MICROBIAL CHARACTERIZATION OF SULFATE-REDUCING COLUMNS REMEDIATING ACID MINE DRAINAGE[1]

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Abstract. Sulfate-reducing permeable reactive zones (PRZs) are a promising approach for passively remediating mine drainage. PRZs may be applied effectively in the field in anaerobic wetlands, sulfate-reducing bioreactors, or permeable reactive barriers. However, maintenance difficulties, such as variation of sulfate-reducing activity, are not well understood. To solve these problems, a better understanding of microbial communities in PRZs is essential. DNA based techniques provide a powerful means of characterizing microbial composition of PRZs. In this study, we developed a suite of methods specific to PRZ communities and analyzed samples from columns simulating PRZs that were operated at the Colorado School of Mines. Our objective was to determine the differences in microbial community structure between highly active columns, columns with reduced activity, inactive columns, and columns operated for over one year. Denaturing gel gradient electrophoresis (DGGE) and single stranded conformation polymorphism (SSCP) were performed to compare the microbial community structures of the columns with different activity levels and operation times. Sequencing results from DGGE and SSCP suggested that gram positive microorganisms belonging to the *Clostridium* group were dominant in all columns. This group includes cellulose degraders, fermenters, and sulfate reducers: all critical to PRZ function. In order to target sulfate-reducing groups directly, quantitative real-time PCR (Q-PCR) methods were developed for three genera: *Desulfobacterium, Desulfotomaculum,* and *Desulfovibrio*. Quantification of all three groups demonstrated that a decline in sulfate-reducing activity does not necessarily indicate a decline in SRB populations, suggesting that previous steps in the PRZ functional pathway would be wiser targets for improving performance. The methods developed in this study will be useful as diagnostic tools for PRZs in the field and may assist in developing optimized PRZ inocula.

Additional Key Words: microbiology, acid mine drainage, sulfate reduction, permeable reactive zones, passive treatment

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Introduction

Sulfate-reducing permeable reactive zones (PRZs) applied in anaerobic wetlands, sulfate-reducing bioreactors, or permeable reactive barriers, provide an attractive means of passive treatment of acid mine drainage. They are low in cost and require minimal maintenance, thus they are ideal for remote areas with restricted access (Waybrandt et al., 2002; Berghorn & 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 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 known cause (Waybrandt et al., 2002). To address this issue, our work has generally focused on better understanding the role of organic matter and microbial populations in PRZ function (Logan et al., 2003; Logan, 2003). The purpose of this study was to develop and apply molecular biological tools specific to PRZ microbial communities in order to gain a fundamental understanding of the microbiology of PRZs as a basis for improved design. A better understanding of such factors may help establish PRZ design criteria for increased lifespan, improved reliability, and optimized sulfate reduction.

In previous work we have presented a model for carbon flow through a PRZ (Logan et al., 2003). In this model, complex organic matter, mainly in the form of cellulose, is converted to cellobiose and other intermediates by cellulolytic microbes. Cellobiose is further broken down into sugars before being fermented to form by-products such as lactate and acetate. Sulfate-reducing bacteria (SRB) use these fermentation by-products as a carbon source and excess sulfate present in the mine drainage water as an electron acceptor to form sulfides. It is these sulfides that are ultimately responsible for binding heavy metals and precipitating them out of the aqueous phase. SRB, however, cannot use complex organic matter directly and therefore their function is critically dependent upon previous steps in the pathway. Column and batch studies conducted by Logan (2003) have suggested, in fact, that cellulose hydrolysis is the rate-limiting step, rather than sulfate reduction. If true, this represents an important contribution and indicates that efforts to improve PRZ performance would be most productive in targeting cellulose degradation, rather than the more intuitive sulfate-reduction step that performs the ultimate PRZ function.

In this study a suite of molecular biology tools was developed specifically for the study of PRZ microbial communities. These tools were applied to the analysis of the columns operated by Logan in order to provide a fundamental understanding of the microbial community dynamics relating to variations in column activity and operation with time.

