ANALYSIS OF MICROBIAL BIOFILM COMMUNITY COMPOSITION WITHIN CONSTRUCTED WETLANDS

by

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APPROVAL

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ABSTRACT

Constructed wetlands (CWs) are ecologically-based water treatment systems that provide cost-effective amelioration of waterborne pollutants. Fundamental understanding of removal mechanisms, especially microbial processes, limits greater usage of constructed wetlands as a wastewater treatment system. The influence of plant species selection, season, and organic load rate on pollutant removal was previously linked to the redox condition of the sub-surface wetland environment. The goal of this research was to determine which of these environmental variables (including spatial location within the CW) influenced the dominant microbial populations and/or the activity of various sub-populations. Once identified, a constructed wetland might be optimized for growth of microorganisms involved in removal of a specific pollutant.

To assess environmental factors, microbial population samples were taken in six locations (effluent, 3 root and 2 gravel areas) within replicate unplanted microcosms and wetland microcosms planted with *Deschampsia cespitosa* or *Leymus cinereus* during the summer (24°C) and winter (4°C) seasons. Microcosms were fed a synthetic domestic wastewater in 20-day batches for at least 12 months prior to sampling. The most recent techniques in molecular biology including denaturing gradient gel electrophoresis (DGGE) and quantitative PCR were utilized and included treatment with and without propidium monoazide (PMA) to distinguish between "live" and "dead" microbial communities. Primer sets targeted the entire bacterial community (16S rDNA) and two functional groups, nitrifying bacteria (*amoA* gene) and sulfate reducing bacteria (*dsrB* gene).

Results indicated that overall microbial community structure (16S rDNA) was affected by general location within the microcosm (effluent, root, gravel) as well the plant species present. Specific microbial groups appeared to be affected differently with relative gene quantities of sulfate reducing bacteria and nitrifying bacteria being influenced by a combined effect of plant species and season. For *dsrB*, *D. cespitosa* had the lowest relative gene quantities overall. Both genes were more abundant in the summer season, indicating seasonal importance. Location within the microcosms was also important, with anoxic environments (column bottom) being more important for *dsrB* presence and a diverse population of cultivated sulfate reducers. The roots were an important location for both microbial diversity and activity for all genes investigated.

CHAPTER 1

INTRODUCTION

The focus of this dissertation was to elucidate the microbial communities and processes involved in the degradation of wastewater in constructed wetland (CW) treatment systems. CWs are artificial wetland systems specifically designed to remediate a variety of wastewater types by filtration, settling, and bacterial decomposition. They are low cost, ecologically-based water treatment systems that provide amelioration of waterborne pollutants originating from a wide variety of sources. Other benefits include pleasing aesthetics and wildlife habitat which make this technology highly attractive for agricultural producers, small municipalities, and other rural inhabitants. There are a variety of CW designs, including vertical flow CW, surface flow CW, and subsurface flow CW, among others. The research in this dissertation specifically investigated subsurface flow CW microcosms operated in the Montana State University Plant Growth Center.

CW research has long inferred the presence of active microbial groups based upon the disappearance of specific pollutants (such as sulfate). It has been believed that CW systems would be most useful in climates where seasonal temperatures did not vary largely from summer to winter, as decreases in temperature negatively influenced CW performance (USEPA, 1988; WPCF; 1989, Reed et al., 1995). Additionally, performance results (based upon effluent water quality) in temperate regions did not indicate plant species as an important factor in designing a CW (Kadlec and Knight, 1996; USEPA, 2000; Scholz and Lee, 2005). As a result, plant species selection in CW

has been driven more by convention, aesthetics, professional experience, and broad guidelines than by research or other biological rationales. This dissertation sought to provide microbial evidence to support observations about CW performance and the importance of plant species selection.

Our research group at Montana State University has investigated CW performance in cold climate regions such as those observed in Montana. This research considered seasonal temperature variation and plant species. Results revealed seasonal performance differences (as determined by chemical oxygen demand (COD), sulfate removal, and redox potential) according to plant species in the wetland, with some plant species yielding higher COD removal efficiencies and redox readings in the winter season than the summer season (Borden et al., 2001; Allen et al., 2002; Hook et al., 2003; Stein and Hook, 2005; Taylor et al., 2010). It was hypothesized that these observed performance differences could be attributed to the microbial biofilm communities present in the wetlands and that these communities were influenced by plant presence and plant species. It was also hypothesized that different microbial functional groups could be detected at different locations within the wetlands. To address these questions, denaturing gradient gel electrophoresis (DGGE) was used to visualize the microbial communities present and quantitative PCR (qPCR) was used to quantify the microbial groups of interest. These molecular methods were used to assist in determining any differences between the microbial communities within the microcosms with regard to season and/or plant species.

Chapter 2 of this dissertation was published in Ecological Engineering (Faulwetter et al., 2009) and summarizes the current literature in CW research as it applies to microbial processes. This review addresses the molecular techniques currently employed in CW research as well as the major biogeochemical processes studied to date. It identifies the importance of linking CW performance with the microbial communities present and active within these systems.

The research presented in Chapter 3 describes the initial work conducted on our CW systems focused primarily on sulfate reducing bacteria (SRB) present within one unplanted control column utilizing cultivation based and molecular approaches. The goal of this study was to enrich and characterize the total cultivable SRB community as well as to develop our DGGE method for primers targeting a single functional group. This work showed unique community profiles for SRB cultivated on a variety of different growth media and was presented as a poster at the 11th International Conference on Wetland Systems for Water Pollution Control in Indore, India and published in the conference proceedings (Faulwetter et al., 2008).

Chapter 4 is an in depth explanation of the statistical approach taken to analyze the data in the remainder of the dissertation. It is divided between methods used for DGGE data analysis (utilizing Gel Compar II and R software) and quantitative polymerase chain reaction (qPCR) data analysis (utilizing Minitab® software). DGGE data was rigorously analyzed with data packages designed by Dr. David W. Roberts, Montana State University, Department of Ecology (R software libraries labdsv and

optpart) and statistical analyses for the qPCR data was designed, implemented, and interpreted with the assistance of Dr. Albert Parker, Center for Biofilm Engineering.

The research in Chapter 5 compares the 16S rDNA DGGE community profiles across season (summer to winter), plant species (unplanted, *Deschampsia cespitosa*, and *Leymus cinereus*), sample type (effluent, rhizosphere, and gravel), as well as six specific sample locations within the CW microcosms. This study was performed to answer the initial hypotheses: 1) plant presence (and species) altered the microbial community structure; 2) season altered the microbial community structure; and 3) the rhizosphere cultivated a biofilm community that was unique from the surrounding gravel. An intensive sampling procedure was developed and implemented to examine regions of presumed importance within the CW microcosms and rigorous statistics applied to analyze the results. Since a novel approach to statistical analysis is being applied to the data, results and/or interpretations presented in this dissertation may be updated prior to publication. This work is being prepared for submission to the International Society for Microbial Ecology Journal.

Chapter 6 is being prepared for submission to Microbial Ecology and focuses on the molecular work performed on functional genes. The genes investigated in this paper are the beta subunit of the dissimilatory sulfite reductase gene (*dsrB*) and the ammonia monooxygenase gene (*amoA*). The *dsrB* gene is essential to dissimilatory sulfate reduction and found in every organism capable of sulfate reduction. Likewise, the *amoA* gene is essential for the conversion of ammonia to nitrite (the first step in the nitrification process), and thus is a useful indicator of this process and the ammonia oxidizing

organisms involved. These genes were selected because of sulfate and ammonia removal observed within our microcosms and the importance of sulfate reduction and nitrification in biological nutrient cycling. Both processes are well documented within CW systems, however, little research has focused on direct microbial analysis. The CW microcosms used were maintained and destructively sampled in the same manner as described in Chapter 5. In an effort to characterize both abundance and diversity of these communities across plants species, season, etc., a combination of qPCR and DGGE methodologies were used for each gene. Propidium monoazide (PMA) treatment was also incorporated to focus on the active members of the microbial community. Treatment with PMA results in the PCR inhibition of DNA from cells with compromised cell membranes leaving only DNA from intact cells to be amplified and analyzed. The data generated were analyzed as described in Chapter 4.

Chapter 7 is the conclusion of the body of the dissertation and suggests possible future research directions.

Appendix A is a paper on the application of the functional DGGE approach as applied to floating island technology for wastewater treatment. Floating islands are a form of treatment wetland characterized by a mat of synthetic matrix at the water surface into which macrophytes can be planted and through which water passes. This research focused on nitrifying and denitrifying biofilm communities cultivated on the surfaces of several floating island substrates. All experiments concentrated on optimizing for simultaneous nitrification and denitrification within these substrates and in the absence of plants. This work was presented in October 2010, as a poster at the 12th International

Conference on Wetland Systems for Water Pollution Control in Venice, Italy and has been submitted to Water Science and Technology for publication.

Contribution of Authors and Co-authors

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CHAPTER 2

LITERATURE REVIEW

MICROBIAL PROCESSES INFLUENCING PERFORMANCE OF TREATMENT WETLANDS: A REVIEW

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Abstract

This review summarizes the microbial mechanisms responsible for removal of carbon, nitrogen, and sulfur compounds in treatment wetlands (TWs) and identifies, categorizes and compares various techniques, from plate count to more modern genomic

methods used to elucidate these mechanisms. Removal of a particular pollutant is typically associated with a specific microbial functional group, therefore employment of design and operational methodologies that enhance the activity of that group will better optimize performance. Redox condition is a manipulable parameter that can be used to optimize growth of a targeted functional group, therefore factors influencing the TW redox condition and its influence on organic carbon removal mechanisms are emphasized. Environmental factors influencing growth and activity of N and S cycling microbes (including temperature, pH, salinity, plant species selection and availability of organic carbon and/or inhibiting substances) are discussed with particular attention to factors that might be manipulated. This information is used to offer design and operational methodologies that might enhance growth of a desirable microbial functional group and project what additional microbially-focused research is required to better optimize TW performance.

Introduction

Treatment wetlands (TWs), also known as constructed wetlands, are engineered systems designed to remove pollutants from contaminated water. Use of these systems over the last quarter century has developed rapidly and TWs are now successfully employed to remove a diverse array of pollutants originating from almost every conceivable contamination source. Understanding of the removal mechanisms responsible for water treatment has expanded concurrently with TW usage. Better understanding has led to a great variety of designs and configurations in an effort to optimize the removal of a specific pollutant, e.g. nitrate, or a consortium of pollutants

typical to a specific source, e.g. primary treated domestic wastewater or acid mine drainage (AMD). While a variety of removal mechanisms including sedimentation, filtration, precipitation, volatilization, adsorption and plant uptake are well documented (Kadlec and Knight, 1996), recognition that removal of most pollutants in treatment wetlands is due primarily to microbial activity has been a cornerstone of the technology almost from the beginning (Hatano et al., 1994; Reddy and D'Angelo, 1994; Kadlec and Knight, 1996). This is certainly true for removal of organic carbon (OC), especially soluble labile forms that dominate primary treated domestic wastewater. While plant uptake is a minor nitrogen removal mechanism, microbial transformations provide the majority of total nitrogen (TN) removal (Kadlec and Knight, 1996). Sulfate reduction has been recognized as an important mechanism for metals removal (Dvorak et al., 1992; Machemer and Wildeman, 1992), but it may also play an important part in OC removal; and sulfide oxidation may also be an important process in TWs. Thus, pollutant removal and microbial activity in TWs are closely tied to the cycling of carbon, nitrogen and sulfur.

Early publications typically assumed the influence of microbial processes in TWs, based primarily on inference from known processes in other wastewater treatment systems and/or natural wetlands. A large body of literature uses circumstantial evidence to corroborate these basic assumptions based primarily on measurement of changes in water chemistry, but lacks direct evidence of specific microbial consortia at work. Relatively recent advances in qualitative and quantitative microbial techniques make direct evidence for the presence of specific microbial species or functional groups

influencing pollutant removal possible. As shown below, literature employing these techniques to the study of TWs have recently begun to proliferate, but what we "know" about microbial activity in TWs is still largely based on assumption and circumstantial evidence.

In this paper we try to summarize the body of knowledge on microbially influenced pollutant removal mechanisms in treatment wetlands. Given the overwhelming body of literature on C, N, and S cycling in wetlands, the diversity of microbial consortia involved in these cycles, and the rapid advances in techniques used to quantify microbial activity, we could not possibly review every manuscript that is pertinent. In addition, the great variety of designs and configurations of treatment systems that can be collectively termed a treatment wetland makes this an even more daunting task. Therefore we limit our focus to the practical, summarizing microbial methods that have been employed in TW research and discussing what microbial functional groups are most likely to be involved in the removal of pollutants typically found in TW influent. In the next section we briefly discuss and categorize the various techniques that have been employed to the study of microorganisms in TWs. Additional sections will expand upon: (a) the influence of TW redox on determining which microbial functional groups will dominate and how redox can be manipulated, with specific focus on OC removal; (b) bacterial populations critical to nitrogen removal and factors influencing these microbes; and (c) microbial populations affecting sulfur transformations and the importance of these in TW performance.

Techniques for Assessing Microorganisms in TWs

Pioneering studies enumerating microbial populations within treatment wetlands generally used the plate count method, e.g. Hatano et al. (1993). More recent studies have taken advantage of new molecular technologies and genetic techniques such as polymerase chain reaction followed by denaturing gradient gel electrophoresis (PCR-DGGE) (Muyzer et al., 1993) or fluorescence in-situ hybridization (FISH) probes (Manz et al., 1992). We found more than 50 peer-reviewed articles employing microbial techniques to the study of TWs. These studies can be classified into three general types: those dealing primarily with the removal of infectious organisms and/or total bacteria counts; those dealing with global microbial processes in TWs by coupling several molecular and biochemical techniques to estimate a global level of microbial density, activity and/or diversity; and those with an emphasis on a single activity or on a specific microbial functional group such as nitrifiers, denitrifiers, methanogenic archaea, or sulfate reducers. We focus primarily on those referring to global microbial processes as well as those focusing on a specific activity, referring readers to Ghermandi et al. (2007) for a recent review of pathogenic organism activity and removal in TWs. We divide the studies based on whether they are focused on microbial density, activity, or diversity; then by the specific techniques used to achieve those measures.

Microbial Density Studies

Microbial density refers to a measure of the overall quantity of microbes, or the quantity of a specific type of microbe, within or removed by the TW. Most TW microbial studies fall under this category. Initial studies focused on removal of

pathogenic bacteria (or indicator organisms) between the TW inlet and outlet or within the TW matrix (e.g. Hill and Sobsey, 2001; Stenstrom and Carlander, 2001). Molecular methods have been increasingly employed with success (Table 2.1).

Plate counts are a well established method, especially to estimate pathogenic or indicator bacteria removal. Plate counts are very useful to estimate bacteria and fungi in the main flow through of a TW (Sleytr et al., 2007), and were observed to be correlated with molecular techniques when measuring microbial communities residing within the TW (Truu et al., 2005). However, utility is limited to a quantitative estimation of specific bacterial numbers as the majority of microorganisms cannot be cultivated, and other techniques are usually employed to determine the community structure (Vacca et al., 2005). The most probable number (MPN) statistical method is applied to culture-based techniques (plate or tubes) to reduce the standard deviation when counting bacterial colonies.

Epifluorescent microscopy is commonly used to determine total bacterial counts in TWs. This relatively simple method couples fluorescent staining to identify specific bacteria groups with direct observation under a microscope. The technique has been employed to estimate bacterial numbers at the inlet and outlet (Decamp and Warren, 2001), or within the TW matrix (Munch et al., 2005; Tietz et al., 2007a). Staining samples using BacLight^{TM} allowed for separation between living and damaged bacteria (Decamp and Warren, 2001).

Table 2.1. Methods employed to assess microbial density in treatment wetlands.

Measured parameter	Method	References	Advantages	Disadvantages
Direct bacteria, protozoa, and fungi counts	Plate count	Chong et al., 1999; Hallberg and Johnson, 2005; Hatano et al., 1993; Hill and Sobsey, 2001; Liang et al., 2003; Ottova et al., 1997; Sleytr et al., 2007; Stenstrom and Carlander, 2001; Truu et al., 2005; Vacca et al., 2005; Vymazal et al., 2001a; Zhang and Lampe, 1999	Does not require complex instrumentation. By using different media, applicable to a wide range of bacteria types.	Can lead to errors both by the large number of dilutions required and by the method itself (CFU). Limited to several type of bacteria and fungi.
	MPN	Fortin et al., 2000; Kyambadde et al., 2004, 2006; Truu et al., 2005; Vacca et al., 2005	Reduces the standard deviation of the culture-based method	Needs a large number of replicates to narrow the confidence intervals. As few as 10% of soil bacteria are able to grow
	Epifluorescence microscopy (DAPI, BacLight, SYBR, Sytox)	Decamp and Warren, 2001; Hallberg and Johnson, 2005; Ishida et al., 2006; Larsen and Greenway, 2004; Münch et al., 2005; Nicomrat et al., 2006b; Silyn-Roberts and Lewis, 2003; Sleytr et al., 2007; Tietz et al., 2007b	Very accurate cell counts especially when using a specialized software coupled to the microscope	Organic matter can interact with staining. Need to "detach" groups of bacteria from the biofilm
	Flow cytometry	Gagnon et al., 2007; Scholtz et al., 2001	Does not require many dilutions compared to plate count or epifluorescence	Interferences associated with EPS and other organic compounds contained in samples can lead to errors
Indirect biomass	DNA	DeJoutnett et al., 2007	Complete community snapshot	Also included inactive/dead cell DNA
	Protein	Gagnon et al., 2007; Larsen and Greenway, 2004; Ragusa et al., 2004	Very practical method, easy to estimate biomass, does not require instrumentation	Compounds such as sulfide can interfere reducing overall utility
	Phospholipids fatty acid (PLFA)	Jin and Kelly, 2007; Mentzer et al., 2006; Ragusa et al., 2004	Very accurate estimation of micro-organism biomass	Complex protocol
	Polysaccharide and extracellular polymeric substance (EPS)	Larsen and Greenway, 2004; Lazarova and Manem, 1995; Ragusa et al., 2004	Very simple to extract. Well correlated with protein content	Recurrent overestimation of the biomass
	Chlorophyll-a	Toet et al., 2003	Very easy to measure, enables a quick estimation	Only applicable in surface wetlands (presence of algae). Samples need to be preserved in the field
	N Biomass (Fumigation)	Nguyen, 2000; Tietz et al., 2007a; Truu et al., 2005	A technology well employed in soil biology	High variability between samples. Needs replications
	Substrate induced respiration (SIR)	Nurk et al., 2005; Tietz et al., 2007b; Truu et al., 2005	Enable a good estimation of biomass C in the sample	A better estimation rather than an accurate measurement tool due to large standard deviation
	ATP	Lazarova and Manem, 1995; Nurk et al., 2005; Tietz et al., 2007b	ATP values remain constant after freezing and relatively prolonged storage of samples	Complexity of the analytical procedure, extreme sensitivity to the extraction technique, not selective for bacteria

Flow cytometry is a powerful technology that allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of single cells (bacteria or protozoa) flowing through an optical and/or electronic detection apparatus (Scholz et al., 2001; Gagnon et al., 2007). Once the flow cytometer is properly set up, this technique enables a very quick count.

Measuring DNA, protein, PLFA, EPS, polysaccharides, chlorophyll-a or other specific compounds associated with bacteria cells to infer biomass was shown to be a very efficient approach when applied to TWs. However, they are used mainly in association with other direct and indirect biomass estimation techniques and often serve as a reference for activity measurements (Table 2.1). For further explanation of these techniques, please see section on microbial diversity studies.

C and N bacterial fumigation and substrate induced respiration (SIR) are alternative methods to estimate biomass. In fumigation extraction, the cell membrane is broken, usually by exposing the sample to a caustic vapor, e.g. chloroform, and comparing the quantity of carbon and/or nitrogen to an unfumigated sample. The SIR method measures CO_2 production of a sample exposed to a readily available substrate (Table 2.1).

Microbial Activity Studies

Microbial activity refers to a measure of the microbially-driven biological processes occurring in a TW. Activities can be measured *in situ,* often by estimating a specific gas production, e.g. CO_2, N_2, CH_4, or *ex situ* where the term potential activity is

usually employed. *Ex situ* studies are more common as environmental parameters can be better controlled, and often include a characterization of microbial density. Studies focused on microbial activity are summarized in Table 2.2 and the techniques are summarized below.

Potential Activity Potential respiration can be estimated either by soil basal respiration which is similar to the SIR method (the sample is placed in a closed environment but in this case substrate is not added) (Nurk et al., 2005; Truu et al., 2005) or by placing a sample in a respirometer (Gagnon et al., 2007). Both techniques can be applied to anaerobic as well as aerobic activities by measuring anaerobic gaseous by-products such as methane and sulfide (Edwards et al., 2006; Caselles-Osorio et al., 2007). Measured potential values can be compared to water quality measures of TW efficiency but results are usually not scalable to field conditions. The respiration rate is often over estimated in subsurface flow systems, and the contribution by plant root respiration is never taken into account (Chazarenc et al., 2007).

Enzymatic Activity An estimation of the production (or consumption) of enzymes used in various biological processes important to water treatment can shed light on those processes. Generic enzymatic activities are frequently estimated and can give information on the global activity in the sampled TW. Targeting specific enzymatic activities can help to better understand degradation mechanisms of a variety of pollutants and specific pathways within the carbon, nitrogen, phosphorus and sulfur cycles (Kang et al., 1998).

Table 2.2. Methods employed to assess microbial activity in treatment wetlands.

Measured parameter	Method	References	Advantages	Disadvantages
Potential activity	Respiration	Gagnon et al., 2007; Hernandez and Mitch, 2007; Nguyen, 2000; Nurk et al., 2005; Truu et al., 2005	Provides a good estimation of the TW potential degradation rate	Can over-estimate the real value Plant effects are not considered
	Nitrification	D´Angelo and Reddy 1999; Edwards et al., 2006; Eriksson and Andersson 1999; Kyambadde et al., 2004, 2006; Münch et al., 2005; Nurk et al., 2005; Sundberg et al., 2007a, 2007b; Truu et al., 2005;	Direct evidence for existence of specific types of bacteria and a rough estimate of their density	
	Anaerobic	Edwards et al., 2006; Mulder et al., 1995		
	Denitrification (Acethylene)	Bastviken et al., 2003; Hernandez and Mitch, 2007; Hunt et al., 2003; Ishida et al., 2006; Lin et al., 2007; Münch et al., 2005; Sirivedhin and Grey 2006; Smialek et al., 2006; Sundberg et al., 2007b; Toet et al., 2003; Xue 1999		
Anaerobic activity	Methane production	Inamori et al., 2007; Smialek et al., 2006	The production of CH_4 is a good indicator of anaerobic activity in the TW	Great variability generally observed
Enzymatic activity	Phosphatase, Glucosidase (p-nitropheol)	Kang et al., 1998; Mentzer et al., 2006; Shackle et al., 2000; Zhou et al., 2005	Blends well with separate density measurements	Sometime associated with complex protocols that generate errors and large standard deviations
	Protease	Zhou et al., 2005		
	Catalase	Zhou et al., 2005		
	Urease	Liang et al., 2003; Zhou et al., 2005		
	Cellulase	Mentzer et al., 2006; Zhou et al., 2005		
	Ammonia oxidation	Münch et al., 2005		
	Denitrifying enzyme activity	Münch et al., 2005; Smialek et al., 2006		
	Arylsulfatase	Kang et al., 1998; Shackle et al., 2000		
	FDA	Gagnon et al., 2007	Correlates well with other activity measurements such as potential respiration rate	Can be affected by very low redox conditions
Electron transport system activity	Dehydrogenase (INT, TTC)	Gagnon et al., 2007; Kang et al., 1998; Ragusa et al., 2004; Zhou et al., 2005	Relatively high precision and applicable to all respiration types	
Radioactive tracer	^{14}C Leucine	Baptista et al., 2003; Tietz et al., 2007b	High precision and can follow plant - microbe interactions	Difficult to apply to full scale TW
	^{14}C urea	Thoren, 2007		
	^{15}N	Bastviken et al., 2003; Xue 1999		
	DNA precursor	Pollard et al., 1995		
	$^{32}S^{2-}$	Baptista et al., 2003		

Electron Transport System Activity The use of a tetrazolium salt, e.g. INT, TTC, to estimate dehydrogenase activities has been successfully applied to a wide range of micro-organisms. Virtually all important TW electron transport, i.e. redox, transformations including oxygen respiration have been documented by this technique. Use of tetrazolium salt combined with potential respiration measurements provides a first estimation of several degradation processes.

Isotope Tracers Organic molecules with unnatural isotope ratios of important elements, e.g. ^{14}C, ^{15}N, can be introduced to the TW and tracked through various metabolic pathways to determine activity of various microbial consortia. This technique is very precise but potential environmental hazards when studying elements where only radioactive isotopes exist typically limit application to small controlled experiments. Use of the stable isotope ^{15}N has, however, provided useful insight into N cycling processes in wetlands.

Microbial Diversity Studies

Microbial diversity refers to a measure of the number of specific species and/or functional groups existing within the TW. These can be made by direct microscopic observation, community log profiling (microplate) techniques, or by using molecular genetics tools like PCR-DGGE or FISH probes. Combined with assessments of activity, the availability and standardization of the new molecular technologies to identify functional groups will undoubtedly revolutionize our understanding of microbial processes occurring within TWs. However, relatively few studies employing these

techniques have been published to date. A summary of diversity studies is found in Table 2.3.

Microscopic Observation Observation of "fresh" samples under a traditional optical microscope has been used to identify protozoan and metazoan communities (Puigagut et al., 2007a; 2007b), however issues with sampling and identification have hindered application to bacteria. Electron microscopy was applied to the observation of biofilm development on gravel surfaces (Larsen and Greenway, 2004).

CLPP Analysis The community-level physiological profiling (CLPP) method, using microplates with multiple sole-carbon sources, has become a popular tool for the comparison of microbial communities with respect to their functional potential. CLPP has been successfully adapted to the study of complex microbial communities (Garland and Mills, 1991) and has been used for rapid assessment of the variation in TW microbial community structure (Osem et al., 2007). However, contradictory conclusions can be drawn if sophisticated statistical analyses are poorly applied (Weber et al., 2007).

PFLA Profile Phospholipids fatty acid profile (PLFA) enables an examination of microbial communities and population changes over time. This method provides an indirect density analysis as well as an estimate of diversity (Table 2.3).

Genomic Methods These are the most employed techniques to assess microbial diversity and relative abundance over the last 5 years. PCR-DGGE seems to be the most popular technique to estimate global diversity of bacteria or to estimate bacterial communities involved in N-removal, or in methanogenic activities (Table 2.3). This

Table 2.3. Methods employed to assess microbial diversity in treatment wetlands.

Measured parameter	Method	References	Advantages	Disadvantages
Microscopy	Light microscopy	Puigagut et al., 2007a, 2007b; Scholz et al., 2001; Vymazal et al., 2001b	Enables frequent observations, provides a good assessment of community profile	Requires knowledge to identify protozoa and metazoa
	Environmental scanning electron microscopy	Larsen and Greenway, 2004	Helps the understanding of biofilm formation on substrates	Difficult to obtain a representative sample
CLPP	Biolog Ecoplate	Collins et al., 2004; Nurk et al., 2005; Osem et al., 2007; Weber et al., 2007	Accurate differentiation of bacterial communities	Limited to relative comparisons between samples
Cell membrane of microorganisms	Phospholipid Fatty-acid Analysis (PLFA)	Jin and Kelley, 2007; Mentzer et al., 2006	Good comparison of community change with time in the same system	Provides a rough estimate. Complex protocol
Genomic methods				
Semi-quantitative PCR	Ammonia oxidizing bacteria	Silyn-Roberts and Lewis, 2001	Accurately followed development of Nitrosomas sp. in the biofilm	Crude method that could be refined
Real-time PCR	Quantification	Ibekwe et al., 2002b	Successfully applied to quantify small communities	Limited by the need of culture to compare with
Amplified ribosomal DNA restriction analysis, ARDRA PCR-cloning	Phylogenic analysis	Brofft et al., 2002; McGarvey et al., 2004; Walsh et al., 2002	Successfully applied to determine microbial diversity in a TW	Must be coupled with a quantitative method
"touchdown" PCR	Acidophilic microorganisms	Hallberg and Johnson, 2005	Successfully applied to determine a wide range of acidophiles microbes	Difficult to obtain a quantitative result thus requires complementary tests
LH-PCR	Diversity analysis	Ahn et al., 2007	Enabled both a diversity and relative abundance estimation	Further work required to assess community structure
T-RFLP	Relative diversity and abundance	Ishida et al., 2006; Lloyd et al., 2004	Similar in some aspects to PCR-DGGE	
PCR-DGGE	Total bacteria community	Baptista et al., 2003; DeJournett et al., 2007; Ibekwe et al., 2007, Jin and Kelley, 2007; Nicomrat et al., 2006a; Truu et al., 2005	Enabled very detailed phylogenic analysis	Difficult to summarize results especially when using different primer pairs
	Ammonia oxidizing bacteria	Ibekwe et al., 2003; Silyn-Robert and Lewis, 2001; Sundberg et al., 2007a; Truu et al., 2005		
	Ammonium monooxygease	Truu et al., 2005		
	Methanotroph	DeJournett et al., 2007	Observed species missed by culture-based method	Required a large number of samples
	Acidophilic microorganisms	Nicomrat et al., 2006a	Observe and identify community members and composition	Included previous enrichment steps which may bias results
FISH probes	Most Bacteria	Criado and Bécares, 2005; Inamori et al., 2007; Nicomrat et al., 2006b; Polprasert and Sawaittayothin, 2006	Simultaneous estimation of diversity and abundance (according to the selected probes). Observation of fine architecture of biofilms	Can have non-specific binding of probes to non-target organisms or groups
	N- cycle bacteria	Criado and Bécares, 2005; Dong and Sun 2007; Polprasert and Sawaittayothin, 2006; Schmid et al., 2000; Silyn-Roberts and Lewis, 2001; Sliekers et al., 2002		
	S- cycle removal	King et al., 2002; Polprasert and Sawaittayothin, 2006		
	Methanotroph	Inamori et al., 2007; Polprasert and Sawaittayothin, 2006; Zhu et al., 2007		
	Acidophilic bacteria	Nicomrat et al., 2006b		

method can be used to determine the relative diversity of a sample by using a phylogenetic analysis based on a data bank, or the presence of specific bacteria, or groups of bacteria, by using specialized primer pairs. Fluorescence *in situ* hybridization (FISH) probes enable both the isolation and enumeration of specific bacteria populations. By choosing adapted FISH probes, it is possible to stain two or more specific populations of bacteria differently, e.g. nitrifying and denitrifying, and to observe the results within a biofilm using a microscope.

In this section we have merely identified and organized the studies employing standard microbial methods to the study of TWs and highlight the techniques employed. In the following sections we focus on microbial processes responsible for TW efficiency considering studies employing microbial techniques, circumstantial evidence, inference from other systems or some combination.

Importance of Redox on Microbial Processes

Respiration and fermentation are the major mechanisms by which microorganisms break down organically-derived pollutants into assumed harmless substances such as carbon dioxide (CO_2), nitrogen gas (N_2) and water (H_2O). Furthermore, end products, such as sulfide, generated by some types of respiration can enable other known removal mechanisms, such as precipitation and sequestration of heavy metals within the wetland matrix (Dvorak et al., 1992; Machemer and Wildeman, 1992). In respiration, the microbe induces a transfer of electrons from a donor compound of higher energy state (typically organic carbon, OC) to an electron acceptor of lower state using the energy differential for growth and reproduction. The prevailing

respiration process and the associated terminal electron acceptor compound, and therefore pollution removal, depends on the oxidation-reduction (redox) conditions prevailing in the wetland environment. High redox potential is associated with an oxidized environment and promotes aerobic processes such as nitrification. In contrast, lower redox potentials are linked to reduced conditions and promote anaerobic processes such as sulfate reduction and methanogenesis. The type of microbial respiration follows a predictable sequence within specific ranges of redox potential (Table 2.4). The quantity of energy available to the microbe follows a somewhat similar path. In general, the higher energy yields of aerobic processes lead to faster microbial growth and reproduction (Mitsch and Gosselink, 2000) and, provided the appropriate electron acceptor is available and redox is maintained within the proper range, the rate of OC consumption decreases as one moves down Table 2.4.

Table 2.4. Selected types of microbial oxidation-reduction reactions

Process	Electron Acceptor (EA)	End Products	Moles of e^- per mole of EA	$\Delta G°$ (kJ/mole of electron)	Redox Potential (mV)
Aerobic respiration	O_2	H_2O	4	-125.1	300 to 700
Nitrate reduction	NO_3^-	N_2, NO_x	5	-118.8	100 to 350
Manganese reduction	Mn^{+4}	Mn^{+2}	2	-94.5	-100 to 300
Iron reduction	Fe^{+3}	Fe^{+2}	1	-24.3	-100 to 200
Sulfate reduction	SO_4^{-2}	S^{-2}	8	-25.4	-200 to -100
Methanogenesis	CO_2	CH_4, CO_2	8	-23.2	-350 to -100

Adapted from: Laanbroek, 1990; Reddy and D'Angelo, 1994; Kadlec and Knight, 1996; IWA, 2000; Sahrawat, 2004; Szogi et al., 2004; Diakova et al., 2006

Treatment wetlands for domestic wastewater can be designed to favor a wide range of redox conditions, therefore enhancing a variety of biological processes and removal of multiple pollutants in the same TW bed. Conversely, they can be engineered to favor a limited range of redox conditions targeting a specific microbial respiration process optimizing for removal of a specific pollutant. For example, a TW designed primarily for OC removal will be more efficient, and hence smaller, if it can be designed to promote aerobic processes; whereas one designed for sulfate reduction and subsequent metals removal requires the exclusion of oxygen to promote low redox conditions. Manipulation of redox conditions in treatment wetlands can be achieved by altering the organic loading rate (if possible), the hydraulic design, mode of operation, and possibly by plant species selection. In addition, aerobic conditions can be induced by forced aeration of the TW matrix (Nivala et al., 2007).

