MICROBIOLOGY OF SULFATE-REDUCING PASSIVE TREATMENT SYSTEMS[1]

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Little is known about the microbiology of passive mine drainage treatment systems, such as sulfate-reducing permeable reactive zones (SR-PRZs). We have recently developed a suite of molecular biology tools in our laboratory for characterizing the microbial communities present in SR-PRZs. In this study our suite of tools is used to characterize two different field bioreactors: Peerless Jenny King and Luttrell. Both bioreactors are located near the Ten Mile Creek Basin near Helena, MT, and both employ a compost-based substrate to promote the growth of sulfate-reducing bacteria (SRB) for production of sulfides and precipitation of metals. In summer, 2005, the reactors were sampled at multiple locations and with depth. DNA was extracted from the compost material and followed by cloning of polymerase chain reaction (PCR) amplified 16S rRNA genes, restriction digest screening, and DNA sequencing to provide insight into the overall composition of the microbial communities. To directly examine the SRB populations, a gene specific to SRB, *apsA*, was PCR-amplified, cloned, and sequenced. This revealed that *Desulfovibrio* spp. were prevalent in both Luttrell and Peerless Jenny King. At Peerless Jenny King, one *Desulfovibrio* spp. found was noted to be particularly aerotolerant. This analysis also revealed that *Thiobacillus denitrificans* were common at Peerless Jenny King. This is an organism that oxidizes sulfides in the presence of nitrate, which is undesirable for biozone function. In order to quantify SRB, quantitative real-time PCR (Q-PCR) was used targeting two specific groups of SRB, *Desulfovibrio* and *Desulfobacteria*. These results indicated that these two SRB groups, which have distinct substrate requirements, vary in distribution between the two bioreactors and with depth. It is hoped that an improved understanding of the microbiology of these systems will help to improve design and operation of passive treatment systems employing sulfate reduction.

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Introduction

Mining influenced water (MIW) is a major environmental issue in the United States and other countries with estimated costs of treatment in the tens of billions of dollars (Benner et al., 1999). MIW is associated with mining activities that expose sulfide minerals such as pyrite (FeS_2) to air and water. The oxidation of sulfide minerals releases acidity, sulfates ($SO_4^{-2)}$ and Fe(II) which contaminate the water. The acidity generated also mobilizes toxic trace metals.

Sulfate-reducing permeable reactive zones (SR-PRZs) as applied in anaerobic wetlands, sulfate-reducing bioreactors, or permeable reactive barriers, provide an attractive means of "passive" treatment of mining influenced water. Being passive, they are low in costs and require minimal maintenance; thus they are ideal for remote areas with restricted access (Waybrant et al., 2002; Berghorn and Hunzeker, 2001). However, because fundamental aspects of these systems are poorly understood, there is a lack of clear and reliable design criteria for their implementation. Some SR-PRZs are known to operate well in the field without interruption, while others fail after clogging (Kamolpornwijit et al., 2003), freezing events, or for no apparent reason (Waybrandt et al., 2002).

For many years, SR-PRZs have been treated as black boxes without any thorough understanding of the members or roles of the microorganism involved. Since the operation of SR-PRZs is highly dependent on microbial activity, a better understanding of the role of the microbial community in these systems will help improve their design and performance (Hallberg and Johnson, 2005, Kaksonen et al., 2004, Labrenz and Banfield, 2004). From the standpoint of this study, a complex community is responsible for MIW remediation (Logan et al., 2003). SRB are obligate anaerobes that use short chain organic compounds or H_2 as electron donors. Thus, SRB depend on microorganisms that have the enzymes to degrade cellulose, lignin, and other polymers to monomers and on fermenters that transform these short chain compounds into even simpler compounds. Our previous batch and column studies have shown a relationship between performance and the type of microbial inoculum. Through this work, a suite of molecular biology tools was developed specifically for the study of SR-PRZ microbial communities. In this study, we apply these molecular techniques to compare the microbial communities of two active field SR-PRZs. Both systems are located in the Ten Mile Creek Basin near Helena, MT. This study provides one of the first insights into the composition of microbial communities in a field-scale SR-PRZ using molecular methods.

Methods

Sample Sites

Peerless Jenny King (PJK)In Fig. 1 is the schematic of the PJK bioreactor. The influent water to PJK is typically low in SO_4^{-2} (~100 mg/l), slightly acidic in pH (~6.0), and contains ~2 mg/l Zn, 5 mg/l Mn, and trace levels of Fe and Cd. Mine drainage exits the adit and flows through a wetland in order to provide pre-treatment and precipitate Fe oxides. Hay bales are used as baffles in the wetland to help slow the flow rate and to increase biological activity. Pre-treated water then passes through a limestone rock channel before entering four compost-based serpentine SO_4^{-2}-reducing biozones in series. The depth of the compost varies from 0.7 m to 1 m in the four biozones. Sulfate concentrations are typically about 100 mg/L and the pH is ~6.0 at the influence of the first bioreactor.