Materials and Methods

Column Operating Conditions

All sulfate-reducing columns simulating PRZs were operated at Colorado School of Mines. For detailed description of column construction and operation, see Logan (2003). In brief, columns were packed with: 15% walnut wood shavings, 10% alfalfa pellets, 20% dairy manure, 5% wetland sediment kindly provided by T. Wildeman from the Big Five mine drainage treatment wetland (Idaho Springs, CO), 5% limestone rock (#10-#20 mesh; Pioneer Sand Co., Golden, CO), and 45% #8 mesh silica sand. Walnut shavings and alfalfa pellets were processed to 4 mm in a Wiley mill. The columns consisted of glass and were 30 cm long and 5 cm in diameter with a total volume of 589 mL and a reactive mixture volume of 324-399 mL. The

mass of reactive mixture packed in the columns was 150 g. The influent to the columns was simulated mine drainage water consisting of: 1000 mg/L sulfate, 50 mg/L zinc, 50 mg/l manganese at a pH of 6.0. The flow rate to the columns was 90 mL/day during start-up and was reduced to 30 mL/day subsequently. It was found that start up with 1000 mg/L sulfate in de-ionized (DI) water (without metals) initially before beginning the mine drainage water led to improved performance. The hydraulic retention time was determined to be an average of 4.13 days.

Columns 5a and 6a. Columns 5a and 6a were operated in parallel initially under the above conditions except that 50 mg/L of nickel was also present in the feed and the columns were started directly on simulated mine drainage water rather than DI water with sulfate only. The average sulfate reduction rate was lower than expected from previous column studies, averaging 0.48 moles SO_4^{2-}/m^3/day, and thus column operation was terminated after 38 days. Microbial analysis of these columns was conducted with the goals of gaining insight into the microbial community structure in inhibited columns and comparing them with the more active columns described below. Microbial analysis of these two columns was conducted individually to investigate possible differences between duplicate columns.

Columns 1b and 7b, 3b and 4b, 5b and 6b. Columns 1b and 7b, 3b and 4b, 5b and 6b were operated in parallel under the conditions described, without nickel and using 1000 mg/L sulfate in DI water during start-up at a flow rate of 90 mL/day before final operation at 30 mL/day with simulated mine drainage water. The sulfate reduction rate was steady at 0.6 moles SO_4^{2-}/m^3/day on day 41 when columns 1b and 7 b were sacrificed. On day 99, when columns 3b and 4b were sacrificed, the sulfate reduction rate had decreased to 0.3 moles SO_4^{2-}/m^3/day. Finally, after one year of operation, when columns 5b and 6b were sacrificed, flow through the columns was notably hindered and the sulfate reduction rate was negligible. Microbial analysis of this column set allowed for comparison of the microbial community present in columns during a period of high sulfate-reducing activity, lowered activity, and severely hindered activity following extended operation. For simplification, duplicate columns sacrificed in this set were combined for microbial analysis. Table 1 provides a summary of the performance of the columns analyzed in this study.

Microbial Community Analyses

Two parallel methods were used to profile and compare the microbial communities present in the columns: denaturing gradient gel electrophoresis (DGGE) and single stranded conformation polymorphism (SSCP). Both methods detect "dominant" organisms (greater than 1% of the total population) (Brüggemann et al., 2000) and provide a visible pattern, or "fingerprint," that allows for direct comparison. As a complementary approach, real-time PCR was used to target and quantify specific groups of interest. DNA extraction was required prior to all molecular analyses and is described below.

DNA extraction. Upon column sacrifice, the reactive mixture was frozen at -20 ºC for no more than 2 weeks prior to transport from Colorado School of Mines to Colorado State University where they were then stored at -80 ºC prior to DNA extraction. DNA was extracted using the FastDNA Spin Kit for soil (QBiogene, Irvine, CA) according to manufacturer protocol. The mass of soil was carefully recorded in order to allow quantitative analyses. Extracted DNA was further cleaned using the GeneClean Spin Kit (QBiogene) and stored at -20 ºC.

Table 1: Summary of Column Performance.