Influence of Hydraulic Design on Redox Condition

There are numerous types of treatment wetlands that are usually classified by their hydraulic design (Vymazal, 2007). Each type of TW can promote a specific redox condition or alternatively a wide range of redox conditions varying with time of treatment and/or spatially within the wetland matrix. Variable conditions enable the formation of ecological niches favorable to the development of microbial biofilms with functionally different respiration processes and pollution removal capacities (Wiessner et al., 2005b).

Vertical Flow Treatment Wetland This type of TW is generally considered to be a highly aerobic system, since wastewater drains vertically through the planted matrix,

allowing for unsaturated conditions and excellent oxygen transfer (IWA, 2000). We found no information on direct redox measurements in the literature, but circumstantial evidence strongly suggests that vertical flow wetlands have high redox potentials that favor aerobic microbial processes. For example, BOD removal and nitrification were significantly higher in vertical flow compared to surface and subsurface flow wetlands, but denitrification was low (Cooper et al., 1996; Vymazal, 2007; Li et al., 2008). Furthermore, direct microbial measurement has shown that the microbial density and activity were maximized in the first 5-10 cm of the vertical flow filter (Ragusa et al., 2004; Tietz et al., 2007a). This distribution of microorganisms is presumed to be caused by the high nutrient content and the ample oxygen supply in the upper zone of the vertical flow wetlands (Tietz et al., 2007a). The influence of plant oxygen release on redox potential in vertical flow wetlands is considered negligible, due to high amount of oxygen already permeating during the draining period (IWA, 2000).

Horizontal Subsurface Flow Treatment Wetlands This type of TW has both oxidized and reduced zones, but overall is generally considered an anoxic system (IWA, 2000). Numerous design factors can influence the redox condition in subsurface wetlands including the length and depth of the wetland. Redox potential usually increases from the inlet to the outlet (length) due to progressive pollution biodegradation (Garcia et al., 2003; Headley et al., 2005) and the mechanical filtration of suspended solids. Furthermore, redox potential usually decreases with depth, where higher redox in the surface zone (5-20 cm) is possibly caused by plant oxygen release and passive oxygen diffusion (Allen et al., 2002; Garcia et al., 2003). However, redox variation with depth

may not always be present, due to vertical mixing of water through the wetlands matrix (Headley et al., 2005). In addition, the vertical profile of the redox gradient varies according to the wetland length, with less vertical variation of redox near the inlet than the outlet (Kadlec and Knight, 1996; Garcia et al., 2003). It was also reported that shallower (0.27-0.5 m) wetlands had generally higher redox potentials than deeper beds (0.5-1.0 m), consequently being more efficient in removal of COD, BOD, NH_3 and dissolved reactive phosphorus (Garcia et al., 2003; Headley et al., 2005).

Surface Flow Treatment Wetland This type of TW is generally considered anoxic, with a thin aerobic layer at the surface due to passive aeration of the water (IWA, 2000). Direct measurements have indeed shown that dissolved oxygen and redox potential decreased with water depth (Tao et al., 2006). Within the sediment, a study by Gao et al. (2000) showed that the redox potential was lowest at the soil/water interface (5 cm; *E*h = -200 mV) but then increased with depth with values as high as 300 mV at a depth of 15 cm below the soil surface. Lower redox potential in the surface soil is possibly caused by sedimentation of particulate organic matter, creating a zone with high oxygen demand due to greater microbial activity (Fox and Doner, 2003). Higher redox potential with depth could be explained by a low diffusion of pollutant through the soil matrix (Fox and Doner, 2003) and oxygenation of the rhizosphere by plants (Brix, 1997). However, plant oxygenation is species dependent and highly reduced conditions have been measured in the soil matrix of surface flow wetlands (Szogi et al., 2004).

While it is possible to draw a general assessment of the relation between the type of TW and redox potential, other factors strongly influence redox potential within the

wetland matrix, so it is difficult to predict the redox potential by TW type exclusively. Compounding factors include the mode of operation, type of macrophytic plant species and/or species diversity, and season.

Influence of the Mode of Operation on Redox Condition

Given a specific TW design, the redox condition can be managed by selecting a suitable mode of operation, especially in subsurface flow systems. The most important operating factors are feeding mode (how water is applied), hydraulic loading rate and retention time. Feeding mode can be classified following three distinct categories depending on the wastewater management strategy.

Batch Feed This is a sequential process in which the TW is filled with wastewater for a determined period of time and then completely drains before the next batch of effluent is applied (Caselles-Osorio and Garcia, 2007). Alterations to this design include reciprocating or tidal flow wetlands (Tanner et al., 1999; Behrends et al., 2001; Austin et al., 2003). This type of feeding creates a temporal redox variation in the wetland, with, at first, a major drop of the redox potential when the wastewater is added and then a gradual increase of the redox condition with time and pollution removal (Allen et al., 2002). Thus, the alternation between reduced and oxidized conditions may fundamentally influence microbial consortia by favoring robust aerobic facultative biofilms that can operate under varying nutrient concentrations and fluctuating redox conditions (Stein et al., 2003). Furthermore, it was generally observed that batch feed

promotes greater oxidized conditions and therefore better performance for COD removal than continuous flow feeding (Stein and Kakizawa, 2005).

Intermittent Flow Feed This is similar to batch feed but the wetland is not drained before a fresh batch of wastewater is added to the system. Caselles-Osorio and Garcia (2007) showed that intermittent feeding produced a higher redox potential, greater ammonium removal and lower sulfate removal than continuous flow feeding of a similar system, even though COD removal showed no difference. A tracer study revealed the presence of vertical mixing throughout the length and depth of an intermittently fed wetland (Headley et al., 2005). Mixing of the anaerobic zone at the bottom of the wetland with the aerobic and anoxic micro-sites in the rhizosphere might explain the higher redox conditions (Caselles-Osorio and Garcia, 2007). Intermittent feeding is also presumed to create a temporal redox variation as observed in batch mode (Allen et al., 2002), as well as spatial redox variation resulting in more equal pollutant removal throughout the length of the wetland (Headley et al., 2005).

Continuous Flow Feeding This is simplest and therefore the most common technique. Generally, it results in a lower redox potential and has recently been considered less effective than batch and intermittent flow mode for aerobic pollutant removal (Stein et al., 2003; Caselles-Osorio and Garcia, 2007). Continuous flow feeding tends to generate a spatial gradient, with higher redox potential and lower pollution concentration toward the outlet of the wetland. Therefore, it is assumed that microbial communities follow an analogous pattern, with microbes selected to thrive under high

nutrient load and anoxic conditions near the inlet and those favoring low nutrient load and more aerobic conditions toward the outlet (Stein et al., 2003).

Regardless of feeding mode, pollution removal in constructed wetlands depends greatly upon the hydraulic retention time (HRT) and the hydraulic loading rate (HLR) (Toet et al., 2005). These hydraulic variables affect the duration of contact between pollutants and the microbial population within the wetland system. It has been shown that longer HRT generate higher redox potentials and greater pollution removal. For example, in an intermittently fed horizontal subsurface wetland, redox potential varied between -92 and +103 mV when the nominal HRT was of 10.1 days but from -109 to +186 mV when the nominal HRT was of 16.1 days (Headley et al., 2005). In a batch feed experiment, Allen et al. (2002) showed that redox potential and COD concentration varied with treatment time, with redox potential around -150 mV at day one to a range of 0-300 mV, depending on plant species, after 20 days.

Influence of Plants on Redox Condition

Plants are the most notable feature of treatment wetlands and their presence has been reported to improve pollution removal (Fraser et al., 2004; Picard et al., 2005). Tanner (2001) measured a higher redox potential in the root zone of a horizontal subsurface flow wetland compared to an unplanted one. Other studies have shown that microbial density, activity, and diversity are enhanced in the plant rhizosphere regions of subsurface flow TWs (Hatano et al., 1993; Ottova et al., 1997; Collins et al., 2004; Munch et al., 2005; Gagnon et al., 2007), suggesting that plants enhance the establishment of microorganisms responsible for pollution removal.

Wetland plants transfer oxygen to their root system and release a fraction of this oxygen into the rhizosphere (Brix, 1997) promoting the formation of an oxidized layer around the root and creating a redox gradient ranging from $E_h \approx$ 500mV very near the root surface to $E_h \approx$ -250 mV at a distance of 1-20 mm from the root surface (Wiessner et al., 2002; Bezbaruah and Zhang, 2004; Munch et al., 2005). However, oxygen loss varies by root type and location; greatest loss occurs in the sub-apical region of young adventitious and secondary roots, particularly at the base of fine lateral rootlets, but very little is lost from old roots and rhizomes (Armstrong and Armstrong, 1988). As a result, the rhizosphere exhibits a mosaic of strong redox gradients enabling the formation of many ecological niches that promote a multitude of microbial processes. Experiments made by Munch et al. (2005) revealed that roots of *Phragmites australis* enhanced nitrification and denitrification activity within 30-40 mm from the root surface. Because the average root-to-root distance was 35 mm, this root influence extended over the entire rooted part of the TW (Munch et al., 2005). Gagnon et al. (2007) reported that the microorganisms present in the TW rhizosphere had a significantly higher aerobic respiration rate potential compared to an unplanted control TW, suggesting that the root oxygen release influences the type of microorganisms present in the rhizosphere. These results tend to confirm the conventional wisdom that when aerobic processes are desired, the optimal depth for horizontal subsurface wetlands should be based on the nominal rooting depth of the dominant macrophyte plant species (Reed et al., 1995).

Oxygen loss, and hence redox potential and the diversity of the rhizosphere microbial community, likely varies by macrophyte plant species and other plant and

environmental conditions, but results are not consistent from study to study. As reviewed by Brix (1997) and Stottmeister et al. (2003) root oxygen release differs according to plant species. For example, oxygen release rates were observed to be highest in *Typha latifolia* (1.41 mg h^{-1} $plant^{-1}$), followed by *Phragmites australis* (1.0 mg h^{-1} $plant^{-1}$), *Juncus effusus* (0.69 mg h^{-1} $plant^{-1}$), and *Iris pseudacorus* (0.34 mg h^{-1} $plant^{-1}$) (Wiessner et al., 2002). Redox measurements in a full scale surface wetland showed that a cell planted with a mix of *Juncus* spp. and *Schoenoplectus* spp. had only moderately reduced soil conditions (+100 mV < E_h < +300 mV) while one planted with a mix of *Typha* spp. and *Sparganium* sp. had reduced (-100 mV < E_h < +100 mV) to highly reduced soil conditions (E_h < -100 mV) (Szogi et al., 2004). Stein and Hook (2005) found seasonally averaged redox values and COD removals were greatest in TWs planted with *Carex utriculata* followed by *Schoenoplectus acutus*, *Typha latifolia*, and unplanted TWs.

Reported variation between studies in species root oxygen release and redox potential may be due to other environmental factors. Some, but not all plant species generate redox potential diurnal fluctuations in response to variation in light intensity. For example, Sorrell (1999) showed that root oxygen release in *Juncus effusus* was positively influenced by light intensity, but not in *Juncus inflexus*. At low light intensity, the rhizosphere of *Juncus effusus* exhibited, on average, reduced redox conditions (E_h ≈ -250 mV), but reached moderately oxidized status (E_h ≈ 230mV) at high light intensity (Wiessner et al., 2005a). Higher oxygen transfer was also observed in *Phragmites australis* in response to higher light intensity, lower humidity and higher temperature, and

this increase was attributed to the enhancement of convective oxygen transport mechanisms to the root system (Armstrong and Armstrong, 1990, 1991).

Root oxygen release is also dependent on the redox state of the wetland matrix. Wiessner et al. (2002) found that the oxygen release rate was specific to the plant species under reduced conditions ($E_h \approx$ -400 mV to +200 mV) but depended on the physiological status of the plant at higher redox potential (more than +200 mV). Root oxygen release ceased when the immediate surroundings of the roots reached a highly oxidized redox condition. The relation between root oxygen release rate and the redox state of the environment was not linear and the highest release rate was in the range -250 mV < E_h < -150 mV. Reduction or stabilization of the oxygen release rate at highly reduced conditions was observed in other studies (Kludze and Delaune, 1994; Sorrell, 1999) and might be explained by physiological limitations to oxygen release and the finite quantity of root area permeable to oxygen diffusion (Sorrell, 1999; Bezbaruah and Zhang, 2004). A threshold capacity of plant oxygen release to compensate the demand at low redox potential might explain the generally low nitrification rates observed in horizontal subsurface wetlands treating domestic wastewater high in organic carbon.

Influence of Season on Redox Condition

The seasonal influence on TW performance can be particularly important in a cold climate, as near or below freezing temperatures and plant dormancy may affect important processes. It is generally known that microbial activity is linked to temperature, with bacterial growth and metabolic rates strongly reduced with decreasing temperature (Atlas and Bartha, 1998). For example, early studies reported that

nitrification activity is inhibited around 6-10°C and denitrification activity was detected only above 5°C (Herskowitz et al., 1986; Brodrick et al., 1988; Werker et al., 2002). Additionally, plant oxygen transport by convective flow, which is assumed to be the most efficient mechanism, greatly decreases at low temperature (Armstrong and Armstrong, 1991). Thus, most early TW design publications (e.g. USEPA, 1988; Reed et al., 1995) assumed poor pollution removal would occur during the winter season. However, numerous studies as reviewed by Kadlec and Knight (1996) and Kadlec and Reddy (2001) have shown that seasonal temperature variation did not always significantly affect COD and BOD removal. Several hypotheses have been proposed to explain this better-than-expected winter removal efficiency (Jenssen et al., 1993), including enhancement of aerobic (and more efficient) microbial degradation of organic matter due to increased redox potential with colder temperature (Stein and Hook, 2005).

Allen et al. (2002) demonstrated that diurnally-averaged redox potential was higher in wetland microcosms at 4°C compared to those at 24°C, when planted with *Carex utriculata* or *Schoenoplectus acutus*, but not for *Typha latifolia* and unplanted control microcosms. Consistent results were found at intermediate temperatures (Stein and Hook, 2005), indicating that seasonal variation in oxygenation of the rhizosphere is species-specific. Furthermore, COD removal did not decrease with decreased temperature in *C. utriculata* and *S. acutus* but did for *T. latifolia* and unplanted controls, suggesting that a shift to more aerobic microbial respiration in response to increased redox masked any temperature effect on microbial utilization of organic matter (Stein and Hook, 2005). Similar results were obtained in surface flow wetlands, where higher redox

potential was measured in the winter period (minimum 5°C) in cells planted with a mix of *Juncus* spp. and *Schoenoplectus* spp. However, cells planted with a mix of *Typha* spp. and *Sparganium* sp. always had low redox potential (Szogi et al., 2004). Higher winter redox potential associated with a particular plant species could be the result of low root respiration at cold temperatures, and therefore a greater quantity of oxygen transported to the roots reaches the rhizosphere (Stein and Hook, 2005). However, the reason some plant species demonstrate higher redox potential at low temperature and the underlying biological or physical mechanisms responsible for these processes remain unknown.

Redox potential is a broad indicator of the diversity and activity of various microbial populations. Manipulation of redox potential by variations in the design and/or operation of TWs is probably the best method available to encourage growth of desired microbial functional groups associated with the removal of a specific pollutant. Organic carbon removal occurs by all respiration types, but the rate, and hence efficiency, can be increased by promoting aerobic conditions. As discussed in the following sections, removal of nitrogen species and sulfur transformations important in metals removal are restricted to specific microbial respiration pathways and manipulation of redox is particularly important for these processes. Vertical flow and forced aeration wetlands clearly promote aerobic conditions and enhance removal of organic carbon and reduced nitrogen species, but they would be poor options for promotion of anaerobic/anoxic processes such as denitrification and sulfate reduction. Manipulation of feeding mode and plant species selection, especially in horizontal subsurface flow wetlands, also influences TW redox and there is a growing body of evidence that these manipulations

influence performance (positively or negatively depending on which respiration pathway is promoted compared to treatment objective) and temper typical seasonal influences on microbial growth. However, much more research is required to quantify the effects of these design variables so that their influence can be optimized for a specific treatment objective.

Nitrogen Removal

The importance of nitrogen removal is probably second only to organic carbon removal for TWs treating domestic wastewater. However, forms of influent nitrogen, e.g. organic N, ammonia, urea, and nitrate, are quite variable depending on source and level of pre-treatment, and removal expectations vary by geographical and political regions. In many cases, removal of ammonium through nitrification is considered satisfactory. Subsequent removal of nitrate through denitrification may also be required. There is some concern as to the extent to which denitrification proceeds to N_2 or whether there are significant emissions of the greenhouse gas N_2O. Also of interest is anaerobic ammonium oxidation (anammox) because of its potential to convert ammonium directly to N_2. However, removal of ammonium and nitrate are generally thought of as operationally separate processes (temporally or spatially) requiring aerobic and anaerobic conditions, respectively. Components of the nitrogen cycle in TWs have been well studied, and there is consensus that microbial processes dominate the transformations, but surprisingly little research has focused on direct microbial analysis; instead most studies rely on indirect measures such as inflow/outflow water quality to infer the presence and

activity of N transforming microbes. This section reviews what is known about microbial transformations of nitrogen in TWs.

Nitrification

Ammonium removal by nitrification has been documented in TWs differing in design and purpose, including municipal sewage treatment using surface flow TWs (Sundblad, 1998; Andersson et al., 2005) and vertical flow TWs (Cooper and Griffin, 1999), landfill leachate treatment in overland flow systems (Sundberg et al., 2007b), and in a constructed filter bed with an open pond system (Renman and Kietlinska, 2000; Sundberg et al., 2007a). Autotrophic nitrification consists of two successive aerobic reactions, the conversion of ammonium to nitrite by ammonium oxidizing bacteria (AOB, *Nitroso*-) and the conversion of nitrite to nitrate by nitrite oxidizing bacteria (NOB, *Nitro*-) (Hooper et al., 1997; Koops and Pommerening-Roser, 2001). AOB and NOB use CO_2 and bicarbonate for cell synthesis and ammonium or nitrite as the energy source (Hooper et al., 1997). AOB belonging to β-Proteobacteria include two genera, *Nitrosospira* and *Nitrosomonas*, which are divided in nine different phylogenetic clusters based on 16S rRNA gene (rDNA) sequences (Stephen et al., 1996; Purkhold et al., 2000; Purkhold et al., 2003). The genus *Nitrosospira* is included in clusters 0-4 and *Nitrosomonas* in clusters 5-8.

Complete nitrification stoichiometry requires 4.6 kg oxygen per kg NH_4^+-N and dissolved oxygen (DO) concentrations of 1 mg l^{-1} are sufficient for oxidation of ammonium (Hammer and Hammer, 2001). However, it has been observed in wastewater treatment systems that at DO concentrations lower than approximately 2.5 mg l^{-1} nitrite

oxidation is inhibited, leading to its accumulation (Paredes et al., 2007b). The oxygen transfer rate may be as important as the actual concentration. As discussed in the redox section, plants provide an oxygenated zone around the roots enhancing nitrification (Zhu and Sikora, 1995; Johnson et al., 1999; Munch et al., 2005), but in less aerated systems the transfer rate varies by plant species and other environmental and operational factors. Indeed, ammonium removal in subsurface TWs has been shown to vary with plant species (Kadlec and Knight, 1996; Eriksson and Andersson, 1999; Riley et al., 2005; Bojcevska and Tonderski, 2007) and a subsurface flow wetland had a high nitrification capacity when operated in tidal flow mode (Renman and Kietlinska, 2000), but a low activity when permanently flooded (Sundberg et al., 2007a). Pre-aeration did enhance ammonium removal in another study (Noorvee et al., 2007). However, no significant differences in quantity of microbial biomass or purification performance between planted and unplanted vertical flow TWs were detected by Tietz et al. (2007a), presumably due to higher oxygen levels in these unsaturated systems.

Seasonal differences in TW nitrification are notable (Kuschk et al., 2003; Song et al., 2006) and, like most biological processes, nitrification is sensitive to temperature (Painter, 1986; Hammer and Hammer, 2001). The optimal temperature for nitrifying bacteria is 28-36°C, however, significant TW nitrification has been observed at temperatures between 0 and 5°C (Sundblad and Wittgren, 1991; Sundberg et al., 2007b). Cookson et al. (2002) suggested that nitrifying communities can adapt to temperature changes and may maintain their activity at lower temperatures by metabolic adaptation. However, other studies have shown that nitrification is inhibited by water temperatures

<10°C and drops off rapidly below 6°C (Herskowitz et al., 1986; Xie et al., 2003). Alleman (1985) observed the accumulation of nitrite at cold temperatures, indicating that only ammonium oxidation was proceeding. Complete nitrification to nitrate is a desirable component of all treatment systems, including cold climate TWs (Jenssen et al., 2005).

Organic mass loading rates are supposed to affect nitrification in TWs due to competition for oxygen and inorganic nitrogen between heterotrophic and nitrifying bacteria (Thompson et al., 1995; Grunditz et al., 1998; Truu et al., 2005) which should be more intense at elevated OC concentrations (Prosser, 1989; van Niel et al., 1993). However, Tanner et al. (2002) found that nitrification and denitrification occurred concurrently with COD removal in early stages of a cascade mesocosm wetland receiving relatively organic rich wastewater. In batch-fed subsurface flow TWs, Riley et al. (2005) observed better ammonium removal at higher organic load rates in winter but poorer removal with higher organic loadings in summer, suggesting organic loading influences on nitrification interact with temperature and other factors. Nevertheless, there is evidence of competition between nitrifiers and heterotrophs in TWs. *Nitrosomonas* was one of the initial colonizers when rocks were placed in a TW (Silyn-Roberts and Lewis, 2001) but once the biofilm matured, heterotrophic bacteria outnumbered nitrifying bacteria. Heterotrophic bacteria were shown to reduce the AOB population in a TW biofilm (Okabe et al., 1996; Schramm et al., 1996) and in another system they collapsed an AOB biofilm by forming a thick layer on its surface (Nogueira et al., 2002).

A variety of environmental factors including temperature, pH, and salinity, as well as inhibiting substances such as ammonia and organic carbon loading may influence the diversity of nitrifiers (Schramm et al., 1996; Stephen et al., 1998; Kowalchuk et al., 2000; Webster et al., 2002). The distributions of AOB populations in a horizontal subsurface flow TW (Truu et al., 2005) and a filter bed/pond system (Sundberg et al., 2007a) were correlated with depth, total soil nitrogen, and total soil phosphorous. Nurk et al. (2005) observed higher potential nitrification close to the outlet compared to the inlet, perhaps due to a decrease of toxic substances and/or organic carbon during treatment.

AOB populations may diversify with different types of biofilm attachment sites. Greater numbers of nitrifying bacteria and higher activity were detected on macrophyte roots compared to the bulk matrix (Kyambadde et al., 2006) and differences were detected between AOB populations growing on living and detritus macrophyte tissue (Flood et al., 1999). AOB may also diversify within the TW biofilm itself and different organisms can be active in response to large micro-scale variations in the physiochemical environment as observed in unmanaged soil (Bruns et al., 1999; Ibekwe et al., 2002b; Webster et al., 2002).

Nitrosomonas spp. have a lower substrate affinity but higher maximum activity than *Nitrosospira* spp. (Schramm et al., 1996), therefore *Nitrosospira* spp. are likely more prevalent in low ammonia environments (Kowalchuk et al., 2000; Bäckman et al., 2003) and may be better at withstanding physicochemical variations (Purkhold et al., 2000; Bäckman et al., 2003). Additionally, high ammonia concentrations seem to create

systems dominated by single *Nitrosomonas* species (Schramm et al., 1996; Juretschko et al., 1998; Okabe et al., 1999). However, Sundberg et al. (2007b) found a more robust AOB community containing a *Nitrosomonas oligotropha*-like sequence, a *Nitrosococcus mobilis*-like sequence, as well as other *Nitrosospira*-like sequences (Purkhold et al., 2003) in an overland flow TW treating water high in ammonium. Similarly, Tietz et al. (2007b) found that a vertical flow TW receiving municipal wastewater supported a diverse AOB community that appeared to be unaffected by strong temperature changes. This community appeared to be comparable to other communities already observed in horizontal subsurface flow TWs (Ibekwe et al., 2003). In contrast, a TW consisting of a filter bed connected to an open pond supported a less diverse AOB community with *Nitrosospira*-like sequences (Sundberg et al., 2007a).

Denitrification

The large decrease in nitrate observed in most TW (excluding VF systems) suggests that denitrification is an important process (Billore et al., 1999; Xue et al., 1999; Lin et al., 2002; Kadlec, 2005; Sirivedhin and Gray, 2006; Vymazal, 2007). Denitrification in TWs was estimated to account for as much as 90% of overall N removal (Xue et al., 1999; Lin et al., 2002). Denitrification is a four-reaction process converting NO_3^- to N_2 via intermediaries ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) (Myrold, 1999). Gaseous NO and N_2O may be released during denitrification, but the major product is N_2 (Paul and Clark, 1989). Most denitrifying bacteria are facultative anaerobic chemoheterotrophs using organic compounds as electron donors and as a source of cellular carbon and nitrogen oxides (in ion and gaseous form) as terminal electron

acceptors (Hauck, 1984; Vymazal, 2007). The genera *Bacillus*, *Micrococcus* and *Pseudomonas* are most common in soils while *Pseudomonas*, *Aeromonas* and *Vibrio* are more common in aquatic environments (Grant and Long, 1981). It has been shown that the ammonia oxidizing bacteria *Nitrosomonas eutropha* is able to denitrify by using hydrogen as an electron donor, NO_2^- as an electron acceptor and producing NO and N_2O under anoxic conditions (Bock et al., 1995). Detection of denitrifying bacteria in the environment has generally targeted *nirK/nirS* genes (Braker et al., 1998; Angeloni et al., 2006) and *nosZ* genes (Gomez-Villalba et al., 2006); molecular markers for the conversion of NO_2^- to NO and N_2O to N_2, respectively.

Environmental factors that influence denitrification include the absence of oxygen, availability of organic matter and nitrate, appropriate redox potential, temperature, pH, soil type, and degree of moisture saturation (Focht and Verstraete, 1977; Vymazal, 1995). Denitrification activity increases with increasing temperature up to 60-75°C (Toet et al., 2003; Burchell et al., 2007). Denitrification rates were not only suppressed at low temperature in TWs (5°C) (Brodrick et al., 1988; Werker et al., 2002; Burchell et al., 2007), but major low temperature products are usually greenhouse gases N_2O and NO (Bremner and Shaw, 1958; Broadbent and Clark, 1965). Optimal pH is 6-8 (Paul and Clark, 1989) and the activity is low at pH 5 and absent in pH below 4 (Vymazal, 2007). Potential denitrification (when electron donors and acceptors are not limiting) was higher in an organic soil than in a mineral soil when the soils were incubated as wetland sediments (D'Angelo and Reddy, 1999; Davidsson and Stahl, 2000). Usually, the addition of organic carbon has been shown to increase denitrification activity

in TWs (Gersberg et al., 1983; Lin et al., 2002); however, at winter temperatures (7.5°C) denitrification activity did not respond to added organic matter (Burchell et al., 2007). Higher C:N ratios led to more complete denitrification, to N_2 (Hunt et al., 2003) and N_2O emissions from a TW were also correlated with oxygen consumption in the rhizosphere, regardless of season and plant growth (Inamori et al., 2007).

Plants may influence denitrification by supplying organic carbon through rhizodeposition of C substrates; significantly more nitrate removal was detected in a planted versus unplanted microcosm (Lin et al., 2002) and the effect was species-specific. Nitrate removal was highest in TWs planted with *Penniserum purpureum* (Lin et al., 2002). Bachand and Horne (2000) also reported differences in nitrate removal rates between different emergent macrophyte communities. Potential denitrification varied significantly between vegetation communities (Toet et al., 2003; Bastviken et al., 2007), and between biofilm attachments sites within a TW. Bastviken et al. (2003) measured the highest potential in sediments, with rates three times higher than on old pine and spruce twigs, and 40 times higher than on shoots of Eurasian watermilfoil (*Myriophyllum spicatum* L.). Denitrification was also higher on *Potamogeton perfoliatus* shoots with a higher periphyton abundance than on those with low abundance (Weisner et al., 1994).

Higher activity was detected in the upper layer in a filter bed (Sundberg et al., 2007a), but in open water, activity increased with depth (Hernandez and Mitsch, 2007). Higher potential denitrification was detected in stands of emergent macrophytes than in open water (Hernandez and Mitsch, 2007). However, ten times higher nitrogen removal was detected in a surface flow constructed wetland with 50% plant cover compared to

one with 100% plant cover. This difference was attributed to possible short-circuiting due to litter accumulation (Ibekwe et al., 2007).

The potential denitrification in soil has been shown to increase significantly when it is converted to a wetland. A linear relationship between denitrification activity and sediment organic matter content was detected, but only in the upper layer (Hernandez and Mitsch, 2007; Sundberg et al., 2007a). Denitrification activity in sediment was correlated with nitrate concentrations in the overlying water (Toet et al., 2003) or in the sediment itself (Hunt et al., 2003; Toet et al., 2003). The activity was increased by the addition of nitrate but it was unaffected by addition of C, even at the high levels of soil nitrate (Toet et al., 2003).

Relatively few studies have looked at denitrifier density and diversity. T-RFLP analysis detected differences in bacterial community structure between the TW inlet and outlet (Ishida et al., 2006). Kjellin et al. (2007) found the least complex DGGE pattern of *nosZ* genotypes close to the inlet of a TW. They suggested that longer residence times result in more complex communities due to decreasing nitrogen and carbon levels in the sediments. In the upper layer of a filter bed and pond system treating landfill leachates, the denitrifying bacterial community increased in numbers and diversity during the growing season (Sundberg et al., 2007a).

Anammox

Anammox is an anaerobic ammonium oxidation reaction converting NO_2^- and NH_4^+ to N_2 (Mulder et al., 1995; Van de Graaf et al., 1996; Jetten et al., 1998). Ammonium is the electron donor and NO_2^- is the electron acceptor under anaerobic

conditions at a redox level around +200 mV. Anammox is almost as energetically favorable as the aerobic nitrification reaction (Mulder et al., 1995) and is performed by planctomycete-like bacteria *Candidatus Brocadia anammoxidans* which dominate in wastewater treatment and *Candidatus Kuenenia stuttgartiensis* which often dominate in bacterial biofilms (Schmid et al., 2000; Sliekers et al., 2002). These bacteria were detected by FISH in wastewater treatment systems with large nitrogen removal (Schmidt et al., 2002). Compared to true nitrifiers, anammox bacteria more easily coexist with heterotrophic bacteria because heterotrophic consumption of oxygen creates a more anoxic environment beneficial to anammox bacteria but in competition with nitrifiers. Based on the environmental factors favoring anammox bacteria, it seems likely that they would exist in TWs, however direct evidence is limited (Dong and Sun, 2007). A distinct advantage of anammox in TWs is the potential to accomplish ammonia removal with N_2 as the primary product. In an anammox reactor, about 85% of the ammonia is converted to N_2, 15% to NO_3^-, and less than 0.1% to N_2O. Denitrifiers could presumably convert the created NO_3^- to N_2 (Dong and Sun, 2007), suggesting anammox as a process for the complete conversion of ammonia to N_2 without generation of excessive greenhouse gases.

As with conventional wastewater treatment systems, the literature suggests promotion of aerobic zones within a TW increases the overall rate of nitrification and decreases the rate of denitrification. Lower temperature typically decreases the nitrification rate, but if lower temperature enhances other environmental factors beneficial to nitrifiers, such as increased redox potential, the temperature effect may be

limited. Many studies suggest that differences in nitrification between systems might be due to differences in nitrifier microbial diversity and/or temporal shifts in diversity due to changes in environmental factors. However, far too few studies have been performed to predict how nitrifier diversity might be manipulated to enhance performance. Even fewer studies have employed microbial techniques to assess the process of denitrification in TWs. This is probably because the process is often considered less essential for water purification and horizontal subsurface flow systems appear to have acceptable denitrification rates. The overall environmental conditions of a TW may be favorable for growth of anammox bacteria, but very little research into this promising group has been conducted.

Sulfur Cycling

Sulfur cycling in treatment wetlands is an important process as sulfur is present in considerable amounts in all forms of wastewater, especially acid mine drainage (AMD). Depending on conditions, sulfur cycling can vary greatly within different zones of a single TW (Scholz and Lee, 2005). Though microbial activity is not the only means by which sulfur is transformed in TWs (Sturman et al., 2008) most of the extensive research conducted on sulfur cycling within TWs has acknowledged the importance of microbial populations, particularly sulfate reducing bacteria (SRB). However, as with the carbon and nitrogen cycles, few studies have employed microbial techniques.

Many factors can affect microbial sulfur cycling including carbon availability, the presence of more energetically favorable elements and redox conditions (Table 2.4). The relative concentration of sulfate to other electron acceptor compounds will determine

which microbial processes are occurring. For example, denitrification is more energetically favorable with sulfate reduction occurring only after all the nitrate has been removed (Whitmire and Hamilton, 2005) and methanogens are known to compete with sulfate reducers for available carbon at similar redox levels (Omil et al., 1998). It has been shown that oxygen released from the roots may be used to re-oxidize reduced metabolites formed in the sulfur and iron cycles (Brune et al., 2000).

Sulfate Reduction

Sulfate reducing bacteria (SRB) are among the most ubiquitous organisms on the planet. These organisms utilize sulfate as a terminal electron acceptor in the anaerobic oxidation of organic substrates (Hsu and Maynard, 1999). SRB are critically important as they are the only known organisms to perform this function. Their role in the geochemical cycling of sulfur is vital to many biological processes and is also important to the generation of alkalinity in TW systems (Kalin et al., 2006).

