Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms

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Influence of Season and Plant Species on the Abundance and Diversity of Sulfate Reducing Bacteria and Ammonia Oxidizing Bacteria in Constructed Wetland Microcosms

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Abstract

Constructed wetlands offer an effective means for treatment of wastewater from a variety of sources. An un-derstanding of the microbial ecology controlling nitrogen, carbon and sulfur cycles in constructed wetlands has been identified as the greatest gap for optimizing performance of these promising treatment systems. It is suspected that op-erational factors such as plant types and hydraulic operation influence the subsurface wetland environment, especially redox, and that the observed variation in effluent quality is due to shifts in the microbial populations and/or their activ-ity. This study investigated the biofilm associated sulfate reducing bacteria and ammonia oxidizing bacteria (using the dsrB and amoA genes, respectively) by examining a variety of surfaces within a model wetland (gravel, thick roots, fine roots, effluent), and the changes in activity (gene abun-dance) of these functional groups as influenced by plant species and season. Molecular techniques were used includ-ing quantitative PCR and denaturing gradient gel electro-phoresis (DGGE), both with and without propidium monoazide (PMA) treatment. PMA treatment is a method for excluding from further analysis those cells with compro-mised membranes. Rigorous statistical analysis showed an interaction between the abundance of these two functional groups with the type of plant and season ($p<0.05$). The richness of the sulfate reducing bacterial community, as indicated by DGGE profiles, increased in planted vs. unplanted microcosms. For ammonia oxidizing bacteria, season had the greatest impact on gene abundance and diversity (higher in summer than in winter). Overall, the primary influence of plant presence is believed to be related to root oxygen loss and its effect on rhizosphere redox.

Introduction

Constructed wetlands (CW) are a technology utilizing engi-neered wetland systems for the treatment of a variety of wastewaters ranging from domestic sources to storm runoff. Although the operation of CW is relatively well understood, there is a lack of understanding of the microbial impact on performance [21]. Many studies have investigated micro-bially mediated processes in CW by focusing on net changes in the concentration of specific chemicals or waste constit-uents [78, 79, 93]. Microbial communities have been shown to effectively contribute to the removal of chemical pollu-tants in CW [34, 71]; however, microbiology remains the most underrepresented area of research in this field. Further-more, the role of biofilms and their function within these systems have been virtually ignored. Presently, these gaps in knowledge may impede the effective design and operation of CW [21, 81]. An understanding of the community struc-ture of microorganisms involved in biogeochemical process-es, their location and distribution within the CW, and the extent of seasonal population shifts are important for ad-vancing CW technology.

Microbial biofilm communities are found on every avail-able surface in CW, including roots, as biofilms are capable of creating stable, protected environments for microbial survival [31]. It is also possible for oxygen gradients to develop within the biofilm due to the activity of the biofilm microorganisms themselves [12, 80]. Therefore, biofilms may contain microenvironments that allow for the simulta-neous presence and growth of both anaerobic and aerobic

organisms. Root surfaces are very dynamic and heterogeneous, which promotes colonization by varying populations of microorganisms. Plant roots support large biofilm populations primarily because they are sites of oxygen release and root exudation [6, 13, 53]. Chemical exudates released by plant roots are generally plant species specific, and plants are known to selectively enrich the rhizosphere with microorganisms accordingly [13]. Because of their effects on rhizosphere biofilm populations, root oxygen loss (ROL) and root exudation may ultimately affect effluent water quality.

Sulfate reducing bacteria (SRB) are important in CW 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, are important for many biological processes and for the generation of alkalinity in CW [41]. SRB utilize sulfate (SO_4^{-2}) as a terminal electron acceptor in the anaerobic oxidation of organic substrates [35] and are critically important since they are the only known organisms to perform this function. SRB improve effluent water quality by removing sulfate from the aqueous phase. However, precipitates of sulfide remain within the system and CW probably accumulate sulfur over time. SRB 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 [12, 18, 72]. 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 [3, 9, 35, 76, 79].

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 [17, 82, 83]. In bacteria, nitrification occurs via two aerobic reactions: ammonia to nitrite, by ammonia oxidizing bacteria (AOB), and nitrite to nitrate, by nitrite oxidizing bacteria (NOB). Ammonia oxidizing archaea (AOA) have been detected in wastewater treatment [62], natural wetlands [74] and CW [73]. The oxic conditions required for nitrification can limit transformation of ammonia in traditional wastewater treatment systems [61], but plants in CW may be able to provide sufficient oxygen for nitrification through their roots [10, 11, 39, 71, 96].

Previous research in our laboratory has long inferred the presence of a robust SRB population within CW microcosms [3, 9, 79]. Most recently, Taylor et al. [86] showed that microcosms planted with *Deschampsia cespitosa* had high removal efficiencies for organic carbon (COD) (nearly 100 %) and seasonally dependent removal of sulfate (highest in summer) while unplanted microcosms and those planted with *L. 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 removal performance (determined by effluent water quality) were assumed to be linked to both plant presence (vs. unplanted control) and plant species selection. Redox data revealed very consistent values in the range of −200 to −250 mV for the unplanted control and *L. cinereus* microcosms regardless of season. *D. cespitosa* microcosms also tracked in this redox range in summer, but had redox readings during successive winters in the 0 to +400 mV range [84, 85]. The effect of *D. cespitosa* on increasing redox in the entire bulk water during winter is attributed to ROL, and was the primary reason it was selected for study in the current research.

Molecular methods are increasingly being used to investigate microbial diversity and activity in environmental systems such as CW, because culture-based methodologies are severely limiting [4, 69]. An overall perspective of the microbial diversity can be obtained by targeting the 16S rRNA gene, but this approach has limited utility for investigating specific microbial groups. For this reason, it is becoming more common to target specific functional genes. Furthermore, since DNA from nonviable cells has been shown to remain intact for a certain time in the environment [40], DNA-based PCR technologies cannot normally distinguish between DNA extracted from live (potentially active) and dead (inactive) cells. Differentiating between microbial populations that are merely present in biofilms vs. those that are potentially active may provide a better link between biogeochemical processes and community structure [42, 51]. The live–dead continuum continues to be debated, however, one criterion for a live cell is an intact cell membrane, which can be determined experimentally as the ability to exclude from the cell membrane-impermeable dyes [30, 56]. Recently, Nocker et al. [57, 58] introduced a method designed to distinguish between cells with intact vs. compromised membranes based on treatment with propidium monoazide (PMA) prior to DNA extraction. PMA is capable of entering the cells of bacteria with compromised or damaged cell membranes and binding to the DNA. Exposure to light during processing causes PMA to become irreversibly bound to the DNA, which is then permanently inhibited from subsequent PCR amplification. This process leaves only DNA from cells with intact cell membranes for further analysis using PCR methodology [57]. PMA treatment also excludes extracellular DNA from subsequent amplification.