Constituent	*Column Set 2*	*Column Set 3*			
	5a and 6a	1b and 7b 3b and 4b 5b and 6b		3b and 4b 5b and 6b*	5b and 6b**
		Day 1-24	Day 24-41	Day 41-99	Day 99- 13 months
Average Sulfate-reduction rate (moles SO_4^{2-}/m^3/day)	0.48	0.6	0.6	0.3	N/A
Effluent pH	< 7	Steadily increased between 7 - 8.5			N/A
Zinc	not monitored	completely removed			N/A
Manganese	not monitored	appeared < 25 mg/L after day 37			N/A

* After column 1b and 7b were sacrificed
** After column 3b and 4b were sacrificed

DGGE. Microbial community DNA extracted from the columns was polymerase chain reaction (PCR) amplified using primers specific to the hypervariable V3 region of the 16S rRNA gene as described in Watanabe et al. (2001). PCR products were resolved by DGGE with a gradient of 20% to 55%, as described in Muyzer et al. (1993). Gels were stained with SYBR Gold nucleic acid stain (Molecular Probes, Inc., Eugene, OR) and images were captured using a UVP BioChemi Gel documentation system with Labworks Software (Upland, CA). Dominant bands were excised and stored in 36 mL of purified water prior to reamplification for DNA sequencing. DNA was sequenced off-site by Davis Sequencing, Inc. (Davis, CA).

SSCP. Fluorescently-labeled PCR primers were used to amplify the same ~200 bp V3 region of the 16S rRNA gene according to the methods described by Dabert et al. (2001). SSCP is similar to DGGE, except that a peak profile is produced rather than a banding profile. The fluorescently labeled primers allow the PCR products to be visualized by a detector as they are separated through a capillary based on their conformation as single strands. This conformation is dictated by DNA sequence properties which are unique for each organism present in the sample. The identity of peaks was determined by analyzing clones from the sample in parallel, and sequencing clones with matching peaks.

Real-time PCR. Real-time PCR (Q-PCR) provides a means to quantify specific groups of interest. Protocols were developed for quantifying three different groups of sulfate-reducing bacteria: *Desulfotomaculum* in the Clostridiales family of the Firmicutes (gram positive) Phylum, and two δ-Proteobacteria groups: *Desulfobacterium,* and *Desulfovibrio*. The methods developed for *Desulfotomaculum* and *Desulfovibrio* allowed for relative comparison (i.e. Sample A has significantly more/less than sample B), whereas the method for *Desulfobacterium* allows for direct quantification of the organism in the columns.

Q-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA) using a SYBR Green (Molecular Probes, Inc.) approach as implemented according to manufacturer protocol using the QuantiTect SYBR Green Kit (Qiagen, Valencia, CA). Briefly, a known mass of extracted column DNA was added to the reaction mixture with the appropriate primer pairs for the target group. Primers for *Desulfotomaculum* and *Desulfovibrio* were obtained from the literature (Daly et al., 2000). Primers for *Desulfobacterium* were designed specifically for this project by downloading appropriate 16S rDNA sequences from the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp), aligning them using ClustalX (Thompson et al., 1997), and finding optimal primer pair options from the alignment consensus using FastPCR (www.biocenter.helsinki.fi). Potential primer pairs were compared with all available sequences in the NIH NCBI database using the Blast alignment tool (http://www.ncbi.nlm.nih.gov/BLAST/) in order to ensure specificity of the primers for *Desulfobacterium*. The specificity of the primers was further investigated by using various foreign template DNAs, including: *Bordetella*, *Escherichia coli*, *Enterococcus faecalis*, *Desulfotomaculum*, *Desulfovibrio*, and calf thymus DNA. It was found that at the optimal annealing temperature of 62ºC, only the target *Desulfobacterium* DNA was amplified.

A calibration curve was prepared for *Desulfobacterium* from a serial dilution of *Desulfobacterium autotrophicum* genomic DNA (obtained from American Type Culture Collection, ATCC # 43914) with a 30 to 100 ng of "carrier" DNA (non-target) added per reaction. The log of the concentration of DNA added versus the threshold cycle for amplification (C_T value) was plotted to generate the calibration curve. In Fig. 1 the calibration curve for *Desulfobacterium* is presented. The C_T values for *Desulfovibrio* and *Desulfotomaculum* in the different samples were determined directly and allowed for a relative comparison between column samples. Absolute quantification was not carried out on these groups.