Historically, SRB have been considered strict anaerobes and extremely sensitive to low temperature, but more recent research has begun to question these paradigms. Some species have been discovered to persist in oxic conditions and survive extended periods of oxygen exposure (Brune et al., 2000; Cypionka, 2000; Sigalevich et al., 2000; Holmer and Storkholm, 2001). Sageman et al. (1998) showed SRB grow well at low temperatures suggesting that sulfate reduction may be more dependent on substrate concentration and supply than temperature alone. Low temperatures reduced SRB establishment in laboratory AMD bioreactors, but SRB communities established at room

temperature showed no decline in sulfate reduction when temperatures were later decreased to 6°C (Tsukamoto et al., 2004).

Many studies have assumed SRB activity in TWs based on the following statements: high sulfate input, minimal sulfate uptake by TW plants (Whitmire and Hamilton, 2005), low redox conditions, low sulfate output and higher sulfide concentrations (Song et al., 2001; Stein et al., 2007). Considering the ubiquity of SRB and uniqueness of the reaction, these assumptions seem warranted.

System age, plant species and season influence the activity of SRB in TWs. Less effective and more variable sulfate removal rates were observed in newly created TWs with insufficient time for microbial community establishment or acclimatization (Kalin and Chaves, 2003; Wiessner et al., 2005a). Baptista et al. (2003) and Weber et al. (2008) used microbial techniques to identify SRB communities in TWs planted with *Phragmites australis*. Baptista et al. (2003) attributed 25% of the carbon removal to the SRB community, while Weber et al. (2008) found different and more robust microbial communities (including SRB) in mesocosms planted with *Phragmites australis* than in unplanted mesocosms. The microbial community within the planted mesocosms was more capable of handling disturbances than the community in the unplanted mesocosms. Stein et al. (2007) estimated the SRB contribution to COD removal in summer and winter for two plant species and unplanted TW microcosms. Summer (24°C) and winter (4°C) values were: unplanted microcosms, 25%/40%; *Typha latifolia* 30%/15% and *Schoenoplectus acutus* 30%/0%, at the same sulfate and carbon loading. The system was never sulfate limited.

Though a few studies, e.g. Fortin et al. (2000), have demonstrated better sulfate removal in winter and in higher redox conditions, results across most TW studies seem reasonably conclusive that warmer temperatures and lower redox potentials foster conditions ideal for larger and more robust SRB populations and therefore better sulfate removal (Hsu and Maynard, 1999; Borden et al., 2001; Stein and Hook, 2005). Plant effects seem most prevalent in the winter months when plant activity is at its lowest and redox potential within the TW sediments is highest (Borden et al., 2001; Holmer and Storkholm, 2001; Stein and Hook, 2005; Stein et al., 2007). However, plant effects are reversible if the carbon loading rate is increased to create more anoxic conditions (Borden et al., 2001; Stein and Hook, 2005; Wiessner et al., 2005a; Stein et al., 2007). *Typha* spp. seem to be the desired species for consistent year round sulfate removal as they have been shown to maintain the lowest redox conditions regardless of season (Hsu and Maynard, 1999; Allen et al., 2002). High sulfate reduction rates have also been observed in the rhizosphere of natural wetlands planted with *Spartina alterniflora* (Bahr et al., 2005).

Only a few studies have used traditional microbial or molecular methods to assess SRB density and diversity within TWs. King et al. (2002) showed diversity changes with depth and different plant species. *Desulfobacter* was prominent in upper sediment layers (0-90 mm) planted with *Scirpus californicus* while *Desulfovibrio*, *Desulfobulbus*, *Desulfococcus*, and *Desulfobacterium* were present in high numbers at depths greater than 90 mm. In TWs planted with both *S. californicus* and *Potamogeton pusillus*, fewer SRB were found in the upper layers and all SRB populations increased with depth. The

presence of both *S. californicus* and *P. pusillus* seemed to limit the quantity of SRB found at the surface of the TW but diversified the SRB population found at lower depths. *Desulfovibrio* species have also been isolated from other wetlands being used to treat AMD (Russell et al., 2003; Lloyd et al., 2004) and appear to have symbiotic relationships with other microbes. Russell et al. (2003) showed that *Desulfovibrio* (most closely related to *D. alcoholovorans*) and another unnamed SRB were capable of degrading algal biomass as their sole carbon source and were identified in a coculture with a potentially new genus related to *Clostridia*. Lloyd et al. (2004) identified *Desulfovibrio* spp. utilizing the by-products of sucrose fermentation, generated by *Clostridium acetobutylicum* and *Clostridium butyricum*, as their electron donors.

Sulfur Oxidation

The exact mechanisms and magnitude of sulfur oxidation within the wetland sulfur cycle are not well understood (Holmer and Storkholm, 2001). Known sulfide oxidation processes include chemical oxidation with O_2, anoxic chemical oxidation, bacterial oxidation under oxic conditions (by aerobic chemolithotrophic organisms such as *Acidithiobacillus*), phototrophic oxidation (by photosynthetic organisms such as *Chromatium* and *Chlorobium*), and bacterial oxidation under anoxic conditions (by filamentous organisms such as *Beggiatoa*) (Holmer and Storkholm, 2001). In the case of many sulfur oxidizing bacteria (SOB), sulfide is oxidized to sulfur utilizing an electron acceptor other than oxygen, followed by the migration of these microbes to more oxic layers where the sulfur is completely oxidized to sulfate (Brune et al., 2000).

In marine ecosystems SOB have been shown to use nitrate to oxidize sulfide (Fossing et al., 1995; Bonin, 1996; Philippot and Hojberg, 1999; Zopfi et al., 2001). Whitmire and Hamilton (2005) observed a similar increase in sulfate concurrent with a decrease in nitrate in freshwater wetlands. It remains unclear whether the marine SOB are denitrifiers or produce ammonium in a form of dissimilatory reduction of nitrate to ammonium (DNRA), but it is possible that they are capable of switching between these two pathways. If the DNRA pathway is utilized, the nitrogen remains biologically available in contrast to the denitrification pathway (Whitmire and Hamilton, 2005). Zhang and Lampe (1999) showed that the SOB *Acidothiobacillus thiooxidans* and autotrophic denitrifiers were responsible for sulfate production in the presence of nitrate (in batch reactors), with *A. thiooxidans* solely responsible for sulfate production when nitrate had been completely consumed. Based on these studies, it is conceivable that SOB play a role in denitrification in TWs.

Only a few studies address SOB activity in treatment wetlands. One study, by Nicomrat et al. (2006b), investigated the microbial profiles of a TW planted with *Typha* spp. treating AMD using DGGE. Sequencing results revealed two bands matching the iron oxidizer *Acidithiobacillus ferrooxidans* with another band matching the SOB *Acidithiobacillus thiooxidans*. *A. thiooxidans* is a chemolithotrophic, aerobic, acidophile that is capable of growth on elemental sulfur as an energy source (Zhang and Lampe, 1999). Additional bands also matched the eubacterium *Alcaligenes* sp. and the aresenite oxidizing bacterium *Bordetella* sp. It is suspected that the *Alcaligenes* and *Bordetella* were probably a wildlife contribution, as they have no known physiological function in

AMD. DGGE profiles showed higher microbial diversity in the TW sediments compared to the profiles of the enrichment cultures, as would be expected. There were three bands common in all enrichment DGGE profiles indicating that these organisms are dominant bacteria within the system (Nicomrat et al., 2006b). In addition, *Thiomonas* (an SOB) was identified in the effluent of a TW also treating AMD and has been implicated in the oxidation of ferrous iron (Hallberg and Johnson, 2005).

Sulfur Transformation

Interactions with Other Processes

Microbial sulfate reduction and metal precipitation are closely related, and several review articles are available on the subject including Kosolapov et al. (2004) focusing specifically on treatment wetlands. Therefore, factors influencing SRB activity; including insufficient amounts of available carbon, excess oxygen (or other more favorable terminal electron acceptors), or low temperatures may limit metal sulfide precipitation (Stein and Hook, 2005). Depending on conditions, certain factors may be more influential than others. For example, species and seasonal variation in redox potential was more important than variation in carbon loading for zinc removal rates (Borden et al., 2001). As predicted by information in Table 2.4, microbial iron reduction dominated over other anaerobic carbon metabolic pathways such as sulfate reduction and methanogenesis in brackish tidal marshes (Neubauer et al., 2005). Increased microbial iron reduction appeared to be seasonal and led to increased concentrations of soluble Fe^{2+}. Similarly, Kalin et al. (2006) showed that in bioreactors treating AMD with high concentrations of dissolved iron and sulfate, sulfate reduction occurred only after microbial activity had fully reduced Fe^{3+} to Fe^{2+}.

Of particular note are the TW studies associated with the Wheal Jane site in Cornwall, UK for AMD treatment and metals removal. This site uses five aerobic reed beds planted with *Typha*, *Phragmites*, and *Scirpus* to remove iron and arsenic, followed by anaerobic cells for sulfate reduction and zinc, copper, cadmium, and iron removal, and finally, aerobic rock filters to promote algal growth and assist in manganese precipitation (Hallberg and Johnson, 2005; Whitehead et al., 2005). The AMD is the source of most of the reducing bacteria present within the TW system. Increasing numbers of acidophilic iron-oxidizers were observed as the pH of the water decreased across the five aerobic cells. Sediment samples had a greater diversity of acidophiles than surface water samples. Aerobic cell sediments were dominated by moderately to extremely acidophilic organisms but no particular sub-group dominated. More alkaline wetland effluent had a higher fraction of moderate acidophiles and the next most common group of organisms in the effluent was heterotrophic acidophiles. Organisms identified within the Wheal Jane TW were: the extremely acidophilic iron oxidizing microbes *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, the moderately acidophilic iron-oxidizers most closely related to *Halothiobacillus neapolitanus* and *Thiomonas intermedia*, an organism related to the iron-oxidizing heterotroph *Ferrimicrobium acidiphilum*, heterotrophs belonging to genus *Acidiphilium*, and others related to genus *Frateuria*, and finally, heterotrophs highly related to *Propionibacterium acnes* and *Acidobacterium capsulatum* (Hallberg and Johnson, 2005). Overall, the heterotrophs tended to outnumber the iron-oxidizing autotrophs. *Thiomonas* (an SOB, mentioned previously) was also found in the effluent of the Wheal Jane TW. The presence of this organism may

potentially reverse the AMD remediation process by catalyzing the oxidation of ferrous iron back to ferric iron (Hallberg and Johnson, 2005).

Sulfate reducers from within this TW precipitated metals much more slowly than SRB isolated from natural wetland systems and differences may be related to variation in the ability of SRB to use available substrates and hence hydrogen sulfide production (Webb et al., 1998). As expected by MnS solubility, manganese was not removed by any of the SRB isolated from the TW. Additionally, metal removal in the Wheal Jane system does not appear to be solely driven by the presence or concentration of sulfide; other unknown mechanisms appear to be in play (Webb et al., 1998).

Microorganisms other than SRB may have potential benefits for metal removal. One such organism is the iron-reducer, *Geobacter metallireducens*. When added to TW samples, the presence of this bacterium resulted in a 77% reduction in iron concentration in rhizosphere samples from *Typha latifolia* and a 30% reduction in soil samples (Weiss et al., 2004). The ecological effects on removal rates of other metals within TWs have also been studied. These include zinc (Borden et al., 2001; Song et al., 2001; Collins et al., 2004; Stein et al., 2007), copper (Gammons and Frandsen, 2001; Scholz et al., 2001), lead (Scholz et al., 2001; Song et al., 2001; Scholz and Xu, 2002), aluminum (Collins et al., 2004), and selenium (Azaizeh et al., 2003) among others. The accumulation of dissolved metals in the litter zone of TWs has been shown to lead to a reduction in bioactivity (as determined by the presence of ciliated protozoa and zooplankton) suggesting potential negative effects for the system's overall ecology (Scholz et al.,

2002). The long-term performance of metal removing TWs may be compromised if important bacteria are similarly affected.

Microbial sulfur transformations within TWs are very dynamic and, with the partial exception of SRB communities, are poorly understood with regard to the actual organisms responsible for the majority of the occurring processes. However, the general success of TWs for metals removal and comparison of sulfate and organic carbon removal rates in many other TWs suggests that SRB activity is an important component of the carbon as well as sulfur cycle (Sturman et al., 2008). Though not yet proven, circumstantial evidence suggests that SOB activity may be important in the carbon, nitrogen and metals cycles within TWs (Whitmire and Hamilton, 2005; Nicomrat et al., 2006b; Sturman et al., 2008). Considering the importance of the sulfur cycle in its own right, and its potential influence on other important transformations within a TW, more research emphasis on the microbial populations driving sulfur transformations seems warranted.

Summary

The microbiology of treatment wetlands is the most important factor influencing the removal of pollutants in these systems. However, its complexity has forced scientists and engineers to largely ignore the underlying details of microbial processes and use a "black box" approach to the design and operation of these systems. Quite recent application of newer molecular and genetic microbial techniques to the study of TWs has opened a new era of treatment wetland research. Results to date generally confirm the existence of the appropriate microbial functional groups, e.g. nitrifiers, denitrifiers, SRB,

SOB etc., responsible for removal of specific pollutants in TWs. We believe future research will shift from mere identification and spatial location of these functional groups to environmental factors influencing the activity of them. When we understand what controllable factors turn critical functional groups on and off we will be able to fully optimize performance for removal of a specific pollutant, or perhaps still be able to achieve the "perfect" TW system that can satisfactorily remove virtually all pollutants from domestic wastewater, AMD or other sources.

Nevertheless, it is clear that our current black box approach to TW design has allowed us to shift the internal TW environment to favor one functional group over another. Research confirms that manipulation of redox by use of different TW types, e.g. vertical flow, horizontal subsurface flow, forced aeration, can shift dominance between various microbial functional groups and affect performance. Vertical flow and forced aeration systems will favor aerobic microbial populations and pollutant removal mechanisms while horizontal subsurface flow systems will typically favor anoxic or anaerobic populations. Feeding mode, e.g. batch, intermittent, continuous, especially within the most commonly used subsurface TW type, appears to have a secondary influence on performance with batch feeding favoring more aerobic processes and continuous feeding favoring more anaerobic processes. More research into microbial population density and diversity heterogeneity, both spatially and temporally, between feeding modes will help to further optimize design of horizontal subsurface systems.

Less certain is the role of plants and the overriding influence of season on redox status and appurtenant microbial populations. Research seems fairly conclusive that

plants increase the likelihood of occurrence for aerobic processes compared to unplanted "wetlands" and there is evidence to suggest that some plants create a more aerobic root zone than others. However, results are inconclusive as to the potential ranking of species. The factors which influence how aerobic a plant might make its root zone and the mechanisms by which it does so are quite speculative. Limited research indicates some plants induce a seasonal shift to more aerobic conditions in winter which helps mitigate the expected negative effect of temperature on organic carbon removal. Considering the well-established interactions between plants and microbes in agronomic settings, it seems quite probable that manipulation of plant species might be as important for the enhancement of desirable microbial functional groups as wetland type and is an area ripe for additional research.

Contribution of Authors and Co-authors

Chapter 3: Characterization of sulfate reducing bacteria in constructed wetlands

Author: Jennifer L. Faulwetter
Contributions: Designed and conducted the experiments included as well as analyzed the resultant data. Compiled and organized the data into the publication and was actively involved in the editing and submission process.

Co-author: Mark D. Burr
Contributions: Assisted in data analysis and publication compilation, as well as contributed to revision and editing of the paper. Was also a co-PI and advisor on the project.

Co-author: Otto R. Stein
Contributions: Assisted in editing the publication as well as presented the paper and poster at the India conference. Also was a PI and advisor on the project.

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CHAPTER 3

CHARACTERIZATION OF SULFATE REDUCING BACTERIA IN CONSTRUCTED WETLANDS

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Abstract

The types, densities, and distributions of microbial communities present in constructed wetland (CW) environments are poorly understood. Information gaps exist with regard to microbial community structure and function and their relationship to plant species, season, and wastewater type. One functional group of particular interest in CWs is sulfate reducing bacteria (SRB) because they can remove Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) in anoxic environments. Furthermore, the sulfide by-product of SRB activity can sequester heavy metals. SRB are critically important since they are the only known organisms to reduce sulfate to sulfide. The goal of this research was to characterize and identify the SRB present in a CW that had been previously used to treat simulated acid-mine wastewater. Samples were taken from an

unplanted control CW, since it maintained the most anoxic conditions throughout the year. Samples were enriched for SRB using a variety of electron donors and the enrichment cultures were analyzed using molecular techniques to identify key members of the sulfate reducing community. SRB community composition appeared to be affected by the enrichment culture media used. Closest BLAST relatives of the SRB clones sequenced included *Desulfobulbus rhabdoformis*, *Desulfomicrobium apsheronum*, *Desulfotomaculum nigrificans*, and *Desulfotomaculum alkaliphilum.*

Keywords: Constructed wetlands, Denaturing Gradient Gel Electrophoresis (DGGE), sulfate reducing bacteria (SRB)

Introduction

Sulfur cycling in constructed wetlands (CW) is an important process since sulfur is present in considerable amounts in all forms of wastewater, especially acid mine drainage (AMD). Depending upon conditions, sulfur cycling can vary greatly within different zones of a single CW (Scholz and Lee, 2005). Though microbial activity is not the only means by which sulfur is transformed in CW (Sturman et al., 2008), most of the extensive research into sulfur cycling within CW has confirmed the importance of microbial populations, particularly sulfate reducing bacteria (SRB). SRB also contribute to wastewater treatment because they are capable of removing Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) in anoxic environments.

SRB are among the most ubiquitous organisms on the planet. They utilize sulfate as a terminal electron acceptor in the anaerobic oxidation of organic substrates (Hsu and

Maynard, 1999). The basic chemical reaction for sulfate reduction is (Odom and Singleton, 1993):

$$2CH_2O + SO_4^{2-} + H^+ \rightarrow 2CO_2 + 2H_2O + HS^-$$

SRB are critically important since they are the only known organisms to perform this function. Their role in the geochemical cycling of sulfur is vital to many biological processes and is also important to the generation of alkalinity in CW (Kalin et al., 2006).

SRB are mainly found below the soil (or water) surface where anoxic environments are ideal for sulfate reduction. Different species of sulfate reducers have been found in different environments, such as water-logged soils rich with organic matter as well as the spermosphere and rhizosphere of plants. The most efficient sulfate reduction occurs at redox potentials between -100 mV and -250 mV. Historically, SRB have been considered strict anaerobes, but more recent research has shown that SRB can persist in oxic conditions and survive extended periods of oxygen exposure (Brune et al., 2000; Cypionka, 2000; Sigalevich et al., 2000; Holmer and Storkholm, 2001).

Many studies have assumed SRB activity in CW based on observations of high sulfate input, low sulfate output, high sulfide concentrations, minimal sulfate uptake by CW plants (Whitmire and Hamilton, 2005), and low redox conditions (Song et al., 2001; Stein et al., 2007). Stahl et al. (2007) extensively reviewed the use of molecular techniques to study SRB in the environment, but these methods have not been applied to CW. In this study, we report enrichment culture and molecular evidence for the presence of SRB in a model CW microcosm. SRB were enriched using a suite of separate electron donors. Denaturing gradient gel electrophoresis (DGGE) was used to analyze the PCR

products amplified from an SRB-specific gene (*dsr*). DGGE separates PCR products from different members of a microbial community based on sequence differences in PCR products amplified using conserved primers (Muyzer et al., 1993). DGGE was also used to display the SRB diversity contained in clone libraries of *dsr* PCR products. The migration patterns of individual *dsr* clones were used to differentiate them. In addition, pools of clones were created and analyzed by DGGE in order to estimate overall SRB diversity within the CW (Burr et al., 2006).

Methods

Gravel samples (1-5 mm diameter) were extracted from the base of an unplanted wastewater treatment column (15 cm diam. x 30 cm PVC pipe) immediately following draining. The column had received synthetic wastewater (Taylor et al., 2008) simulating secondary domestic effluent. The wastewater contained ~490 mg/L COD, ~0.8 mg/L NO_3, ~8 mg/L PO_4, and ~14 mg/L SO_4 (Taylor et al., 2008). Columns were filled, incubated for 20 days, drained, and refilled. The sample column had received wastewater for more than one year by the time it was sampled. Columns had been maintained in a greenhouse in which the temperature was set to mimic natural seasonal ambient temperatures (Bozeman, MT, USA 46^{o} N, 111^{o} W). Starting on approximately January 1, the annual temperature regime consisted of the following ~60-day increments: 4, 8, 16, 24, 16, and 8 ^{o}C. Gravel samples, collected at 4 ^{o}C, were immediately placed into sterile 50 mL screw cap tubes and sealed to minimize oxygen exposure. Therefore, the only exposure was from the oxygen in the tube headspace during the time of transport to an anaerobic glove box (~45 min.).

In an effort to culture as much SRB diversity from the wetland microcosm as possible, twelve types of media were formulated for cultivation by modifying Postgate Medium B (Postgate, 1984) (Table 3.1). Six different carbon sources/electron donors (lactate, acetate, butyrate, propionate, benzoate, palmitate) were added to the basic medium. In addition, one set of media contained ascorbic and thioglycolic acid while a second set was made without these compounds because these vitamins have been shown to have either positive or negative effects on cultivation of specific SRB (Postgate, 1984). The individual electron donors were also supplemented with yeast extract to achieve the same total carbon content as in the original Postgate Medium B. Media were dispensed in 9 mL volumes into 10 mL serum bottles in an anaerobic glove box and the serum bottles were crimped and autoclaved.

Table 3.1. Constituents of the 12 SRB enrichment media. Each medium contained all of the mineral constituents of Postgate Medium B (Column 1), one of six electron donors (Column 2), and supplemental yeast extract (corresponding row, Column 3). In addition, each medium was formulated both with and without ascorbic acid (0.1 g/L) and thioglycolic acid (0.1 g/L).

Postgate Medium B (mineral salts only)		Electron Donors		Yeast Extract
(1)		(2)		(3)
KH_2PO_4	0.5 g/L	sodium lactate (60%)	3.0 g/L	1.0 g/L
NH_4Cl	1.0 g/L	sodium acetate•$3H_2O$	2.0 g/L	0.67 g/L
$CaSO_4$•$2H_2O$	1.0 g/L	n-butyric acid	0.8 g/L	0.27 g/L
$MgSO_4$•$7H_2O$	2.0 g/L	propionic acid	0.7 g/L	0.23 g/L
$FeSO_4$•$7H_2O$	0.5 g/L	p-aminobenzoic acid	0.5 g/L	0.17 g/L
		n-palmitic acid	0.5 g/L	0.17 g/L

In an anaerobic glovebox, 4-5 pieces of gravel were transferred into a sterile 15 mL screw cap tube that had previously been filled, also under anaerobic conditions, with fine sterile sand to the 3 mL mark and sterile 0.85% saline up to the 5 mL mark. The tube was then vigorously shaken for 30 s to help facilitate removal of bacterial biofilms colonizing the gravel surface. The debris was allowed to settle and the supernatant was collected from each tube and placed into a sterile 50 mL screw cap tube. It was necessary to repeat the process seven times in order to collect at least 30 mL of supernatant. Serum bottles containing 9.0 mL of each of the 12 types of media were inoculated, in duplicate (24 total), by asceptically injecting 1 mL of the supernatant from the biofilm extraction through the septum. Serum bottles were incubated at room temperature. Cultures positive for SRB were indicated by a black precipitate (FeS_2) forming at the bottom of the bottle (Figure 3.1).

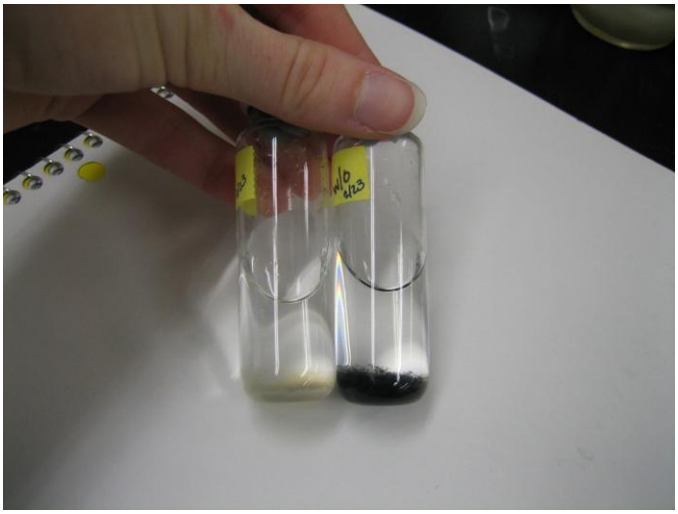

Figure 3.1. Serum bottles of sulfate reducing medium following incubation to detect SRB activity. The bottle on the left is an uninoculated control. The bottle on the right indicates an SRB-positive sample (accumulation of FeS_2).

Duplicate 1.0 mL aliquots were anaerobically extracted from positive serum bottles and placed into 1.5 mL centrifuge tubes. Tubes were centrifuged at 10,000 x g for 10 minutes and the supernatant discarded. Pellets were resuspended in 0.5 mL DEPC water and the duplicate samples were combined. Tubes were again centrifuged at 10,000 x g for 10 minutes and the supernatant was again discarded. Pellets were resuspended in 1.0 mL of DEPC water and centrifuged (as before) a total of 3 times to thoroughly wash the cell pellets. Pellets were resuspended in 0.2 mL DEPC water and transferred to MO BIO PowerBead Tubes (MO BIO PowerSoil™ DNA Isolation Kit). The PowerSoil™ DNA Isolation Kit was used to complete the DNA extraction as described in the manufacturer's protocol with the exception of that PowerBead tubes were placed into the FastPrep® Instrument (Qbiogene, Inc.) at speed 5 for 30 s. DNA yield was estimated on an agarose gel with ethidium bromide staining, serial dilutions were performed for PCR, and the DNA preparations were stored at -20°C.

Oligonucleotide primers were synthesized by Integrated DNA Technologies (www.idtdna.com). PCR primers DsrBF (5'CAACATCGTYCAYACCCAGGG 3', Geets et al., 2006) and Dsr4R (5'GTGTAGCAGTTACCGCA 3', Bahr et al., 2005) target the sulfite reductase gene (*dsr*, required for sulfate reduction). Presumptive presence of the *dsr* gene was indicated on an agarose gel by a 350 bp PCR product. Primer DsrBF was also synthesized with a 5' 40-bp GC clamp (5'CGCCCGCCGCGCCCCGCGCCCG GCCCGCCGCCCCCGCCCC 3', Ferris et al., 1996) and was paired with primer Dsr4R for amplifying fragments to be analyzed by DGGE. PCR reactions (20 or 50 μL) were performed using 2X PCR Master Mix (www.promega.com). For 20 μL reactions, the

PCR reaction mixture consisted of 10 μL 2X PCR Master Mix, 3 μL DEPC-treated water, 1 μL 12.5 μM forward and reverse primers, and 5 μL unquantified template DNA. For 50 μL reactions, the PCR reaction mixture consisted of 25 μL 2X PCR Master Mix, 19 μL DEPC-treated water, 2 μL 12.5 μM forward and reverse primers, and 5 μL template DNA. All PCR amplifications were performed on an Eppendorf Mastercycler® ep thermal cycler (Eppendorf North America, www.eppendorfna.com) using the following program. An initial denaturation for 4 min at 94°C was followed by a total of 35 cycles of amplification consisting of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s. The program ended with an extension step at 72°C for 10 min (Geets et al., 2006). PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide and were either used for cloning or were analyzed by DGGE (Burr et al., 2006).

PCR products were cloned into plasmid vector pCR®2.1 using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. White colonies indicative of a successful cloning reaction were used to inoculate sterile microcentrifuge tubes containing 0.5 mL of Luria-Bertani (LB) broth plus 50 mg mL^{-1} ampicillin or kanamycin. The tubes were incubated overnight at 35°C in a shaking incubator. Pools of clones were obtained by pipetting 20 μL stationary phase LB broth from each of 10 clones into a single 2 mL tube. Plasmid DNA was purified from the pools or from individual clones using the Wizard Plus SV Minipreps kit (www.promega.com) and stored at -20°C. Plasmid DNA was amplified for DGGE analysis using the same PCR reagents and conditions.

DGGE was performed on PCR products from community chromosomal DNA, from individual clone plasmid DNA, or from the plasmid DNA of pools of clones, using a DCode™ system (www.biorad.com) and reagents from Sigma-Aldrich (www.sigmaaldrich.com). Gels had a gradient of denaturant concentrations from 40% at the top of the gel to 70% at the bottom, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained an 8 to12% polyacrylamide gradient from top to bottom (Girvan et al., 2003). Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr®Gold (www.molecularprobes.com) and documented using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com). Bands in DGGE images were identified visually on a presence–absence basis. Band intensities were not physically measured, but visually prominent bands were considered to represent numerically significant members of the SRB community and were candidates to be matched with individual clones. Clones were sequenced in both directions from purified plasmid DNA with either primer M13F or M13R (TOPO® TA Cloning kit) using the Big Dye™ Terminator Cycle Sequencing kit (www.appliedbiosystems.com). Sequencing reaction products were analyzed on an ABI310 DNA sequencer (www.appliedbiosystems.com). Edited sequences were compared with known *dsr* sequences in the GenBank database using the Basic Local Alignment Sequence Tool (BLAST) (www.ncbi.nlm.nih.gov).

Results and Discussion

An unplanted gravel column that had been operated as a model CW microcosm was chosen as a likely environment from which to culture SRB because unplanted columns had previously been shown to be more anoxic than planted columns (Stein et al.,

2007). CW plants are known to release oxygen through their roots, and this effect may actually be enhanced in cold weather when plants are dormant (Stein and Hook, 2005). Although there was an attempt to limit exposure of the sample to oxygen during processing, sampling in ambient air took about 15 minutes. The sample was also exposed to oxygen in the sample tube headspace for about 45 minutes during transport. Therefore, the protocol may have been biased against selection of strict anaerobes. However, SRB have been shown to survive extended periods of oxygen exposure (Brune et al., 2000; Cypionka, 2000; Sigalevich et al., 2000; Holmer and Storkholm, 2001).

A primary objective of this experiment was to test different enrichment media for their effectiveness at culturing SRB from a model CW microcosm and to maximize discovery of SRB diversity. The basic medium was chosen for its simplicity and its inclusion of $FeSO_4$, which served as an indicator of SRB activity (Figure 3.1). In this experiment, blackening occurred in all 24 enrichment bottles within two days to two weeks, which was typical of SRB culture (Postgate, 1984). In general, turbidity was not observed in SRB-positive enrichments. The precipitate formed in the liquid media apparently provided a suitable surface for biofilm attachment and growth, and probably made it possible to culture biofilm bacteria in the enrichment bottles without gravel as a solid surface. Postgate (1984) reported a debate in the literature regarding the addition or omission of ascorbic and thioglycolic acids to media for SRB enrichment. The author recommended their addition to enrich for members of the genera *Desulfovibrio* and *Desulfotomaculum*, but noted that these components might also select against other genera. In order to cultivate as many SRB from the CW as possible, each carbon source

was prepared with and without ascorbic and thioglycolic acids. Of the media used (Table 3.1), benzoate amended with ascorbic and thioglycolic acids and butyrate without amendment resulted in the greatest amount of SRB activity (judged by the extent of blackening).

Although all broth enrichments produced evidence of SRB activity (blackening) and DNA was extracted from one of each duplicate serum bottle (12 total), PCR products using *dsr* primers could be amplified from only eight of those. The profiles of *dsr* PCR products from those eight DNA extracts were displayed using DGGE (Figure 3.2). Not surprisingly, the different broths resulted in overall community differences despite a uniform inoculum. Although all the media were supplemented with yeast extract, the differences in community profiles suggest that the different electron donors (Table 3.1, Column 2) were a primary influence on selective enrichment of SRB. In general, fewer than 12 bands could be distinguished per profile and the differences in band intensity suggested communities consisting of 2-4 dominant genotypes and 6-8 minor genotypes. Rare genotypes are not likely to be detected by this method. The SRB communities enriched on palmitate (Lane 1) and acetate amended with ascorbic and thioglycolic acids (Lane 2) appeared to have less diversity, with highly skewed distributions consisting of 1-2 dominant genotypes. There were also some similarities among profiles. Arrows 1-5 indicate bands common to several of the SRB communities. Lanes 3-7 shared a prominent band (Arrow 2). This band was also present but noticeably fainter in Lanes 2 and 8 (profiles of SRB enriched on acetate), suggesting an SRB phenotype that may be relatively less able to use acetate as an electron donor. Lanes 2, 3, 4, 5 (faint), 6 and 8

also share a band indicated by Arrow 1, which is absent in Lanes 1 and 7. This result suggests a phenotype relatively less able to use palmitate.

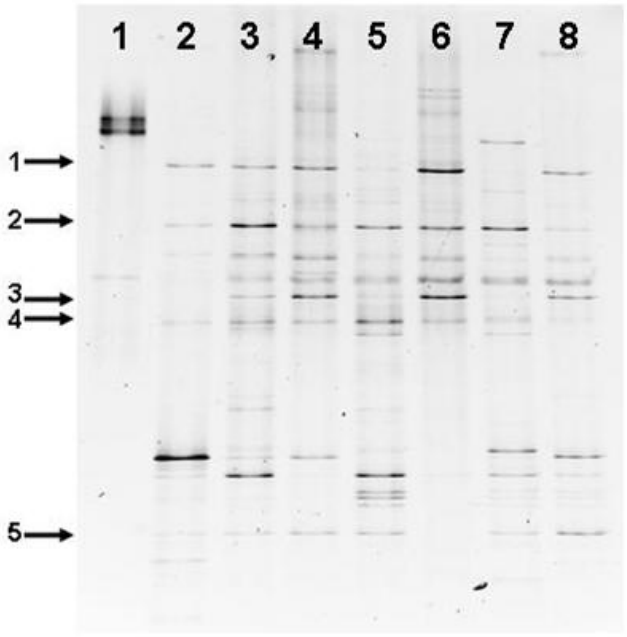

Figure 3.2. DGGE profile of the SRB communities from different broth enrichments. Lane 1 – palmitate. Lane 2 – acetate with ascorbic and thioglycolic acids. Lane 3 – butyrate. Lane 4 – lactate. Lane 5 – benzoate. Lane 6 – propionate. Lane 7 – palmitate with ascorbic and thioglycolic acids. Lane 8 – acetate. Arrows indicate examples of bands that are common to several enrichment broth communities.