Luttrell (LUTR) In Fig. 2 is the schematic of the Luttrell bioreactor. The influent water to Luttrell is typically high in SO_4^{-2} (~1000 mg/l), slightly acidic in pH (~6.0), and also contains Mn (~40 to 170 mg/l) and Zn (~40 to 200 mg/l), along with trace amounts of Al, Cu, Cd, Fe, and Ni. Mine drainage flows from the top to the bottom of a ~1 m layer of compost. A layer of ~3 cm diameter rocks is emplaced on top of the compost to prevent floating of the compost. The bottom of the bioreactor is lined with an impermeable geotextile. Flow exits the bioreactor through a network of perforated 8 cm pipes at the bottom of the compost. Effluent is held in an underground storage tank and subject to land application disposal (LAD) per EPA standards.

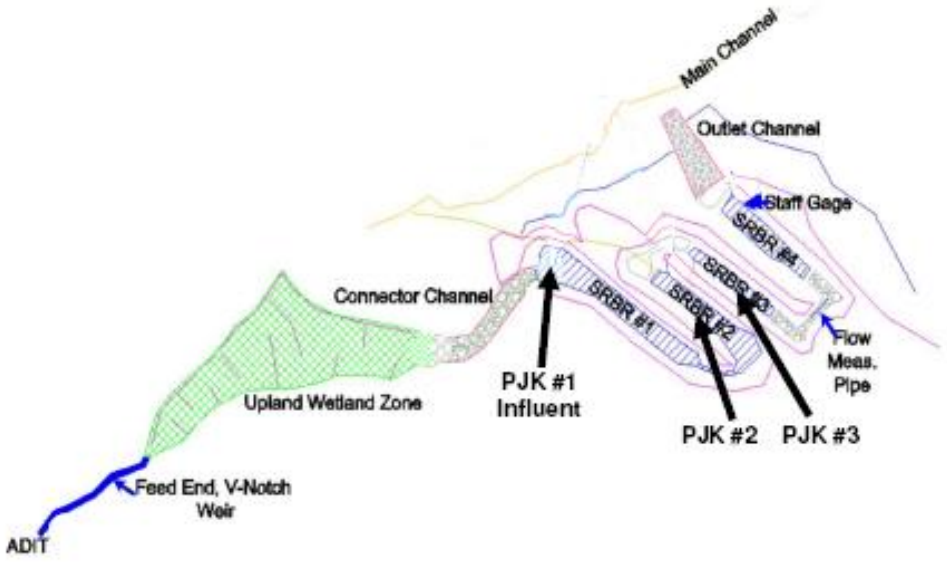

Figure 1. Overview of Peerless Jenny King wetland and serpentine sulfate-reducing biozones. Arrows indicate three sampling locations that were the focus of this study, though other sites were also sampled.

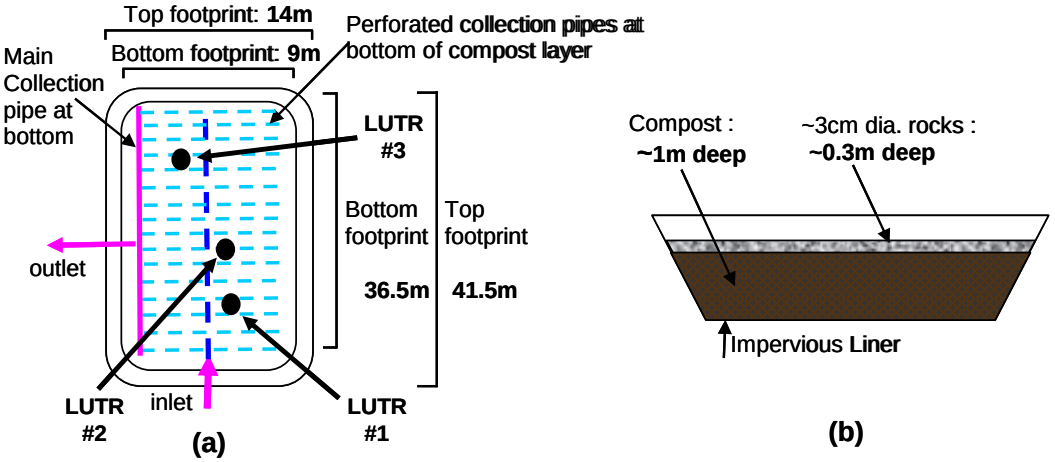

Figure 2. Luttrell Bioreactor and dimensions. (a) plane-view, with approximate sampling locations for this study indicated by the two black circles (b) side view.