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 CW, optimization of effluent water quality is the main objective; therefore, bulk water sampling

would seem appropriate for understanding the community dynamics within. However, most nutrient removal in CW very likely takes place in root and matrix surface biofilms, yet these environments are difficult to sample. The plant rhizosphere in CW and other vegetated soils has been found to have a different microbial community compared to the bulk substratum [46, 75]. Furthermore, composite sampling methods (such as soil cores) can underestimate actual microbial diversity because they tend to combine regions of high and low diversity [42, 44]. Exactly how a CW should be sampled is still unknown.

The goal of this study was to determine the diversity and activity of SRB and AOB communities within CW microcosms by evaluating the relative DNA copy numbers by quantitative PCR (qPCR) of two functional genes, *dsrB* (involved in sulfate reduction) and *amoA* (involved in nitrification). *dsrB*, the smaller fragment of the dissimilatory sulfite reductase gene (*dsrAB*), was selected as it is essential for anaerobic sulfate reduction and has been found in all dissimilatory sulfate-reducing prokaryotes examined thus far [1, 5, 27, 51]. AOB were targeted by examining the beta proteobacterial 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 [19, 38, 45, 67, 95]. The potentially active community was defined as consisting of cells with intact cell membranes as determined by PMA treatment. The diversity within each of these genes was also evaluated as the number of bands (richness) in denaturing gradient gel electrophoresis (DGGE) profiles.

CW microcosms were planted with one of 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 fed a synthetic wastewater that simulated post-primary domestic wastewater effluent. Microcosms were destructively sampled in summer and winter seasons to investigate the effects of plant species and season on the SRB and AOB functional communities. To investigate whether specific locations within the CW were ecologically more relevant habitats for AOB and SRB than others, i.e., selectively enriched, samples were collected from bulk effluent water, gravel (two depths) and roots (thick, thin, ultra-fine). Effluent water quality analyses were also performed to assess reduction of sulfate and removal of ammonia as well as used to correlate CW performance (by pollutant removal) with the relative quantities of the targeted genes.

Methods

Constructed Wetland Operation

This research was conducted in the same facilities used in earlier studies [78, 79, 86]. CW 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). Four replicates each of unplanted controls and monocultures of *D. cespitosa* and *L. cinereus* were planted in model subsurface wetlands consisting of 15 cm diameter by 30 cm tall polyvinyl chloride columns filled with 1–5 mm diameter 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 [86]. There were three 20-day batches at each temperature. Between batches, the microcosms were completely drained. Periodic measurement of sulfate, ammonia, and COD in the bulk water was by standard methods as reported previously [85]. 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 CW microcosm experiments [84, 86]. The objective was to compare two species at opposite ends of the spectrum in terms of their apparent effects on nutrient removal (carbon and sulfate) and root oxygen release in the CW. Plants were selected based upon COD removal and oxygen release because these are standard criteria for evaluating CW performance [71]. CW columns planted with *D. 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 [84-86]. 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 *L. cinereus* provided poor COD removal and undetectable levels of root oxygen release [84, 86]. 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.

The plants were transplants from microcosms used in earlier experiments and were 12 months old at the time of the first sampling and 18 months old at the second sampling. In addition, the gravel used in each microcosm was also obtained from earlier experiments and was plant-species specific, that is, only gravel from earlier *D. cespitosa*

microcosms was used for replanting this species, and so on. There is no standard for such studies, and the literature reports a wide range of plant ages. Molle et al. [52] allowed plants to mature for 3 years to minimize plant aging effects. In contrast, Gagnon et al. [26] sampled plant microcosms that were less than 6 months old. Iasur-Kruh et al. [36] used 3-month-old microcosms to model a 3-year-old CW. Wang et al. [92] used plantings that were at least 2 years old, but noted that even with mature plants, there were some differences in plant performance between senescent plants in the fall compared to fast growing plants in the spring.

Microcosm Destructive Sampling

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. First, the column was completely drained and the water was collected as effluent (E1). Next, the plant and root ball were removed and separated from any residual rootbound gravel by swirling the plant in a container of tap water. Roots of two diameters were then aseptically excised from the plant, thicker and older roots near the crown (~1–2 mm diameter, R2), and thinner and younger roots near the tip (~0.5–1 mm diameter, R3). Ultra-fine roots that had detached from the plant during processing (R4, < ~0.5 mm diameter) were found floating in the container of tap water and were skimmed from the surface. Finally, gravel from the top of the column (G5) and from the bottom (G6) were collected.

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. The purpose of this intensive sampling was to determine whether certain sample locations were selectively enriched for the target genes (*amoA* or *dsrB*) and/or more responsive to seasonal or plant species differences.

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 aseptically divided in half for PMA-treated and untreated processing. For PMA treatment, clear 1.7 ml microcentrifuge tubes (www.biotang.com) were used for maximum transmittance of light (see PMA protocol below). Untreated samples were transferred directly to DNA extraction tubes (MO BIO PowerSoil™ DNA Isolation Kit; www.mobio.com). Thin (0.03 g) and thick roots (0.05 g) were collected in duplicate and aseptically transferred to 1.7 ml clear tubes or to DNA extraction tubes. Ultra-fine roots were skimmed into a 100 ml volume of water, filtered (41 Whatman paper filter (www.whatman.com)), and the filter contents were also transferred to either clear tubes or DNA extraction tubes. To remove biofilm from the gravel surface, each of the gravel samples collected (7.5 ml) was vortexed with phosphate buffered saline (pH 7.2) and 10 ml sterile sand for 60 s. For PMA treatment, 3 ml of the supernatant was transferred in 0.5 ml aliquots to six clear 1.7-ml microcentrifuge tubes. For the untreated sample, six 0.5-ml aliquots were sequentially pelleted into DNA extraction tubes.