Quantification of total bacterial DNA was also important in order to have an idea of the overall concentration of bacteria in the columns. This was done using a TaqMan Q-PCR approach as described in Suzuki et al. (2000).

Results and Discussion

Microbial Community Profiling

Microbial community profiling by DGGE (Fig. 2) and SSCP (Fig. 3) provided a comparable picture of the microbial communities present in the columns through time. Significant differences were noted in the overall community structure of the more active set of columns (1b and 7b, 3b and 4b) and the less active columns (5a and 6a). Also, in the DGGE gels a progression could be viewed with time in which some bands remained consistent with time, some appeared with time, and others disappeared with time. This is representative of changes in community structure in the columns with time. These dominant organisms present possible targets for optimizing the microbial aspect of PRZ design and performance.

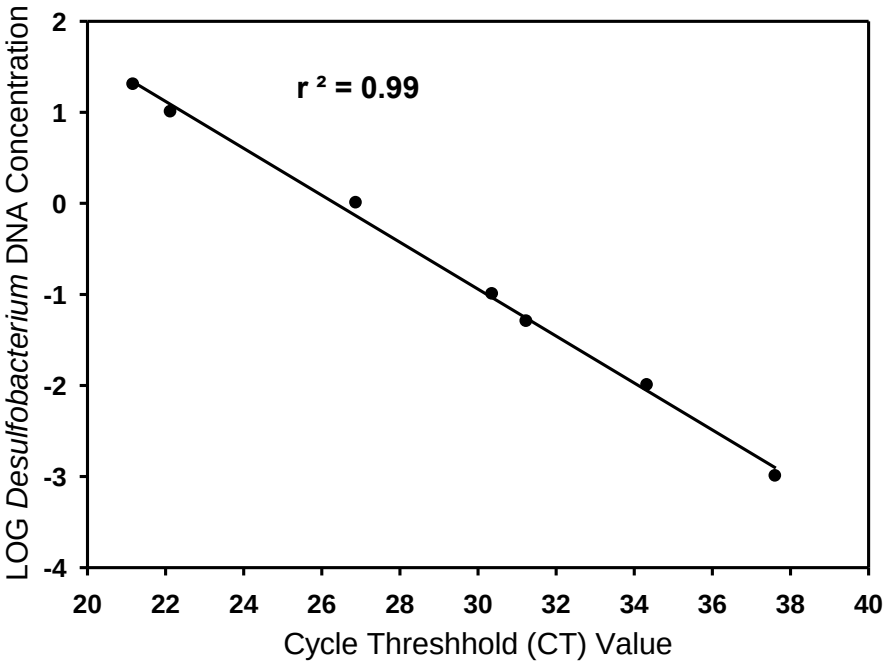

Figure. 1: Real-time PCR (Q-PCR) Calibration curve obtained for *Desulfobacterium*. Points are average of 6 replicates.

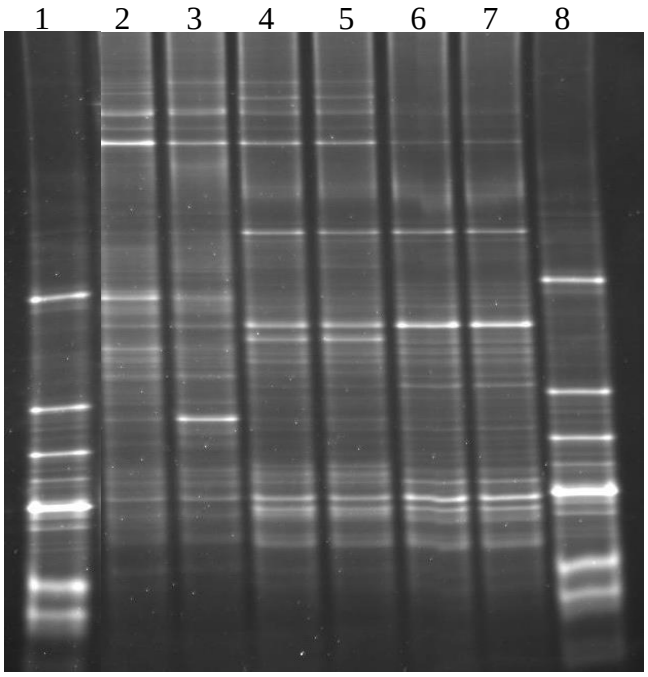

Figure 2: DGGE profile of column microbial communities. 1, 8: standards 2, 3: column 5a and 6a (inhibited activity) 4, 5: column 1b and 7b (high sulfate-reduction rate) 6, 7: column 3b and 4b (reduced sulfate-reduction rate)**.**