Sample Collection The first sampling event took place June 13-17, 2005. The week prior to sampling was characterized by significant snow-melt, which was already meeting discharge

standards, and therefore was diverted from the bioreactors in preparation for sampling. Approximately 0.3 m of standing water above the rock layer at Luttrell was pumped off prior to sampling.

The second sampling event took place on August 30th, 2005 and focused only on PJK because there was concern for disrupting operation at Luttrell. This second sampling event followed a warm spell; however, there was significant snow fall on the actual day of sampling.

Samples were collected with depth using a fabricated "cleaver" device (constructed by Sonle Technical Engineers) which was pounded into the substrate using a sledge hammer. The downstream side was then exposed by removing the substrate with a shovel and pumping the water level down within the exposed hole with a sump pump. Sample compost material was then collected using a sterile spatula and a sterile Whirl-Pak sampling bag. Approximately 1 kg of material was collected for each sample in order to provide a sufficient amount for other work. A small portion (~10 g) was sub-sampled and transferred to a fresh Whirl-Pak bag for transport. All samples were vacuum sealed within 12 hours of collection and samples for molecular analysis were placed on dry ice for shipment and were stored at -80ºC once they reached the laboratory. At PJK, samples were collected at a range of 2-11 cm below the surface ("top") and just above the gravel layer at the bottom ("bottom") which varied in depth between the cells. All four cells were sampled approximately at the middle and an additional set of samples was taken at the influent point of the first cell. In the second PJK sampling event, these sites were off-set by ~1 m upstream. Three different locations ("#1 (front)," "#2 (middle)," and "#3 (end)") were sampled at Luttrell.

Microbial Community Analysis

DNA extraction DNA was extracted using the MO BIO UltraClean™ Soil DNA Kit (Carlsbad, CA). Eight replicate extractions (~1 g each) of compost were performed for each sample collected and combined in order to ensure that downstream analyses were representative of the heterogeneous material. DNA extracts were stored at -80ºC.

Cloning of 16S rRNA genes 16S rRNA gene sequences are commonly used to identify bacteria and thus they were amplified by polymerase chain reaction (PCR) from the DNA extracts and cloned into *Escherichia coli* using the Invitrogen TOPO TA Cloning Kit for Sequencing (Carlsbad, CA). PCR of 16S rRNA genes was carried out with primers 8F and 1492R, as described previously (Weisburg et al., 1991). Clones were screened for the presence of the 16S rRNA gene inserts using PCR with vector-specific M13 primers. M13 PCR products were digested with the restriction enzyme *MspI* in order to identify unique patterns and characterize the overall diversity (number of different kinds of microorganisms present) in the samples. Clones with restriction digest patterns occurring two or more times were sequenced in order to determine the identity of dominant microorganisms in the samples. Sequences were analyzed using the National Institute of Health Blast database (http://www.ncbi.nlm.nih.gov/BLAST), in order to find the highest percent matches, as compared with cultured and uncultured bacterial sequences available in the database.

Cloning of *apsA* genes The *apsA* functional genes are widely distributed across phylogenetically distinct SRB and thus provide a good target gene for focusing analysis on SRB (Friendrich, 2002). Also, cloning of *apsA* genes at the Quaking House compost wetlands in England has been done previously (Hallberg, submitted) and provides a means to compare with the work of others. A 900 bp region of the *apsA* genes was PCR amplified using primers as described in

Friendrich (2002). *ApsA* PCR products were cloned, screened by restriction enzyme digestion, and sequenced as described above for 16S rRNA genes.

Real-time quantitative PCR (Q-PCR) In order to obtain quantitative information regarding SRB, recently developed Q-PCR protocols were applied targeting the 16S rRNA genes of two gram negative genera of SRB: *Desulfovibrio* and *Desulfobacteria*. Q-PCR is a modified version of PCR in which the product amplification is monitored in real-time by fluorescence detection. Calibration curves can then be generated by determining the threshold cycle (Ct) values of standard DNA at known concentrations. The Ct value corresponds to the number of PCR cycles necessary before log amplification is achieved. Thus, for high initial copy numbers of target genes, fewer cycles are be needed to achieve amplification, and for low initial copy numbers, more cycles are necessary. PCR primers used were described in Daly et al., (2000) and protocols were run on a Cepheid SmartCycler using Qiagen SybrGreen MasterMix. SybrGreen emits a signal when bound to double-stranded DNA, thus providing a means to detect the PCR product as it is amplified. Total bacterial 16S rRNA genes were also quantified using a TaqMan approach described previously by Suzuki et al. (2000).