PMA Treatment

A total volume of 0.5 ml for each sample was used for PMA treatment. For the effluent samples and all root samples, 0.5 ml of sterile effluent filtrate was added to the clear tubes. 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 by Nocker et al. [58], except as modified below. To each tube, 1.5 μl of PMA (20 mM stock solution prepared in 20 % dimethyl sulfoxide; Biotium, Inc., Hayward, CA) was added and each tube was shaken vigorously. Tubes were incubated in the dark on ice for 5 min with the exception of bottom gravel samples which were exposed to PMA for 7 to 10 min. PMA was then inactivated by exposing the tubes to light for 2 min using a 650-W halogen light source (sealed beam lamp, FCW 120 V, 3,200 K; GE Lighting, General Electric Co., Cleveland, OH). Samples were shaken during light exposure. Root samples were exposed to light for 4 min 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. Aliquots were combined and concentrated by sequential centrifugation for 5 min at 5,000×*g* into a DNA extraction tube.

DNA Extraction

The MO BIO PowerSoil™ DNA Isolation Kit 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

Conventional PCR was performed to screen for the target genes and to obtain products to be analyzed by DGGE. In

some cases, primers for both genes were combined in a single multiplex PCR reaction, primarily to screen for the target genes and to conserve sample DNA and PCR reagents. All PCR reactions (20 μl) were performed using 2× GoTaq® Green Master Mix (www.promega.com). The PCR reaction mixture consisted of 10 μl 2× GoTaq® Green Master Mix, 0.5 μl ultrapure bovine serum albumin (BSA) (50 mg/ml; Ambion, www.ambion.com), 2.5 μl nuclease-free 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 1. Multiplex reactions were adjusted to contain 10 μl 2× GoTaq® Green Master Mix, 0.5 μl ultrapure BSA (50 mg/ml; Ambion, www.ambion.com), 0.5 μl nuclease-free 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 program specified in Table 2. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide.

Sulfate Reducing Bacteria (for DGGE)

PCR primers DsrBF and Dsr4R (Table 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 by Geets et al. [27] (Table 2) was used for amplification. Products were analyzed by DGGE.

Multiplex PCR

PCR primers DsrBF and Dsr4R (Table 1) were combined with primers RottF and RottR (Table 1). Presumptive presence of the genes was indicated on an agarose gel by 370- and 491-bp PCR products. The PCR program specified by Bahr et al. [5] (Table 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 DGGE, with Multiplex Above)

PCR primers RottF and RottR (Table 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 by Bahr et al. [5] (Table 2) was used for amplification. Reamplified multiplex PCR products were used for DGGE analysis.

DGGE

DGGE was performed on functional PCR products (*dsrB* and *amoA*) (with and without PMA treatment) from community DNA using a DCode™ system (www.biorad.com) and reagents from Sigma-Aldrich (www.sigmaaldrich.com).

Table 1 Primer sequences used in this study

Primer	Target	Sequence (5′ to 3′)	Method	Reference
DsrBF	*dsrB* gene (*dsr* β-subunit)	CAACATCGTYCAYACCCAGGG	PCR[a]-DGGE/qPCR	[27]
Dsr4R	*dsrB* gene (*dsr* β-subunit)	GTGTAGCAGTTACCGCA	PCR[a]-DGGE/qPCR	[91]
RottF	*amoA* gene	GGGGTTTCTACTGGTGGT	PCR[a]-DGGE/qPCR	[67]
RottR	*amoA* gene	CCCCTCKGSAAAGCCTTCTTC	PCR[a]-DGGE/qPCR	[67]
VectF	pCR®2.1-TOPO plasmid	AGTGTGCTGGAATTCGCC	DGGE Marker	[14]
VectR with GC	pCR®2.1-TOPO plasmid	ATATCTGCAGAATTCGCC	DGGE Marker	[14]
Eub341F	16S rRNA V3 region	CCTACGGGAGGCAGCAG	qPCR	[55]
Eub534R	16S rRNA V3 region	ATTACCGCGGCTGCTGGC	qPCR	[55]
GC Clamp	Attach at 5’ end of primer	CGCCCGCCGCGCCCCGCGCCCG GCCCGCCGCCCCCGCCCC	PCR-DGGE	[22]

[a] Used in multiplex PCR screening reactions without the GC clamp

Table 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 min.	[27]
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.	[5]
16S rRNA 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 [1]
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.	[28]

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 [29]. 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 unidentified clones according to the method described by Burr et al. [14], data not shown) 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.

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 2: 16S rRNA gene program was modified from Agrawal and Lal [1], *dsrB* from Geets et al. [27] (see qPCR modifications in Table 2), *amoA* from Geets et al. [28]. Data were acquired using the FAM/Sybr detection channel during the extension step. For each sample, C_t values, the number of cycles required for the signal to exceed background fluorescence, were converted to initial DNA template concentrations using a standard curve. The standard curve was a plot of C_t values vs. standard template concentrations. Standards and samples were prepared in triplicate and appropriate negative controls containing no template DNA were included to ensure that no contamination had occurred. Melt curve analysis was also performed after thermal cycling to verify PCR products. Melt curve analysis was performed from 65 °C to 95 °C in 0.3 °C increments held for 5 s with an initial pre-melt hold for 90 s at the first step.

