan Holzem was born in Sheboygan, Wisconsin on November 10, 1983. The universities attended, degrees obtained, publications, and academic honors are listed below.

EDUCATION

Ph.D., Civil and Environmental Engineering (March 2014)

Certificate in Biomolecular and Tissue Engineering

Certificate for College Teaching

Duke University, Durham, NC

Dissertation: Elucidating the impacts of biosolids-derived emerging organic contaminants on microbial community function and structure in agricultural soils.

M.S., Civil and Environmental Engineering (July 2008)

University of Wisconsin - Madison, Madison, WI

Thesis: Turbidity as an Indicator of Unidirectional Flushing Frequency and Prioritization

B.S., Civil and Environmental Engineering, *Magna cum Laude* (December 2006)

University of Wisconsin - Platteville, Platteville, WI

PUBLICATIONS

Holzem, R. and C.K. Gunsch. "Determining the Ecological Impacts of Organic Contaminants in Biosolids Using a High-Throughput Colorimetric Denitrification Assay: A Case Study with Antimicrobial Agents. *Environmental Science and Technology*, 2014, 48(3): 1646-1655.

Ikuma, K., **R. Holzem**, and C.K. Gunsch. "The effects of organic carbon substrate availability and recipient genetic characteristics on the conjugal transfer of the TOL plasmid in soil slurry batches." *Chemosphere*, 2012, 89(2): 158-163.

S. Wang, **R. Holzem**, and C.K. Gunsch, "Inhibitory Effects of Pharmaceutically Active Compounds on Mixed Microbial Community Originating from a Municipal Wastewater Treatment Plant", *Environmental Science and Technology*, 2008, 42(4):1091-1095.

AWARDS AND NOTABLE RECOGNITIONS

Ryan was part of a team selected as a 2013 Duke Start-Up Challenge Second Round Qualifiers. Ryan received the ASM Student Travel Award in 2012. Ryan was an accepted attendee of the 2012 NC State Building Future Faculty Conference. Also in 2012, Ryan was nominated as a Predoctoral Fellow in the Center for Biomolecular and Tissue Engineering's NIH Training Program. As the project mentor, Ryan's students were awarded the 2011 EWB-USA Premier Project. In addition, when Ryan started at Duke he received a Civil and Environmental Engineering Department Fellowship. While completing his Master's, Ryan was awarded the L.B. Polkowski Fund Fellowship.