# MICROBIAL ACTIVITY IN THE PEERLESS JENNY KING SULFATE REDUCING BIOREACTOR SYSTEM.[1]

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**Abstract:** The Peerless Jenny King treatment system is a series of four sulfate reducing bioreactor cells installed to treat acid mine drainage in the Upper Tenmile Creek Superfund Site located in the Rimini Mining District, near Helena MT. The system consists of a wetland pretreatment followed by the four cells connected in a serpentine manner. The mining impacted water flows from the wetland through each cell before discharge. Sulfate reducing bioreactors mitigate acidity and metal contamination through the microbial production of sulfide. The produced biogenic sulfide precipitates metals, and the microbial process of reducing sulfate to sulfide produces alkalinity.

The health of the entire microbial community present in such systems is important for remediation to be effective. Classes of microbes generally present in such systems include fermenters, methanogens and sulfate reducers. The health can be measured in terms of active microbial populations and positive interactions between populations for the support of sulfate reduction. The goal of this research is to measure the activity of each class utilizing analyses that quantify the groups by their function, as opposed to the traditional molecular techniques of identifying bacteria. Gas chromatography, HPLC- DAD, and ICP-AES are used to identify and quantify the end products of metabolism. The microbial activity can then be characterized and changes can be monitored over time. Results from 2005 sampling of Cell 3 within the system indicate that the activity of sulfate reducing bacteria is much higher than the numbers present would indicate. These results combined with those from 2006 sampling indicate that methanogenesis is a minor process within this cell. The calculation of the stoichiometry of carbon utilization by SRB is much higher than what would be predicted from known stoichiometric ratios of carbon used per sulfate reduced.

**Additional Keywords:** acid mine drainage, microbial populations, stoichiometry

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[1] Paper was presented at the 2007 National Meeting of the American Society of Mining and Reclamation, Gillette, WY, 30 Years of SMCRA and Beyond June 2-7, 2007. R.I. Barnhisel (Ed.) Published by ASMR, 3134 Montavesta Rd., Lexington, KY 40502.

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Proceedings America Society of Mining and Reclamation, 2007 pp 109-122
DOI: 10.21000/JASMR07010109
http://dx.doi.org/10.21000/JASMR07010109

## Introduction

Throughout the United States, acid mine drainage (AMD) generation is a serious environmental issue. Coal mining is the primary cause of AMD in the eastern states, while hardrock mining is a significant cause in the western states (Cohen, 2006). The silver and gold rushes of the nineteenth century left a legacy of abandoned mines across the western U.S. with 40% of the water bodies in the region impacted by AMD, the majority from abandoned mines (Center for the American West, 2006). Acid mine drainage is characterized by net acidic water, with elevated concentrations of dissolved metals and high levels of sulfate (Johnson and Hallberg, 2005). This AMD not only impacts the native species that inhabit the aquifer, but also may impact human health and activities and thus restrict water usage.

Passive treatment options are often more appealing than active treatments because they are relatively less expensive to install, and require little maintenance. Since many of the sites are remote, and without access to power, active treatment is not a viable option. Biotic passive treatment systems rely on microbial processes to remediate acidity and dissolved metals, and include aerobic and anaerobic wetlands and bioreactors (Cohen, 2006). Sulfate reducing bioreactors (SRBRs) rely on the microbially mediated reduction of sulfate to sulfide. This process generates alkalinity and the biogenic sulfide can precipitate dissolved metals as highly insoluble solids (Cabrera et al., 2006; Kaksonen et al., 2006).

Sulfate reducing bioreactors rely primarily on the metabolism of sulfate reducing bacteria (SRB) for the process of metal precipitation; however these systems are populated by an entire community of microorganisms that coexist and interact. Much work has been done in describing the microbial communities responsible for the generation of acid mine drainage (Johnson and Hallberg, 2003; Benner et al., 2000; Baker and Banfield, 2003). The role of SRB in the remediation process has also been well elucidated (Chang et. al, 2000; Christensen et al., 1996; Dvorak et al., 1992). Relatively few studies have been done, however, in characterizing the microbial community present in SRBRs (Hallberg and Johnson, 2005; Pruden et al., 2006), and specifically the active microbial populations. The purpose of this research was to measure the microbial activity of the PJK system, through quantification of the microbial end products of metabolism. Three major classes of microbes were focused upon: fermenters, sulfate reducing bacteria, and methanogens. A comparison of the activity for two times points over a period of fourteen months was conducted for Cell 3 in the system.

## Materials and Methods

### Sample Collection

The Peerless Jenny King (PJK) treatment system is an example of a SRBR installed to treat mining impacted waters. The system was installed in 2003 within the Ten Mile Creek Basin, in the Rimini Mining District, near Helena, Montana. The watershed is utilized by the City of Helena for drinking water. The site contains a number of abandoned and inactive hardrock mines that previously produced Pb, Cu, Au, and Zn (EPA Fact Sheet, 2006). The mining impacted water exits through an adit and is directed first into an upper wetland pretreatment system, followed by a rock channel. The flow enters a series of sulfate reducing cells before discharge (Fig. 1). The water saturation of the cells varied by location; Cell 1 where the water enters the system is fully saturated, whereas Cell 4 may experience dry periods.

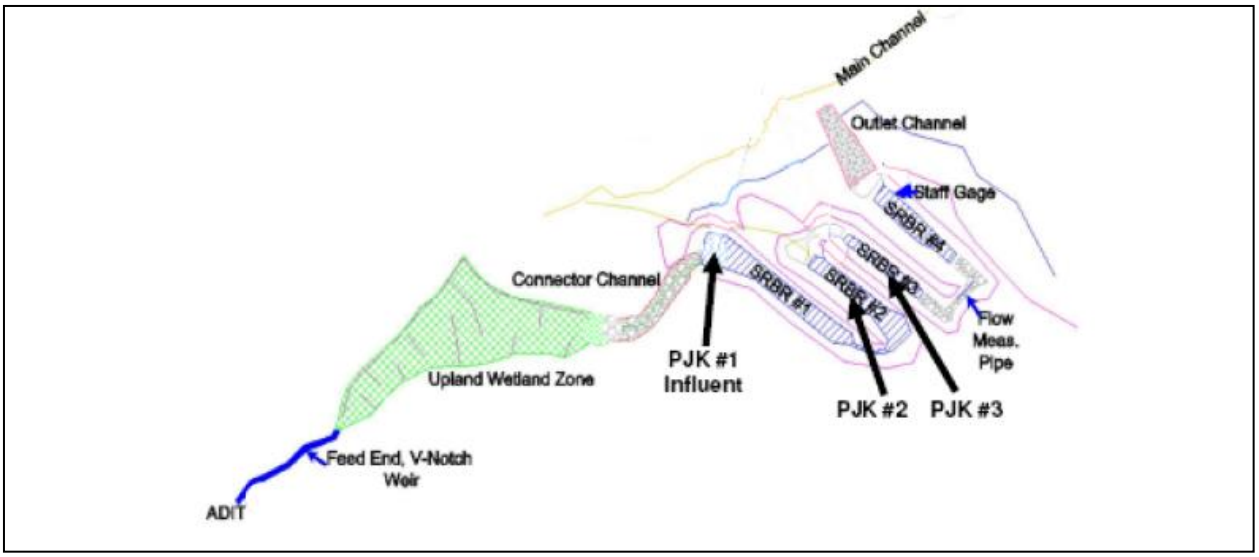


Figure 1: A schematic of the PJK SRBR treatment system (Pruden et al., 2006).