The 16S rRNA gene was quantified and used to normalize the amounts of *dsrB* and *amoA* genes in each sample location. 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 nuclease-free 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.

qPCR data were expressed in terms of relative gene abundance by normalizing absolute copy numbers to copy numbers of the 16S rRNA gene from the same sample. This allowed for comparison among samples in which different amounts of DNA were extracted, and seemed to be the only way to compare qPCR results for such different sample materials as water, gravel, and root biomass. It also allowed a straightforward assessment of the extent to which a sample location was enriched for either functional gene compared to other sample locations. A limitation of this approach is that it does not determine the absolute contribution of SRB and AOB associated with effluent vs. gravel vs. roots. It was not possible to relate gene abundance to cell numbers of AOB and SRB because the intracellular copy number for each of the genes investigated is variable. The 16S rRNA gene can range from 1 to 15 copies per cell [23, 28, 32], and the *amoA* gene from 2 to 3 copies per cell [16, 28, 59, 88]. The *dsrB* gene has been reported to have a single copy per cell [43, 48]. Samples below the level of detection were distorted when the copies of 16S rRNA genes from that same location were also low. This occurred with only one sample (from the ultra-fine roots in a summer *D. cespitosa* microcosm). PCR primers DsrBF and Dsr4R (Table 1) were used for amplification of SRB and RottF and RottR were used for amplification of AOB. 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 nuclease-free water, 1 μl of forward and reverse primers (12.5 μM), and 8 μl undiluted template DNA.

qPCR Standard Curve Preparation

Purified (16S rRNA gene, *dsrB*, *amoA*) PCR products obtained from DNA extracted from CW samples were cloned into plasmid vector pCR®2.1-TOPO® using the TOPO® TA Cloning kit (Invitrogen, www.invitrogen.com) following the manufacturer's protocol. 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). The 16S rRNA gene sequence was 99 % identical to the 16S rRNA gene of an uncultured beta proteobacterium clone (accession no. FJ535165). The *dsrB* sequence was 84 % identical to the *dsrB* gene of a *Desulfatiferula* sp. isolate (accession no. HE613445). The *amoA* sequence was 99 % identical to the *amoA* gene of an uncultured bacterium from an environmental sample (accession no. JN177542).

Standard curves were obtained with serial dilution of the quantified standard plasmids carrying the target 16S rRNA gene, *dsrB*, or *amoA* gene. Standard curves were generated for each gene, 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 the 16S rRNA gene, 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. Subsequent statistical analyses of the presence-absence data were performed using R software (Version 2.11.1) libraries labdsv [65] and optpart [66] (www.r-project.org). Dissimilarity matrices were calculated using the Sorensen method [50, 87].

Hierarchical clustering (HC) analyses from the Sorensen dissimilarity matrices were performed on DGGE band patterns profiles for each of the functional genes, and dendrograms were generated using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) [8, 37]. 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 [2], while the silhouette width measures the mean similarity of each sample in the cluster to the mean similarity of the next most similar cluster [68]. The optimized dendrogram was sliced (according to the optimized partana ratio) and chi-square tests performed on the sliced trees to determine impact of the environmental parameters (season, plant type, sample type) on the clusters (<0.05 indicated a significant relationship).

Separately, principal coordinate analysis (PCO) was applied to the DGGE dissimilarity matrix. 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, in two dimensions, important partitions of the data with respect to the environmental variables (e.g., summer vs. winter, planted vs. unplanted) for each of the functional genes (R software library labdsv [65]). 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 (maximum was 1) indicated 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 (the one with the highest D^2 value) was selected and the dataset subdivided according to this parameter (e.g., season). Subsequent analysis of these two subgroup datasets determined the next most influential variable (e.g., plant species or sample type). Surface analysis is capable of comparing two categories at a time; for those with variables with three categories, one was compared to the remaining two as was relevant to the CW system (e.g., unplanted (control bands) vs. planted (*D. cespitosa* and *L. cinereus* bands considered together)). This process was repeated until all environmental variables had been assessed and subgroups created and analyzed, or surface analysis no longer revealed associations between the PCO data and the environmental parameter applied. Results from surface analyses were compared with HC results to determine whether similar conclusions could be drawn using a second independent approach.

Quantitative PCR

Quantitative PCR results were analyzed using analysis of variance (ANOVA) and multivariate ANOVA (MANOVA) in Minitab (version 15). All data were normalized to 16S rRNA gene quantity [1, 19, 28] and transformed to the log scale to satisfy the normal assumption of the statistical models. The Benjamini–Hochberg correction was used to maintain a false discovery rate of either 5 % or 10 % [7]. Due to the limited number of data points, MANOVA analysis could not be performed on all six sample types simultaneously. Effluent (E1) was analyzed with an ANOVA. The other five responses were separated into root (R2, R3, R4) and gravel (G5, G6) subsets and these two groups were

analyzed separately. The factors in these models were Plant, Season, and the two-way interaction. If there was no statistically significant interaction, the main effects were interpreted directly. That is, an overall effect due to Plant Type could be interpreted across all of the Seasons. Otherwise, a "follow up" MANOVA was performed with a single factor with levels across all of the combinations of Plant and Season in order to examine the interactions between Plant and Season. The linear discriminate functions (LDF), which are the directions of maximum discriminability of the group means produced by the MANOVA [64], were calculated, rounded to an integer value, and interpreted with regard to our CW system. The coefficients of each LDF were standardized by multiplying by the within group standard deviation. By comparing the magnitude of the standardized coefficients, we ranked the importance or contribution of the original gravel and root measurements to the discriminability of the group means [64]. Statements of significance are made based on Wilks' likelihood ratio test.

Results

The combined approach of incorporating PMA, DGGE and qPCR with functional gene primers allowed for a comprehensive analysis of membrane-intact microorganisms that had *amoA* and *dsrB* genes. PMA removed the DNA from cells with compromised cell membranes (and extracellular DNA); DGGE was a measure of the diversity of the community in each sample, and qPCR quantified gene copy number. In this way, we attempted to determine if diversity and/or abundance of a particular gene within the membrane-intact microbial community varied with time and sample location.