DGGE profiles should be interpreted with caution because they are invariably a mix of artifact and real diversity. Individual bands are generally assumed to represent individual genotypes, but only DNA sequencing can confirm this. The total number of bands in a profile is a rough estimate of diversity and the intensity of a band is a rough estimate of the prominence of the corresponding genotype in the microbial community (Muyzer et al., 1993). Obtaining DNA sequence information from bands in DGGE profiles can be problematic. Typically, bands are excised from gels and the desired

sequences are re-amplified with PCR to obtain a pure fragment that can then be sequenced. However, cloning is required for bands that cannot be purified by re-amplification alone. As an alternative, our laboratory has developed a protocol in which the original PCR products are directly cloned prior to DGGE analysis and then DGGE is used to display the diversity contained in the resulting clone libraries (Burr et al., 2006). The DGGE mobility of individual clones can be compared with profiles of the whole community in order to select clones for sequencing. These may be clones with unique positions in the gel or clones aligning with dominant bands in the whole community profile. The rationale for creating pools of clones is to estimate the total SRB diversity in the clone library. If diversity is low, there is a high probability that two random clone pools containing a small number of clones (e.g., 10 clones each) will contain most of the same *dsr* sequences. Furthermore, as a clone pool becomes larger, its DGGE profile begins to resemble that of the whole community obtained directly by PCR amplification of extracted DNA, without cloning. Therefore, our approach has been to estimate diversity by comparing DGGE profiles of different random pools of clones to each other and to the direct DGGE profile.

This approach was applied to the two enrichment broths in which blackening was the most pronounced, butyrate without amendment (Figure 3.3, Lanes 1-6) and benzoate amended with ascorbic and thioglycolic acids (Figure 3.3, Lanes 7-13). Individual clones (Lanes 4-6 and 10-13) generally produced distinct bands and could be aligned with bands in the direct profiles (Lanes 3 and 7) and with bands in the clone pools (Lanes 1-2 and 8-9). Profiles of the clone pools in Lanes 1-2 generally resemble each other and the direct

DGGE profile (Lane 3). This result suggests that most of the diversity within the butyric acid enrichment would likely be discovered by sequencing 10-20 clones. The clone pools in Lanes 8-9 appear less similar, suggesting a more diverse SRB community enriched on benzoic acid supplemented with ascorbic and thioglycolic acids.

In general, DNA sequences of SRB clones obtained in this study poorly matched sequences in the GenBank database, suggesting that previously undiscovered SRB diversity exists in the model CW. Clones in Lanes 4 and 11 have the same apparent mobility in DGGE (Figure 3) and were 100% identical at the DNA sequence level. They share 94% DNA sequence identity with three uncultured *dsr* clones from an Asian aquifer impacted by landfill leachate. Their nearest named BLAST relative is *Desulfobulbus rhabdoformis* (GenBank accession AJ250473, 82% identity). Clones in Lanes 5 and 10 share >98% DNA sequence identity, and are 83% and 80% identical to *Desulfotomaculum nigrificans* (AF482466) and *Desulfotomaculum alkaliphilium* (AF418195), respectively. The clone in Lane 12 is 94% identical to *Desulfomicrobium apsheronum* (AB061529). Clones in Lanes 6 and 13 were poorly related (generally <85%) to sequences of uncultured SRB clones. Although our sequencing dataset was small (seven clones sequenced), it may be surprising that *Desulfovibrio* spp. were not among those sequences identified, however, there are two possible explanations. Dsr primers used in this study may be biased against *Desulfovibrio* spp. and *Desulfovibrio* spp. may not be as dominant in marsh and wetland environments as other SRB (Bahr et al., 2005). On the other hand, *Desulfobulbus* spp. have previously been detected within CW environments, but only at depths greater than 9 cm (King et al., 2002). It is

important to note that enrichment culturing is selective and that SRB enrichment broth communities would probably have a quite different species distribution compared to the original biofilm community in the CW that was used as inoculum. That biofilm community was not analyzed.

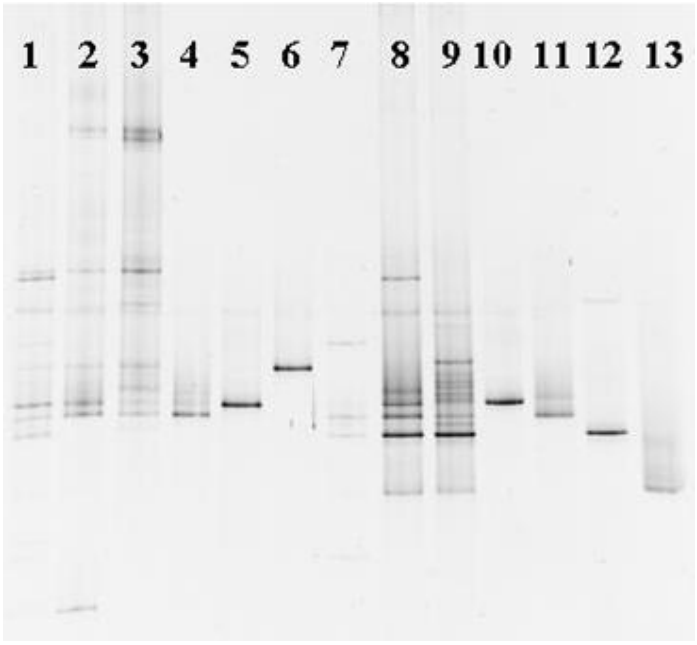

Figure 3.3. DGGE analysis of SRB communities, clones, and pools of clones from enrichments on butyric acid broth (Lanes 1-6) and benzoic acid broth (amended with ascorbic and thioglycolic acids) (Lanes 7-13). From the butyric acid broth SRB enrichment, Lanes 1-2 – separate pools of 10 clones, butyric acid broth community. Lane 3 – direct profile, butyric acid broth community. Lanes 4-6 – single sequenced clones, butyric acid broth community. Lane 7 – direct profile, benzoic acid broth community. Lanes 8 and 9 – separate pools of 10 clones, benzoic acid broth community. Lanes 10-13 – single sequenced clones, benzoic acid broth community. The nearest named BLAST relative of the clones were: Lanes 4 and 11 – *Desulfobulbus rhabdoformis*; Lanes 5 and 10, *Desulfotomaculum* spp.; Lane 12, *Desulfomicrobium apsheronum*. Lanes 6 and 13 were not closely related to any named SRB species in the GenBank database.

Conclusions

In this study, we demonstrated that a protocol combining enrichment culturing with molecular methods was able to recover viable SRB from a model CW operated at 4°C at the time of sampling and to characterize the diversity of the SRB community. We chose to target a functional gene common to all known SRB (*dsr*), rather than the commonly studied phylogenetic marker 16S rDNA. We were able to display *dsr* gene diversity within different enrichment cultures using DGGE. The diversity of the enriched SRB communities varied with different electron donors in the media even though the inoculum was uniform for all enrichments and all media also contained yeast extract. SRB communities as judged by DGGE profiles generally consisted of fewer than 12 dominant and minor genotypes. Rare genotypes were probably not detected. In general, sequenced *dsr* clones from the enrichments had low DNA sequence identity with sequences deposited in GenBank, suggesting that much novel SRB diversity exists within the model CW.

Acknowledgments

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CHAPTER 4

STATISTICAL APPROACH FOR MOLECULAR DATA ANALYSES

A variety of statistical techniques were applied to the datasets generated for each experimental method employed and each gene of interest. Denaturing gradient gel electrophoresis (DGGE) band profiles were analyzed using GelCompar II v. 6.1 (Applied Maths) and R v. 2.11.1 utilizing similarity and dissimilarity matrices (respectively), principal coordinate analysis (PCO) with general surface plotting (logistic regression using a general additive model), as well as hierarchical clustering, displayed as dendrograms. Quantitative PCR data were analyzed using Minitab v. 15 utilizing ANOVA and MANOVA techniques.

DGGE Analysis

GelCompar II

Each DGGE gel experiment included three standard marker lanes (containing 7 bands) distributed evenly throughout the gel (about every 6 lanes) to allow for comparison within and between gels (Figure 4.1, "M"). Gels were stained using SYBR Gold ® (Invitrogen) and imaged using FluorChem^{TM} 8800 (AlphaInnotech). Initial gel processing and normalization was performed using GelCompar II v. 6.1 (Applied Maths) software, according to recommended guidelines. Only band presence and absence, not fluorescent intensity, was considered in the subsequent analyses.

Every attempt was made to minimize the over processing of DGGE gels (such as the inclusion of false bands). Automated background subtraction and spot removal

corrections were not used as they may have caused distortion of the gel images in downstream processing. (Background subtraction was later used in spectral analysis when concerns of distortion were not an issue. Using this correction early in gel processing risks permanent image distortion to downstream processing, for example, when determining densiometric curves.) Band location corrections were also not used to correct sloped or "smiling" bands. Tone correction was used with the linear curve method for all gels, as recommended by the GelCompar II software manual. When defining the densiometric curves for each gel, spectral analysis was used to determine the optimum least square filters and background subtraction to be applied. Based on the conditions discussed above, GelCompar II automatically identified likely bands according to densiometric intensity. The presence of these bands was verified visually and uncertain bands, or those determined to be artifacts (Figure 4.1), were removed from consideration.

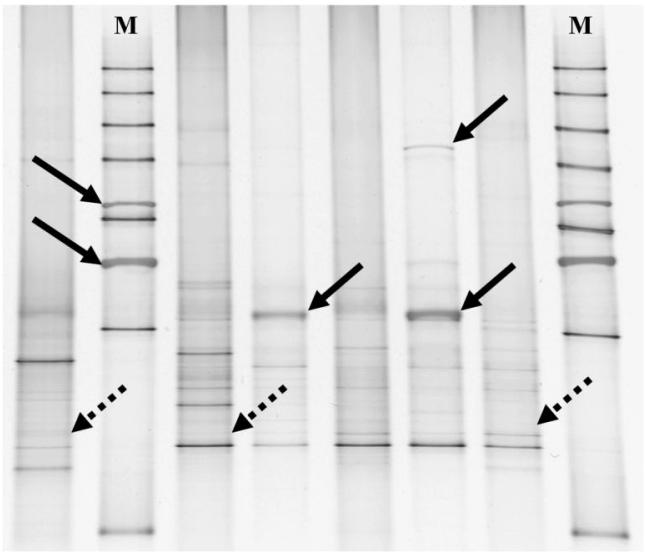

Figure 4.1. DGGE of 16S rDNA showing examples of artifacts ("smiling" or distorted bands, solid arrows) and uncertain bands (recommended by densiometric intensity but not visible on the image, dashed arrows) which were excluded from subsequent analyses. Lanes labeled "M" are marker lanes.

Presence-absence tables were generated from the normalized DGGE gels and exported to Excel (Microsoft Office 2007) in Comma Delimited Format (CDL) for use in R v.2.11.1. Bands that were uncertain or determined to be artifacts were not included in the tables and subsequent analyses (Figure 4.1). In addition, similarity matrices and dendrograms were generated using the Dice calculation (same as Sorensen, described below) and Unweighted Pair Group Method with Arithmetic Mean (UPGMA, described below).

R

Data were exported from GelCompar II and separated into two CDL Excel files to appropriately match the format requirements for analysis in R v. 2.11.1. One file contained unique sample numbers designated for each row containing the DGGE band profile data and a separate Excel file was created to contain the individual environmental parameters for each unique sample number (Table 4.1).

Table 4.1. Example of DGGE Excel file designating environmental conditions. S = summer, C = control, Dc = *Deschampsia cespitosa*, E1 = effluent, G5 = top gravel.

Sample	**Season**	**Plant**	**Location**	**Sample Type**	**Replicate**	**PMA**
DBK001	S	C	E1	E	A	-
DBK002	S	Dc	G5	G	B	+

Dissimilarity Matrices Files were loaded into R and dissimilarity matrices generated. Similarity and dissimilarity matrices were generated using the same calculations with both being determined by the pair wise comparison of two samples (ex. DGGE band profiles (Figures 4.2 and 4.3)). A dissimilarity matrix value was calculated

by subtracting a similarity matrix value from 1. Figure 4.2 diagrams a simplified example of a dissimilarity matrix being generated for a DGGE band profile and Figure 4.3 shows a more complex example. Both examples were calculated using the Jaccard calculation for similarity, an unweighted analysis (Diez et al., 2001). Our actual analyses used the Sorensen calculation (labdsv, Roberts, 2009; Van der Gucht et al., 2001), which weights similarity between groups more heavily by adding a coefficient of 2 for bands common to both lanes. The Sorensen calculation is commonly used for such comparisons.

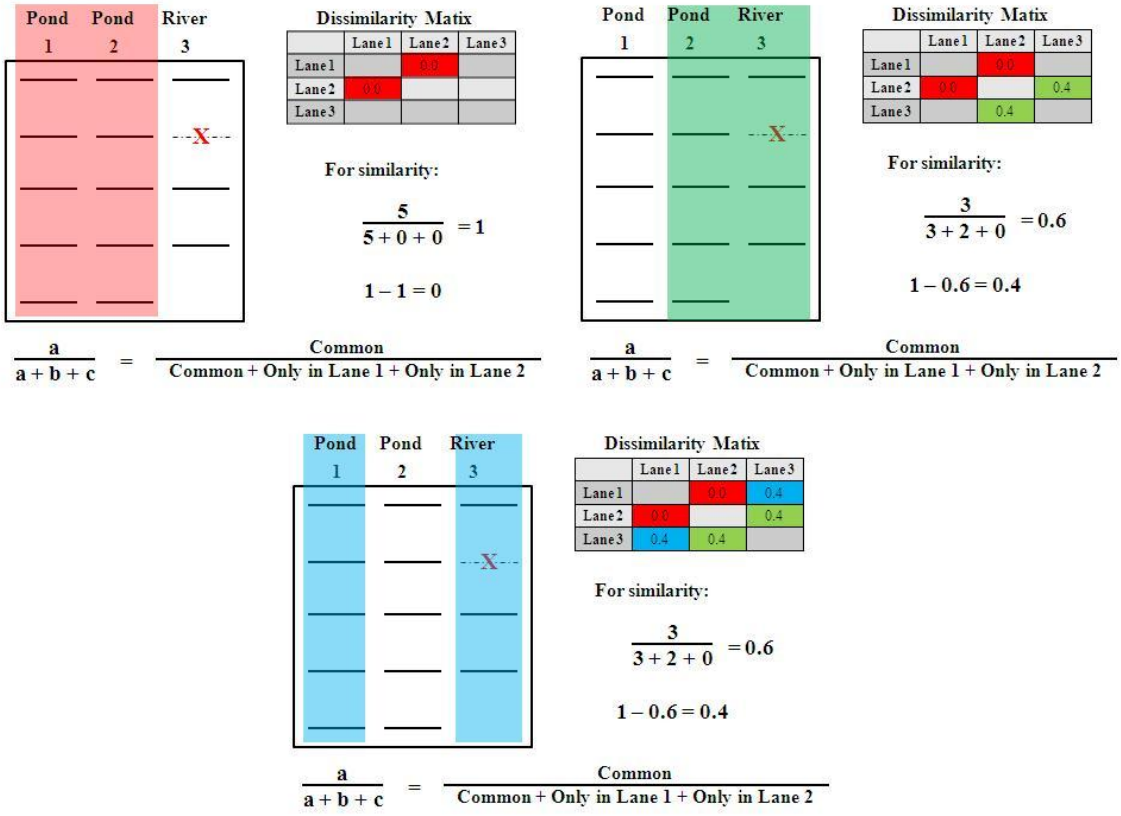

Figure 4.2. Example 1: Simple dissimilarity calculation, using Jaccard. Pairwise comparisons are in the shaded lanes. Dashed line with "X" through it shows an uncertain band that was excluded from the subsequent analyses and dissimilarity matrix.

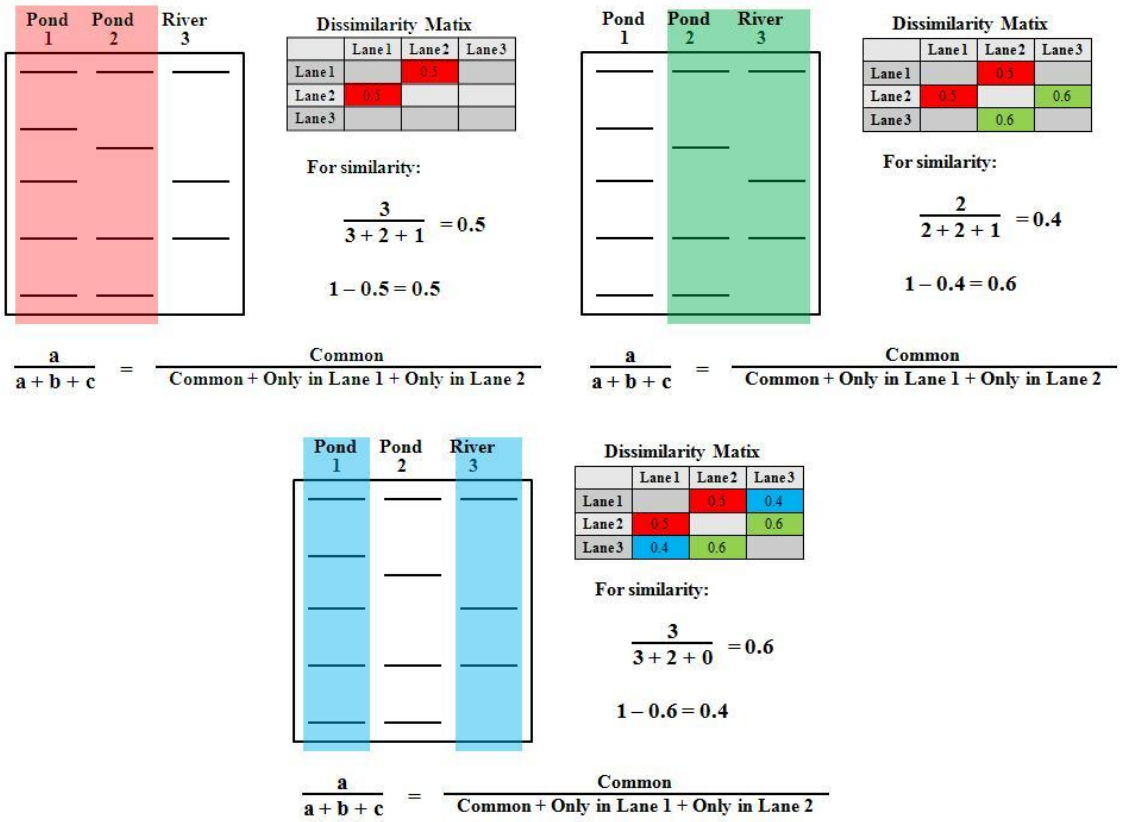

Figure 4.3. Example 2: A more complex dissimilarity calculation with unique band patterns, using Jaccard.

Jaccard calculation:

$$\frac{a}{a+b+c} = \frac{\text{Common}}{\text{Common + Only in Lane 1 + Only in Lane 2}}$$

Sorensen calculation:

$$\frac{2a}{2a+b+c} = \frac{2(\text{Common})}{2(\text{Common}) + \text{Only in Lane 1 + Only in Lane 2}}$$

Principle Coordinate Analysis Principal coordinate analysis (PCO) (McCune and Grace, 2002) was performed on the resultant dissimilarity matrices (labdsv, Roberts, 2009). PCO, not principal component analysis (PCA), was performed because presence-

absence data was used to calculate the dissimilarity matrix (a unitless, non-quantitative measure). If quantitative data were being analyzed, PCA could have been utilized which uses a correlation (when the data have different units) or covariance matrix (when the data have the same units of measure). PCO analyses can represent the DGGE data in the two dimensions that best separate data points from each other, essentially collapsing the data onto the two most informative dimensions. These new dimensions (axes) are unknown "variables" (calculated from eigenvectors corresponding to the two largest eigenvalues of the dissimilarity matrix) that are calculated based on the maximum dissimilarity of the original DGGE data (Figure 4.4).

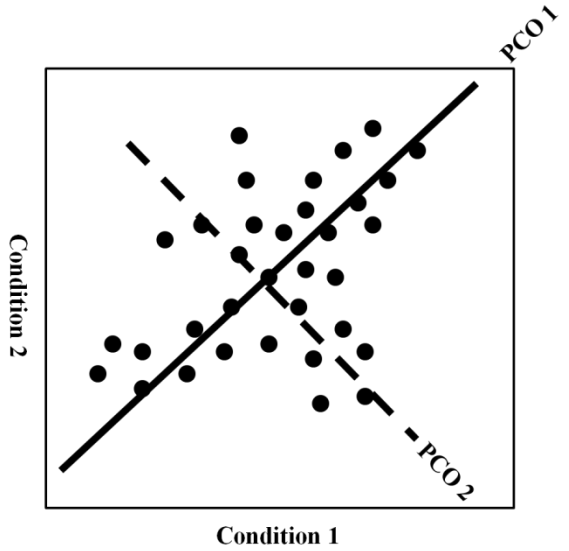

Figure 4.4. Example of principal coordinate analysis plot generation from a raw dataset. PCO 1 is the direction of the maximal dissimilarity in the data, while PCO 2 is the second most maximal direction and is always orthogonal to PCO 1.

General Surface Plotting Once PCO plots had been generated for the dataset, general surface plotting (surf) was performed on the PCO plots and D^2 values recorded (Roberts, 2009). Surf utilizes a logistic regression with a general additive model (GAM)

rather than a general linear model (GLM) to fit the PCO plot data to one of the categorical environmental responses (e.g. season). A GLM fits a parameterized function, linear in the parameters, to the data and the fit of this line is reported as an R^2 value when the response is quantitative. The R^2 value is the percent of variability of the quantitative response explained by the model. The GAM fits a logistic regression to the data using a smoother with a D^2 value reporting its fit. The percent of deviances explained by the logistic regression model is reported as D^2 (analogous to R^2). Large D^2 values indicate a good fit of the logistic regression of the two most informative PCOs to the environmental partitioning of the data. Surface plots resemble topographical maps with higher values ("elevations") indicating a higher probability that a sample point would belong to the environmental group being tested (e.g. control vs. planted). Our surf analyses are visual representations of the PCO plots which can be used to detect the effect of the environmental variables (e.g. season, plant species, sample location and type, etc.) on the PCO data spread. Examples of potential PCO plots and surf analyses from dissimilarity matrices calculated previously (Figures 4.2 and 4.3) are shown (Figure 4.5).

As the actual datasets were more complex than the examples included here, a hierarchical approach was taken for PCO surf analysis. Initially, the entire dataset was analyzed using surf. The most influential environmental factor (highest D^2 value, generally above 0.75) was selected and the dataset subdivided according to this parameter (e.g. season). For example, if a D^2 value of 0.9785 for season was larger than the D^2 values for either sample type or plant species, then this would result in the data being divided into summer and winter subgroups and reanalyzed as a new dataset to determine

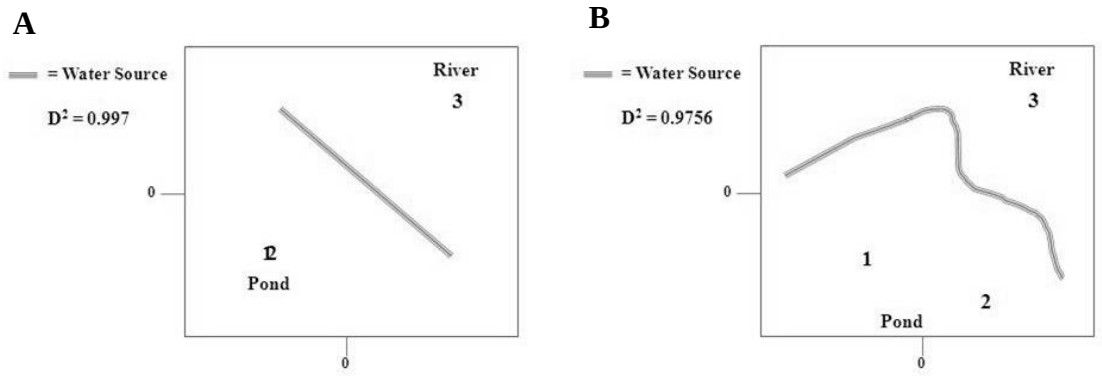

Figure 4.5. Principle coordinate plot and surf analysis of Examples 1 and 2 (A and B, respectively). Numbers (1-3) on the PCO plots represent the DGGE lane profile (from Figures 4.2 and 4.3). Large D^2 values indicate a strong separation of the data according to source water. Since only two unique points are represented in Example 1 (A), surf analysis generates a straight line. As additional data points are represented (Example 2, B), the surf analysis may be represented by a non-linear form.

the next most influential variable (out of plant species or sample type). This process was repeated until all environmental variables had been assessed and subgroups created and analyzed, or surf analysis no longer revealed associations between the PCO data and the environmental parameter applied (Figure 4.6). Parameters with low D^2 values, less than 0.60, were generally not pursued with subsequent analyses. Results from surf analyses were compared with hierarchical clustering results to determine whether similar conclusions could be drawn using a second independent approach. Ultimately, we wanted to determine which environmental variables most influenced the community structure.

Hierarchical Clustering Hierarchical clustering analyses were performed using the Sorensen dissimilarity matrices calculated previously. Results were displayed using a graphical representation of the dataset, or dendrogram (also referred to as a "tree"), where branches merge according to the similarity of the observed data. We used the

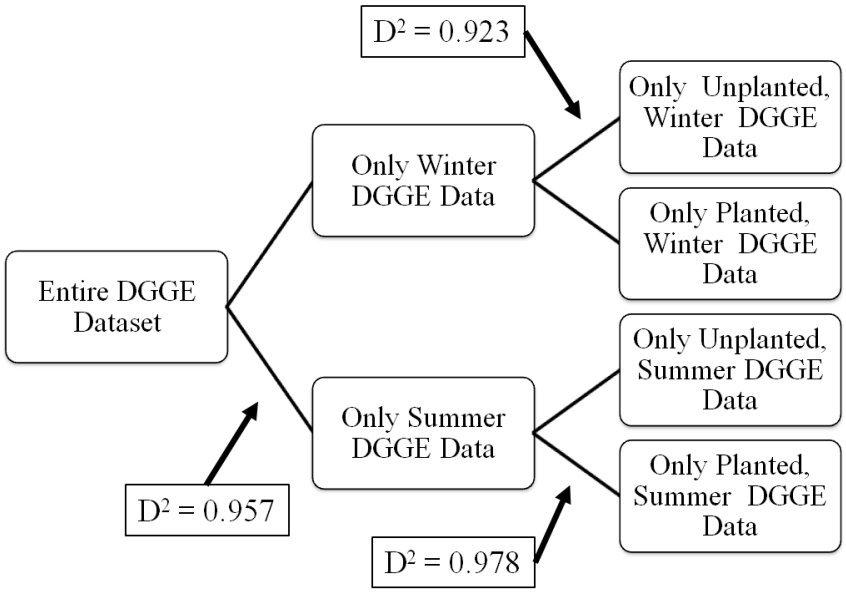

Figure 4.6. Hypothetical example of the approach taken for dividing and analyzing the DGGE data using surf analysis. The entire dataset was considered for each of the environmental variables and surf analysis performed for each variable (season, plant, etc.). Season had the greatest influence on the spread of the data (largest D^2 value of variables examined, $D^2 = 0.957$). The data was divided into winter and summer subsets and reanalyzed in the same manner. The highest D^2 value within these data sets indicated that presence vs. absence of plants was the next most informative separation of the data ($D^2 = 0.978$ for summer data and $D^2 = 0.923$ for the winter data). Additional subdivisions continued (considering the remaining variables) until no further separations were possible or informative.

Unweighted Pair Group Method with Arithmetic Mean (UPGMA) which averages the distances between pairs of objects to generate the resultant tree (Ibekwe et al., 2001; Boon et al., 2002). Samples more similar to one another are closest together on the dendrogram. Figure 4.7 illustrates this for the two examples described previously.

Hierarchical clustering results were analyzed to identify the informative clusters formed. To achieve this, both the global (related to all other samples) and local (related to the neighboring samples) effects of the samples on each other were investigated using stride analysis (optpart, Roberts, 2010; unpublished). Stride creates dendrograms of different cluster sizes using the dissimilarity matrix and calculates a global and

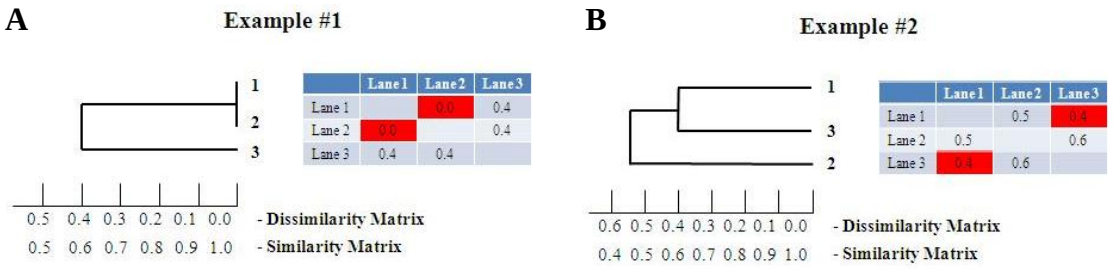

Figure 4.7. Dendrogram results for Example 1 and 2 (A and B, respectively). A – Example 1 shows lanes 1 and 2 as being 100% similar or 0% dissimilar (as shown on the scale bar below). Lane 3 is equally dissimilar to the two groups and branches off accordingly. B – Example 2 shows lanes 1 and 3 being least dissimilar to one another. The dissimilarity of lane 2 to lanes 1 and 3 is averaged and branches accordingly.

local value for each cluster result. These values are displayed in a stride plot which shows the global (partana ratio) and local (silhouette width) values for the cluster analysis (Figure 4.8). The partana (partition analysis) ratio evaluates within-cluster similarity

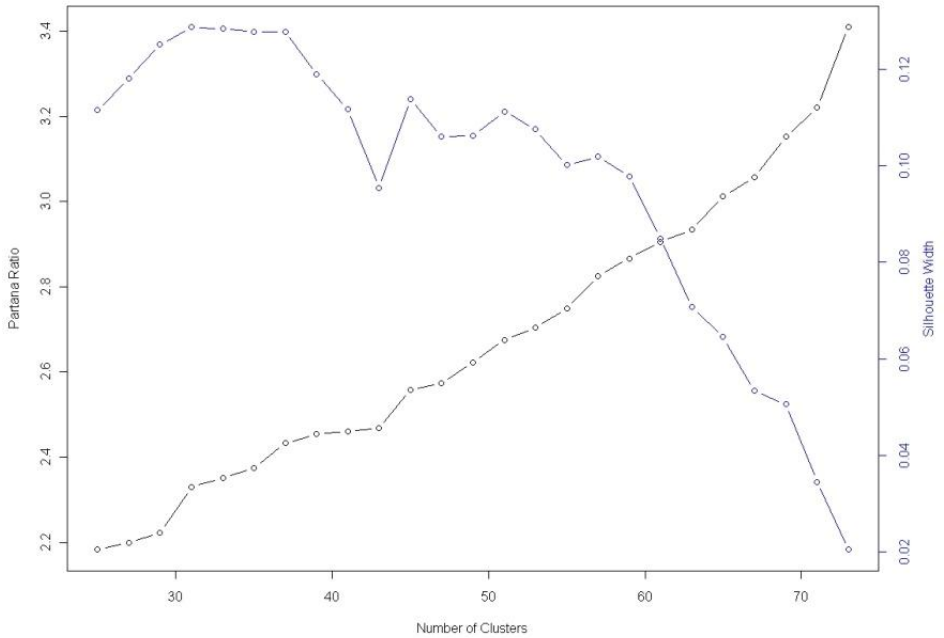

Figure 4.8. Stride plot for 16S rDNA DGGE profiles. The x-axis is the number of clusters. The y-axis on the left is the partana ratio and the y-axis on the right is the silhouette width. Cluster numbers are selected and simultaneously optimized to find the highest value for both the partana ratio and silhouette width (generally where the two cross, the optimum for this dataset was 59 clusters representing the DGGE profiles).

with among-cluster similarity and is a tool to measure the cluster validity (Aho et al., 2008; Foy, 2008). The silhouette width measures the mean similarity of each sample in the cluster to the mean similarity of the next most similar cluster (Rousseeuw, 1987). Optimization of both of these measures for their maximum values assisted in identifying the number of clusters within each dendrogram that were most informative.

