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The effect of sulfide inhibition and organic shock loading on anaerobic biofilm reactors treating a low-temperature, high-sulfate wastewater

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THE EFFECT OF SULFIDE INHIBITION AND ORGANIC SHOCK LOADING ON ANAEROBIC BIOFILM REACTORS TREATING A LOW-TEMPERATURE, HIGH-SULFATE WASTEWATER

by

Heather Brown McDonald

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Civil and Environmental Engineering
in the Graduate College of
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Thesis Supervisor: Professor Gene F. Parkin

ABSTRACT

In order to assess the long-term treatment of sulfate- and carbon- rich wastewater at low temperatures, three anaerobic biofilm reactors were operated at 20°C, a hydraulic retention time (HRT)of two days and fed a synthetic wastewater containing lactate and sulfate. The reactors were operated for over 900 days. DNA was extracted from the reactors around days 180 and 800. Three clone libraries, methanogenic archaea (MA), sulfate reducing bacteria (SRB), and bacteria, were constructed and quantitative PCR analysis was performed with the DNA. It was found that anaerobic biofilm reactors can be operated at 20°C with an organic load rate (OLR) of 1.3 g-chemical oxygen demand (COD)/L-day or less and an sulfur load rate (SLR) of 0.2 g-S/L-day with no significant deterioration in process performance. With long acclimation periods, OLR as high as 3.4 g COD/L-d and SLR of 0.3 g/L-d can be tolerated, producing effluent volatile-acid COD levels consistently less than 200 mg/L. Effluent dissolved sulfide and hydrogen sulfide levels were around 600 mg S/L and 150 mg S/L, respectively, during this period. In addition to long term operation, the effect of organic shock loading was assessed. The reactors were able to recover from one but not two lactate spikes of approximately 5,000 mg COD/L. It was determined that long-term stability could be achieved in reactors that contained well balanced, stable populations of lactate- and propionate-degrading SRB and aceticlastic methanogens. Significant populations of fermenters present resulted in an imbalance which caused lactate to be routed through an additional pathway where propionate was formed. Greater numbers of MA than bacteria were found in all reactors. This may be attributed to the availability of acetate in the reactors for MA consumption and to using the immobilized fixed bed reactor type. Aceticlastic methanogens were the dominant methanogen, and were observed to remove nearly all acetate produced in all reactors. SRB were observed to remove lactate in microbially balanced reactors, whereas fermenters degraded lactate in reactors with less balanced populations.

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CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

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LIST OF ABBREVIATIONS

aMA	aceticlastic methanogenic archaea
ARC	primer set or primer/probe set for archaea
BAC	primer set or primer/probe set for bacteria
COD	chemical oxygen demand
cSRB	complete-oxidizing sulfate reducing bacteria
Ct	threshold cycle
DS	dissolved sulfide
dsDNA	double-stranded DNA
EPT	external positive control
H_2S	free hydrogen sulfide
hMA	hydrogenotrophic methanogenic archaea
HPLC	high pressure liquid chromatography
HRT	hydraulic retention time
IC	ion chromatograph
IPC	internal positive control
iSRB	incomplete-oxidizing sulfate reducing bacteria
LEC	lactate enrichment culture
LH-PCR	length heterogeneity polymerase chain reaction
MA	methanogenic archaea
MBT	*Methanobacteriales*
MCC	*Methanococcales*
MMB	*Methanomicrobiales*
MPN	most probable number
MSL	*Methanosarcinales*
OLR	organic load rate
PCR	polymerase chain reaction
qPCR	quantative polymerase chain reaction
rDNA	ribosomal DNA
SEC	sulfate enrichment culture
SEM	scanning electron microscope
SLR	sulfate load rate
SRB	sulfate reducing bacteria
TRFLP	terminal restriction fragment length polymorphism
UASB	upflow anaerobic suspended bed reactor
VFA	volatile fatty acids
VA-COD	volatile acid chemical oxygen demand

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1 Background

Many industries, including fermentation, food processing, and paper mills, produce wastewater high in organic and sulfate content. Anaerobic biological treatment of these wastewaters can result in the formation of methane by methanogenic archaea (MA) and sulfide as sulfate reducing bacteria (SRB) reduce the sulfate to sulfide. Recovery of the energy content in the form of methane is advantageous, as is recovery or removal of the sulfur if sulfide is formed (Khanal and Huang 2003; Martin et al. 2002). Unfortunately, sulfide can inhibit the activity of both SRB and MA; both unionized hydrogen sulfide (H_2S) and total dissolved sulfide ($DS = H_2S + HS^- + S^{-2}$) are inhibitory.

Previous research has shown that biofilm processes can withstand higher levels of sulfide (up to 200 mg-H_2S-S/L and 1,000 mg-DS/L), while suspended growth systems showed deteriorating performance at much lower levels (60 mg-H_2S-S/L and 150 mg-DS/L) (Maillacheruvu et al. 1993). The sulfide tolerance of biofilm reactors is not well understood, but could be due to superior attachment properties of the more sensitive MA (Isa et al. 1986a), or perhaps the MA are located deeper in the biofilm and protected from high levels of H_2S and DS. There have been no systematic studies investigating the composition of biofilms developed when treating wastewaters containing sulfate. Perhaps a better understanding of the composition of SRB and MA in the reactor over time will yield clues to this advantage.

The general objective of this work is to develop a better understanding of how the consortium of microorganisms in an anaerobic biofilm reactor develops, acclimates, and

adapts to sulfide inhibition caused when treating high sulfate wastewaters. If sulfide inhibition can be better understood and controlled, wastewaters with high organic and sulfate contents can be more effectively treated. Laboratory column reactors were seeded with microbial enrichment cultures containing active MA and SRB. These columns were fed a sulfate-containing wastewater to ascertain the effect of sulfide inhibition. Several molecular techniques were used to assess the effect of column operation on the development of the microbial community (e.g., location and type of *Bacteria* or *Archaea*) within each reactor.

1.2 Hypotheses

There are two general hypotheses that were tested during this research.

1.2.1 Hypothesis 1

It is possible to develop an anaerobic biofilm reactor capable of treating a low-temperature sulfate-rich wastewater with hydrogen sulfide levels of at least 200 mg-S/L and dissolved sulfide levels of 1,000 mg-S/L.

Biofilm reactors using a plug-flow regime were constructed and inoculated with organisms from stock enrichment cultures. The reactors were fed sulfate rich synthetic wastewater using lactate as the carbon source. Sulfate and lactate concentrations in the feed stream were gradually increased and the operational responses of the reactors were monitored using parameters such as pH, volatile fatty acids, sulfate, and sulfide. Also investigated was the reactor performances under varying chemical oxygen demand

(COD) loads, sulfate loads, COD/S ratios, organic shock loading conditions, and sulfide levels.

1.2.2 Hypothesis 2

The microorganism populations located in the reactor will shift over time.

At startup, the organisms begin to establish themselves within the reactor and shifts of the different groups of MA and SRB will be observed until a steady state is reached. A change in the reactor conditions, such as sulfate load, COD load, or hydraulic retention time (HRT) will also affect the organism populations. Increasing sulfate concentrations in the influent will cause an increase in the relative quantity of SRB and a decrease in MA until a steady state is reached. The relative populations of aceticlastic and hydrogenotrophic MA may shift with increasing H_2S and DS levels; as the SRB population grows, a decrease in hydrogenotrophic MA will be observed. While incomplete-oxidizing SRB will be present in the reactor initially, complete-oxidizing SRB will require more time to become established.

1.3 Thesis organization

Chapter II provides a literature review, including background information about sources of sulfate- and carbon-rich wastewater, MA, SRB, and techniques used to study microbial population dynamics. Chapter III examines temporal reactor data over the 935 day run. This chapter will be submitted for peer review to the journal *Water Environment Research.* Chapter IV discusses the various clone libraries and phylogenetic trees constructed from genomic DNA obtained from the reactors at two different time periods.

This chapter will be submitted to *FEMS Microbiology Ecology*. Chapter V discusses the results obtained from quantitative PCR studies performed on the genomic DNA obtained from the two time periods, and Chapter VI summarizes significant findings of this work.

Several appendices have been included to supplement the main body of work. Appendix A includes photographs of the reactors during different stages of development. and Appendix B describes the results of a Mössbauer analysis of the black precipitate found in all of the reactors. Appendix C reports the findings from a series of microcosm studies performed after the completion of the reactor study using reactor material. These microcosm studies were developed in order to determine how the removal of components such as lactate, acetate, propionate, and sulfate varied between reactors. Appendix D contains micrographs of reactor pumice granules obtained using a scanning electron microscope (SEM).

CHAPTER II

LITERATURE REVIEW

2.1 Sources of high sulfate wastewater

High-sulfate wastewater originates from numerous industrial processes. Such wastewater often has a high organic content as well. Examples of processes that can contain high COD and sulfate can be seen in Table 2.1. Industries with such wastewater include pulp and paper processing, food processing, peroxide production, and tanneries. In addition, domestic sewage also has high enough sulfate concentrations to allow for the growth of SRB.

Due to the high COD content of these industrial wastewaters, aerobic biological treatment would require an excessive amount of aeration and treatment costs could be prohibitive. Anaerobic processes have the potential to treat these high COD/high sulfate effluents in a more cost-effective way. However, successful application requires a better understanding of the microbial interactions and processes involved in removing sulfate and organic matter simultaneously.

2.2 Biochemistry and phylogeny of methanogens

MA must obtain their energy for growth from the conversion of substrates into methane gas (Table 2-2 reactions 3-4). The methanogens that have been described by various researchers have been divided into three nutritional categories: CO_2 reducing, methylotrophic, and aceticlastic (Boone et al. 1993). Of the 83 species mentioned by Garcia et. al (2000), (i) 61 species are considered hydrogenotrophs (hMA) that utilize H_2 and CO_2, 38 of which are also formatotrophs; (ii) 20 species are methylotrophs that can

use methyl compounds; and (iii) nine species are aceticlastic MAs that degrade acetate (aMA), two of which are obligate acetotrophs. Since about 72% of the methane formed in anaerobic digestion originates from the cleavage of acetate (McCarty and Smith 1986), the small number of species that degrade acetate to methane in an anaerobic process are critical to the overall health of the system.

The conversion of the above substrates to methane has a small free energy change (Table 2-2). Indeed, only about one mole of ATP is produced per mole of methane created (Whitman et al. 1992). Such low energy production leads to long generation times that are anywhere from 0.18-3.9 days, depending on the substrate (Speece 1996).

It is also important to note the thermodynamically unfavorable ΔG°' of the conversion of propionate to acetate and hydrogen (Table 2.1 equation 1). This reaction is only possible at a hydrogen partial pressure below 10^{-4} atm. The conversion of this hydrogen to methane is favorable only above concentrations of 10^{-6} atm, requiring hMA to operate in a narrow hydrogen concentration range of 10^{-4} to 10^{-6} atm (Speece 1996).

Methanogens have been classified within the kingdom *Archaeobacteria* into five orders (Boone et al. 1993; Garcia et al. 2000). The order *Methanopyrales* contains one hyper-theromphilic methanogen, while order *Methanococcales* contains hydrogenotrophic MAs that have been isolated from marine and coastal habitats. These orders are unlikely to be found in typical anaerobic waste treatment systems. The order *Methanobacteriales* contains the genus *Methanobacterium*, whose 13 species can all utilize H_2 and CO_2 for growth. Order *Methanomicrobiales* contains three families of hygrogenotrophic methanogens that have been isolated from various environments. Almost all species from this order require acetate as a source of cell carbon (Boone et al.

1993). The last order, *Methanosarcinales*, contains all the acetotrophic and all the methylotrophic methanogens in two families. The first family, *Methanosaetaceae*, includes two species of obligate acetotrophs; the second, *Methanosarcinaceae*, contains MAs that are methylotrophic and can also utilize H_2. This family appears to have the most metabolic, physiological, and environmental diversity of all methanogens (Galagan et al. 2002), and they appear to predominate in many anaerobic ecosystems. Due to their higher affinity for acetate, *Methanosaetaceae* are expected to predominate in systems with low acetate levels, while higher concentrations allow for *Methanosarcinaceae* to become the dominant aMA (Griffin et al. 1998).

2.3 Biochemistry and phylogeny of sulfate reducers

SRB are distinguished by their ability to utilize sulfate as an electron acceptor for growth and convert it to sulfide (Table 2-2 equations 5-8). Two metabolic groups of SRB exist; the first includes those species that incompletely oxidize their substrate to acetate (iSRB), the second contains those that can completely oxidize their substrates to carbon dioxide (cSRB). The incomplete oxidizers appear to grow faster than the complete oxidizers (Widdel 1988). Studies of SRB in sulfate-fed reactors have found that incomplete oxidizers predominate (Hilton and Oleszkiewicz 1988) and that complete oxidizers take long periods of time to become established (Omil et al. 1998).

SRB can utilize a broad range of electron donors, including lactate, propionate, acetate, and hydrogen (Widdel and Bak 1992). Lactate can be consumed by iSRB as well as most cSRB, while hydrogen can be consumed by iSRB but not by most cSRB.

Acetate is consumed only by cSRB, while propionate is consumed by some iSRB (*Desulfobulbus)* and some cSRB (*Desulfococcus)*.

In the oxidation of organic material via sulfate reduction, 0.67 g COD can be oxidized per 1 g of sulfate. Therefore, for waste streams with a COD/sulfate ratio of 0.67, there is in theory sufficient sulfate present to completely remove the organic matter present with sulfate reduction. For ratios exceeding 0.67, complete organic removal can only occur when methanogenesis occurs in addition to sulfate reduction (Lens et al. 1998).

Sulfate reducers belong to the delta subdivision of proteobacteria. Through extensive 16S ribosomal DNA (rDNA) sequence comparison, the phylogenetic relationships among SRB have been established and categorized into genera (Devereux et al. 1989; Devereux et al. 1990; Devereux et al. 1992). These genera include *Desulfovibrio* and *Desulfobulbus*, which are lines of iSRB; *Desulfobacter*, *Desulfobacterium*, *Desulfococcus*, *Desulfosarcina*, and *Desulfobotulus*, which are cSRB; and the mixed-oxidizer Gram positive genus *Desulfotomaculum*. These phylogenetic divisions can be used to characterize populations of SRB in anaerobic systems.

2.4 Inhibition due to sulfide

SRB metabolism produces sulfide, which can inhibit microorganism activities. In its free soluble form, H_2S can permeate cell membranes and form cross-links between polypeptide chains, thus altering cell proteins (Lens et al. 1998; Percheron et al. 1997; Weijma et al. 2002). While H_2S appears to be correlated to MA inhibition, it has been

indicated that DS correlates to inhibition of SRB (Hilton and Oleszkiewicz 1988; Parkin et al. 1991; Visser et al. 1996)

Small changes in pH can affect sulfide inhibition. The optimal pH for MA growth is 6.5-7, and 7-7.5 for MA in granules; the optimal pH for SRB is 8.5-9 (Visser et al. 1996). However, for a given concentration of DS, as the pH decreases the hydrogen sulfide concentration will increase, making the lower pH more likely to cause inhibition for MA. Conversely, at higher pH levels, SRB reactions become less favorable (McCartney and Oleszkiewicz 1993).

Mass transfer also affects the observed inhibition of a system by sulfide. Previous work in our laboratory has shown that chemostats can fail at sulfide levels of greater than 60 mg-S/L and DS levels of 150-200 mg-S/L (Maillacheruvu et al. 1993; Parkin et al. 1990). For reactors with biofilms or granules, only organisms on the outermost layer near the bulk liquid are exposed to bulk sulfide and sulfate levels. Diffusion will cause sulfide and sulfate concentrations to decrease with increasing biofilm depth. The result is that organisms in the biofilm appear to withstand higher levels of bulk sulfide without inhibition and that these systems can be sulfate limited even if the influent has high levels of sulfate (Visser et al. 1996). Maillacheruvu et al. (1993) found that H_2S concentrations up to 200 mg-S/L and DS levels of 800 mg-S/L had little effect on the performance of a biofilm reactor. Isa et al. (1986a, 1986b) found that hMA and aMA in a biofilm reactor were only significantly inhibited at H_2S levels of 1,000 mg-S/L. Previous research on upflow anaerobic suspended bed (UASB) granules found MA to be located in the deeper layers of the granules (Sekiguchi et al. 1999); the existence of MA in this part of the

biofilm may allow protection from high levels of H_2S and DS and explain why biofilm reactors can operate at higher sulfide levels without failure.

2.5 Competition in sulfate-rich systems

The presence of sulfate in a wastewater introduces many complexities into anaerobic treatment. When sulfate is present, it will prompt the growth of SRB. As discussed earlier, the end product of sulfate transformation, sulfide, can have an inhibitory effect on MA. In addition, since many SRB can utilize acetate or hydrogen, they will compete with the MA for growth substrates.

SRB have been shown to out-compete MA for acetate (Colleran et al. 1995; Elferink et al. 1994; Kristjansson et al. 1982), hydrogen (Colleran et al. 1995; Elferink et al. 1994; Kristjansson et al. 1982; O'Flaherty et al. 1998; Omil et al. 1996), and fatty acid mixtures (Omil et al. 1998). SRB can also consume hydrogen below the minimum threshold for MA utilization (Percheron et al. 1997). In addition, the consumption of lactate or propionate by SRB and not acetogens means that hydrogen will not be produced and thus hMA will not be able to proliferate. However, some studies have found that MA can out-compete SRB for acetate in immobilized biomass reactors (Elferink et al. 1994; O'Flaherty et al. 1998; Omil et al. 1996), and that MA may have superior attachment properties (Isa et al. 1986a; Isa et al. 1986b).

Many studies have emphasized the COD/sulfate ratio as an important operational parameter for sulfate fed systems (Choi and Rim 1991; Maillacheruvu et al. 1993; McCartney and Oleszkiewicz 1993; Mizuno et al. 1994; Parkin et al. 1991; Weijma et al. 2002). This ratio gives an indication of the amount of sulfate available for SRB

metabolism. Several studies have focused on how changing the ratio effects a shift in organism populations. In their research with UASB reactors, Choi and Rim (1991) found that SRB and MA were competitive at COD/sulfate ratios from 1.7-2.7. At ratios less than 1.7, acetotrophic SRB were predominant, while at ratios greater than 2.7, aMA prevailed. Similar results were found by McCartney and Oleszkiewicz (1993); a ratio of 3.7 did not result in significant sulfate reduction, while ratios less than 1.6 showed evidence of sulfate reduction.

In the above studies, varying outcomes in SRB and MA competition and sulfide inhibition are due to the differing characteristics of the experiments. These include different reactor types (suspended growth, granular sludge bed, attached biofilm, etc.), carbon sources (industrial wastewaters, one or more fatty acids, etc.), and organism seed compositions (methanogenic, sulfidogenic, or both), as well as the use of different indirect measurement techniques (cell counts, mass balances, sludge activity assays). Different combinations of operational variables can result in the carbon flow shifting towards either SRB or MA. For example, Hilton and Oleszkiewicz (1988) found that SRB were more inhibited by DS while higher H_2S levels caused inhibition of MA activity. They suggest a minimization of DS would increase sulfate reduction at the expense of methane production. For increased methanogenic activity, they recommend maintaining an elevated pH and allowing for an increase in DS levels above 400 mg-S/L while maintaining H_2S levels below 200 mg-S/L.

2.6 Molecular analysis of anaerobic systems

There are numerous studies that have investigated the relationship between SRB and MA competition, sulfide inhibition (H_2S and DS), pH, and sulfate; for every study there appears to be a different conclusion as to how these parameters are related. This disparity is due to the fact that a majority of the research indirectly examines the organisms using parameters such as activity assays, growth rates, and concentrations of products and reactants. A more direct approach, such as the examination of the microbial population from reactors using molecular techniques, should provide insight into the quantities of MA and SRB in the biofilm and allow for a more complete understanding of MA and SRB interaction.

Until about twenty years ago, the only methods available for microbial identification of environmental samples were isolation of pure cultures, or morphology and most probable number (MPN) techniques. Due to the symbiotic nature of the many microbial communities of interest in the environment, such isolations were often unsuccessful in characterizing all organisms present in a sample. In the past decade, molecular techniques have been used instead of culturing methods to characterize and analyze microorganism samples (Amann et al. 1995).

2.6.1 Examination of anaerobic systems using molecular techniques

Molecular techniques can be used in several different ways to examine anaerobic systems. One way is to use them for characterization; for example, molecular techniques have been used to characterize environmental samples by analyzing for SRB and MA from Antarctic and estuary sediments (Purdy et al. 2003a; Purdy et al. 2003b).

Characterization is also important in anaerobic reactors. Delineating the organism populations present in a reactor can give much insight into why the reactor may be operating in a particular way. In their investigation of a granular sludge reactor treating papermill wastewater with a COD/sulfate ratio of 9.5 and a COD load of 1.7 g/l, Elferink et al. (1998) used probes to determine that acetate was mainly degraded by MA and propionate was the preferred substrate for SRB. It was also found that *Methanosaeta* were the dominant aMA, *Methanobacterium* were the dominant hMA, and *Desulfobulbus* was important for propionate degradation. Raskin et al. (1995b) analyzed samples from 21 anaerobic sludge digesters. They determined that *Methanosaeta* were the dominant MA in most samples and *Desulfovibrio* and *Desulfobulbus,* both iSRB, were the dominant SRB groups. Since these groups cannot degrade acetate, in was concluded that aMA were responsible for acetate degradation.

A second way to use molecular methods is to examine population dynamics of reactors. Several studies have investigated suspended-growth systems (Angenent et al. 2002; Griffin et al. 1998; McMahon et al. 2001), with quantities of a particular group of MA usually expressed as a percentage of total MA nucleic acid levels. When examining a reactor at startup, Griffin et al. (1998) found that as acetate levels increased, the aMA population shifted from the *Methanosaeta* predominant in the inoculum that had low acetate levels to *Methanosarcina* in reactors with high VFA levels. Similar patterns were observed by McMahon (2001) and Angenent (2002). Griffin also found that while *Methanobacteriaceae* were the dominant hMA, not enough MA were present for a conversion of organics to methane, as evidenced by a buildup of fatty acids in the reactor. In addition, Angenent (2002) found that as the total ammonia levels increased, there was

another shift from *Methanosarcina* towards *Methanomicrobiales*, which are less sensitive to ammonia than aMA. Hence, changes in the operational characteristics of the reactor could be explained by observing how the microorganism community changed over time.

2.6.2 Specific molecular techniques: clone libraries

16S rDNA is a gene frequently used in molecular analysis to determine phylogenetic relationships between organisms. Several reasons for the use of 16S rDNA include the fact that it exists in all organisms, it has the same function in all organisms (homology) and it is large (about 1500 bases). Some regions of rDNA are among the most highly conserved and appear in all organisms, while other regions are unique to a particular organism or group (i.e. kingdom, family, genera, etc.) of organisms. By using a molecular probe that hybridizes to and identifies the unique regions of the 16S rDNA sequences, the microbial community may be delineated from mixed samples without the need for cultivation (Amann et al. 1990; Amann et al. 1995).

One powerful application of 16S rDNA sequences is using them to construct a graphical representation of the evolutionary relationship between organisms, i.e. a phylogenetic tree. The branch lengths of a phylogenetic tree are proportional to the predicted evolutionary time between sequences, or in other words, the sequence similarity. Thus, the sequence similarity becomes a predictor of the relatedness between sequences in a tree (Figure 2-1).

In order to construct a phylogenetic tree, the sequences used to make a tree must first be aligned. Alignment for multiple sequences is accomplished by computer algorithms that maximize the number of bases that match by shifting sequences and

inserting gaps into the sequences. Biologically, these gaps are assumed to represent insertions or deletions that occurred as the sequences diverged from a common ancestor (Hall 2004). After the alignment is created, the phylogenetic tree can be generated. In order to determine the reliability of the groupings in the tree, bootstrapping is often used. Bootstrapping takes a sub-sample of the sites in an alignment and creates trees based on those sub-samples. The process is iterated multiple times (usually 1,000) and the results are compiled to allow an estimate of the reliability of a particular grouping (Hall 2004). For example, a bootstrap value of 900 would mean that the grouping would appear as seen on the tree 900 out of 1,000 times, indicating a reliable arrangement, whereas 250 would indicate a less reliable arrangement. Interpretation of the bootstrap value depends on how important the particular branch of the tree may be. For instance, when comparing sequences with a small evolutionary distance between them, a high bootstrap value may not necessarily be significant.

2.6.3 Specific molecular techniques: RTQ-PCR

The polymerase chain reaction (PCR) has enabled the qualitative description of microbial diversity, usually by the detection of 16S rDNA in mixed samples. Results are determined using end point detection of the PCR products, usually by agarose gel resolution. Due to biases introduced during the amplification processes of mixed-species DNA (Smith et al. 2006) and the imprecise nature of agarose gel resolution, quantitation of components of mixed DNA from environmental samples is not practical using PCR. However, in the last few years, quantitative PCR (qPCR) has become widely available. Whereas PCR is sensitive enough to detect about a 10-fold difference between samples,

qPCR has the ability to detect differences as low as 2-fold (Applied_Biosystems 2006). This is accomplished by monitoring the progress of amplification while it is taking place and collecting data in real time, compared to only at the end point of the reaction, as in PCR.

While other quantitative techniques exist, such as terminal restriction fragment length polymorphism (TRFLP) analysis and length heterogeneity PCR (LH-PCR), they rely on quantitation after a number of replication cycles have been completed. This type of quantification has been found to allow for the biasing of proportions of different amplicons in a mixture (Suzuki and Giovannoni 1996). In addition, since identification of microbes in TRFLP and LH-PCR is based on a diagnostic fragment size, identification is presumptive, not determinative, as in qPCR (Suzuki et al. 2000).

Two main ways of performing qPCR are SYBRGreen chemistry and hydrolysis probe based chemistry (TaqMan®). SYBRGreen is a dye that binds to all double-stranded DNA (dsDNA). As amplification proceeds and the amount of dsDNA increases, the autofluorescence of the SYBRGreen does as well. Advantages of this chemistry are that it can be used in conjunction with any primer set and is not as costly as TaqMan®. However, since SYBRGreen is a non-specific binding dye, it binds to all dsDNA indiscriminately, making the specificity of primers of utmost importance for accurate quantification. In addition, it had been observed that when applied to complex environmental samples, most primer pairs might produce unspecific products (Zhang and Fang 2006).

TaqMan® chemistry can be more expensive than SYBRGreen and the design of an additional probe is required. However, this method allows for a higher degree of

specificity, especially for mixed population samples. The probe used in TaqMan® is an oligonucleotide labeled with a fluorescent reporter dye on the 5' end and a quencher dye on the 3' end that reduces the fluorescence of the reporter dye when in close proximity to it. It is designed to anneal to a specific sequence of interest downstream of the primer site. After this occurs, the probe is cleaved by the 5' nuclease activity of Taq DNA polymerase as DNA is transcribed. The probe can be cleaved only if it is hybridized to the target sequence. When this occurs, the quencher dye is separated from the reporter dye, unmasking the signal of the reporter. Since this process continues during each qPCR cycle, the signal of the reporter dye is increased as more amplicons are produced from the qPCR, leading to an increase in fluorescence intensity proportional to the amount of PCR product. This signal is detected in a specialized qPCR thermocycler designed to measure fluorescence.

In the preliminary cycles of the qPCR, the fluorescent signal is low and is defined as the baseline region (Figure 2-2), when only background signal is detected. As the signal increases above this baseline, the target sequence is detected. A fixed threshold is set above the baseline. The threshold cycle (Ct), determined for each qPCR reaction, is the fractional cycle number at which the fluorescence passes the fixed threshold and begins an exponential growth phase of the PCR. Higher starting amounts of a target sequence will produce an increase in fluorescence sooner and will therefore have lower Ct values, whereas targets not present in large numbers will produce higher Ct values. Quantification of the starting template in a particular sample may be achieved by converting the Ct values found from qPCR into absolute quantities using standards produced from known amounts of the amplicons of interest (Figure 2-3).

Table 2-1 Sources of high COD, high sulfate wastewater

Industrial Wastewater Type	**COD, mg/L**	**Sulfate, g-S/L**	**Reference**
Pulp and paper effluent	7.5-10.4	1.2-1.5	Lens et al. 1998
Pulp and paper aggregate	1.7	0.18	Elferink et al. 1998c
Pulp and paper aggregate	0.8-3.1	0.34-0.85	Rintala and Lepisto 1998
Sugar beet molasses	30	2.5-4.5	Lens et al. 1998
Cane molasses alcohol production	50.6	2.9	Lens et al. 1998
Citric acid production	N/A	4.5	Rajczyk 1993
Rum distillery	95	6	Colleran et al. 1995
Seafood processing	10-60	0.6-2.7	Omil et al. 1995
Seafood processing	12.4-16.9	2.1-2.7	Lens et al. 1998
Edible oil processing	40-50	40-50	Colleran et al. 1995
Organic peroxide production	15-40	12-35	Silva et al. 2002
Tannery wastewater	4.8-8.0	1.2-2.0	Lens et al. 1998
Domestic sewage	N/A	0.02-0.5	Fukui et al. 2000

Table 2-2 Anaerobic reactions of interest.

Acetogenic Reactions[a]	$\Delta G^{\circ'}$, kJ/mol [b]
(1) $Propionate^- + 3\ H_2O \longrightarrow Acetate^- + HCO_3^- + H^+ + 3\ H_2$	+ 76.1
(2) $Lactate^- + 2\ H_2O \longrightarrow Acetate^- + HCO_3^- + H^+ + 2\ H_2$	- 4.2
Methanogenic Reactions	
(3) $4\ H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3\ H_2O$	-33.9
(4) $Acetate^- + H_2O \longrightarrow CH_4 + HCO_3^-$	- 31.0
Sulfate Reducing Reactions	
(5) $4\ H_2 + SO_4^{-2} + H^+ \longrightarrow HS^- + 4H_2O$	- 38.1
(6) $Acetate^- + SO_4^{-2} \longrightarrow 2\ HCO_3^- + HS^-$	- 47.6
(7) $Propionate^- + 0.75\ SO_4^{-2} \longrightarrow Acetate^- + HCO_3^- + 0.75\ HS^- + 0.25\ H^+$	- 37.7
(8) $Lactate^- + 0.5\ SO_4^{-2} \longrightarrow Acetate^- + HCO_3^- + 0.5\ HS^- + 0.5\ H^+$	- 80.0
Propionate Production Reaction	
(9)[c] $Lactate^- \longrightarrow 0.66\ Propionate^- + 0.33\ Acetate^- + 0.33\ HCO_3^- + H^+$	- 54.9

[a] Source: Elferink et al. 1994

[b] free energy change at standard conditions (T = 25 ºC, P = 1 atm), at pH = 7 and with all unit activities equal to one.

[c]Source: Mccartney and Oleszkiewicz 1991

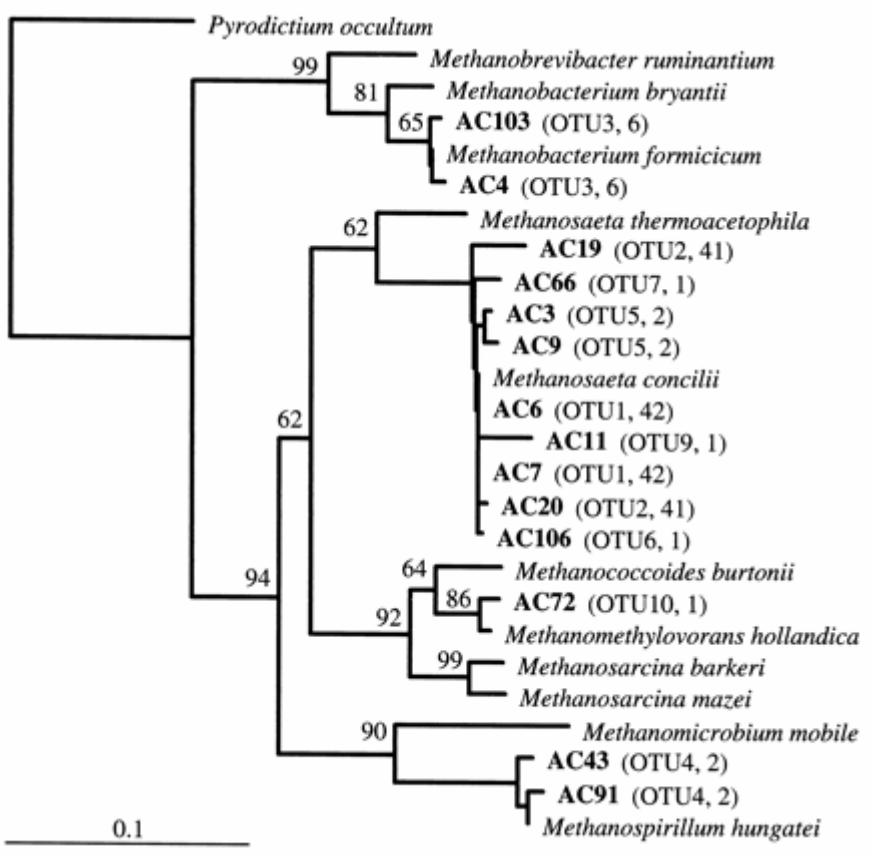


Figure 2-1 Example of a phylogenetic tree with scale bar and bootstrap values out of 100 (Plumb et al. 2001).

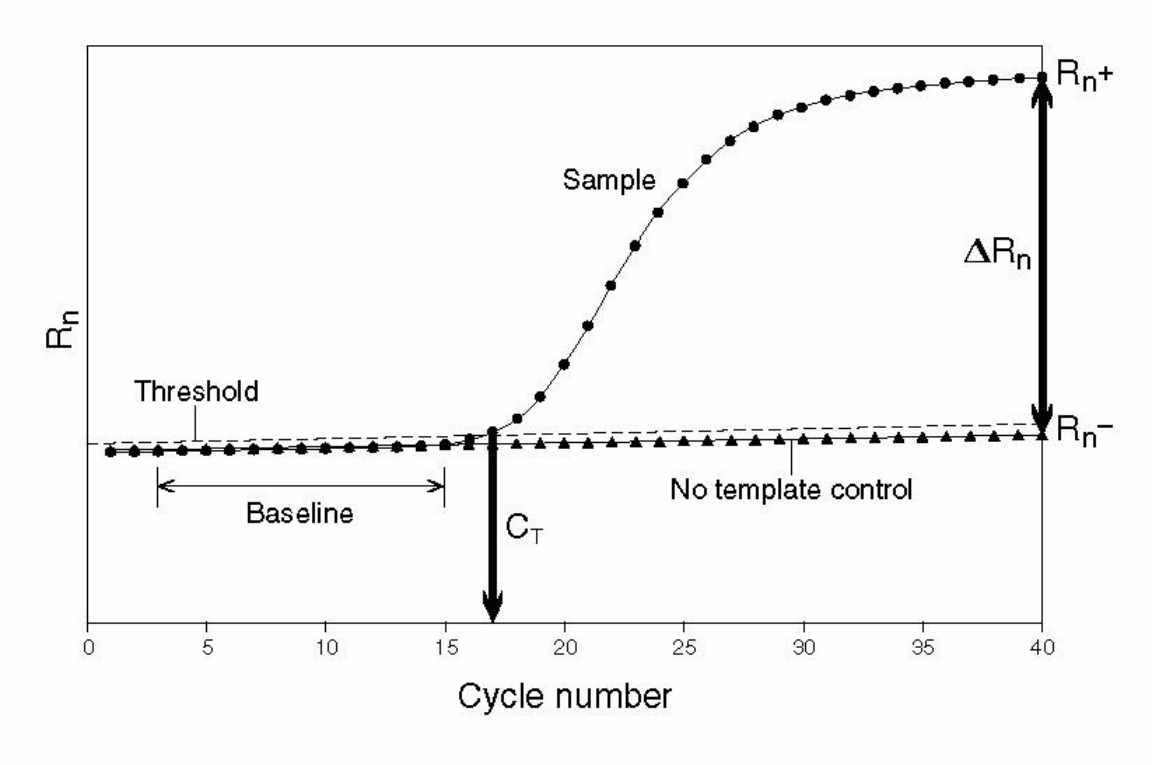


Figure 2-2 Quantitative PCR detection (Applied_Biosystems 2002).

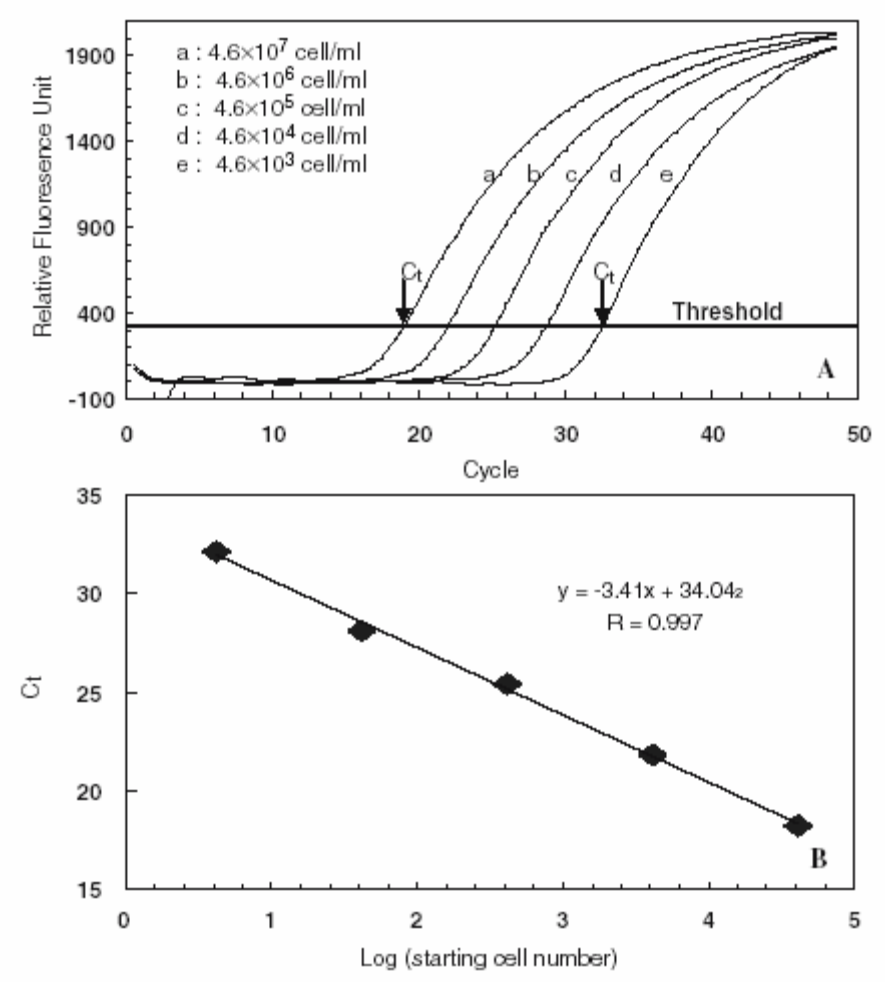


Figure 2-3 Example of the construction of a standard curve for qPCR absolute quantification (Zhang and Fang 2006). A: Relative fluorescence intensity of five standard solutions through qPCR amplification; B: Standard curve for qPCR measurement, showing concentration as a function of Ct.

CHAPTER III
TEMPORAL ANALYSIS OF SULFATE-FED ANAEROBIC BIOFILM REACTORS

3.1 Introduction

Currently, almost all full scale anaerobic wastewater treatment systems operate at temperatures above 18 ºC, even though many industrial wastewaters are discharged at low ambient temperatures (Lettinga et al. 1999). Low temperature treatment would reduce or eliminate the need to heat influent wastewaters and thereby increase the economic feasibility of anaerobic treatment. This possibility has led to considerable research on the application of low-temperature anaerobic treatment to a wide variety of wastewater types (Collins et al. 2005; del Pozo et al. 2006; Enright et al. 2005; Luostarinen and Rintala 2006; McHugh et al. 2006; Singh et al. 2006). While there has been extensive research on the anaerobic treatment of high sulfate wastewaters, there have been no systematic studies investigating the effect of high sulfide levels in the long term anaerobic treatment of sulfate-rich wastewater at lower temperatures.

Given the involvement of MA, SRB, and fermenters (Table 2-2, reactions 1-2) in consuming the electron donors present in a complex wastewater stream, it has been difficult to determine the role of these different groups in anaerobic treatment of a sulfate-rich wastewater. There is also conflicting information on the levels of sulfide or H_2S that are inhibitory to sulfate wastewater treatment, and which organism groups are actually inhibited by sulfide. In addition, there is a general lack of research on treatment of sulfate-rich wastewaters at low temperatures.

Therefore, the specific objectives of this portion of the research were (1) to establish anaerobic biofilm reactors operating at 20 ºC that can treat a high sulfate and high COD wastewater; (2) to assess the effect of high sulfide levels on the operational performance of mature biofilm reactors; (3) to observe how organic shock loading affects reactor performance; (4) to better understand the interaction between MA and SRB in mature biofilm reactors exposed to high sulfide levels.

3.2 Materials and methods

3.2.1 Biofilm reactors

Three reactors (ID 3 in., length 36 in.) were constructed from 0.25 in. thick acrylic. Five sample ports were located along the length of the reactor at 3, 6, 14, 23, and 32 in. from the bottom. A 20-L feed bottle was used to pump mineral media into the reactors with a peristaltic pump. Reactors were fed in an upflow regime (Figure 3-1). For all reactors, a tee before the inlet allowed lactate from a syringe pump to be combined with the feed media right before entry. After exiting the top of the reactor, effluent flowed into a gas trap.

Four liters of pumice (diameter 2-4 mm) were added to each reactor as a support material for the biomass. Pumice has been used as a microbial carrier in previous studies (Balaguer et al. 1997; Minami 1994; Patel et al. 1995; Petrozzi et al. 1993). In order to wet the pumice and fill the voids between particles with liquid, distilled water was pumped into the reactors from the bottom. During this process, the void volume of the reactors was found to be 2.6 L. The reactors were then seeded using a combination of two stock enrichment cultures maintained our lab: LEC, a lactate enrichment culture, and

SEC, a lactate enrichment culture fed sulfate. Reactor R1 was seeded with 2.6 L of LEC, R2 with 2.6 L of SEC, and R3 with 1.3 L each of LEC and SEC. After seeding, the reactors were allowed to sit for two days in order to promote microbial attachment to the pumice carrier. At the end of this time period, feeding of the reactors commenced. Mineral media was pumped from the feed storage bottle and combined at the tee near the inlet with lactic acid from a syringe pump. The composition of the mineral media was as follows: 400 mg/L each of NH_4Cl, $MgCl_2$, KCl; 80 mg/L $(NH_4)_2HPO_4$; 40 mg/L $FeCl_2 \cdot 4H_2O$; 25 mg/L $CaCl_2 \cdot 2H_2O$; 10 mg/L $(NaPO_3)_6$; 2.5 mg/L each of $CoCl_2 \cdot 6H_2O$, KI; and 0.5 mg/L each of $MnCl_2 \cdot 4H_2O$, NH_4VO_3, $ZnCl_2$, $Na_2MoO_4 \cdot 2H_2O$, H_3BO_3, $NiCl_2 \cdot 6H_2O$. On day 475 , $FeCl_2 \cdot 4H_2O$ was removed from the media solution in order to prevent the formation of a precipitate in the feed storage bottle. Sulfate and bicarbonate were also added to the media in varying amounts. The bicarbonate concentration in the feed media was adjusted in order to maintain an effluent pH of 7.0-7.5.