Results & Discussion

Cloning of 16S rRNA genes

Clones of 16S rRNA genes with a restriction enzyme digest pattern occurring two or more times (out of a sample size of 120 to 180 clones) were sequenced in order to determine their identity. This provided an overview of the microbial species present in the bioreactors estimated to be above 2% of the total microbial community. Only one of the four samples analyzed, PJK 3 bottom, contained an SRB above this threshold. Others have noted that SRB tend to comprise a relatively low percentage of the total community (Morales et al., 2005, Pereyra et al., 2005), though it is possible that a consortium of SRB exists in which the individual species are below 1%, but the consortium as a whole is higher. Nevertheless, this emphasizes the critical roles that other functional groups in compost-based biozones play. As has been demonstrated in our previous research and that of our collaborators (Logan et al., 2003), cellulose-degradation, rather than sulfate-reduction, is the rate-limiting step in these systems. Summaries of the bacteria, their relative frequencies, and the highest percent identities found in the Blast database for representative PJK and Luttrell samples are provided in Fig. 3 and Fig. 4, respectively.

Cloning also provided a means to assess the relative diversity (number of different kinds of bacteria) of the samples. This is of particular interest because one hypothesis is that high diversity is desirable because this provides a "robust" community capable of adjusting to changing conditions. Figure 5 provides the accumulation curves of two of the PJK and one of the LUTR samples, which give an indication of overall diversity. It was noted (qualitatively) that the overall diversity was high in all three samples. This observation was based on the high number of screened clones necessary for the accumulation curves in Fig. 5 to begin to reach asymptotic behavior (i.e. 120 to 180 clones screened revealed 60 to 100 bacterial types). Based on Fig. 5, the overall diversity is similar among the three samples, but PJK #3 bottom was the highest.

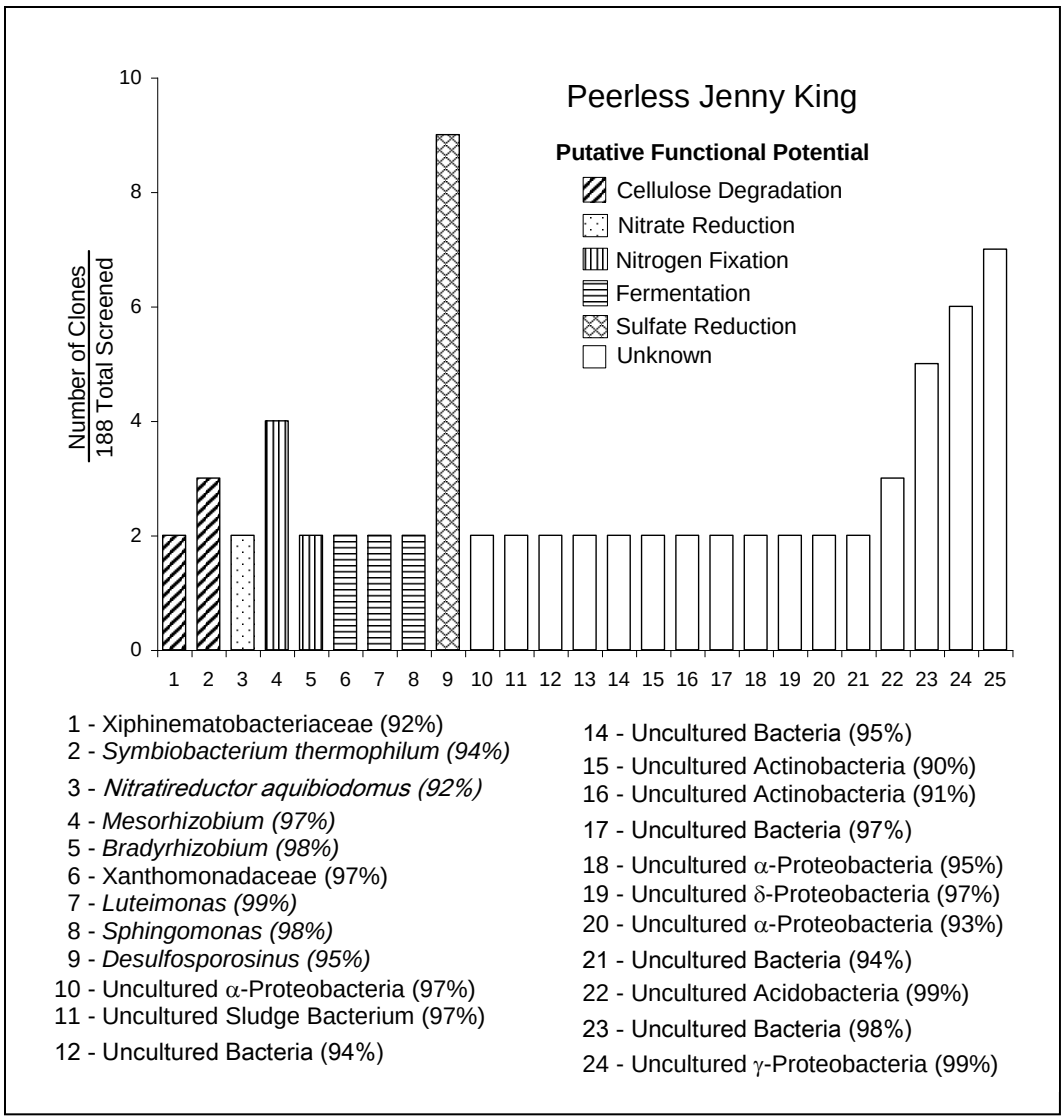

Figure 3. Summary of bacteria identified, their relative frequency of observation, and highest percent match within the Blast database as determined by cloning of 16S rRNA gene followed by restriction fragment length polymorphism (RFLP) screening and sequencing of a representative PJK sample (PJK #3 Bottom).