The Peerless Jenny King system was sampled in June of 2005. At this time, a snow event had occurred the day previous to sampling. Substrate sampling was achieved by hammering a sampling plate into the cell and holes chiseled into the plate allowed for sampling at depth (Fig. 2). The process was conducted in air, but the samples were placed in sterile Whirlpak bags, and sealed with a commercial Seal-A-Meal® to minimize further exposure to oxygen. The samples were kept on ice for return to the lab, and kept at 4° C for a 7 day period before the batch experiments were performed. Within the PJK system, Cell 1 was sampled at the inflow and within the middle of the cell. The remaining cells were all sampled in the middle of the cell, and samples were taken from the top and bottom of the sampling hole at that location. The results only for Cell 3 will be presented here.

The Peerless Jenny King system was sampled again in August of 2006, following the same protocol as listed above. By August, it was assumed the system was in a state of quasi-equilibrium, after the snow melt had subsided. Two months prior to sampling, the cells were covered with a fresh layer of wood chips. This extra layer was then called the "new top". Also it was decided at that time, that the front/influent of Cell 1 would not be sampled for microbial activity batch experiments, as the water was aerated as it entered, and anaerobic microbial activity would be inhibited.

Batch experiments

Batch experiments were conducted in effort to assess the microbial activity of the field site substrate. The batch experiments were carried out with adaptations to those used for assessment of bench scale treatment systems (Logan et al., 2005). Four of the substrate samples taken from the SRBR cells were chosen for carbon-substrate supplemented, detailed batch studies, while the remaining substrate samples were not supplemented for batch studies. The substrate representing the front and middle of Cell 1, bottom; and the middle of Cell 3, top and bottom, was chosen for the supplemented experiment. The experiments were conducted in duplicate, with a total of eight serum bottles per sample. Approximately 5 grams of wet weight substrate was measured and placed in 160 mL sterile serum bottles. The substrate was heterogeneous,

composed of large pieces of wood chips in varying degrees of decay, manure, and hay, as well as pieces of vegetation that had taken root in the bioreactors. The material for the batch experiments was limited to particles that would fit through the mouth of the serum bottle, which is approximately 12mm in diameter. To support the sulfate reducing bacterial population, a sterile 1000 mg/L sulfate solution was added, making the total liquid volume 80 mL. The serum bottles were sealed with a thick rubber septum, and crimped with an aluminum seal. The headspace was purged for 35 minutes with argon, to ensure an anaerobic environment. Bottles were kept static in the dark for the duration of the experiment.

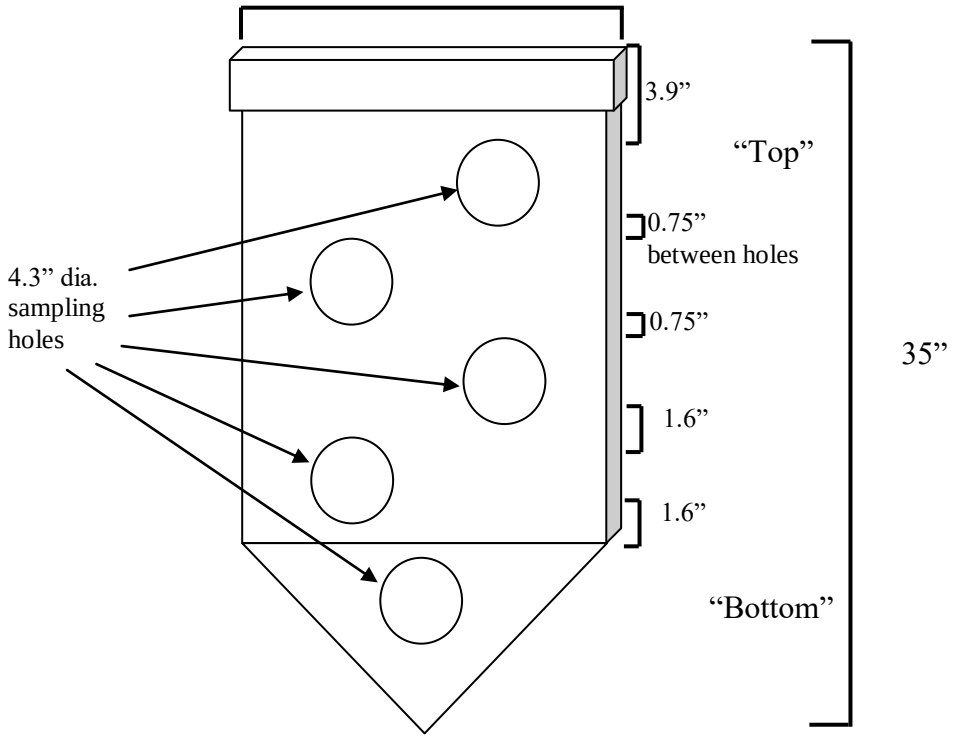


Figure 2. Illustration of the sampling plate used for substrate sampling.

In the 2005 experiment, supplements of glucose (Aldrich α-D anhydrous glucose), lactate (Sigma DL-lactic acid, 60% w/w), and acetate (Fisher anhydrous sodium acetate) were added to the serum bottles to achieve a 2mM concentration. The supplements were chosen to extract particular information about the microbial populations, and were expected to be preferred carbon sources for fermentative, sulfate reducing, and methanogenic populations, respectively. Duplicates without a supplement were run in parallel to serve as a control. A schematic of the experimental design is shown in Figure 3.