DNA sequencing of dominant DGGE bands (bright bands visible in Fig. 2) allowed organisms represented by these bands to be identified. All organisms identified by this method belonged to the same class of bacteria: Clostridiales (data not shown). This gram positive group contains several of the guilds critical to PRZ function, including cellulose degraders, fermenters, and sulfate reducers. Dominant peaks revealed by SSCP were sequenced and most also belonged to Clostridiales, including *Acetivibrio cellulolyticus*, an anaerobic cellulose degrader (Table 2). Bacteriodetes (fermenters) were the other major bacterial group identified by SSCP.

Table 2: SSCP dominant peak/clone sequencing results from column 3b and 4b.

Clone	Archae
A3	*Methanobrevibacter* sp. (Euryarchaeota, Methanobacterium Group)
A4	*Methanosarcina* (Euryarchaeota, Methanosarcinales Group)
A6	*Methanosarcina* Euryarchaeota (Euryarchaeota, Methanosarcinales Group)
A8	*Methanobrevibacter* sp. (Euryarchaeota, Methanobacterium Group)
	Bacteria
B12	*Bacterioides*- (99%) (CFB- Elbe River snow isolate 9_3)
B16	*Bacterioides* sp. (99%) (Bacteriodetes- Elbe River snow isolate 9_3)
B17	*Clostridium* sp. (98%) (Firmicutes, Clostridium- UnID anoxic Eubacterium)
B18	*Bacterioides* sp. (99%) (CFB- uncultured bacterium mle1-2)
B25	*Clostridium* sp. (93%) (Firmicutes, Clostridium, clone GOUTA13)
B27	*Clostridium* sp. (96%) (Firmicutes, Clostridium, clone p-2506-18B5)
B28	*Clostridium glycolicum* (98%) (Firmicutes, Clostridium)
B29	*Acetivibrio cellulolyticus* (98%) (Firmicutes, Clostridium)

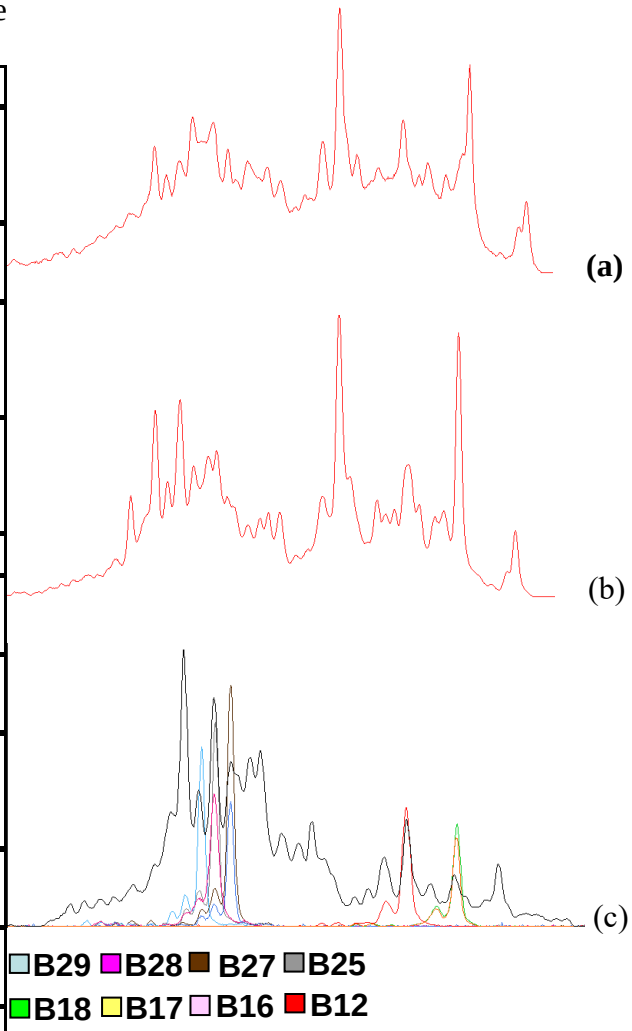

Figure 3: SSCP profiles of column bacterial microbial communities. (a): column 5a and 6a (b): column 1b and 7b (c): 3b and 4b with clones matching dominant peaks. Identity of clones/dominant peaks is found in Table 2. Archae profiles and clones are not shown.