ApsA Gene Cloning

Results of *apsA* gene cloning are summarized in Fig. 6. The SRB detected in the Luttrell samples all belonged to the gram negative *Desulfovibrio* genus. Most of these were identified as *Desulfovibrio burkinensis.* In contrast to PJK, the distribution of SRB detected in the top and bottom of the sampling hole at Luttrell were relatively similar. At PJK, *Desulfovibrio aerotolerans* and *Desulfovibrio africanus* were found to be most prevalent at the top of the sampling hole, while an unidentified *Desulfovibrio* was found in the bottom. *Desulfovibrio aerotolerans*, like the spore-forming *Desulfosporosinus* identified by 16S rRNA gene cloning, is an aerotolerant SO_4^{-2} reducer. These results suggest that these systems are not strictly anaerobic and that occasional exposure to O_2 has selected for specific kinds of sulfate-reducers. This is

supported by the common observation at PJK that the upper layers of substrate are not saturated during times of low-flow. This could be of significant engineering interest considering that this may or may not be desirable for overall system function. For example, resistance to harsh and changing conditions is obviously a desirable attribute for SR-PRZs which are typically located at high altitudes. However, do aerotolerant sulfate-reducers reduce sulfate at a high rate or a low rate? This will be an important question to address in future research.

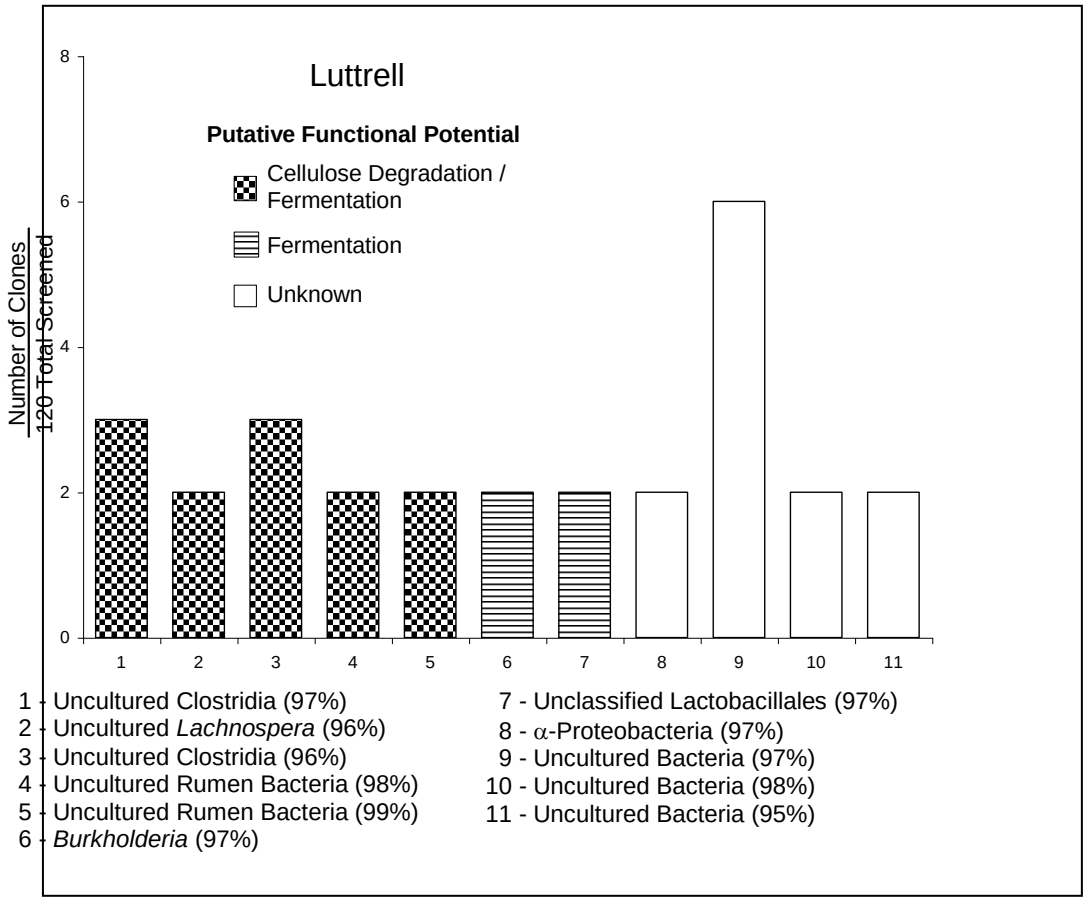

Figure 4. Summary of bacteria identified, their relative frequency of observation, and highest percent match within the Blast database as determined by cloning of 16S rRNA genes followed by restriction fragment length polymorphism (RFLP) screening and sequencing of a representative LUTR sample (LUTR #1 Bottom).