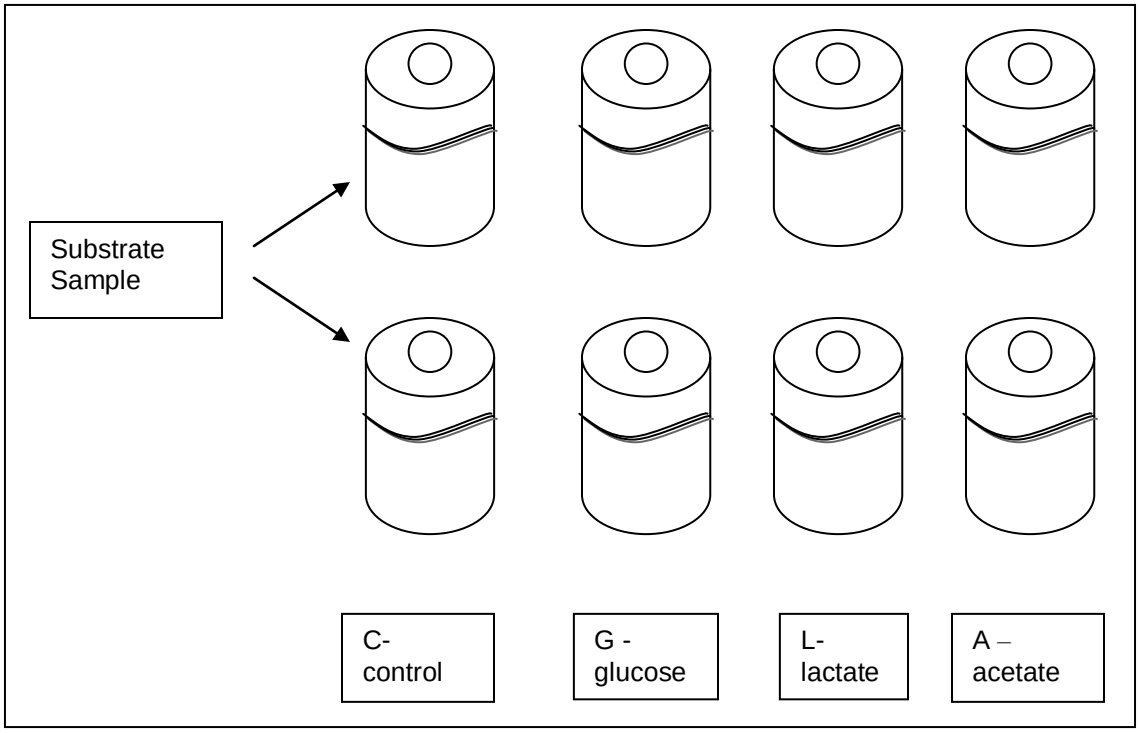


Figure 3. Illustration of the experimental supplemented batch studies.

The supplemented batch experiment for the August 2006 samples was modified slightly, in part because of the results from the 2005 experiment. Again, one set of control duplicates was run in parallel to three supplemented sets of serum bottles with the substrate from four sample locations within the PJK system. The supplements were changed to reflect what was inferred from prior results. Glucose was still added as a 2mM solution. Another supplement investigated was 2mM glucose plus N and P composed at a 10:1:0.2 mass ratio of C:N:P, where 14.4 mg/L of N as $NH_4Cl$ and 2.88 mg/L of P as $K_2HPO_4$ were added with the glucose supplement. The mass ratio was derived from the stoichiometric requirement of nitrogen per mole of carbon utilized for aerobic heterotrophs, while the P requirement was estimated at 1/5 of the N requirement (Grady and Lim, 1980). The final supplemental variation was an addition of mixed organic acids, to investigate whether it was a fermentation product other than acetate that was being utilized by SRB. A mixture of lactate, butyrate, and propionate was added as a supplement such that the total concentration of carbon was 12mM, in order to make a comparison to the molar carbon concentration of glucose. The reactor vessels were also modified to better accommodate the particle sizes of the substrate. Sterilized 240 mL nominal Mason jars were used; the measured liquid volume the jars held was 231 mL. The volume of sulfate solution added was adjusted such that the total volume of solution was 100 mL, to create a comparable headspace to previous experiments. The lid to the jar was punched with a 12 mm hole for the rubber septum to be inserted. The lids were lightly sanded and the septa were sealed with all-purpose epoxy sealant applied to the lid.

Headspace Analysis

At a time point between three to fours weeks into the experiment, the headspace gas was sampled and analyzed for composition on an Agilent Micro GC, with a thermal conductivity

detector (TCD), using helium as the carrier gas. Methane, carbon dioxide, and hydrogen sulfide gas standards were used to create calibration curves. Detection limits for the standard gases are 0.15% $CO_2$; 0.01% $H_2S$ and $H_2$; and 0.03% for $CH_4$. Hydrogen gas was analyzed; however it was never detected in the headspace samples. The gases were sampled with a sterile 3 mL syringe fitted with a LuerLok valve and a sterile 21G1 needle. The gas within the syringe was assumed to be at atmospheric pressure, which in Golden CO is 0.8 atm. The gas was introduced to the instrument columns by a vacuum. The gas sample is split and run through parallel columns, A and B, within the instrument. Column B, a PoraPlot U column, was used for identification of the three gases being analyzed.

Solution Analysis

At the same time point of headspace sampling, the aqueous solution was also sampled for analysis of organic acids and sulfur. One milliliter of solution was taken for HPLC analysis and centrifuged at 14,000 G for 10 minutes. The supernatant was transferred to a new vial and frozen for later HPLC analysis of organic acids. The HPLC analysis was conducted with an Agilent HPLC fitted with a Biorad Aminex HPX-87H analytical column, and a diode array detector set at 210 nm for detection. The eluent was a 0.003N $H_2SO_4$ solution prepared with HPLC grade water. A set of organic acid standards were created for common microbial fermentation organic acid products: lactate, acetate, butyrate, formate, propionate, and succinate.

Another milliliter of solution was taken and introduced to 100 µL of 0.1M zinc acetate (Spear et al., 1999; Machemer et al., 1993). The zinc acetate is known to precipitate out dissolved sulfide. The solution was also centrifuged at 14,000 G for 10 minutes, and the supernatant was taken and stored frozen until ICP analysis. The ICP instrument was a Perkin Elmer 3000, with a scandium internal standard. The limit of detection for sulfur for this instrument is 0.05 mg/L. The results of the analysis indicated the sulfate remaining after microbial sulfate reduction, without the presence of any dissolved sulfide species.

## Results and Discussion

The cells in the PJK system vary in water saturation levels with location and depth. The first two cells are saturated year round, while the third cell is less saturated than the first two, and the last cell periodically becomes dry. For comparison, the results for Cell 3 only will be discussed. Within the following figures, the control is indicated by "C", while glucose, lactate, and acetate supplements are indicated, respectively by "G", "L", and "A", and each replicate is represented. The flow for this system is horizontal, and therefore the saturated bottom portions of the cells are anaerobic, while the top, especially for Cells 3 and 4 can become aerobic. Accordingly, methanogenic activity would be expected in the bottom samples of the cells. The headspace analysis results in 2005, however, indicated the top of Cell 3 produced methane gas, while the bottom of Cell 3 did not (Fig. 4 and 5).