The reactors were kept at a temperature of 20 ± 2°C. The HRT of the reactors was maintained at two days. The initial organic load rate (OLR) and sulfur load rate (SLR) were 0.3g COD/L-day and 9 mg-S/L-day, respectively. On day 104, the reactors were operating at pseudo-steady state conditions with respect to volatile acid COD (VA-COD and sulfate (effluent VA-COD and sulfate were less than 20 mg-COD/L and 20 mg-S/L, respectively, for at least 20 days). The OLR was then doubled to 0.6 g COD/L-day. The OLR was subsequently increased to 1.5 g COD/L-day on day 245, 2.6 g COD/L-day on day 506, and reached a maximum on day 560 when the it was increased to 4.0 g COD/L-day. The SLR was increased in approximately the same proportion as the OLR. Starting around day 600, a series of lactate spikes were applied to the reactors. R3 was

spiked with lactate on days 594-599, R2 was spiked on day 640, and all reactors were spiked on days 688-691.

3.2.2 Analytical methods

Approximately every two days, effluent samples were collected from the effluent gas traps, filtered with a 0.22 μm syringe filter, and analyzed for volatile fatty acids (VFAs) and sulfate. Dissolved sulfide analysis was performed once per week; anaerobic liquid samples were removed from the top of each reactor and injected immediately into an anaerobic solution of 2% zinc acetate in order to sequester sulfide and prevent oxidation prior to analysis. Sulfate analysis was carried out using a Dionex ICS-2000 Ion Chromatography (IC) System. The IC was equipped with an Automatic Self-Regenerating Suppressor Ultra 4mm suppressor column at a current of 80 Ma. Injections of 15 mL were made with a AS50 autosampler. Separation was achieved with a Dionex Ion-Pac AS18 Anion Exchange Column using an eluent of 39 mM KOH flowing at 1mL/min. Peak areas were integrated with Chromeleon software. Analysis for VFAs, including lactate, acetate, propionate and butyrate, was performed using a Hewlett Packard series 1100 high pressure liquid chromatograph (HPLC) equipped with a UV/visible light detector operating at 210 nm. Injections of 100 μL were made by a Hewlett Packard series 1100 sampler. Separation was achieved with an Alltech 150 mm, 6.5 mm ID anion exchange column and an eluent of 0.01 N sulfuric acid flowing at 1 mL/min. Peak areas were integrated by Hewlett Packard ChemStation software. Sulfide analysis was performed using a methylene blue spectrophotometric assay (Truper and

Schlegel 1964). pH was detected using a Fischer Scientific Accumet Basic AB15 pH meter with an accuracy of 0.01 pH. The meter was calibrated prior to each use.

3.3 Results

In subsequent discussion, times are divided into the following experimental periods: Startup (days 0-244), Increasing OLR (days 245-506), High OLR to Spike (days 507-691 for R1, and days 507-640 for R2 and R3), Spiking (days 641-691 for R2 and R3 only), and Spike Recovery (days 692-935). Appendix A displays photographs of the reactors as they became populated over time. Appendix B describes the analysis of black precipitate found in the reactors using Mössbauer spectroscopy. Figures 3-2 through 3-4 show the effluent VFA-COD and sulfide for each reactor and Figures 3-5 through 3-7 show the effluent VFA levels. Tables 3-1 and 3-2 show averages and standard deviations for various parameters for each time period. Although standard deviations are quite large for some parameters during some time periods, their values are useful in describing the variability in reactor performance and framing the following discussion.

3.3.1 Reactor startup and acclimation

Figures 3-2 through 3-4 show the effluent VFA-COD and sulfide for each reactor. By day 104 when all reactors exhibited very low effluent VFA-COD and sulfide, the OLR was doubled from 0.3 to 0.6 g COD/L-day. By this time, significant amounts of black precipitate were observed in all reactors (Figure A-3), compared to the initial gray color of the pumice prior to startup (Figure A-1) and the smaller quantity of black precipitate observed after seeding (Figure A-2). After the increase in OLR, effluent VFA-

COD continued to be low, with effluent VFA-COD less than 25 mg/L and effluent sulfide was below 15 mg-S/L. The highest average effluent VFA-COD and sulfide levels for all reactors during the startup period (days 0-244) was 55 mg-COD/L and 5 mg-S/L, respectively (Tables 3-1, 3-2). Sulfate removal for all reactors during this period was over 80%.

In order to determine the composition of the black precipitate observed in the reactors, a sample of the precipitate was removed on day 205 and analyzed using transmission Mössbauer spectroscopy (Appendix B). This precipitate was found to be mackinawite (FeS). Although $FeCl_2 \bullet 4H_2O$ was removed from the feed solution on day 475, FeS was observed in all reactors throughout the study.

The reactors exhibited different responses after the OLR was increased to 1.5 g COD/L-day on day 245. R1 effluent VFA-COD remained low through day 460, with the exception of one excursion around day 400. R2 and R3 effluent VFA-COD levels rose after the OLR was increased, but then returned to low levels by days 380 and 278, respectively. During the period of increasing OLR (days 245-506), effluent sulfide increased; average effluent sulfide levels were above 160 mg-S/L in all reactors. In addition, increased amounts of mackinawite were observed in all reactors (Figures A-4 through A-6).

Average H_2S levels for R1, R2, and R3 also increased from near zero levels in the prior time period to 82, 70, and 65 mg-S/L, respectively. Both COD and sulfate removals remained high during this period; average COD removal was 90% or greater and average sulfate removal was 89% or greater for all reactors.

Figures 3-5 through 3-7 illustrate the COD composition for each reactor. Throughout days 245-506, R1 exhibited low VFAs until propionate levels began to increase on day 459. At the beginning of this time period, R2 effluent VFA-COD was composed of higher levels of both acetate and propionate than the other reactors during this time, but after about 140 days, effluent VFAs were very low. R3 VFAs also rose after the OLR was increased on day 245, but the elevated VFAs decreased to low levels about 20 days after the perturbation. Butyrate was rarely observed.

Due to the large amounts of unquantifiable makinawite present, a complete mass balance of S in the system was not possible. However, a mass balance on S was performed in order to assess the effect of Fe removal from the mineral media. Figures 3-8 through 3-10 display the mass balances, where S_{in} is equal to sulfate in the feed in mg-S/L and S_{out} is equal to the summation of effluent sulfate and sulfide in mg-S/L. During the period where Fe was added to the mineral media, large amounts of FeS were formed, as indicated by the larger amount of S_{in} than S_{out}. After Fe was removed from the mineral media, the mass balance was generally closer. Since no Fe was added in the feed solution, it is possible that some FeS would have dissolved, leading to several data points with a larger S_{out} than S_{in}.

3.3.2 Increasing OLR and sulfate reduction

On days 507 through 565, the OLR was increased incrementally from 1.5 to 4.0 g COD/L-day. R2, while previously exhibiting higher effluent VFA-COD levels compared to R1 and R2, appeared to have very stable operation. While R1 and R3 average COD were 665 and 187 mg-COD/L, respectively, R2 average COD was 44 mg-COD/L. R1

was the least stable of all reactors as shown by effluent VFA-COD rising slowly over this time. Effluent VFA-COD on day 507 was 78 mg-COD/L, but was 858 mg-COD/L by day 687. Average propionate and acetate levels were 617 and 28 mg-COD/L, respectively, indicating the COD was mostly propionate. R3 effluent VFA-COD increased during this time period but then decreased; as with R1, the COD was composed of mostly propionate. Overall COD removal remained high even with the increased OLR; average COD removal remained over 90% for all reactors. Effluent sulfide continued to increase during this time period. R1 had the highest average sulfide (390 mg-S/L), while R2 and R3 also increased (329 and 259 mg-S/L). Average sulfate removal dropped during this time period for all reactors, with removals for R1, R2, and R3 dropping to 71%, 73%, and 67%, respectively.

3.3.3 Lactate spiking and recovery

The first reactor spiked with lactate was R3, which was spiked on days 594-599 and 688-691. R2 was spiked with lactate on day 640 and days 688-691. Finally, R1 was spiked on days 688-691. After the single lactate spike in R1, effluent VFAs rose, but returned to pre-spike levels after about 30 days. For R2 and R3, the lactate spikes caused increased amounts of fatty acids in the effluent. Effluent lactate levels decreased shortly after the first spike and effluent acetate and propionate remained elevated, although prior to the second spike, R2 and R3 effluent VFA-COD appeared to be decreasing somewhat. During this spiking period (days 641-691 for R2 and R3), average effluent VFA-COD was over 2,300 mg/L for these reactors. Average acetate levels were similar in R2 and R3 (about 1,000 mg-COD/L), but propionate levels differed; R2 average propionate was

1,386 mg-COD/L, while R3 average propionate was 977 mg-COD/L. Sulfide levels in R2 and R3 decreased sharply to around zero following the spike, but increased rapidly afterwards. However, average sulfate removals for R2 and R3 during spike recovery were only 64% and 49%, respectively.

3.4 Discussion

There are a variety of pathways (and organisms) involved in anaerobic lactate biodegradation in the presence of sulfate. The major ones thought to be relevant in this research are summarized in Figure 3-11 and provide the framework for some of the discussion that follows.

3.4.1 Effect of seeding

The effect of seeding appeared to be minimal, probably because the OLR and SLR were increased gradually during the first two experimental periods. However, there were some small differences. Both seed cultures, LEC and SEC, contained populations of MA and SRB, although it is likely that SEC had more SRB than did LEC. Based on SLR and general performance of SEC it is estimated that a maximum of about 4.6% of the electron flow went through sulfate reduction while LEC had no sulfate added in its feed. Prior to lactate spiking, R2 and R3, both seeded with SEC (R2 with 100% SEC), showed stable performance with VFA-COD levels below 100 mg/L for the most part. Conversely, effluent VFA-COD levels for R1 were consistently above 500 mg/L during the period prior to spiking.

However, after spiking, behavior of R2 and R3 was much more erratic than that of R1. In fact, it may be that the high propionate levels present in R1 prior to spiking provided the selective pressure to develop more propionate-degrading SRB (Path 1 in Figure 3-11) which would enhance post-spiking stability. Such behavior is discussed in more detail in a subsequent section. Regardless, after two years of operation, it is unlikely that original seeding exerted a significant influence on reactor behavior.

3.4.2 Stable operation at low temperature

A major objective of this work was to define conditions under which an anaerobic biofilm reactor could operate stably when treating a low-temperature (20 ^{o}C), high-sulfate organic wastewater. In general, effluent VFA-COD levels of less than 200 mg/L are thought to represent such conditions (Parkin and Owen 1986; Speece 1996). It does appear that as long as OLR is kept below 1.5 g COD/L-d in combination with an SLR less than 0.2 g S/L-d, effluent VFA-COD levels will be less than 200 mg/L. In fact, R2 had an extended period prior to spiking where average OLR and SLR were 3.6 g COD/L-d and 0.27 g S/L-d, respectively, while maintaining effluent VFA-COD levels consistently around 100 mg/L. During this period, effluent H_2S-S averaged 150 mg/L and effluent DS averaged 329 mg/L. Previous work in our laboratory (Maillacheruvu et al. 1993) showed that stable operating conditions could be maintained at 35 ^{o}C with OLR of 5 g COD/L-d and SLR of 0.6 g/L-d. Effluent DS levels averaged around 800 mg S/L with corresponding H_2S-S levels of about 160 mg/L. As expected, performance was less robust at lower temperatures. Nonetheless, these low-temperature results are encouraging.

It should be noted that R1 performed well after recovering from its single spike. Data shown in Figure 3-2 indicate that after about day 760, effluent VFA-COD levels were in general less than 200 mg/L. Average OLR and SLR were 3.4 g COD/L-d and 0.3 g S/L-d, respectively. During this period, effluent DS and H_2S-S levels were around 600 mg/L and 150 mg/L, respectively. These results indicate that given sufficient time for acclimation, perhaps OLR, SLR, effluent VFA-COD, and effluent sulfide levels could be attained at 20 ^oC that approach those reported by Maillacheruvu et al. (1993) for 35 ^oC treatment.

3.4.3 Sulfide inhibition and recovery from spiking

In the period prior to spiking (High OLR to Spike), DS and H_2S levels were highest in R1 with average levels of 395 and 185 mg S/L respectively. Concentrations were lower in R2 (329 and 150 mg S/L, respectively) and lowest in R3 (259 and 106 mg S/L). It should be noted that even though data were highly variable, DS and H_2S levels for R1 were statistically greater than those for R2 and R3 (95% confidence level). H_2S levels for R2 were higher than for R3 (90% confidence level), while DS concentrations for R2 were higher at a confidence level of only 85%. During this time period, effluent VFA-COD concentrations were lowest for R2 (44 mg/L) with R3 (187 mg/L) and R1 (665 mg/L) having significantly higher levels (95% confidence level). It is likely that sulfide inhibition was at least partially responsible for the elevated VFA-COD levels in the effluent from R1.

After the first spikes to each reactor, recovery patterns were quite different and more complex. It is likely that while sulfide inhibition played a role in recovery patterns,

other factors were more important, at least initially. After the initial spike of lactate, the major VFA produced in R1 was propionate with little acetate production observed. Conversely, significant concentrations of both propionate and acetate were produced in R2 and R3. In R1, effluent sulfide levels dropped significantly, but quickly recovered indicating that sulfate reduction was minimally impacted. However, sulfide levels dropped precipitously in R2 and R3 (to near zero) indicating that sulfate reduction was dramatically impacted. These results indicate that the microbial communities were significantly different in the three reactors.

The fairly quick recovery of sulfate reduction in R1 suggests a healthy population of lactate-degrading SRB in balance with acetate-utilizing methanogens (Path 3 in Figure 3-11) and perhaps propionate-degrading SRB (Path 1). Propionate levels did increase after the spike, but subsequently decreased rapidly. In fact, microcosm studies with R1 fed lactate and sulfate showed complete removal of these substrates and no propionate build-up, while some acetate was produced (Appendix C, Figure C-1). While these microcosm studies were done some time after the Spike Recovery period, they provide another line of evidence for a balanced population.

Conversely, significant quantities of propionate and acetate were produced in both R2 and R3 while sulfate reduction was dramatically reduced, and these reactors did not appear to be recovering prior to the second spike. Clearly the populations of fermenters, acetogens (Path 2 in Figure 3-11), lactate- and propionate-degrading SRB, and methanogens were not as balanced as in R1. The reasons for this are unclear from these data. Microcosms developed from R2 and R3 samples fed lactate and sulfate were similarly unbalanced and behaved differently from R1 microcosms (Appendix C, Figures

C-2 and C-3). Lactate and sulfate were completely removed from R2 microcosms, but unlike R1 microcosms, significant concentrations of propionate were formed and persisted. In R3 microcosms, lactate was completely removed but no sulfate removal was observed. Significant concentrations of propionate and acetate were formed and persisted. pH was always above 6.7 in the microcosms; however, free volatile acid toxicity may have been a problem (Parkin and Owen 1986). It is also possible that H_2 levels were high enough to prevent further conversion of propionate to acetate and H_2. Unfortunately, H_2 concentrations were not measured.

After the second lactate spike to R2 and R3, performance continued to be highly variable and effluent VFA-COD remained high, in general in excess of 1,000 mg/L. DS levels were consistently higher in R2, ranging between 500 and 700 mg/L after day 800 while levels between 300 and 500 mg/L were observed in R3. Interestingly, effluent VFA-COD concentrations were somewhat lower and less variable in R2. As noted above, microcosm studies with R2 and R3 indicated slow and incomplete removal of sulfate with persistent presence of propionate and acetate. Clearly establishment of a stable, balanced population of SRB is key to recovery from organic overload in these systems. Why such populations were apparently developed in R1 but not R2 or R3 is not known at this time. It should be noted, however, that it is possible that both R2 and R3 would have recovered like R1 had not a second lactate spike been administered.

3.4.4 Long-term performance and implications

Based on results from over 900 days of operating R1, R2, and R3, and from microcosm studies, a few general observations can be made about long-term treatment of

high-sulfate wastewaters. It appears that long-term stability depends on the development stable and balanced populations of lactate-degrading SRB and aMA, and perhaps propionate-degrading SRB. The dominant pathway of lactate degradation fostering the most stable operation appears to be pathway 3 in Figure 3-11. Similar behavior was also reported in biofilm reactors fed propionate and sulfate (Maillacheruvu and Parkin 1996; Maillacheruvu et al. 1993); there, propionate-degrading SRB and aMA were suggested as the dominant populations. The primary reason given was sulfide inhibition of other groups of bacteria as evidenced by their corresponding inhibition coefficients (K_I – Table 3-3).

The reasoning is that K_I values for organisms converting propionate to acetate and H_2 (Table 2-2 reaction 1) and acetate-utilizing SRB (Table 2-2, reaction 6) are quite low making these organisms much more sensitive to sulfide inhibition than competing organisms (propionate-degrading SRB (Table 2-2, reaction 7) and acetate-utilizing methanogens (Table 2-2, reaction 4). That being the case, very little H_2 would be produced and thus H_2-utilizing methanogens would be in small numbers, while H_2-utilizing SRB would consume substrates other than H_2, such as lactate. The relative unimportance of H_2 in this scenario has practical implications in that, if true, thermodynamic inhibition of acetogenesis by high levels of H_2 should not occur.

Perhaps a similar scenario is true for competition for lactate (pathway 1 vs. pathway 2 vs. pathway 3 in Figure 3-11). Experimental data from biofilm reactors and microcosms support this hypothesis. R1 appeared to have more lactate- and propionate-degrading SRB compared to fermenters and acetogens while R2 had populations of fermenters and SRB competing for lactate and R3 had more fermenters as compared to

lactate- and propionate-degrading SRB. McCartney and Oleszkiewicz (1993) reported similar behavior when comparing responses of cultures fed lactate with and without sulfate (acclimated to sulfate and unacclimated, respectively). They found significant build-up of propionate in unacclimated cultures due to the increased presence of propionate-producing lactate degraders in the unacclimated cultures. The key role of propionate-degrading bacteria in the stability of anaerobic reactors has been also been described by others (McMahon et al. 2001; Xing et al. 1997a). Why such differences developed in R1, R2, and R3 over time is not yet clear, but does indicate the variability inherent in biological treatment systems.

3.5 Summary and conclusions

The general objectives of this aspect of the research were to (1) assess the performance of anaerobic biofilm reactors fed high concentrations of lactate and sulfate, and operated at 20 °C (2) determine the effect, if any, of seeding with different microbial enrichments, and (3) determine the effect of organic shock load shocks. Three biofilm reactors (R1, R2, R3) were operated at and HRT of two days and seeded with varying amounts of enrichment cultures fed lactate with and without sulfate. Reactors were operated for over 900 days, with OLD starting at 0.3 g-COD/L-day and reaching a maximum of about 4.0 g-COD/L-day. Sulfate loading rates started at 0.009 g-S/L-day and reached a maximum of about 0.3 g-S/L-day.

Other than this study with lactate and that of Maillacheruvu et al. (1993) with propionate and acetate, we know of no other long-term studies (> 900 days) with anaerobic biofilm reactors fed high concentrations of sulfate. O'Flaherty (1998)

performed a five-year study of a full scale packed reactor treating citric acid wastewater; however, the operating temperature was 37 °C and their focus was population structure, not analysis of long term data trends. The following are general conclusions that can be drawn from this work.

- Anaerobic biofilm reactors can be operated at 20°C with an OLR of 1.3 g-COD/L-day or less and an SLR of 0.2 g-S/L-day with no significant deterioration in process performance.
- With long acclimation periods, OLR as high as 3.4 g COD/L-d and SLR of 0.3 g/L-d can be tolerated, producing effluent VA-COD levels consistently less than 200 mg/L. Effluent DS and H_2S levels were around 600 mg S/L and 150 mg S/L, respectively, during this period.
- Initial seeding does not appear to make a significant difference in long-term reactor performance.
- Reactors appear to be able to recover from one lactate spike of approximately 5,000 mg COD/L but not two spikes of this magnitude.
- The key to long-term stability appears to be the development of large, stable populations of lactate- and propionate-degrading SRB and aceticlastic methanogens. It may be that bioaugmentation with these organisms could speed up acclimation to sulfate.

Table 3-1 Summary of COD data for various experimental periods.

Time Period	Days	OLR[a]	Total VFA-COD[b]	Acetate[b]	Propionate[b]	% COD Removal
R1						
Startup	0-244	0.3-0.6	23 ± 68 (118)	10± 31 (118)	7 ± 21 (118)	96 (120)
Increasing OLR	245-506	0.6-2.6	78 ± 159 (121)	12 ± 29 (121)	61 ± 134 (121)	97 (121)
High OLR to Spike	507-691	2.6-3.9	665 ± 323 (84)	28 ± 21 (84)	617 ± 320 (84)	91 (84)
Spike Recovery	692-935	3.9-4.0	368 ± 445 (87)	30 ± 30 (87)	307 ± 400 (87)	94 (88)
R2						
Startup	0-244	0.3-0.6	55 ± 120 (118)	33 ± 71 (118)	17 ± 46 (118)	91 (120)
Increasing OLR	245-506	0.6-2.6	254 ± 455 (121)	121 ± 182 (121)	113 ± 219 (121)	90 (121)
High OLR to Spike	507-640	2.6-3.9	44 ± 104 (63)	19 ± 55 (63)	14 ± 39 (63)	99 (63)
Spiking	641-691	3.9-1.9	2,618 ± 759 (20)	1,012 ± 336 (20)	1,386 ± 531 (20)	61 (20)
Spike Recovery	692-935	1.9-3.0	1,339 ± 630 (88)	664 ± 359 (88)	620 ± 330 (88)	74 (88)
R3						
Startup	0-244	0.3-0.6	18 ± 55 (119)	8 ± 29 (119)	6 ± 22 (119)	97 (120)
Increasing OLR	245-506	0.6-2.6	63 ± 143 (121)	27 ± 67 (121)	31 ± 76 (121)	98 (121)
High OLR to Spike	507-640	2.6-3.9	187 ± 238 (42)	26 ± 32 (42)	151 ± 203 (42)	97 (42)
Spiking	641-691	3.9-1.9	2,134 ± 947 (41)	1,014 ± 497 (41)	977 ± 403 (41)	60 (38)
Spike Recovery	692-935	1.9-2.5	1,834 ± 972 (88)	589 ± 495 (88)	1,156 ± 704 (88)	65 (84)

[a] starting and ending g-COD/L-day for the time period

[b] mg-COD/L ± standard deviation for (n) samples

Table 3-2 Summary of sulfate and sulfide data for various experimental periods.

Time Period	Days	SLR[a]	COD/S[b]	Sulfate[b]	Sulfide[c]	Hydrogen Sulfide[c]	% Sulfate Removal
R1							
Startup	0-244	0.04 ± 0.03 (121)	24 ± 24 (120)	17 ± 30 (117)	4 ± 5 (36)	2 ± 2 (36)	83 (112)
Increasing OLR	245-506	0.21 ± 0.07 (121)	7 ± 3 (121)	39 ± 80 (121)	173 ± 79 (36)	82 ± 42 (36)	91 (121)
High OLR to	507-691	0.27 ± 0.08 (84)	14 ±3 (84)	157 ± 121 (84)	395 ± 97 (21)	185 ± 41 (21)	72 (83)
Spike Recovery	692-935	0.30 ± 0.07 (86)	12 ± 3 (76)	147 ± 141 (86)	514 ± 137 (36)	165 ± 60 (36)	75 (86)
R2							
Startup	0-244	0.04 ± 0.03 (121)	24 ± 24 (120)	16 ± 28 (118)	5 ± 5 (36)	3 ± 3 (36)	82 (113)
Increasing OLR	245-506	0.21 ± 0.07 (121)	7 ± 3 (121)	43 ± 75 (121)	162 ± 74 (36)	70 ± 36 (36)	89 (121)
High OLR to	507-640	0.27 ± 0.09 (63)	15± 4 (63)	138 ± 131 (63)	329 ±110 (13)	150 ± 40 (13)	75 (63)
Spiking	641-691	0.28 ± 0.03 (21)	13 ±4 (21)	413 ± 125 (21)	77 ± 85 (7)	35 ± 33 (7)	29 (18)
Spike Recovery	692-935	0.30 ± 0.07 (86)	9 ± 3 (76)	220 ± 146 (86)	478 ± 155 (36)	219 ± 108 (36)	64 (85)
R3							
Startup	0-244	0.04 ± 0.03 (121)	24 ± 24 (120)	14 ± 27 (117)	3 ± 4 (36)	1 ± 2 (36)	87 (115)
Increasing OLR	245-506	0.21 ± 0.07 (121)	7 ± 3 (121)	38 ± 81 (121)	163 ± 76 (36)	65 ± 38 (36)	92 (121)
High OLR to	507-640	0.27 ± 0.09 (63)	13 ±5 (63)	73 ± 75 (42)	259 ± 165 (8)	106 ± 75 (8)	87 (42)
Spiking	641-691	0.28 ± 0.03 (21)	11 ± 4 (21)	376 ± 123 (42)	215 ± 115 (11)	173 ± 78 (11)	35 (38)
Spike Recovery	692-935	0.30 ± 0.07 (86)	8 ± 3 (76)	320 ± 137 (86)	343 ± 126 (36)	183 ± 78 (36)	49 (84)

[a] g-S/L-day ± standard deviation for (n) samples

[b] g-COD/g-S

[c] mg-S/L ± standard deviation for (n) samples

Table 3-3 Sulfide inhibition constants (K_I) at 35 ºC for various groups of bacteria.

Organism Group	**K_I (mg DS/L)***	**K_I (mg H_2S-S/L)***
HPr fermenters	53	25
HPr SRB	681	194
aMA	222	108
aSRB	35	8
hMA	1,430	625
hSRB	422	140

*Source: Maillacheruvu et al. 1993

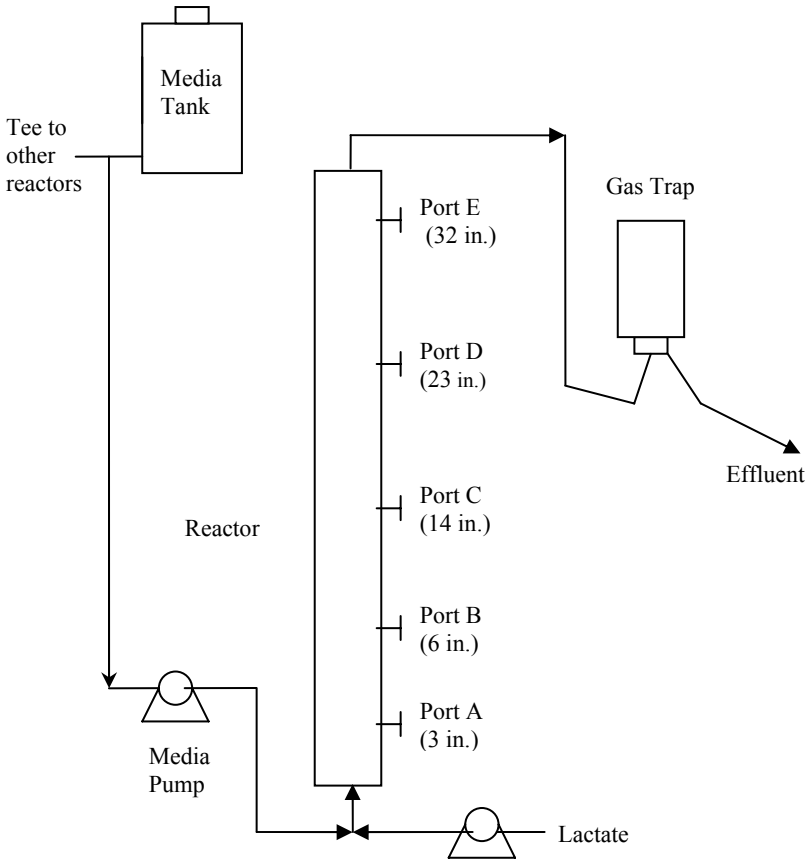


Figure 3-1 Reactor schematic.

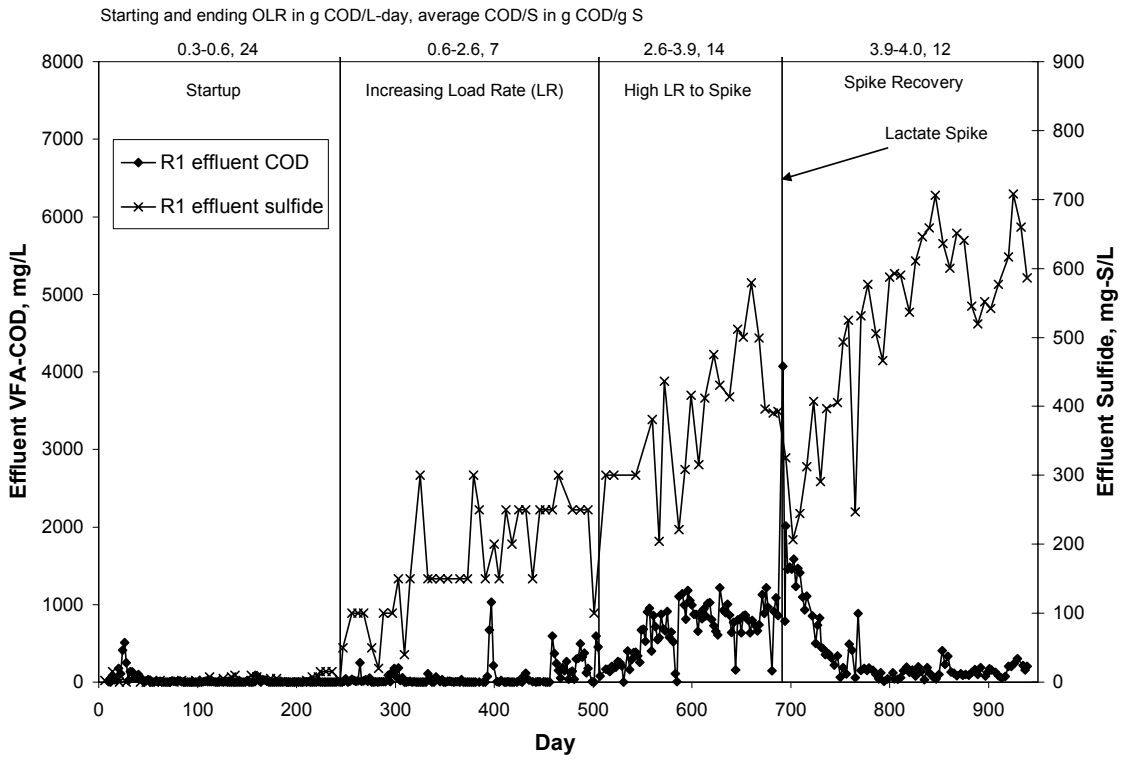


Figure 3-2 Performance of R1: effluent VFA-COD and sulfide vs. time.

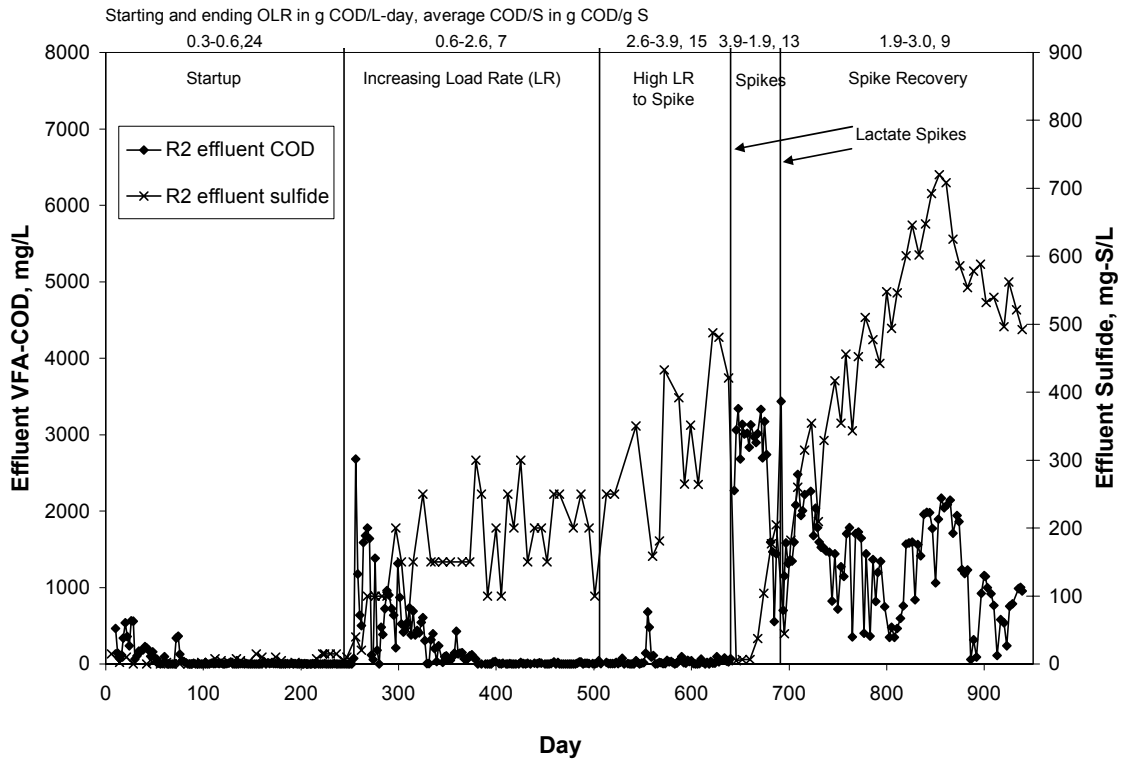


Figure 3-3 Performance of R2: effluent VFA-COD and sulfide vs. time.

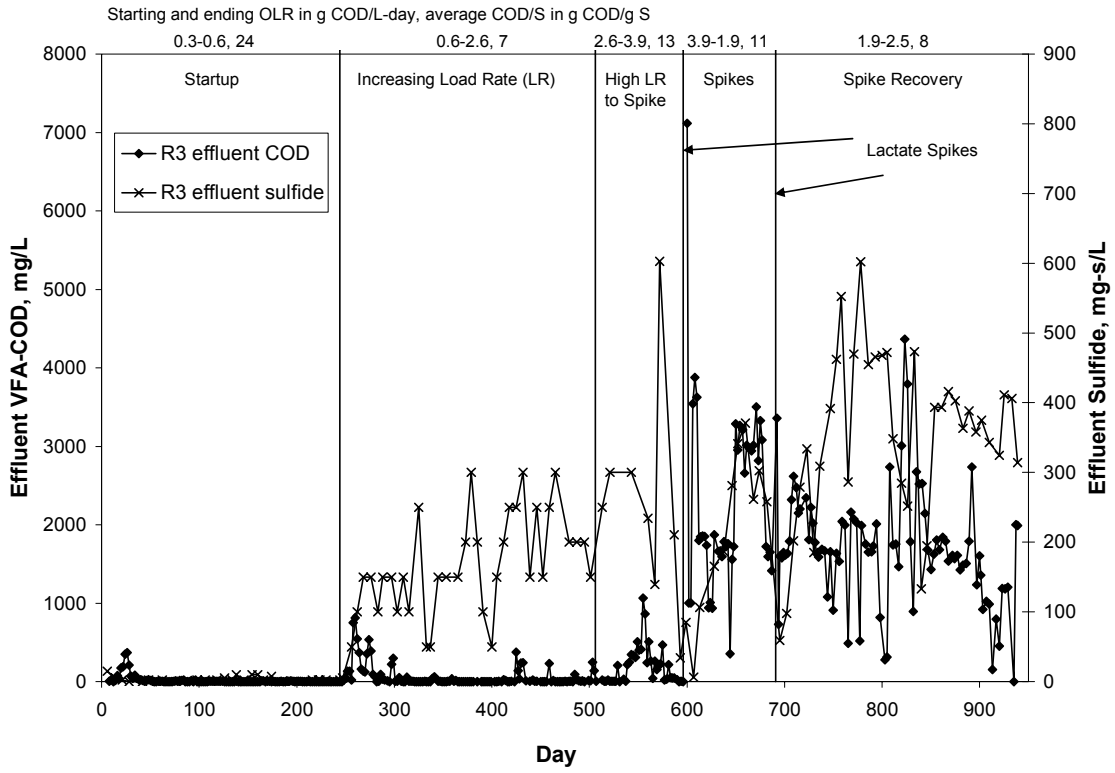


Figure 3-4 Performance of R3: effluent VFA-COD and sulfide vs. time.

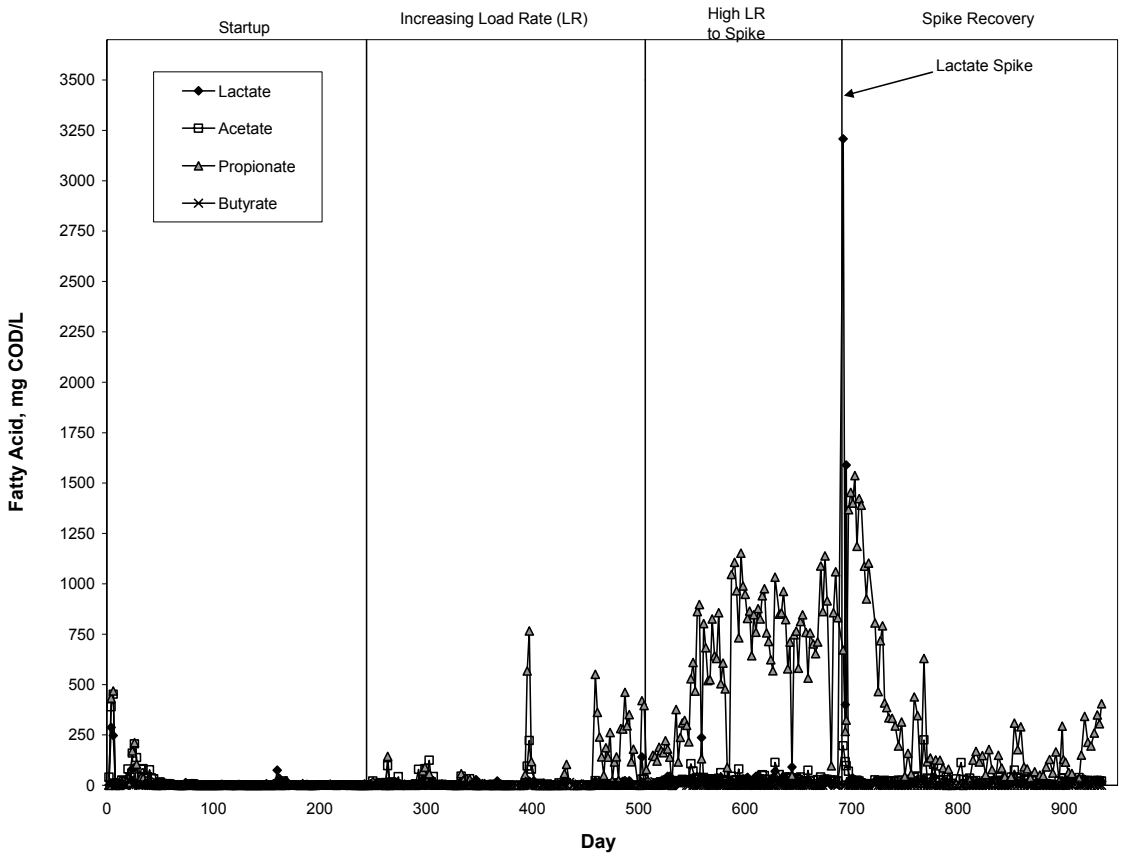


Figure 3-5 Performance of R1: volatile acids vs. time.

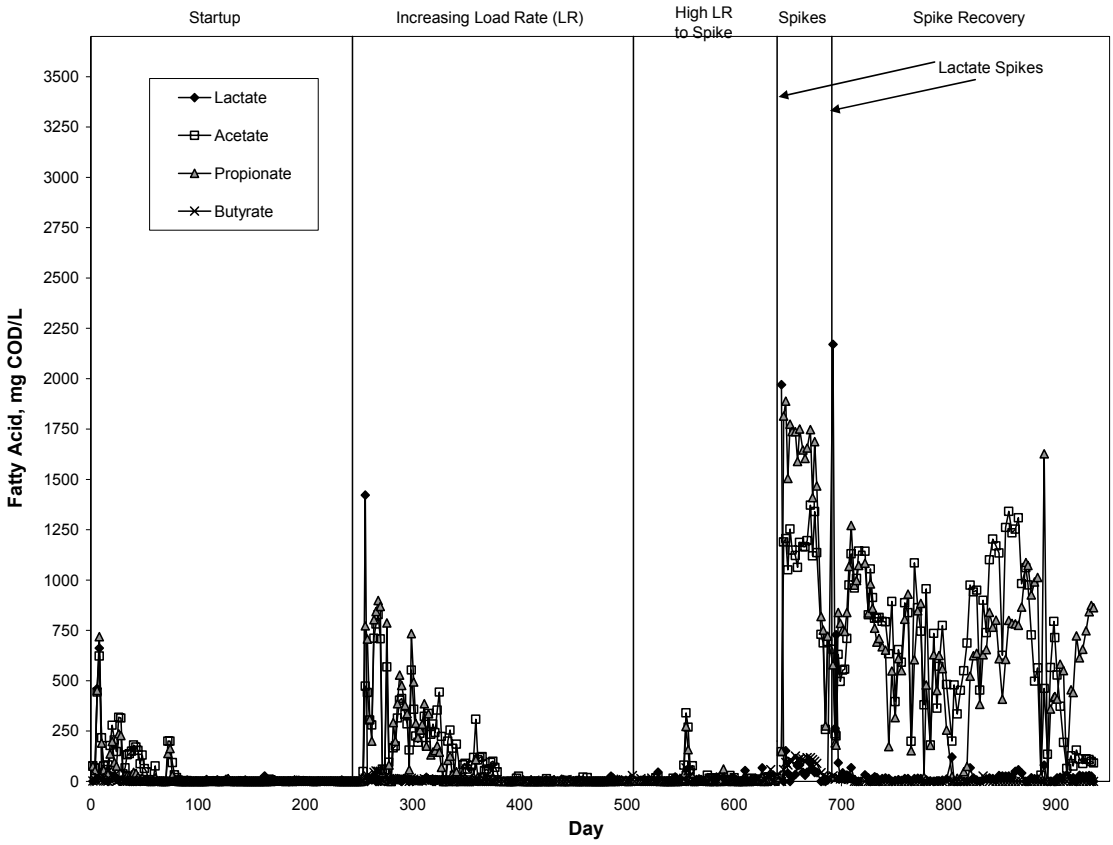


Figure 3-6 Performance of R2: volatile acids vs. time.