Suprisingly, *Thiobacillus denitrificans* was found to be prevalent at PJK, especially in the bottom of the 3rd cell sampling hole. This chemolithotrophic microorganism is a facultative anaerobe and can use either O_2 or NO_3^{-1} as an electron acceptor and inorganic reduced sulfur compounds as electron donors. Thus in the presence of O_2 or NO_3^{-1} this organism has the potential to reverse the desired function of sulfate-reduction. This organism was also identified at the Quaking House wetlands site when *apsA* genes were cloned and analyzed there (Hallberg, submitted). The fact that facultative anaerobe *T. denitrificans* was more prevalent at PJK than at Luttrell suggests the presence of NO_3^{-1} or O_2 at PJK.

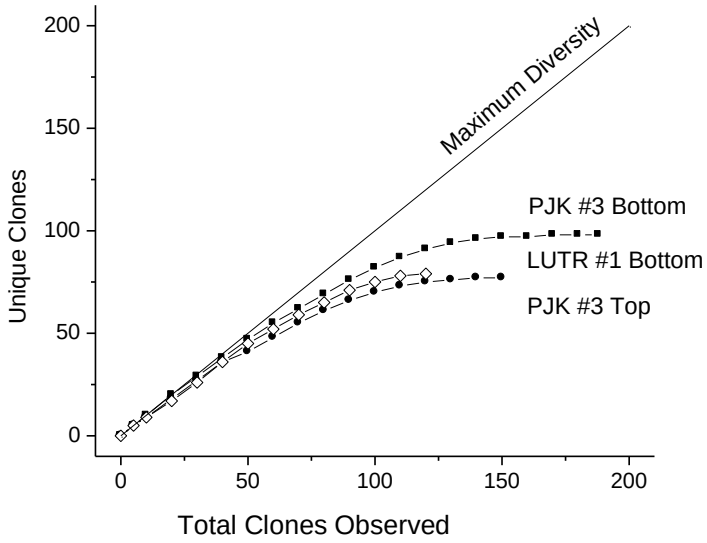

Figure 5. Accumulation curves representing relative diversity of two PJK samples and one LUTR sample as determined by cloning of 16S rRNA gene

Q-PCR Quantification of SRB

Desulfovibrio, *Desulfobacteria*, and total bacterial 16S rRNA genes were quantified by Q-PCR. Normalizing the relative *Desulfovibrio* and *Desulfobacteria* numbers to the total bacteria provided a means to compare these groups proportionally. Figure 7 and Fig. 8 summarize the results obtained for *Desulfovibrio* and *Desulfobacteria*, respectively. It was apparent from these results that *Desulfobacteria* were more prevalent at PJK while *Desulfovibrio* were more prevalent at Luttrell. *Desulfobacteria* are known to be more versatile in terms of their substrate-utilization capability than *Desulfovibrio*, which suggests a positive attribute for PJK bioreactor function. These results support the need for future research regarding the relative roles of different SRB and focused studies to determine which SRB are most desirable given the site-specific circumstances of the bioreactor.

Differences were also found with respect to the distribution of these two groups with depth in the two bioreactors. Interestingly, heterogeneity with depth was especially noted in PJK, as was also observed with respect to the *apsA* gene profiling of SRB. Theoretically, PJK would not be expected to exhibit stratification with depth given that it is a horizontal plug-flow reactor. However, in actuality, the depth of water is continually changing depending on the conditions. For example, at the time PJK was sampled, it was completely saturated because of the recent snow-melt. At other times the flow can decrease drastically, and it has been reported by the operators that the third and fourth cells dry out from time to time. Clogging and the development of preferential flow-paths are also possibilities, though obvious signs of this have not been reported by the field operators.

Though PJK has higher concentrations of the more versatile *Desulfobacteria*, it was the Luttrell bioreactor that was superior to PJK in terms of total bacteria present as represented by total bacterial 16S rRNA genes (data not shown). This could be an advantage, considering that non-SRB bacterial groups, notably cellulose degraders, are just as critical, if not more critical, for bioreactor function. These are the groups that convert the compost into a form that SO_4^{-2} reducers can subsequently use. A higher total bacterial population suggests higher populations

of cellulose degraders and fermenters. One possible reason that Luttrell may have higher total populations is because the compost substrate is constantly submerged, in contrast to PJK, which experiences periods of low flow and exposure of the substrate. This may also be why more aerotolerant SRB were found in PJK.