Methanogenic bacteria belonging to Archae were confirmed to be present in the columns using an Archae-specific SSCP method (profiles not shown, dominant peak/clone sequencing results are presented in Table 2).

Q-PCR provided a means to specifically target, quantify and/or compare the relative quantities of sulfate-reducing groups in the columns. Quantities of *Desulfobacterium* DNA per mass of column material are plotted in Figure 4 and provide direct quantification of this group. Here it is demonstrated that quantities of this sulfate-reducing group were actually higher in the less active columns (5a/6a) than in the more active columns (1b/7b). It also should be noted that though columns 5a/6a and 1b/7b were not run in parallel, they were sacrificed after approximately the same number of days (38 days and 41 days, respectively), which facilitates comparison. Also, *Desulfobacterium* decreased by only about one half after one year of operation, even though sulfate reduction was essentially negligible at the time of sacrifice. These results suggest that a deficiency in sulfate-reducing populations is not the cause for degraded performance. It seems that sulfate reducers are available, but not active because of some other factor. Our hypothesis is that the cellulose-degradation step becomes hindered with time in PRZs and thus slowly diminishes available carbon for sulfate-reducing bacteria. This hypothesis is supported by our previous investigations with organic substrates in which it was demonstrated that cellulose degradation is the rate-limiting step in these columns (Logan et al., 2003; Logan, 2003). This is significant because it indicates that efforts at improving the performance of PRZs will be most fruitful in targeting cellulose degradation rather than sulfate reduction.

Relative quantification of *Desulfotomaculum* and *Desulfovibrio* was also carried out and the results are presented in Figure 5. *Desulfotomaculum* was of particular interest because this genus belongs to the Clostridiales group which was found to be dominant by the DGGE and SSCP profiling methods. Similar to the results obtained with *Desulfobacterium*, these groups did not show notable differences between active, less active, or declining columns. *Desulfotomaculum* even increased slightly in columns 3b and 4b, which were sacrificed on day 99 when sulfate reduction had decreased to about half the rate of what was observed on day 41 when columns 1a and 7a were sacrificed. These results further support the conclusion that although sulfate reducers were readily available in the columns, previous steps in the carbon pathway were limiting their activity.

Quantification of total bacterial populations (Fig. 6) showed that population levels decreased slightly from the period of high sulfate-reducing activity to lower activity, but remained stable at time of sacrifice one year later. However, columns 5a and 6a (inhibited) had by far the highest overall bacterial populations. This indicates that a high level of total bacteria is not necessarily an indicator of high rates of sulfate reduction.

Conclusions

The suite of molecular biological tools applied in this study, including quantitative techniques, provided a means of evaluating the microbial communities present in sulfate-reducing PRZs remediating heavy metals. These tools have significant potential to aid in the design and monitoring of PRZs in the field, as well as in optimizing the composition of the inoculum implemented in PRZ start-up. In this study, the tools developed were applied to sulfate-reducing columns simulating PRZs and demonstrated that a decline in sulfate-reducing performance does not correlate with a decline in sulfate-reducing populations. This finding is

significant and will allow optimization efforts to be better targeted at steps prior to sulfate reduction for improving PRZ performance.