Upon determining the number of clusters (by optimizing for partana and silhouette width), dendrograms were "sliced" to visualize and analyze the environmental parameters most affecting the cluster distribution. A sliced dendrogram has a red horizontal line across the resultant clusters. Clusters above the line were different from one another while clustered samples below the line were too similar to be clustered further (Figure 4.9). Chi-squared tests were performed on the sliced trees (clusters above

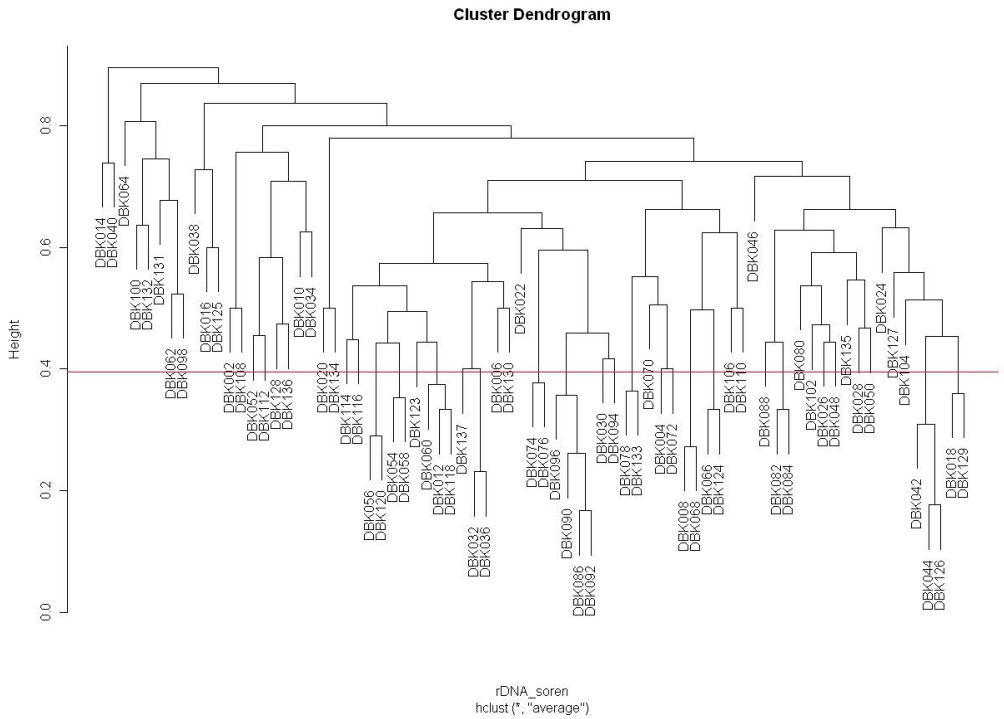

Figure 4.9. Hierarchical cluster dendrogram for 16S rDNA DGGE profiles sliced to show 59 clusters. Samples clustered below the red line are too similar to be distinguished from one another and are grouped as a single entity.

the slice line) to determine the impact of the environmental parameters on the resultant clusters. All environmental variables were considered and tested. Small p-values indicated which parameters had the most significant influence on the hierarchical clustering results.

Principal coordinate analyses with general surface plotting and hierarchical clustering were performed for all of the DGGE data included in this dissertation.

Quantitative PCR Analysis

Quantitative PCR results (C_t values, number of qPCR cycles required for the fluorescent signal intensity to cross the specified threshold value) from each run were exported from the Rotor Gene 6000 Series software package 1.7 to Microsoft Excel (2007). The qPCR level of detection was 678 copies/µL for *dsrB* and 267 copies/µL for *amoA.* This was determined by averaging the y-intercepts from all of the qPCR runs performed for each gene and calculating the copy number for the threshold C_t value. C_t values that were below the level of detection were amended to these values for subsequent analyses. No adjustments were required for the 16S rDNA gene. Quantitative PCR reactions were performed in technical triplicate (C_t values were all within one cycle of each other). These values were averaged together to a single value for each sampled location. The sample data for each functional gene were normalized to the 16S rDNA data from the same corresponding sample location (i.e. *dsrB* summer effluent from control column replicate A normalized to 16S rDNA summer effluent from control column replicate A). Minitab software v. 15 was used for all of the statistical analyses.

As a first step, an ANOVA was fit to the normalized C_t values and the residuals were checked for normality and constant variance (Figure 4.10A) and found not to meet the requirements of the general linear model. As a result, all of the data were log transformed to: $\log_{10}$(copies/µL) for 16S rDNA and $\log_{10}$((copies/µL gene)/(copies/µL 16S rDNA)) for each of the functional genes. After transformation (Figure 4.10B), the data appeared more evenly spread and fell along the expected line in the normal probability plots. The symmetry observed in the residual "versus fits" plots was because there were only two replicates per condition sampled.

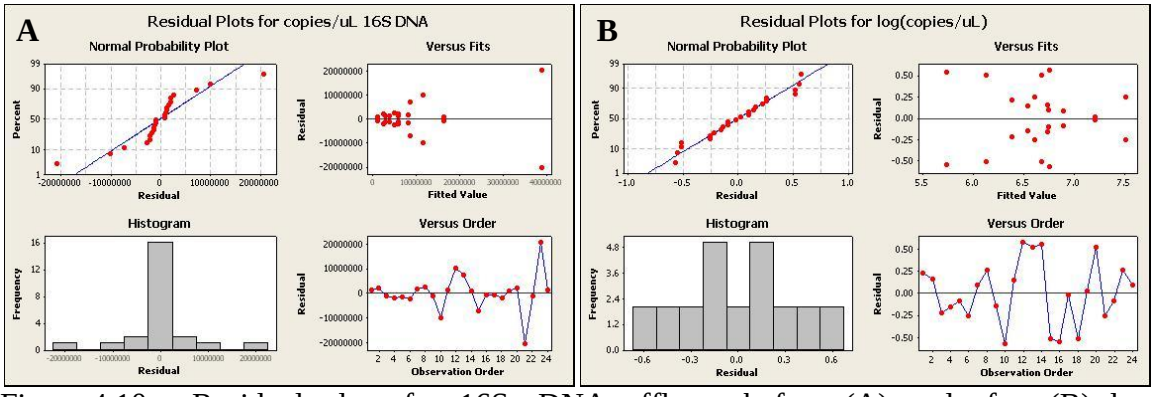

Figure 4.10. Residual plots for 16S rDNA effluent before (A) and after (B) log transformation of data. After transformation, the data more closely resembled a normal distribution and were more evenly spread in the residual "versus fits" plots.

Analysis of Variance (ANOVA)

Univariate analysis of variance using a general linear model was performed on the data set for each location (E1-G6, Table 4.2) for each of the genes. This analysis compared the variability of the means between groups with the variability of the data within groups to reveal any significant effects of the environmental variables examined (e.g. season,

plant). Significant effects were observed when the mean differences between groups were much greater than the differences within groups.

Table 4.2. Sample identity for constructed wetland columns.

Sample ID	Location
E1	Drained effluent
R2	Thick roots (nearest the crown of plant)
R3	Fine roots (furthest from the crown of the plant)
R4	Ultra-fine roots (torn off during destructive sampling and recovered by skimming and filtering)
G5	Top gravel (root associated for planted columns)
G6	Bottom gravel (bottom of the column)

The response tested for each sample location was the $\log_{10}$(copies/µL) (for 16S rDNA) and $\log_{10}$((copies/µL gene)/(copies/µL 16S rDNA)) (for each of the functional genes, *dsrB* and *amoA*) compared to season, plant and a plant-season interaction (PMA was also included as a factor in 16S rDNA analysis). Main effects and interaction plots were generated for each analysis performed to visualize the data (Figure 4.11). The p-value was recorded for each variable tested.

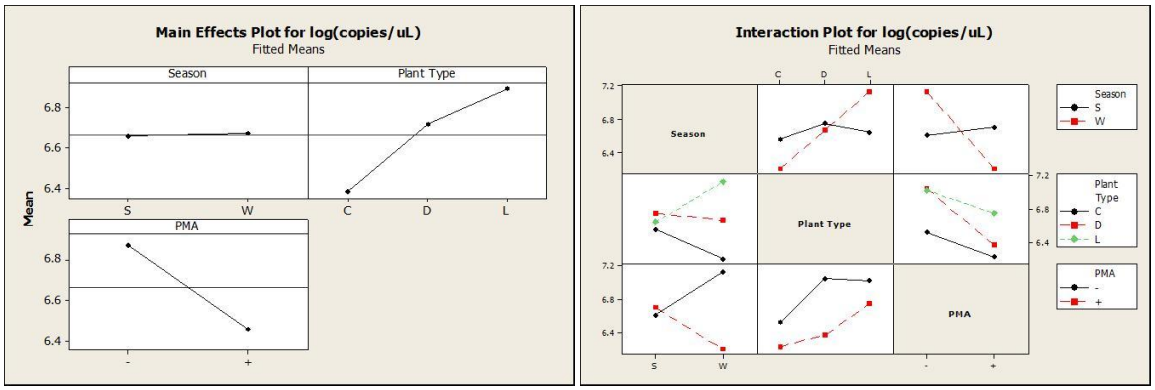

Figure 4.11. Examples of effluent 16S rDNA ANOVA main effects (left) and interaction plots (right).

Since many statistical analyses were performed, corrections against false positives were also taken into account using the Benjamini Hochberg method to maintain a family-wise 5% or 10% false discovery rate (Benjamini and Hochberg, 1995). The Benjamini Hochberg method can be summarized as in Table 4.3 (an example for the six tests for season). Recorded p-values from the ANOVA models were entered into the table from largest to smallest. Once the first p-value was smaller than the value in the comparison column, that and all subsequent tests were significant.

Table 4.3. Example of Benjamini Hochberg comparison table for 16S rDNA ANOVA of season. Bolded values are significant as the largest bold value is of lesser value than its comparison value (far left).

Benjamini Hochberg Comparison Value		**Season**		
for 5% false discovery rate	for 10% false discovery rate	p-values, from largest to smallest		
0.05	0.1	0.945	E1	
0.0417	0.0833	0.752	R2	
0.0333	0.0667	0.516	R3	
0.025	0.05	0.072	G6	
0.0167	0.0333	**0.002**	**G5**	**significant at 5%**
0.0083	0.0167	**0.000**	**R4**	**significant at 5%**

Multivariate Analysis of Variance (MANOVA)

Multivariate analysis of variance (MANOVA) is a useful method to analyze the data so that multiple responses (ex. G5 and G6) can be analyzed simultaneously. One hypothetical example would be to plot the data for the two gravel samples measured in these experiments (G5 and G6) and label them according to plant species. The plot of these two responses does not reveal a clear separation of the means for each plant species

(Figure 4.12). When a one way MANOVA is applied (Figure 4.13), the MANOVA is able to clearly separate the plant species from each other by determining the linear discriminant function (LDF). The LDF is the direction in which the means of the data are maximally separated from each other after taking into account the variability within each group. In this example, LDF 1 would be: LDF 1 = 0.5(G5) + 0.5(G6). Since the coefficients for both G5 and G6 are the same, we can interpret both locations to be equally important for separating plant species from one another.

It was suspected that there would be correlations between sample locations within the columns. To model those correlations, the three root measurements (R2-R4) and the two gravel measurements (G5-G6) were analyzed separately using a balanced MANOVA. All six samples (E1, R2-R4, G5-G6) could not be analyzed

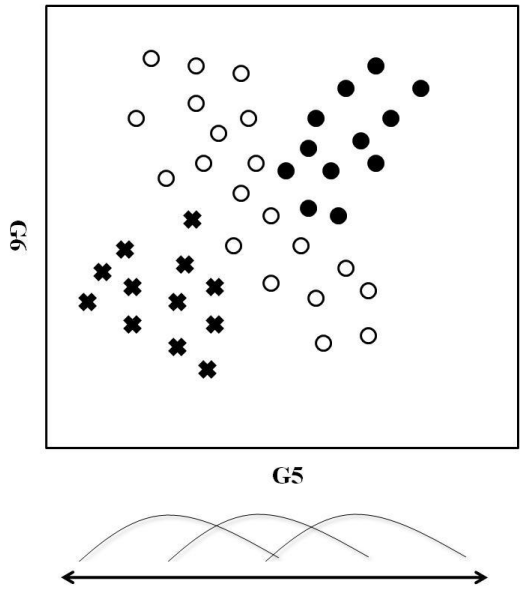

Figure 4.12. Example showing three distinct groups of data (ex. three plant species) plotted according to G5 and G6. Each data set appears to be different from the others, but the means of each of the groups (plotted below condition G5) are not significantly different by univariate ANOVA.

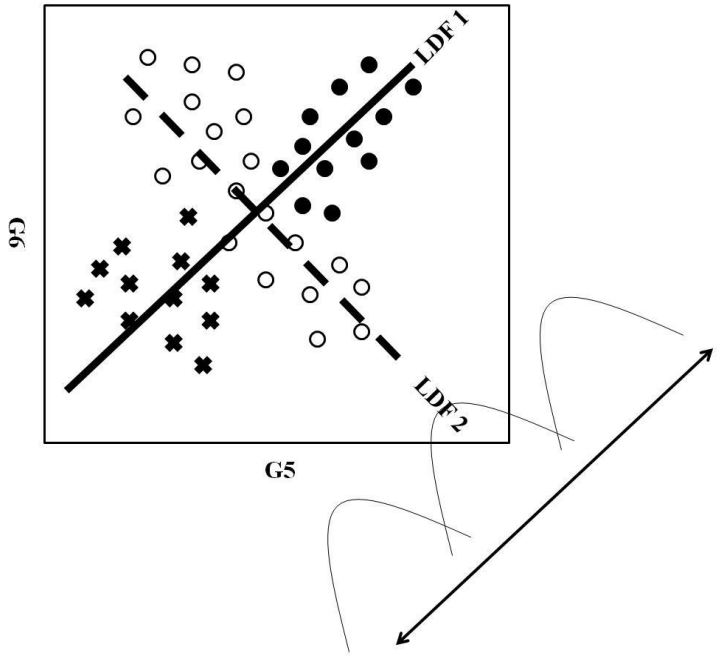

Figure 4.13. Example showing three distinct groups of data (ex. three plant species). The linear discriminant function is the equation which best separates the means of each of the groups (plotted in the lower right of the graph) from one another.

simultaneously because only duplicate samples were taken for each condition so that there were not enough data points for a full multivariate analysis. Since effluent samples contained only a single data measurement, they were simply considered using univariate ANOVA.

A two-way MANOVA considering plant and seasonal effects with an interaction was performed. If a plant-season interaction was significant, a second "follow-up" MANOVA was performed to determine the type of interaction. This follow-up analysis was done combining plant and season as a single factor. If no interactions were observed, the original MANOVA was used for further analysis of the main effect (i.e. season or plant).

MANOVA used eigen analyses to determine the linear discriminate function (LDF) that best discriminated the group means in the data set. LDFs are equations that show the best discriminability of the mean values of the data (Figure 4.13). Computed LDFs were plotted to visualize the data. In all cases for these data, the first LDF captured at least 85% of the discriminability of the group means. ANOVA using a general linear model was performed on the dominant LDF (LDF 1) compared to plant and season. Tukey tests were performed to determine the significant differences between location sampled and the season-plant groups. LDFs were simplified for ease in interpretation without significantly altering the statistical results. The biological relevance of the new LDFs were interpreted with regard to the constructed wetland system, as shown in the example at the beginning of this section.

The statistical approaches explained in this chapter were used for the data analyses presented in the remainder of this dissertation.

Contribution of Authors and Co-authors

Chapter 5: The effect of plant species and sample location on bacterial biofilm communities associated with constructed wetland microcosms

Author: Jennifer L. Faulwetter
Contributions: Designed and conducted the experiments included as well as analyzed the resultant data. Compiled and organized the data into the publication and was actively involved in the editing and submission process.

Co-author: Mark D. Burr
Contributions: Assisted in data analysis and publication compilation, as well as contributed to revision and editing of the paper. Was also a co-PI and advisor on the project.

Co-author: Albert E. Parker
Contributions: Assisted in the design of statistical analyses for the data, interpretation of results and editing of the related manuscript sections.

Co-author: Otto R. Stein
Contributions: Was a PI and advisor on the project as well as assisted in editing the publication.

Co-author: Anne K. Camper
Contributions: Was a co-PI and advisor on the project as well as assisted in editing the publication.

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CHAPTER 5

THE EFFECT OF PLANT SPECIES AND SAMPLE LOCATION ON BACTERIAL BIOFILM COMMUNITIES ASSOCIATED WITH CONSTRUCTED WETLAND MICROCOSMS

Running Title: Plant and surface effects on CW biofilm

Subject Category: Microbial population and community ecology

Contributors:

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Abstract

As the field of microbial ecology advances, studies are trending more toward the use of high throughput molecular methodologies. While advantageous in the amount of information yielded, the abundance of data may make the task of data analysis more difficult. In this study we investigated the biofilm communities within constructed wetland microcosms using denaturing gradient gel electrophoresis (DGGE) and a macro ecological approach to the statistical procedures. To obtain a more detailed look at the microbial communities present throughout the microcosm, an intensive destructive sampling protocol was implemented to investigate six distinct locations (effluent, three root locations, and two gravel locations). DGGE analysis was performed using universal primers targeting the 16S rDNA gene and statistical analyses performed using the R software package. Results revealed unique biofilm communities attached to the gravel, rhizosphere and effluent regions of the microcosms as well as unique communities as a result of wetland plant species. Community distinctions along the root surface (attributed to aging and exudation) were also detected as well as differences in the communities according to gravel depth in the microcosms.

Keywords: 16S rDNA/Biofilm/Constructed Wetland/DGGE/Rhizosphere

Introduction

Constructed wetlands (CWs) are engineered systems designed to provide low cost, low maintenance solutions for removing pollutants from contaminated point and non-point water sources. CWs are artificial wetland systems specifically designed to

remediate a variety of wastewater types by filtration, settling, and bacterial decomposition. The body of research investigating CW performance has focused primarily on the importance of effluent water quality and assumed the influence and involvement of microbial processes. There is a vast amount of evidence to imply microbial involvement in water quality improvement, but little direct evidence supporting these assumptions.

The presence of plants within CW has long been believed to enhance microbial activity, and thus performance of CW systems, but the reasons for the observed improvements have not been well investigated. Some nutrient removal may be due to plant uptake; however, the main contribution has long been attributed to microbial activity (Hatano et al., 1994; Reddy and D'Angelo, 1994; Kadlec and Knight, 1996). Another way in which plants may influence microbial communities within CW is through the release of oxygen to the rhizosphere (the root surface and environment directly influenced by the root) which could enhance the activities of specific microbial functional groups to promote organic carbon degradation. Some studies have reported an increase in microbial activity and density within the rhizosphere (Gagnon et al., 2007; Munch et al., 2007). Most recently, studies have incorporated molecular techniques to determine the effect of plants on the microbial dynamics within CW systems but most of what we "know" is still largely based on inference (Faulwetter et al., 2009).

CWs have generally been considered poorly suited for cold climates (USEPA, 2000) and seasonality also greatly affects the performance of CWs (Allen et al., 2002; Stein and Hook, 2005; Taylor et al., 2010). It is generally known that microbial activity

is linked to temperature, with bacterial growth and metabolic rates strongly reduced with decreasing temperature (Atlas and Bartha, 1998). Interestingly, the best performing CW plants in microcosm studies showed the most efficient organic carbon removal and had the highest redox conditions during plant senescence in the winter season (4°C) (Allen et al., 2002; Hook et al., 2003; Stein and Hook, 2005). Increased redox suggested that more oxygen was transported to the roots and rhizosphere, which could promote aerobic microbial degradation of organic matter (Stein and Hook, 2005). Taylor et al. (2010) also observed seasonal performance trends and concluded that some wetland plant families (sedges, *Cyperaceae* and rushes, *Juncaceae*) were well suited for use in CW systems as they were able to effectively reduce chemical oxygen demand (COD) and sulfate concentrations (indicative of root zone oxygenation) while other families (grasses, *Poaceae*) were poorly suited for CW systems, with the notable exception of *Deschampsia cespitosa*, which was a top performer overall for COD removal. This research led to the belief that there was a link between plant presence, temperature, and microbial activity within these systems.

Previous studies have disagreed about whether or not there are differences in the microbial communities of CW based on plant presence and performance. Some studies have shown that microbial density, activity, and diversity are enhanced in the plant rhizosphere regions of subsurface flow CWs (Hatano et al., 1994; Ottova et al., 1997; Wieland et al., 2001; Collins et al., 2004; Vacca et al., 2005; Gagnon et al., 2007; Munch et al., 2007), suggestive that plants enhance the establishment of microorganisms, while

others have demonstrated no effects due to plant presence (Ahn et al., 2007; Gorra et al., 2007; Tietz et al., 2007b; Baptista et al., 2008).

Molecular advances have greatly enhanced the ease with which researchers are able to collect data for a wide variety of environmental systems, including systems like CWs. CWs have been investigated using several different molecular techniques, most involving the 16S rDNA gene in some form and most focusing on composite samples collected from CWs. For these analyses, bulk samples have been collected and analyzed. It is possible that this method of broad composite sample collection may mask true differences present within the microbial communities present. Historically, most microbial ecology studies have been based on the analysis of ribosomal DNA (rDNA), the standard prokaryote phylogenetic marker (Amann et al., 1995). Once widely investigated, the 16S rDNA gene has more recently been passed over as too broad to provide useful information for a given system, at least from a functional perspective. Likewise, denaturing gradient gel electrophoresis (DGGE), commonly used to visualize a community profile at a specific time for a given gene (Muyzer et al., 1993), has also begun to be replaced with higher throughput technologies such as pyrosequencing and microarrays. Interpretation of DGGE gels has been highly subjective and the profile patterns observed were often too complex to interpret and compare, especially when analyzing 16S rDNA. However, when used appropriately, DGGE can be an informative tool for profiling microbial communities.

Because of the earlier observations on the importance of seasonal differences and plant type in CW performance (Taylor et al., 2010), this study was done to detect

microbial community differences as a function of plant species, season (summer vs. winter), location on the plant root (tip vs. mature root), and surface sampled (rhizosphere biofilm, biofilm removed from the gravel, bulk water). To maximize the likelihood of detecting these differences, an intensive localized destructive sampling protocol was done to determine differences in community composition that may be present at the microscale or niche level. It is well accepted that natural conditions can provide numerous microenvironments, especially with respect to microbial environments (Grundmann and Gourbiere, 1999; Kang and Mills, 2006; Konopka, 2009). An intensive, systematic approach was evaluated and was crucial to adequately capture and determine the complexity of the microbial community.

The 16S rDNA gene was used in this study to visualize the entire bacterial community present within the system. A rigorous statistical analysis of the 16S rDNA DGGE was applied to profiles taken from a variety of distinct locations within constructed wetland microcosms. It was hypothesized that each planted condition would have a unique microbial community, that the biofilm community attached to the gravel surface would be different than the rhizosphere community, and that the communities would change from season to season. This approach shows the power of sampling microsites within an environmental system as well as what may be accomplished when a thorough statistical analysis of the data is performed.

Methods and Materials

Constructed Wetland Microcosm Operation

Constructed wetland microcosms (columns) were maintained in a greenhouse at the Plant Growth Center at Montana State University in Bozeman, MT (46˚N, 111˚W). The temperature sequence during the experimental period was 24, 16, 8, 4, 8, 16, and 24˚C. Greenhouse temperature was changed every 60 days to mimic natural seasonal cycles. Supplemental lighting was not used. Model subsurface wetland microcosms consisted of 15 cm diameter by 30 cm tall polyvinyl chloride columns filled with 1-5 mm diameter gravel. Microcosms were fed synthetic wastewater (SWW) simulating post-primary effluent (Taylor et al., 2010) with three 20 day batches at each temperature. At the end of each batch, the columns were completely drained and refilled.

The study utilized a total of 18 microcosms; six replicates of unplanted controls and six monocultures each of *Deschampsia cespitosa* and *Leymus cinereus.* Both species belong to the same family (*Poaceae*). The plant species were selected based upon their performance in previous studies (Taylor et al., 2010). In those studies, *Deschampsia cespitosa* significantly improved organic carbon removal from the synthetic wastewater compared to unplanted controls, while columns planted with *Leymus cinereus* behaved similarly to unplanted controls. Patterns of natural light and controlled temperature induced normal seasonal cycles of plant growth and dormancy. The microbial communities were allowed to develop naturally and there was no deliberate inoculation of specific organisms.

Microcosm Destructive Sampling

Plants were grown for a minimum of 20 months prior to the first sampling date. Sampling was in summer 2007, winter 2007-2008, and summer 2008. Microcosms were destructively sampled, in duplicate, at the end of the third 20-day batch at 24°C (summer) or 4°C (winter). Six sample locations were collected within each microcosm (Figure 5.1): effluent (E1), thick roots (near the crown) (R2), fine roots (R3), ultra-fine roots (root hairs, torn off during removal of the plant from the column) (R4), top gravel (G5) and bottom gravel (G6).

The effluent was completely drained from the column and mixed (~1.5 L), and a 250 mL sample was vacuum filtered through a 0.2 µm polycarbonate membrane (47 mm diameter, Poretics®). The membrane was shredded and placed into a MO BIO PowerBead tube (MO BIO PowerSoil™ DNA Isolation Kit) for DNA extraction. The entire plant was then removed from the column and root samples (thick and thin) were removed directly from the plant using a sterile razor blade and forceps, and transferred to MO BIO PowerBead tubes. Approximately 0.05 g and 0.03 g of material was collected for thick and fine roots, respectively. Each of the gravel samples was collected in a 50 mL conical centrifuge tube (www.biotang.com) containing 10 mL of sterile sand. Gravel was added up to the 17.5 mL mark on the tube. A 9 mL volume of phosphate buffered saline was added and the tube was vortexed for 60 seconds (to remove the attached biofilm). The supernatant of each sample was concentrated (in 2 mL volumes, up to 10 mL total) by sequential centrifugation at 5,000 x g for 5 minutes into a MO BIO PowerBead Tube. Ultra-fine roots (R4) were collected by filling a container with the

gravel that remained in the column when the plant was removed and swirling it with tap water. Root hairs floating on the surface of the water were collected by skimming the water surface with a 50 mL Falcon tube. A total of 100 mL of skimmed water was collected. The root hairs were vacuum filtered through a 41 Whatman paper filter (www.whatman.com), collected, and transferred into MO BIO PowerBead Tubes (MO BIO PowerSoil™ DNA Isolation Kit) for DNA extraction. Six samples were collected from each planted column sampled; only three samples were collected from unplanted columns as they contained no root material. Columns were destructively sampled in duplicate for summer and winter seasons although only a single column for each plant type was sampled in summer 2008, amounting to: 3 seasons x 3 plants x 6 locations x 2 replicates (except summer 2008).

DNA Extraction

The MO BIO PowerSoil™ DNA Isolation Kit (www.mobio.com) was used for DNA extraction as described in the manufacturer's protocol with the exception that instead of vortexing, PowerBead tubes were placed into the FastPrep® Instrument (Qbiogene, Inc.) at speed 5.5 for 45 s. DNA yield was estimated on an agarose gel with ethidium bromide staining, serial dilutions were performed for PCR, and the DNA preparations were stored at -20°C.

PCR

Oligonucleotide primers were synthesized by Integrated DNA Technologies (www.idtdna.com). PCR primers 8F (Amann et al., 1995) and 1492R (Liu and Huang, 2002) target bacterial 16S rDNA. These primers amplified a near full-length fragment

(~1500 bp). Products from this PCR were diluted 1:100 and used as template for a second (nested) PCR reaction using primers 1055F and 1392R with a 5' 40-bp GC clamp (Ferris et al., 1996). The amplicon from the nested PCR was ~370 bp. All PCR reactions (20 µL) were performed using 2X GoTaq® Green Master Mix (www.promega.com). The PCR reaction mixture consisted of 10 µL of 2X GoTaq® Green Master Mix, 3 µL of DEPC-treated water, 1 µL of forward and reverse primers (12.5 µM), and 5 µL of 1:10 diluted (unquantified) template DNA for 8F-1492R reactions and 5 µL of 1:100 diluted 8F-1492R product for 1055F-1392R+GC reactions. All PCR amplifications were performed on an Eppendorf Mastercycler® ep thermal cycler (www.eppendorfna.com) using the following program. An initial denaturation for 2 min at 94°C was followed by a total of 25 cycles of amplification consisting of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. The program ended with an extension step at 72°C for 7 min. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide.

DGGE

DGGE was performed on products from the second PCR reaction (7 µL/lane) using a DCode™ system (www.biorad.com) and reagents from Sigma-Aldrich (www.sigmaaldrich.com). Gels had a gradient of denaturant concentrations from 40% at the top of the gel to 70% at the bottom, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained an 8 to 12% polyacrylamide gradient from top to bottom (Girvan et al., 2003). Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr®Gold (www.invitrogen.com) and documented using a FluorChem™ 8800

fluorescence imager (www.alphainnotech.com). Three marker lanes were included in each DGGE gel so that gel-to-gel comparisons could be made. Marker lanes consisted of bands from five unspecified clones.

DGGE Data Analysis

DGGE gels were compared and normalized using the GelCompar II software (Version 6.1, Applied Maths Inc.). Bands in DGGE images were identified on a presence–absence basis; band intensities were not considered for statistical analysis. Subsequent statistical analyses of the presence-absence data were performed using R software (Version 2.11.1) libraries labdsv (Roberts, 2009) and optpart (Roberts, 2010) (www.r-project.org) to determine any important differences in the 16S rDNA community profiles. Dissimilarity matrices were calculated using the Sorensen method (El Fantroussi et al., 1999; Van der Gucht et al., 2001; McCune and Grace, 2002), which compared DGGE lane band patterns using a pair-wise method.

Hierarchical clustering (HC) analyses were performed for each of the functional genes and dendrograms were generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Ibekwe et al., 2001; Boon et al., 2002). To optimize the dendrograms to the most informative number of clusters, a stride plot was generated which showed the global (partana ratio) and local (silhouette width) values for the cluster analysis. The partana (partition analysis) ratio evaluates within-cluster similarity with among-cluster similarity and is a tool to measure the cluster validity (Aho et al., 2008), while the silhouette width measures the mean similarity of each sample in the cluster to the mean similarity of the next most similar cluster (Rousseeuw, 1987). The optimized

dendrogram was sliced and chi-squared tests were performed on the sliced trees to determine impact of the environmental parameters on the clusters. Significance tests of the relationship between each of the environmental variables and the HC clusters were performed for each environmental variable using a chi-squared test. A small p-value (<0.05) from the chi-squared test indicated a significant relationship.

In completely separate but parallel analyses, principal coordinate analysis (PCO) with general surface plots were generated to visualize important partitions of the data with respect to the environmental variables for each of the functional genes (R software library labdsv; Roberts, 2009). The general surface plots use a logistic regression with a general additive model to categorically compare specified environmental variables. The goodness of fit of the surface plots was reported by D^2, which is analogous to R^2 (used when the response is quantitative). Large D^2 values indicate a good fit of the logistic regression of the two most informative PCOs to the environmental partitioning of the data. Surface plots resembled topographical maps with higher values ("elevations") indicating a higher probability that a sample point would belong to the environmental group being tested (e.g. control vs. planted). Environmental variables investigated were plant species (or unplanted control), season, and the sampling location within the microcosm. Results from surf analyses were compared with hierarchical clustering results and used to visualize the results as well as to determine whether similar conclusions could be drawn using two independent approaches.

Results

Initial PCO analysis was performed on the dissimilarity matrix calculated for the entire DGGE data set. The dissimilarity matrix was calculated by comparing the profile for each DGGE lane with every other lane in the dataset, in a pair-wise manner. Samples included profiles from microcosms containing each of two plant species (and unplanted controls) as well as for two summers (summer 2007 and 2008) and the intervening winter (winter 2007-2008). In addition, microcosms were sampled in six locations (only three for unplanted controls) to determine if some sampling locations were more informative than others or selected for unique microbial communities. Subsequent analyses were usually performed on increasingly narrow subsets of the data, selected with the guidance of earlier results. This produced a hierarchy of factors that were found to affect the bacterial community profiles. The hierarchy was described by p-values from the HC analyses (dendrograms not shown). Following HC, general surface plots were created and D^2 values (0 – 1) computed for the comparisons between DGGE profiles. Each statistical method (HC and PCO) was performed independently of the other. The D^2 values reported for the PCO surface analysis do not express statistical significance in that they are not directly associated with the p-values reported, however, the D^2 values indicate the degree of dissimilarity between DGGE banding patterns.

HC analysis of the entire 16S rDNA DGGE data set showed that sample type (gravel, effluent, roots) within the CW microcosm and plant species, were the main factors discriminating bacterial communities ($p<0.001$ for both). General surface plots (PCO) showing the data distribution revealed that gravel was the most unique sample

type (gravel vs. not gravel) and control to be the most unique plant species (unplanted vs. planted) (D^2=0.612 and 0.595 respectively; Figure 5.2). While HC showed both sample type (gravel, effluent, and roots) and plant species (unplanted control, *D. cespitosa*, and *L. cinereus*) to have an equal effect on the microbial community structure (as indicated by similar p-values), surface plots suggest that sample type had a stronger relationship than plant presence or absence (as demonstrated by the higher D^2 value). To further investigate these effects, the dataset was subdivided according to sample type and plant species.

Analyses were first considered for sample type (gravel, roots, and effluent), using HC and PCO. The separation of the gravel communities from effluent and rhizosphere communities (Figure 5.2a) suggested that gravel presented a distinctly different surface for biofilm formation (gravel surfaces are likely inert) compared to a root surface and was also a different environment from the effluent. When gravel samples were excluded from subsequent analysis, rhizosphere and effluent communities could be discriminated from each other (HC, p=0.001; PCO, D^2=0.8223). Since each sample type (gravel, roots, and effluent) appeared to cultivate a unique microbial community, the data was divided accordingly and further analyzed.

Analysis of the gravel community profiles indicated a plant effect ($p<0.001$) with unplanted gravel communities being unique from planted gravel communities (D^2=1, Figure 5.3a). When gravel from unplanted columns was excluded from the analysis a unique community profile was also observed for each of the plant species with *D. cespitosa* separating from *L. cinereus* (p=0.01, D^2=1; Figure 5.3b).

Analysis of the root communities (only *D. cespitosa* and *L. cinereus*) also showed the rhizosphere community profiles of the two species as separate from one another ($p<0.05$, $D^2=0.3472$). Interestingly regardless of plant type, more significant community differences were associated with root sample location (i.e. thick roots, thin roots, ultra-fine roots) ($p<0.01$, $D^2=0.7649$ for R2 (thick roots) and $D^2=0.9969$ for R4 (ultra fine roots); Figure 5.4). The hierarchical clustering results showed that the thick root samples grouped together on the dendrogram but separately from the fine and ultra fine roots, which clustered more together regardless of plant species.

Gravel sample location also had an effect on the microbial community ($p=0.10$, $D^2=0.9994$). This was true for all gravel samples, planted and unplanted. Gravel higher in the column (generally considered to be a more aerobic environment (Stein and Hook, 2005; Taylor et al., 2010), and associated with the roots in planted treatments) had biofilm communities distinct from those lowest in the column (in anaerobic conditions) and furthest from plant roots (Figure 5.5).