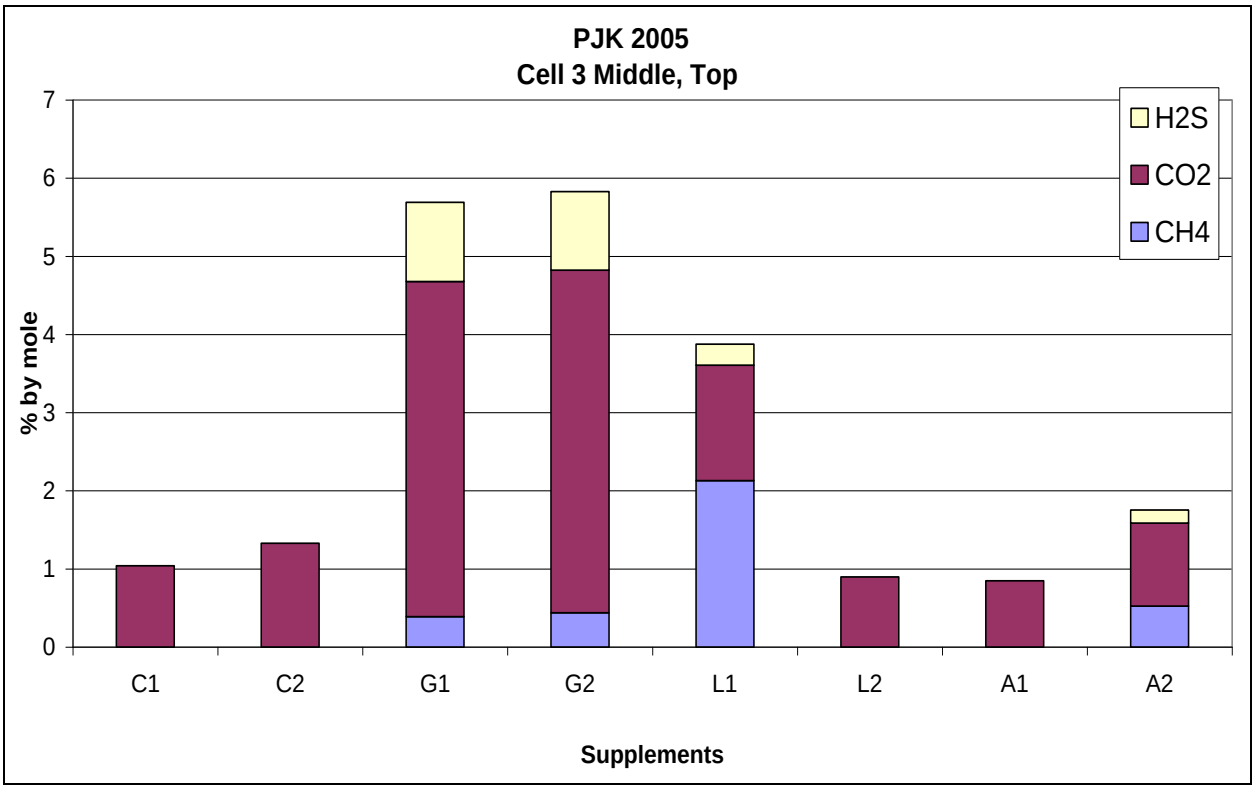


Figure 4. Headspace composition for the 2005 top of the middle sample location of Cell 3.

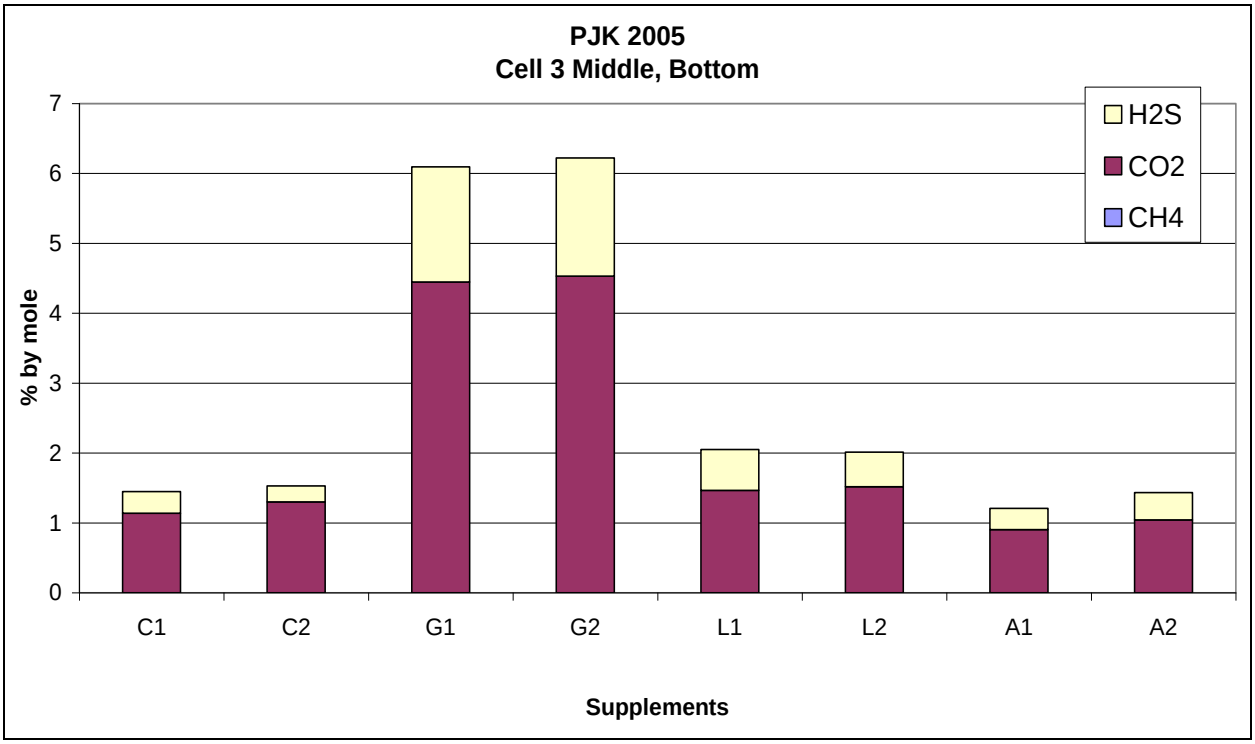


Figure 5. Headspace composition for the 2005 bottom of the middle sample location of Cell 3.