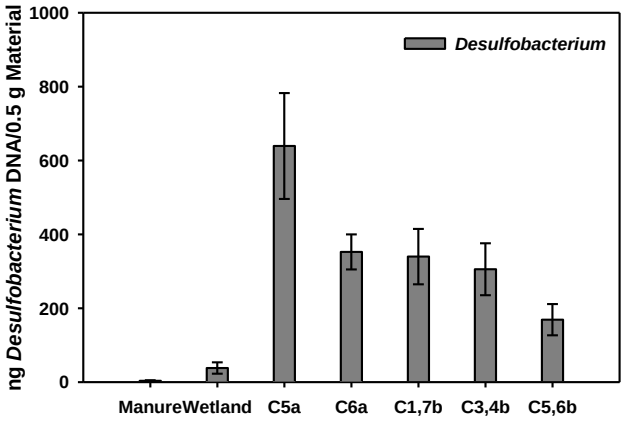

Figure 4: Real-time PCR quantification of *Desulfobacterium* in the columns and their inocula plotted as average and standard deviation of 6 replicates. There are approximately 7.14 x 10^8 *Desulfobacterium* cells per ng of DNA.

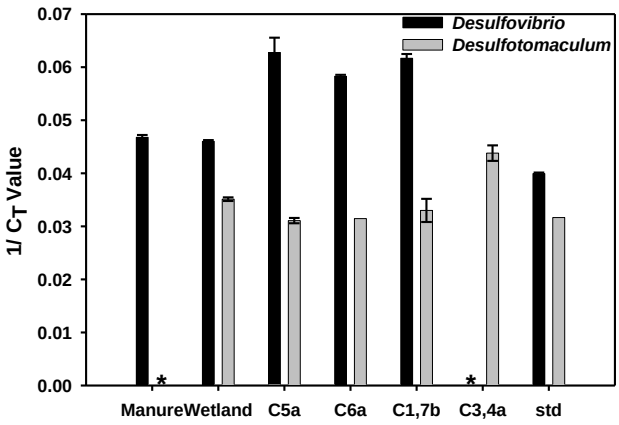

Figure 5: Real-time PCR relative quantification of *Desulfovibrio* and *Desulfotomaculum* in the columns and their inocula. Higher inverse C_T values indicate higher concentrations of target DNA. The standards (std) ωερε 10 νγ/μL. * indicates sample not analyzed.

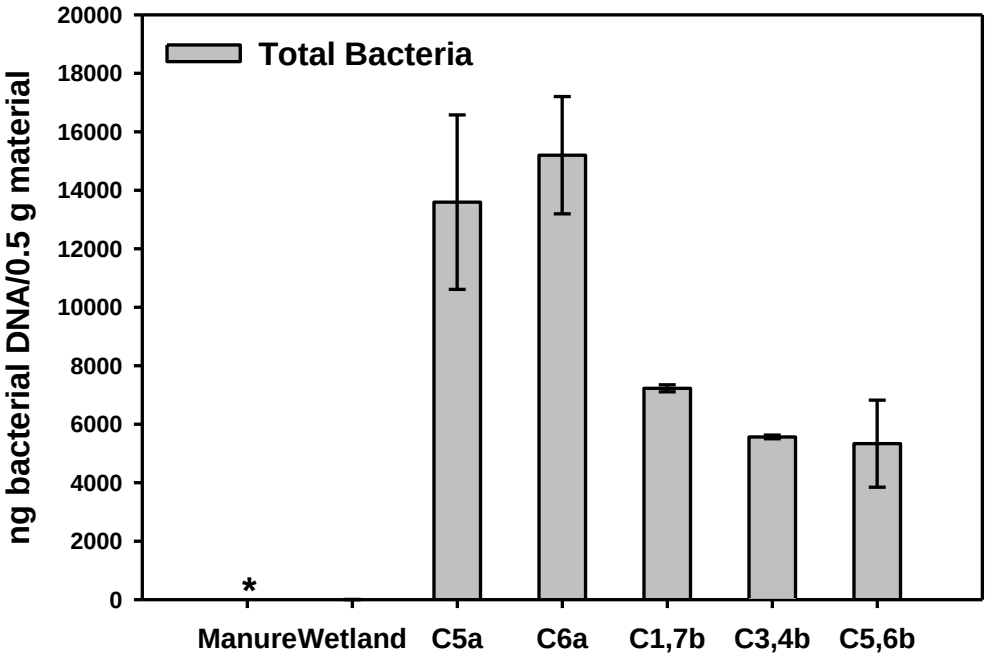

Figure. 6: Real-time PCR (Q-PCR) quantification of total bacteria in columns expressed as ng/0.5 g column material.
* indicates sample not analyzed.

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