Complex Biochemical Reactors for Selenium and Sulphate Reduction: Organic Material Biodegradation and Microbial Community Shifts

by

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ABSTRACT

Biochemical reactors using complex organic materials for treatment of mine-affected waters are attractive low-cost solutions, but their widespread adoption is severely limited by poor reliability and limited longevity. This is in part due to lack of guidance on which organic materials to use, their degradation over time, and how this affects the microbial community composition, which in turn influences reactor performance. Continuous-flow column bioreactors containing differing ratios of a wood, hay and manure mixture were operated for 159 to 430 days including successful and decline phases of performance. Reactor performance, detailed organic matter composition and microbial community structure were measured for reactors with different wood to hay ratios and after different times of operation. Reactors with more hay than wood reduced sulphate from 500 mg/L to less than 100 mg/L and selenium from 20.3 µg/L to less than 0.2 µg/L with a retention time of 14 days for the whole period of operation. Whereas reactors with a high wood to hay ratio operated successfully for 100-200 days after which their performance fluctuated. Increase in more readily available organic compounds with decrease in recalcitrant fibrous materials was charted over time and correlated with changes in microbial community composition. More hemicellulose and α-cellulose were consumed in the bioreactors with more hay content. Lignin content remained the same for the wood rich bioreactors, and increased in the hay-rich columns. Ash content in bioreactors with either organic mixture increased over time. The labile components, determined as neutral detergent and water soluble compounds, fluctuated cyclically. The microbial communities that evolved in the bioreactors were distinctly different from those present initially. At the early stages, the communities were rich in organic matter degraders classified in the Bacteroides, Parabacteroides and Ruminococcaceae taxonomic groups. There was a shift towards Methanogens and Mollicutes and Spirochaetes classified groups for the longer running bioreactors. Sulphate reducing bacteria were mostly Desulfobulbus and Desulfovibrio related and they were more prevalent in the presence of high sulphate throughout the reactor history.
PREFACE

Part of the work in Chapter 2 has been published as two conference papers:


The Sequential washes were done by undergraduate students Melissa Liu, Resza Boenavan, Leila Saeedi and Frank Su.

Part of DNA isolation and PCR were done by the undergraduate student Adrian Chu.
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NOMENCLATURE

BR: Bioreactor

BR1: Initial bioreactor with 40% woodchips, 30% hay and 30% manure
BR2: Young duplicate bioreactor with 20% woodchips, 50% hay and 30% manure
BR3: Old duplicate bioreactor with 20% woodchips, 50% hay and 30% manure
BRT1: Young bioreactor with 40% woodchips, 30% hay and 30% manure
BRT2: Old bioreactor with 40% woodchips, 30% hay and 30% manure
BRT3: Old bioreactor with 40% woodchips, 30% hay and 30% manure
BRTT1: Young bioreactor with 20% woodchips, 50% hay and 30% manure
BRTT2: Young bioreactor with 20% woodchips, 50% hay and 30% manure
BRTT3: Old bioreactor with 20% woodchips, 50% hay and 30% manure

CDBC: Cellulose degrading bacteria culture

DO: Dissolved oxygen

DOC: Dissolved organic matter

dw: Dry weight

HRT: Hydraulic retention time

NDF: Neutral detergent fiber

NDS: Neutral detergent soluble

NDNS: Neutral detergent non-soluble

OM: Ashless dry organic material (gram)

ORP: Oxidation-reduction potential (mV)

TOC: Total organic carbon
SRB: Sulphate-reducing bacteria
SRBC: Sulphate-reducing bacteria culture
SMD: Synthetic mine drainage,
STPW: Synthetic tailings pond water
PB: Postgate B growth medium
SRR: Sulphate removal rate (mg/L.day)
ww: Wet weight
WNS: Water non-soluble organic material (gram)
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DEDICATION

I dedicate this work to my family.