The addition of glucose appeared to stimulate $CO_2$ production as well as $H_2S$ production over that of the control, and the other supplements. First of all, this indicated that the fermentative population may have been carbon limited. Secondly, it may be inferred that it is an organic acid fermentation product other than lactate or acetate that was the preferred carbon source for this population of sulfate reducing bacteria. Glucose is not used directly by SRB (Faque, 1995). Finally, the lack of methane production coupled with an increase in $H_2S$ production of the bottom of this sample location suggested that SRB are not in competition with methanogens for carbon sources.

A solution phase sample was obtained at the same time point as the headspace analysis for organic acids which would be products of microbial metabolism, or would indicate supplement that was not utilized. As shown in Fig. 6, when a suite of organic acids were analyzed for, only acetate was detected in solution. Very little soluble carbon was present at this time point, suggesting that the microbial population was carbon limited, and utilized whatever labile carbon that was available.

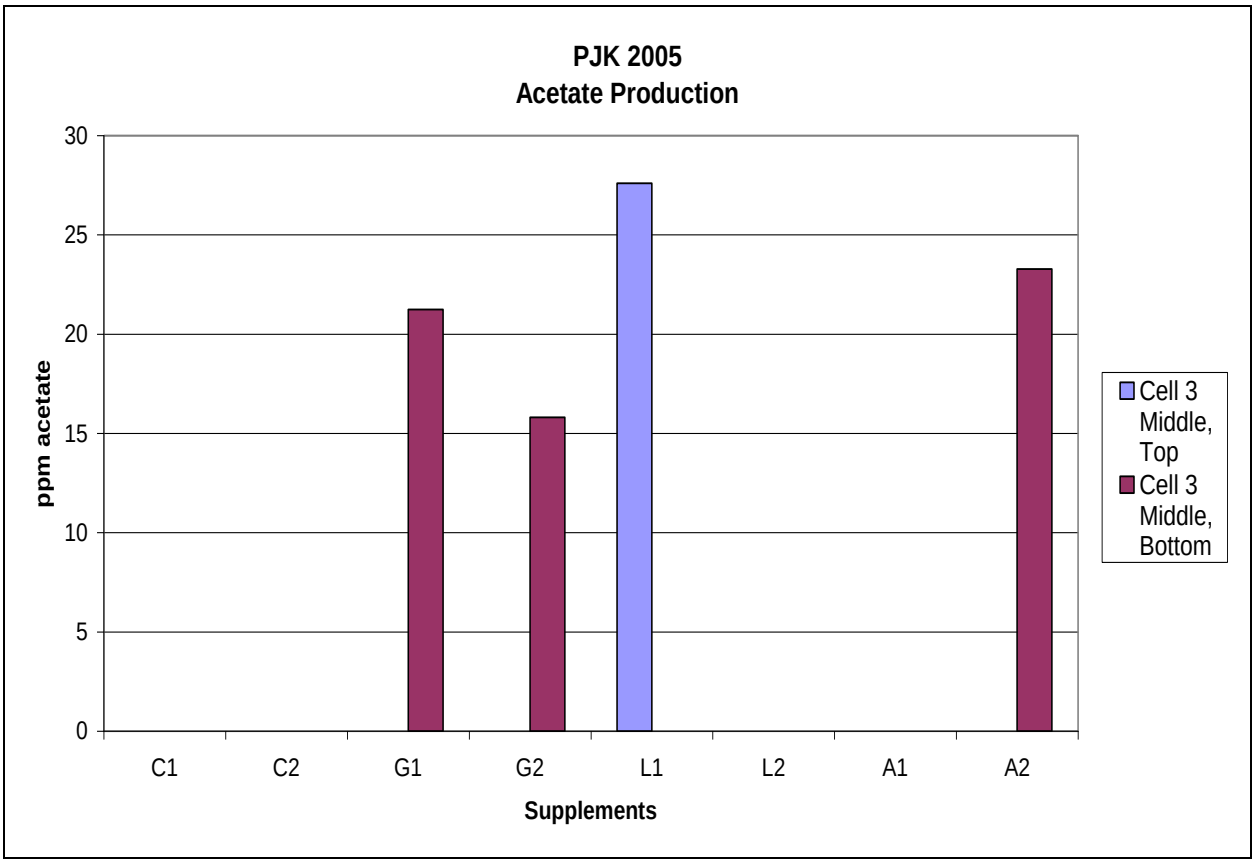


Figure 6. Organic acid production comparison for the 2005 top and bottom of the middle sample location for Cell 3.

The sulfate removal was comparable between the top and bottom of the middle sample location (Fig. 7). It is clear, however, that the glucose supplement best supported sulfate

removal, in comparison to the other supplements. Glucose is not a carbon source that is directly utilized by SRB, thus indicating that a healthy, synergistic microbial community is required for the success of SRBRs.