In spite of the observed differences in the biofilm communities, analysis by HC of the effluent communities in the CW showed no significant separation of the profiles according to any of the environmental variables (season or plant species) (all $p>0.4$). Surface analysis of the effluent samples could not be performed because there were too few data points. This result indicates that microbial community analysis of CW effluent was not a reliable means for determining community structure or detecting differences.

The dataset was also partitioned and analyzed according to plant species (control, *D. cespitosa*, and *L. cinereus*) in the same manner as was done for sample type. With this

approach, the greatest differences in microbial communities were attributed to the growth surface available (i.e. root, gravel, effluent; $p<0.01$ for each plant species). Once again, effluent was generally the most dissimilar cluster making it the most different of all the sample types.

Discussion

In this study, we demonstrated that 16S rDNA DGGE profiles were informative for detecting microbial community shifts as well as comparing communities from a variety of samples, including season, plant species, and location. Although admittedly not directly relatable to function, 16S rDNA based methodologies are more frequently being considered to be too general to be informative. Rigorous statistical analyses were applied to overcome these obstacles and all of the hypotheses posed were re-evaluated.

First, the biofilm communities from the gravel, rhizosphere, and effluent were distinct from each other, indicating that the growth surface had the greatest effect on the resultant microbial biofilm composition. This result was supported by hierarchical clustering analysis as well as principle coordinate analysis with general surface plots. This further demonstrated that the location from which a sample was taken needs to be evaluated to determine whether or not it appropriately represents the community of interest (discussed below).

Second, there was a plant specific effect on the microbial communities detected by both methods used. Hierarchical clustering showed plant species to have a significant effect on biofilm community structure overall. Other researchers have also reported observing plant effects on the microbial community structure in CWs (Tanner, 2001;

Stottmeister et al., 2003; Vacca et al., 2005). Within sample type, plant species also impacted the microbial community, which resulted in a distinct community profile. It should be noted that when gravel samples were compared, there was greater similarity among communities from planted CW microcosms (*D. cespitosa* and *L. cinereus*) than to the community from the unplanted microcosm. This result implied that the presence or absence of any plant significantly impacts the microbial community composition. Root surfaces are known to supply exudates and oxygen to rhizosphere bacteria (Wieland et al., 2001). Similarly, focus on the rhizosphere communities revealed that each plant species also cultivated a unique community. Location along the root most greatly affected the rhizosphere community observed, with distinct populations observed from the rhizosphere of thick (R2) and ultra-fine (R4) roots. Rhizosphere biofilm communities attached to the thick roots (R2) nearest to the crown of the plant had unique communities compared to the thinner roots (R3) sampled and the ultra-fine roots (detached during sampling and observed to be leaking oxygen, R4) also had unique rhizosphere communities. The thin roots (R3) between these two locations (R2 and R4) did not have a distinct community of their own indicating a community gradient from thickest (older) to thinnest (youngest) roots. One study focused on rhizosphere microscopy found an increase in bacterial density with distance from the root tip and a high morphological similarity of organisms observed at the root tips (Munch et al., 2007). Other studies have indicated that plant age and growth stage affects the rhizosphere community structure (Grayston et al., 1997; Westover et al., 1997; Miethling et al., 2000; Marschner et al., 2001; Kowalchuk et al., 2002; Marschner et al., 2004) while others did not show a

correlation (Duineveld et al., 1998; Wieland et al., 2001). It has been reported that the highest amount of root oxygen loss occurs at the root tips of the youngest roots (Brix, 1994, 1997), and that increases in root exudation occur near the apical roots (Marschner et al., 2004), which can influence the communities (Yang and Crowley, 2000).

Gravel sample location also affected biofilm community structure (regardless of plant species) with the biofilm attached to the top gravel having a distinct community from the biofilm attached to the bottom gravel. The presence of roots in the top gravel of planted microcosms likely influenced the associated gravel with increased oxygen release and exudation. Top gravel samples from the unplanted column were collected from the upper portion of the column and were likely to be more aerobic than the deeper locations. The bottom gravel samples of all columns were without roots and considered highly anaerobic with extensive blackening and hydrogen sulfide odors observed during destructive sampling.

Third, in regard to the hypothesis of a seasonal effect, we did not observe a seasonal shift in the microbial community from summer to winter as expected. This indicated that the established communities are robust and not significantly impacted by the seasonal temperature shifts encountered. It is believed that the diversity of a population of organisms, such as observed in this instance, causes it to be more resilient to stress and environmental changes (Kirk et al., 2004). Although the age of the plant appears to affect the rhizosphere communities along the length (age) of the root, these differences don’t appear to translate to a detectable community shift with respect to CW age or seasonal cycling (summer to winter or year to year).

Overall the most important factors influencing microbial community structure were plant species and sample type (effluent, gravel, roots). These results have previously been observed in agricultural soil environments (Miethling et al., 2000; Marschner et al., 2001; Kent and Triplett, 2002). Some researchers that have analyzed large composite samples consisting only of gravel and effluent and have not observed a plant effect on the microbial community structure (Tietz et al., 2007b; Baptista et al., 2008). Other groups have also found no effect of plant species on microbial community composition (Ahn et al., 2007; DeJournett et al., 2007; Gorra et al., 2007; Tietz et al., 2007b). This may be due to the composite sampling methods used or the plant species being investigated.

It is well known that a variety of microenvironments can exist in close proximity to one another (Kang and Mills, 2006) and that several samples need to be collected to adequately assess the microbial diversity in soil habitats (Grundmann and Gourbiere, 1999). This work has demonstrated the importance of separately investigating these microenvironments so that appropriate analyses of the communities can be performed.

Care must be taken to appropriately sample the system of interest and to take a thorough and thoughtful statistical approach to the data. It is also important to note that DGGE is an inherently qualitative method and although rigorous statistics may be applied, over interpretation of gels during initial processing may detrimentally bias the final results. Great care was taken to disregard uncertain bands and artifacts and to only utilize statistical methods focused on the presence or absence of bands. In addition, band intensity was not considered a factor in the statistical calculations to avoid incorporating

PCR biases (e.g. preferential amplification). This is a unique approach as research has generally incorporated band intensity assuming it to be directly related to the corresponding phylotypes represented (Murray et al., 1996). This approach assumes no biases during extraction or amplification and results in the incorporation of these biases into the reported results (Fromin et al., 2002).

This is a novel approach to DGGE data analysis, as such it is important to note that these techniques and results may be updated prior to publication. In this paper we've applied advanced statistical methods have been applied to determine the variables contributing most to differences observed between 16S rDNA band profiles in constructed wetland microcosm systems. These methods may be useful for prescreening environmental samples to determine where more in depth molecular methods can be applied most advantageously. New technologies such as the PhyloChip and pyrosequencing yield immense quantities of data that can be difficult to analyze. Using these statistical methods, we were able to determine that sample type and plant species contributed most greatly to community shifts. As a result, future research could focus subsequent molecular analyses on these particular samples to determine the microorganisms most influencing these communities.

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FIGURES

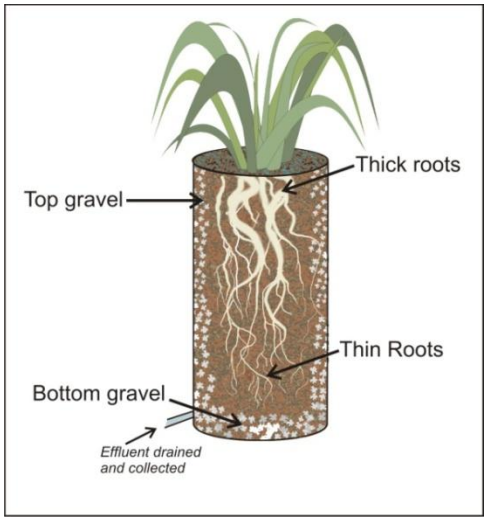

Figure 5.1. Location of microcosm samples. Ultra-fine roots are not shown.

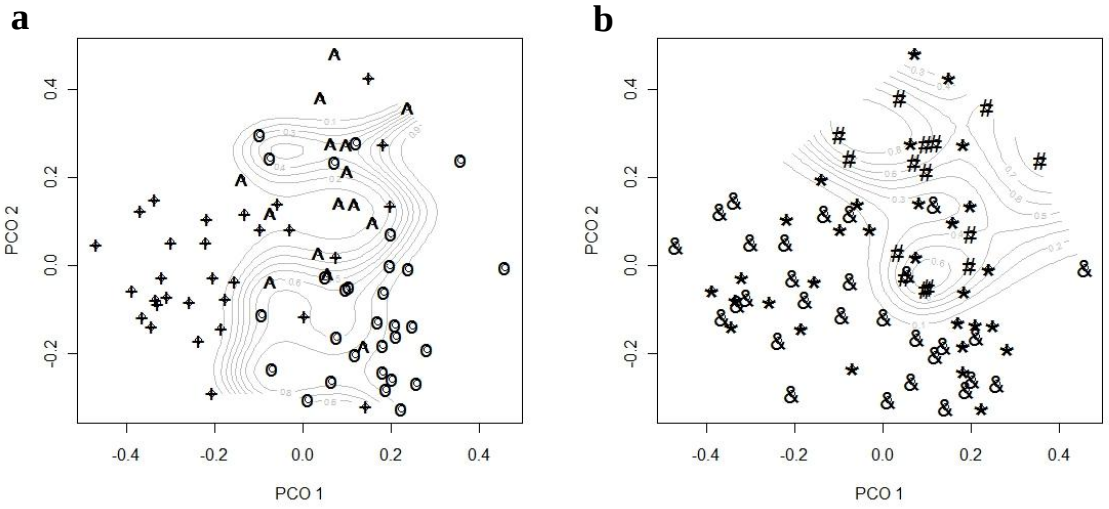

Figure 5.2. PCO surface analysis of the whole 16S rDNA dataset according to sample type (2a) and the plant species (2b). 2a – Plotted lines indicate separation of gravel communities from rhizosphere and effluent communities, **o** = gravel, + = roots, ^ = effluent. 2b – Plotted lines indicate separation of control communities from planted communities, # = unplanted, * = *L. cinereus*, **&** = *D. cespitosa*.

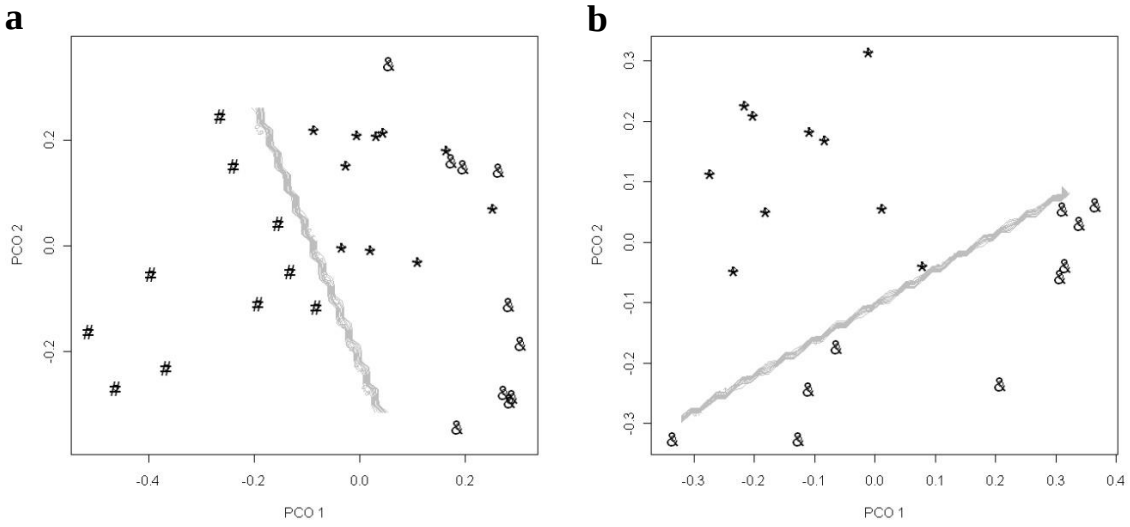

Figure 5.3. Analysis of biofilm communities attached to gravel (3a) and to gravel in planted microcosms only (3b). 3a – Plotted lines indicate separation of planted from unplanted microcosms. 3b – Plotted lines indicate separation of plant species *L. cinereus* from *D. cespitosa* from one another. # = unplanted control, * = *L. cinereus*, **&** = *D. cespitosa*.

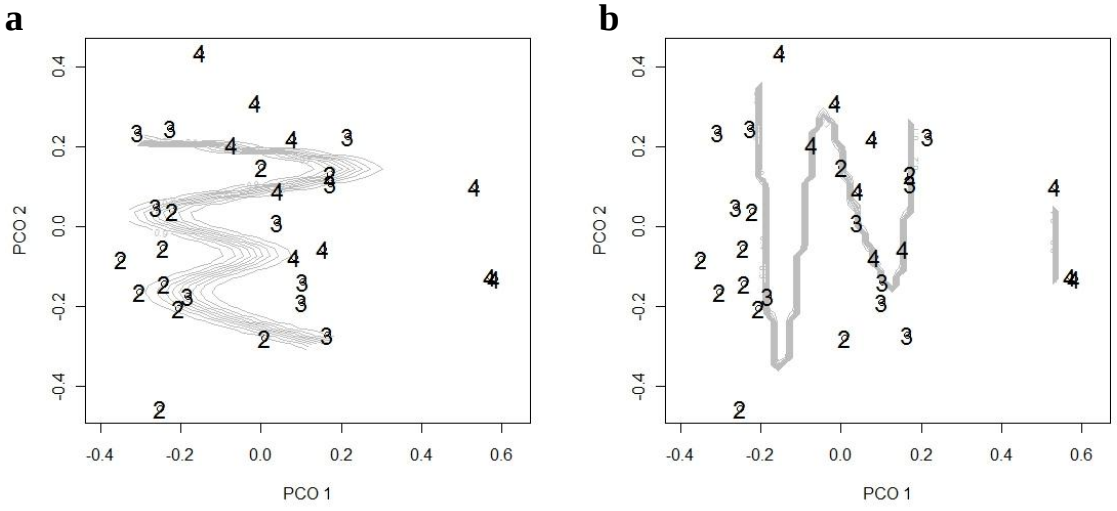

Figure 5.4. Distinction of the rhizosphere biofilm communities according to sample location along the root surface regardless of plant type. 4a – Plotted lines indicate separation of the thick roots (R2) from the remaining root samples ($D^2 = 0.7649$). 4b – Plotted lines indicated separation of the ultra fine roots (R4) from the remaining root samples ($D^2 = 0.9969$). 2 = thick roots (R2), 3 = fine roots (R3), 4 = ultra fine roots (R4).

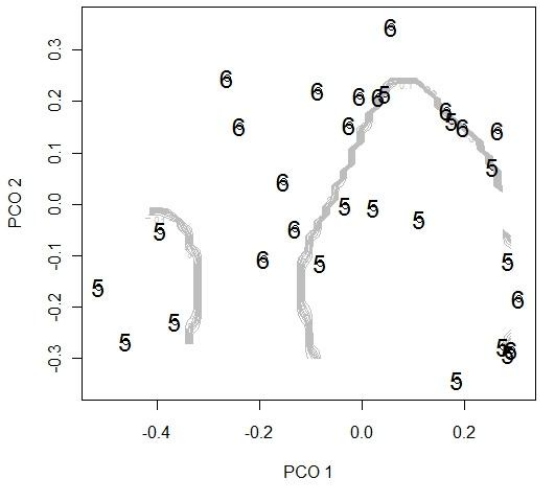

Figure 5.5. Analysis of biofilm communities attached to gravel regardless of plant species. Plotted lines indicate separation of top and bottom gravel communities. 5 = top gravel (G5), 6 = bottom gravel (G6).

Contribution of Authors and Co-authors

Chapter 6: The influence of sulfate reducing bacteria and ammonia oxidizing bacteria on nutrient cycling in constructed wetland microcosms

Author: Jennifer L. Faulwetter
Contributions: Designed and conducted the experiments included as well as analyzed the resultant data. Compiled and organized the data into the publication and was actively involved in the editing and submission process.

Co-author: Mark D. Burr
Contributions: Assisted in data analysis and publication compilation, as well as contributed to revision and editing of the paper. Was also a co-PI and advisor on the project.

Co-author: Albert E. Parker
Contributions: Assisted in the design of statistical analyses for the data, interpretation of results and editing of the related manuscript sections.

Co-author: Otto R. Stein
Contributions: Was a PI and advisor on the project as well as assisted in editing the publication.

Co-author: Anne K. Camper
Contributions: Was a co-PI and advisor on the project as well as assisted in editing the publication.

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CHAPTER 6

THE INFLUENCE OF SULFATE REDUCING BACTERIA AND AMMONIA OXIDIZING BACTERIA ON NUTRIENT CYCLING IN CONSTRUCTED WETLAND MICROCOSMS

Short title: Influence of SRB and AOB on CW nutrient cycling

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Abstract

Constructed wetlands (CW) offer an effective means for treatment of wastewater from a variety of sources. Current research is focused on a better description of the carbon, nitrogen and sulfur cycles; the characterization of the microbial ecology controlling these cycles has been identified as the greatest research need to increase performance of these promising treatment systems. It is suspected that operational factors, such as plant species selection and hydraulic operation influence the sub-surface wetland environment, especially redox, and that the observed variation in effluent quality is due to shifts in the microbial populations and/or the activity of these various sub-populations. This study investigates sulfate reducing bacteria and ammonia oxidizing bacteria (using the *dsrB* and *amoA* genes, respectively) by examining a variety of locations within the wetland (gravel, thick roots, fine roots etc.), and the changes in activity (gene presence) of these functional groups as influenced by plant species selection and season. A variety of molecular techniques were used including quantitative PCR and denaturing gradient gel electrophoresis (DGGE) both with and without propidium monoazide (PMA) treatment. Results showed an overall plant and seasonal interaction with respect to the relative gene abundance for both functional groups investigated ($p<0.05$). DGGE profiles of sulfate reducing communities appeared to be most impacted by the presence/absence of a plant with communities in planted microcosms also being affected by season. Ammonia oxidizing community profiles appeared to be equally affected by season and sample type (gravel, roots, or effluent).

Introduction

Constructed wetlands (CW) are a promising technology utilizing engineered wetland systems for the treatment of a variety of wastewaters ranging from domestic sources to storm run-off. Although the operation of CWs is relatively well understood, there is a lack of understanding of the microbial impact on performance (Faulwetter et al., 2009). Many studies have investigated microbially-mediated processes in CWs by focusing on net changes in the concentration of specific chemicals or waste constituents (Stein and Hook, 2005; Whitmire and Hamilton, 2005; Stein et al., 2007). Microbial communities have been shown to effectively contribute to the reduction of chemical pollutants in CWs (Hatano et al., 1994; Scholz and Lee, 2005); however, microbiology remains the most underrepresented area of research in this field. More specifically, the role of biofilms and their function within these systems has been virtually ignored. Presently, these gaps in knowledge of the microbial ecology may impede the effective design and operation of constructed wetlands (Faulwetter et al., 2009). An understanding of the microorganisms involved in specific biogeochemical processes and where the organisms involved are located (as well as how the community structure and population changes from summer to winter etc.) is important for the design of CWs, as well as for other bioremediation applications.

The microbial diversity found in natural environments is an asset that can be utilized for bioremediation, but the diversity also makes the study of microbial ecology in these environments difficult. In CWs, optimization of effluent water quality is the main objective; as a result, bulk water sampling would seem to be an appropriate approach to

understand the community dynamics within. However, composite sampling methods (such as soil cores) can underestimate actual microbial diversity (Kirk et al., 2004; Konopka, 2009); implementation of similar methods also makes characterization of the microbial diversity in CWs a challenge. To overcome this issue, an intensive destructive sampling protocol was developed to better characterize the diversity present in microenvironments within the CW microcosms. In addition to gravel and effluent samples, focus was placed on the rhizosphere biofilms since the plant rhizosphere in CWs, and other vegetated soils, has been found to have a different microbial community compared to the bulk substratum (Smalla et al., 2001; Kowalchuk et al., 2002; Chapter 5).

Microbial biofilm communities are found on every available surface in CWs, as biofilms are capable of creating stable, protective environments for microbial survival and are an ideal location for all forms of microorganisms (Hall-Stoodley et al., 2004). Due to the active microbial populations within these biofilms, it is possible for oxygen gradients to develop within the biofilm (Brune et al., 2000; Stewart and Franklin, 2008). These microenvironments allow for the simultaneous presence and growth of both anaerobic and aerobic organisms. It is important to not only identify the organisms present within these biofilms, but to also investigate the microorganisms that are active (viable) within these environments so that a link between the biogeochemical processes and the population, structure and function of the microbial community responsible can be made (Minz et al., 1999; Kirk et al., 2004). These dynamic populations of microbes are

responsible for various biogeochemical cycling processes such as sulfate reduction (an anaerobic process) and nitrification (an aerobic process).

Molecular methods are being increasingly used to investigate microbial diversity and activity in environmental systems, such as CWs, because culture-based methodologies are severely limiting (Amann et al., 1995; Saleh-Lakha; 2005). An overall perspective of the microbial diversity can be obtained utilizing molecular markers targeting the 16S rDNA gene, but these have limited utility for investigating specific microbial groups. In order to focus on specific microbial populations of interest, functional primers targeting genes involved in specific metabolic pathways were employed. Since DNA is known to be stable in the environment (Josephson et al., 1993) and PCR based technologies cannot distinguish between DNA extracted from live (active) and dead (inactive) cells, samples were treated with propidium monoazide (PMA) prior to DNA extraction (Nocker et al., 2006; Nocker et al., 2007). PMA is capable of entering the cells of bacteria with compromised or damaged cell membranes and binding to the DNA. Exposure to light causes PMA to become permanently bound to the DNA-PMA complex, which is permanently inhibited from subsequent PCR amplification leaving only DNA from the living cells (with intact cell membranes) to be amplified (Nocker et al., 2006).

Previous research had long inferred the presence of a robust sulfate reducing bacteria (SRB) population within our CW microcosms (Borden et al., 2001; Allen et al., 2002; Stein et al., 2007). Most recently, Taylor et al. (2010) showed that microcosms planted with *Deschampsia cespitosa* had high removal efficiencies of organic carbon

(COD) (nearly 100%) and seasonally dependent removal of sulfate (highest in summer) while unplanted microcosms, as well as those planted with *Leymus cinereus*, had slightly lower overall COD removal (80%) but constant sulfate removal, regardless of season. Since constant sulfate removal was observed in the unplanted control and *L. cinereus* microcosms, the fluctuations in seasonal sulfate performance (determined by effluent water quality) were assumed to be linked to the plant species selection. Redox data revealed a near constant values of -200 mV or less for the unplanted control and *L. cinereus* microcosms, with high redox values (up to +400 mV) observed for *D. cespitosa* microcosms (with the exception of the summer season where redox values dropped to -200 mV) (Taylor et al., 2008; Taylor, 2009). These data show that conditions appropriate for both aerobic and anaerobic processes exist with these microcosms. The redox variation within these microcosms is likely the result of root oxygen loss (ROL) from the root surface into the rhizosphere.

The root surface is very dynamic and heterogeneous, making it an ideal surface for colonization by varying populations of microorganisms. Plant roots support large populations of microorganisms as more nutrients are available at the root surface (Burgmann et al., 2005; Bais et al., 2006; Munch et al., 2007) making it an ideal location for a variety of bacterial species. Additionally, chemical compounds released from roots, such as oxygen, are generally plant species specific and plants are thought to selectively enrich the rhizosphere with microorganisms accordingly (Burgmann et al., 2005). This process of ROL and exudation is likely to enrich for unique microbial assemblages within CW microcosms and affect the effluent water quality.

Sulfate reducing bacteria are important in CW systems because sulfate is a common pollutant in a variety of wastewater types including domestic wastewater and acid mine drainage. SRB have a vital role in the geochemical cycling of sulfur, important for many biological processes and also for the generation of alkalinity in CWs (Kalin et al., 2006). SRB utilize sulfate (SO_4) as a terminal electron acceptor in the anaerobic oxidation of organic substrates (Hsu and Maynard, 1999) and are critically important since they are the only known organisms to perform this function. These organisms are found mainly below the soil (or water) surface where anoxic environments are best suited for sulfate reduction. Historically, SRB have been considered strict anaerobes, but more recent research has shown that SRB can persist in oxic conditions and survive extended periods of oxygen exposure (Brune et al., 2000; Cypionka, 2000; Sigalevich et al., 2000). Many studies have assumed SRB activity in CW based on observations of high sulfate input, low sulfate output, high sulfide concentrations, minimal sulfate uptake by CW plants, and low redox conditions (Hsu and Maynard, 1999; Borden et al., 2001; Song et al., 2001; Allen et al., 2002; Stein et al., 2007). This study focused on the dissimilatory sulfite reductase gene (*dsrAB*), specifically the second smaller fragment (*dsrB*). This gene was selected as it is essential for anaerobic sulfate reduction and has been found in all dissimilatory sulfate-reducing prokaryotes thus far (Minz et al., 1999; Bahr et al., 2005; Geets et al., 2006; Agrawal and Lal, 2009).

Ammonia is another common pollutant found in wastewaters (e.g. domestic and agricultural wastewater) and can have detrimental effects on the environment when discharged. Nitrification plays a critical role in the biogeochemical cycling of nitrogen

and has been documented in a variety of CW systems (Sundblad, 1998; Cooper and Griffin, 1999; Sundberg et al., 2007b). Nitrification occurs via two aerobic reactions: ammonia to nitrite, by ammonia oxidizing bacteria (AOB), and nitrite to nitrate, by nitrite oxidizing bacteria (NOB). The oxic conditions required can limit transformation of ammonia in traditional wastewater treatment systems (Paredes et al., 2007a), but the presence of plants in CWs may be able to provide the required oxygen concentrations through their roots (Brix, 1994; Zhu and Sikora, 1995; Brix, 1997; Johnson et al., 1999; Scholz and Lee, 2005). AOB were targeted by examining the ammonia monooxygenase gene (*amoA*) as this gene is responsible for the initial rate limiting step in nitrification and has been used in a variety of ecological studies investigating AOB (Rotthauwe et al., 1997; Kowalchuk et al., 2000; Ibekwe et al., 2003; Yin et al., 2009; Dang et al., 2010).

The goal of this study was to determine the active communities (using PMA) of SRB and AOB, within CW microcosms, by evaluating the relative DNA copy numbers (by quantitative PCR) of two functional genes, *dsrB* (involved in sulfate reduction) and *amoA* (involved in nitrification). The diversity within each of these genes was also evaluated using denaturing gradient gel electrophoresis. CW microcosms were fed a synthetic wastewater that simulated post-primary domestic wastewater effluent. Effluent water quality analyses were performed to assess removal of sulfate and ammonia as well as used to correlate performance (by pollutant removal) with the relative quantities of the targeted genes. Microcosms were either planted with two different macrophyte species or left unplanted, and maintained in a greenhouse with temperature regulation that simulated natural ambient conditions in a seasonally cold climate. Microcosms were

destructively sampled in summer and winter seasons to investigate the effects of plant species and season on the functional communities of interest.

Methods

Constructed Wetland Operation

This research was conducted in the same facilities used in earlier studies (Stein and Hook, 2005; Stein et al., 2007; Taylor et al., 2010). Constructed wetland microcosms were used to simulate an operational subsurface flow CW and were maintained in a greenhouse at the Plant Growth Center at Montana State University in Bozeman, MT (46˚N, 111˚W). Six replicates of unplanted controls and monocultures of *Deschampsia cespitosa* and *Leymus cinereus* were planted in model subsurface wetlands consisting of 15 cm diameter by 30 cm tall polyvinyl chloride columns filled with 1-5 mm gravel. Greenhouse temperature was changed every 60 days to mimic natural seasonal cycles. The annual temperature sequence was 4, 8, 16, 24, 16, 8, and 4˚C. Supplemental lighting was not used. Patterns of natural light and controlled temperature induced normal seasonal cycles of plant dormancy and growth. Microcosms were fed synthetic wastewater simulating post-primary domestic wastewater effluent (Taylor et al., 2010). There were three 20-day batches at each temperature. Between batches, the microcosms were completely drained. Plants were grown for a minimum of 12 months prior to the first sampling date. All sampling for this research was done during one winter (4˚C) and the following summer (24˚C).

Plant Species Selection

The two plant species investigated in this research were selected from a list of 19 species based upon their performance in earlier constructed wetland microcosm experiments (Taylor et al., 2008; Taylor et al., 2010). The objective was to compare two species at opposite ends of the spectrum in terms of their apparent effects on nutrient removal (carbon, oxygen release, sulfate) in CW. Plants were selected based upon COD removal and oxygen release because these are standard criteria for evaluating CW performance (Scholz and Lee, 2005). CW columns planted with *Deschampsia cespitosa* were very effective at carbon removal, as demonstrated by reductions in chemical oxygen demand (COD). *D. cespitosa* also readily released oxygen from its roots regardless of season (Taylor et al., 2008; Taylor, 2009; Taylor et al., 2010). Thus, it had the potential to create oxic microenvironments immediately surrounding the root in the otherwise anoxic depths of the CW. In contrast, CW columns planted with *Leymus cinereus* provided poor COD removal and undetectable levels of oxygen release from roots (Taylor et al., 2008; Taylor et al., 2010). It was also advantageous that both species belonged to the same family (*Poaceae*), because potential differences in plant physiology were minimized. Unplanted control columns (containing only gravel) were also included in the experimental design.

Wastewater Analysis

Chemical oxygen demand (COD), and concentrations of ammonia, sulfate, nitrite and nitrate were measured to confirm that microcosms were performing as in earlier studies with respect to removal of carbon, nitrogen, and sulfate (Taylor et al., 2010) prior

to destructive sampling for microbial analysis. During the third 20-day wastewater batch of the winter (4°C) and summer (24°C) season, water was collected from the wastewater mixing tank before filling the microcosms (day 0), and afterwards from the microcosms on days 3, 6, 9, and 20. Microcosm water samples were collected via a vinyl tube with an intake at 15 cm depth. Chemical oxygen demand and ammonia were measured colorimetrically (dichromate method, 20-1500 mg/L COD range and salicylate method, 0.4-50 mg/L NH_3-N, respectively, HACH, Loveland, CO). SO_4, NO_2, and NO_3 were measured by ion chromatography (Dionex Corp., Sunnyvale, CA).

Microcosm Destructive Sampling

Sampling was essentially as reported previously (Chapter 5). CW microcosms were destructively sampled, in duplicate, at the completion of the third batch (day 20) at the specified temperature (4°C, winter, and 24°C, summer). Six samples were obtained from each microcosm, for the ultimate purpose of DNA extraction: effluent (E1), thick roots near the crown of the plant (R2), thin roots near the tip (R3), ultra-fine root hairs that were skimmed from the surface after gravel was washed with tapwater (R4), gravel from the top of the column (G5), and gravel from the bottom (G6). The purpose of this intensive sampling was to determine whether certain samples were more descriptive of the microbial community than others. The gravel from the bottom of the column was considered anoxic and interesting for its potential to harbor anaerobic (or facultative) microorganisms. For that reason, it was immediately transferred to an anaerobic chamber to protect anaerobes against oxygen exposure. Duplicate samples were collected from each column location, one for PMA treatment, the other untreated. Briefly, the effluent

sample was filtered (250 mL), and the filter was asceptically divided in half between PMA-treated and untreated DNA extraction tubes (MO BIO PowerBead Tube, MO BIO PowerSoil™ DNA Isolation Kit). Thin and thick roots were asceptically transferred to DNA extraction tubes. Ultra-fine roots were skimmed into a 100 mL volume of water, filtered, and the filter contents were transferred to a DNA extraction tube. Gravel was vortexed as described in Chapter 5. For PMA treatment, 3 mL of the supernatant was transferred in 0.5 mL aliquots to six DNA extraction tubes. For the untreated sample, six 0.5 mL aliquots were sequentially pelleted for DNA extraction. Untreated samples were placed directly into a MO BIO PowerBead Tube (MO BIO PowerSoil™ DNA Isolation Kit) for DNA extraction. PMA treated samples were placed into a clear 1.7 mL microcentrifuge tube for treatment (see protocol below).

PMA Treatment

A total volume of 0.5 mL for each sample was used for PMA treatment. For the effluent and all root samples, 0.5 mL of sterile effluent filtrate was added to the clear 1.7 mL tube (www.biotang.com) containing the sample to be treated. Since PMA treatment is based upon the integrity of the cell membrane, sterilized filtrate was used to minimize any osmotic shock or artificial membrane damage to the cells prior to treatment with PMA. PMA treatment was as described in Nocker et al. (2007), except as modified below. To each tube, 1.5 µL of PMA solution was added and each tube was shaken vigorously. Tubes were incubated in the dark on ice for 5 minutes with the exception of bottom gravel samples which were exposed to PMA for 7 to 10 minutes. PMA was inactivated by exposing the tubes to light for 2 minutes using a 650 W halogen light

source (sealed beam lamp, FCW 120V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH). Samples were shaken during light exposure. Root samples were exposed for 4 minutes to minimize the effect of shadowing by root material. Upon completion of PMA treatment, samples were transferred to MO BIO PowerBead Tubes (MO BIO PowerSoil™ DNA Isolation Kit) for DNA extraction. Gravel samples were processed in six 0.5 mL aliquots and after PMA inactivation, each sample was concentrated by centrifugation sequentially (in 2 mL volumes, total of up to 3 mL) for 5 minutes at 5,000 x g into a MO BIO PowerBead Tube.

DNA Extraction

The MO BIO PowerSoil™ DNA Isolation Kit (www.mobio.com) was used to complete the DNA extraction as described in the manufacturer's protocol with the exception that instead of vortexing, PowerBead tubes were placed into the FastPrep® Instrument (Qbiogene, Inc.) at speed 5.5 for 45 s. DNA yield was estimated on an agarose gel with ethidium bromide staining, serial dilutions were performed for PCR, and the DNA preparations were stored at -20°C.