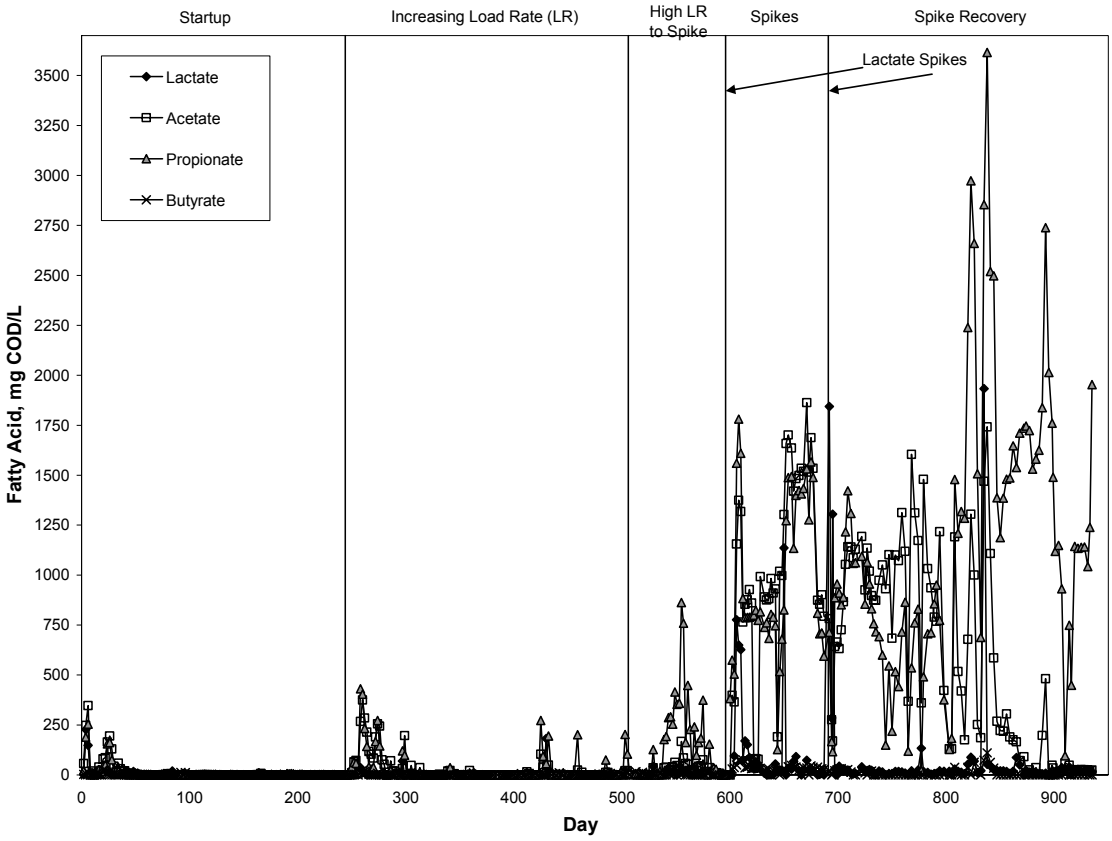


Figure 3-7 Performance of R3: volatile acids vs. time.

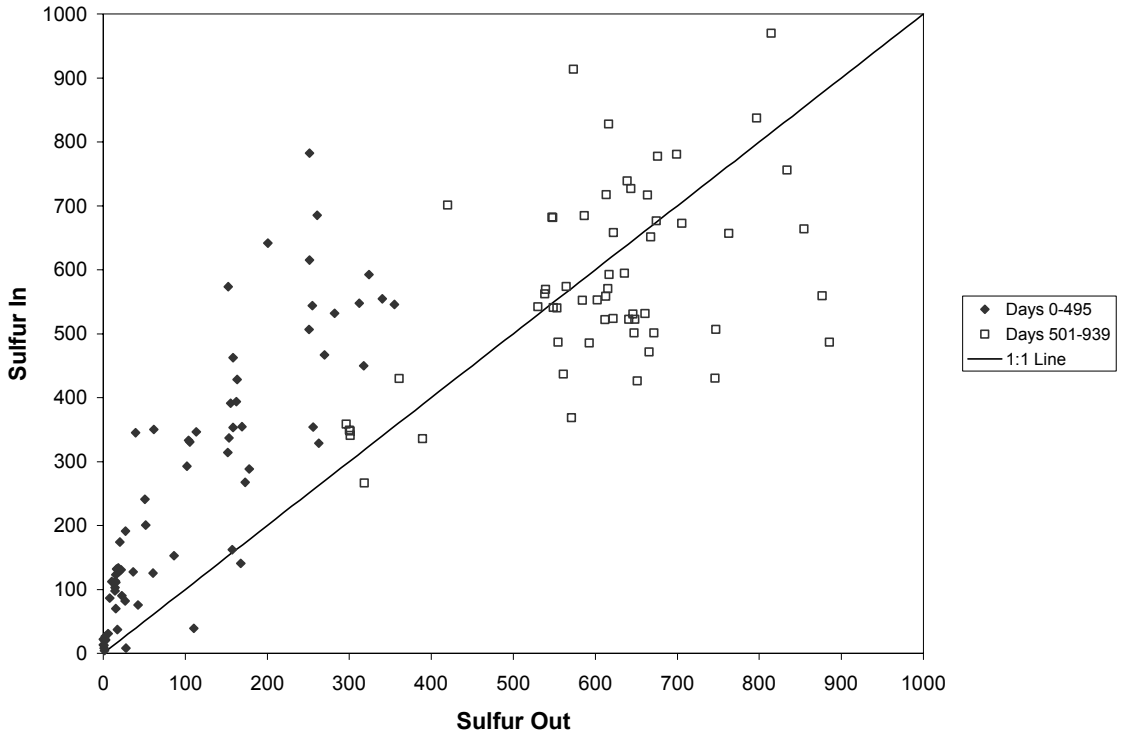


Figure 3-8 Sulfur mass balance for R1.

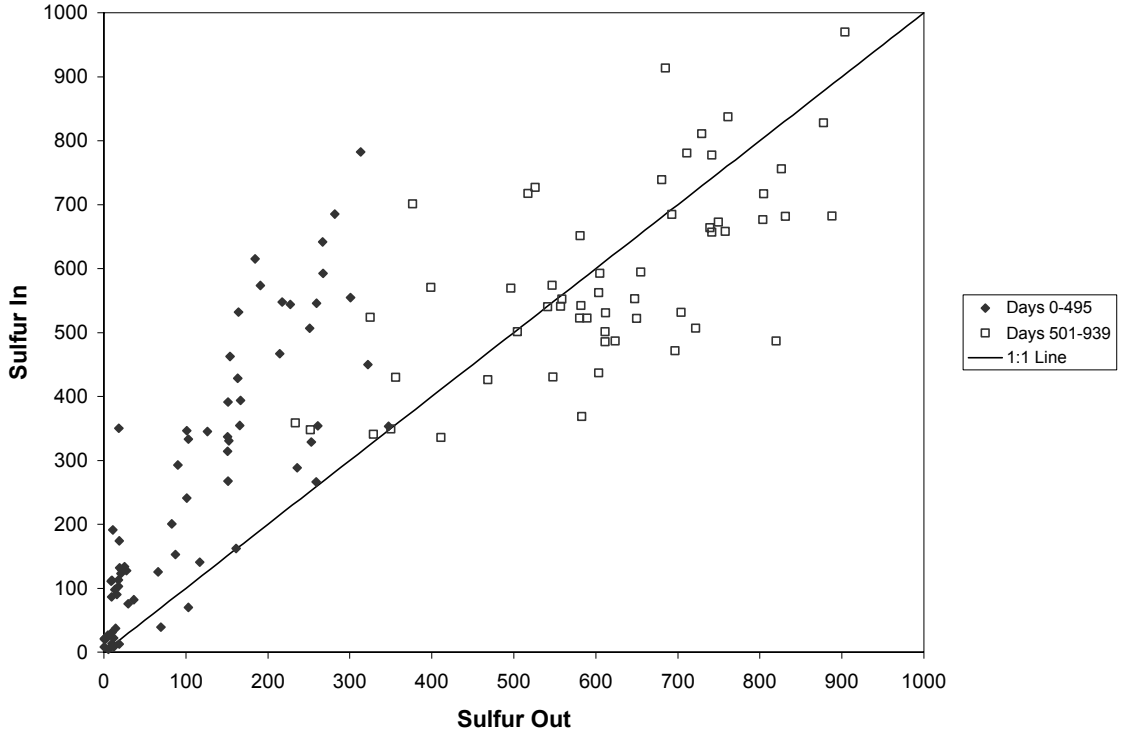


Figure 3-9 Sulfur mass balance for R2.

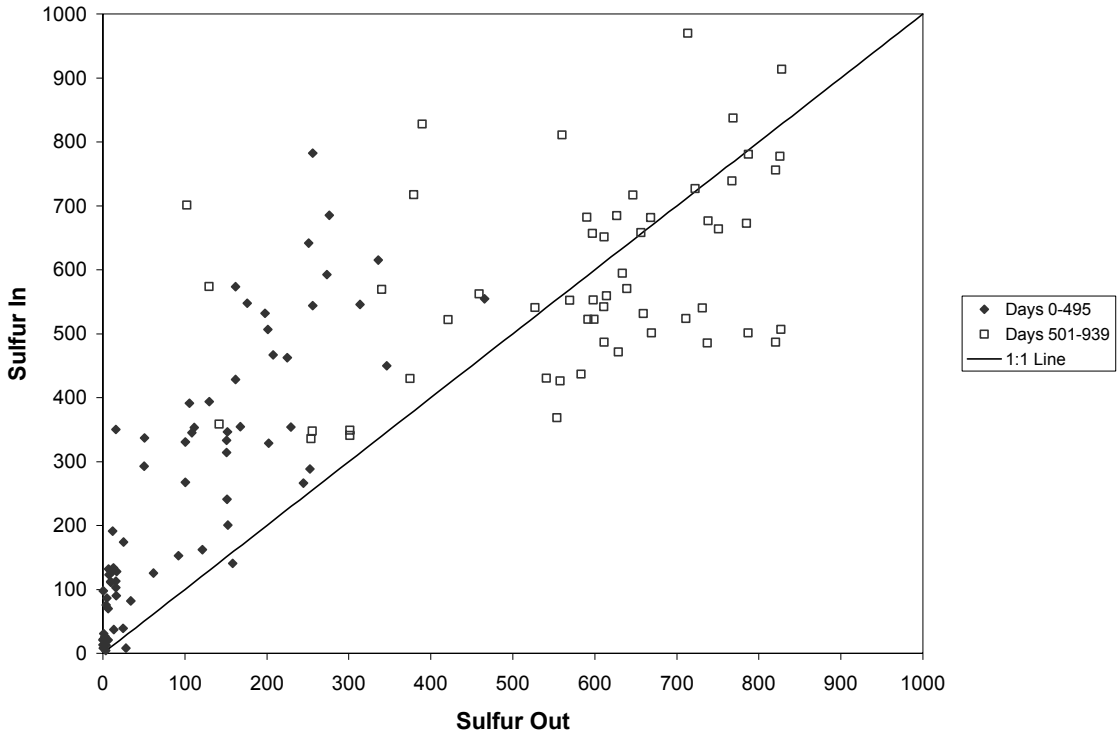


Figure 3-10 Sulfur mass balance for R3.

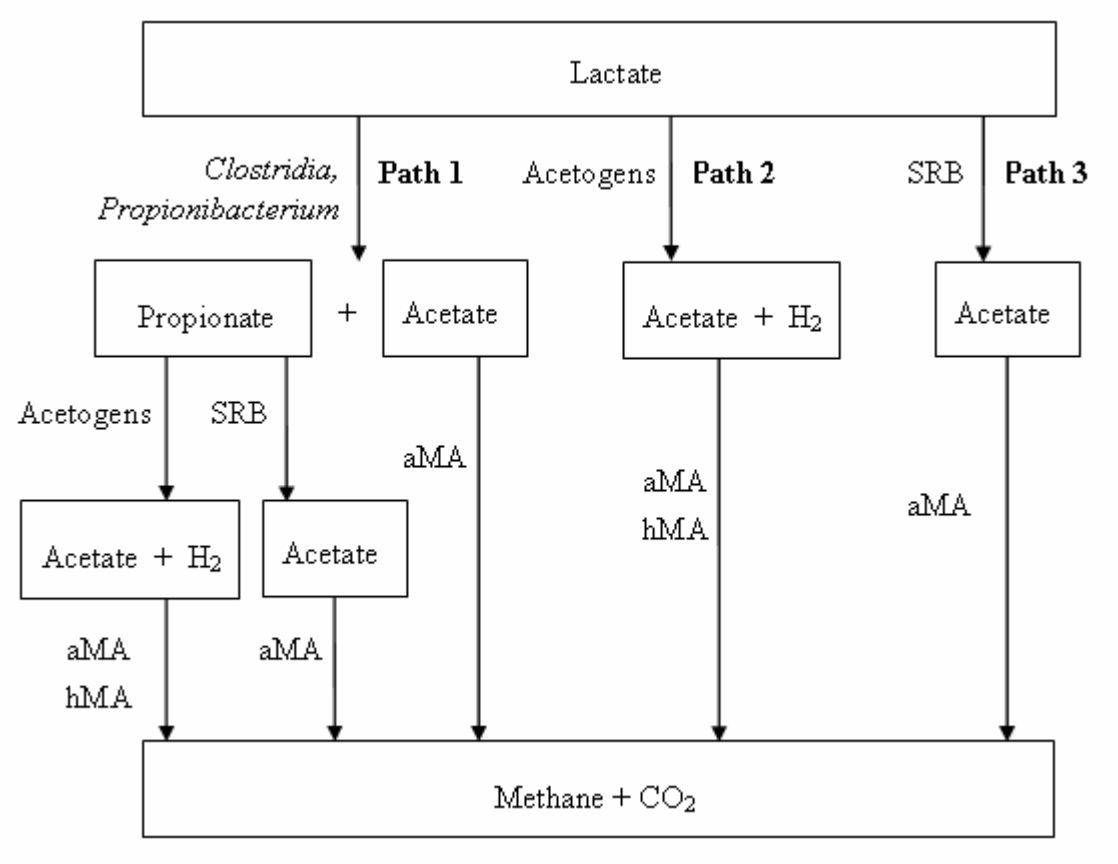


Figure 3-11 Proposed lactate biodegradation pathways for experimental reactors.

CHAPTER IV

CHARACTERIZATION OF SULFATE-REDUCING BACTERIA AND METHANOGEN POPULATIONS IN SULFATE-FED ANAEROBIC BIOFILM REACTORS USING CLONE LIBRARIES

4.1 Introduction

Clone libraries and phylogenetic trees have previously been used to assess the microbial ecology of natural environments where MA and SRB play an important role. In some cases, studies focused on only MA (Chin et al. 2004; Go et al. 2000; Marchesi et al. 2001; Struchtemeyer et al. 2005) or SRB (Kaksonen et al. 2004), but several studies produced phylogenetic trees for both MA and SRB or bacteria (Labrenz and Banfield 2004; Mills et al. 2003; Purdy et al. 2003b). Fewer phylogenetic studies of MA and SRB in engineered environments have been done, however. MA phylogeny has been studied in industrial dye effluent treatment (Plumb et al. 2001), and both MA and bacteria phylogeny has been investigated in anaerobic digesters (Godon et al. 1997) and granular sludges (Sekiguchi et al. 1998). However, no phylogenetic studies have been performed on both MA and SRB in sulfate-fed wastewater treatment systems.

Research investigating microbial communities using techniques other than phylogenetic trees has been done on sulfate-fed anaerobic reactors. While these studies provide insight into the nature of the microbial communities involved in sulfate wastewater treatment, the studies have focused on characterizing either MA (Griffin et al. 1998; Raskin et al. 1994; Zheng and Raskin 2000), or SRB communities (Dar et al. 2007; Elferink et al. 1998a; Elferink et al. 1998c; Kaksonen et al. 2004; Okabe et al. 1999; Santegoeds et al. 1998). Some studies have examined both MA and SRB populations

(Ney et al. 1990; Raskin et al. 1995a; Raskin et al. 1996), but they did not address the impact of long term operation and how the community changes over time. In addition, low-temperature sulfate-fed systems have not been thoroughly investigated.

Given that there is a need for more information regarding the phylogeny of MA and SRB in low temperature sulfate-fed systems and how the community may change over time, the goals of this portion of the research were to (1) observe the microbial community composition in sulfate-fed anaerobic reactors operating at 20 ºC at low and high COD, SLR, and sulfide conditions and (2) to relate community composition to reactor performance under such conditions, especially in light of the variability between reactors that was observed in the operational analysis described in Chapter 3.

4.2 Materials and methods

4.2.1 Reactor operation

Three anaerobic biofilm reactors were constructed and operated as previously described in section 3.2.1.

4.2.2 Sample collection

Reactor samples of 5 g each were collected from all sample ports (Figure 3-1) for each reactor at two different times. R1 was sampled on days 182 and 797, R2 was sampled on days 180 and 799, and R3 was sampled on days 181 and 800. These times correspond to different operating conditions in the reactors (Table 4-1); the first sampling period was characterized by low COD and sulfate loading and low effluent sulfide, while

the second sampling period had high COD and sulfate loading and high effluent VA-COD and sulfide.

4.2.3 DNA extraction

DNA was extracted from all samples on the same day of their removal from the reactors. The extraction was based on a method for DNA extraction from soil (Zhou et al. 1996), with a few modifications. A sample containing the pumice carrier was ground with a sterile mortar and pestle. The sample was then mixed with 13.5 mL of extraction buffer (100 mM Tris-HCl [Ph 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl) and 100 µL proteinase K (10 mg/mL) in sterile 50 mL centrifuge tubes by horizontal shaking at 225 rpm for 30 min. at 37 ºC. Next, 1.5 mL of 20% sodium dodecyl sulfate were added and the sample was incubated in a 65 ºC water bath for two hours with end over end inversions every 15 min. After a centrifugation at 6,000 x *g* for 10 min. at room temperature, the supernatant was collected and transferred to a new sterile 50 mL centrifuge tube. The pellet was extracted two more times by adding 4.5 mL of extraction buffer and 0.5 mL of 20% sodium dodecyl sulfate, vortexing for 10 sec., incubation at 65 ºC for 10 min., and centrifuging as before. Next, supernatants from the three extractions were combined and an equal volume of chloroform-isoamyl alcohol (24:1, vol/vol) was added and mixed by gently inverting the tubes. The aqueous phase was obtained by centrifugation and precipitated with 0.6 volume of iso-propanol at room temperature for 1 hour. A pellet of nucleic acids was obtained after centrifugation at 16,000 x *g* for 20 min. at room temperature, washed with 70% ethanol, and re-suspended in 50 µL of sterile Tris buffer (10 mM [pH 8.5]).

For clone library analysis, a composite DNA sample for each reactor at each of the two time points was made by adding equal volumes of DNA from each port. In other words, for any given reactor, 1.5 µL of DNA from each port was combined with 7.5 µL nuclease-free water to obtain a 15 µL composite sample representative of the entire reactor. For each sample, the DNA concentration was determined and a dilution of 20 ng/µL was made for use in PCR. All DNA concentrations were measured at a wavelength of 260 nm using a Cary 50 UV-Vis spectrophotometer loaded with Cary WinUV software (Varian Inc., Palo Alto, CA).

4.2.4 PCR amplification and cloning

Genomic DNA was extracted from the seed cultures LEC and SEC using the Qiagen DNeasy® Tissue Kit (Valencia, CA). PCR amplifications were performed on each seed culture DNA sample using the archaeal (ARC) and six group specific SRB primer sets (Table 4-2). The primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). All PCR reactions were carried out using a *Taq* PCR Core Kit (Qiagen) at the following concentrations: 1x Qiagen PCR buffer, a 200 µM concentration of each deoxynucleoside triphosphate, 0.025 u/µL *Taq* DNA polymerase, 200 nM of each forward and reverse primer, and 20 ng/µL DNA template. Thermal cycling was performed with an Eppendorf Mastercycler (Westbury, NY) as follows: initial denaturation at 94 ºC for 3 min. followed by 30 cycles of denaturation at 94 ºC for 1 min., annealing at 50 ºC for 1 min., extension at 72 ºC for 1 min., and final extension at 72 ºC for 10 min. The reaction products were visualized with 1% agarose gel electrophoresis.

PCR amplification of the composite reactor DNA was also performed using the ARC and SRB primer sets. At a later time, a bacterial (BAC) clone library was constructed with the same composite DNA using the BAC primer set. All reactions were carried out using the same materials and PCR cycling used for the seed culture DNA. After viewing the reaction products with 1% agarose gel electrophoresis, PCR products were then purified with the QIAquick PCR Purification Kit (Qiagen).

For each of the primer sets, negative and positive controls were performed. For the negative controls, template DNA that was outside of the target group (*Desulfovibrio desulfuricans* genomic DNA for ARC and *Methanococcus maripaludis* genomic DNA for BAC and the SRB groups) was used with the primer sets in a PCR reaction. No bands were observed upon visualization using agarose gel electrophoresis. For the positive controls, genomic DNA that was expected to be amplified by a particular primer set was used as the template for a PCR reaction; all reactions resulted in amplicons when visualized with agarose gel electrophoresis.

For cloning, the purified PCR products were inserted into the PCR4-TOPO cloning vector using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), then transformed into TOP10 *E. coli* cells with kanamycin selection using the manufacturer's instructions. In order to ensure the kit was functioning properly, control reactions for the kit were performed according to the manufacturer's instructions prior to cloning. The plasmids were purified from liquid culture using the QIAprep Spin Miniprep Kit (Qiagen) according to the kit protocol. Selected plasmids were digested with *EcoRI* and visualized by agarose gel electrophoresis to verify the size of the inserts.

4.2.5 Sequencing and phylogenetic analysis

Plasmid inserts were sequenced using reverse M13 primers and labeled dye terminators on an Applied Biosystems (Foster City, CA) Model 3730 DNA sequencer at the University of Iowa DNA Facility. The partial 16S rDNA gene sequences were compared to those in the NCBI database using BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST) (Altschul et al. 1990). Sequence alignments were done using ClustalX v1.81 (Thompson et al. 1997) and corrected with manual inspection. Phylogenetic trees were created from the alignments using the neighbor-joining method (Saitou and Nei 1987). Bootstrap analyses (Felsenstein 1985) of 1,000 replicates were performed in order to assess the reproducibility of all trees.

4.2.6 Nucleotide sequence accession numbers

The 212 nucleotide sequences obtained from the ARC and SRB clone libraries were deposited in the GenBank database under the accession numbers EF592612 to EF592823. The 112 nucleotide sequences obtained from the BAC clone library were deposited in the GenBank database under the accession numbers EU104743 to EU104854.

4.3 Results and discussion

While amplicons were obtained for R1 and R2 for both time periods, no amplicons were obtained for R3 on day 181, even after several attempts. This may have been due to the fact that a smaller amount of DNA was extracted from the reactor samples in R3 than in R1 and R2.

PCR amplification results from R1 and R2 at the two time points and from R3 at the second time point indicated the presence of archaea and SRB from Groups 2 (*Desulfobulbus*), 5 (*Desulfococcus)*, and 6 (*Desulfovibrio, Desulfomicrobium)* (Table 4-2). These amplification products were used to construct the MA and SRB clone libraries. As previously mentioned, bacterial clone libraries were constructed at a later date. Due to limited funds, bacterial clone libraries were only produced for R1 and R2 at the two time points.

All phylogenetic trees (Figures 4-1 through 4-14) were constructed using a neighbor-joining analysis of partial 16S rDNA sequences. Numbers in parenthesis next to a clone name indicate the frequency of appearance of the sequence, if greater than one. Numbers at nodes represent bootstrap values for the nodes out of 1,000 times re-sampling. All trees are unrooted. The scale bar represents a sequence divergence of 10%.

In addition to characterization using clone libraries, reactor samples were examined using microscopy (Appendix D). Samples were taken from the reactors on day 260 and were analyzed prior to constructing the clone libraries. The micrographs provided initial information that the reactors contained a complex, multi-species biofilm.

4.3.1 Seed culture PCR results

Based on agarose gel electrophoresis, both LEC and SEC seed culture DNA samples produced amplicons for archaea, *Desulfovibrio, Desulfococcus,* and *Desulfobulbus*. An amplicon for *Desulfobacterium* was also found in the LEC seed culture.

4.3.2 Archaeal clone libraries

Archaeal clone libraries (Figures 4-1 through 4-3) indicate that several clones were similar to hydrogen utilizing MAs, but most clones were similar to *Methanosaeta concilii*, an MA that uses acetate as its sole electron donor. This evidence agrees with the suggestion in Chapter 3 that aMA play a significant role in acetate removal in the reactors. aMAs were the dominant MA in all reactors under conditions of low and high COD and SLR. Since effluent sulfide was also elevated during the second sampling, especially for R1, it appears that increased sulfide does not cause a change in MA composition.

Regarding other types of MA, in R2, several clones similar to the acetate/hydrogen utilizer *Methanosarcina mazei* were found at both time periods. This is in keeping with previous research, which did not find many *Methanosarcina*-like clones in full-scale anaerobic reactors treating a several types of wastewater (McHugh et al. 2003). Very few hMA clones were found in any reactor at either sampling time. The lack of hMA clones at either time point may be due to the role of SRB in these systems. Unlike acetogens, which generate hydrogen in the consumption of lactate, SRB do not. If SRB consume a majority of the lactate compared to acetogens, hydrogen levels in the reactors will be especially low. Thus, the small populations of hMA present could have been due to a lack of substrate. In fact, SRB were found to dominate the bacterial kingdom in the reactors, as discussed later in section 4.3.4.

These results are consistent with previous research on different anaerobic environments. In natural environments, *M. concilii* were the dominant MA in Antarctic

sediments (Purdy et al. 2003b), chlorinated-solvent contaminated aquifers (Dojka et al. 1998), and gas-condensate contaminated aquifers with high levels of sulfate (Struchtemeyer et al. 2005). *M. concilii* were also found to predominate in engineered systems treating sewage or municipal solid waste (Griffin et al. 1998; McMahon et al. 2001; Raskin et al. 1995b; Zheng and Raskin 2000), and industrial effluents (Plumb et al. 2001). In one study, *M. concilii* were found to make up 90% of the archaeal community in an anaerobic digester (Merkel et al. 1999). In the one study known to have investigated MA in high-sulfate wastewater treatment, it was found that *Methanosaeta* sp. were dominant in a full-scale reactor treating paper mill wastewater (Elferink et al. 1998c).

The aforementioned treatment systems were operated at either 35 ºC or 55 ºC. Similarities in the results from this study to those at elevated temperatures or temperatures found in the natural environment indicate that temperature does not appear to affect the predominance of aMA in anaerobic environments.

It has been found that *Methanosaeta* is dominant in systems operating under low acetate, while under high acetate levels, *Methanosarcina* dominates (Griffin et al. 1998; McMahon et al. 2001; Raskin et al. 1995b; Zheng and Raskin 2000). At low acetate levels, *Methanosaeta* have a competitive advantage over *Methanosarcina* due to the lower half-velocity coefficient and minimum threshold values for acetate (Speece 1996). However, in our study, the R2 and R3 clone libraries at the second time point indicated that *Methanosaeta* were still dominant, even though the average acetate levels were high. This observation is most likely due to the fact that by the time R2 and R3 were operating under higher acetate levels, the *Methanosaeta* population was much greater relative to the

Methanosarcina. *Methanosarcina* may have then had a competitive advantage and started to grow as a result of high acetate, as indicated by a small increase in *Methanosarcina* clones found in the R2 library. However, since *Methanosaeta* were already established as the dominant MA, *Methanosarcina* growth probably would not have affected the population balance.

Nearly all of the studies that have investigated MA population composition in anaerobic biofilm reactors have found *Methanosaeta* to be the dominant MA, not *Methanosarcina*, including some recent studies that employed new microbiological identification techniques (Baloch et al. 2007; Boonapatcharoen et al. 2007; Diaz et al. 2006; Rizzi et al. 2006). While few studies have investigated the long-term operation of anaerobic reactors, there is some evidence that *Methanosaeta* have the ability to maintain a population dominance; in a UASB treating paper mill wastewater, *Methanosaeta* were the dominant MA over a three year time period (Roest et al. 2005).

This fact could be due the way a biofilm develops over time (Zheng et al. 2006). When a new layer develops on the biofilm surface, this layer is dominated by fermenting and sulfate reducing bacteria. When the layer matures and becomes thicker, fermenters and SRB that are located deeper in the biofilm decay due to mass transfer limitation of substrate. The decay of these organisms leaves voids that the MA, located in the deepest layers of the biofilm, are able to fill. Thus, *Methanosaeta*, already established deep in the biofilm, have room for growth in an environment that is protected from the high acetate concentrations seen in the bulk liquid. *Methanosarcina,* present in very low quantities, (if at all), simply cannot gain a foothold in this environment. Thus, even under conditions of high acetate, it may be possible that once *Methanosaeta* establishes a place

within the biofilm, it can continue to be the dominant MA under conditions of both low and high acetate.

4.3.3 Sulfate reducing bacteria clone libraries

It should be noted that the SRB libraries referred to in the following discussion are a combination of three separate clone libraries (*Desulfofibrio-Desulfomicrobium, Desulfococcus,* and *Desulfobulbus*) that were combined to make the aggregate SRB libraries. This method of combining the separate libraries means that comparisons of number of clones between the groups cannot be made. However, comparisons between the two sampling times of the number of clones within a particular group can be made.

During the first sampling time, since COD and SLR in the reactors were low, competition would have been likely for electron donors (lactate and hydrogen) and the electron acceptor (sulfate). All of the SRB types present in the reactors could utilize lactate, hydrogen, and sulfate (Figures 4-4 through 4-6). In addition to lactate and hydrogen, some of the SRB groups can utilize electron donors like acetate and propionate. However, since acetate and propionate levels were low in the reactors during the first sampling, factors such as sulfate affinity and sulfate utilization rates and not metabolic flexibility would have made an impact on the SRB population.

At the first time point in R1, many clones were found in the *Desulfomicrobium* and *Desulfovibrio* group (Figure 4-4a). A second group of clones were similar to the genus *Desulfobacterium*, and another cluster was found to be similar to the genus *Desulfobulbus*. The *Desulfovibrio- Desulfomicrobium* group and *Desulfobulbus* have previously been found to be the main SRB groups in anaerobic sludge digesters (Raskin

et al. 1995b) and lab-scale biofilm reactors treating sulfate wastewater at 20 ºC (Alvarez et al. 2006).

As in R1, the R2 clone library from the early time point shows many clones from the *Desulfomicrobium* and *Desulfovibrio* (Figure 4-5a) group. However, no clones relating to *Desulfobacterium* were found in R2. This may due to the differences in the initial seed inoculum between the reactors. The LEC used to seed R1 contained *Desulfobacterium*, while the SEC used to seed R2 did not.

The presence of *Desulfomicrobium-Desulfovibrio* and *Desulfobacterium* under conditions of low or no sulfate has also been found in previous research (Raskin et al. 1996). *Desulfobacterium* can be found in engineered systems operating with very low influent sulfate (0.4 mg/L) (Raskin et al. 1995a; Raskin et al. 1995b), or in natural systems where sulfate is also usually low (Labrenz and Banfield 2004; Purdy et al. 2003a). It appears that *Desulfomicrobium-Desulfovibrio* and *Desulfobacterium* have the ability to utilize electron acceptors other than sulfate. Thus under low sulfate conditions, they are likely to have a competitive advantage over other SRB groups.

It has also been shown that in a competition for sulfate among *Desulfobacter, Desulfovibrio,* and *Desulfobulbus*, *Desulfovibrio* consumed sulfate fastest, followed by *Desulfobulbus,* then *Desulfobacter* (Laanbroek et al. 1984). The presence of *Desulfovibrio-Desulfomicrobium* in the reactors at the first sampling could be explained by their higher affinity for sulfate compared to other organisms (at least compared to *Desulfobulbus*), and also possibly their ability to out-compete other organisms like fermenters for lactate. However, a review of previous studies on SRB groups did not

reveal any data comparing lactate utilization rates of the different SRB groups, so this hypothesis cannot be proven at this time.

Results from the second clone libraries indicate that unlike the MA populations, which did not appear to change over time, the SRB populations changed significantly. In contrast to the first sampling period when many *Desulfobacterium* clones were found, there were no *Desulfobacterium* clones found in R1, R2, or R3 for the second sampling. Since previous findings have shown that *Desulfobacterium* can be found in low sulfate environments, perhaps an increase in SLR may have caused *Desulfobacterium* to decrease in population. While no kinetic data comparing the affinity of different SRB for sulfate has been found, it may be the case that *Desulfobacterium* have a high affinity for sulfate but a low maximum specific utilization rate. In addition, it may be the case that other SRB groups were able to out-compete *Desulfobacterium* for lactate. Although *Desulfobacterium* can also use acetate as an alternate electron donor, providing it with added possible substrates at the later time when acetate was higher in R2 and R3, it is likely that *Desulfobacterium* could not take advantage of this because aMA were utilizing a majority of the acetate. Evidence of this may be found in the acetate microcosm study results (Appendix C), which indicate that for all reactors, acetate removal occurs without the concurrent removal of sulfate (Figures C-8 to C-12).

While *Desulfobacterium* clones disappeared between the two samplings, many *Desulfovibrio-Desulfomicrobium* clones were found in all reactors at the second sampling. *Desulfobulbus*-like clones were found to increase in R1 and decrease slightly in R2, and many *Desulfobulbus* clones were also found in R3. At the time of the second sampling, the lactate feed concentration was over 5 g-COD/L. It is likely that fermenters

such as *Clostridia* and *Propionibacterium* consumed some of the lactate, as evidenced by the production of propionate in the reactors. These higher propionate levels most likely stimulated the growth of propionate-utilizing *Desulfobulbus*.

Desulfococcus-like clones, which were not found in R1 or R2 at the first sampling, were found in all reactors during the second sampling. Although *Desulfococcus* is a cSRB, it is likely that they were consuming lactate or propionate and not acetate, as indicated by the acetate microcosm study results (Appendix C). This finding is consistent with several other studies that have found cSRB to play little, if any, role in acetate degradation compared to aMA (Elferink et al. 1998b; O'Flaherty et al. 1998; Visser et al. 1993). cSRB such as *Desulfococcus* grow slower than iSRB (Widdel 1988) and take longer periods of time to become established in anaerobic reactors (Omil et al. 1998). This may be especially true when sulfate levels are low during startup, as in this study. Lactate consumption for cSRB requires more sulfate than iSRB; the ratio of required mols of sulfate to lactate is 3/2 for cSRB and 2/2 for iSRB (Widdel 1988). Thus, it follows that *Desulfococcus* did not become a significant part of the SRB population until the reactors were mature and operating at a higher SLR.

Elevated sulfide levels may also have had an effect on the types of SRB found in the clone libraries. Acetate utilizing SRB had a very low K_I of 35 mg DS/L (Table 3-3), but since the acetate utilizing *Desulfococcus* were found not to be degrading acetate, the role of sulfide inhibition for cSRB is not noteworthy. However, the K_I values do indicate that propionate utilizing SRB have a higher inhibition constant than hydrogen/lactate utilizing SRB (681 vs. 422 mg DS/L, respectively). Thus, as sulfide levels rose over

time, propionate utilizing SRB may have been less affected by sulfide and were able to grow faster under these conditions compared to the lactate utilizing SRB.

4.3.4 Bacterial clone libraries

Due to the large number of clones sequenced for the bacterial libraries, two phylogenetic trees were constructed for each reactor at a particular time point. The clones were divided into two groups: those belonging to the phylum *Proteobacteria* and all other bacterial clones. While the relative amount of SRB clones could not be compared in the SRB libraries, the use of a common primer set in the bacterial library allows for direct comparison of the number of different SRB groups and other bacterial clones.

For all bacterial clone libraries, the majority of clones belonged to the phylum *Proteobacteria* with SRB dominating the *Proteobacteria* in all libraries (Figures 4-7 through 4-14). About 80% of the clones from both sampling times in R1 and the first sampling in R2 were *Proteobacteria*, but only 50% in the second R2 library were *Proteobacteria.*

In both R1 and R2, the first sampling showed that *Desulfovibrio-Desulfomicrobium* was the principal SRB group, while the second sampling showed that the *Desulfovibrio-Desulfomicrobium* group decreased in size while *Desulfobulbus* increased to become the dominant SRB in both reactors. This shift is probably not due to sulfate affinity, since *Desulfovibrio* have a higher affinity for sulfate than *Desulfobulbus*, and sulfate was not limiting due to the increasing SLR. Sulfide inhibition is not likely, because *Desulfovibrio* appear to have a high tolerance for sulfide. In a pure culture

sulfide toxicity study, it was found that sulfide levels as high as 1100 mg-S/L (240 mg-S/L H_2S) did not inhibit the degradation of lactate by *Desulfovibrio desulfuricans* (Figure 4-15).

Lactate spiking may have caused the change in SRB population composition. Excess lactate allowed fermenters to consume lactate and produce propionate, which was then consumed by *Desulfobulbus* and *Desulfococcus* to a lesser extent. The alternative substrate allowed for rapid growth of these groups that were already present in the reactors, but in small numbers relative to *Desulfovibrio*. After the *Desulfobulbus* population size was comparable to *Desulfovibrio,* perhaps they began to out-compete *Desulfovibrio* for lactate. Deprived of substrate, the population began to diminish. While this cannot be proven since no lactate affinity data could be found for confirmation, it may explain the shift in the SRB composition.

Other bacterial groups that were found in the reactors include the propionate producing *Clostridia* and *Propionibacterium* (Table 2-2, reaction 9). For R1, 45 out of 70 clones in the first library were SRB and 11 out of 70 clones were *Propionibacterium*. By the second sampling, 56 of the 69 clones were SRB and there were no propionate producing bacteria found. While the R1 library became enriched in SRB, the R2 clone libraries shifted in the opposite direction. SRB clones made up 52 out of 64 clones in the first library and 3 out of 64 clones were either *Clostridia* or *Propionibacterium*. By the second time point, an increase in propionate-utilizing clones was found. Only 27 out of the 65 clones were SRB, compared to 22 *Clostridia* clones. The presence of *Clostridia* in R2 explains why elevated propionate was found in the R2 effluent after the lactate spikes (Figure 3-6). By the second time point, SRB were in competition with *Clostridia* for

lactate. In contrast, SRB and not fermenters were using nearly all of the lactate added to R1 after the lactate spikes (Figure 3-5), resulting in low propionate in the effluent.

In addition to fermenters, a few types of syntrophs and *Bacteroides* clones were observed. It is unclear why any *Bacteroides* clones were found, since these anaerobic organisms are typically found in mammalian guts. Regarding the syntrophs, their small numbers is in keeping with the low number of hMAs found in the *Archaea* library, since little hydrogen would be produced by the syntrophic population for consumption by hMA. In fact, previous research has found that propionate-degrading syntrophic methanogenic consortia are of little importance in sulfate-fed reactors (Visser et al. 1993). This is probably due to the fact that specific growth rates for propionate degrading SRB such as *Desulfobulbus propionicus* can be much higher than for propionate degrading syntrophic organisms in such as *Syntrophobacter wolinii* (2.64 vs. 0.2 day^{-1}, respectively) (Elferink et al. 1994).

4.3.5 Effect of microbial populations on reactor performance

SRB were the dominant bacterial clone in both R1 libraries. Lactate utilizing SRB made up 63% of the bacterial clones in the first library. While lactate utilizing SRB only made up 16% of the bacterial population in the second library, propionate utilizing SRB made up 65% of the bacterial clones. In R2, while the first bacterial library was dominated by lactate utilizing SRB (81%), the second library showed fewer lactate utilizing SRB (6%), and roughly even amounts of propionate utilizing SRB (35%) and fermenters (34%).

Some fermenters such as *Propionibacterium* were found in R1 at the first sampling (16% of the first bacterial clone library), but none were found at the second time. The presence of a few propionate-producers early in the reactor history indicates that the rise in propionate starting around day 450 in R1 (Figure 3-5) could have been due to these *Propionibacterium*. When the lactate shock load was applied to R1 (Figure 3-2), *Propionibacterium* were present to degrade the excess lactate in the reactor, as indicated by the presence of propionate after the spike. This propionate allowed *Desulfobulbus* to grow, as evidenced by a concomitant rise in effluent sulfide concentration when effluent propionate levels decreased. However, after the propionate was consumed by *Desulfobulbus*, this microbial group probably started to consume lactate. Growth with lactate may be faster than with propionate (Samain et al. 1984; Widdel 1988), probably due the greater free energy change associated with lactate degradation by SRB than propionate degradation (Table 2.1 equations 7 and 8). Thus the fermenter population might have diminished due to competition with SRB for lactate.

In contrast, R2 did not have as many *Propionibacterium* clones at the first sampling, indicating a smaller propionate-producing population. Indeed, little propionate was seen in R2 or R3 until after the lactate spiking, when the presence of so much lactate may have allowed the growth of propionate producers, as evidenced by the existence of a large number of *Clostridium* clones in R2 for the second sampling (Figure 3-11 path 1). When *Clostridia* produced propionate in R2 (Figure 3-6), *Desulfobulbus* began to grow as a result. Since this process occurred later in time in R2 compared to R1, *Desulfobulbus* comprised a smaller percentage of the bacterial clones in R2 than in R1

(35% vs. 65%). Since the *Desulfobulbus* population in R2 was small, propionate continued to remain high well after lactate spiking.