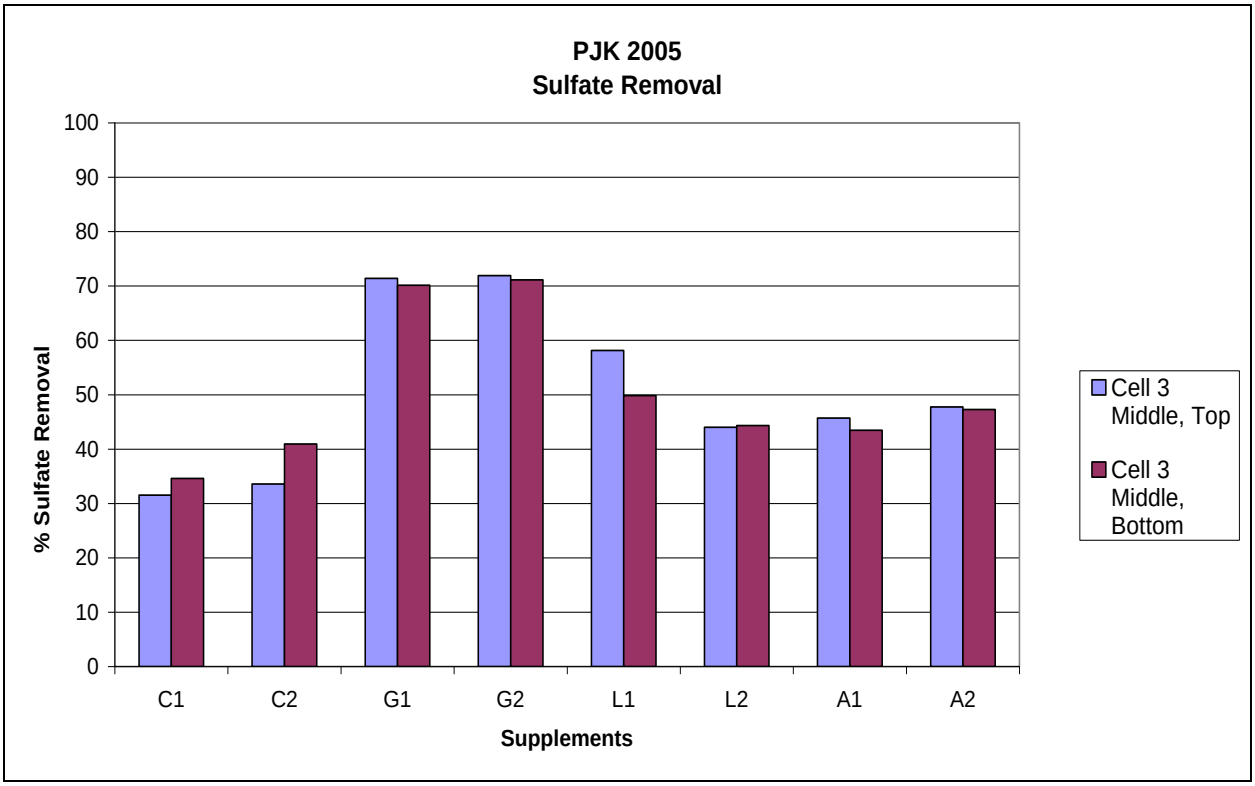


Figure 7. Percent sulfate removal comparison for the 2005 top and bottom of the middle sample location of Cell 3.

The experiments were repeated a year later, and again the results for the top and bottom of the middle sample location of Cell 3, in 2006, are shown for comparison (Fig. 8 and 9). The supplements were modified, and are symbolized in the following figures as "G" for glucose, "GN" for glucose with added nutrients of nitrogen and phosphorus, and "MO" for the supplement of mixed organic acids. Again, methanogenic activity was detected in the top of the sample location, and also at the bottom of the cell. Sulfate reduction was enhanced at the top of the cell by added nutrients, and the sulfate reducing bacteria appeared to out-compete the methanogenic population at the bottom of the cell when the carbon source was a mixture of readily utilizable organic acids. $H_2S$ production was increased and $CH_4$ production was repressed with the addition of N and P, over that of the control or glucose alone.

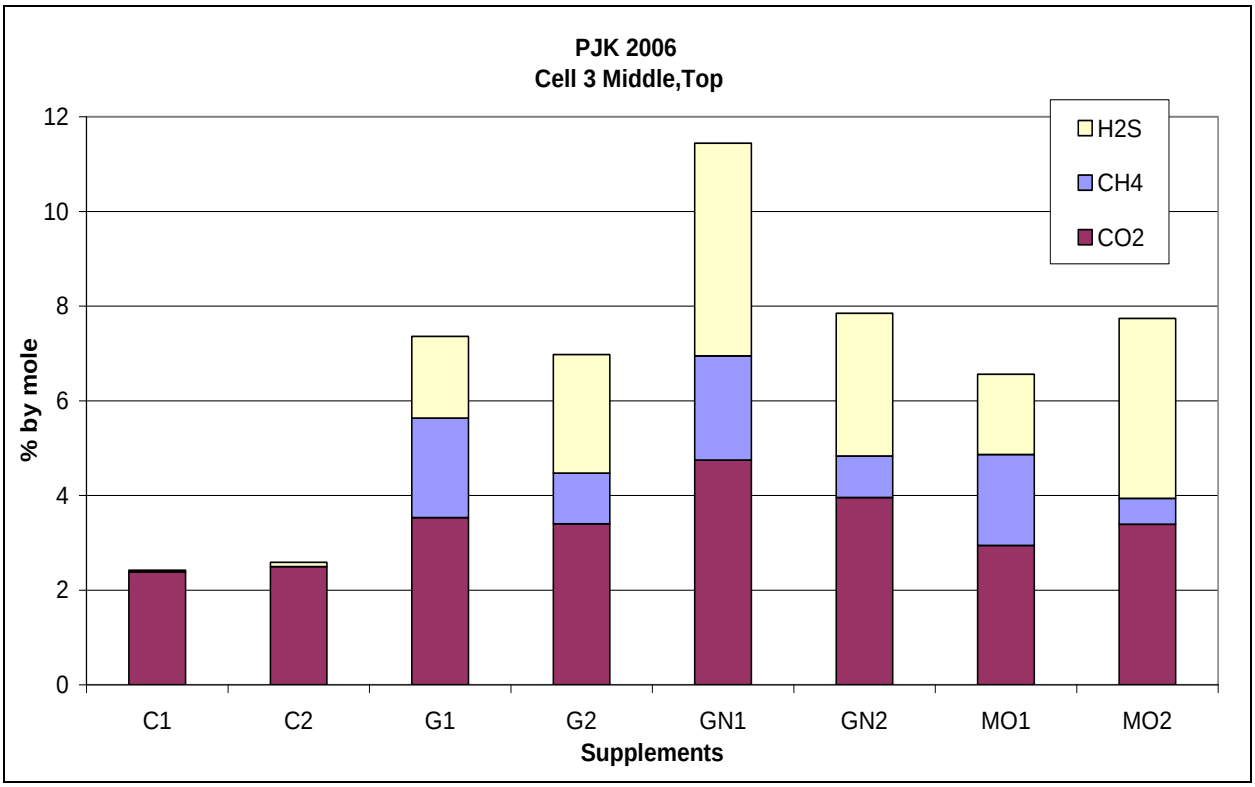


Figure 8. Headspace analysis for the 2006 top of the middle sample location of Cell 3.