I am here because of all their love and support throughout my life.
CHAPTER 1

BACKGROUND AND LITERATURE REVIEW

1.1 INTRODUCTION

Mining activities create polluted waters that are harmful to the environment. Each year mining companies dump more than 180 million tonnes of hazardous waste into water bodies worldwide. In Canada alone roughly 2 million tonnes of mine waste are produced every day. The sources of pollution are the mining processing units or the water running through the voids of the mines, waste rocks and tailings. Collectively these mine-affected water streams are referred to as mine drainage. The polluted waters might contain elevated concentrations of heavy and trace metals such as aluminum (Al), magnesium (Mg), copper (Cu), iron (Fe), selenium (Se) and arsenic (As). Also, they might contain sulphate in concentrations of hundreds to thousands of mg/L. These metals are harmful to both human and aquatic life and must be removed before they are released into the environment (Levings et al., 2004; Pagnanelli et al., 2008; Gray & Delaney, 2010). Mine drainage treatment processes can be active or passive. Active treatment uses engineered systems requiring ongoing human operation, monitoring, and energy consumption (Johnson & Hallberg, 2005; Kuyucak, 2006). On the other hand passive treatment is based on natural geochemical and biological processes. These approaches can be classified further into biotic and abiotic. Abiotic treatment involves addition of chemicals such as limestone or soda lime to increase the pH of mine drainage so that metals precipitate as hydroxides or carbonates (Larsen et al., 1973). Although this is a straightforward approach, it is associated with some problems. The sludge produced is bulky and thus difficult and costly to dispose of. In addition, consumption of chemicals
makes it expensive. The high-density sludge (HDS) process using lime neutralization is the current Industry standard (Aubé & Zinck, 2003). Passive abiotic treatment methods include aeration units, surface catalyzed oxidation of ferrous iron (SCOOFI) and anoxic limestone drains (ALD). In aeration units, ferrous iron (Fe$^{2+}$) is oxidized to ferric iron (Fe$^{3+}$), which will be precipitated at circum neutral pH (Kirby et al., 2009). The same concept is used in SCOOFI except that in this method, ochre is used as a catalyst to enhance oxidation of ferrous iron to ferric iron (Younger et al., 2002). In ALD iron is removed as ferrous by passing the mine water through a bed of limestone that makes it alkaline (Cravotta III & Trahan, 1999; Watten et al., 2005). Abiotic methods are costly. In addition, the focus of these methods is on increasing the pH and removal of iron and aluminum. Mine drainage from some mines might include sulphate and metals such as cadmium, copper, lead, nickel and zinc. It is very hard to remove these metals with abiotic methods either due to the high pH required for their precipitation, or the slow removal rate (Wang et al., 2004).

Biological or biotic approaches are introduced as a more appropriate and efficient approach for removal of these metals (Cohen, 2006). These methods are based on the ability of some of microorganisms to immobilize metals. One example of an active biological method is the Thiopaq process (Paques Corporation, Balk, The Netherlands). In this process, hydrogen sulphide is produced biologically in one reactor, and then comes into contact with mine drainage in another reactor so as to precipitate metals as metal sulphides (Cline et al., 2003). Active biotic treatments are also expensive requiring reagents and continuous supervision, whereas passive systems are more cost effective. Constructed wetlands and compost bioreactors are by far the most frequent biological passive systems that have been in use. Aerobic wetlands are suitable for treatment of alkaline mine drainage and they are generally constructed for the removal of ferrous iron and arsenic (Johnson & Hallberg, 2005). Anaerobic compost wetlands are based on the ability of sulphate-reducing bacteria (SRB) to generate hydrogen sulphide, increase alkalinity and immobilize metals as metal sulphides (Stalker & Michaud, 1996). The
advantages of passive systems are that once they are built, they need little maintenance, and the fact that chemicals are not used reduces water treatment costs (Neculita & Zagury, 2008).

Although anaerobic passive treatment systems have been used at many mine sites (Doshi, 2006; Nordwick, 2008), there are limited data available on their long-term performance and reliability. Some design criteria are available (Gusek, 2009; Gusek & Figueroa, 2009) but most cases involve trial and error. The organic matter used in anaerobic wetlands sustains microbes that are key to successful treatment. Sustainable operation depends on the availability and degradation of the organic material. Although many different organic materials have been tested for their ability to remove metals and sulphate only few studies have addressed the change in their composition over time and the impact on treatment performance (Prasad et al., 1999; Thompson et al., 2001). Moreover, despite these being biologically based processes little is known about the microbiological communities involved, their activities, and how they change as the system ages.