Conventional PCR

All PCR reactions (20 μL) were performed using 2X GoTaq® Green Master Mix (www.promega.com). The PCR reaction mixture consisted of 10 μL 2X GoTaq® Green Master Mix, 0.5 μL Ultrapure BSA (50 mg/mL, Ambion, www.ambion.com), 2.5 μL DEPC-treated water, 1 μL each of forward and reverse primers (12.5 μM), and 5 μL 1:10 diluted (unquantified) template DNA. Oligonucleotide primers were synthesized by Integrated DNA Technologies (www.idtdna.com) and described in Table 6.1. Multiplex

reactions were adjusted to contain 10 µL 2X GoTaq® Green Master Mix, 0.5 µL Ultrapure BSA (50 mg/mL, Ambion, www.ambion.com), 0.5 µL DEPC-treated water, 1 µL of each forward and reverse primer(12.5 µM), and 5 µL 1:10 diluted (unquantified) template DNA. All PCR amplifications were performed on an Eppendorf Mastercycler® ep thermal cycler (Eppendorf North America, www.eppendorfna.com) using the specified program below. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide.

Sulfate Reducing Bacteria (for Cloning and DGGE) PCR primers DsrBF and Dsr4R (Table 6.1) target the β-subunit of the dissimilatory sulfite reductase gene (*dsrB*, required for sulfate reduction). Presumptive presence of the *dsrB* gene was indicated on an agarose gel by a 370 bp PCR product. Primer DsrBF was also synthesized with a 5' 40-bp GC clamp and was paired with primer Dsr4R for amplifying fragments to be analyzed by DGGE. The PCR program specified in Geets et al. (2006, Table 6.2) was used for amplification. Products were used for either cloning or were analyzed by DGGE.

Multiplex PCR PCR primers DsrBF and Dsr4R (Table 6.1) were combined with primers RottF and RottR (Table 6.1). Presumptive presence of the genes was indicated on an agarose gel by 370 and 491 bp PCR products. The PCR program specified in Bahr et al. (2005, Table 6.2) was used for amplification. Products were diluted and used for additional PCR amplification with the Rott primers and analysis by DGGE.

Ammonia Oxidizing Bacteria (for Cloning and DGGE, with Multiplex Above)

PCR primers RottF and RottR (Table 6.1) target the ammonia monooxygenase gene (*amoA*, required for ammonia oxidation to nitrite). Presumptive presence of the *amoA* gene was indicated on an agarose gel by a 491 bp PCR product. Primer RottR was also synthesized with a 5' 40-bp GC clamp and was paired with primer RottF for amplifying fragments to be analyzed by DGGE. Multiplex PCR product was diluted 1:100 and 5 µL was used as template in the reactions subsequently analyzed by DGGE. For PCR products to be cloned and used as qPCR standards, 5 µL 1:10 diluted (unquantified) template DNA was used for each reaction. The PCR program specified in Bahr et al. (2005, Table 6.2) was used for amplification. Products were used for cloning and reamplified multiplex products were used for DGGE analysis.

DGGE

DGGE was performed on functional PCR products (*dsrB* and *amoA*) with and without PMA from community DNA using a DCode™ system (www.biorad.com) and reagents from Sigma-Aldrich (www.sigmaaldrich.com). Gels had a gradient of denaturant concentrations from 40% at the top of the gel to 70% at the bottom, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained an 8 to 12% polyacrylamide gradient from top to bottom (Girvan et al., 2003). Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr®Gold (www.invitrogen.com) and documented using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com). Three marker lanes (generated from five pooled clones) were included in each DGGE gel so that cross-comparison between gels would be possible. Bands in DGGE images were

identified using GelCompar II software (Version 6.1, Applied Maths Inc.) and confirmed visually.

DGGE Marker DGGE marker was generated by pooling five unidentified clones. The clones were individually screened using clone/DGGE (Burr et al., 2006) and selected based upon their different migration distances in DGGE to span the entire length of the gel from top to bottom. A DGGE marker pool was created by combining 250 µL of each individual clone broth culture, purifying the combined plasmid DNA from the marker pool using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com), and storing the plasmid DNA at -20°C. Marker DNA was amplified using VectF and VectR+GC primers (Table 6.1), which target the plasmid vector pCR®2.1-TOPO® immediately flanking the vector cloning site (Burr et al., 2006). PCR reactions (20 µL) were prepared as described above and the program described in Bahr et al. (2005, Table 6.2) was used for amplification. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide and were then used in DGGE.

Quantitative PCR

All PCR amplifications were performed in a Rotor-Gene 3000 real time PCR cycler (QIAGEN, formerly Corbett Life Science, www1.quiagen.com) in a 72-well rotor using the programs described in Table 6.2: 16S rDNA program was modified from Agrawal and Lal (2009), *dsrB* from Geets et al. (2006, see qPCR modifications in Table 6.2), *amoA* from Geets et al. (2007). Data was acquired using the FAM/Sybr detection channel during the extension step. For each run, a standard curve of C_t values was

incorporated and samples calculated against the standard curve. Standards and samples were prepared in triplicate and appropriate negative controls containing no template DNA were included to ensure no contamination had occurred. Melt curve analysis was also performed after thermocycling to verify PCR products. Melt curve analysis was performed from 65-95°C in 0.3°C increments held for 5 s with an initial pre-melt hold for 90 s at the first step.

16S rDNA PCR primers Eub341F and Eub534R target the variable V3 region of the 16S rDNA gene. Quantitative PCR reactions (25 µL) were performed using Power SYBR® Green PCR Master Mix (www.appliedbiosystems.com). The PCR reaction mixture consisted of 12.5 µL Power SYBR® Green PCR Master Mix, 0.5 µL Ultrapure BSA (50 mg/mL, Ambion), 2 µL DEPC-treated water, 1 µL of forward and reverse primers (12.5 µM), and 8 µL 1:10 diluted template DNA. These primers amplified a 193 bp fragment of the 16S rDNA gene.

Sulfate Reducing Bacteria and Ammonia Oxidizing Bacteria PCR primers DsrBF and Dsr4R (Table 6.1) were used for amplification of sulfate reducing bacteria and RottF and RottR (Table 6.1) were used for amplification of ammonia oxidizing bacteria. Quantitative PCR reactions (25 µL) were performed using Power SYBR® Green PCR Master Mix (www.appliedbiosystems.com). The PCR reaction mixture consisted of 12.5 µL Power SYBR® Green PCR Master Mix, 0.5 µL Ultrapure BSA (50 mg/mL, Ambion, www.ambion.com), 2 µL DEPC-treated water, 1 µL of forward and reverse primers (12.5 µM), and 8 µL undiluted template DNA. The DsrB primers amplified a 370 bp fragment of the *dsrB* gene and the Rott primers amplified a 491 bp fragment of the *amoA* gene.

Cloning and Sequencing (for qPCR Standards and DGGE Marker)

Purified (16S rDNA, *dsrB*, *amoA*) PCR products were cloned into plasmid vector pCR®2.1-TOPO® using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. White colonies indicative of a successful cloning reaction were used to inoculate sterile tubes containing 10 mL of Luria-Bertani (LB) broth plus 50 mg mL^{-1} ampicillin. The tubes were incubated overnight at 37°C in a shaking incubator. Plasmid DNA was purified from individual clones using the Wizard Plus SV Minipreps DNA Purification System (www.promega.com) and stored at -20°C. Plasmid DNA was quantified using the NanoDrop ND-1000. Clones were sent to the Research Technology Support Facility (RTSF) at Michigan State University and were sequenced using the M13F primer. Edited sequences were compared with known sequences in the GenBank database using the Basic Local Alignment Sequence Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

qPCR Standard Curve Preparation

Standard curves were obtained with serial dilution of the quantified standard plasmids carrying the target 16S rDNA, *dsrB*, or *amoA* gene. Standard curves were generated for each gene of interest, in triplicate, according to the protocol described above. The copy number of the standard plasmids carrying the targeted genes ranged from 1.09×10^7 to 1.09×10^2 copies/µL for 16S rDNA, 3.12×10^7 to 3.12×10^2 copies/µL for *dsrB*, and 2.53×10^7 to 2.53×10^2 copies/µL for *amoA*.

Statistical Analyses

DGGE DGGE gel images were processed and normalized using the GelCompar II software (Version 6.1, Applied Maths Inc.). Bands in DGGE images were identified on a presence–absence basis. Band intensities were not considered during statistical analysis. This is a unique approach as previous research has incorporated band intensity assuming it to be directly related to the quantities of the corresponding phylotypes (Murray et al., 1996). The above approach assumes no biases during extraction or amplification and results in the incorporation of these biases into the reported results (Fromin et al., 2002). Subsequent statistical analyses of the presence-absence data were performed using R software (Version 2.11.1) libraries labdsv (Roberts, 2009) and optpart (Roberts, 2010) (www.r-project.org). Dissimilarity matrices were calculated using the Sorensen method (Van der Gucht et al., 2001; McCune and Grace, 2002).

Hierarchical clustering (HC) analyses were performed for each of the functional genes and dendrograms were generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Ibekwe et al., 2001; Boon et al., 2002). To optimize the dendrograms to the most informative number of clusters, a stride plot was generated which shows the global (partana ratio) and local (silhouette width) values for the cluster analysis. The partana (partition analysis) ratio evaluates within-cluster similarity with among-cluster similarity and is a tool to measure the cluster validity (Aho et al., 2008), while the silhouette width measures the mean similarity of each sample in the cluster to the mean similarity of the next most similar cluster (Rousseeuw, 1987). The optimized dendrogram was sliced and chi-squared tests performed on the sliced trees to determine

impact of the environmental parameters on the clusters. Significance tests of the relationship between each of the environmental variables and the HC clusters were performed for each environmental variable using a chi-squared test. A small p-value (<0.05) from the chi-squared test indicated a significant relationship.

Separately, principal coordinate analysis (PCO) with general surface plots (i.e. logistic regression with the first two PCOs as predictors and one of the categorical environmental variables as the response) were generated to visualize important partitions of the data with respect to the environmental variables for each of the functional genes (R software library labdsv; Roberts, 2009). The goodness of fit of the surface plots was reported by D^2, which is analogous to R^2 (for quantitative responses). Large D^2 values indicate a good fit of the logistic regression of the two most informative PCOs to the environmental partitioning of the data. The most influential environmental factor (highest D^2 value) was selected and the dataset subdivided according to this parameter (e.g. season). Analysis of these two subgroup datasets determined the next most influential variable (e.g. plant species or sample type). This process was repeated until all environmental variables had been assessed and subgroups created and analyzed, or surf analysis no longer revealed associations between the PCO data and the environmental parameter applied. Results from surf analyses were compared with hierarchical clustering results to determine whether similar conclusions could be drawn using a second independent approach.

Quantitative PCR Quantitative PCR results were analyzed using ANOVA and MANOVA in Minitab (version 15). All data were normalized to 16S rDNA gene

quantity (Geets et al., 2007; Agrawal and Lal, 2009; Dang et al., 2010) and transformed to the log scale to satisfy the normal assumption of the statistical models. The Benjamini Hochberg correction was used to maintain a family false discovery rate of either 5% or 10% (Benjamini and Hochberg, 1995). Due to the limited number of data points, MANOVA analysis could not be performed on all 6 responses simultaneously (see Table 6.3). The 5 responses were thus separated into root (R2, R3, R4) and gravel (G5, G6) subsets and analyzed separately. If there were no interactions, the main effects were interpreted directly. If interactions were observed, a "follow up" MANOVA was performed to examine the interactions. The linear discriminate functions (LDF) which are the directions of maximum discriminability of the group means outputted by the MANOVA were calculated and interpreted with regard to our CW system.

Results

The combined approach of incorporating PMA, DGGE and qPCR with functional primers allowed for a comprehensive analysis of the active community of these functional groups. PMA removed the DNA from those cells with compromised cell membranes; DGGE showed the diversity of the community in each sample and qPCR was able to quantify the gene copy number in the sample. In this way we were able to determine whether or not both diversity and abundance of a particular gene varied with time and sample location, or if one was more constant than the other.

Water Quality

Water quality results for COD and sulfate agreed closely with earlier experiments (Taylor et al., 2010). There was a seasonal trend in COD removal for both the unplanted control and *L. cinereus*, ranging from 70-80% in winter and up to 90% in summer. *D. cespitosa* COD removal was typically highest in winter (near 100%) and slightly reduced in summer (about 90%). When the summer *D. cespitosa* sampling results for this study were averaged together, COD removal was reduced to about 70%. This was due to increased turbidity in the sample effluent (samples were unfiltered) of one of the microcosms sampled, which contributed to the increased COD readings. Plant health of this replicate appeared to be equitable to the other replicate sampled, which had high performance as previously reported. Sulfate data also correlated well with earlier experiments (Taylor et al., 2010) for all treatments, with nearly 100% removal in summer and winter for *L. cinereus* and the unplanted control, and nearly 100% sulfate removal in the summer and 50% removal in the winter for *D. cespitosa*. The winter sampling results for this study showed lower sulfate removal (~20%) for one of the *L. cinereus* replicates. The health of the plant in this microcosm appeared to be declining (this was the more poorly performing species), this was likely the effect of shading by other macrophytes in the greenhouse. The other replicate sampled was in good health and performed as expected. Ammonia was most efficiently removed by the *D. cespitosa* columns (near complete removal in both summer and winter) followed by *L. cinereus* (75% removal in summer, 60% in winter) and the unplanted control (~50% removal in summer and winter).

Quantitative PCR

For each DNA extract, copy numbers of 16S rDNA and the two functional genes, *dsrB* and *amoA*, were calculated from their respective qPCR standard curves. The functional gene copy numbers were then normalized to the 16S rDNA copy number as ratios (copies *dsrB* or *amoA*/copies 16S rDNA) (Geets et al., 2007; Agrawal and Lal, 2009; Dang et al., 2010). This calculation expressed functional gene abundance relative to the total bacterial population, and allowed comparisons between different samples in which DNA yield might have varied. For samples below the level of detection, the qPCR threshold value was substituted for the respective functional gene (678 copies/µL for *dsrB* and 267 copies/µL for *amoA*) since the statistical analyses required a numerical value for each sample. These values were determined by averaging the y-intercepts from the qPCR runs performed for each gene and calculating the copy number for the threshold C_t value.

Each sample was split between PMA treated and untreated fractions prior to DNA extraction. PMA treatment prevents PCR amplification of DNA from membrane-compromised ("inactive") cells. The effects of PMA treatment were tested on 16S rDNA. C_t values for the 16S rDNA treated with PMA (relative to the untreated samples) indicated that removal of DNA was no more than 10% and was consistent for all sample locations. Furthermore, the difference between PMA treatment vs. no treatment was only significant for the ultra fine root samples and top gravel (ANOVA, 95%). Because there was no statistical difference between PMA treated and untreated samples, all subsequent qPCR data for *dsrB* and *amoA* is reported for the PMA treated samples only.

Analysis of Variance (ANOVA) ANOVA was performed for each of the individual locations sampled (see Table 6.3) to see how season and plant species affected relative gene abundance. The ultra fine roots and the top gravel layers were most informative locations for both the *amoA* and *dsrB* genes. Both locations showed seasonal variation for AOB and SRB with significantly higher gene ratios observed in the summer (95% and 90% confidence, respectively). Overall, *D. cespitosa* had the lowest quantities of SRB (for all sample locations except the ultra fine roots). *D. cespitosa* also had the highest redox potential of the CW microcosms investigated except in summer when redox was comparable to *L. cinereus* and the unplanted control (Taylor et al., 2008; Taylor, 2009). The unplanted control column and *L. cinereus* had similar relative SRB quantities regardless of sampled location with the effluent and bottom gravel samples containing the highest relative quantity of *dsrB*. This correlates well with the similarly efficient sulfate removal rates observed for both of these treatments. For *amoA*, both planted species contained similar relative quantities of AOB throughout the columns, regardless of sample location.

Multivariate Analysis of Variance (MANOVA) Multivariate analysis of variance (MANOVA) was performed for a more comprehensive examination of the qPCR data for environmental responses. There were too few data points to perform a MANOVA on all six locations (see Table 6.3) for each gene, thus the data was subdivided into root (R2, R3, R4) and gravel (G5, G6) subsets. Effluent data was analyzed by standard ANOVA separately with no significant results. Both *dsrB* and *amoA* had significant plant and season interactions within the root samples ($p<0.05$). For *dsrB*, 87% of the variability of

the group means was discriminated by the equation: log(R2)-log(R3)+0.5log(R4). Similarly, 86% of the variability of the group means for *amoA* was discriminated by the equation: log(R2)-2log(R3)+0.5log(R4). Side by side comparison of these equations reveals that the fine roots (R3) are twice as important a location for AOB as they are for SRB. Since the R4 location had the smallest coefficient of both equations, it was suspected that this term could be removed in order to simplify the equations. Removal of the ultra fine root term (R4) altered the results revealing that this sample location could not be removed and was an important contributor to both functional groups. *L. cinereus* had the highest relative quantities of SRB on its roots in the summer season, whereas *D. cespitosa* had minimal seasonal variation in the relative quantity of SRB present on its roots.

The results for gravel data depended on the gene investigated. For *amoA*, only a seasonal effect was observed (no plant involvement) with respect to relative gene quantity ($p<0.10$) with more AOB observed in the summer and 100% of the variability of the group means discriminated by the equation: log(G5). This indicated that only the gravel nearest the surface of the columns (and associated with the roots of the planted columns) had an effect on the quantity of AOB observed. It also implied that the bottoms of the microcosms are relatively unimportant with respect to nitrification, which was intuitive as the bottoms of the columns are assumed to be the most anaerobic regions in the microcosms (as evidenced by blackening and odor upon destructive sampling). To preserve the conditions within the bottom gravel, samples were processed within an anaerobic chamber which may have negatively impacted any AOB that might have been

present. Similar to the root samples, *dsrB* showed a plant and seasonal interaction within the gravel ($p<0.05$) and 86% of the variability of the group means was discriminated by the equation: log(G5)+2log(G6). This relationship shows the bottom (anaerobic) gravel to be twice as important, in relation to the top gravel (more aerobic), with regard to SRB quantity within the columns. In addition, *D. cespitosa* gravel was always observed to have lower quantities of SRB in the summer season.

DGGE Analysis

For consistency in comparing the DGGE data with the qPCR results, only those samples that were PMA treated and above the level of detection for qPCR analysis were included in DGGE analysis. For each of the functional genes, hierarchical clustering analysis (HC) and principal coordinate analysis (PCO) combined with general surface plotting were performed on the DGGE community profiles for each of the functional genes (16S rDNA was not analyzed by DGGE). DGGE images appeared to indicate differences in the SRB community profiles from planted and unplanted microcosms as well as from summer to winter seasons among the planted microcosms (Figure 6.1). Statistical analyses were performed to determine if the apparent differences were significant. Even though no visible differences were observed in the *amoA* DGGE community profiles, statistical analyses were also performed.

Hierarchical clustering (HC) analysis of the *dsrB* gene revealed differences between the community profiles of planted and unplanted microcosms ($p<0.005$). It also indicated that sample type (gravel, roots, effluent) within the column affected community structure ($p=0.01$). Principal coordinate (PCO) surface analysis also detected a difference

between the planted and unplanted SRB communities (D^2=0.5799; Figure 6.2). When analysis was limited to data only from planted microcosms, there was a significant seasonal effect on SRB community structure ($p<0.05$, D^2=0.3852; Figure 6.3). No seasonal effect was observed within the control column communities ($p>0.5$). Hierarchical clustering analysis of the planted columns also showed sample type to be important in determining the community structure ($p<0.05$), with the effluent samples being the most different of any of the other locations within the microcosm.

Analysis of the *amoA* community profiles by HC indicated both a seasonal effect on AOB community structure as well as an effect due to sample type (effluent, roots, gravel; $p<0.01$). However, PCO surface analysis revealed no remarkable effects on the community structure by the tested environmental variables. In summary, hierarchical clustering showed sample type to select for unique communities for both *amoA* and *dsrB*. Season also was revealed to affect microbial community composition for both genes. For *dsrB*, only planted columns were seasonally affected; whereas the *amoA* gene indicated a seasonal effect for both planted and unplanted microcosms.

Discussion

In this study, relative gene quantities were reported for each of the functional genes and cannot be directly related to the AOB and SRB population abundance. This is because the intracellular copy number for each of the genes investigated is variable. The 16S rDNA gene can range from 1 to 15 copies per cell (Fogel et al., 1999; Geets et al., 2007; Hallin et al., 2009), the *amoA* gene with either 2 or 3 copies per cell (Chain et al., 2003; Okano et al., 2004; Geets et al., 2007; van der Wielen et al., 2009), and the *dsrB*

gene only reported to have a single copy per cell (Klein et al., 2001; Leloup et al., 2009). Samples below level of detection were distorted when the copies of 16S rDNA from that same location were also low. This was only a problem with one sample from the ultra fine roots in a *D. cespitosa* microcosm which resulted in odd results for *dsrB* and *amoA* for that location. Efficiency for all qPCR reactions was near 0.9 with the standard curves for all genes having linearity over 6 orders of magnitude ($R^2 > 0.99$). As a result, we believed we were justified comparing the results from the functional genes to the 16S rDNA results.

Using this comparison, the ratios of functional genes were closely related to the ranges previously reported in the literature. *Dsr*/16S rDNA ranged from 0.013% to 7.5% (removing the inflated normalized R4 value) and 0.002% to 13% for *amoA*. Other researchers have shown similar quantities of SRB in the environment, generally not representing more than 5% of the total microbial community present (Devereux et al., 1996; Scheid and Stubner, 2001; both compared SRB rRNA with total rRNA). Most recently, Dang et al. (2010) showed the ratio of *amoA*/16S rDNA in marine sediments to be between 0.003% and 0.07%.

So that activity in terms of wetland performance (removal of sulfate and ammonia) could be determined, the targets were the active cells within the CW microcosms, thus PMA treatment was integrated into the sampling protocol. A 10% difference between PMA treated and untreated samples was observed for 16S rDNA; only 16S rDNA was investigated because all other samples were normalized to this gene. As a result, subsequent analyses were performed using only PMA treated sample results.

ANOVA of the PMA untreated functional genes normalized to PMA untreated 16S rDNA was also performed to see if PMA treatment affected the results. The untreated samples yielded the same results reported for PMA treatment. This may imply that PMA treatment had no significant effect on the outcome for this system; however, with PMA treatment, we were able to focus on the active portion of the community.

Quantitative PCR allows the quantification of genes from natural and engineered systems that is otherwise impossible using conventional PCR (Saleh-Lakha et al., 2005; Geets et al., 2007). Overall, plant and seasonal interactions affected both the AOB and SRB communities for the root and gravel samples ($p<0.05$), with the exception that the AOB gravel community was only affected by season ($p<0.10$).

All three root samples were important in quantifying the populations present. For SRB, the attached roots (R2 and R3) had higher impacts on the population numbers present, but the ultra fine roots (R4) could not be completely discounted without altering the outcome of the results. SRB appeared to be a more robust community across season as few samples were below detectable levels. Increased SRB presence in the unplanted control and *L. cinereus* indicated that these treatment conditions were more appropriate and less variable for treating wastewater with high levels of sulfate. *D. cespitosa* had the lowest quantity of SRB of all the CW microcosms tested (for all sample locations except the ultra fine roots (R4)), which correlated well with the observed redox and sulfate data (Taylor et al., 2010). Higher quantities of SRB within the bottom gravel (compared to top gravel) also correlated well with the expected anaerobic conditions in these locations, which are likely to be enhanced in the unplanted or *L. cinereus* conditions (lowest redox

values). Conversely, another study (in a wetland lake system) reported the increased presence of SRB (MPN methodology) in the rhizosphere compared to surrounding sediments (Vladar et al., 2008).

It was unexpected that the ultra fine roots (R4) of *D. cespitosa* in summer would simultaneously support a large SRB and AOB community, as reported by ANOVA. As mentioned previously, correction for samples below the level of detection resulted in this location being misrepresented as artificially high for both genes because the copies of 16S rDNA were also low for this location in summer. Overall, *D. cespitosa* had the lowest SRB quantity with very little seasonal variation in relative gene abundance. This implies a stable SRB community but it had been hypothesized that an increase in the SRB population in summer would occur in response to increased CW performance in summer (with regard to sulfate removal). It is possible that the increased redox within the *D. cespitosa* microcosms in winter is high enough to inhibit sulfate reduction but not high enough to affect the abundance of the SRB community present. Alternatively, the sampling method developed for these microcosms may have excluded the preferred habitat for these organisms.

The attached roots were of greatest impact on the AOB communities with the fine roots (R3) affecting the population twice as much as for the SRB. Similarly, the ultra fine roots (R4) were equally important for AOB and could not be removed from analysis without affecting the outcome observed. It was difficult to characterize the AOB community as thoroughly as the SRB because many of the samples were below the level of detection by qPCR. These organisms were most frequently detected in the summer

season and generally attached to root surfaces. Although not significant for the MANOVA results, AOB were detected in the bottom gravel of all the CW microcosms, confirming their ability to survive under anaerobic conditions (Geets et al., 2007). It has also been reported that AOB and anammox organisms can coexist (Pathak et al., 2007), although screening (by conventional PCR) for anammox organisms in our systems did not yield any positive results (data not shown).

There was a similarity between the relative gene abundances of *amoA* in *D. cespitosa* and *L. cinereus* microcosms. This was surprising as the overall redox and performance for *D. cespitosa* was much greater than *L. cinereus* with respect to ammonia removal. *L. cinereus* roots did not penetrate deeply into the gravel substrate, potentially causing any oxygen released to be concentrated within the uppermost portion of the column and supporting a higher proportion of AOB. Additionally, this may be due to many samples being below the level of detection and normalized to similar values.

Quantitative PCR results showed that for SRB, the sampled root locations were, in general, equally informative, but for gravel, the bottom gravel was most informative. Ultimately, SRB were found in all of the microcosm locations sampled supporting previous evidence that these organisms are capable of existing in both anaerobic and aerobic environments (Brune et al., 2000; Cypionka, 2000; Fortin et al., 2000). For AOB, the fine roots (R3) were the most informative location being twice as important in describing the variability of the means, making this a potential location of interest in future study. It was interesting to find that although the ultra fine root data could not be removed from analysis without altering the results, they did not have a greater impact on

the statistical outcome. This location was specifically sampled with the reasoning that these root surfaces were the youngest and most dynamic. It is possible that the population at this location was still under development and did not have high quantities of the groups investigated in this paper. The top gravel was very important for AOB with ANOVA and MANOVA both implicating this location to be most informative making it a targeted location for future AOB investigation. Effluent samples were the least informative for both *dsrB* and *amoA* with no significant differences observed regarding relative gene abundance. DGGE analysis always indicated that the effluent samples were least representative of the microbial biofilm communities within the microcosms.

Interactions between plant and season were observed in the DGGE results. This was best illustrated for the *dsrB* root communities where MANOVA clearly demonstrated a plant and season interaction and the DGGE data revealed a seasonal effect on community structure when planted profiles were examined. Other sample locations and types also demonstrated these trends. These results indicated the importance of plants (due to root activity in the subsurface likely influencing redox conditions) and temperature (season) on the overall functioning of CW systems for both aerobic and anaerobic microbial processes.

Season also influenced the microbial community structure with effects observed both for the *dsrB* and *amoA* genes. Although strong seasonal effects were observed for AOB communities with respect to DGGE, the *amoA* gene was generally below the level of detection for most winter samples affecting the analysis. This was not surprising given the literature has often reported a strong temperature effect on nitrification (Painter, 1986;

Hammer and Hammer, 2001; Kuschk et al., 2003; Song et al., 2006). Although decreased quantities of *amoA* were observed in these winter samples, water quality analyses indicated that nitrification was still occurring. This may be due to archaeal ammonia oxidizers or anammox organisms; however screening several samples for the genes of these organisms, by conventional PCR, and did not detect any (data not shown). Another CW research group also found a seasonal shift in AOB DGGE profiles, but were investigating changes from autumn to spring (Yin et al., 2009).

A difference in the SRB community DGGE profile was observed depending on plant presence or absence with unplanted control columns having unique SRB communities. This was also observed in other wetland studies (Vladar et al., 2008; Weber et al., 2008). Since water quality and qPCR data consistently matched for both unplanted microcosms and *L. cinereus* planted microcosms, a difference due to plant species was expected to separate *D. cespitosa* from the remaining treatments. This result may implicate the rhizosphere of *L. cinereus* to be leaking oxygen. Since *L. cinereus* had such shallow roots, the oxygen released may not be enough to impact sulfate to the extent observed for *D. cespitosa*. This could indicate that the SRB community structure was dependent on plant presence or absence and that sulfate removal efficiency did not predict the differences in SRB community profiles.

Statistical analyses showed *dsrB* to be present in higher ratios for *L. cinereus* roots than for *D. cespitosa* roots; however DGGE showed similar communities for the roots of both of these plants. This shows that variation in relative gene abundance does not necessarily indicate a difference in community structure, but density. In general, the

DGGE community structure for SRB appeared to be most greatly influenced by the presence or absence of a plant while the gene quantities appeared to be most greatly influenced by a combination of plant species and seasonal variation.

The majority of CW research has focused on the microbial population associated with the gravel and neglects those associated with the roots. The root surface is very dynamic and heterogeneous, making it an ideal surface for colonization by varying populations of microorganisms such as SRB (Vladar et al., 2008) and AOB (Brix, 1994, 1997; Scholz and Lee, 2005). One study found that high efficiency nitrification and sulfate reduction was possible in CW microcosms planted with *Juncus effusus*. It was postulated that plant presence, and thus ROL, limited sulfide accumulation and toxicity within the microcosms (Wiessner et al., 2008). Our results for the root samples were different depending on the gene investigated. It is possible that each plant species affected the microbial community abundance by uniquely altering the redox environment within the microcosm.

In these experiments, both AOB and SRB were present in the same sample locations, confirming that both aerobic and anaerobic organisms can exist in close proximity in CW biofilms (Stewart and Franklin, 2008). The results of this study begin to illustrate the interactions occurring within CW and can be applied to a variety of environmental systems and genes for a more in depth understanding on microbial processes. Quantification of controllable parameters and their influences on the microbial ecology will lead to better design and operation of constructed wetlands systems. Optimization of the microbial community structure and function should be a

priority for the effective design of wastewater treatment systems (Geets et al., 2007). The research reported here was focused on a fundamental understanding of wetland ecology at the microbial level and the influence of that ecology on critical chemical cycles; it has far-reaching applications in natural wetland biogeochemistry, release of potential greenhouse gasses (CH_4, NO_x) to the atmosphere and climatic effects on wetland ecosystems.

Acknowledgments

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FIGURES

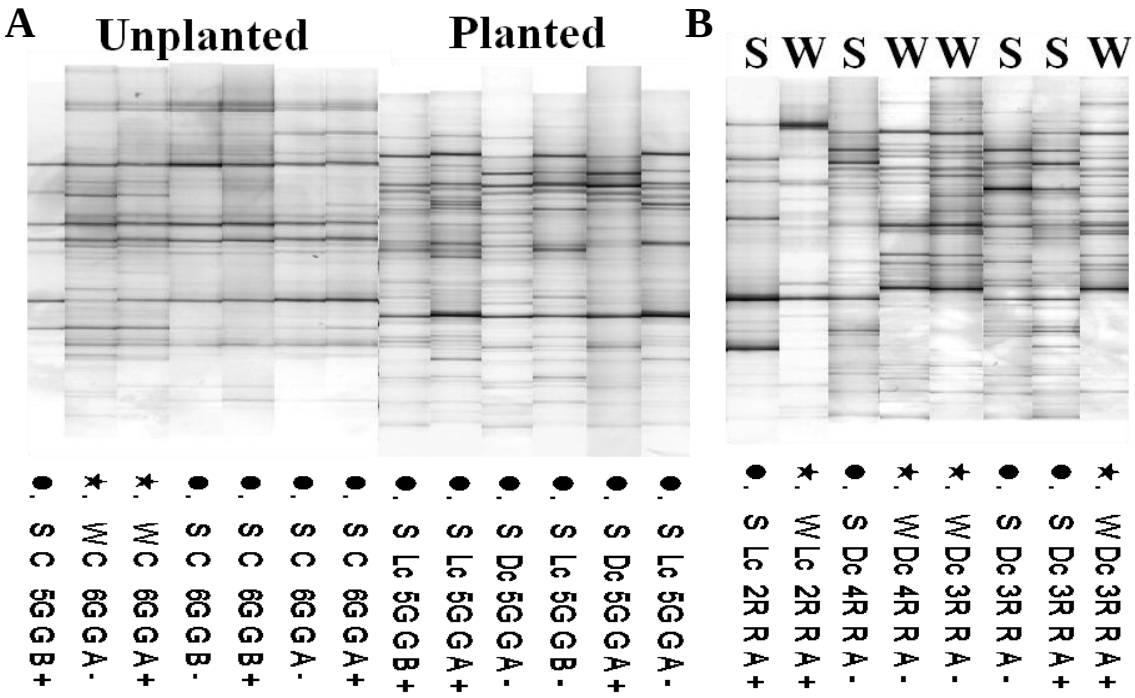

Figure 6.1. DGGE profiles of SRB communities (*dsrB* gene). A – SRB community profiles comparing gravel samples from unplanted (left) and planted (right) microcosms. B – SRB community profiles comparing summer and winter seasons from rhizosphere (R2, R3, R4) biofilm samples. Images were created using GelCompar II software v. 6.1. S = summer, W = winter, C = unplanted control, Dc = *D. cespitosa*, Lc = *L. cinereus*, A/B = replicate, +/- = PMA treatment.