CW performance, as determined by effluent water quality, had been the focus of previous research [86] with COD and sulfate removal closely examined. To ensure that the CW microcosms were performing as anticipated, water quality was evaluated prior to destructive sampling with methods used in the previous study [86]. For water quality data, there was good agreement between the previous and current research. In the current research, there was a seasonal trend in COD removal for both the unplanted control and *L. cinereus*, ranging from 70 % to 80 % in winter and up to 90 % in summer. *D. cespitosa* COD removal was typically highest in winter (nearly 100 %) and slightly reduced in summer (about 90 %). Sulfate data also correlated well with previous research for all treatments, with nearly 100 % sulfate removal in summer and winter for *L. cinereus* and the unplanted control, and for *D. cespitosa* nearly 100 % sulfate removal in the summer and 50 % removal in the winter. 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

The efficiency of qPCR was near 0.9 and the standard curves for all genes were linear over six orders of magnitude (R^2>0.99). For each DNA extract, copy numbers of the 16S rRNA gene 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 rRNA gene copy number as ratios (copies *dsrB* or *amoA*/copies 16S rRNA gene) [1, 19, 28]. 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 (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. The effects of PMA treatment were used to evaluate 16S rRNA gene copy number when compared to untreated samples. C_t values for the 16S rRNA gene treated with PMA indicated that removal of DNA was no more than 10 % when compared to the untreated samples. Furthermore, the difference between PMA treatment vs. no treatment was only significantly different for the ultra-fine root samples and top gravel (ANOVA, 5 %). Therefore, all subsequent qPCR data for *dsrB* and *amoA* are reported for the PMA treated samples only.

Dissimilatory Sulfite Reductase Gene (dsrB)

ANOVA was performed for each of the six individual locations to see how season and plant species affected relative gene abundance. The ultra-fine roots and the top gravel layers showed seasonal variation for SRB with significantly higher gene ratios observed in the summer (10 % significance; Tables 3, 4 and 5). Overall, *D. cespitosa* had the lowest relative *dsrB* abundance (for all sample locations except the ultra-fine roots; Tables 3–5). In previous studies, *D. cespitosa* microcosms also typically had the highest winter redox potential; in summer redox was comparable to *L. cinereus* and the unplanted control [84, 85]. The unplanted control column and *L. cinereus* had similar relative *dsrB* abundance for all sample locations, with the effluent and bottom gravel samples containing the highest relative abundance of *dsrB*. This correlates well with the similarly efficient sulfate removal rates observed for both of these treatments.

Table 3 Average (n=2) relative gene abundances for *dsrB* and *amoA* compared to the16S rRNA gene, separated by plant species: unplanted control

	Unplanted Control					
	Summer			Winter		
	E1	G5	G6	E1	G5	G6
Copies/μl						
AOB	6.6E+02	2.4E+04	8.4E+02	7.0E+02	1.3E+04	1.3E+03
SRB	1.8E+04	2.7E+03	1.7E+04	2.1E+03	4.4E+03	5.2E+04
16S	5.9E+06	5.4E+05	3.1E+06	1.0E+06	1.1E+07	1.0E+07
Relative abundance						
AOB/16S (%)	0.011	4.560	0.027	0.067	0.118	0.013
AOB/16S (log10)	−3.95	−1.34	−3.57	−3.17	−2.93	−3.89
SRB/16S (%)	0.311	0.495	0.541	0.203	0.041	0.502
SRB/16S (log10)	−2.51	−2.31	−2.27	−2.69	−3.39	−2.30

E1 effluent, *R2* ~1–2 mm diameter roots from near the plant crown, *R3* ~0.5–1 mm diameter roots near the root tips, *R4*< ~0.5 mm diameter ultrafine roots detached during plant processing, *G5* gravel from column top, *G6* gravel from the column bottom

MANOVA was performed for a more comprehensive examination of the qPCR data for environmental responses. Effluent data (E1) was analyzed separately by standard ANOVA with no significant results. There were significant plant and season interactions within the root samples (p<0.05). For *dsrB*, 87 % of the variability of the group means (for the different plant and season combinations) was discriminated by the equation: log(R2)−log(R3)+0.5 log (R4). This discriminant function can be used to transform the measurements on each sample that were actually measured, R2, R3 and R4, to a new variable which yields maximal discriminability between the group means. Although the coefficients for log(R2) and log(R3) have the same magnitude, standardization of these coefficients show that log(R3) is the single most important root measurement to monitor in order to ascertain mean *dsrB* differences amongst plants and seasons. *L. cinereus* had the highest relative *dsrB* abundance on its roots in the summer season, whereas *D. cespitosa* had minimal seasonal variation in the relative *dsrB* abundance in root samples. 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 more important in explaining plant and seasonal mean differences in relative *dsrB* abundance, compared to the top gravel (more aerobic). In addition, *D. cespitosa* gravel was always observed to have lower relative *dsrB* abundance in the summer season compared to the unplanted control or *L. cinereus* gravel locations (Tables 3, 4 and 5).

Table 4 Average (n=2) relative gene abundances for *dsrB* and *amoA* compared to the16S rRNA gene, separated by plant species: *D. cespitosa*

	D. cespitosa											
	Summer						Winter					
	E1	R2	R3	R4	G5	G6	E1	R2	R3	R4	G5	G6
Copies/μl												
AOB	5.3E+02	3.5E+02	3.8E+02	2.7E+02	3.5E+03	1.2E+03	2.7E+02	3.1E+02	2.7E+02	2.7E+02	2.7E+02	3.0E+03
SRB	1.4E+04	7.5E+02	2.2E+03	6.8E+02	2.8E+03	9.4E+03	6.8E+02	6.8E+02	8.1E+03	2.4E+03	8.5E+03	3.9E+04
16S	4.9E+06	2.9E+06	1.0E+06	9.6E+04	2.9E+06	1.0E+07	2.4E+06	1.3E+06	9.7E+06	4.7E+06	1.1E+07	8.9E+06
Relative abundance												
AOB/16S (%)	0.011	0.012	0.037	0.279	0.119	0.011	0.011	0.024	0.003	0.006	0.002	0.034
AOB/16S (log10)	−3.97	−3.92	−3.43	−2.55	−2.92	−3.94	−3.96	−3.63	−4.56	−4.24	−4.61	−3.47
SRB/16S (%)	0.278	0.026	0.221	0.708	0.096	0.093	0.028	0.051	0.083	0.051	0.077	0.433
SRB/16S (log10)	−2.56	−3.59	−2.65	−2.15	−3.02	−3.03	−3.56	−3.29	−3.08	−3.29	−3.11	−2.36

E1 effluent, *R2* ~1–2 mm diameter roots from near the plant crown, *R3* ~0.5–1 mm diameter roots near the root tips, *R4*<~0.5 mm diameter ultrafine roots detached during plant processing, *G5* gravel from column top, *G6* gravel from the column bottom