1.2 BACKGROUND

1.2.1 Passive Treatment Systems and the Mechanism for Sulphate-Reduction and Metal Removal

Anaerobic passive treatment systems or biochemical reactors (BR) are bioreactors in which the water to be treated flows upwards or downwards through an organic-rich layer where sulphate is reduced and metals are immobilized. Clean water leaves through a collection pipe on the surface, or at the bottom. These are also called subsurface flow or vertical flow wetlands. The size of these wetlands can be calculated using the following formula (Younger et al., 2002):
\[ A = \frac{Q_d(C_i - C_t)}{R_A} \]

Where:

- \( A \): required BR area (m\(^2\))
- \( Q_d \): mean daily flow rate (m\(^3\)/d)
- \( C_i \): Mean daily influent concentration (mg/L)
- \( C_t \): Concentration of contaminant in final discharge (mg/L)
- \( R_A \): contaminant removal rate (g/m\(^2\)/d)

Many anaerobic passive treatment systems have a dry cover on top where an impermeable membrane or natural material, such as glacial till, prevents the contaminated water from puddling on the surface and avoids oxygen penetration into the treatment system (Younger et al., 2002). The surface is often covered with soil and plants so that the treatment system has a natural appearance (Figure 1.1). Other types of anaerobic passive treatment systems are covered with water as in the new pilot system constructed at Mount Polley Mine (Figure 1.2). Some anaerobic passive treatment systems have internal baffles and membranes so that the water flows in a serpentine fashion approximating plug flow with minimal short-circuiting (Gusek & Figueroa, 2009).

The factors that are important in the design of BRs include size of the treatment system, hydraulic conductivity, variations in temperature, hydraulic retention time, prevention of channeling and short-circuiting and type of organic materials. The minimum suggested depth of the organic material in a BR is about 0.5 m (Watzlaf et al., 2003). The permeability of the organic bed is an important factor. The organic bed must be able to retain its hydraulic conductivity once it is self-loaded to a depth of 0.5 m, with additional loading of 0.1-0.2 m due to water. At the same time, it should not be too compact to make
the passage of water difficult and causes channeling. In a horizontal flow passive treatment cell in Highmount valley (Logan Lake, B.C.) the initial hydraulic conductivity of the organic bed was $3.5 \times 10^{-4}$ cm/sec, which dropped by 50% during the course of 6 years of operation due to the flooding and freshets. But in the same site, a second vertical flow passive treatment started with the initial hydraulic conductivity of $1.6 \times 10^{-3}$ cm/sec and they did not measure a significant change in the permeability after 3 years of operation (Doshi, 2006).

The size of the treatment system is another important factor. In the design of these systems, the fluctuation in the metal loading rates and the change in the flowrate during the spring or drought seasons must be taken into account. In the Lilly Orphan Mine (Elliston, Montana), the performance of their passive treatment was lowest during the spring as a result of increased flow rates (Sobolewski, 2002). The increased flow rates cause a reduction in retention time and decreases the efficiency of microbial degradation. In addition, the high flow rate forms channels in the organic bed that reduces the effectiveness of the system. Therefore it is important to provide an adequate safety margin in the design of these systems.