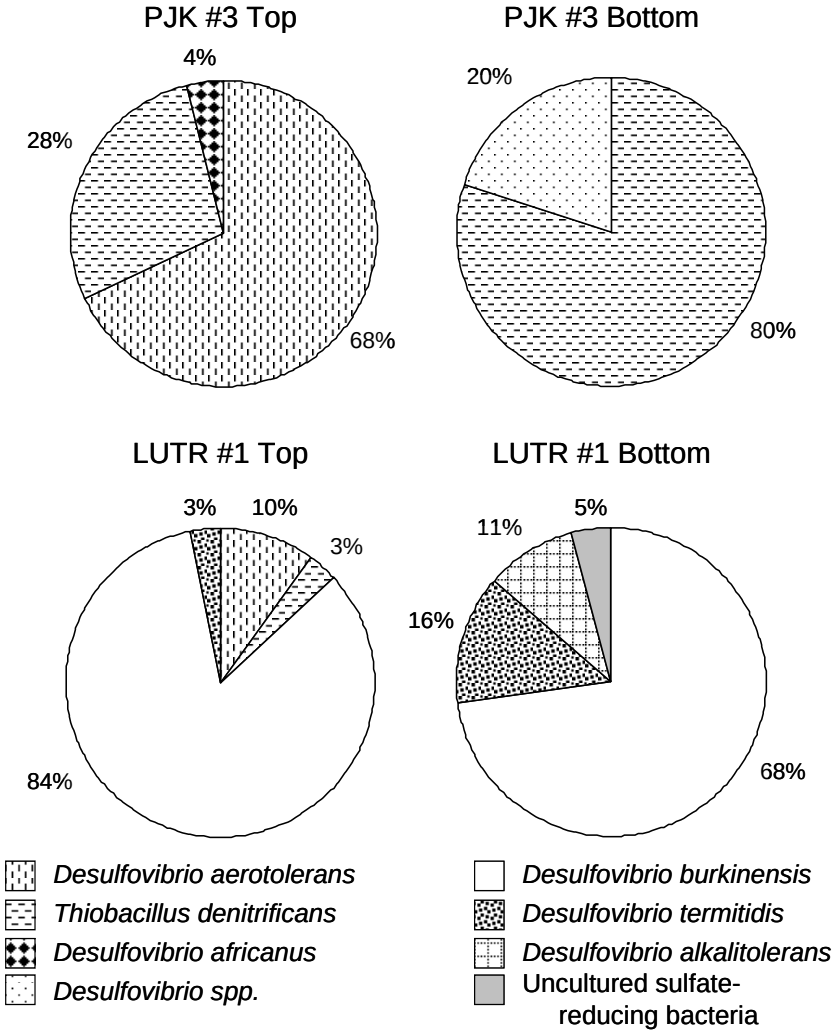

Figure 6. Summary of results obtained by *apsA* gene cloning for two LUTR and two PJK samples.

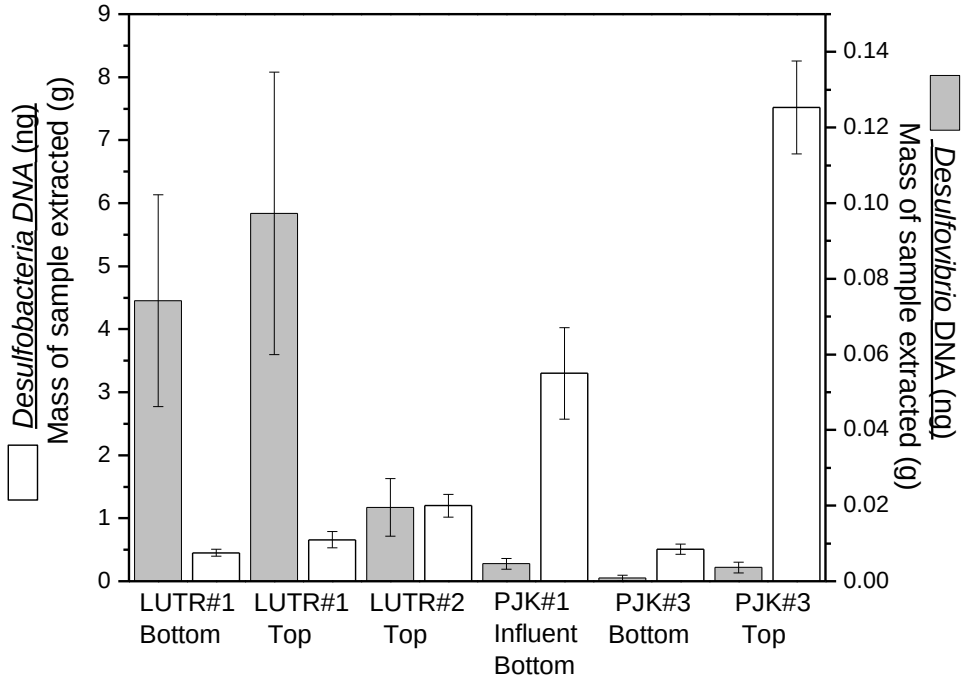

Figure 7. Quantification of *Desulfovibrio* and *Desulfobacteria* by Q-PCR, normalized to mass of sample used for DNA extraction, for three PJK and three Luttrell samples.