Table 5 Average (n=2) relative gene abundances for *dsrB* and *amoA* compared to the16S rRNA gene, separated by plant species: *L. cinereus*

	L. cinereus											
	Summer						Winter					
	E1	R2	R3	R4	G5	G6	E1	R2	R3	R4	G5	G6
Copies/µl												
AOB	2.6E+03	4.5E+03	3.8E+03	1.7E+03	4.9E+03	3.8E+03	6.7E+02	2.3E+03	4.2E+03	2.7E+02	7.2E+03	8.0E+03
SRB	1.6E+04	9.0E+03	3.9E+03	1.1E+03	1.1E+04	3.3E+04	3.1E+04	1.9E+04	3.3E+04	3.2E+03	1.9E+04	2.7E+04
16S	1.1E+07	1.6E+06	1.5E+06	2.1E+05	2.2E+06	4.9E+06	5.8E+06	7.3E+06	4.4E+06	2.4E+07	2.3E+07	1.1E+07
Relative abundance												
AOB/16S (%)	0.022	0.276	0.257	0.783	0.219	0.076	0.012	0.032	0.096	0.001	0.031	0.070
AOB/16S (log10)	−3.65	−2.56	−2.59	−2.11	−2.66	−3.12	−3.94	−3.49	−3.02	−4.95	−3.50	−3.16
SRB/16S (%)	0.143	0.548	0.268	0.539	0.471	0.664	0.528	0.263	0.750	0.014	0.084	0.239
SRB/16S (log10)	−2.85	−2.26	−2.57	−2.27	−2.33	−2.18	−2.28	−2.58	−2.12	−3.87	−3.07	−2.62

E1 effluent, *R2* ~1–2 mm diameter roots from near the plant crown, *R3* ~0.5–1 mm diameter roots near the root tips, *R4*<~0.5 mm diameter ultrafine roots detached during plant processing, *G5* gravel from column top, *G6* gravel from the column bottom

Ammonia Monooxygenase Gene (amoA)

The ultra-fine roots and the top gravel layers showed seasonal variation for AOB, with significantly higher gene ratios observed in the summer (5 % significance; Tables 3, 4 and 5). In addition, both planted species contained similar relative *amoA* abundance for each sample location throughout the columns. MANOVA indicated that for the *amoA* gene there were significant plant and season interactions within the root samples (p<0.05) with 86 % of the variability of the group means for the different plant and season combinations was discriminated by the equation: log(R2)−2 log(R3)+0.5 log(R4). For gravel samples, only a seasonal effect was observed (no plant involvement) with respect to relative gene quantity (p<0.10), with relatively higher *amoA* abundance 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 mean *amoA* abundance 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).

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, HC analysis and PCO combined with general surface plotting were performed on the DGGE community profiles for each of the functional genes (the 16S rRNA gene was not analyzed by DGGE). Figure 1 shows representative DGGE profiles for *dsrB*. Profiles for both PMA-treated and untreated samples are included to demonstrate that there was no visible difference between them. DGGE profiles 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 (Fig. 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 (data not shown), statistical analyses were performed on these profiles also.

Richness of *dsrB* and *amoA* genes was measured as the number of bands in the DGGE profiles. For *dsrB*, the most interesting observation was that of 72 total bands detected (all *dsrB* DGGE profiles combined), profiles from planted microcosms contained 71of these, while profiles from unplanted controls contained only 62 bands. The most interesting result for *amoA* DGGE profiles was the comparison between summer and winter samples. There were a total of 44 bands in winter profiles and 45 in summer profiles; however, seven or eight bands were only detected in one of the seasons, suggesting a slight shift in the AOB community.

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). PCO surface analysis also detected a difference between the planted and unplanted SRB

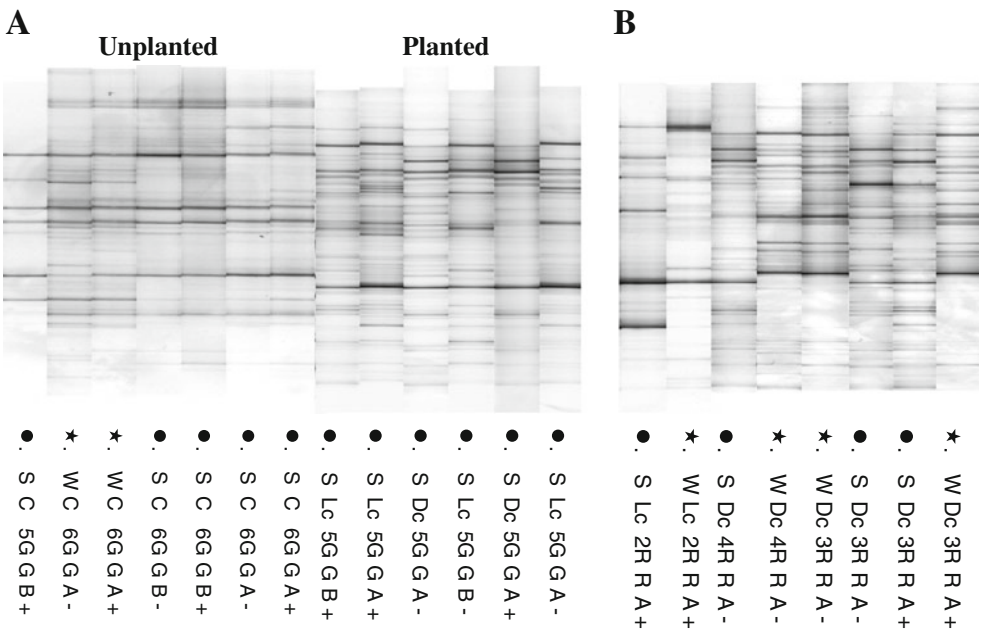

Figure 1 Representative DGGE profiles of SRB communities (*dsrB* gene). **a** SRB community profiles comparing gravel samples from unplanted (*left*) and planted (*right*) microcosms. **b** SRB community profiles comparing summer and winter seasons from rhizosphere (R2, R3, R4) biofilm samples. Images 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 microcosms, +/− PMA treatment

communities (D^2=0.5799; Fig. 2). The figure provides a visual representation of the surface plot analysis performed on the data, with the contour lines illustrating the separation of the data in a third dimension (analogous to a topographic map) according to the presence or absence of plants. When analysis was limited to data only from planted microcosms, there was a significant seasonal effect on SRB community structure (p<0.05, as determined by chi-square analysis; D^2= 0.3852, as determined by surface analysis). No seasonal effect was observed within the control column communities (p> 0.5). HC 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 from any of the other locations within the microcosm.