Sulphate-reducing bacteria can tolerate temperatures between -5°C to 75°C (Postgate, 1984; Barton, 1995). As other biological activities, the change in temperature impacts the activity of SRB and their activity will reduce at lower temperatures. Moosa et al. (2005) studied the kinetics of sulphate reduction in a continuous flow reactor in the presence of acetate and in the temperature range of 20-35°C. Their results indicated that at higher temperatures the rate of sulphate reduction was higher (Moosa et al., 2005). They developed the following mathematical model relating the rate of sulphate reduction to temperature:

$$r_s = \frac{\mu^{[s]}_{\text{max}}}{K_s[S_0][X] + [S]} \frac{[X]}{Y} - k_d$$
\[ k_d = K_0 e^{-\frac{E_a}{RT}} \]

Where \( r_s \) is the reaction rate (Kg/m³/h), \([X]\) the biomass concentration (Kg dry weight/m³), \([S]\) the residual concentration of sulphate (Kg/m³), \([S_0]\) the initial concentration of sulphate in the feed (Kg/m³), \(\mu_{\text{max}}\) the maximum specific growth rate (1/h), \(K'_s\) the apparent saturation constant (Kg/dry weight/m³), \(k_d\) the decay coefficient (1/h), \(Y\) is the yield (Kg bacteria/Kg sulphate), \(K_0\) Arrhenius constant (1/h), \(T\) temperature (K), \(R\) universal gas constant (KJ/K/mol) and \(E_a\) activation energy (KJ/mol).

Since passive treatment systems for mine drainage are located in cold climate areas, it is important to know how these systems work in cold winters. There are not many studies on the performance of passive treatment in cold climates. Stein et al. (2002) investigated the seasonal influence on SRB. The results of their study indicated that sulphate reduction in wetland mesocosms was slower during winter (4 °C) and higher during summer (24 °C) (Stein et al., 2007).

In the actual mine sites, the influent to the BR and the BR itself are subject to extreme changes in the weather. This is especially of importance in cold winters, since the drop in temperature slows down the biological reactions and consequently the inefficiency of the BR. In the vertical flow passive treatment system in Highmount Valley, mentioned above, the efficiency of sulphate and metal removal was higher in the downflow mode because the flow had a chance to warm up before entering the SRB bed. Proposed solutions to reduce the effect of cold temperatures are to insulate the piping and to cover them under the ground. In addition, covering the BR with a layer of rock or soil helps to keep the treatment system warm (Gusek & Figueroa, 2009).

The chemical and biological reactions responsible for metal and sulphate removal occur within the organic rich layer. Here a consortium of microbes exists through degradation of the organic matter. Some of these microorganisms are essential for successful treatment of mine drainage. One very important
group is the sulphate-reducing bacteria (SRB) that reduce sulphate to sulphide (Postgate, 1984). SRB use sulphate as an electron acceptor to generate energy:

\[ 2 \text{CH}_2\text{O} + \text{SO}_4^{2-} \rightarrow 2\text{HCO}_3^- + \text{H}_2\text{S} \]

CH\(_2\)O represents the organic carbon source that is required as an electron donor. SRB prefer low molecular weight compounds such as lactate and ethanol as carbon sources.

The sulphide that is produced by SRB reacts with metals to form metal sulphide precipitates:

\[ \text{M}^{2+} + \text{HS}^- \rightarrow \text{MS} + \text{H}^+ \]

M is a cationic metal such as Fe, Mn, Cd or Zn.

An additional benefit to this treatment is the production of bicarbonate and carbonate by SRB, which increases alkalinity allowing some of the metals to precipitate as carbonates and hydroxides.