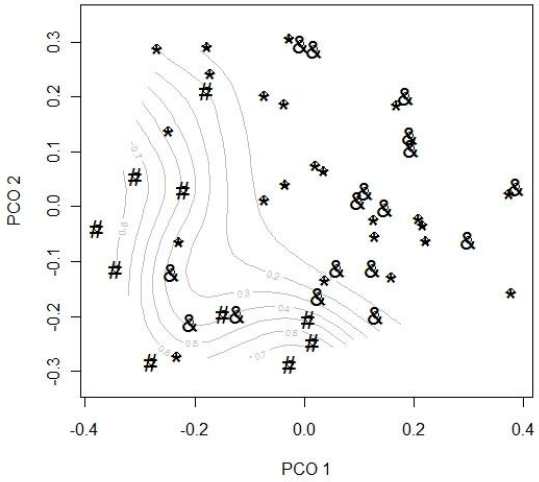

Figure 6.2. Analysis of SRB biofilm community profiles from planted and unplanted microcosms post PMA treatment. Plotted lines indicate separation of unplanted and planted microcosm communities. # - unplanted control, * = *L. cinereus*, **&** = *D. cespitosa*.

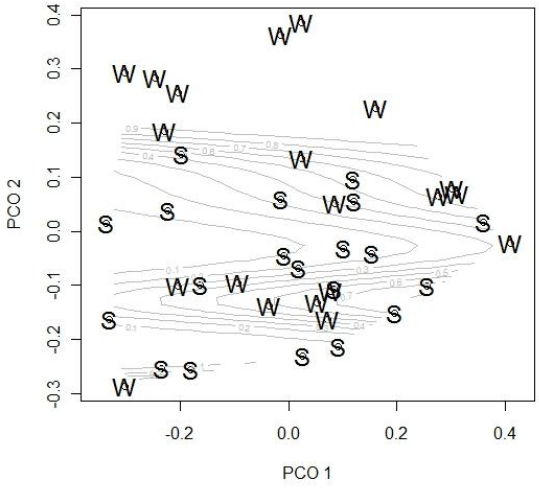

Figure 6.3. Analysis of SRB biofilm community profiles from planted microcosms post PMA treatment. Plotted lines indicate separation of communities present in the summer from those detected in the winter. S = summer, W = winter.

Table 6.1. Primer sequences used in this study. *Used in multiplex PCR screening reactions without the GC clamp

Primer	Target	Sequence (5' to 3')	Method	Reference
DsrBF	*dsrB* gene (*dsr* β-subunit)	CAACATCGTYCAYACCCAGGG	PCR*-DGGE/ qPCR	Geets et al., 2006
Dsr4R	*dsrB* gene (*dsr* β-subunit)	GTGTAGCAGTTACCGCA	PCR*-DGGE/ qPCR	Wagner et al., 1998
RottF	*amoA* gene	GGGGTTTCTACTGGTGGT	PCR*-DGGE/ qPCR	Rotthauwe et al., 1997
RottR	*amoA* gene	CCCCTCKGSAAAGCCTTCTTC	PCR*-DGGE/ qPCR	Rotthauwe et al., 1997
VectF	pCR®2.1-TOPO plasmid	AGTGTGCTGGAATTCGCC	DGGE Marker	Burr et al., 2006
VectR with GC	pCR®2.1-TOPO plasmid	ATATCTGCAGAATTCGCC	DGGE Marker	Burr et al., 2006
Eub341F	16S rDNA V3 region	CCTACGGGAGGCAGCAG	qPCR	Muyzer et al., 1993
Eub534R	16S rDNA V3 region	ATTACCGCGGCTGCTGGC	qPCR	Muyzer et al., 1993
GC Clamp	Attach at 5' end of primer	CGCCCGCCGCGCCCCGCGCCC GGCCCGCCGCCCCCGCCCC	PCR-DGGE	Ferris et al., 1996

Table 6.2. PCR programs used in this study.

Application	PCR Program	Reference
dsrB Cloning, DGGE, qPCR	Initial denaturation for 4 min at 94°C, followed by 35 cycles of: 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. The program ended with an extension step at 72°C for 10 min. qPCR program run for 40 cycles, denaturation temperature increased to 95°C, the initial denaturation step increased to 10 minutes.	Geets et al., 2006
multiplex PCR, *amoA* and DGGE Marker amplification	Initial denaturation for 60 s at 94°C, followed by 35 cycles of: 94°C for 60 s, 54°C for 60 s, and 72°C for 3 min. The program ended with an extension step at 72°C for 10 min.	Bahr et al., 2005
16S rDNA qPCR	Initial denaturation for 10 min at 95°C, followed by 45 cycles of: 95°C for 10 s, 60°C for 15 s, and 72°C for 20 s.	modified from Agrawal and Lal, 2009
amoA qPCR	Initial hold at 50°C for 2 min and denaturation for 10 min at 95°C, followed by 40 cycles of: 95°C for 60 s, 50°C for 60 s, and 72°C for 60 s.	Geets et al., 2007

Table 6.3. Sample identity for constructed wetland columns.

Sample ID	**Location**
E1	Drained effluent
R2	Thick roots (nearest the crown of plant)
R3	Fine roots (furthest from the crown of the plant)
R4	Ultra fine roots (torn off during destructive sampling and recovered by skimming and filtering)
G5	Top gravel (root associated for planted columns)
G6	Bottom gravel (bottom of the column)

CHAPTER 7

CONCLUSIONS

The goal of this research was to examine the structure and activity of the microbial communities associated with wastewater remediation in constructed wetland microcosms. Previous work had well established a seasonal variability in CW performance (as determined by effluent water quality) and with the plant species present within the wetland (Borden et al., 2001; Allen et al., 2002; Stein and Hook, 2005; Stein et al., 2007; Taylor et al., 2010). Some plants were found to enhance performance in colder seasons (with regard to organic carbon removal) while other plants showed improved performance in warm seasons (with regard to sulfate removal). Since plant species appeared to impact effluent water quality, it was hypothesized that the microbial communities present within the microcosms were plant specific. The majority of CW literature has presumed microbial activity as evidenced by pollutant removal from inlet to outlet. In an effort to substantiate these claims and to explain the seasonal performance fluctuations observed in the greenhouse microcosms, the work presented in this dissertation sought to identify the microbial communities present and contributing to pollutant removal using a variety of microbiological techniques including both culture-based and molecular methods.

The focus was initially on the sulfate reducing microbial community utilizing a combination of culture-based and molecular techniques (Chapter 3). We developed a variety of growth media to enrich for the maximum recoverable sulfate reducers from an unplanted control column capable of near complete sulfate removal. The SRB

communities cultivated were analyzed using PCR-DGGE with primers specific for the dissimilatory sulfite reductase gene (*dsrB*). The success of these experiments led to the continued investigation and use of functional primers in future experiments (Chapter 6).

Concurrently, work was being done to compare the entire microbial community within the CW microcosms using PCR-DGGE with universal bacterial primers targeting the 16S ribosomal gene (Chapter 5). In addition, an extensive destructive sampling protocol was developed to more thoroughly evaluate the likely microenvironments present within the microcosms. The goal of this universal examination of the bacterial community was to determine the factors contributing to microbial community shifts (and CW performance) from summer to winter and for each of the plant species. It was hypothesized that the rhizosphere of the plants would select for a unique microbial community compared to the gravel substrate. In depth statistical analyses were conducted to evaluate the DGGE community profile data generated (Chapter 4). Results showed that sample type (effluent, root, gravel) and plant species had the greatest impact on the microbial communities observed. These results were encouraging, but since 16S rDNA based methods provide little evidence of microbial function, further investigation was required.

To focus on specific microbial functional groups within the CW microcosms, two functional primer sets were selected, the dissimilatory sulfite reductase gene (*dsrB*; Chapter 3) and the ammonia monooxygenase gene (*amoA*). The *dsrB* gene targeted sulfate reducing bacteria (SRB), responsible for sulfate reduction, while the *amoA* gene targeted ammonia oxidizing bacteria (AOB), responsible for the initial step in

nitrification (Chapter 6). Quantitative PCR was used to quantify the presence of each of these genes within each sampled location of our microcosms and PCR-DGGE was used to visualize and characterize the community present. Since PCR methodologies are incapable of distinguishing between DNA from live or dead cells, treatment with propidium monoazide (PMA) was also incorporated. PMA is a DNA intercalating dye incapable of penetrating intact cell membranes; as a result, treatment with PMA results in the PCR inhibition of DNA from cells with compromised cell membranes leaving only DNA from intact cells to be analyzed. The combined use of these methods allowed for both a quantitative and qualitative view of the active populations of SRB and AOB within our CW microcosms. To account for variations in microbial abundance between samples, the qPCR data for each functional gene was normalized to the 16S rDNA quantity in its corresponding sample. The results from this research revealed an interaction between plant and season to affect the abundances of each functional group. Overall, it was determined that relative gene quantities were higher in summer than in winter for both genes. For SRB, the microcosms capable of the greatest sulfate removal year round (unplanted and *Leymus cinereus*) had the highest relative quantities of SRB with *Deschampsia cespitosa* (poor sulfate removal in winter) having the lowest relative quantities of SRB of all. For AOB, the planted microcosms had the highest quantities with more AOB detected in the summer season than the winter season. DGGE community profiles for SRB revealed a community difference between planted and unplanted microcosms with a seasonal shift also observed within the planted microcosms. This finding correlated with the observed plant and season interaction observed for the

qPCR data. AOB communities were less variable with sample type (effluent, root, gravel) appearing to have the greatest impact on community structure. The combination of both qPCR and DGGE was successful in observing both quantitative and qualitative differences within each of the communities examined.

Overall, this work has shown that there are very complex and dynamic interactions occurring within these systems. It is not only seasonal temperature variation or plant species that is affecting the microbial communities within these systems, but rather some combination of these factors. Differences in the microbial communities can be found by intensive sampling of the system in combination with an assortment of molecular methodologies so that a better understanding of these complex environmental systems can be obtained.

Future Work

More research is required to understand the microbial ecology of CWs. It would be useful to characterize all of the biogeochemical processes and microbial communities involved in the breakdown of important wastewater pollutants. Comparison of the communities cultivated with different CW designs would also be of interest. Certain CW designs, such as vertical flow systems, have been shown to be more effective at nitrification than subsurface flow CWs, studied here. It would be interesting and useful to determine if community composition and abundance are responsible for these operational differences. In conjunction, the study of the microbial communities cultivated when treating alternative wastewaters would also be interesting to see if the results obtained with this work are consistent for other wastewater types.

Similarly, testing the effect of various operational conditions on the microbial communities would be useful. Since most CWs are operated under continuous flow conditions, it would be interesting to investigate the microbial communities present along the length of the CW as well as with depth. Some continuous flow systems have been implicated as being less effective at wastewater treatment compared to batch operated systems. It would be interesting to evaluate the microbial communities involved in each type of CW to determine how these biofilms may be affecting performance.

Finally, implementation of other molecular technologies would also be useful for further identifying the microbial communities present and active in CWs. Molecular analyses using the Phylochip would be a useful follow up to the work presented in Chapter 5 to identify the community members present or absent in different microcosms and on different sample surfaces. This approach would also necessitate the development of RNA methods which would aid in identifying only the active organisms within the wetlands. Alternatively, methods such as pyrosequencing could be used to perform metagenomic analyses on the CW ecosystem. All of these approaches would greatly aid in explaining the complex microbial dynamics occurring in these systems.

The long-term goal of this project has been to elucidate the fundamental processes and controlling factors responsible for water treatment in constructed wetlands and, to improve CW design and operation for better water treatment, expand the geographical extent of their reliable usage, and apply CWs to a greater range of contaminant sources. This research has focused on characterizing the physical, biological, and operational factors that control biogeochemical transformations responsible for water treatment in

constructed wetlands. By understanding, quantifying and predicting microbial relationships within these CWs, it might be possible to optimize these processes to meet specific treatment objectives.

APPENDIX A

FLOATING TREATMENT WETLANDS FOR DOMESTIC WASTEWATER TREATMENT

Contribution of Authors and Co-authors

Appendix A: Floating treatment wetlands for domestic wastewater treatment

Co-author: Jennifer L. Faulwetter
Contributions: Assisted in experimental design, conducted the molecular experiments included as well as analyzed the resultant data. Compiled and organized the molecular data into the publication and was actively involved in the editing and submission process.

Co-author: Mark D. Burr
Contributions: Assisted in experimental design, conducted the water quality experiments included as well as analyzed the resultant data. Compiled and organized the water quality data into the publication and was actively involved in the editing and submission process. Also was a co-PI on the project.

Co-author: Alfred B. Cunningham
Contributions: Assisted in the design and operational conditions of the experiments as well as being one of the principal investigators on the project.

Co-author: Frank M. Stewart
Contributions: Assisted in the design and operational conditions of the experiments as well as being one of the principal investigators on the project.

Co-author: Anne K. Camper
Contributions: Assisted in the design and operational conditions of the experiments as well as being one of the co-principal investigators on the project.

Co-author: Otto R. Stein
Contributions: Assisted in editing the publication as well as presented the paper and poster at a conference in Venice, Italy.

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FLOATING TREATMENT WETLANDS FOR DOMESTIC WASTEWATER TREATMENT

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Abstract

Floating islands are a form of treatment wetland characterized by a mat of synthetic matrix at the water surface into which macrophytes can be planted and through which water passes. We evaluated two matrix materials for treating domestic wastewater, recycled plastic and recycled carpet fibers**,** for COD and nitrogen removal. These materials were compared to pea gravel or open water (control). Experiments were conducted in laboratory scale columns fed with synthetic wastewater containing COD, organic and inorganic nitrogen, and mineral salts. Columns were unplanted, naturally inoculated, and operated in batch mode with continuous recirculation and aeration. COD was efficiently removed in all systems examined (>90% removal). Ammonia was efficiently removed by nitrification. Removal of total dissolved N was ~50% by day 28, by which time most remaining nitrogen was as NO_3-N. Complete removal of NO_3-N by

denitrification was accomplished by dosing columns with molasses. Microbial communities of interest were visualized with DGGE by targeting specific functional genes. Shifts in the denitrifying community were observed post-molasses addition, when nitrate levels decreased. The conditioning time for reliable nitrification was determined to be approximately three months. These results suggest that floating treatment wetlands are a viable alternative for domestic wastewater treatment.

Keywords: Bacteria; Biofilm; COD; Denitrification; Floating Treatment Wetland; Nitrification

Introduction

A type of artificial wetland in which emergent plants are grown either hydroponically or in a media floating on the surface of a pond-like basin has been used for habitat enhancement or contaminant amelioration since at least the mid-1970's (Seidel and Happel, 1986; Hoeger, 1988). As with more conventional surface flow and subsurface flow treatment wetlands, Floating Treatment Wetlands (FTW) have been employed for removal and treatment of a diverse array of contaminants and polluted waters (Headley and Tanner, 2008b). However, due to their ability to float with relatively large fluctuations in water level, treatment of stormwater (Headley and Tanner, 2008a, b) and combined sewer overflow (Van de Moortel et al., 2010b) appear to be the most typical applications. Natural floating islands, which can exist in locations where plant growth exceeds decay rates, maintain buoyancy via trapped gasses within a matrix of partially decayed and living plant material (Mitsch and Gosselink, 2000). Ayaz and Sagin (1996) describe a completely hydroponic system, but most other FTW employ a

superstructure frame constructed of buoyant material (PVC, bamboo, polystyrene, etc.) (Hoeger, 1988; Billore et al., 2008; Van de Moortel et al., 2010a; 2010b), or are supported by cables attached to the bank (Kerr-Upal et al., 2000) or alternatively are constructed from an inherently buoyant planting media (Todd et al., 2003; Headley and Tanner, 2008a; Stewart et al., 2008). Plants are either suspended hydroponically from a lightweight mesh supported by the frame or grown in a planting media (soil, gravel, coconut fiber, plastic) that is either frame-supported or inherently buoyant (plastic). Depending on the thickness of the planting media and species of emergent plants employed, roots can be completely contained within the media or extend through and be exposed directly to the water column below.

Compared to other treatment wetland systems, design of FTW is based on very limited information and most applications seem to be unique for even the most basic parameters such as size, degree of buoyancy, planting media, plant selection, etc. Only a few studies (Headley and Tanner, 2008b; Nakai et al., 2008; Li et al., 2010; Van de Moortel et al., 2010a; 2010b) have attempted to assess performance in replicated experiments and, due to the variety of designs and wastewater types and treatment objectives, performance generalizations are not possible at this time. To further advance understanding of processes important in FTW for domestic wastewater applications, we have been conducting experiments on COD and nitrogen removal and associated microbial populations in FTW. An important criterion in FTW design is the type of planting media (matrix). An inherently buoyant matrix avoids the use of a supporting frame and we have focused attention on various buoyant matrix materials available

through a local commercial provider. This paper summarizes research on the removal of COD and nitrogen species together with the dynamics of microbial communities occurring within two different matrix materials. The non-woven matrix is made of 100% recycled plastic fabricated into floating mats which can be configured, as shown in Figure A.1, to include pumps for recirculation as well as aeration systems at various points within the matrix. By varying flow rate, duration and frequency of recirculation and aeration it is possible to control nutrient loading rates and redox conditions within the FTW matrix.

Previous research with these matrix materials conducted in outdoor ponds provided observations of the substantial disappearance of key waste water constituents including COD, ammonia, nitrate and phosphate (Stewart et al., 2008). Since plants were not incorporated, constituent removal in these outdoor experiments was likely due to the activity of microorganisms growing as biofilms on surfaces within the island matrix. The objective of the current study was twofold: 1) Determine the optimum operational conditions to encourage simultaneous nitrification (ammonia removal) and denitrification (nitrate removal) within a FTW environment by stimulating the appropriate microbial communities and 2) Determine the microbial community response to variations in aeration, matrix material and organic carbon loading. Successful completion of this research will not only provide the basis for improving FTW design and efficacy, but will provide insight into the processes responsible for effective water quality remediation occurring within FTW.

Figure A.1. A 232 m^2 FTW in an aerated lagoon at Rehberg Ranch Subdivision, Billings, MT USA. The FTW is circular, with a radial fountain pump installed in the center. The body of the island is designed to float below the surface in order to maximize constituent update by biofilms growing on the plastic matrix. Parameters being tracked include suspended solids, BOD, nitrogen and phosphorus. The island was installed by Headwaters Floating Island, LLC during November 2009, and planted during the spring of 2010. Periodic water quality testing is being provided by the City of Billings, MT and Floating Island International, LLC.

Methods

Experiments were conducted in laboratory scale systems consisting of 20 cm diameter columns containing matrix material 20 cm thick submerged 10 cm below the water surface. Matrix material was either a very porous commercial mat made from 100% recycled plastic or loose shredded carpet fibers contained within a porous mesh, both supplied by Floating Island International (www.floatingislandinternational.com). Additionally, two otherwise identical columns, one filled with 20 cm pea gravel, the other left as open water, were included for comparison. Columns were each filled with 20 liters of simulated domestic wastewater containing ~500 mg/L COD (mostly from

sucrose), ~15 mg/L NH_4-N and ~15 mg/L NO_3-N, ~30 mg organic N/L from Primatone (Sigma), and other inorganic components (Taylor et al., 2010). All columns were unplanted, inoculated with soil and pond water, and operated in batch mode with continuous recirculation from the bottom of the column (at 20 mL/min) with continuous aeration (unless noted) from aquarium pumps into the surface water 10 cm above the matrix. We summarize data from five consecutive batch runs (B1-B5) that followed a conditioning period of four batches over a three month period. B1, B2, and B3 were run for 28 days. B4 and B5 were run for 42 days. During B4, columns were dosed with 10 g molasses (measured as 820 mg COD/g) on day 25 and again on day 29 in order to provide reducing equivalents for denitrification. There was no aeration during days 29-42.

Water Quality Analysis

Water samples were collected from the recirculating return flow on days 0, 3, 8, 14, 21, and 28 of batches B1- B3. Additional sampling of B4 and B5 was on days 35 and 42. Samples were filtered through a 0.2 µm PES filter and stored in glass scintillation vials (4°C). Samples were analyzed for COD, NH_4^+-N, and total N using HACH (Loveland, CO) methods. NO_2^--N and NO_3^--N were measured by ion chromatography (Dionex Corp., Sunnyvale, CA).

Floating Island Biofilm

Collection and DNA Extraction

Biofilm samples were collected on Day 0 of B3 (by which time columns were well conditioned) and again on Day 0 of B5 (14 days after the second molasses dose

during B4) from three depths within each treatment: top (upper 5 cm of material), center (middle 5 cm of material), and bottom (lower 5 cm of material). To clearly distinguish between depths sampled, a 2.5 cm zone was left undisturbed between each of the locations. The open water column was sampled by vacuum filtering 250 mL of effluent through a 0.2 µm polycarbonate membrane. Field samples from unplanted FTW, operated by Floating Island International, were also provided and analyzed for comparison with our laboratory samples. Materials collected from each treatment were placed directly into MO BIO PowerBead Tubes (MO BIO PowerSoil™ DNA Isolation Kit). The PowerSoil™ DNA Isolation Kit was used to complete the DNA extraction as described in the manufacturer's protocol with the exception that PowerBead tubes were placed into the FastPrep® Instrument (Qbiogene, Inc.) at speed 5.5 for 45 s. DNA yield was estimated on an agarose gel with ethidium bromide staining, serial dilutions were performed for PCR, and the DNA preparations were stored at -20˚C.

PCR Targeting Functional Genes

Ammonia Monooxygenase Gene Oligonucleotide primers were synthesized by Integrated DNA Technologies (www.idtdna.com). PCR primers RottF (5' GGGGTTTCTACTGGTGGT 3', Rotthauwe et al., 1997 - *amoA*-1F) and RottR (5' CCCCTCKGSAAAGCCTTCTTC 3', Rotthauwe et al., 1997 - *amoA*-2R) target the ammonia monooxygenase gene (*amoA,* required for ammonia oxidation to nitrite). Primer RottR was synthesized with a 5' 40-bp GC clamp (5' CGCCCGCCGC GCCCCGCGCCCGGCCCGCCGCCCCCGCCCC 3', Ferris et al., 1996) and was paired with primer RottF for amplifying fragments to be analyzed by DGGE. Presumptive

presence of the *amoA* gene was indicated on an agarose gel by a 531 bp PCR product. PCR reactions (20 μL) were performed using 2X GoTaq® Green Master Mix (www.promega.com). The PCR reaction mixture consisted of 10 μL 2X GoTaq® Green Master Mix, 0.5 μL Ultrapure BSA (50 mg/mL, Ambion), 2.5 μL DEPC-treated water, 1 μL 12.5 μM forward and reverse primer, and 5 μL 1:10 diluted (unquantified) template DNA. PCR amplifications were performed on an Eppendorf Mastercycler® ep thermal cycler (Eppendorf North America, www.eppendorfna.com) using the following program. An initial denaturation for 60 s at 94°C was followed by a total of 35 cycles of amplification consisting of denaturation at 94°C for 60 s, annealing at 54°C for 60 s, and extension at 72°C for 3 min. The program ended with an extension step at 72°C for 10 min (Bahr et al., 2005). PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide and were used for DGGE.

Nitrite Reductase Gene Oligonucleotide primers were synthesized by Integrated DNA Technologies (www.idtdna.com). PCR primers NirS cd3aF (5' GTSAACGTSAAGGARACSGG 3', Michotey et al., 2000) and NirS R3cdR (5' GASTTCGGRTGSGTCTTGA 3', Throback et al., 2004) along with NirK F1aCuF (5' ATCATGGTSCTGCCGCG 3', Hallin and Lindgren, 1999) and NirK R3CuR (5' GCCTCGATCAGRTTGTGGTT 3', Hallin and Lindgren, 1999) target the two forms of the nitrite reductase gene (*nir*, required for nitrite reduction to nitric oxide). Primers NirS R3cdR and NirK R3CuR were synthesized with a 5' 40-bp GC clamp (described above) and were paired with their respective forward primers for amplifying fragments to be analyzed by DGGE. Presumptive presence of the *nirS* and *nirK* genes was indicated on

an agarose gel by a 465 bp or a 502 bp PCR product, respectively. PCR amplifications were performed on an Eppendorf Mastercycler® ep thermal cycler (Eppendorf North America, www.eppendorfna.com) using the following program. An initial denaturation for 2 min at 94°C was followed by a total of 35 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 57°C for 60 s, and extension at 72°C for 60 s. The program ended with an extension step at 72°C for 10 min (Throback et al., 2004). PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide and were used for DGGE.

Denaturing Gradient Gel Electrophoresis

DGGE was performed on PCR products from community DNA using a DCode™ system (www.biorad.com) and reagents from Sigma-Aldrich (www.sigmaaldrich.com). Gels had a gradient of denaturant concentrations from 40% at the top of the gel to 70% at the bottom, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels also contained an 8 to 12% polyacrylamide gradient from top to bottom (Girvan et al., 2003). Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr®Gold (www.invitrogen.com) and documented using a FluorChem™ 8800 fluorescence imager (www.alphainnotech.com). Three marker lanes (generated from five pooled clones) were included in each DGGE gel so that across-gel comparison would be possible. Bands in DGGE images were identified visually on a presence–absence basis. Band intensities were not physically measured, but visually prominent bands were considered to represent numerically significant members of the community.

DGGE Data Analysis

DGGE gels were compared and analyzed for each gene investigated using the GelCompar II software (Version 6.1, Applied Maths Inc.). Subsequent statistical analyses were performed using R software libraries labdsv (Roberts, 2009) and optpart (Roberts, 2010) (www.r-project.org). Similarity and dissimilarity matrices were calculated using Dice (GelCompar II) and Sorensen (R). Hierarchical clusters were generated from these matrices and displayed using the Unweighted Pair Group Method using Arithmetic averages (UPGMA). This method displayed the average similarity between profiles for each sample compared. Hierarchical clusters displayed were generated from the similarity matrix calculated using the GelCompar II software; while more robust statistical analyses were performed using the dissimilarity matrix calculated using the R software. Large D^2 values (similar to R^2) indicate more confidence in the resultant clusters whereas small p-values (<0.05, determined using the chi-squared test) indicate that the clusters generated well represent the dataset.

Results and Discussion

Water Quality

Because all water quality analyses were done on filtered samples (0.2 μm pore size), bacterial cells were excluded. The laboratory columns containing plastic matrix, carpet fibers, pea gravel or open water were all effective at removing COD and nitrogen. There was relatively little difference among treatments (except as noted below). COD removal in all treatments was ~90% within the first two weeks of each batch, i.e., from ~500 mg COD/L initially to < 50 mg COD/L by day 14. Initial total dissolved nitrogen

was ~60 mg N/L, consisting of ~30 mg N/L organic N (from Primatone), ~15 mg NO_3^--N/L, and ~15 mg NH_4^+-N/L. Total dissolved N generally decreased by ~50% within the first two weeks, but leveled off after that. In the first week, removal of NO_3^--N was usually ~90%, probably from denitrification. NH_4^+-N usually increased in the first week, probably from mineralization of organic N. An exception was the gravel column where NH_4^+-N decreased immediately. This behavior may have been the result of adsorption onto the gravel matrix. By days 21-28, NO_3^--N usually began to accumulate again and often accounted for most of the total N. There was usually a corresponding loss of NH_4^+-N during this time period, indicating that nitrification was occurring. Differences in nitrification between new and conditioned plastic matrix suggest that about three months were required to establish an effective nitrifying biofilm community. By day 28 in batches B1-B3, the columns had reached a steady state in which COD was virtually absent, and almost all of the total dissolved N was a NO_3^--N (~20-30 mg N/L). To test the hypothesis that denitrification in these batches had been carbon-limited, we introduced doses of molasses (10 g molasses/column) on days 25 and 29 of B4. Dosing produced a spike in COD (to ~370 mg COD/L), but by the end of the batch two weeks later, >90% of this COD had also been removed. The molasses was effective at increasing denitrification. By the end of the batch on day 42, total dissolved N was <5 mg N/L, NO_3^--N was <4 mg N/L, and NH_4^+-N was <2 mg N/L. COD was <100 mg/L, but this residual COD from the molasses would probably have been removed had the batch been allowed to run beyond day 42. Batch B5 produced results that were similar to

B4. NO_2^--N was not a component of the synthetic wastewater and was usually ≤ 1 mg N/L for all treatments and time points.

Microbial Community Responses

The responses of the nitrifying and denitrifying microbial communities were monitored using DGGE. Interpretation of DGGE profiles should be done cautiously as they are invariably a mix of artifact and real diversity. Individual bands are generally assumed to represent individual genotypes, but only DNA sequencing can confirm this. The total number of bands in a profile is a rough estimate of diversity and the intensity of a band is a rough estimate of the prominence of the corresponding genotype in the microbial community (Muyzer et al., 1993). It should be noted that bands suspected to be artifact were not included in the analysis and as a result, diversity may have been underestimated. Analysis of DGGE gels was performed using GelCompar II software (v. 6.1, Applied Maths Inc.) to visualize and compare gels. Statistical analyses were performed based on band presence/absence within each profile using the R software libraries labdsv (Roberts, 2009) and optpart (Roberts, 2010) (www.r-project.org).

Nitrifying Community The AOB communities within each column were observed to have limited diversity (maximum of 12 bands observed, gravel treatment). The open water column had developed a distinct nitrifying community unlike any of the other treatment conditions (D^2=0.9944). Dosing with molasses and ending aeration did not significantly affect the structure of the nitrifying communities. The community structure of the laboratory versus field samples were distinctly different (D^2=0.9955). Field samples generally contained 5-7 bands, while laboratory samples contained 5-12

bands. It appears as though sample depth within the column was the most significant contributor to community structure ($p<0.001$, Figure A.2) followed by FTW material used ($p=0.01$) with matrix and carpet communities being more similar to one another than to the gravel community.

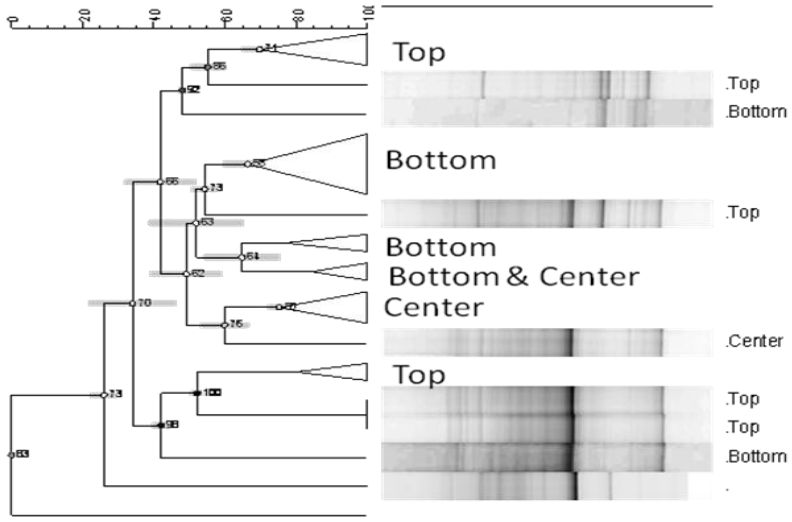
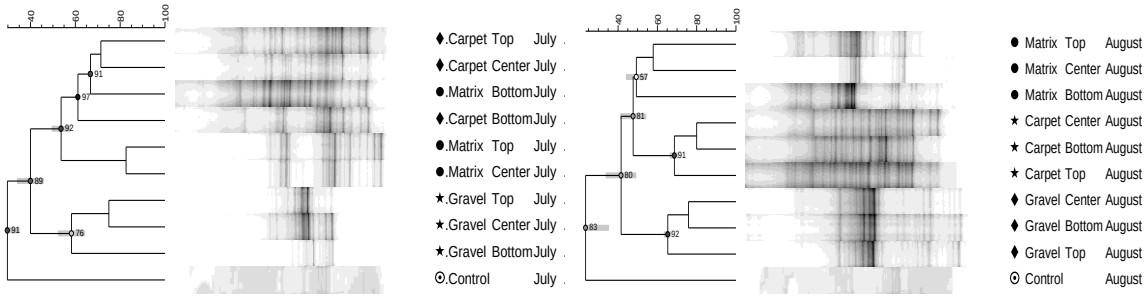


Figure A.2. Shows how the fourteen major *amoA* clusters separated. Depth was the main contributor for the nitrifiers. Triangles indicate that the samples within were similar enough to combine into a single cluster. Top – upper 5 cm of FTW material; Center – middle 5 cm of material; Bottom – lower 5 cm of material; unlabelled rows indicate open water column.

Denitrifying Community In order to investigate the entire denitrifying community present, both the *nirS* and *nirK* genes were characterized. Overall, the denitrifying community profiles were considerably more diverse compared to the nitrifying community profiles. As observed with the nitrifying community profile, the profiles for the open water column had developed unique communities compared to the other treatments ($D^2=1$, for both genes). For the *nirK* gene, the communities were highly diverse, but apparent similarities were specific to the FTW matrix material within the column ($D^2=0.9996$, $p=0.005$). For the *nirS* gene, the FTW matrix material were most

important in determining the community that developed ($p<0.001$) with all of the gravel samples grouping onto a single branch. Adding molasses and ending aeration also appeared to affect the *nirS* denitrifying community, though not as greatly as FTW material had ($p=0.05$, Figure A.3). Finally, the field samples had developed significantly different denitrifying communities for the *nirS* ($p=0.001$) but not the *nirK* gene.



Pre molasses addition Post molasses addition

Figure A.3. Community profile for *nirS* pre- and post-addition of molasses. FTW material tends to dominate community profiles pre-molasses (left), however; post-addition of molasses (right) shows each material to have a distinct *nirS* community as observed with the four grouped clusters.

Conclusions

As expected, all treatments (regardless of material) were able to efficiently reduce the COD. Additionally, communities cultivated in the field versus laboratory conditions developed their own unique consortia for each of the genes investigated.

Distance from the water surface (depth) appears to be most important to the structure of the nitrifying community followed by FTW matrix material employed. Elimination of aeration and addition of molasses did not appear to affect the established nitrifying community indicating that it may be important to first establish an efficient nitrifying community, then optimize for subsequent nitrate removal.

Similarly, FTW matrix material had the largest effect on the denitrifying community present. As observed with the nitrifiers, the elimination of aeration and addition of molasses did not significantly affect the community structure, but did stimulate denitrifying activity and thus nitrate removal.

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