Although a bacterial clone library was not constructed for R3, it probably behaved somewhat similar to R2. Sulfate reduction played a smaller role in lactate removal in R3 compared to R1 and R2, as evidenced by the lactate microcosm studies. While lactate was removed in the R3 studies, this was not accompanied by significant sulfate removal, indicating that organisms such as fermenters played a larger role in lactate removal in R3 (Figure 3-11 paths 1 and 2).

Lower levels of propionate degrading populations have been found in stable anaerobic digesters (Xing et al. 1997b). It has been proposed that such digesters with a history of stable operation are more susceptible to failure during an organic shock load (McMahon et al. 2001). While these studies focused on syntrophic propionate oxidizing bacteria, the role of propionate degrading SRB would be similar in a sulfate-fed system. It appears that R1 showed recovery from the shock load due to the presence of larger population of propionate-degrading *Desulfobulbus*, while R2 (and probably R3), with a history of stable operation prior to the first lactate spike, did not have a significant *Desulfobulbus* population to consume the large quantities of propionate produced by the fermentation of lactate.

In addition to propionate formed in the reactors when lactate was consumed, large amounts of acetate were produced. The presence of high levels of acetate in R2 and R3 may be explained by the concurrently high propionate levels. It has been observed that under conditions of high sulfide and high propionate, acetate degradation is inhibited (Oleszkiewicz et al. 1989). However, in the case of the R1 after the lactate spike, it

appears that even under conditions of high sulfide, there is a significant population of aMA capable of functioning well enough to remove significant portions of COD in the form of acetate.

4.4 Conclusions

This study investigated the methanogenic archaea and sulfate reducing bacteria populations of an anaerobic filter treating sulfate-rich wastewater operating at 20 ºC. The following observations can be made from our research.

- Reactors that contain large populations of lactate- and propionate-utilizing SRB, acetate utilizing MA, and small populations of fermenters appear to have the ability to withstand high levels of sulfide and have a greater resistance to organic shock loading.
- Acetate utilizing *Methanosaeta*-like MA were the dominant MA in all reactors at both sampling times, even under conditions of elevated sulfide.
- In the first sampling in R1, lactate utilizing SRB were the major bacteria found (63%), with small amounts of fermenters present (16%). In R2, lactate utilizing SRB were also dominant (81%), but very few fermenters were observed (5%). These results indicate that the main pathway for lactate biodegradation during the first sampling in R1 and R2 was Path 3 (Figure 3-11).
- The SRB composition changed dramatically over time. In R1, the population shifted away from lactate utilizing SRB and fermenters towards propionate degrading SRB, while in R2, the lactate utilizing SRB population diminished and fermenter and propionate degrading SRB populations were roughly equal. Thus,

the lactate degradation pathway shifted to Path 3 with some routing of lactate to Path 1 in R1, and roughly equal routing of lactate through Paths 1 and 3 in R2.

- R1 exhibited larger populations of the propionate degrading *Desulfobulbus* present than R2 perhaps because it was subjected to perturbations in propionate levels prior to lactate spiking. Evidence suggests that a large amount of *Desulfobulbus* in R1 allowed for a faster recovery from lactate spiking.
- R2 exhibited elevated propionate after lactate spiking due to the combined effect of having a large population of fermenters present to produce propionate and a smaller propionate degrading SRB population compared to R1 present to remove propionate.

Table 4-1 Reactor conditions during sample collection times.

	First Collection[a]			Second Collection[b]		
	R1	**R2**	**R3**	**R1**	**R2**	**R3**
VA-COD in[c]	1,437 ± 727 (201)	1,437 ± 727 (201)	1,437 ± 727 (201)	6,689 ± 955 (74)	5,361 ± 1,089 (74)	2,290 ± 110 (74)
VA-COD out[c]	9 ± 27 (101)	137 ± 453 (101)	45 ± 145 (101)	330 ± 400 (71)	1,415 ± 592 (71)	1,814 ± 673 (74)
Acetate out[c]	3 ± 11 (101)	54 ± 172 (101)	24 ± 70 (101)	28 ± 30 (71)	768 ± 307 (71)	673 ± 453 (71)
Propionate out[c]	3 ± 14 (101)	64 ± 209 (101)	22 ± 72 (101)	294 ± 395 (71)	628 ± 346 (71)	1,108 ± 589 (71)
Sulfate in[d]	139 ± 89 (101)	139 ± 89 (101)	139 ± 89 (101	591 ± 136 (69)	591 ± 136 (69)	591 ± 136 (69)
Sulfate out[d]	21 ± 32 (100)	28 ± 43 (101)	17 ± 29 (101)	125 ± 122 (69)	188 ± 132 (69)	280 ± 116 (69)
Sulfide out[d]	17 ± 30 (31)	14 ± 24 (31)	16 ± 40 (31)	501 ± 136 (30)	482 ± 147 (30)	360 ± 123 (30)

[a] average, days 80-280 ± standard deviation for (n) samples

[b] average, days 700-900 ± standard deviation for (n) samples

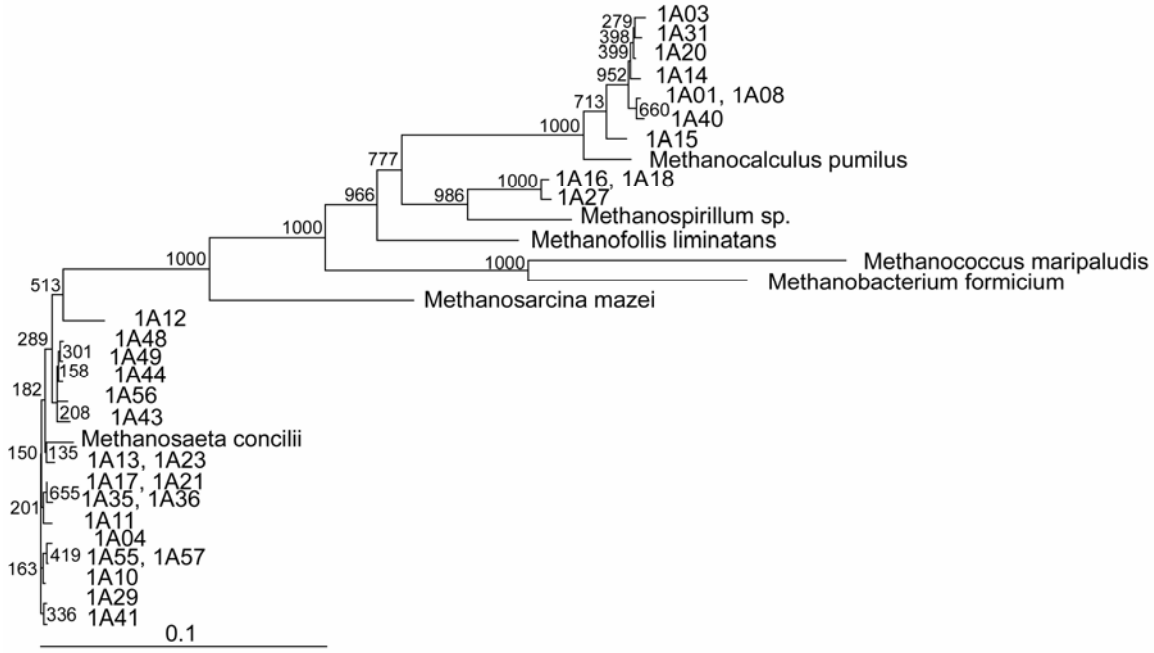
[c] mg-COD/L

[d] mg-S/L

Table 4-2 Oligonucleotide primers used in this study.

Primer Set	Product Size (bp)	5'-3' Sequence	Target Group	Source	Amplicons from time period: Early	Late
1Af 1100Ar	1099	TCYGKTTGATCCYGSCRGAG TGGGTCTCGCTCGTTG	Domain *Archaea*	Embley et al. 1992	+	+
27F 1492R	1465	AGAGTTTGATCCTGGCTCAG GGTTACCTTGTTACGACTT	Domain *Bacteria*	Lane 1991	+	+
DFM140 DFM842	702	TAGMCYGGGATAACRSYKG ATACCCSCWWCWCCTAGCAC	SRB Group 1 *Desulfotomaculum*	Daly et al. 2000	-	-
DBB121 DBB1237	1116	CGCGTAGATAACCTGTCYTCATG GTAGKACGTGTGTAGCCCTGGTC	SRB Group 2 *Desulfobulbus*	Daly et al. 2000	+	+
DBM169 DBM1006	837	CTAATRCCGGATRAAGTCAG ATTCTCARGATGTCAAGTCTG	SRB Group 3 *Desulfobacterium*	Daly et al. 2000	-	-
DSB127 DSB1273	1146	GATAATCTGCCTTCAAGCCTGG CYYYYYGCRRAGTCGSTGCCCT	SRB Group 4 *Desulfobacter*	Daly et al. 2000	-	-
DCC305 DCC1165	860	GATCAGCCACACTGGRACTGACA GGGGCAGTATCTTYAGAGTYC	SRB Group 5 *Desulfococcus*	Daly et al. 2000	+	+
DSV230 DSV838	608	GRGYCYGCGTYYCATTAGC SYCCGRCAYGYRTYCATC	SRB Group 6 *Desulfovibrio/* *Desulfomicrobium*	Daly et al. 2000	+	+

a)



b)

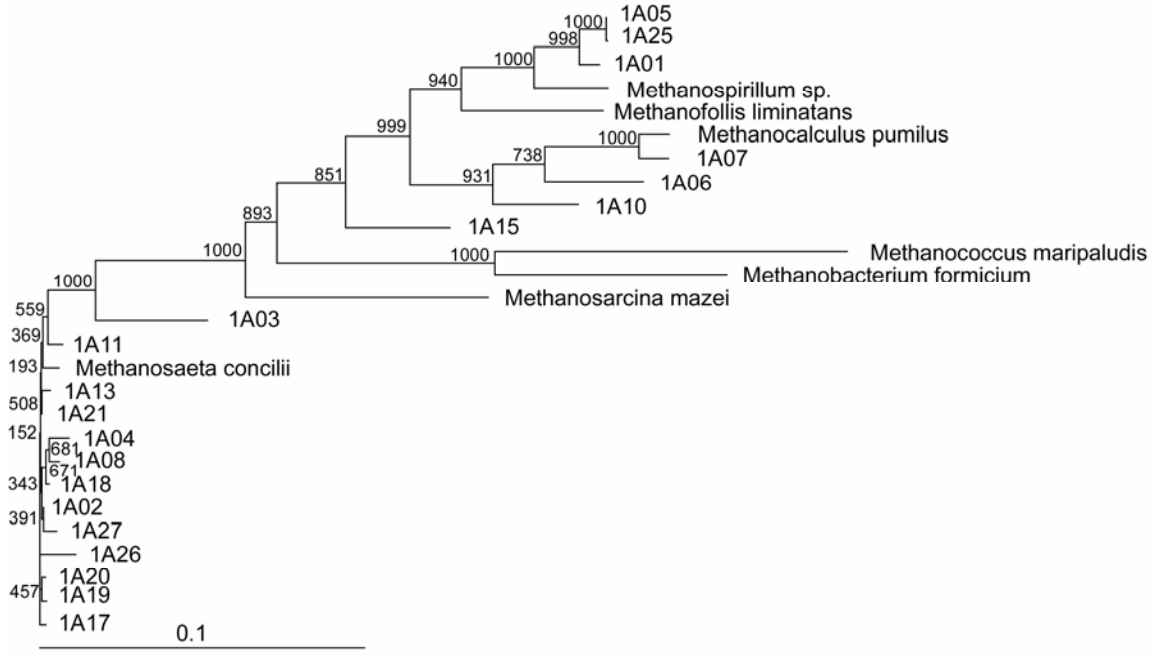
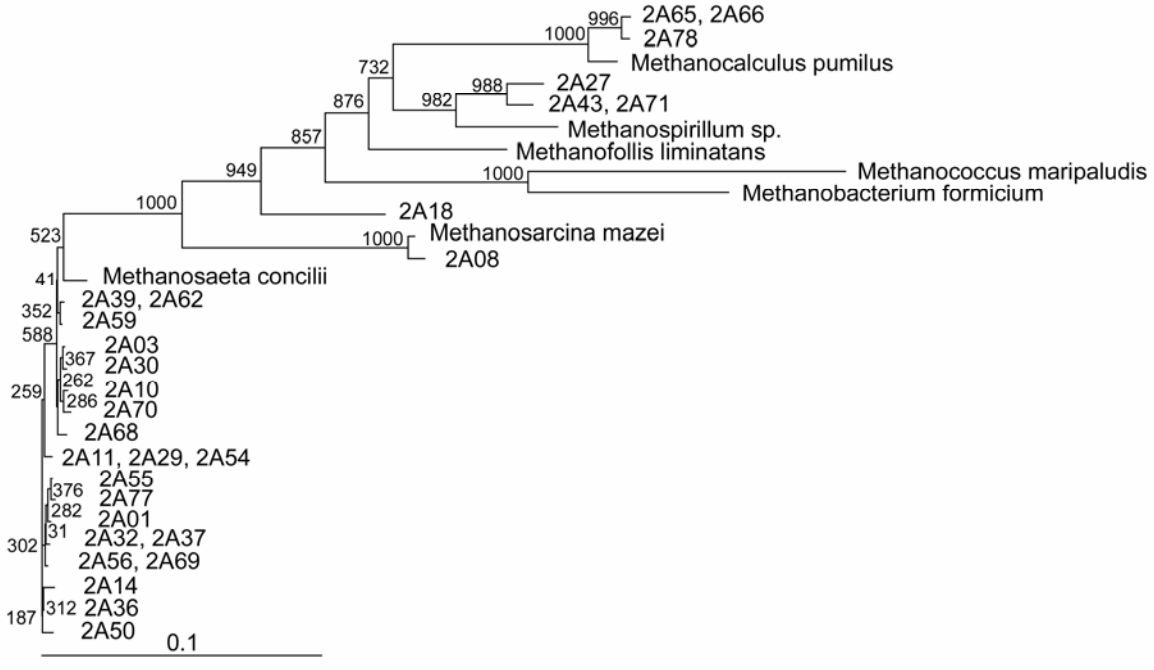


Figure 4-1 Phylogenetic tree of *Archaea* from R1 on a) day 182 b) day 797.

a)



b)

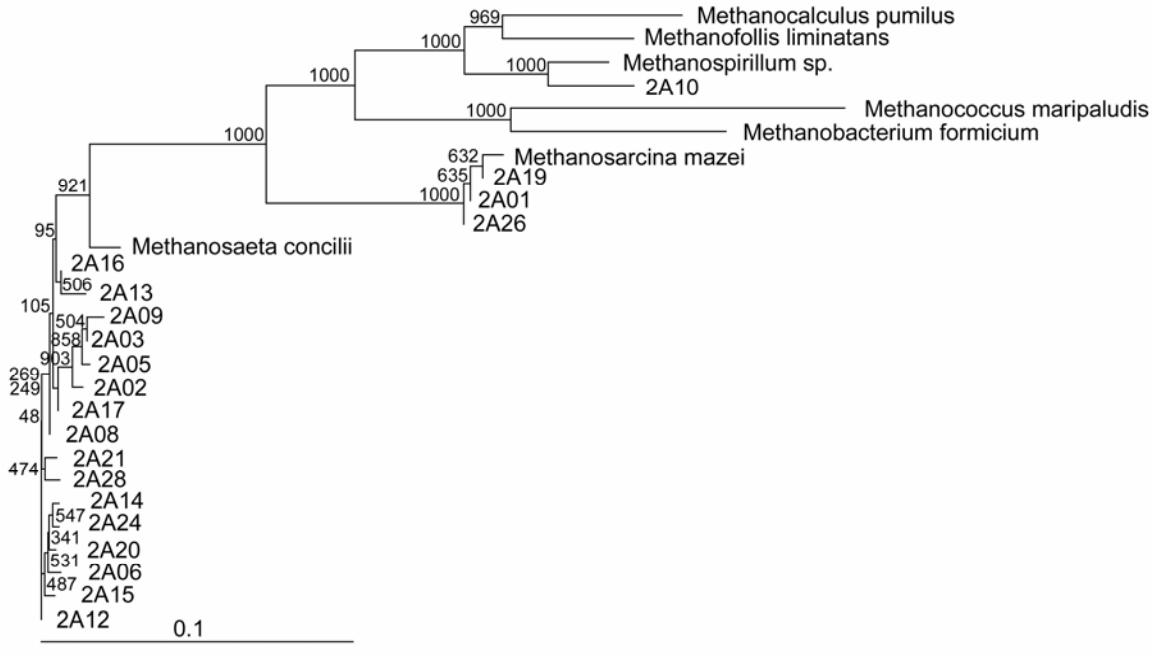


Figure 4-2 Phylogenetic tree of *Archaea* from R2 on a) day 180 b) day 799.

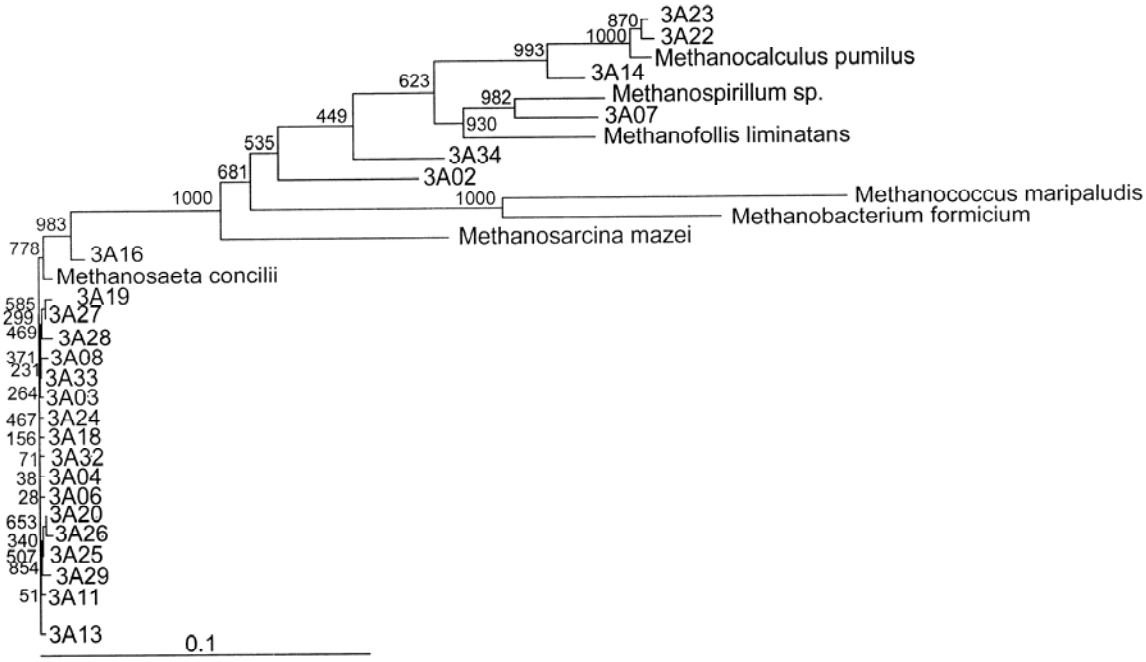


Figure 4-3 Phylogenetic tree of *Archaea* from R3 on day 800.

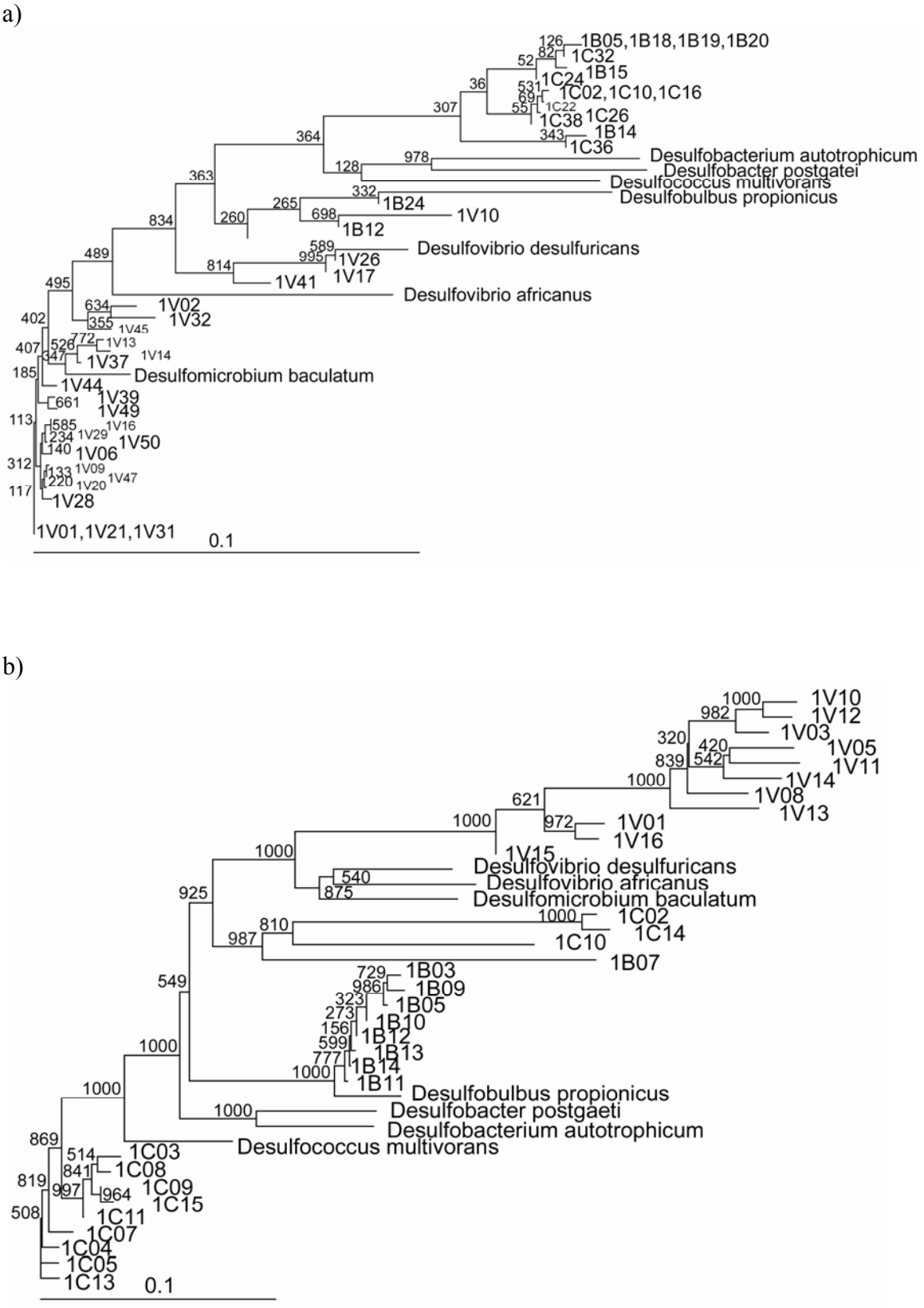
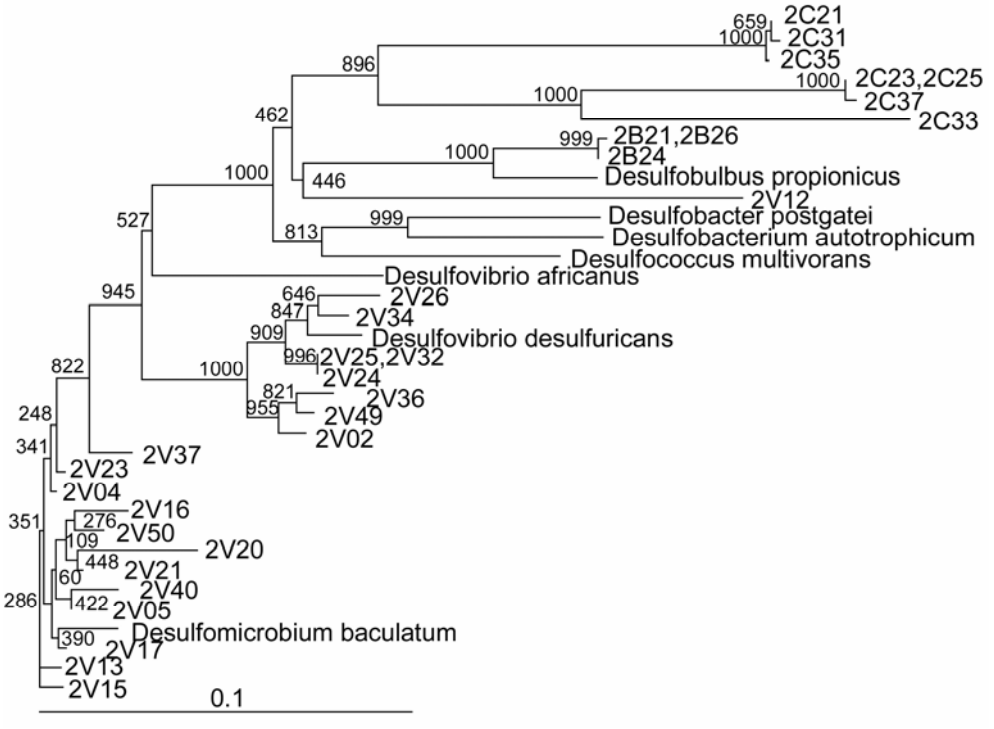


Figure 4-4 Phylogenetic tree of SRB from R1 on a) day 182 b) day 797.

a)



b)

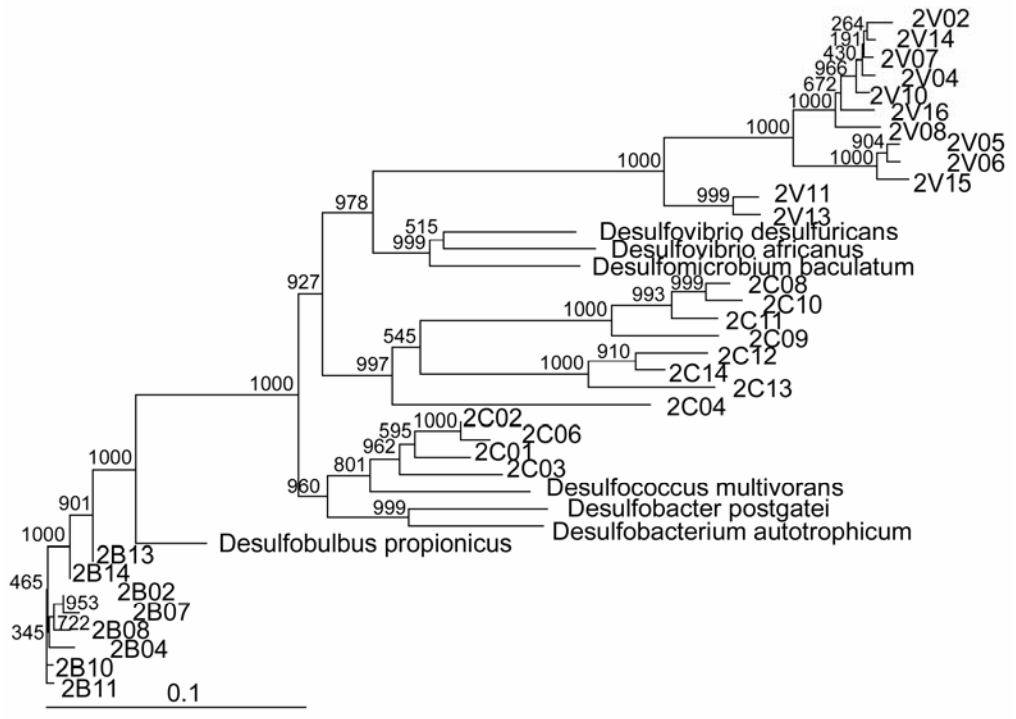


Figure 4-5 Phylogenetic tree of SRB from R2 on a) day 180 b) day 799.

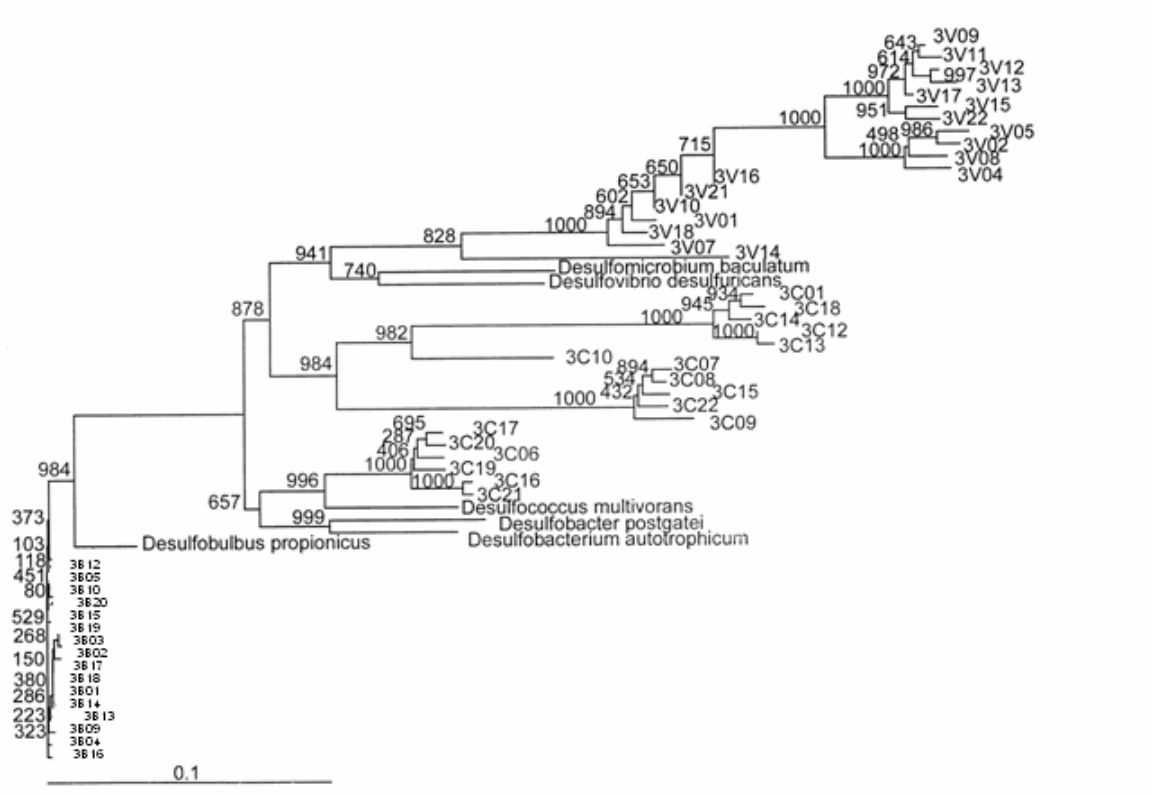


Figure 4-6 Phylogenetic tree of SRB from R3 on day 800.

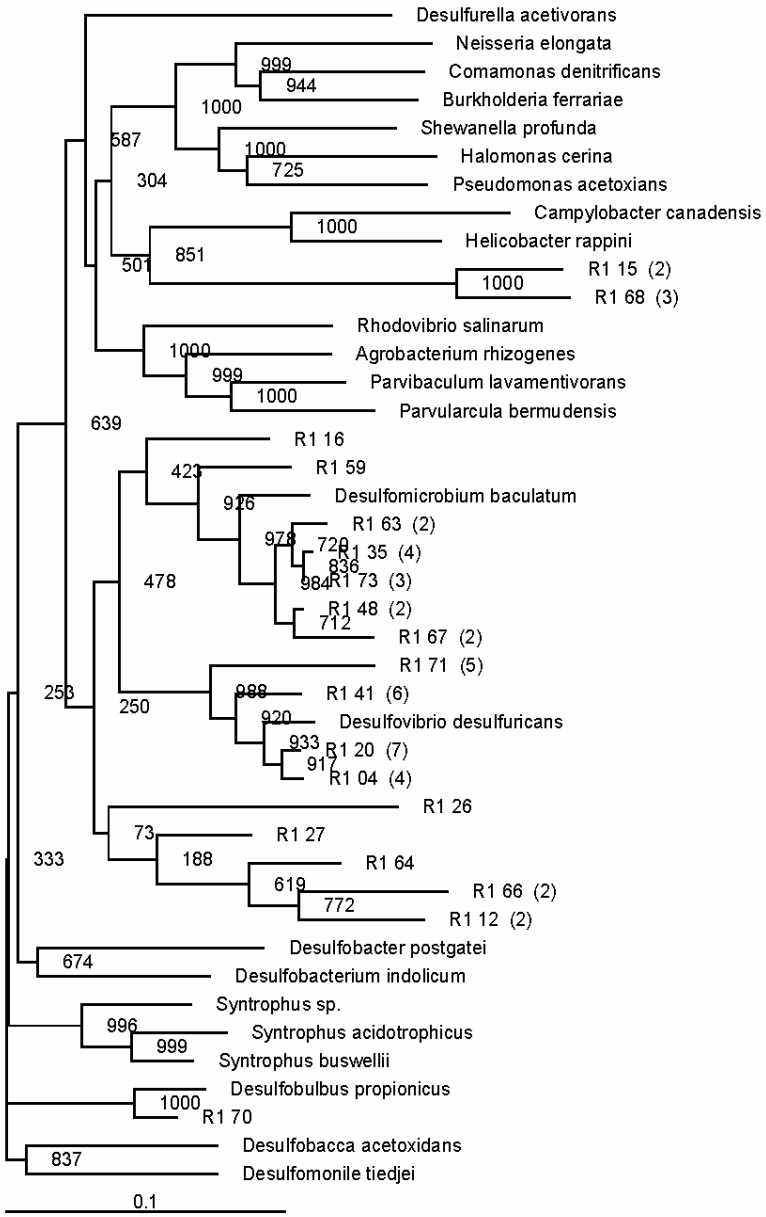


Figure 4-7 Phylogenetic tree of *Bacteria* from R1 on day 182 for phylum *Proteobacteria.*

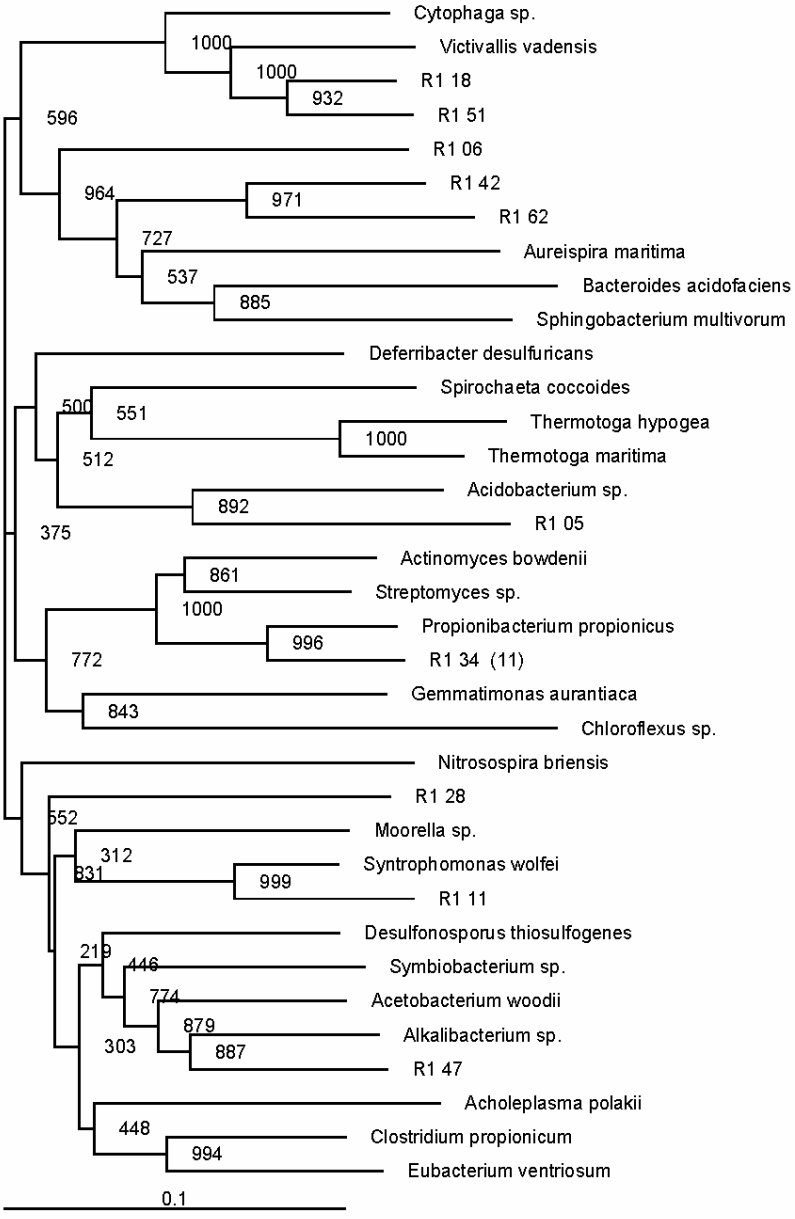


Figure 4-8 Phylogenetic tree of *Bacteria* from R1 on day 182 for non-*Proteobacteria*.

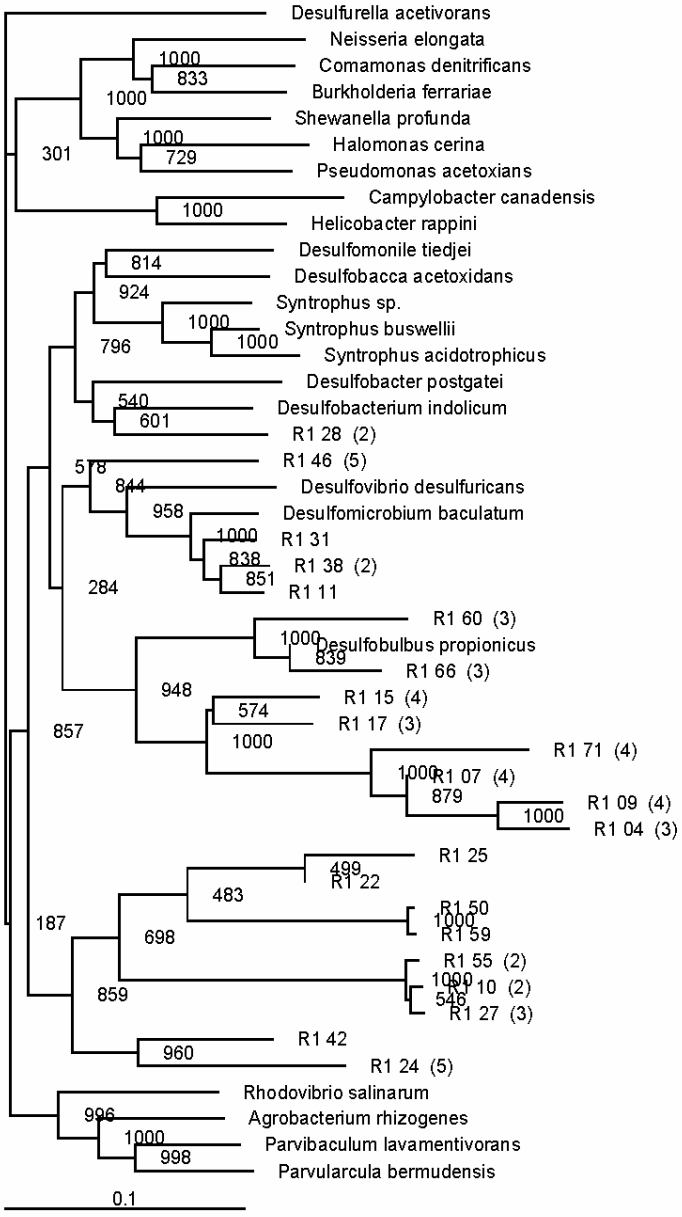


Figure 4-9 Phylogenetic tree of *Bacteria* from R1 on day 797 for phylum *Proteobacteria*.