### 1.2.2 Organic Materials Used in Anaerobic Passive Treatment Systems

Organic carbon sources such as acetate, lactate, propionate and ethanol are the best energy sources for SRB; however using defined carbon sources in a treatment system is too expensive. Complex waste organic materials that are freely available to mining companies, requiring only transportation costs, are more economical. When selecting complex organic materials for a passive treatment system the factors that should be considered are their cost, their degradability, the efficiency and the longevity of the treatment system. In general complex organic materials derived from plants are composed of various components such as structural components (lignin, cellulose, hemicelluloses), lipids, proteins, sugars and other carbohydrates. SRB cannot use these components directly and they rely on other bacteria to break down the larger molecules and polymers to monomers and more available smaller molecules such as volatile fatty acids (acetate, propionate,…), which are consumable by SRB (Figure 1.3). For instance,
Kikuyu grass cellulose is broken down to propionic acid, which can be consumed by SRB directly (Greben et al., 2009a). The rate of degradation of different components varies depending on the size and the type of molecules. Macromolecules such as lignin and cellulose are recalcitrant and the rate of their degradation is very slow (Benner & Hodson, 1985). In contrast, smaller molecules such as proteins and sugars are more degradable (Christ et al., 2000). Selection of suitable organic material for using in a BR has been the subject of much research and many organic materials such as wood chips, compost, leaf mulch, manure, spent mushroom and pulp mill biosolids have been tested (Table 1.1). The consensus of all the studies is that a mixture of organic materials is more effective than using only one type (Waybrant et al., 1998; Cocos et al., 2002; Zagury et al., 2006). In order to find the best organic mixture attempts have been made to correlate initial properties of each material to the sulphate-reduction rate (SRR) and efficiency of BR in removing sulphate. Some of the properties such as high dissolved organic carbon (DOC) content, high total organic carbon content (TOC) and C/N ratio close to 10 were believed to have a positive correlation with SRR (Waybrant et al., 1998; Zagury et al., 2006). In a series of batch experiments using various mixtures of sewage sludge, leaf mulch, woodchips, sheep manure, sawdust and cellulose, Waybrant et al. (1998) did not find a correlation between SRR and total organic carbon (TOC) and nitrogen content of the solid mixtures (Waybrant et al., 1998). In a 150-day batch study a mixture of sawdust, wood chips, poultry manure and leaf mulch with the highest DOC, cellulose, hemicellulose and a C/N ratio close to 10 had the highest sulphate reduction rate (Waybrant et al., 1998). Schmidtova (2011) using silage, compost, molasses and hay, also observed a positive correlation between C/N ratio and SRR, but no positive correlation of SRR with either DOC or the amount of easily degradable material (EDM) was observed (Schmidtova & Baldwin, 2011). In this study the C/N ratio was in a range of 25.4 to 52.5 which is higher than the C/N ratio of 10 suggested by some authors (Bechard et al., 1994; Prasad et al., 1999). The reason for this positive correlation could be the higher production of volatile fatty acids (VFA) at higher C/N ratios (Liu et al., 2008). Some researchers debate the use of C/N on the ground that the entire carbon might not be available for biodegradation or availability of nitrogen in the mine water itself.
(Gibert et al., 2004). Zagury et al. (2006) found that poultry manure, which is high in DOC and EDM and C/N ratio of 3, was not successful at removing sulphate when used alone because of the low permeability (Zagury et al., 2006).

Materials with a higher lipid or protein content, such as dairy whey or alfalfa hay support higher rates of sulphate-reduction (Bechard et al., 1994; Christensen et al., 1996; Martins et al., 2009). In contrast, using materials with more lignin and crude fiber content result in less sulphate reduction (Gibert et al., 2004; Coetser et al., 2006). Wood chips, although they are high in cellulose and lignin, are a suitable source of cellulosic carbon for passive treatment systems since they degrade slowly and they last for a long time (Hulshof et al., 2003). A 41-day batch study with 3% woodchips, 20% poultry manure and 30% leaf compost showed a 153.5 mg/L.day sulphate reduction (Cocos et al., 2002), whereas in a separate study with Neculita et al. using the same mixture, the rate of sulphate reduction was about 40 mg/L.day over 150 days (Zagury et al., 2006). Since leaf compost is more easily degradable it resulted in a higher sulphate removal rate in the short term; however woodchips, which are more recalcitrant than leaf litter, provide a longer term substrate but resulted in a slower sulphate reduction rate. The reduction of SRR with time was also observed in other batch and column studies (Hulshof et al., 2003; Greben et al., 2009a). Fresh wood chips may contain some inhibitory compounds such as resin acids, which slows down their biodegradation in anaerobic environments (Chang et al., 2000). Partially decomposed wood has a lower C/N ratio and more nutrients, such as nitrogen, which makes it more degradable and promotes more sulphate reduction (Thompson et al., 2001; Hulshof et al., 2003).