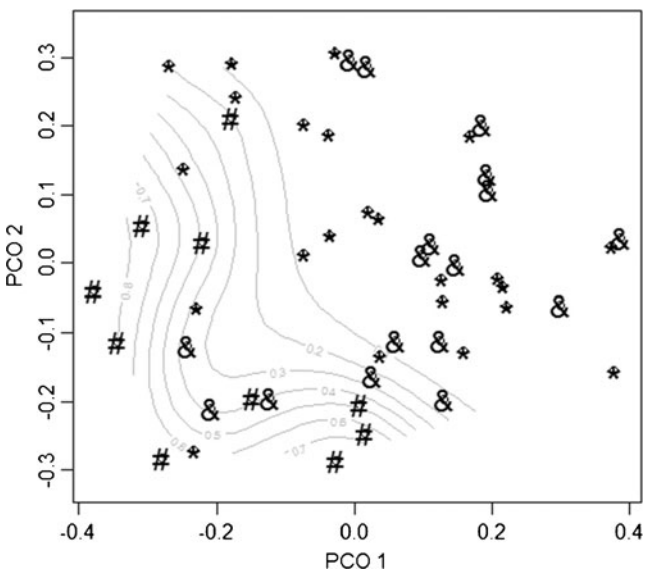

Figure 2 Analysis of SRB biofilm community profiles from planted and unplanted microcosms post PMA treatment. The contours indicate values between 0 (planted) and 1 (unplanted) and offer a visualization of the separation of DGGE results from unplanted and planted microcosm communities. *Pound symbol* unplanted control, *asterisk L. cinereus*, *ampersand D. cespitosa*

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 season, plant species, etc. Although we only used the PCO surface analysis to provide a visualization of possible effects found by HC, since the two methods do not provide consistent results, we do not emphasize the statistically significant differences in the *amoA* community profiles found by HC in this manuscript.

Overall, HC showed sample type (effluent, roots, gravel) to select for unique communities for both *amoA* and *dsrB*. Season also affected 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

The research presented here investigated the effect of two independent variables, season and plants species selection (including unplanted controls), on the diversity and abundance of two functional genes, *amoA* (nitrification) and *dsrB* (sulfate reduction). Both are good model genes because PCR and qPCR methods are well established for them [27, 28, 67, 91]. They are also molecular markers for very important biological transformations in wastewater treatment in CW. Diversity was

measured as presence/absence of bands in DGGE profiles as used in the study by Calheiros et al. [15]. Band intensity was not evaluated since it has been considered a poor indicator of the abundance of the corresponding species [25, 54]. Total gene abundance was then quantified by qPCR.

A secondary objective was to identify sampling locations that were (1) selectively enriched with either gene (greater relative gene abundance or greater diversity) and/or (2) more responsive to the independent variables relative to other sampling locations. Although planktonic bacteria are not thought to be primarily responsible for nutrient removal in CW, investigations typically focus on these easily obtained samples. To ensure that the functional diversity, gene abundance and metabolic activity are more realistically assessed, the communities partitioned into microhabitats in biofilms on gravel and varied root surfaces were investigated. It was hypothesized that these samples might be more responsive to the variables of season and species than bulk water and therefore more sensitive or ecologically relevant. However, even intricate sampling such as that performed in this research cannot separate very different microhabitats that might exist within micrometers to millimeters of each other.

Other research groups have performed similar intensive sampling in full scale CW and/or microcosms. Gagnon et al. [26] combined sand and root samples for analysis of bacterial density, respiration, and enzyme activity. Wang et al. [92] sampled complete below ground biomass, but for microbial enzyme assays, not for DNA-based analysis. Iasur-Kruh et al. [36] separated roots from gravel in a microcosm study, but chose to analyze only the gravel samples for DGGE analysis of the bacterial rRNA gene. Sims et al. [74] sampled water and wetlands soils in their study of seasonal effects on AOB. However, we are not aware of any research in which there was an attempt to determine the relative importance of different sampling locations (including three different root classes) in explaining variations in the data among samples. For example, our statistical analyses indicated that root sample R3 was the most important in explaining seasonal and plant species effects on *dsrB* abundance. Although we have only begun to investigate how a CW should be sampled, we have presented a method and statistical rationale for making that determination.

PMA treatment was integrated into the sampling protocol to target membrane-intact cells within the CW microcosms. The conclusion that the majority of bacterial cells in a community have intact cell membranes does not indicate the extent to which these cells are currently metabolically active or replicating but does focus attention on potentially active cells. Because PMA treatment reduced the copy number of 16S rRNA genes by only about 10 %, subsequent data analyses were performed only on PMA treated sample results. Furthermore, ANOVA of the PMA untreated functional genes normalized to PMA untreated 16S rRNA genes produced results that were very similar to those for PMA treated samples. There are two possible interpretations of these results: (1) the bacterial communities consisted almost entirely (~90 %) of cells with intact membranes, or (2) that PMA treatment was not completely effective at inhibiting PCR amplification of DNA from cells with compromised membranes and/or extracellular DNA. However, since the PMA method [57, 58] convincingly demonstrated the effectiveness of PMA in excluding from PCR amplification the DNA from cells killed by various treatments (e.g., heat, isopropanol), the conclusion that the bacterial communities in these microcosms consisted primarily of cells with intact membranes is probably justified. This result is similar to that of Varma et al. [89], who found little difference in qPCR copy numbers between PMA-treated and -untreated samples from wastewater in the absence of any biocidal treatment. However, when bacteria were heat killed, there was at least a three-log reduction in the qPCR signal in PMA-treated compared to untreated samples. Another possible effect on PMA efficacy is amplicon length. Luo et al. [49] reported that PMA treatment could fail to suppress PCR amplification from membrane-compromised cells when the amplicons were shorter than about 190 bp. In the current study, the amplicon lengths were 193, 370, and 491 bp for 16S rRNA, *dsrB* and *amoA*, respectively.