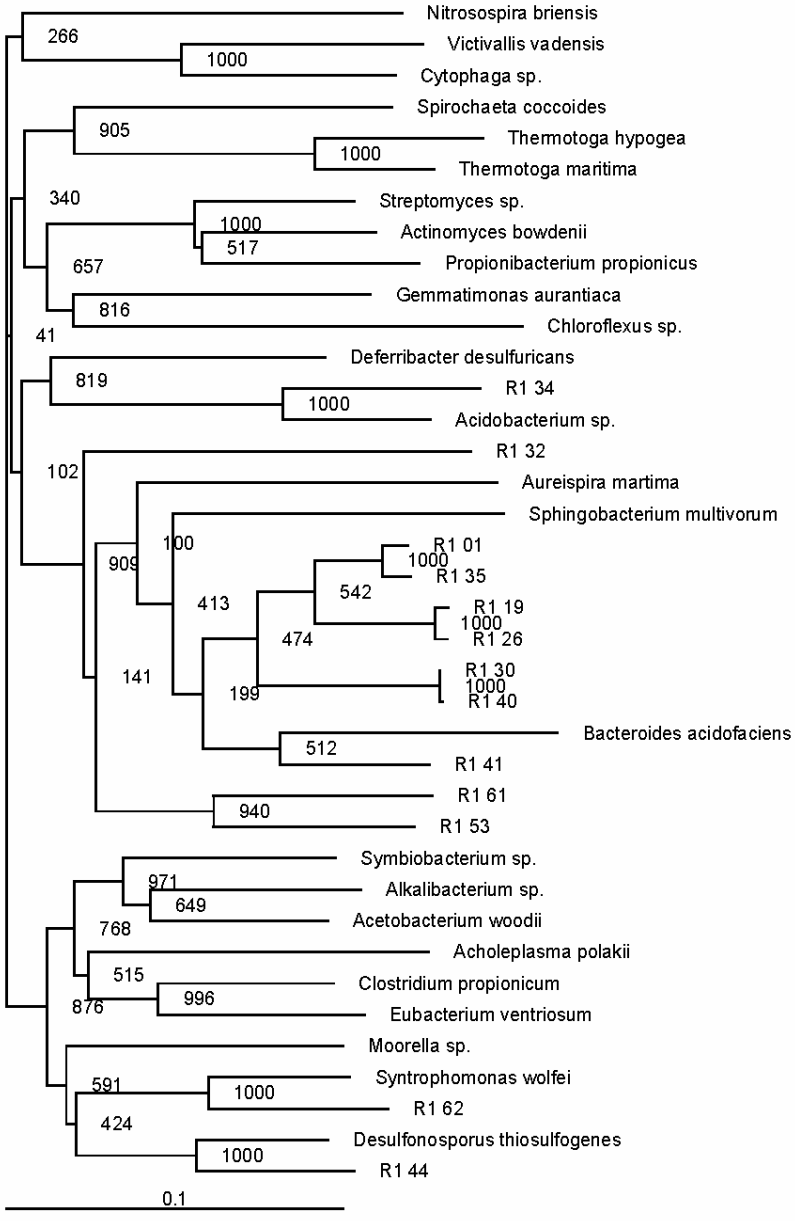


Figure 4-10 Phylogenetic tree of *Bacteria* from R1 on day 797 for non-*Proteobacteria.*

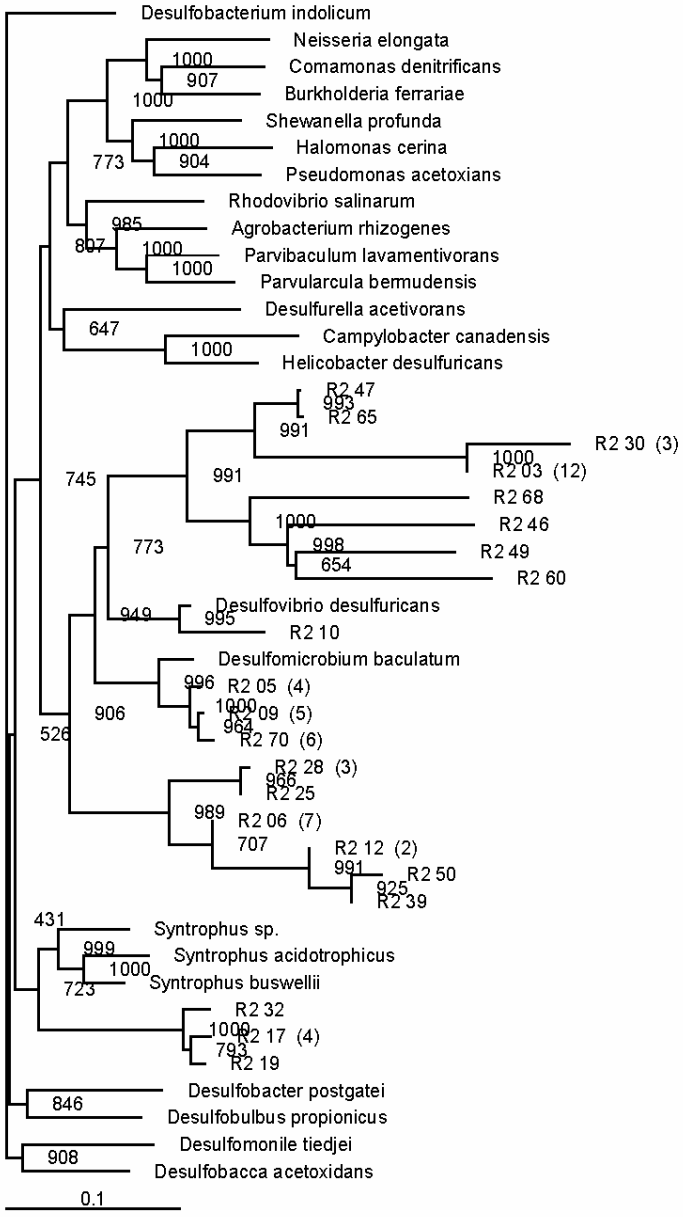


Figure 4-11 Phylogenetic tree of *Bacteria* from R2 on day 180 for phylum *Proteobacteria.*

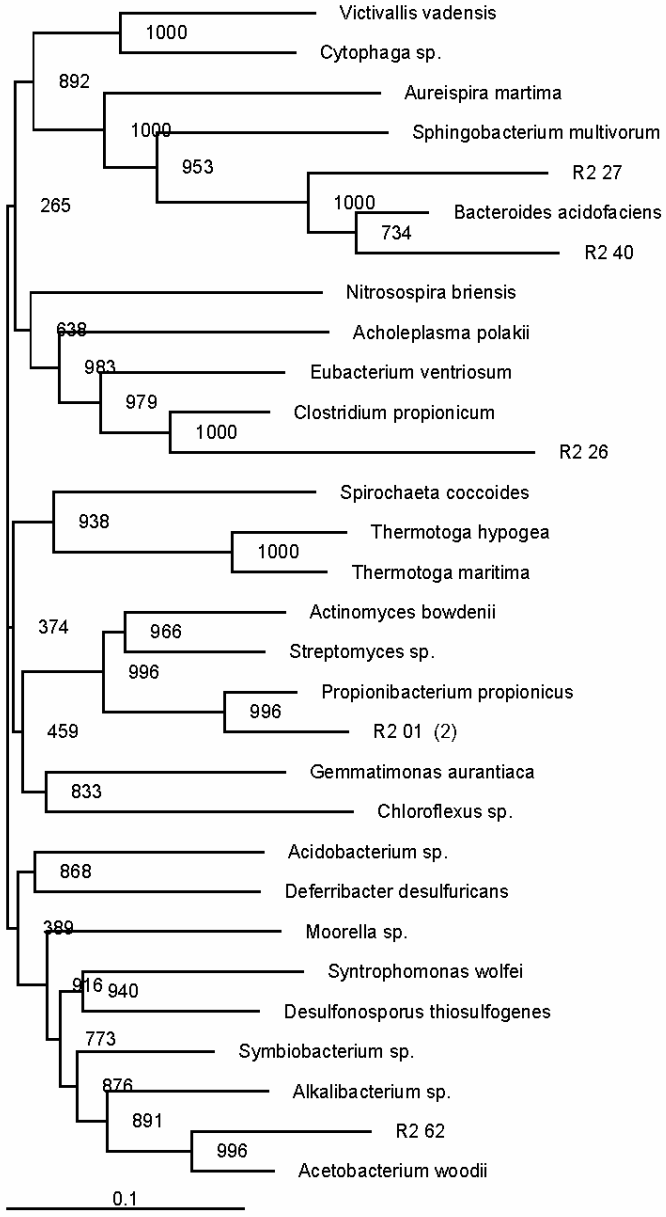


Figure 4-12 Phylogenetic tree of *Bacteria* from R2 on day 180 for non-*Proteobacteria.*

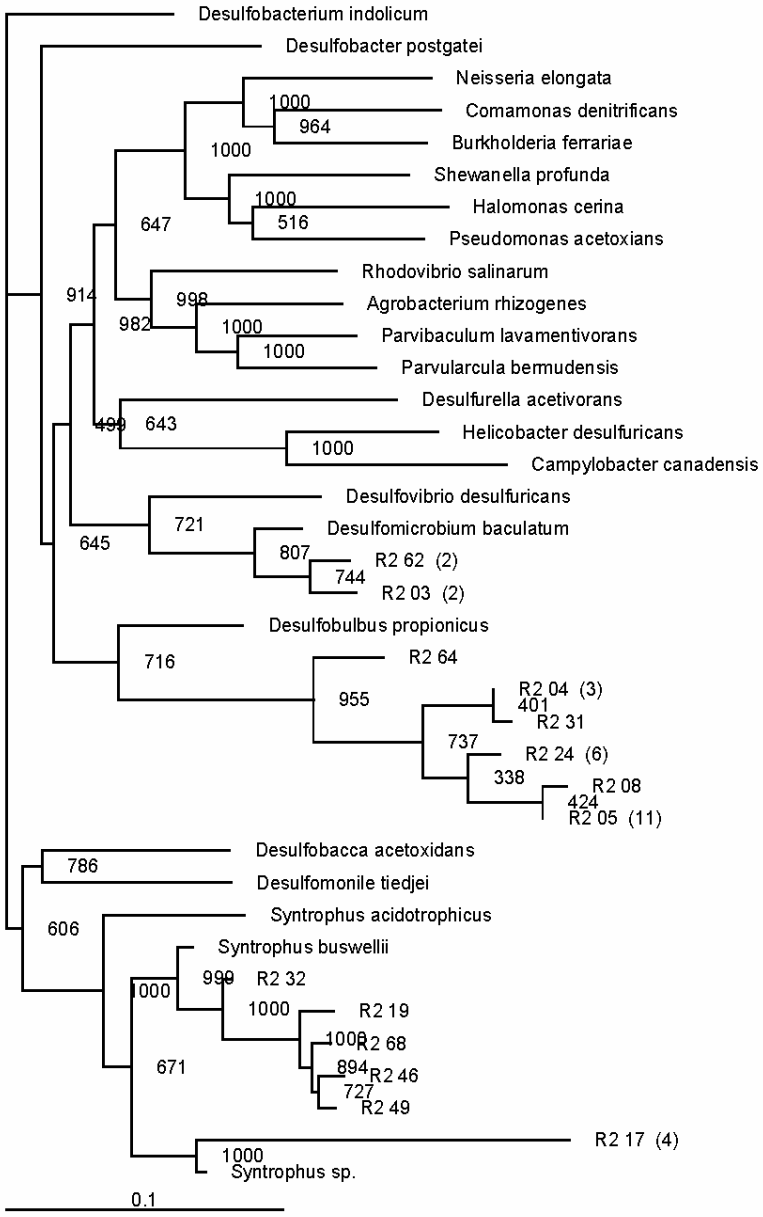


Figure 4-13 Phylogenetic tree of *Bacteria* from R2 on day 799 for phylum *Proteobacteria*.

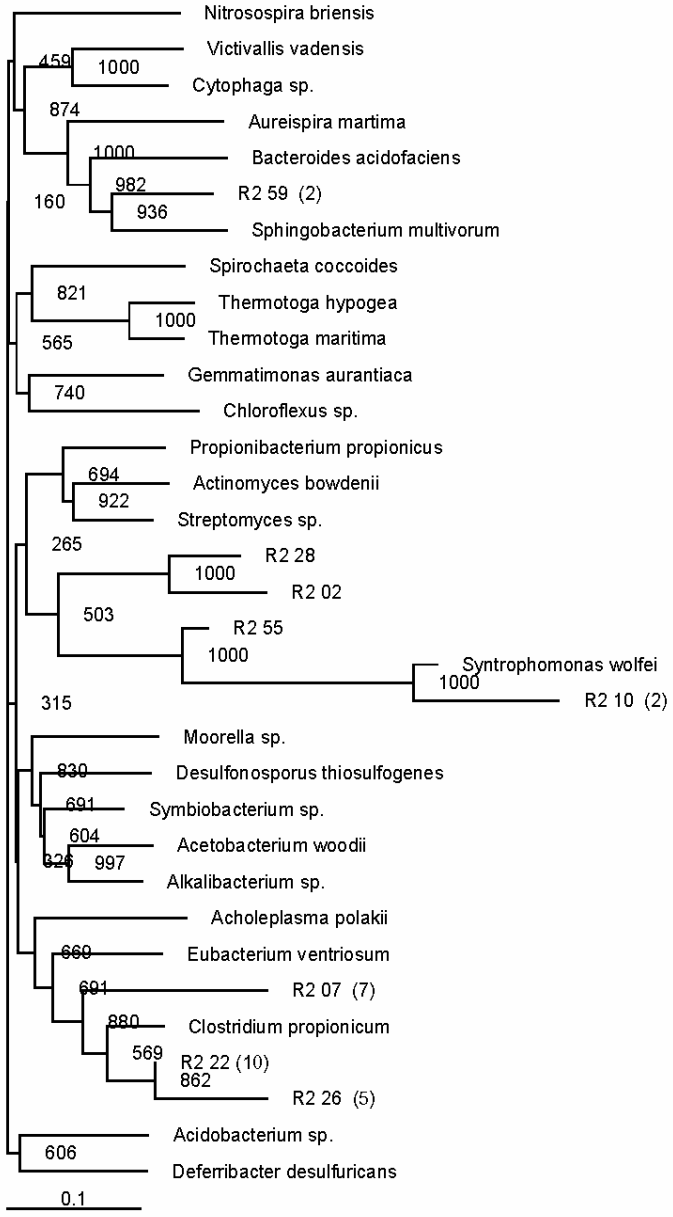


Figure 4-14 Phylogenetic tree of *Bacteria* from R2 on day 799 for non-*Proteobacteria*

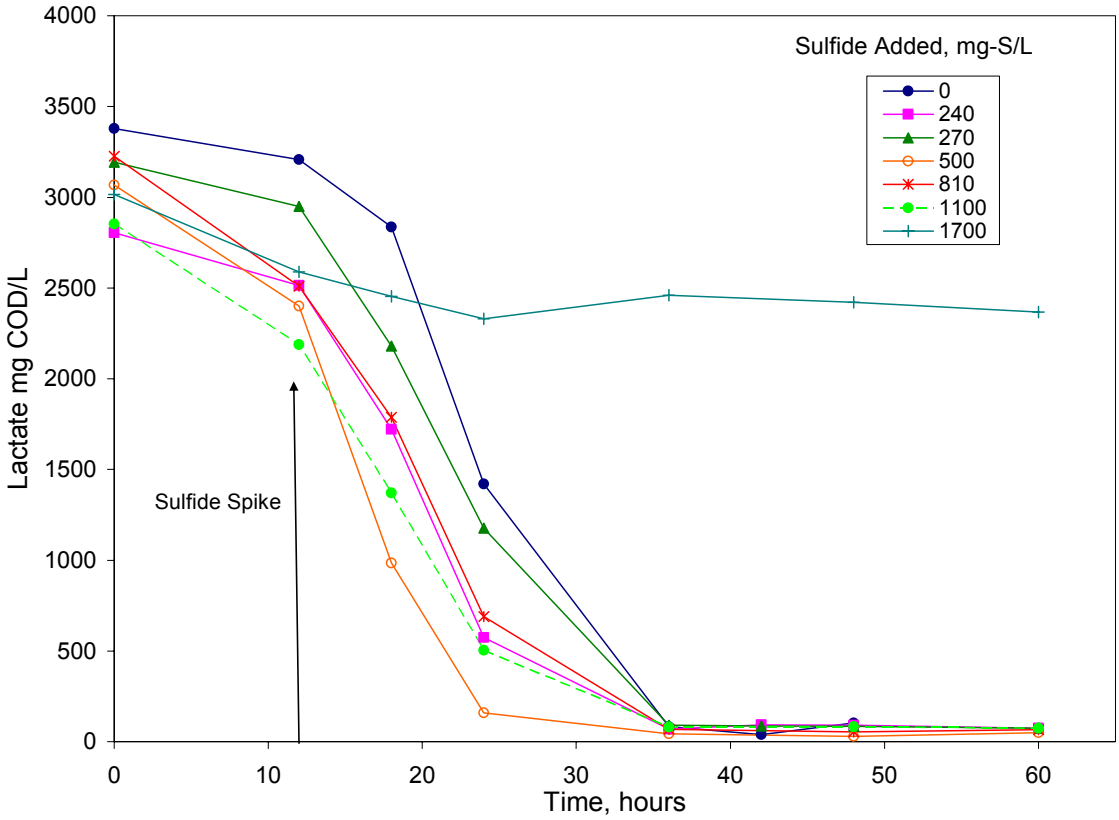


Figure 4-15 Effect of sulfide on lactate degradation for pure cultures of *Desulfovibrio desulfuricans*.

CHAPTER V

QUANTIFICATION OF BACTERIA, ARCHAEA, AND METHANOGEN POPULATIONS IN SULFATE-FED ANAEROBIC BIOFILM REACTORS USING QUANTITATIVE PCR

5.1 Introduction

Due to the relative newness of qPCR, the number of studies using this technique to examine anaerobic wastewater treatment are limited. Several researchers have used absolute quantification by qPCR to investigate aspects of the nitrification/denitrification process (Harms et al. 2003; Nakamura et al. 2006; Okano et al. 2004). qPCR has also been used to characterize MA communities in continuously-fed cultures (Shigematsu et al. 2003; Shigematsu et al. 2004); however, these studies focused on acetate-utilizing MA. Although SRB quantification in natural environments has been done (Stubner 2002; Stubner 2004), using qPCR to quantify SRB in engineered systems has not. In addition, since SYBRGreen chemistry was utilized instead of hydrolysis probe based chemistry (TaqMan®), no SRB-specific probes have been developed.

Recently, Yu et al. (2005b) developed a suite of TaqMan® primer/probe sets that included *Archaea* and four MA subgroups. Separate detection of each MA group may allow for a complete picture of MA composition. The goals for this portion of the research were to: 1) quantify the *Bacteria, Archaea,* and methanogen subgroups present in the reactors, 2) to compare the quantities of these groups at i) different points in time, ii) along the length of a reactor, and iii) between reactors, and 3) to evaluate the usefulness of qPCR for quantifying MA in a complex sulfate-fed wastewater treatment system.

5.2 Materials and methods

5.2.1 Reactor operation, sample collection and DNA extraction

The reactors were operated as previously described in section 3.2.1. Samples were collected as described in section 4.2.2., and DNA was extracted as described in section 4.2.3.

5.2.2 Primers and probes used in this study

PCR and qPCR amplification of 16S rDNA was performed using primer and probe sets developed by Yu et al. (2005b) (Table 5-1); primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA). All probes were labeled with 6-carboxyfluorescein (6-FAM) on the 5' end and Black Hole Quencher (BHQ-1) on the 3' end. It should be noted that since reactor conditions did not favor the growth of non-Methanogenic *Archaea* (halophiles, thermophiles, etc.), the ARC primer/probe set can be used to represent the population of MA.

5.2.3 Construction of standards for quantification

PCR reactions to obtain amplicons for standards were carried out using a *Taq* PCR Core Kit (Qiagen, Valencia, CA) at the following concentrations: 1x Qiagen PCR buffer, a 200 µM concentration of each deoxynucleoside triphosphate, 0.025 u/µL *Taq* DNA polymerase, 200 nM of each forward and reverse primer. Varying templates and template quantities were used for amplification (Table 5-2). Thermal cycling was performed with an Eppendorf Mastercycler (Westbury, NY) as follows: initial

denaturation at 94 ºC for 3 min. followed by 30 cycles of denaturation at 94 ºC for 1 min., annealing for 1 min., extension at 72 ºC for 1 min.; and final extension at 72 ºC for 10 min. Annealing temperatures varied slightly according to the primer set (Table 5-2). The reaction products were visualized with 1% agarose gel electrophoresis then purified with the QIAquick PCR Purification Kit (Qiagen). DNA concentration was measured at 260 nm using a Cary 50 UV-Vis spectrophotometer loaded with Cary WinUV software (Varian Inc., Palo Alto, CA). Copy number was calculated according to the following equation (Yu et al. 2006):

$$Copy\ number\left(copy/\mu L\right) = \frac{16S\ rDNA\ concentration\left(ng/\mu L\right)\times\left(\frac{1g}{10^9 ng}\right)\times 6.022\times 10^{23}\left(copy/mol\right)}{16S\ rDNA\ amplicon\ size\left(bp\right)\times 660\left(g\,16S\ rDNA/mol/bp\right)}$$

Undiluted 16S rDNA copy numbers varied between 8.4 x 10^{10} and 1.4 x 10^{11} copies/µL. Serial dilutions (10^{10} through 10^{1}) for each primer set were made for use in subsequent qPCR assays.

5.2.4 Instrumentation

All qPCR assays were performed on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) equipped with Applied Biosystems 7700 System Sequence Detection Software version 1.2.3.

5.2.5 Optimization of qPCR conditions

The optimal DNA template concentration was determined by varying the amount of template added to each optimization reaction (40, 60, 80, and 100 ng) for all primer/probe sets in triplicate. DNA from R2 port B was used as the template. A quantity of 60 ng/ 25 µL reaction was found to be optimal (i.e to provide the lowest Ct for the smallest amount of DNA template). Next, primer concentration was varied (150, 300, 600, and 900 nM/reaction), then probe concentration was varied (50, 100, 200, and 250 nM/reaction). Based on these results, a standard operating protocol was devised for all subsequent qPCR assays. The protocol consisted of 1x TaqMan® Universal PCR Master Mix (a 2x concentrated mixture of Ampli*Taq* Gold DNA polymerase, uracil-N-glycosylase, deoxynucleoside triphosphates with UTP, passive reference dye, and optimized buffer), 600 µM of the forward and reverse primer, 200 µM of probe, and 2.4 ng/µL DNA template.

5.2.6 Control reactions

Negative controls (Table 5-3) were performed to test the specificity of the primer/probes sets. *Methanococcus maripaludis* genomic DNA was used as a template for the BAC set, and *Desulfovibrio desulfuricans* (both from ATCC, Manassas, VA) was used as a template for the remaining sets, all of which were designed to amplify various *Archaea.* All reactions were preformed in triplicate.

External negative controls (ENC) and internal positive controls (IPC) (Table 5-3) were performed to test for inhibition of the qPCR reaction due to any component in the DNA template that might have been co-purified with the DNA. A primer/probe set

specific for all *Bacteroides* (a microbial group not expected to be present in the reactors in large numbers) was used for the ENC. A PCR was performed using the *Bacteroides* primers and DNA obtained from each of the three reactors using a *Taq* PCR Core Kit (Qiagen, Valencia, CA) at the following concentrations: 1x Qiagen PCR buffer, a 200 µM concentration of each deoxynucleoside triphosphate, 0.025 u/µL *Taq* DNA polymerase, 200 nM of each forward and reverse primer, and 10 ng/µL DNA template. No amplicons were observed using 1% agarose gel electrophoresis, indicating that *Bacteroides* species were probably not present in large quantities in the reactors. Next, a qPCR assay was run in duplicate by adding reactor DNA, *Bacteroides* primers and probe, and 10 ng/µL of *Bacteroides fragilis (B. fragilis)* genomic DNA (ATCC, Manassas, VA). One reaction was also run for each port sample that contained reactor DNA and *Bacteroides* primers and probe, but did not contain *B. fragilis* DNA.

The IPC was performed to determine if the addition of a large amount of foreign DNA to the reaction would inhibit the activity of the primer/probe sets. For a given set, a qPCR assay was run which included the same reactor DNA samples used in the ENC plus *B. fragilis* DNA in the case of the ARC, MBT, MMB, and MSL primer/probe sets, and *Methanococcus maripaludis* DNA in the case of the BAC primer/probe set. Standards for the set were also run in duplicate on the same plate as the IPC samples.

5.2.7 qPCR assays on reactor DNA samples

Thirty different reactor samples were used, corresponding to the different reactors (R1, R2, R3), sample ports (A, B, C, D, and E), and sampling dates (approximately180 and 800 days). For each of the primer/probe sets of interest, a qPCR assay on a 96-well

plate was run for the 15 samples corresponding to a particular sampling date in triplicate. For example, all of the port samples from R1, R2, and R3 obtained on the first sampling using the ARC primer/probe set would be contained on one plate, and R1, R2, and R3 samples from the second sampling using the ARC primer/probe set would be on a separate plate. Each plate contained triplicate standards for the particular primer/probe set ranging from 10^3 to 10^{10} copies/μL and a triplicate non-template control (NTC).

5.3 Results and discussion

5.3.1 Control reactions

No amplification was detected in the negative controls for MMB, MBT and MCC sets. The BAC, ARC, and MSL sets had Ct values of 29.2 ± 3.7, 36.7 ± 2.1, and 32.8 ± 4.0, respectively. Such high Ct values indicate that the primer/probe sets were not amplifying templates outside of their target range.

In the external positive controls, samples which contained reactor template and *Bacteroides fragilis* DNA had an average Ct of 19.34 ± 0.20, while samples without *Bacteroides fragilis* had an average Ct of 23.62 ± 1.84, compared to the average Ct of 34.04 ± 0.21 for the non-template control (NTC). The Ct for samples without *B. fragilis* was high enough compared to the NTC to indicate there might be *Bacteroides* present in the reactor samples (later confirmed by the *Bacteria* phylogenetic analysis in section 4.3.4). However, the small standard deviation for the samples where *B. fragilis* was added indicate the reactor samples did not appear to inhibit the amplification of *B. fragilis* with the *Bacteroides* primer/probe set.

IPCs were performed to evaluate if amplification of the primer/probe sets would be inhibited when large amounts of extraneous DNA was added. Ct values found for a particular IPC sample were compared to original Ct values for the same sample that did not contain any added *B. fragilis* (Tables 5-4 to 5-6). A ΔCt of one would indicate a two-fold difference between samples (2^1) , while a ΔCt of 5 would indicate a 32-fold difference (2^5). For ARC and the MA sets, ΔCt values were low (the largest difference was 2.6). The BAC set showed larger ΔCt's (all were greater than 2 and the largest was over 5), however these differences were still acceptable, since they were different by only about an order of magnitude.

ENC and IPC results indicate there was not an inhibitory substance in the reactor samples that would account for differences in Ct values observed between samples. Therefore, Ct values and the resulting quantities discussed in subsequent sections can be attributed to the biological composition of the samples, not to interfering or inhibiting substances found in the qPCR system.

5.3.2 Conversion of Ct values to absolute quantities

Average Ct values and standard deviations for each sample were calculated from the triplicate reactions for that sample (Table 5-7 and Table 5-8). Ct values were obtained for nearly all samples and primer/probe sets, with the exception of the MCC set. Only two samples in the first extraction and four samples in the second extraction had a measurable fluorescence, indicating that *Methanococcales*-like MA were not present in significant numbers in any of the reactors.

Standard curves converting Ct values to absolute quantities expressed as copies/μL were constructed by the Applied Biosystems sequence detection software, an example of which can be seen in Figure 5-1. Values of the slope, y-intercept, and R^2 for each standard set can be found in Table 5-9. Quantities were transformed to cells/μL by accounting for the average 16S rDNA gene copy numbers in bacteria and archaea, which are 4 and 2.5 copies/cell, respectively (Klappenbach et al. 2001).

It is important to note that the precision of qPCR quantification relies on an assumption that the standards and environmental samples share the same PCR efficiencies. The slope of an ideal PCR amplification is equal to -3.32, and in practice, a reliable standard curve should have an R^2 value of more than 0.95 and a slope of -3.0 to -3.9 (corresponding to PCR efficiencies of 80%-115%, respectively) (Zhang and Fang 2006). As seen in Table 5-9, a majority of the standard curves had slopes within -3.0 to -4.0. Those with slopes outside of this range had efficiencies greater than 115%. This may have been due to the use of degenerate primers and probes in the study (Table 5-1), since degenerate primers are less specific and may have amplified non-target DNA. If this event did occur, then the amount of primer or probe present could be depleted sooner, resulting in an artificially lower quantity value than was actually present.

5.3.3 Quantification of reactor samples

For all reactors at the first sampling, cell counts were significantly higher in the lower reactor ports A and B (Figures 5-2 to 5-4). At the second sampling, cell numbers in the higher ports were comparable to the lower ports, indicating that the reactor was more fully colonized by 800 days.

ARC (or MA) quantities at the first sampling indicate that for a given port, quantities did not vary between reactors. MA numbers did not appear to change much for ports A and B over time, but all other ports had MA numbers comparable to A and B by the second sampling. Even though the reactors were exhibiting different behavior by the second sampling time, MA quantities were about the same for all reactors. Therefore it appears that lactate shock loading, elevated sulfide, or elevated propionate did not cause a decrease in the MA population. This is significant because even if external pressures caused the rate of acetate degradation to slow down, the MA population was stable enough in all reactors to survive such pressures.

More ARC than BAC was present in all ports of the reactors. Previous work on quantification of microbial communities in anaerobic wastewater reactors has reported that MA made up 8-12% of total rRNA in sewage sludge digesters, up to 25% of total rRNA in glucose-fed lab scale reactors, and 76-96% of total rRNA in acetate-fed lab scale chemostats (Raskin et al. 1994; Raskin et al. 1996; Raskin et al. 1995b). In addition, another study found that MA were twice as abundant as bacteria in granular sludge reactors treating paper mill wastewater (Roest et al. 2005). These findings suggest that if significant quantities of acetate are present, it may be possible to establish a microbial population that has relatively more *Archaea* than *Bacteria*.

Higher numbers of ARC than BAC have also been observed in qPCR studies. While *Bacteria* outnumbered *Archaea* in anaerobic digestor sludge and in fluizided reactor biofilms, fixed-bed reactor biofilms contained more *Archaea* than *Bacteria* (2.84 x 10^9 cells/mL vs. 1.53 x 10^8 cells/mL, respectively) (Sawayama et al. 2006; Yang et al.

2004). Thus, fixed-bed biofilm reactors allow for MA numbers comparable or even greater than BAC numbers.

Four MA subgroups were quantified: three groups of hMAs, including *Methanomicrobiales* (MMB), *Methanobacteriales* (MBT), and *Methanococcales* (MCC), and one group which contained all aMAs, *Methanosarcinales* (MSL). Samples from both time points showed that MSL was the dominant MA in all reactors and ports, reaching quantities of 10^6-10^7 cells/mL by the second sampling. The MMB population appeared to increase to around 10^6 cells/mL over time in ports C, D, and E. MBT numbers were also generally low in the first sampling, but increased in some ports to around 10^5 cells/mL by the second sampling. As mentioned previously, very few samples were found to contain MCC.

Dominance of MSL in qPCR analysis is not surprising, given that most acetate in the reactors was utilized by aMAs and that aMAs were the dominant MA group the phylogenetic analysis. However, the phylogenetic analysis indicated that very few hMA were present, while qPCR analysis found populations of hMA. Since only about 30% of methane produced in an anaerobic process originates from hydrogen oxidization (McCarty and Smith, 1986), it appears that unlike clone libraries, the qPCR method is able to detect microbial groups that have a smaller role in lactate degradation. This incongruity is likely due to how these two methodologies work. Primers used in the phylogenetic analysis were designed for all MA. If one type of MA was present in very large numbers compared to other types, it is possible that the clones selected off the agar plates for sequencing could have been skewed to represent mostly that large group. For example, if there are 97 aMA and three hMA among a population of 100 MA clones,

when 20 of those clones are randomly selected, the likelihood finding of a hMA in that group of 20 is low, resulting in skewed phylogenetic results in favor of aMA. qPCR is a more sensitive technique than phylogenetic analysis, since the primer/probe sets were designed to be more specific. Thus, while a phylogenetic analysis is a useful and well-developed tool for defining the dominant groups of MA present, smaller numbers of specific hMA groups can be detected using qPCR.

In addition to the lack of hMA observed in the clone libraries, very few acetogens were found in the R1 and R2 bacterial clone libraries. Acetogens are responsible for the production of hydrogen that would be used as an electron donor for hMA (Equations 1-2, Table 2-2). Thus, the clone library results indicated that this pathway of degradation (Path 2, Figure 3-11) was of little importance. If the qPCR revealed that hMA were present, perhaps it might also have indicated that acetogens were present as well. However, since specific bacterial groups were not investigated in the qPCR due to lack of specific probe/primer sets, acetogens were not quantified.

5.3.4 Effectiveness of qPCR in quantifying mixed DNA samples

The sum of the four MA group quantities did not add up to the cell count for ARC as a whole. It is interesting to note that while Yu et al. (2005b) described the additive property of these primer/probe sets, this summation was never performed (Yu et al. 2006; Yu et al. 2005). Other studies that have utilized some of the Yu primer/probe sets did not use all four MA group sets, making summation impossible.

While the additive property did not work, qPCR did provide useful information on the microbial composition of the reactors. However, qPCR has not yet developed to

the point where it can be an effective tool to completely characterize an anaerobic biofilm reactor. The lack of published findings on reactor characterization is evidence to this fact. Only when thoroughly tested primer/probe sets are developed for both MA and SRB can qPCR provide the desired information on microbial populations.

5.4 Conclusions

. Based on the findings from quantitative analysis using qPCR, the following conclusions can be made:

- MA quantities in the reactors were within the same order of magnitude at the first or second sampling time for a given port. Therefore, factors such as lactate shock loading, elevated sulfide, and elevated propionate did not cause significant changes in MA population.
- Greater numbers of *Archaea* than *Bacteria* were found in all reactors. This may be attributed to the availability of acetate in the reactors for MA consumption and to using the immobilized fixed bed reactor type.
- The acetate utilizing *Methanosarcinales* (which contains the *Methanosaeta* and *Methanosarcina* subgroups) was found to be the type of MA with the largest population. This agrees with the results from Chapters 3 and 4, which indicated that aMA, specifically *Methanosaeta*, were the dominant MA.
- Lower ports A and B contained significantly higher quantities of all organisms than the higher ports at around 180 days. By 800 days, organism quantities in ports C, D, and E increased to quantities similar to ports A and B.

- Quantities of around 10^6 cells/mL hMA were found in the reactors due to the more sensitive nature of the qPCR technique compared to phylogenetic analysis.
- Clone libraries appear to provide information on the major microbial groups in a community and can aid in explaining reactor performance, whereas qPCR has the potential to reveal not only the major microbial groups, but also groups that have smaller but important roles in the community.
- Quantification with qPCR has much potential for analyzing mixed microbial populations, as long as the primer/probe sets of interest can be discriminating.

Table 5-1 Primer and probe sets used in quantative PCR.

Name[a]	Function	Target Group	Sequence (5'-3')	Size, bp
BAC338 BAC516 BAC805	Fwd. Primer Probe Rev. Primer	*Bacteria*	ACTCCTACGGGAGGCAG TGCCAGCAGCCGCGGTAATAC GACTACCAGGGTATCTAATCC	468
ARC787 ARC915 ARC1059	Fwd. Primer Probe Rev. Primer	*Archaea*	ATTAGATACCCSBGTAGTCC AGGAATTGGCGGGGGAGCAC GCCATGCACCWCCTCT	273
MCC495 MCC686 MCC832	Fwd. Primer Probe Rev. Primer	*Methanococcales*	TAAGGGCTGGGCAAGT TAGCGGTGRAATGYGTTGATCC CACCTAGTYCGCARAGTTTA	337
MBT857 MBT929 MBT1196	Fwd. Primer Probe Rev. Primer	*Methanobacteriales*	CGWAGGGAAGCTGTTAAGT AGCACCACAACGCGTGGA TACCGTCGTCCACTCCTT	343
MMB282 MMB749 MMB832	Fwd. Primer Probe Rev. Primer	*Methanomicrobiales*	ATCGRTACGGGTTGTGGG TYCGACAGTGAGGRACGAAAGC TG CACCTAACGCRCATHGTTTAC	506
MSL812 MSL860 MSL1159	Fwd. Primer Probe Rev. Primer	*Methanosarcinales*	GTAACGATRYTCGCTAGGT AGGGAAGCCGTGAAGCGARA GGTCCCCACAGWGTACC	354
AllBac296[b] AllBac375[b] AllBac412[b]	Fwd. Primer Probe Rev. Primer	All *Bacteroides*	GAGAGGAAGGTCCCCCAC CCATTGACCAATATTCCTCACTG CTGCCT CGCTACTTGGCTGGTTCAG	106

[a] Source: Yu et al. 2005b

[b] Source: Layton et al. 2006

Table 5-2 Template sources and PCR conditions for construction of qPCR standards.

Primer Set Name	**Template Source**	**Quantity, ng/µL**	**Annealing Temp. (°C)**
BAC	*Desulfovibrio desulfuricans* DNA[a]	8	48
ARC	*Methanococcus maripaludis* DNA[a]	8	47
MCC	*Methanococcus maripaludis* DNA[a]	8	50
MBT	*Methanothermobacter thermautotrophicus* DNA[b]	8	53
MMB	R1 mixed DNA	20	50
MSL	R1 mixed DNA	20	50

[a] obtained from ATCC, Manassas, VA.

[b] obtained from the laboratory of John Reeve, Department of Microbiology, The Ohio State University, Columbus, OH.

Table 5-3 Control reactions for qPCR.

Control Type	**Probe/Primer Set Used**	**Question**
Negative control	ARC, MBT, MMB, MSL, BAC	Do the primer/probe sets of interest amplify non-target template?
External negative control (ENC)	*Bacteroides*	Does reactor DNA inhibit and independent qPCR reaction?
Internal positive control (IPC)	ARC, MBT, MMB, MSL, BAC	Does adding an external template inhibit the qPCR reactions of the primer/probe sets of interest?

Table 5-4 Internal positive control results for ARC and MSL sets.

Port Sample	ARC		MSL	
	IPC Ct	ΔCt *	IPC Ct	ΔCt *
R2A 1st ext.	19.00 ± 0.28	-1.01	21.29 ± 0.20	-0.36
R2B 1st ext.	17.96 ± 0.02	-1.54	20.34 ± 0.08	-2.37
R2C 1st ext.	21.10 ± 0.51	1.61	24.21 ± 0.52	1.19
R2D 1st ext.	23.72 ± 0.09	2.24	27.05 ± 0.23	2.86
R2E 1st ext.	21.77 ± 0.05	1.92	25.59 ± 0.48	0.93
R1A 2nd ext.	17.71 ± 0.72	0.18	20.75 ± 0.36	-2.45
R1B 2nd ext.	16.92 ± 0.73	-1.55	18.79 ± 0.34	-1.58
R1C 2nd ext.	17.22 ± 0.13	-1.09	19.00 ± 0.03	-1.82
R1D 2nd ext.	17.65 ± 0.42	-0.79	19.44 ± 0.13	-0.12
R1E 2nd ext.	17.09 ±0.26	-0.59	18.90 ± 0.14	0.04
R3A 2nd ext.	20.44 ± 0.13	-0.74	23.68 ± 0.88	-2.02
R3B 2nd ext.	16.01 ± 0.58	-0.39	18.34 ± 0.29	-1.98
R3C 2nd ext.	17.99 ± 0.01	-0.90	19.86 ± 0.26	-1.22
R3D 2nd ext.	17.56 ± 0.03	0.06	19.39 ± 0.07	1.19
R3E 2nd ext.	19.04 ± 0.41	-0.50	20.25 ± 0.56	2.60

* $\Delta Ct = Ct_{sample} - Ct_{IPC}$

Table 5-5 Internal positive control results for BAC and MMB.

Port Sample	BAC		MMB	
	IPC Ct	ΔCt *	IPC Ct	ΔCt *
R1A 1st ext.	19.44 ± 1.70	-2.12	23.19 ± 1.46	0.02
R1B 1st ext.	22.09 ± 0.12	-3.79	22.72 ± 0.96	-0.50
R1C 1st ext.	33.32 ± 0.21	-2.25	37.45 ± 0.72	1.36
R1D 1st ext.	33.11 ± 0.21	-4.22	34.71 ± 0.37	0.22
R1E 1st ext.	N/A	N/A	35.78 ± 0.56	-0.97
R2A 2nd ext.	19.61 ± 0.77	-4.36	29.54 ± 0.06	-1.60
R2B 2nd ext.	18.73 ± 1.44	-5.11	24.59 ± 0.01	-1.53
R2C 2nd ext.	18.96 ± 0.60	-3.44	22.19 ± 0.04	-1.24
R2D 2nd ext.	22.13 ± 0.18	-4.18	23.7 ± 0.08	-0.13
R2E 2nd ext.	23.48 ± 0.34	-4.18	26.35 ± 0.32	1.72
R3A 2nd ext.	20.49 ± 1.49	-4.42	29.62 ± 0.76	-1.06
R3B 2nd ext.	19.33 ± 0.30	-4.93	22.04 ± 0.30	-1.47
R3C 2nd ext.	17.66 ± 0.91	-4.21	24.74 ± 0.25	-1.51
R3D 2nd ext.	21.38 ± 0.59	-3.67	24.35 ± 0.15	0.56
R3E 2nd ext.	22.99 ± 1.49	-3.69	27.07 ± 0.18	1.54

* $\Delta Ct = Ct_{sample} - Ct_{IPC}$

Table 5-6 Internal positive control results for MBT.

Port Sample	MBT	
	IPC Ct	**ΔCt** *
R2B 1st ext.	27.55 ± 0.27	-0.54
R3D 1st ext.	28.70 ± 2.40	-0.04
R3E 1st ext.	24.14 ± 0.47	1.38
R2A 2nd ext.	25.95 ± 0.01	-0.62
R2B 2nd ext.	25.67 ± 0.05	-1.08
R2C 2nd ext.	23.98 ±0.10	-0.61
R2D 2nd ext.	26.04 ± 0.63	-0.59
R3D 2nd ext.	26.68 ± 0.05	-0.29

* $\Delta Ct = Ct_{sample} - Ct_{IPC}$

Table 5-7 Threshold cycle (Ct) values for first sampling period (days 179-182).

Port	BAC	ARC	MCC	MBT	MMB	MSL
R1A	17.22 ± 0.26	16.40 ± 0.05	N/S	33.78 ± 0.19	23.21 ± 0.67	19.67 ± 0.47
R1B	18.30 ± 0.49	16.06 ± 0.31	N/S	32.39 ± 0.56	22.22 ±0.39	18.41 ±0.40
R1C	31.07 ± 1.43	24.79 ± 1.12	N/S	39.30*	38.81± 0.12	30.86 ± 0.93
R1D	28.99 ± 0.44	24.24 ± 0.05	N/S	36.26 ± 0.22	34.93 ± 0.50	28.04 ± 0.31
R1E	29.12 ± 0.26	27.56 ± 1.45	35.83*	N/S	34.81*	36.78 ±1.91
R2A	19.02 ± 0.10	17.99 ± 0.21	N/S	28.96 ± 0.23	24.86 ± 0.48	20.93 ±0.11
R2B	18.02 ± 0.22	16.42 ± 0.32	N/S	27.01 ± 0.30	22.65 ± 0.58	17.97 ± 0.31
R2C	27.13 ± 0.48	22.71 ± 1.34	N/S	34.98 ± 1.01	33.57 ± 1.01	25.39 ±0.15
R2D	32.57 ± 1.43	25.96 ±1.22	N/S	36.45 ± 0.80	38.76*	28.90 ±1.77
R2E	30.39 ± 0.56	23.68 ± 1.87	N/S	29.66 ±1.08	33.87 ± 0.30	26.52 ± 0.64
R3A	19.32 ± 0.46	17.35 ± 0.09	N/S	33.47 ± 0.23	24.60 ±0.86	20.42 ± 0.40
R3B	19.01 ± 0.13	16.70 ±0.20	N/S	31.56 ± 0.13	22.81 ± 0.25	19.22 ± 0.03
R3C	28.28 ± 0.28	23.71 ± 1.95	36.99*	31.85 ± 0.84	36.01 ± 0.71	27.91 ± 0.10
R3D	26.61 ±1.19	21.26 ± 1.62	N/S	28.67 ± 1.27	32.27 ± 0.71	25.00 ± 0.50
R3E	27.52 ± 0.80	21.47 ± 0.56	N/S	25.52 ± 1.13	35.34 ± 2.67	26.37 ± 0.34

N/S: No signal detected

* No standard deviation because only one of three samples had a measurable Ct

Table 5-8 Threshold cycle (Ct) values for second sampling period (days 797-800).