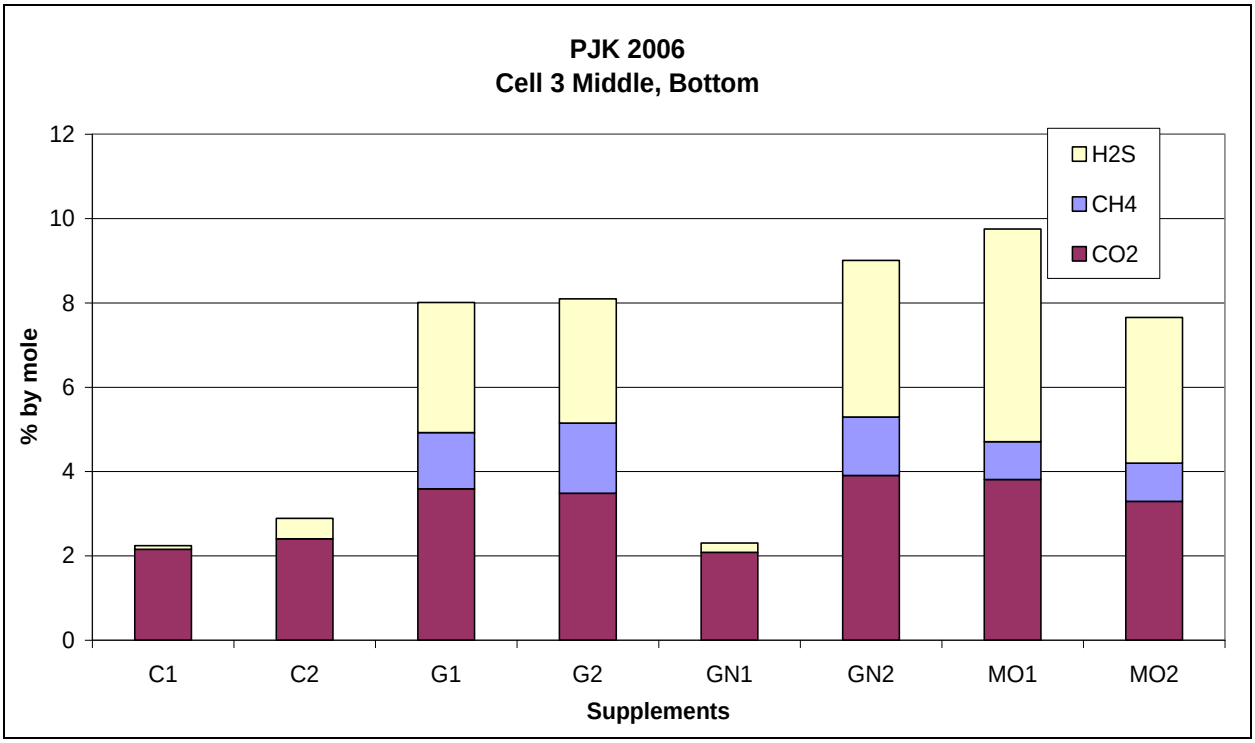


Figure 9. Headspace analysis of the 2006 bottom of the middle sample location of Cell 3.

Figure 10 shows a comparison of the acetate produced for the top and bottom of the middle sample location of Cell 3. Again, the only organic acid detected was acetate, and it was only detected for the mixed organic supplemented bottles. This suggested that the population was still carbon limited, despite the addition of the new wood chips to the top of the cells. The presence of acetate, at a higher concentration than the previous year's (2005) experiment, only in the mixed organic acid bottles possibly suggested a population of incomplete oxidizing SRB active in the cell at during this time.

The percent sulfate removal (Fig. 11), was similar for each type of supplement. The total amount of carbon added as a supplement was 12 mM total C for each supplement, which is reflected in these results.

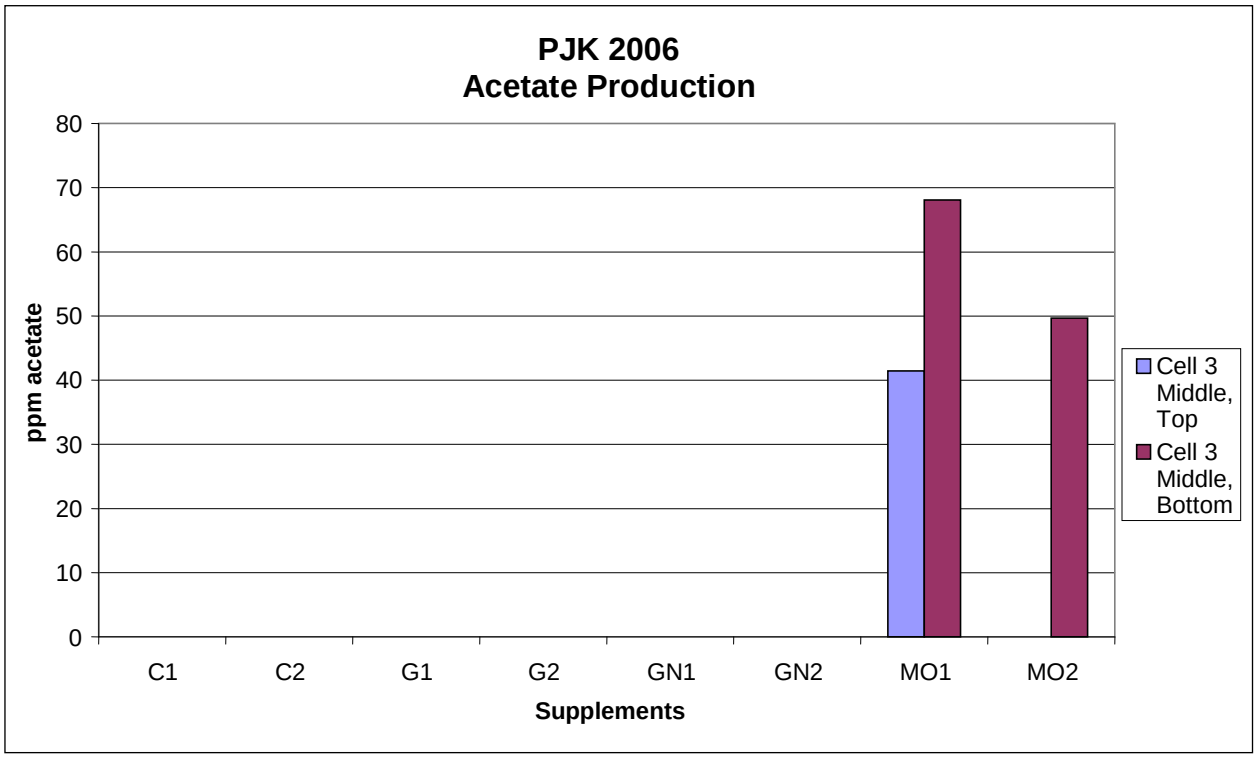


Figure 10. A comparison of the 2006 organic acid production for the top and bottom of the middle sample location of Cell 3.

The quantification of the microbial metabolic products coupled with the known quantity of carbon added allows for the calculation of the stoichiometry for the microbial system active within each serum bottle. A summary of these calculations are presented for the top and bottom of Cell 3, for both the 2005 and 2006 experiments (Fig. 12). The calculations indicate the fraction of carbon utilized for the processes of sulfate reduction, methane production, or other microbial processes.