Using the approaches and rationales described above, the microbial ecology of the wetlands with respect to sulfate reduction and ammonia oxidation were explored. The ratios of the two functional genes to 16S rRNA gene abundance in the CW samples were closely related to the ranges previously reported in the literature from locations such as rice roots [70], marine sediments [19], sludge [28], and oil field production water [1]. Average *dsrB*/16S rRNA genes ranged from 0.013 % to 0.750 % and *amoA*/16S rRNA genes from 0.002 % to 4.6 %. Other researchers have shown similar relative quantities of SRB in the environment, generally not representing more than 5 % of the total microbial community present [20, 70] (both compared SRB rRNA with total rRNA). Most recently, Dang et al. [19] reported the ratio of *amoA*/16S rRNA genes in marine sediments to be between 0.003 % and 0.07 %.

For SRB, the attached roots (R2 and R3) were most important in explaining relative abundance, but the ultrafine roots (R4) could not be completely discounted without altering the results. In general, all the root locations were equally important, but for gravel, the bottom gravel locations were most important. SRB appeared to be a more robust community, compared to AOB, as few samples were below detectable levels. SRB were found in all of the sampled microcosm locations, supporting previous evidence that these organisms are capable of existing in both anaerobic and aerobic environments [12, 18, 24]. H_2S was not measured, but the odor of H_2S was detected during sampling

and there was blackening of the gravel (especially the gravel from the bottom of the column (G6)), indicative of SRB activity.

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. Sulfate, carbon, or redox could limit sulfate reduction. In this research, adequate sulfate and carbon were supplied in the feed water, leaving redox as the variable most likely to limit SRB activity and also the most site specific of these variables. *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 [86]. 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 [90].

Overall, *D. cespitosa* had the lowest relative SRB quantity with very little seasonal variation. This implies a stable SRB community; however, it had been hypothesized that an increase in the SRB population in summer would correspond to increased 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.

In addition to gene abundance, it is important to consider the potential diversity of organisms carrying the *dsrB* gene. 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 [90]. 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 other treatments. This result may suggest that the rhizosphere of *L. cinereus* was leaking oxygen. Since *L. cinereus* had such shallow roots, the oxygen released may not have been sufficient to impact sulfate removal 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 of qPCR showed *dsrB* present in higher ratios for *L. cinereus* roots than for *D. cespitosa* roots; however DGGE showed similar community profiles 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 rather population 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.

For AOB, the attached roots were apparently the most ecologically relevant samples. The fine roots (R3) were more important for AOB compared to SRB in describing the variability of the means. This implicates the "fine root" region as a site of preferential colonization by AOB and an important sampling site. The ultra-fine roots (R4) were equally important for AOB and SRB and could not be removed from analysis without affecting the outcome. 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. AOB were most frequently detected in the summer season and generally attached to root surfaces. The top gravel was also important for explaining AOB abundance (by both ANOVA and MANOVA), whereas the bottom gravel was not important. These results suggest top gravel as another targeted location for future AOB investigation. Although not significant for the MANOVA results, AOB were detected in the bottom gravel of all the CW microcosms, supporting other research indicating their ability to survive under anaerobic conditions [26]. It has also been reported that AOB and anammox organisms can coexist [63], 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 simply explained by many samples being below the level of detection and normalized to similar values.

Although strong seasonal effects were observed for AOB community diversity with respect to DGGE, the quantity of the *amoA* gene was generally below the level of detection for most winter samples. This was not surprising given that the literature has often reported a strong temperature effect on nitrification [33, 47, 60, 77]. Although decreased quantities of *amoA* were observed in these winter samples, water quality analyses indicated that nitrification was still occurring (as measured by loss of ammonia). Ammonia loss could have been due to activity of archaeal ammonia oxidizers (AOA) or anammox organisms; however, screening of several samples for the genes of these two groups of organisms, by conventional PCR, did not detect any (data not shown). Another CW research group also found a seasonal shift in AOB DGGE profiles, but was investigating changes from autumn to spring [95]. Sims et al. [74] generally found AOA in equal or greater numbers compared to AOB (qPCR data)

in wetland soil and water samples. AOB were also more sensitive to winter than AOA.

The majority of CW research has focused on the microbial population associated with the gravel and effluent water 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 [90] and AOB [10, 11, 71]. It was unexpected that the ultra-fine roots (R4) of *D. cespitosa* in summer would simultaneously support a relatively 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 in individual summer samples for both genes because the corresponding copies of 16S rRNA gene were also low. Season also influenced the microbial community structure with effects on the abundance of both the *dsrB* and *amoA* genes. Our results for the root samples differed for the two genes. It is possible that each plant species affected the microbial community abundance by uniquely altering the redox environment within the microcosm.

Although there were differences in gene abundance, and diversity, both AOB and SRB were present at the same sample locations, confirming that both aerobic and anaerobic organisms can exist in close proximity in CW biofilms [80]. This could be due to protected niches in the environment provided by plant species. For example, 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 [94]. Our sampling methods were not capable of separating aerobic from anaerobic regions, since oxygen gradients, especially at root surfaces, are probably on a scale of less than one mm. Because of the predominant anaerobic condition in these columns, there is the potential for denitrification. Although the denitrifying populations were not directly assessed in this study, nitrite and nitrate were monitored in the effluent. The data suggested that denitrification was occurring in the summer in all columns, while nitrite accumulated during the winter in the control and *L. cinereus* columns. The increased redox in the *D. cespitosa* system in winter may have contributed to this phenomenon by limiting the competing SRBs.

The results of this study begin to illustrate the interactions occurring within CW and the methods used herein can be applied to a variety of environmental systems and genes for a more in depth understanding of microbial processes. Quantification of controllable parameters and their influences on the microbial ecology will lead to better design and operation of CW systems. Optimization of the microbial community structure and function should be a priority for the effective design of wastewater treatment systems [28, 81]. 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.

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