Port	BAC	ARC	MCC	MBT	MMB	MSL
R1A	15.10 ± 1.14	17.89 ± 2.16	N/S	31.07 ± 0.46	22.47 ± 0.59	18.29 ± 0.20
R1B	13.56 ± 0.08	15.36 ± 0.08	39.79*	30.62 ± 0.32	20.79 ± 0.51	17.21 ± 0.29
R1C	13.72 ± 1.52	16.13 ± 0.24	36.73*	28.65 ± 0.23	22.57 ±0.24	17.18 ± 0.19
R1D	16.02 ± 0.65	16.85 ± 0.10	N/S	30.03 ± 0.04	23.73 ± 0.86	19.32 ± 1.34
R1E	22.69 ± 4.63	16.50 ± 0.08	N/S	28.93 ± 0.28	23.75 ± 1.13	18.94 ± 1.22
R2A	15.24 ± 0.84	18.16 ± 0.93	38.05	25.33 ± 0.42	27.94 ± 0.36	20.13 ± 0.40
R2B	13.62 ± 1.40	16.18 ± 0.05	N/S	24.59 ± 0.37	23.06 ± 0.62	16.91 ± 0.16
R2C	15.52 ± 0.58	15.44 ± 0.29	34.95*	23.37 ± 0.55	20.95 ± 0.17	17.26 ± 1.06
R2D	17.95 ± 1.54	16.58 ± 0.13	N/S	25.45 ± 0.57	23.57 ± 0.35	21.30 ± 2.04
R2E	23.73 ± 6.26	17.12 ± 0.46	N/S	27.82 ± 0.93	28.06 ± 0.30	18.48 ± 1.68
R3A	16.07 ± 0.28	19.69 ± 0.38	N/S	33.32 ± 0.20	28.55 ± 0.95	21.66 ± 0.58
R3B	14.40 ± 0.50	15.62 ± 0.16	N/S	30.55 ± 0.20	20.57 ± 0.32	16.35 ± 0.26
R3C	13.45 ± 0.67	17.09 ± 0.10	N/S	30.24 ± 0.14	23.23 ± 0.57	18.64 ± 0.20
R3D	19.47 ± 2.48	17.62 ± 0.33	N/S	26.38 ± 0.27	24.90 ± 1.28	20.58 ± 0.48
R3E	24.25 ± 6.99	18.54 ± 0.30	N/S	27.91 ± 2.20	28.61 ± 0.43	22.84 ± 0.48

N/S: No signal detected

* No standard deviation because only one of three samples had a measurable Ct

Table 5-9 Standards data.

Primer/Probe Set	Slope	Intercept	R^2	Linear Range, copies/ µL
BAC 1st	-3.869	48.165	0.995	10^4-10^8
BAC 2nd	-5.001	53.309	0.996	10^5-10^{10}
ARC 1st	-3.789	48.357	0.987	10^4-10^9
ARC 2nd	-3.657	46.713	0.992	10^4-10^9
MCC 1st	-3.918	53.492	0.931	10^4-10^9
MCC 2nd	-4.874	61.396	0.890	10^4-10^9
MBT 1st	-4.046	44.669	0.995	10^3-10^9
MBT 2nd	-4.230	44.460	0.990	10^3-10^{10}
MMB 1st	-4.018	48.359	0.991	10^3-10^8
MMB 2nd	-4.068	48.227	0.997	10^4-10^8
MSL 1st	-3.788	46.054	0.993	10^3-10^8
MSL 2nd	-4.386	48.327	0.989	10^3-10^{10}

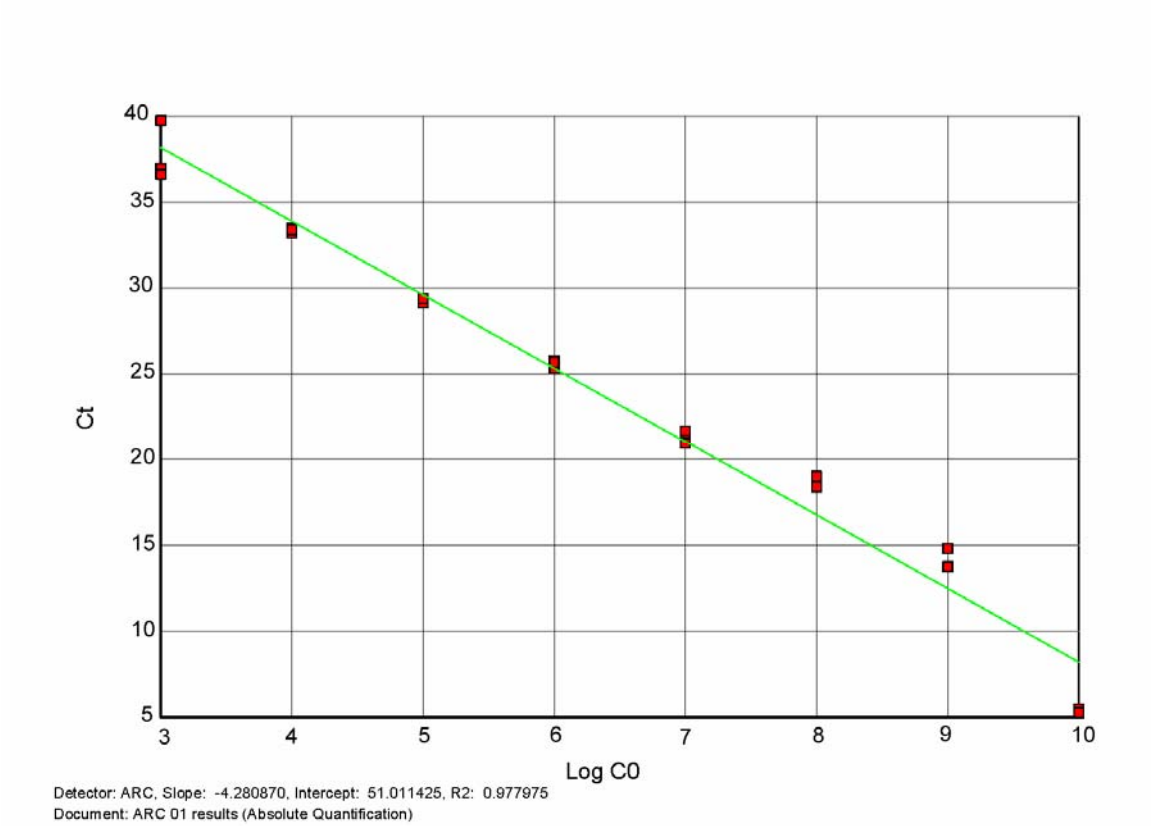
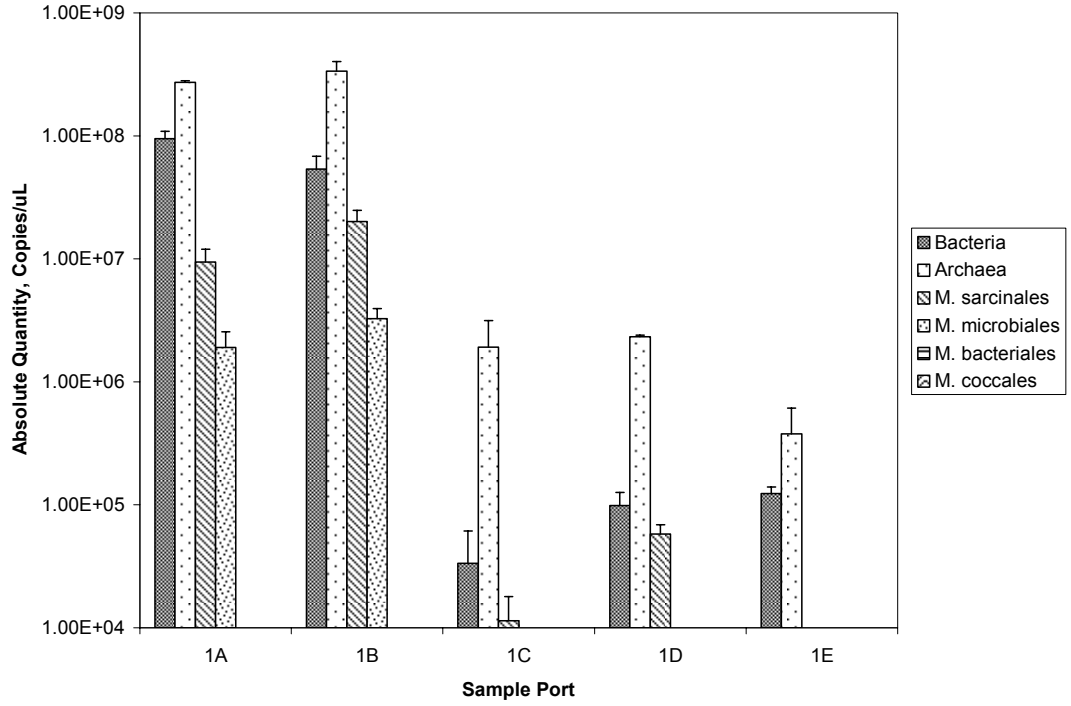


Figure 5-1 Sample plot of linear regression of Ct values found vs. log of the rDNA copy number (C_o) from the assay plate using DNA from the first extraction and the ARC primer/probe set.

a)



b)

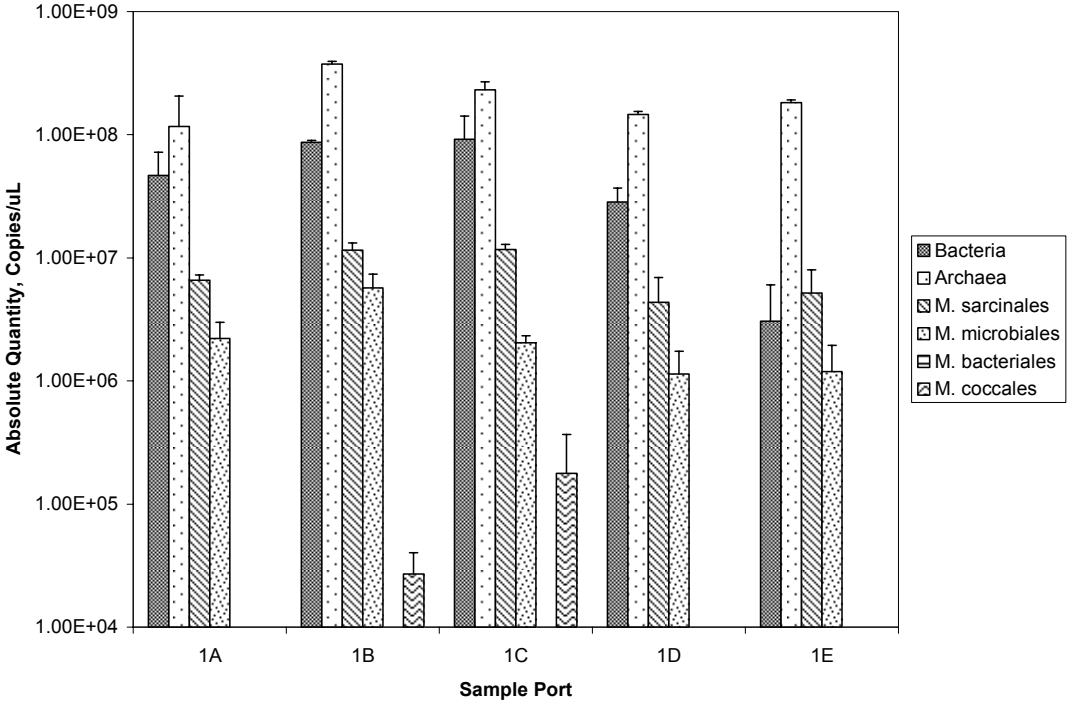
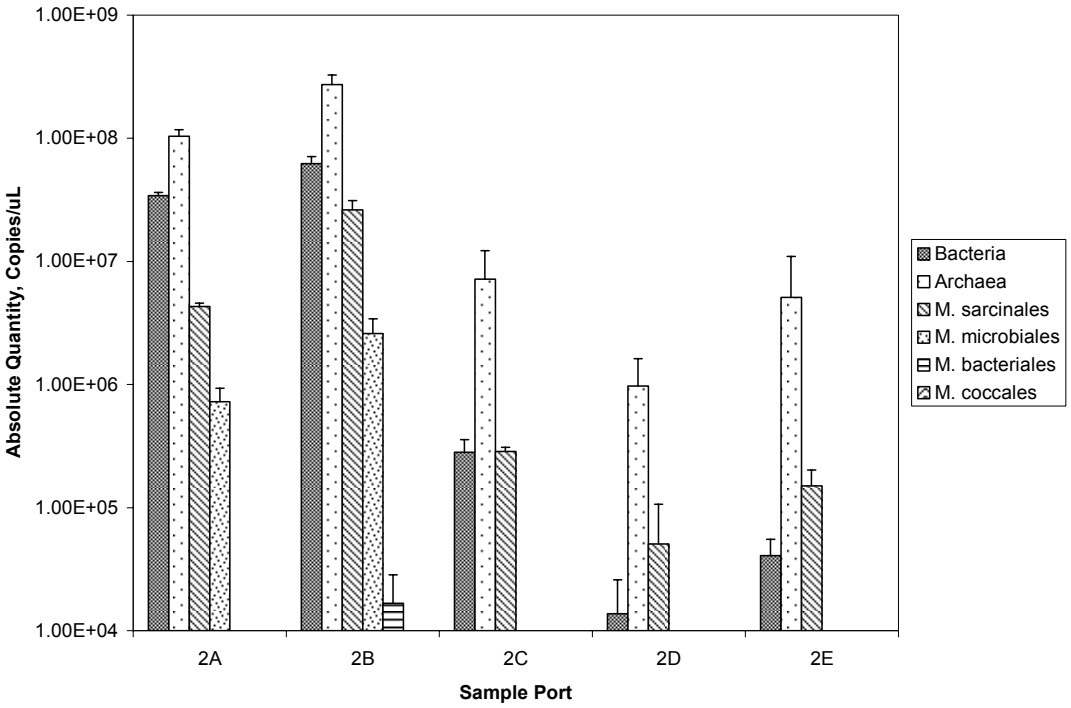


Figure 5-2 Absolute quantities for R1 on a) day 182 and b) day 797.

a)



b)

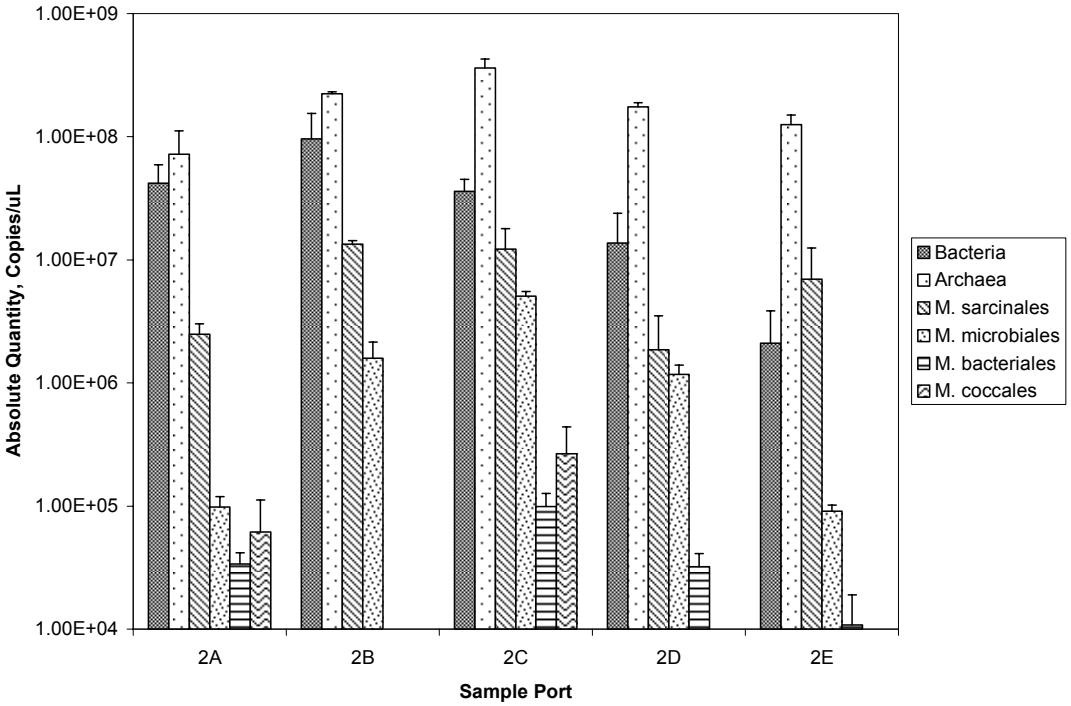
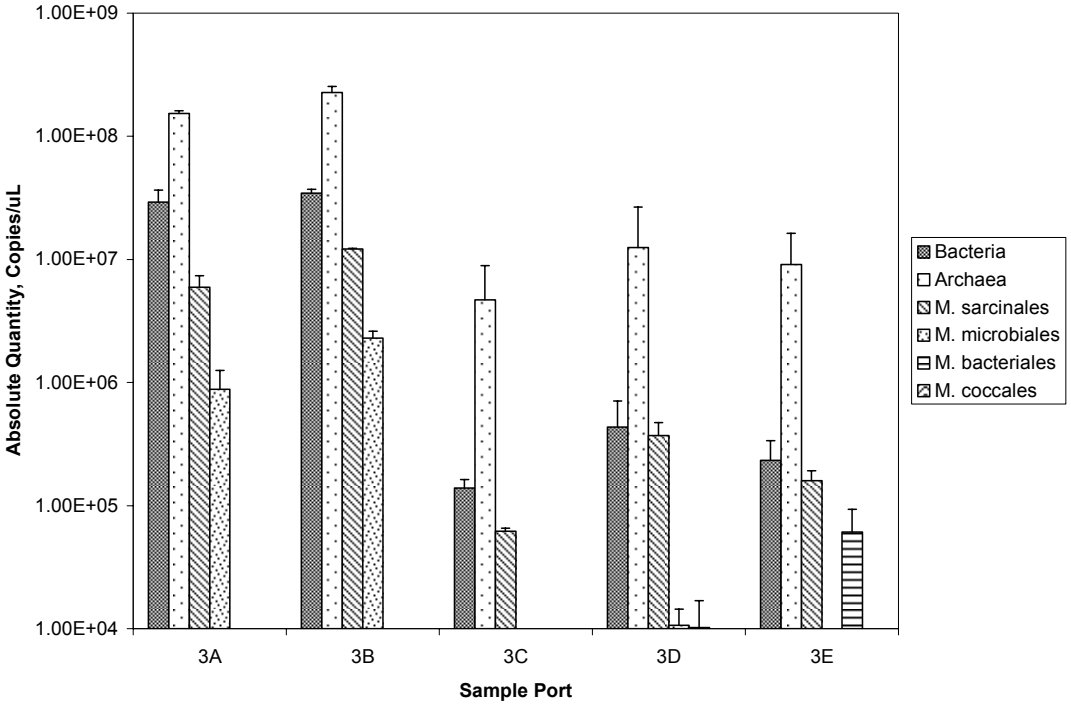


Figure 5-3 Absolute quantities for R2 on a) day 180 and b) day 799.

a)



b)

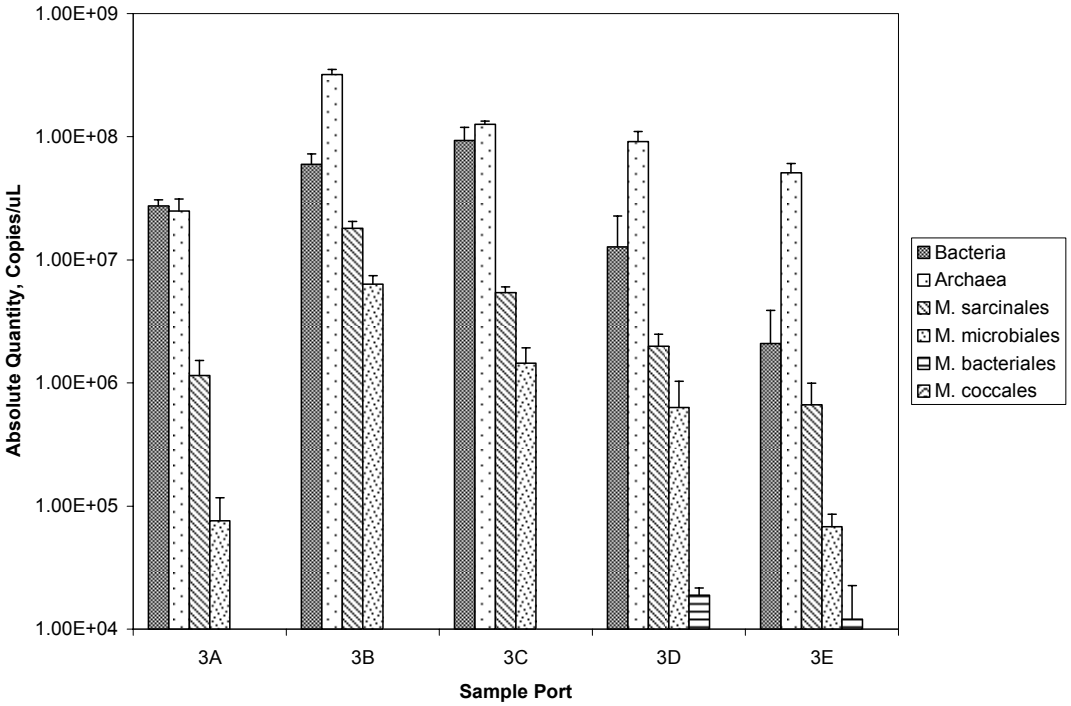


Figure 5-4 Absolute quantities for R3 on a) day 179 and b) day 800.

CHAPTER VI

CONCLUSIONS

Three biofilm reactors (R1, R2, R3) were operated at an HRT of two days and seeded with varying amounts of enrichment cultures fed lactate with and without sulfate. Reactors were operated for over 900 days, with OLR starting at 0.3 g-COD/L-day and reaching a maximum of about 4.0 g-COD/L-day. Sulfate loading rates started at 0.009 g-S/L-day and reached a maximum of about 0.3 g-S/L-day. DNA was extracted from the reactors around days 180 and 800. MA, SRB, and Bacterial clone libraries were constructed and qPCR analysis was performed with the DNA.

The objectives of this research were to (1) assess the long term performance of anaerobic biofilm reactors fed high concentrations of lactate and sulfate operated at 20 °C, (2) determine the effect of seeding with different microbial enrichments, (3) determine the effect of organic shock loads, and (4) determine the microbial composition of the reactors and how this composition changes over time. The following are conclusions that can be drawn from this work.

6.1 Long term performance

- Anaerobic biofilm reactors can be operated at 20°C with an OLR of 1.3 g-COD/L-day or less and an SLR of 0.2 g-S/L-day with no significant deterioration in process performance, as measured by effluent COD and sulfate.
- With long acclimation periods of around 500 days, OLR as high as 3.4 g COD/L-d and SLR of 0.3 g/L-d can be tolerated, producing effluent VA-COD levels

consistently less than 200 mg/L. Effluent DS and H_2S levels were around 600 mg S/L and 150 mg S/L, respectively, during this period.

- The maximum sustainable sulfide levels were lower than the hypothesized achievable levels of hydrogen sulfide and dissolved sulfide (200 mg-S/L and 1,000 mg-S/L, respectively). This is likely due to the lower operating temperature of 20°C, compared to higher temperatures such as 35 °C where higher sulfide levels can be tolerated.

6.2 Effect of Seeding

- Seeding source is not important when starting up sulfate-fed biofilm reactors. Unacclimated cultures may be used if the startup period employs a gradual increase in OLR and SLR.
- Initial seeding does not appear to have a significant effect on long-term reactor performance.

6.3 Effect of organic shock loading

- Reactors appear to be able to recover from one lactate spike of approximately 5,000 mg COD/L but not two spikes of this magnitude.
- qPCR data indicate that shock loading did not cause a decrease in MA population.

6.4 Microbial composition

- The key to long-term stability appears to be the development of large, stable populations of lactate- and propionate-degrading SRB and aceticlastic methanogens.
- In order to achieve a well-balanced reactor capable of treating high OLR/SLR feeds and able to withstand high levels of sulfide or organic shock loading at ambient temperatures, it is critical that sulfate reducers consume most of the lactate fed to the reactor. In this case, the main pathway of lactate degradation will involve sulfate reducers and aceticlastic methanogens (Path 3, Figure 3-11).
- If there is a significant population of fermenters present, the result is an imbalance which causes lactate to be routed through an additional pathway where propionate is formed (Path 1, Figure 3-11). In this case, if a significant population of propionate degrading SRB are present to degrade the propionate, the reactors will remain stable.
- Greater numbers of *Archaea* than *Bacteria* were found in all reactors. This may be attributed to the availability of acetate in the reactors for MA growth and to using the immobilized fixed bed reactor type.
- Aceticlastic methanogens were the dominant methanogen in all reactors, and they were responsible for nearly all acetate removal. This was seen in a low OLR/SLR and low sulfide environment and also in a high OLR/SLR and high sulfide environment.
- Clone libraries appear to provide information on the major microbial groups in a community and can aid in explaining reactor performance, whereas qPCR has the

potential to reveal not only the major microbial groups, but also groups that have smaller but important roles in the community.

6.5 Recommendations for future research

It is recommended that the degradation pathway of propionate in sulfate fed systems be further investigated. This could be achieved by microcosm studies.

It is recommended that the role of hydrogen utilizing MA and SRB in sulfate fed systems be further assessed.

It is recommended that the affinity of different types of SRB for sulfate and electron donors such as lactate and hydrogen be determined. These properties have not been determined for a broad range of SRB and would be useful in assessing competition between the groups. It is further recommended that sulfide inhibition studies on the SRB groups be done as well.

It is recommended that a qPCR technique capable of quantifying the different SRB groups and acetogenic bacteria be developed in order to more fully understand the role of propionate and lactate utilizers.

It is also recommended that additional molecular techniques that can asses the spatial orientation of different microbial groups, such as Fluorescent In-Situ Hybridization, be applied to biofilm structures in sulfate fed systems. Information on the spatial orientation of MA and SRB groups in the biofilm might help to explain the degradation pathways proposed in this study.

Finally, in order to better understand how different populations develop within the reactors, the following experiment is proposed: Seed six attached growth reactors with

the same seed inoccula and use startup loading and operating conditions similar to this experiment, with increasing OLR and SLR over time. Around 250 days, begin to add increased lactate to two reactors, add increased propionate to two reactors, and leave the other two with the normal load rate. Then, monitor the microbial populations of all reactors by using qPCR about every 50 to 100 days in order to detect if there is a difference between the reactors due to exposure to higher levels of fatty acids.

APPENDIX A

REACTOR PHOTOGRAPHS

Figure A-1 Reactors prior to startup, showing initial color of pumice, locations of sample ports, and media and lactate pumps.

Figure A-2 Reactors on day one after seeding with enrichment cultures.

Figure A-3 Reactors on day 117.

Figure A-4 Reactors on day 406.

Figure A-5 Closeup of lower portions of R1 and R2 on day 406.

Figure A-6 Closeup of upper portions of R1 and R2 on day 406.

APPENDIX B

MÖSSBAUER ANALYSIS OF REACTOR PRECIPITATE

A large amount of black precipitate was observed in all reactors during the study. This precipitate caused the reactors to blacken over time, as seen in the photographs of the reactors in Appendix A. In order to determine the oxidation state of the iron in the solid, a sample of this precipitate was collected on day 205 and analyzed using transmission Mössbauer spectroscopy. All Mössbauer work was performed by Phil Larese-Casanova in the University of Iowa Environmental Engineering Laboratory. A Mössbauer spectrum was collected in transmission mode at room temperature. A ^{57}Co source was used with constant acceleration. Data was calibrated with α-Fe(0) spectral line positions and modeled with Recoil software package. The output spectra and model are displayed in Figure D-1. The data can be modeled as one singlet that corresponds well with observed parameters for mackinawite, $FeS_{0.9\text{-}1.0}$. The center shift (CS) is 0.37 mm/s and the quadrupole splitting (QS) is nearly zero (0.001 mm/s). By comparison, (Mullet et al. 2002) reported CS = 0.42 mm/s and QS=0.00 mm/s for synthesized mackinawite.

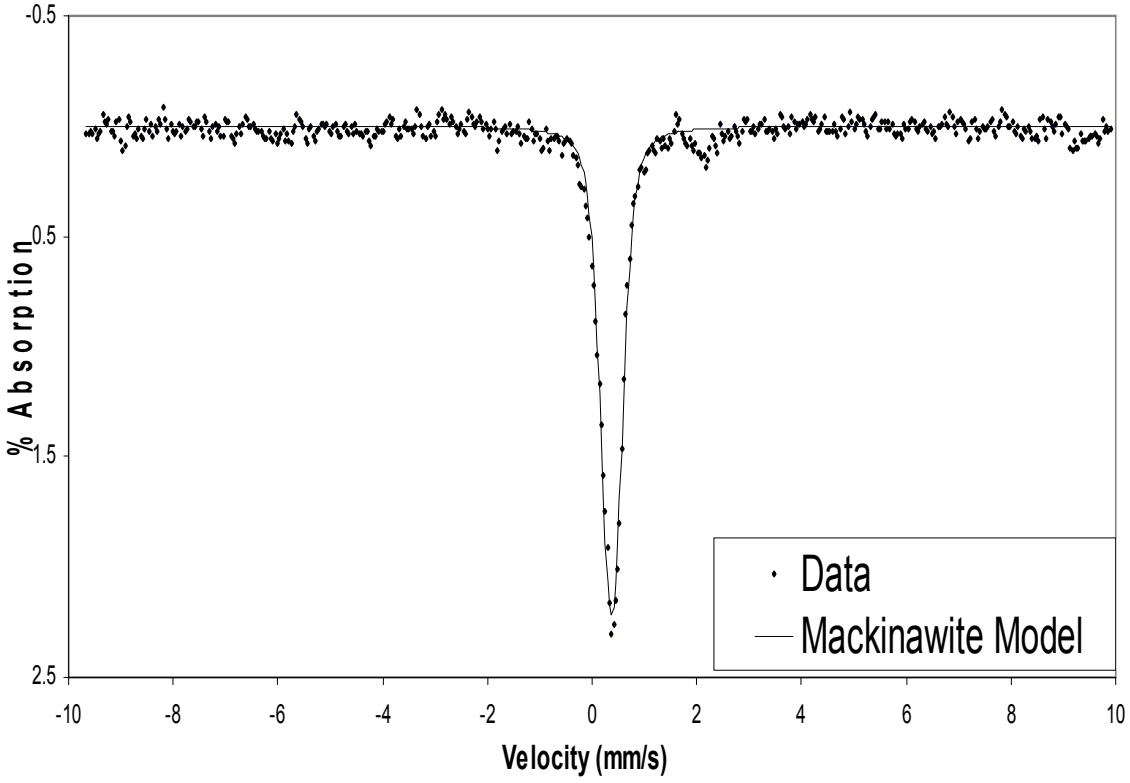


Figure B-1 Mössbauer spectra for iron precipitate found in reactors.

APPENDIX C

MICROCOSM STUDIES

C.1 Experimental procedure

After about 1,010 days of operation, samples were removed from column reactors to perform microcosm studies. Samples were removed from sample ports located along the sides of each reactor at heights of 6 in. (Port B) and 14 in. (Port C) from the bottom, then placed into 125 mL serum bottles with 50 mL of reactor mineral media and pH was adjusted to 7.0. Eight samples of 2 g each were removed from each port. Half of the microcosms were fed lactate (n = 4) and half were fed acetate (n = 4), all in the amount of 1 g COD/L. Sulfate (0.1 g S/L) was added to two of the four bottles fed lactate or acetate. Bottles were placed on a shaker table and shaken at 200 rpm. Samples were removed from the bottles at various times and analyzed for VFAs and for bottles with sulfate added, sulfate and sulfide. Lactate microcosms were run for 3 days, while acetate microcosms were run for 20 days.

In addition, one baseline microcosm was performed with propionate using reactor material from R1 port C. This baseline was run for 14 days. Due to time constraints, no further propionate studies were run.

C.2 Results

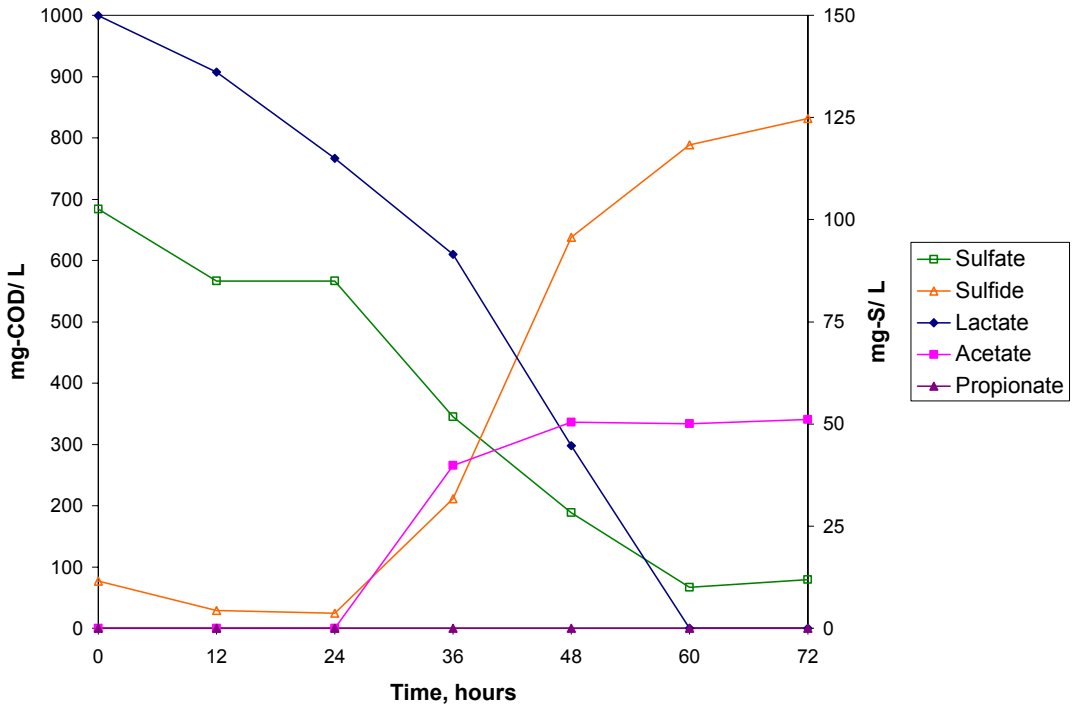
In microcosms fed lactate all lactate was removed within hours. R1 samples with sulfate showed complete sulfate removal as well, with no propionate production, indicating that SRB were responsible for a majority of the lactate degradation (Figure C-

1). However, significant propionate was formed in R2 and R3 microcosms and sulfate removal was less than 100% in R2 Port B and less than 25% in both R3 ports (Figures C-2 and C-3). This indicates that SRB may be competing with fermenters for some of the lactate in R2, but fermenters out-compete SRB for lactate in R3. In the absence of sulfate, all microcosms produced propionate and acetate, indicating the presence of fermenters (Figures C-4 to C-6). The most rapid sulfate removal was observed in R1 followed by R2, with R3 exhibiting very little sulfate removal (Figure C-7).

In the acetate-fed microcosms, only about 20% sulfate removal was observed after 20 days (Figures C-8 to C-11). Complete acetate removal was observed in most microcosms, with few differences between microcosms with and without sulfate (Figure C-12), indicating that aMA, and not cSRB, are responsible for acetate degradation.

The propionate baseline study indicated that while propionate removal did occur in conjunction with sulfate removal in R1, this process was very slow (Figure C-13).

a)



b)

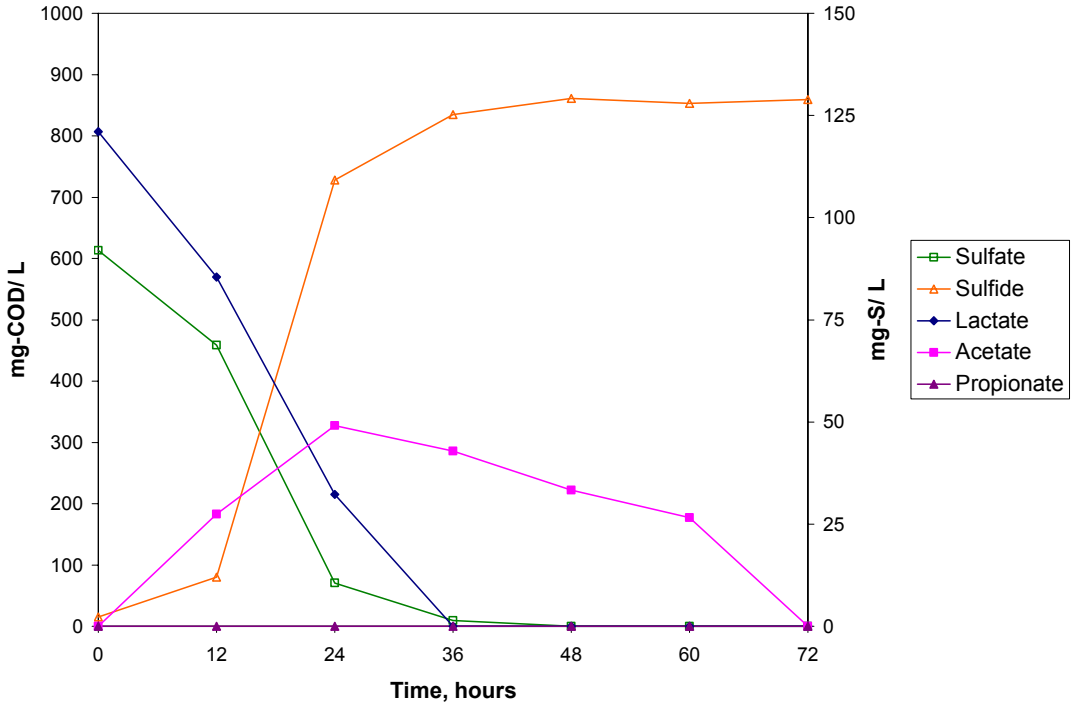
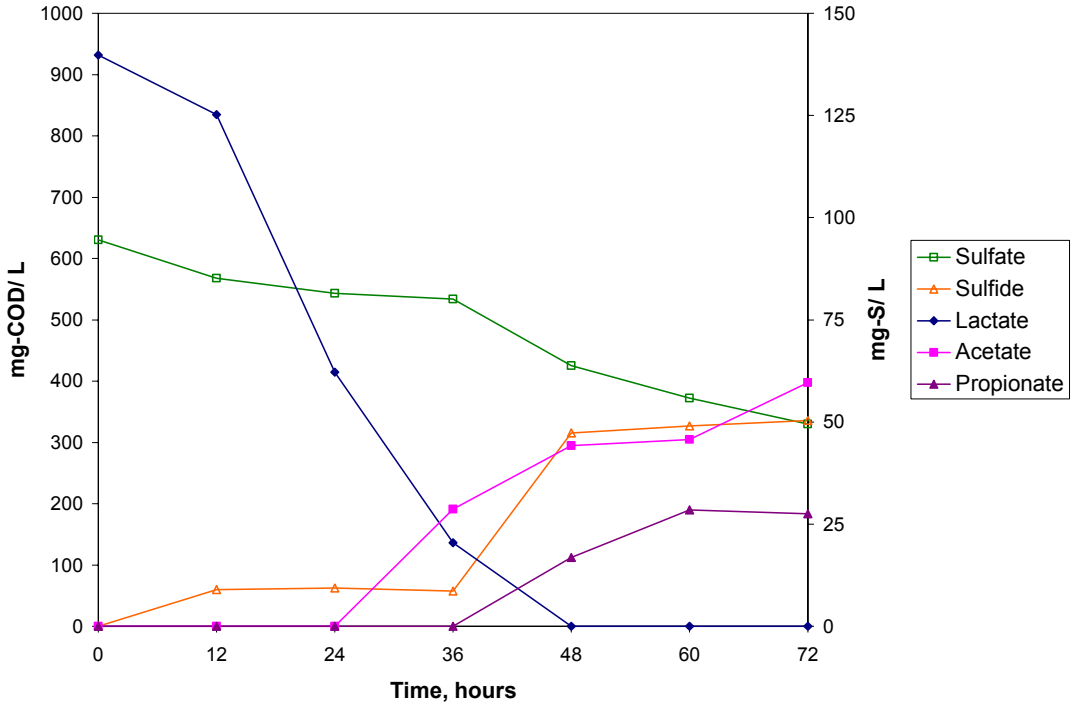


Figure C-1 R1 microcosm results for lactate plus sulfate for a) port B and b) port C.

a)



b)