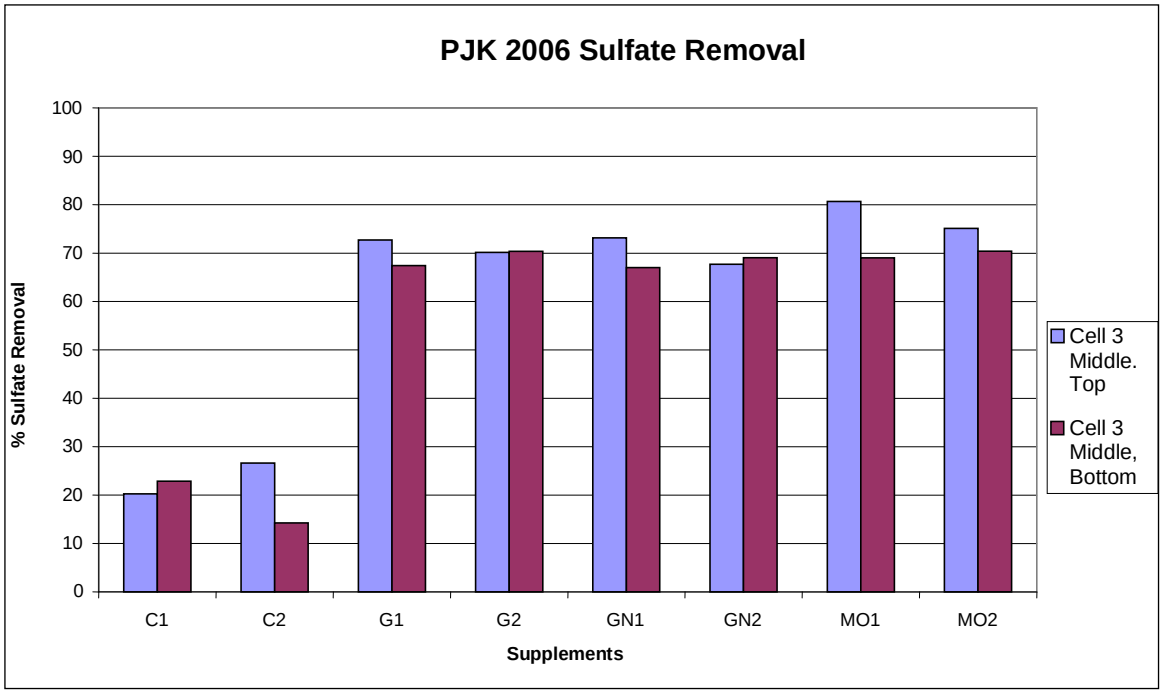


Figure 11. A comparison of the 2006 percent sulfate removal for the top and bottom of the middle sample location of Cell 3.

| PJK 2005 | Carbon fraction for CH4 | Carbon fraction for sulfur removal | Carbon fraction for other pro-cesses | PJK 2006 | Carbon fraction for CH4 | Carbon fraction for S reduced | Carbon fraction for other pro-cesses |
|---|---|---|---|---|---|---|---|
| Cell 3 Middle, Top | | | | Cell 3 Middle, Top | | | |
| G1 | 0.021 | 0.65 | 0.32 | G1 | 0.15 | 0.73 | 0.12 |
| G2 | 0.024 | 0.66 | 0.31 | G2 | 0.076 | 0.69 | 0.24 |
| L1 | 0.52 | 1.9 | -1.5 | GN1 | 0.16 | 0.74 | 0.11 |
| L2 | 0 | 0.64 | 0.36 | GN2 | 0.063 | 0.65 | 0.29 |
| A1 | 0 | 0.67 | 0.33 | MO1 | 0.16 | 1.1 | -0.26 |
| A2 | 0.085 | 0.77 | 0.15 | MO2 | 0.039 | 0.88 | 0.077 |
| Cell 3 Middle, Bottom | | | | Cell 3 Middle, Bottom | | | |
| G1 | 0 | 0.58 | 0.42 | G1 | 0.095 | 0.73 | 0.17 |
| G2 | 0 | 0.59 | 0.41 | G2 | 0.12 | 0.78 | 0.10 |
| L1 | 0 | 0.68 | 0.32 | GN1 | 0 | 0.72 | 0.28 |
| L2 | 0 | 0.37 | 0.63 | GN2 | 0.099 | 0.76 | 0.14 |
| A1 | 0 | 0.29 | 0.71 | MO1 | 0.079 | 1.1 | -0.15 |
| A2 | 0 | 0.60 | 0.40 | MO2 | 0.075 | 1.0 | -0.11 |

Figure 12. Representation of the fraction of carbon utilized for the processes of sulfate reduction, methane production or other microbial processes for Cell 3 top and bottom compared between 2005 and 2006 experiments. (Negative values indicate that solid substrate carbon was utilized as well as the labile supplemental carbon.)

The significance of these calculations is the implication that sulfate reducing bacteria were utilizing a large fraction of the available carbon. Very little of the available carbon was utilized for methanogenesis, implying that little competition existed between methanogens and SRB in this system. From the results, it may also be inferred that acetate is a more efficient carbon source for SRB, in that a larger fraction of carbon could be utilized for sulfate reduction. The addition of nitrogen and phosphorus also enhanced percent removal of sulfate. This process of determining the system stoichiometry is a first step in elucidating the carbon flow within SRBRs.

## Conclusions

Measurement of the microbial activity is an important contribution to the characterization of the microbial ecology present in sulfate reducing bioreactors. Understanding the microbial community and their active functions may lead to a better design and implementation of these systems. A more complete description of the microbial community can be accomplished with microbial activity measurements in conjunction with DNA analysis to identify the microbes present; this work is being conducted by Amy Pruden and her research group at Colorado State University.

The results suggest that the microbial activity is enhanced with a glucose supplement. Increased total gas production was present, including increased hydrogen sulfide production, which may suggest a limitation of free labile carbon in the PJK system. It also indicates that a healthy synergistic functioning community is necessary for optimal activity of the sulfate reducing population. Studies by Pruden et al. (unpublished report) show that SRB are only a small fraction of the total population, numerically, yet the activity measurements indicate that they have a high capacity for the sulfate reduction function. While methanogens are present in the system, this work suggests that under optimal conditions of readily utilizable organic acids, and with sufficient nutrients, sulfate reducers are able to out-compete them for the carbon source. Future work will focus on an effort to elucidate the carbon flow in the system.

## Acknowledgements

This project was funded by the U.S. EPA Office of Research and Development, Engineering Technical Support Center, National Risk Management Research Laboratory, Cincinnati, OH under contract number GS-10F-0130N, through Golder and Associates. Dave Reisman served as the project manager, and he also provided access to the sites, guidance and support. Also, we would like to acknowledge Amy Pruden and her research group at Colorado State University, who are collaborators on this research.

Disclaimer: "The United States Environmental Protection Agency (EPA) through its Office of Research and Development funded and collaborated in this research. It has not been peer reviewed by the EPA and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use."

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