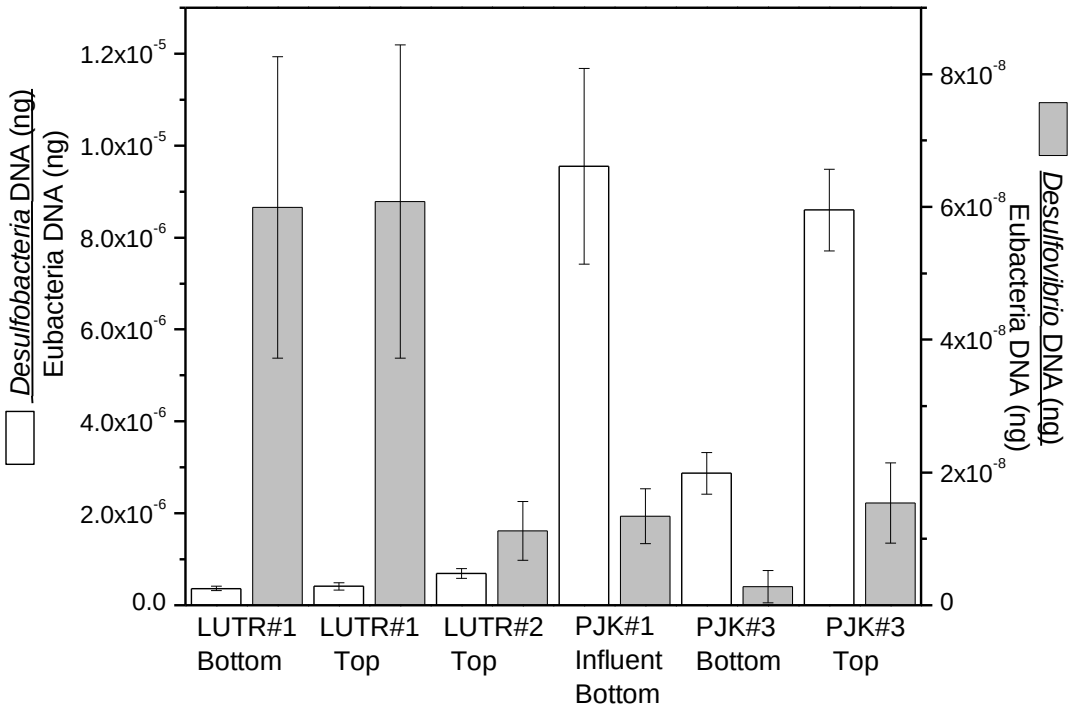

Figure 8. Quantification of *Desulfobacteria* and *Desulfovibrio* by Q-PCR, normalized to total Eubacterial DNA, compared for three PJK and three Luttrell samples.

Conclusions

This study provided insight into the microbial community composition in two passive sulfate-reducing permeable reactive bioreactors operating in the field: Peerless Jenny King and Luttrell. The suite of molecular biology-based tools provided information regarding the diversity of the microbes present that would not be possible with culture-based techniques. The results support previous observations that, individual SRB species are relatively minor members of the community numerically, though they perform the ultimate desired function. Cloning of the *apsA* gene was used to profile directly the species of SRB present and indicated that aerotolerant SRB are dominant in the Peerless Jenny King bioreactor. *ApsA* gene analysis also revealed the presence of *Thiobacillus denitrificans* at Peerless Jenny King. This organism is indicative of the presence of O_2 or NO_3^{-1} and has the potential to reverse the SO_4^{-2} reduction taking place in the biozone by oxidizing the sulfides. Finally, Q-PCR was used to quantify two different groups of sulfate-reducers: *Desulfovibrio* and *Desulfobacteria*. The results indicated that *Desulfobacteria* were more prevalent at PJK, while *Desulfovibrio* were more prevalent at Luttrell. Future work will focus on understanding how factors such as microbial diversity and the kinds of sulfate-reducers present relate to overall function of the biozone. Collaborative work (with Linda Figueroa) at Colorado School of Mines will provide a means to correlate the microbial community composition with the relative sulfate-reducing activity to help answer these questions.

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