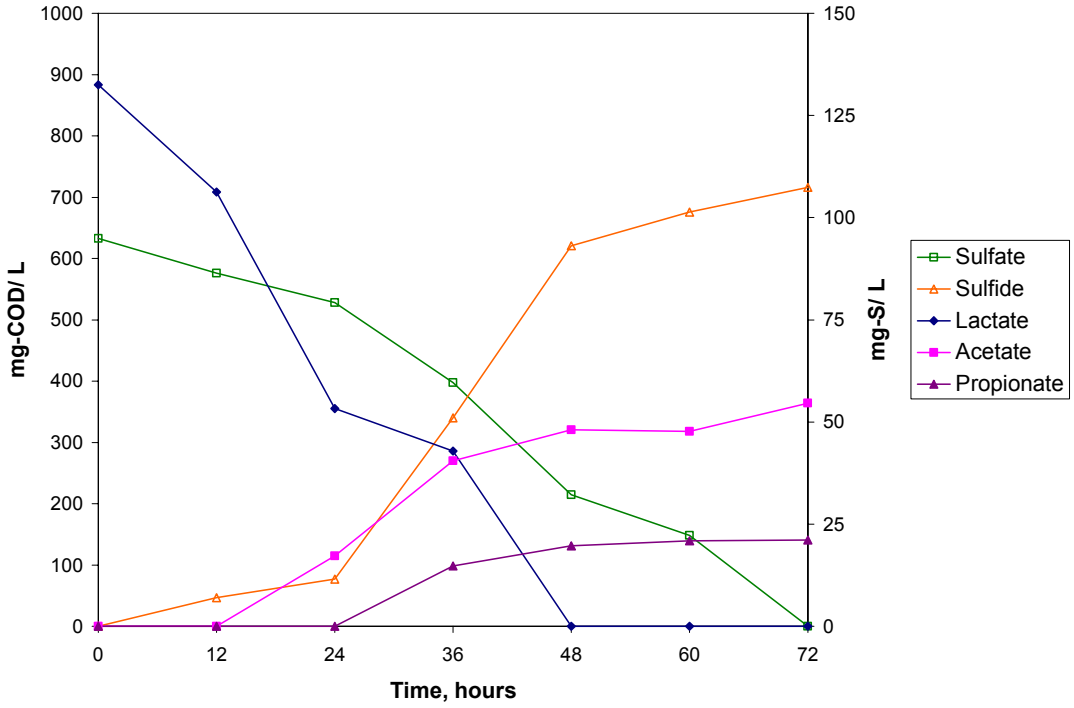
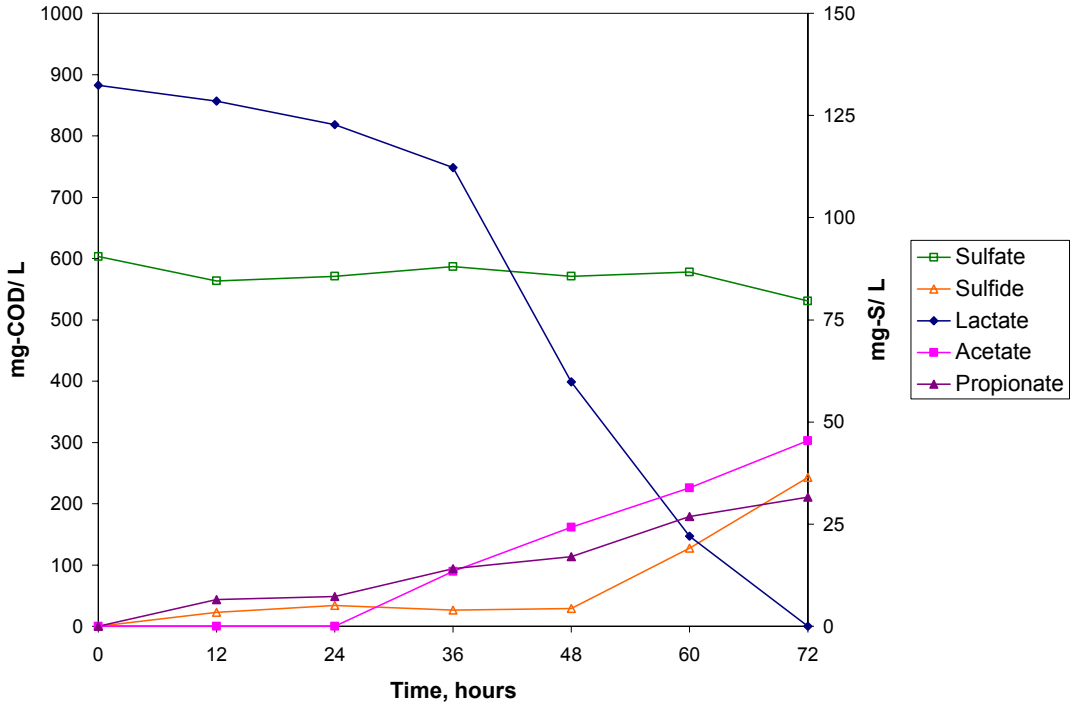


Figure C-2 R2 microcosm results for lactate plus sulfate for a) port B and b) port C.

a)



b)

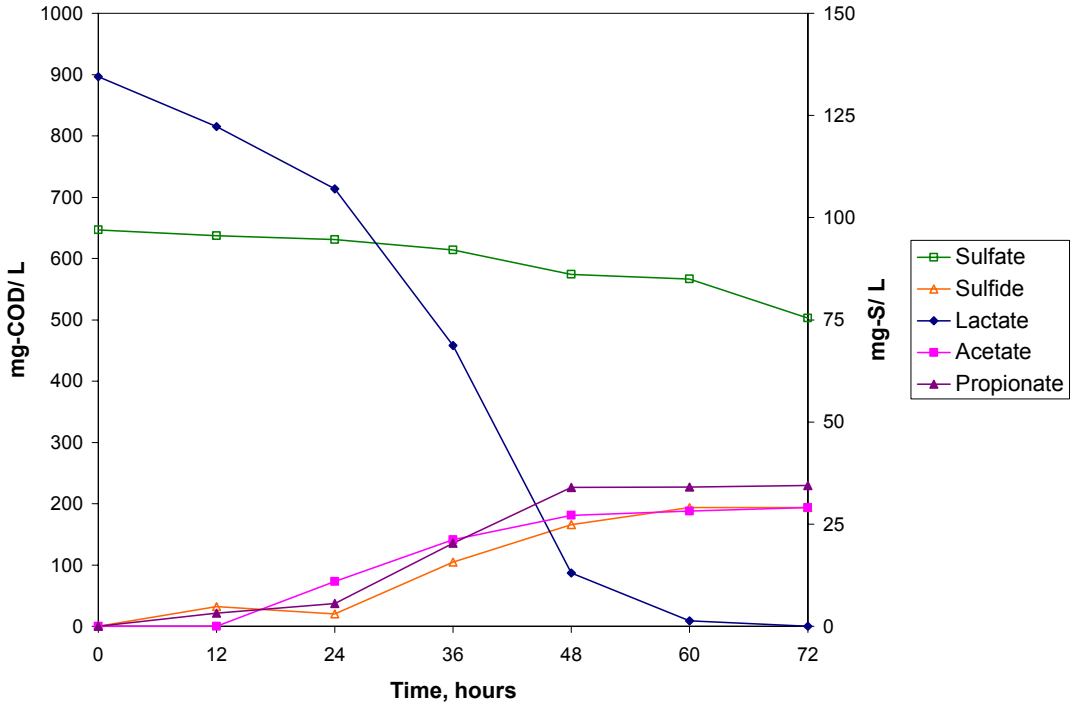
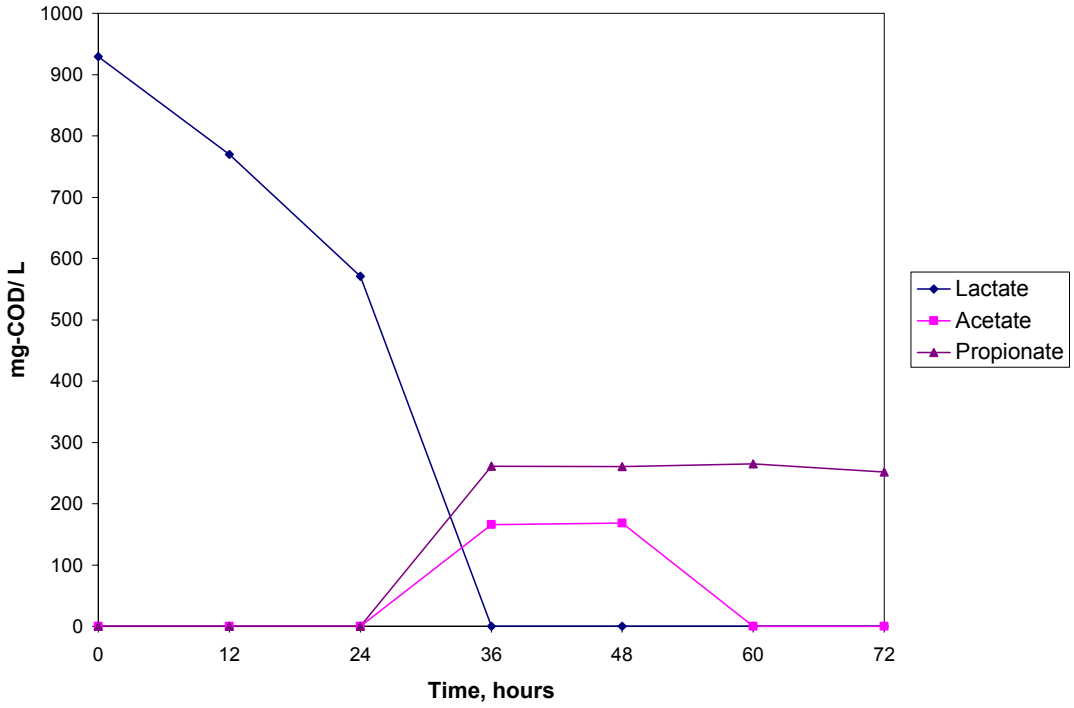


Figure C-3 R3 microcosm results for lactate plus sulfate for a) port B and b) port C.

a)



b)

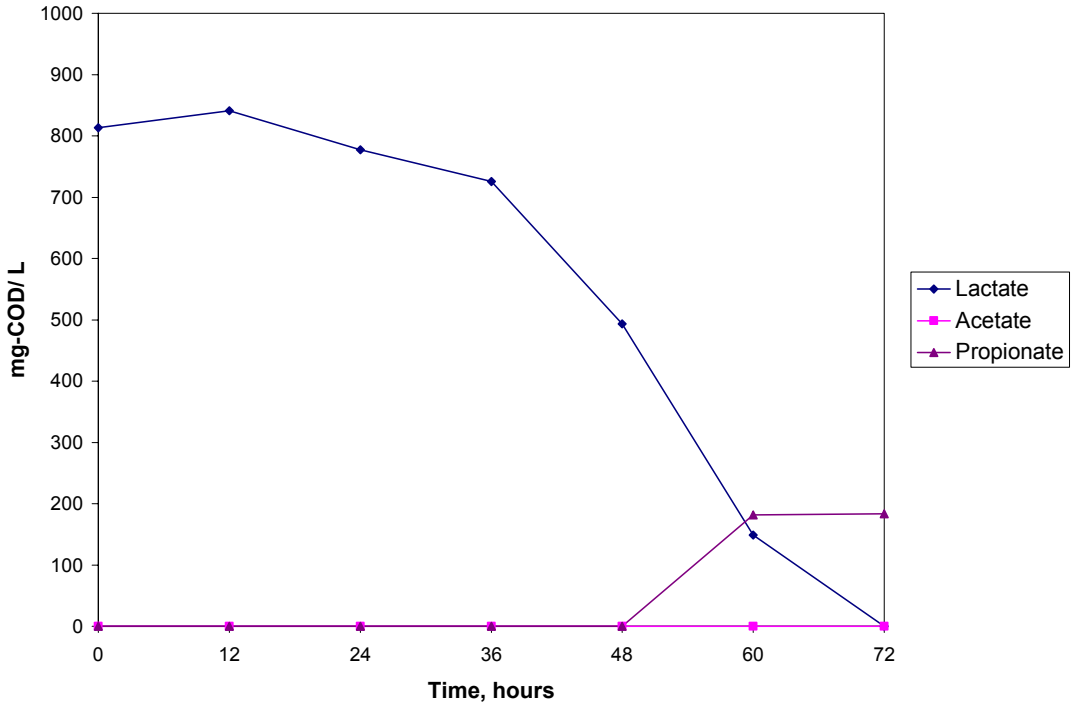
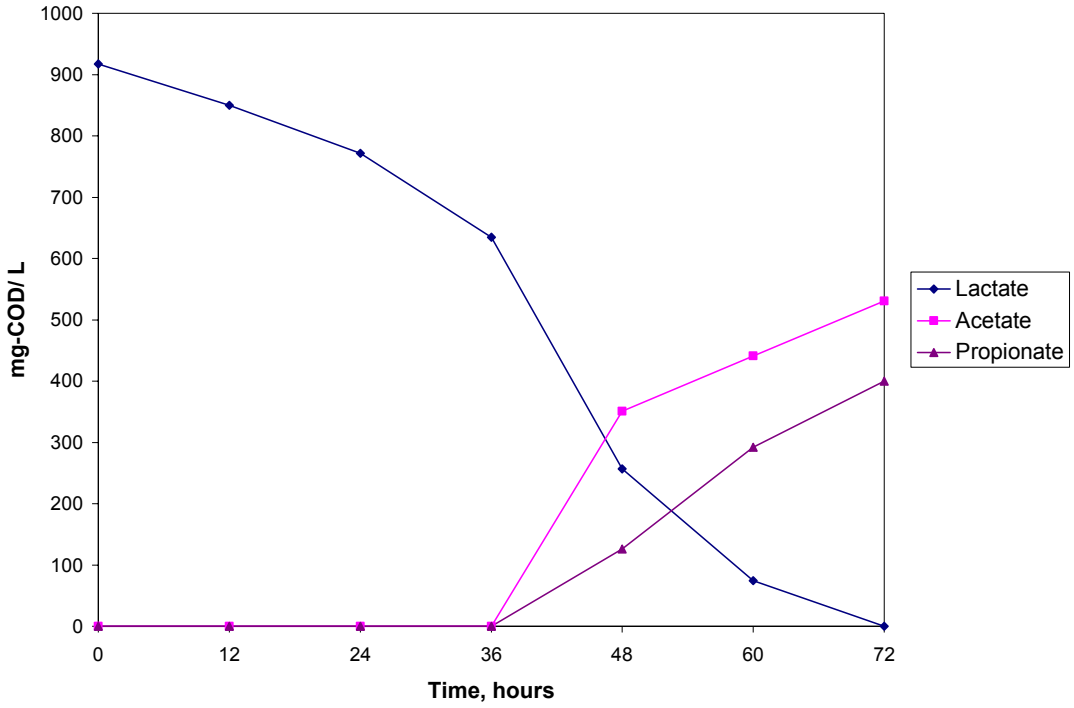


Figure C-4 R1 microcosm results for lactate only for a) port B and b) port C.

a)



b)

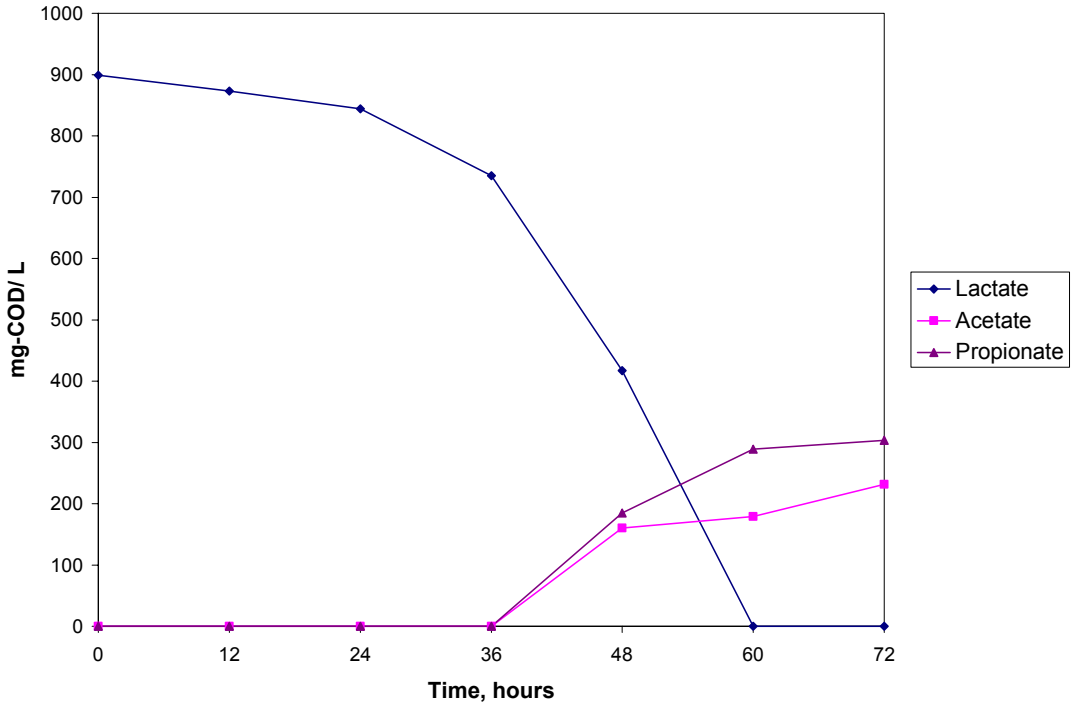
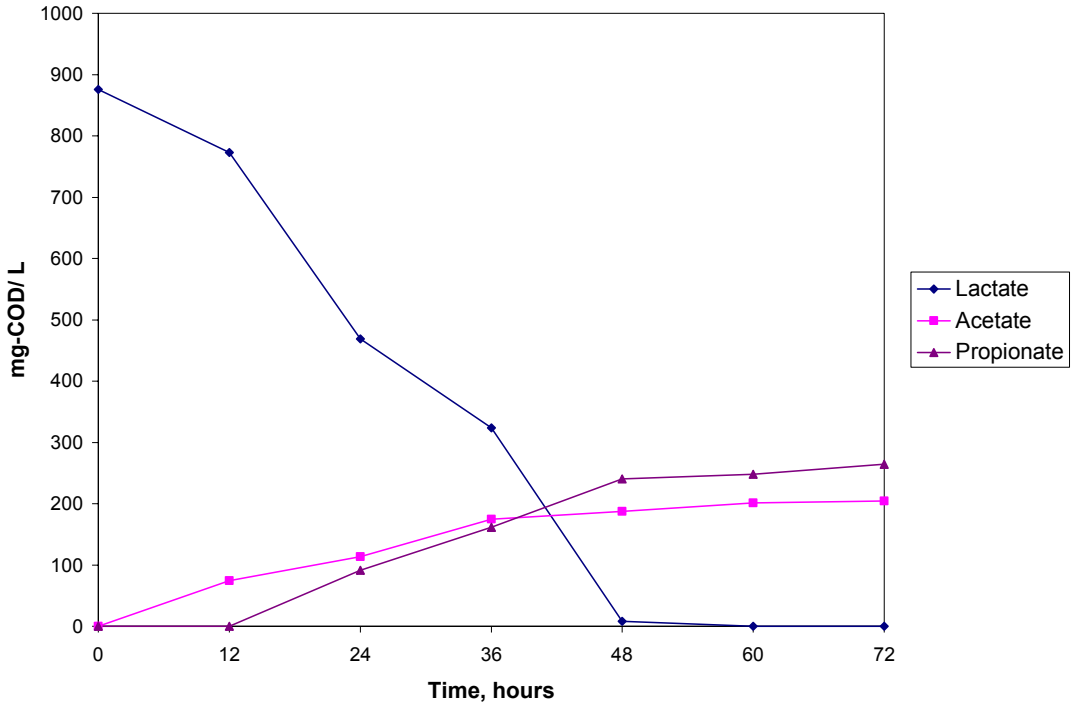


Figure C-5 R2 microcosm results for lactate only for a) port B and b) port C.

a)



b)

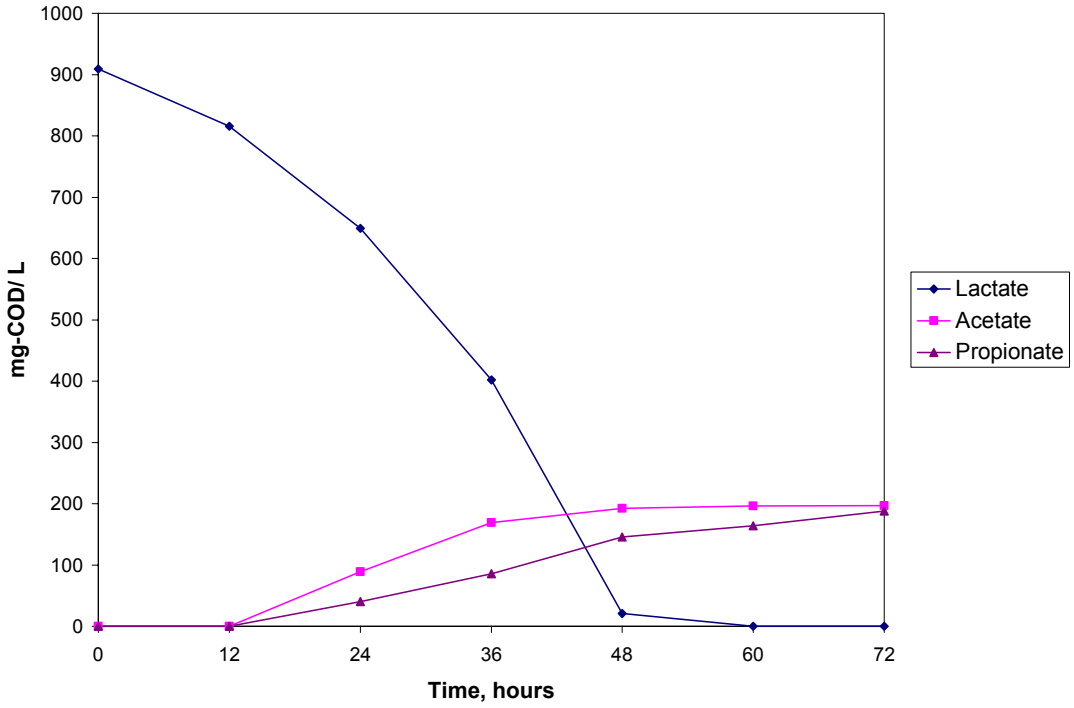


Figure C-6 R3 microcosm results for lactate only for a) port B and b) port C.

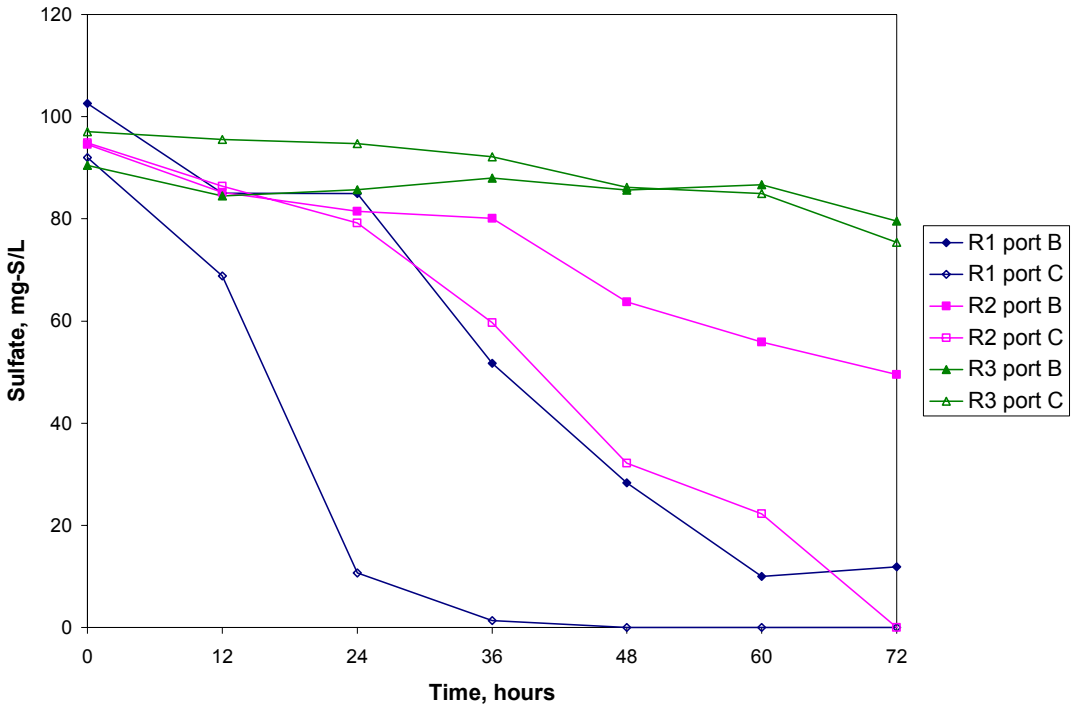
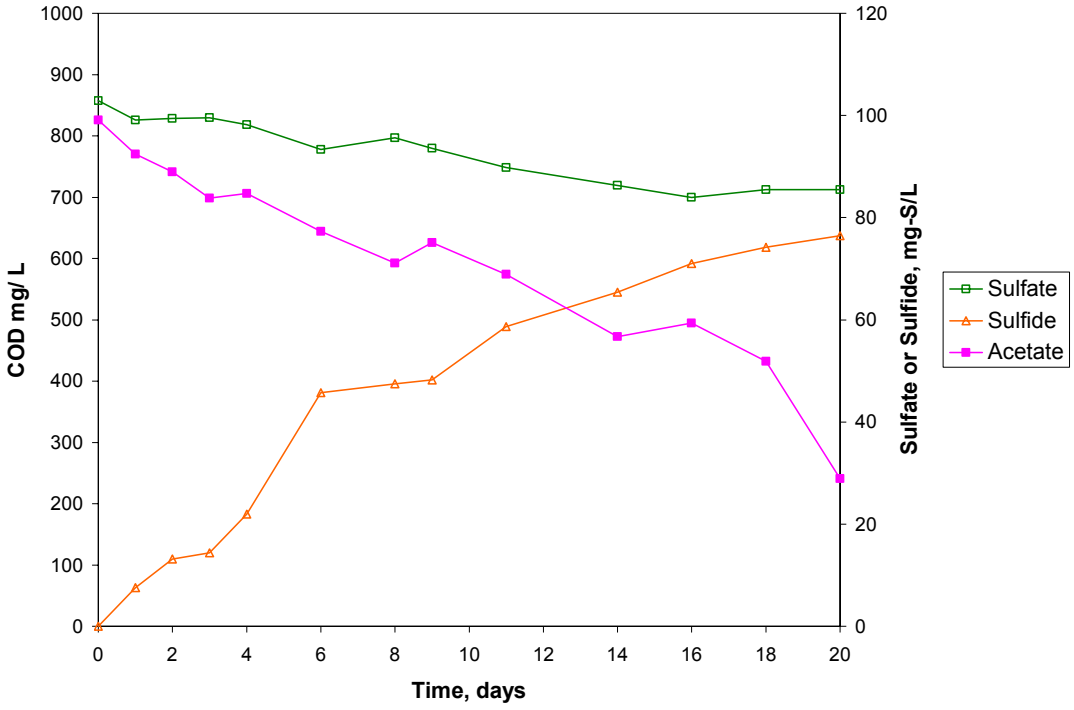


Figure C-7 Sulfate data trends for lactate-fed microcosms.

a)



b)

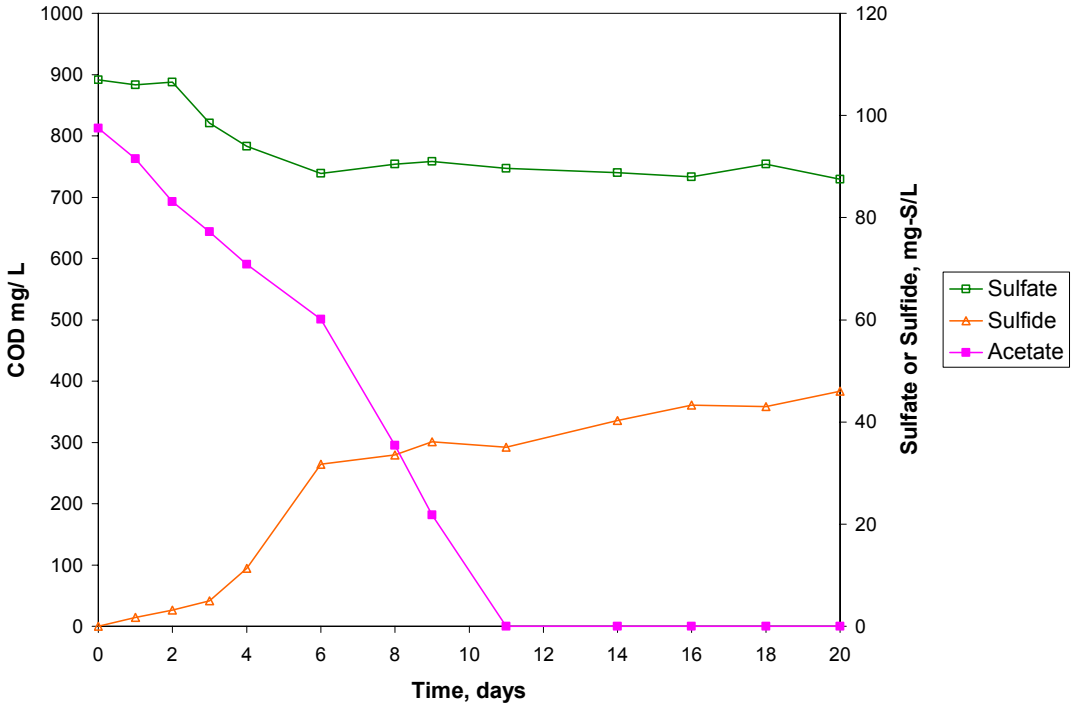
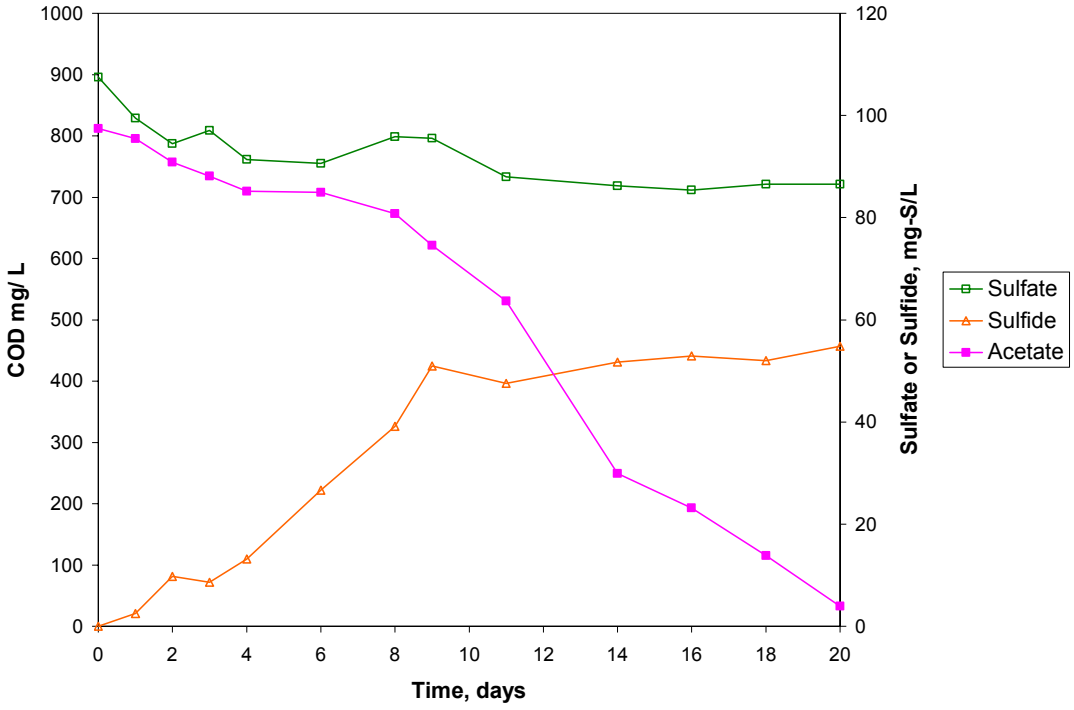


Figure C-8 R1 microcosm results for acetate plus sulfate for a) port B and b) port C.

a)



b)

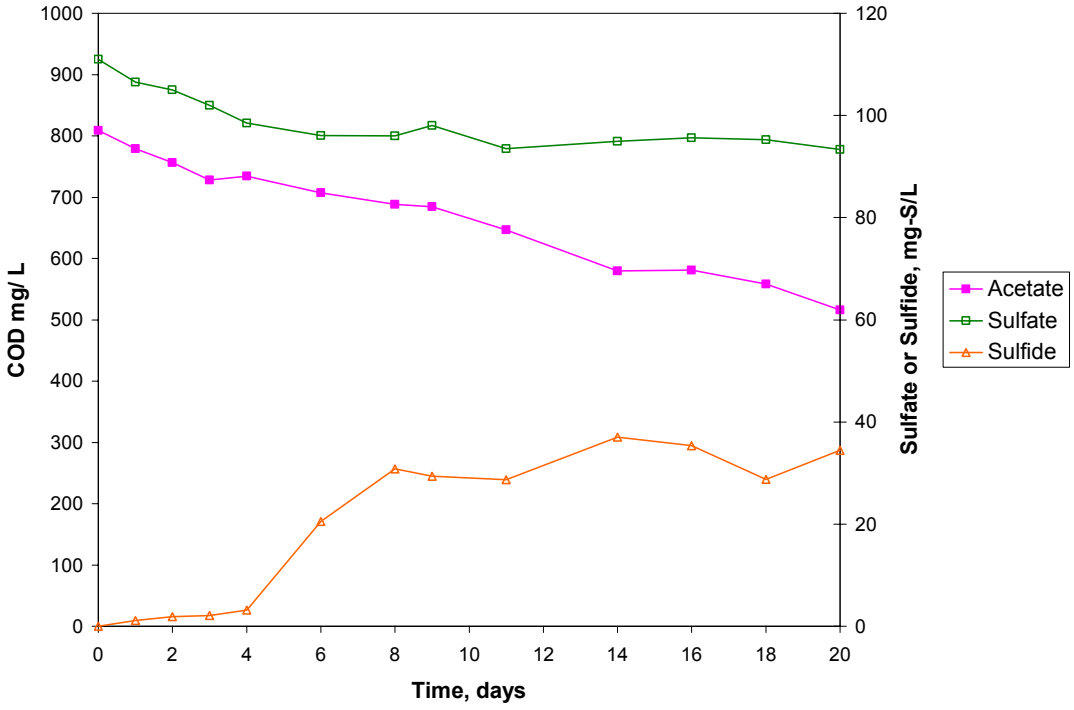
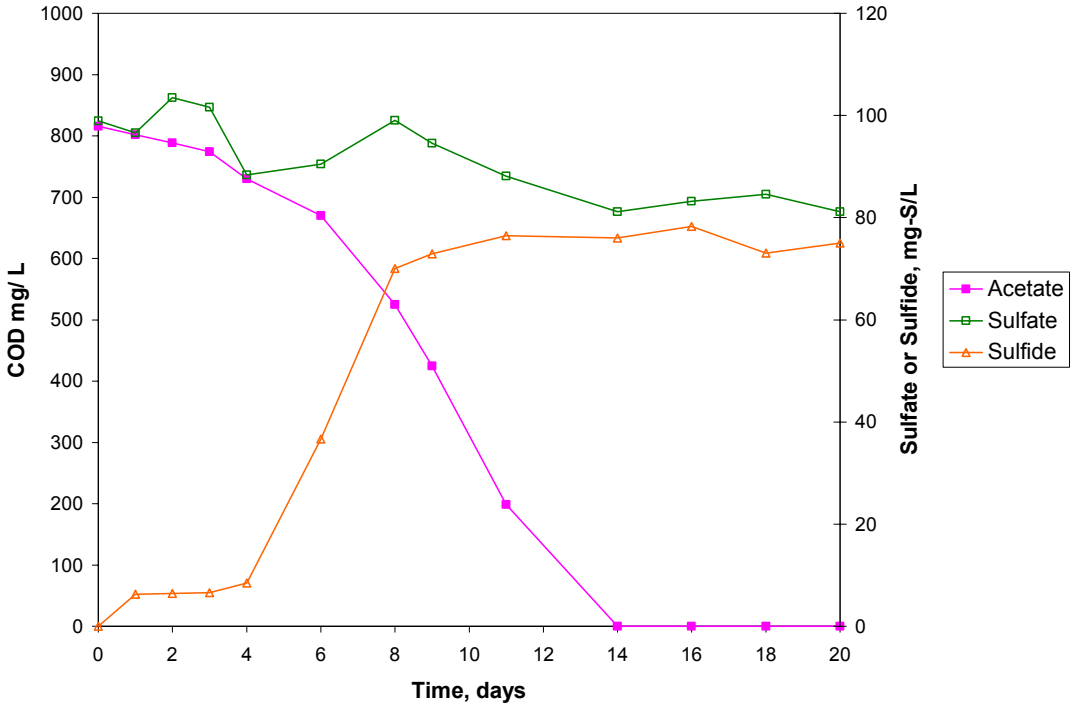


Figure C-9 R2 microcosm results for acetate plus sulfate for a) port B and b) port C.

a)



b)

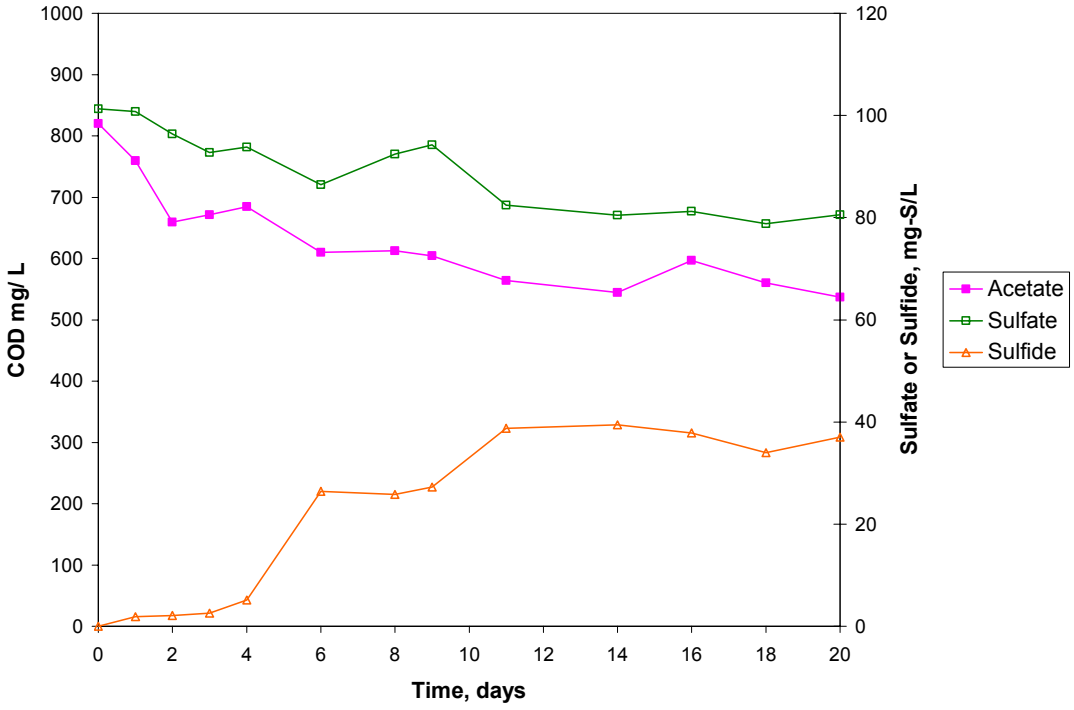


Figure C-10 R3 microcosm results for acetate plus sulfate for a) port B and b) port C.

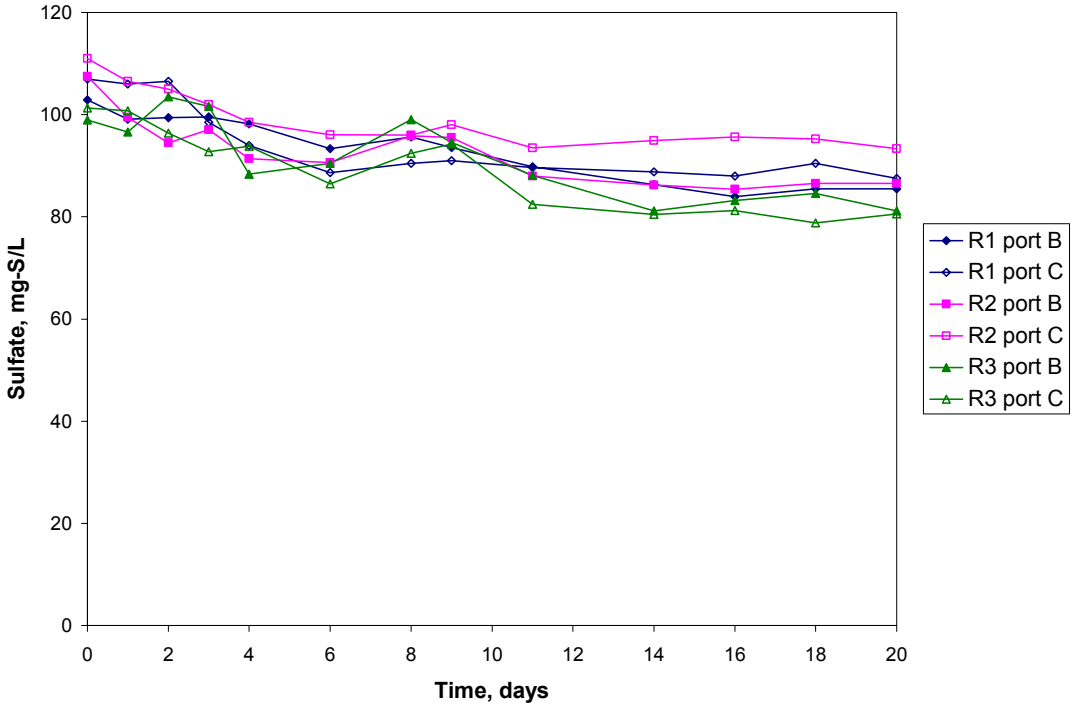
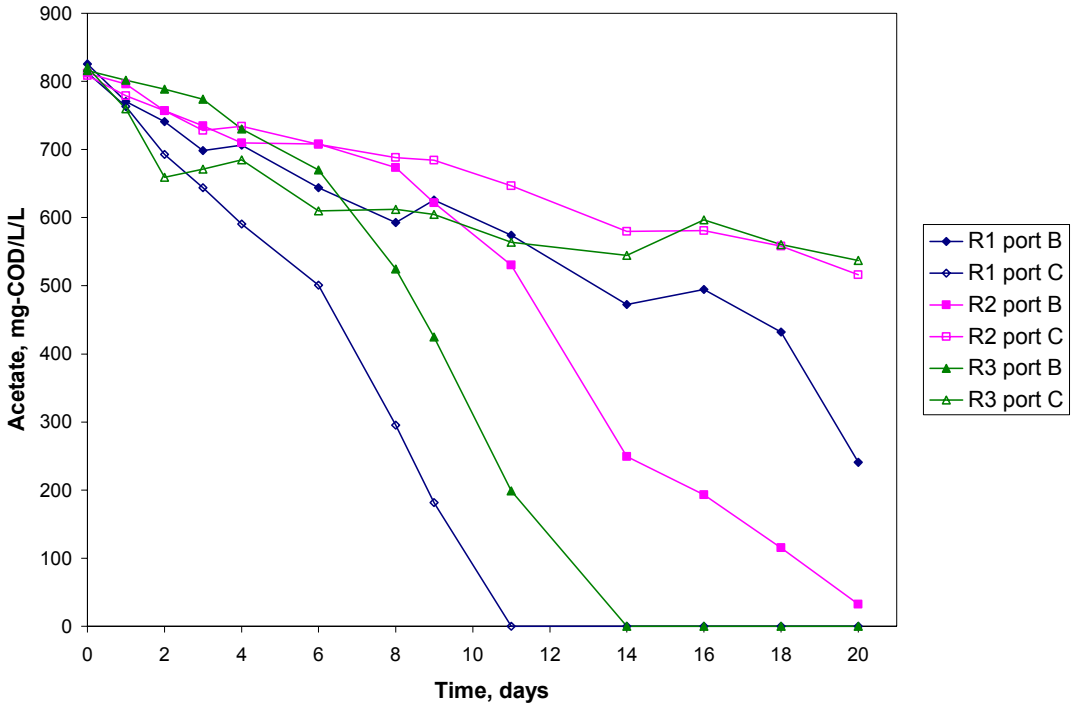


Figure C-11 Sulfate data trends for acetate-fed microcosms.

a)



b)

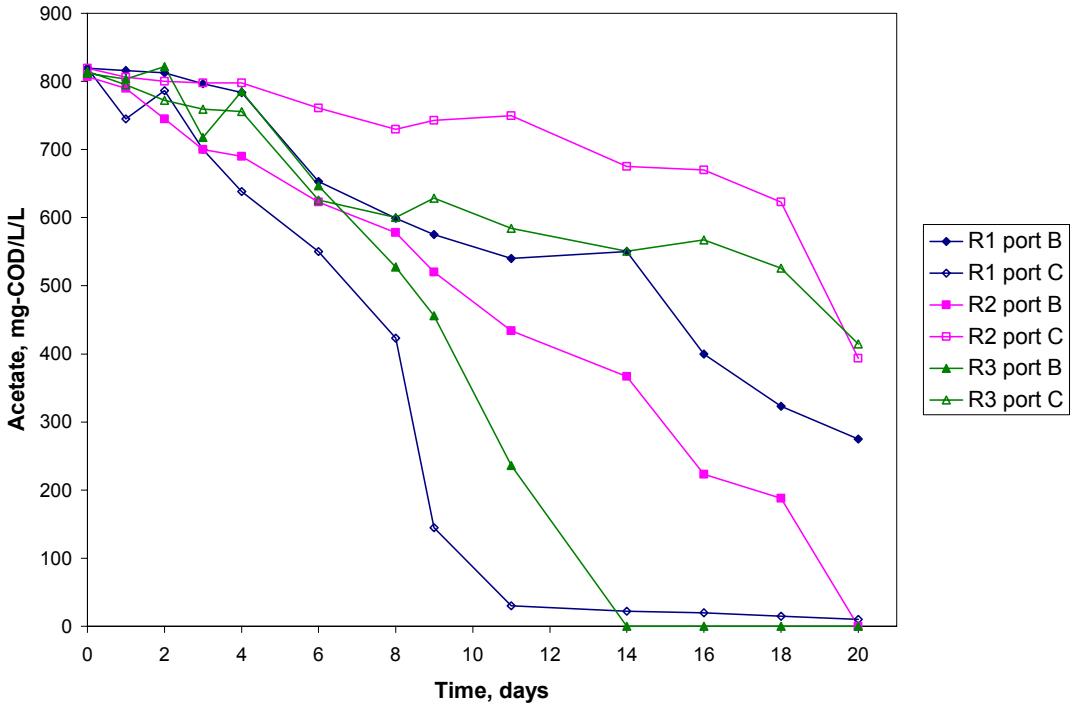


Figure C-12 R1 microcosm results for acetate for a) with sulfate and b) without sulfate.

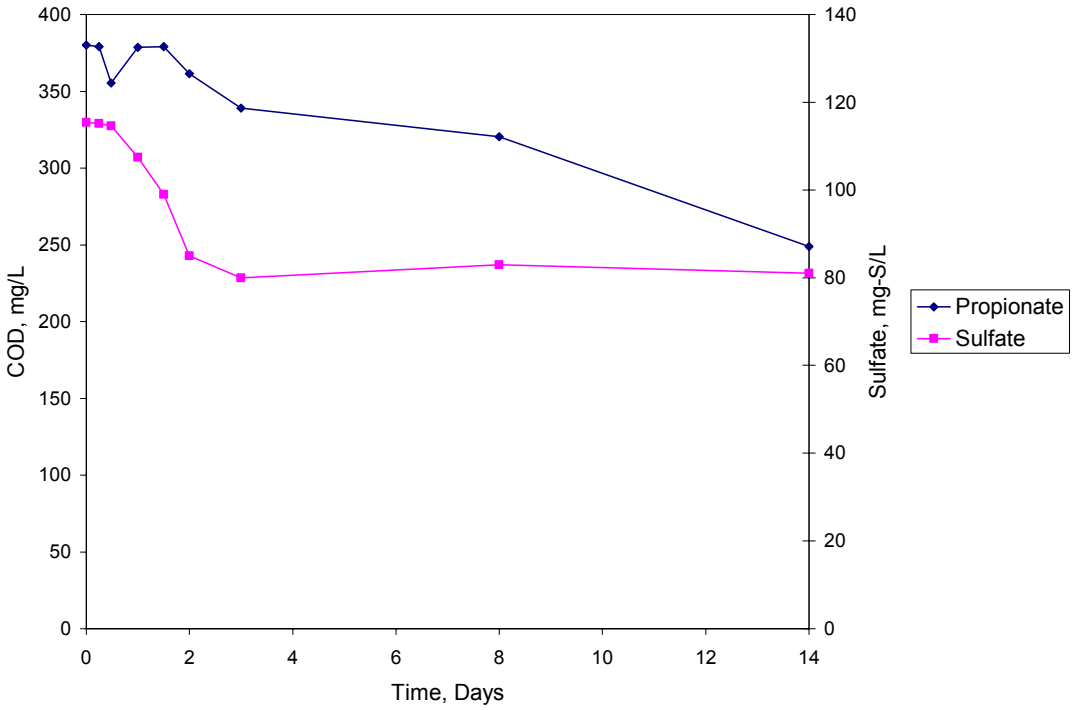


Figure C-13 Propionate baseline study with R1 port C material.

APPENDIX D

MICROSCOPY

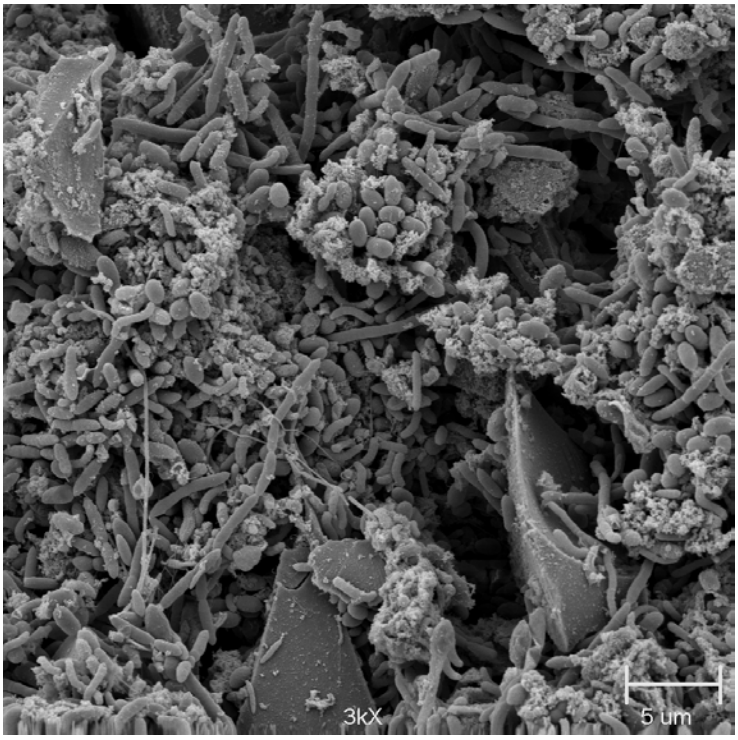
D.1 Experimental procedure

In order to visually characterize the biofilm in the reactors, 2g samples were removed from port A of each reactor on day 260. Samples were then fixed using a perfluorcarbon-OsO_4 fixation method for biofilm (Singh et al. 2000). Briefly, samples were immersed in 5 mL of 2% OsO_4 in perfluorcarbon (Fluorinert FC-72, #M) fixative and placed on a slowly rotating table for one hour. Samples were then rinsed in pure perfluorcarbon by mixing (3 x 5 min) on the rotating table. Dehydration of the samples was achieved by adding 100% ethanol (3 x 15 min) without agitation. Next, HMDS (hexamethyldisilazane) was added (2 x 15 min) and mixed on the rotator table. After decanting the HMDS, samples were left to dry overnight. Finally, selected pumice granules were mounted on SEM stubs, sputter coated, and examined in the scanning electron microscope (SEM).

D.2 Results

Figures D-1 through D-3 illustrate micrographs obtained from the fixed biofilm samples in the reactors. Several different morphologies can be seen in all reactors, indicating a complex, multi-species microbial community.

a)



b)

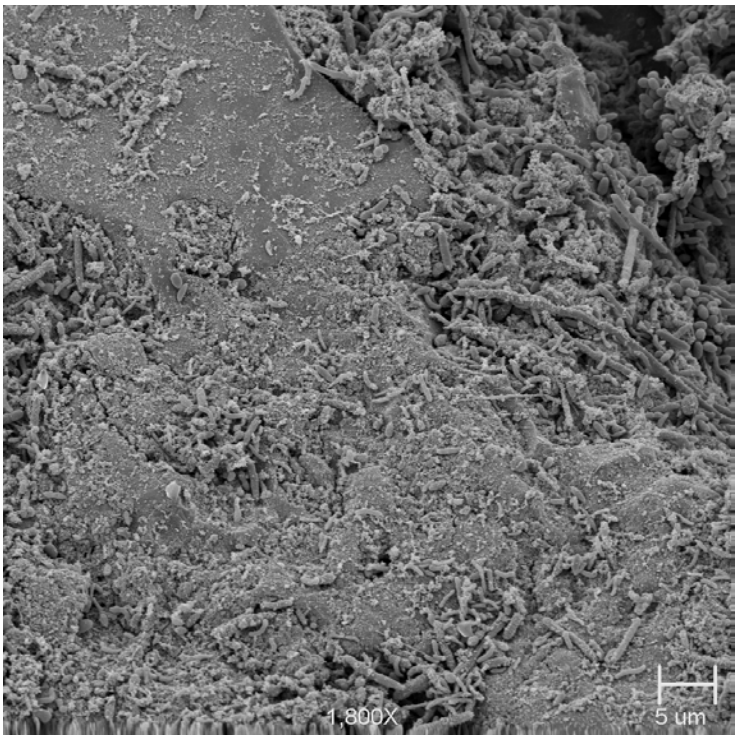
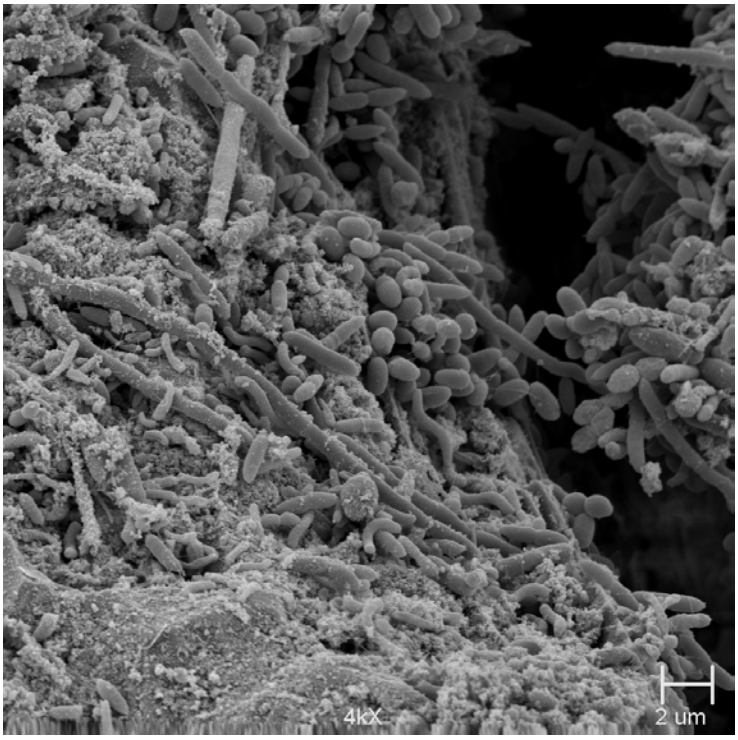


Figure D-1 Reactor R1 micrographs

c)



d)

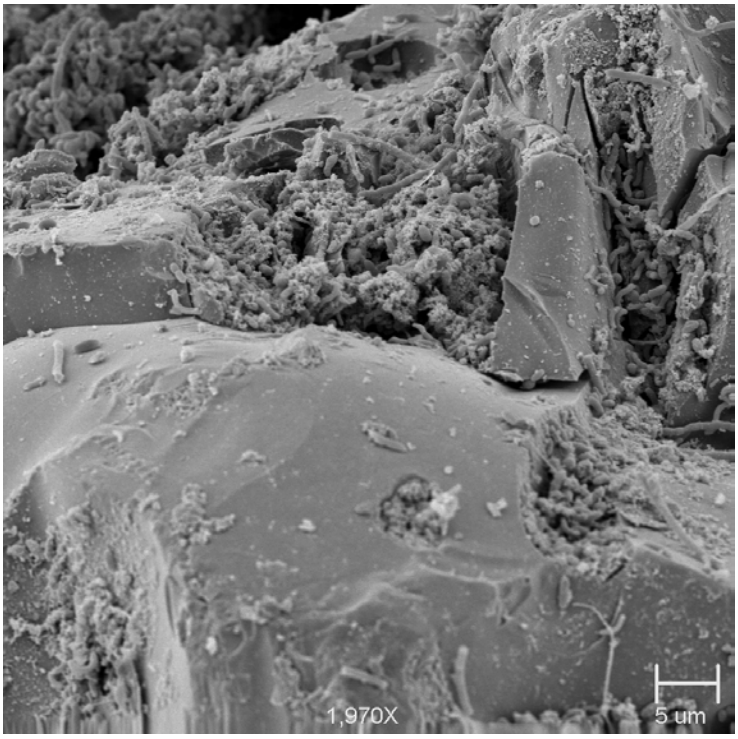
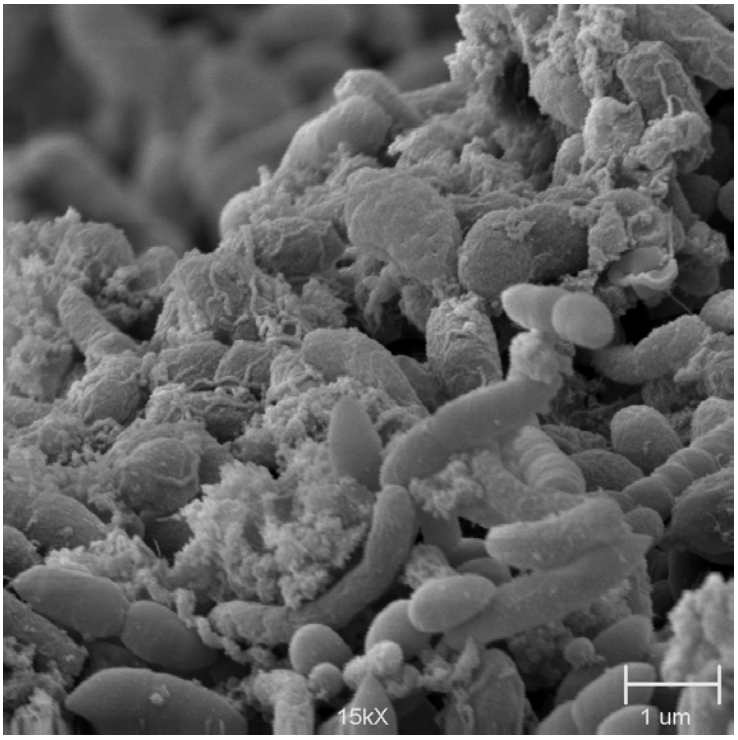


Figure D-1 continued

e)



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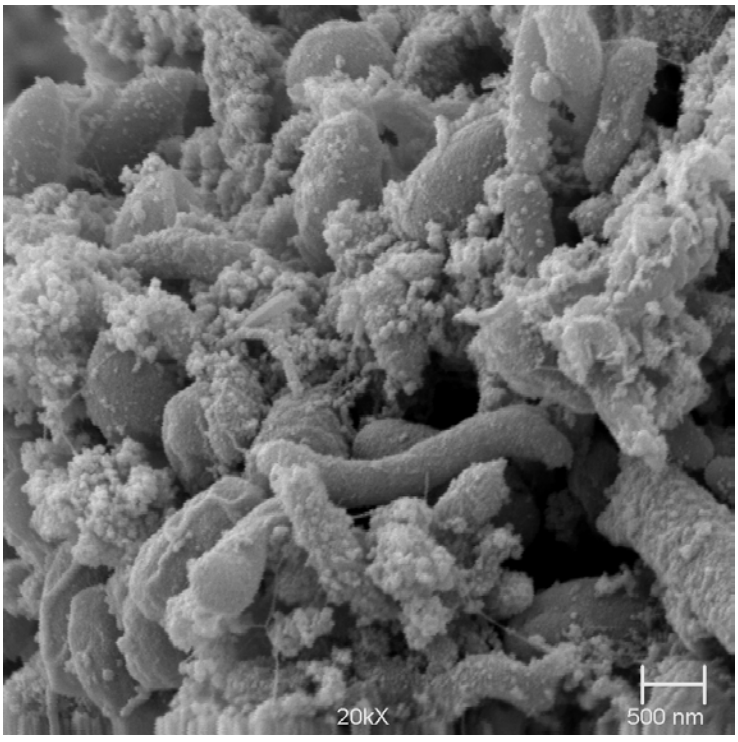

Figure D-1 continued

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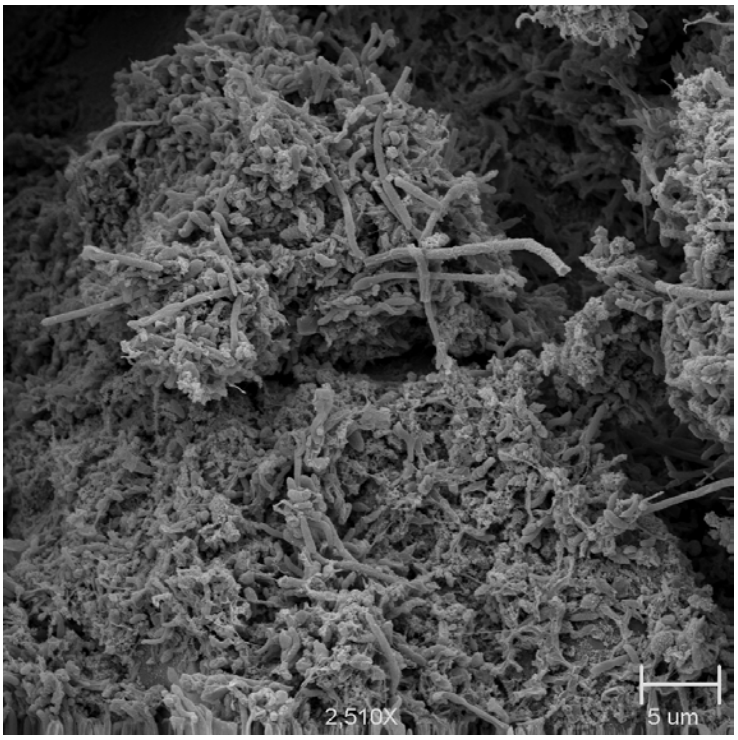

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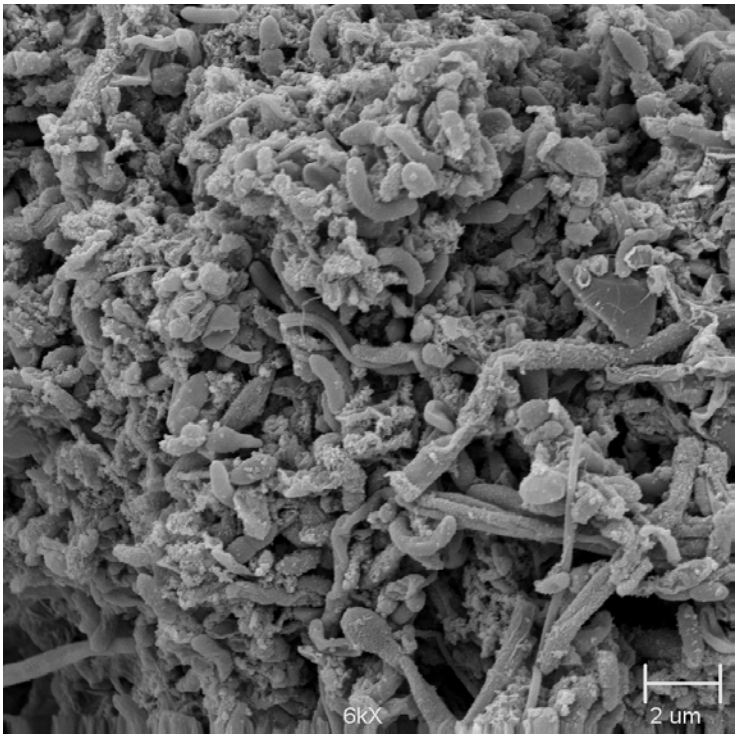
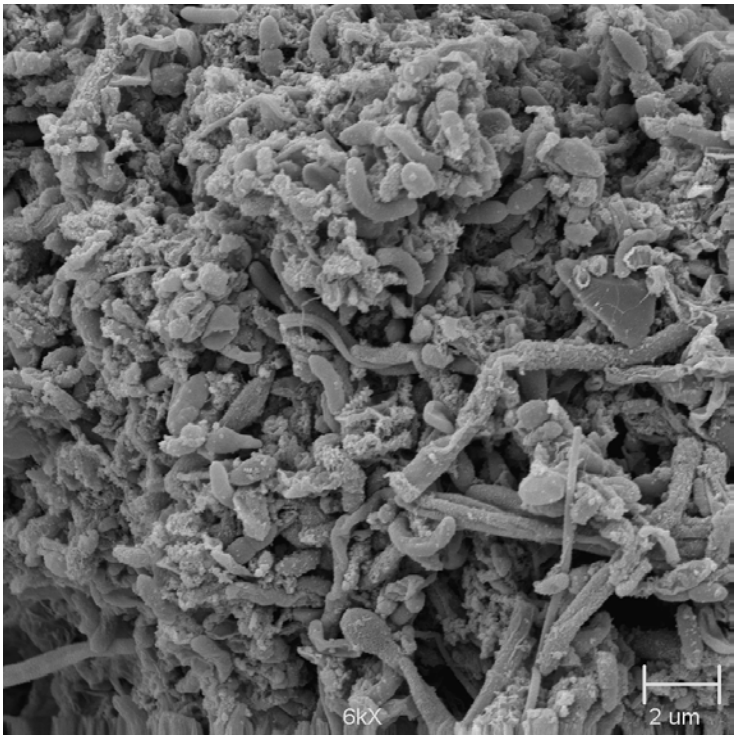


Figure D-2 Reactor R2 micrographs

c)



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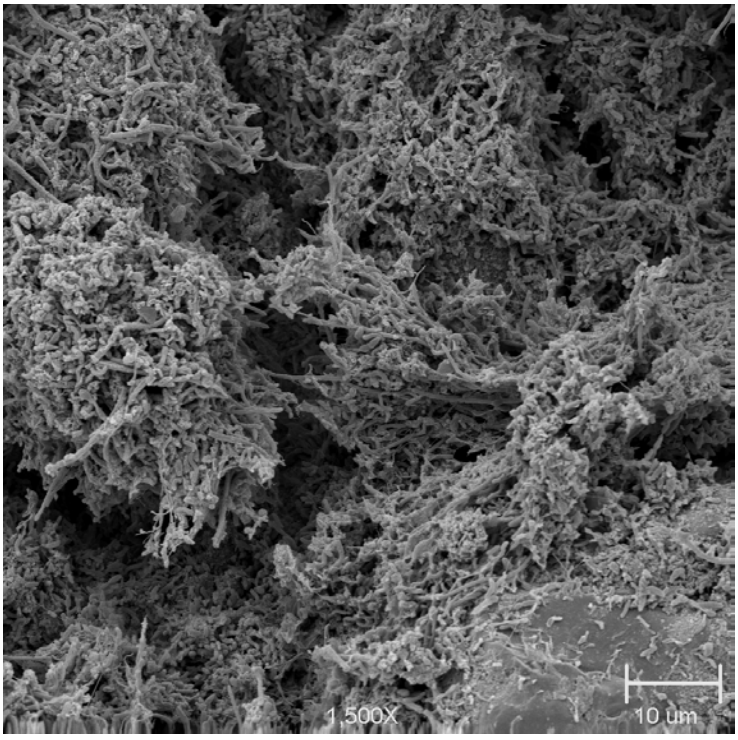

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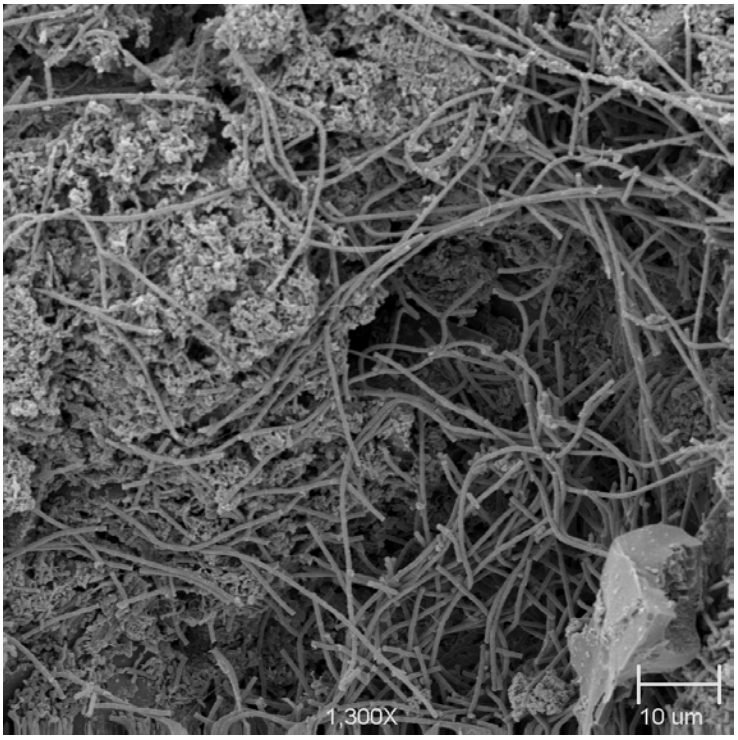

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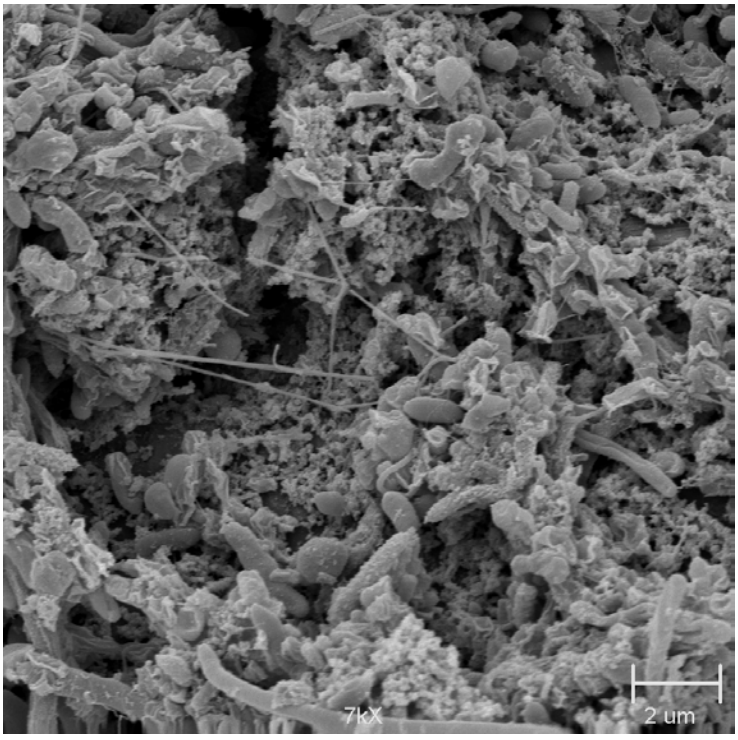

Figure D-2 continued

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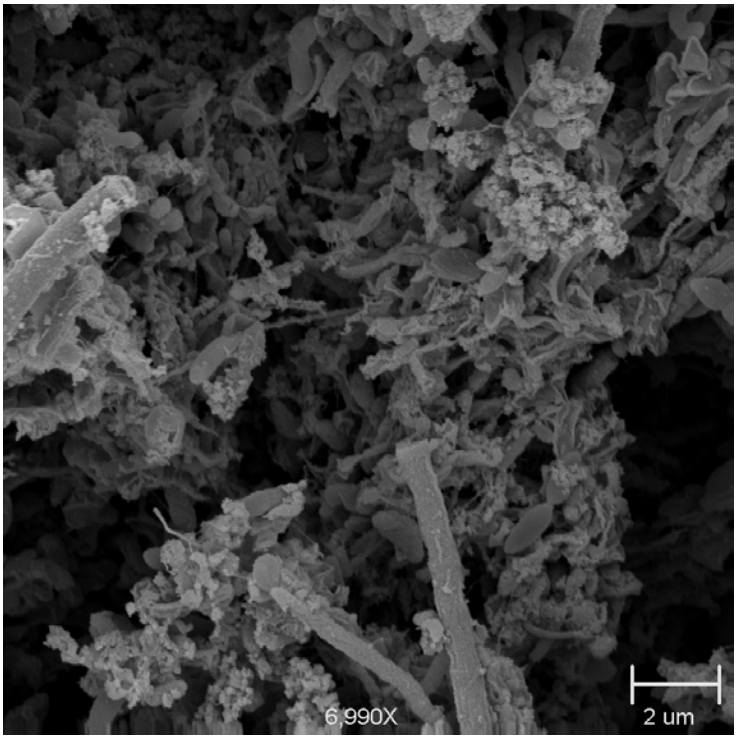

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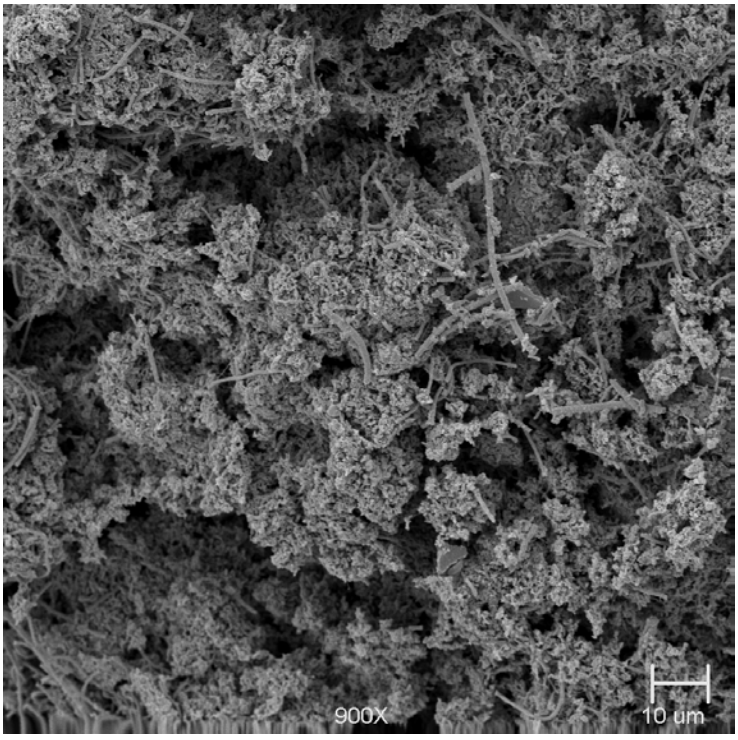
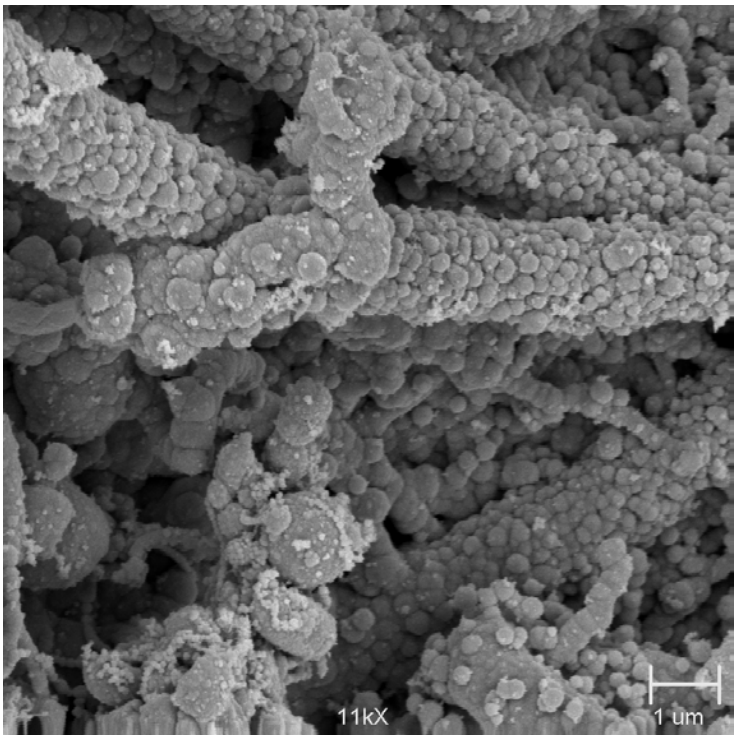


Figure D-3 Reactor R3 micrographs

c)



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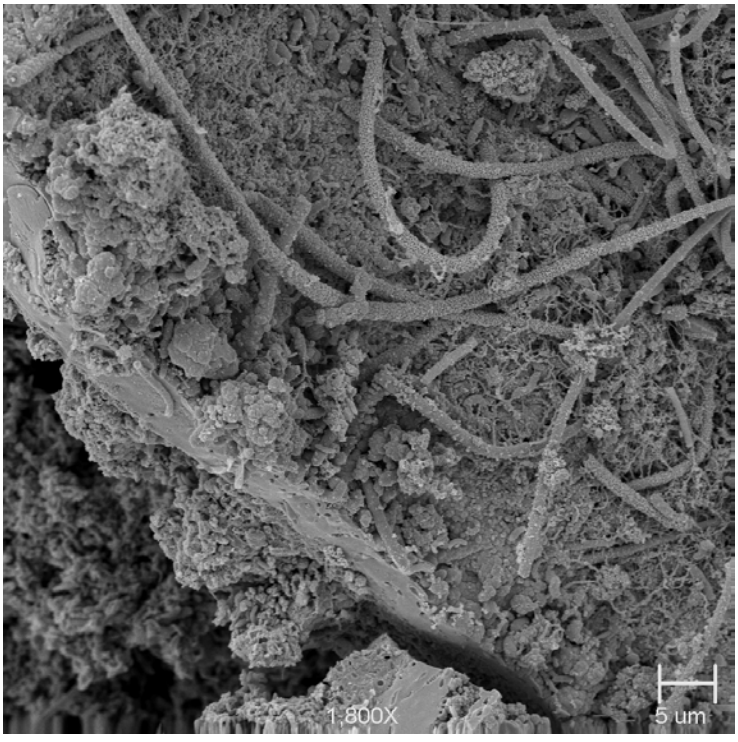

Figure D-3 continued

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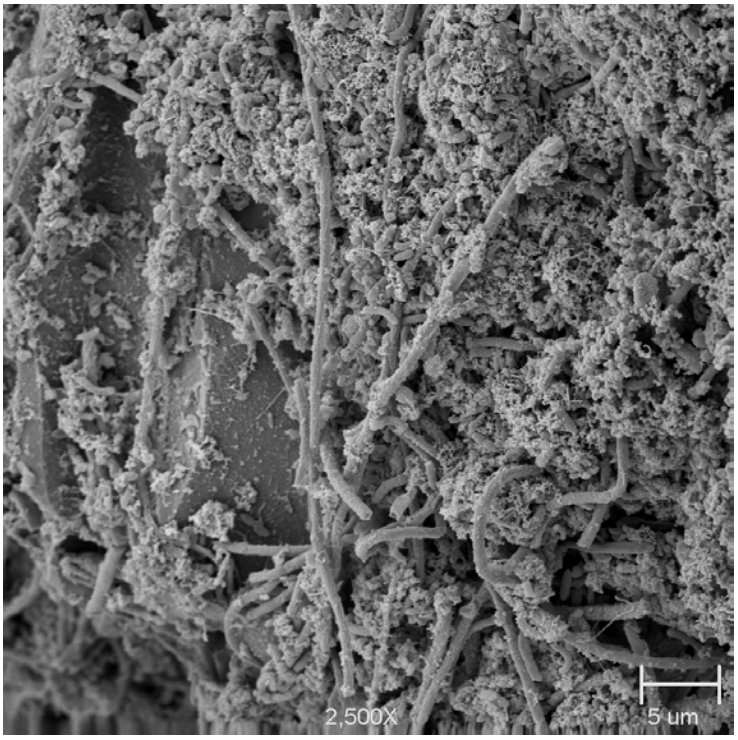

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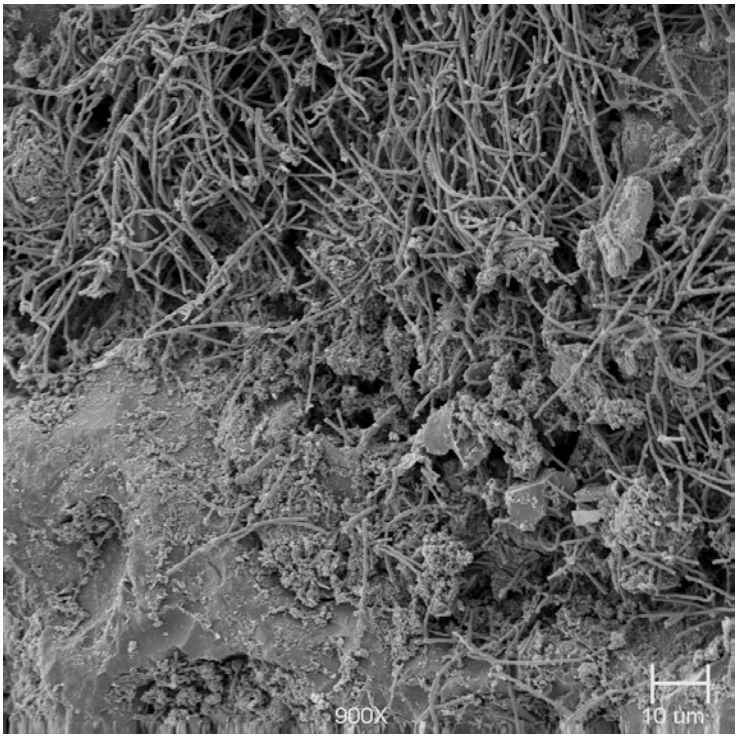

Figure D-3 continued

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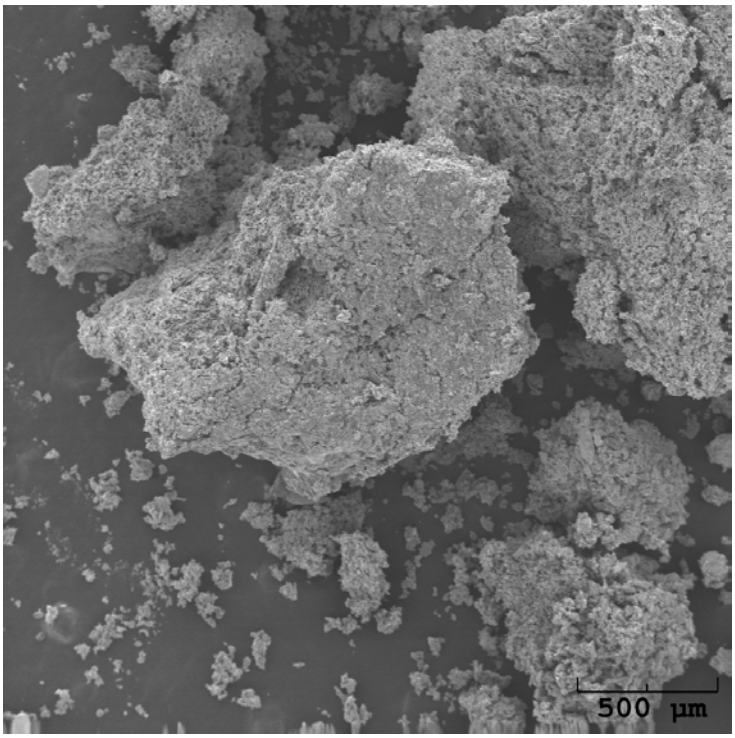

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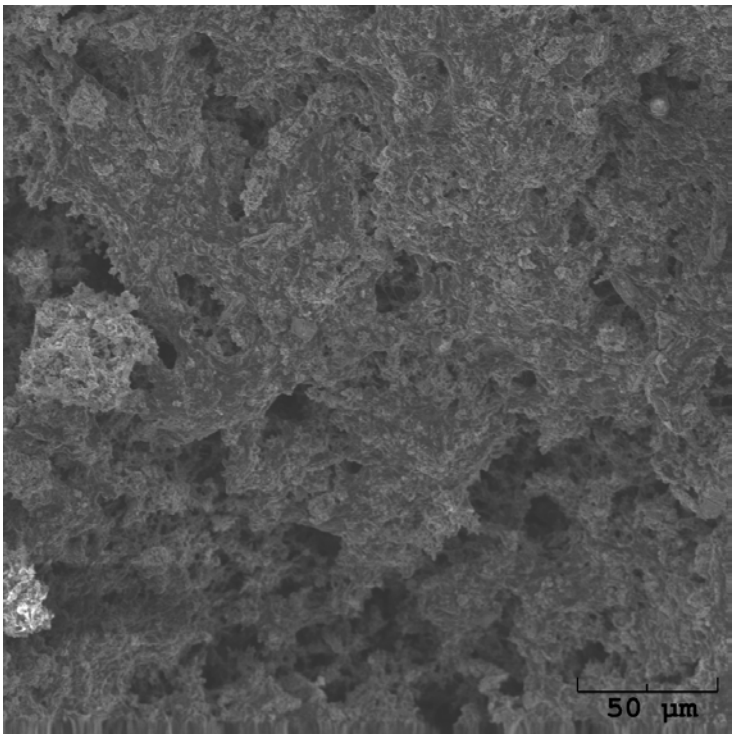

Figure D-4 Micrographs of iron sulfide precipitate from R1

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