The results from previous studies show that we cannot rely on one criterion for selection of an organic carbon source and we should consider a combination of different factors. In addition, most of the previous studies were conducted either in batch mode or over short periods of time. Passive treatment systems work in the continuous flow mode. Although batch studies are very useful in the initial stages of research, they do not reflect the real environment in a passive treatment system. Moreover, in the previous studies
temporal changes in the characteristics of the organic materials were seldom measured. Coetser et al., 
(2006) performed the longest study (371 days) but they related sulphate reduction to only the initial 
characteristics of the organic materials. Therefore it is impossible to accurately predict the longevity of 
passive treatment systems. Conducting a study that monitors the changes in organic materials over time 
can shed light on the rate of biodegradation of recalcitrant components such as cellulose and lignin. This 
can be used to find out the suitable labile: recalcitrant material ratio in an organic carbon source that is 
most effective for sulphate reduction over the long term.

1.2.3 Sulphate-Reducing Bacteria

Sulphate-reducing bacteria (SRB) are anaerobic bacteria that are widespread in anoxic environments. 
They use sulphate as electron acceptor and degrade organic compounds, which results in production of 
hydrogen sulphide. There are two groups of SRB: complete oxidizers and incomplete oxidizers. Complete 
oxidizers are SRB that degrade organic carbon completely to carbon dioxide, whereas incomplete 
odidizers degrade organic compounds to acetate. SRB can grow on a variety of organic compounds. 
There are more than 100 known organic carbons that can be used by SRB (Odom & Singleton, 2011) 
including short chain fatty acids (lactate, propionate, formate, fumarate, pyruvate, malate, succinate, 
butyrate), sugars, amino acids, methanol and ethanol. They can also use aromatic compounds such as 
phenol and benzoate but macromolecules and polymers such as starch, proteins, cellulose and lignin 
cannot be used by SRB directly and SRB are dependent on other microorganisms that breakdown these 
molecules to substrates for SRB. In addition to sulphate, SRB can respire on other electron acceptors such 
as thiosulphate, sulphite and sulphide (Muyzer & Stams, 2008). The optimum temperature and pH for 
SRB growth are 30°C and 5.5-8 respectively. The redox potential must be lower than -100 mv (Postgate, 
1984).
SRB are grouped into seven phylogenetic lineages, five within bacteria and two within Achaeae. Most of the SRB in the bacteria domain belong to the class of Delta-Proteobacteria in phylum Proteobacteria. Others belong to phyla Nitrospirae, Thermosulfobacteria and Thermodesulfobiaceae and Firmicutes. SRB in the domain Achaeae belong to Crenarchaeota and Euryarchaeota (Postgate, 1984).

Sulphate-reducing bacteria compete with methane-producing bacteria (MPB) for acetate and hydrogen and the outcome of the competition depends on various factors such as COD/sulphate ratio, kinetics and growth rates (Omil et al., 1997b). The affinity of hydrogen and acetate-consuming MPB for hydrogen and acetate is lower than SRB. For instance the acetate Michealis-Menten constants (Km) for acetotrophic Desulfbacter postgati, Methanosarcina barkeri and Methanozaeta concili are 0.2 mM, 3 mM and 0.84 mM respectively (Elferink et al., 1994). The Km value for the hydrogenotrophic Desulfovibrio vulgaris and Methanosarcina barkeri are 1.3 µM and 3 µM. In addition, consumption of acetate and hydrogen by SRB is thermodynamically more favorable. COD/sulphate ratio is also a determining factor in the competition of SRB and MPB. At a COD/sulphate ratio of 0.67 all the organic matter can be consumed completely by both SRB and MPB. At ratios higher or lower than 0.67, due to either limited supply of sulphate or excess of sulphate MPB or SRB win the competition respectively (Rinzema et al., 1988).

Acetogens are bacteria that reduce C1 compounds such as H2-CO2 and formate to acetate. In the acetogenesis process acetate is not the only product and other end products such as H2O are produced as well. Acetogens that reduce C2 and C3 compounds such as hexoses to acetate are called homoacetogens. In homoacetogenesis acetate is the only end product (Wiegel, 1995). According to Rinzema et al. (1988) acetate-utilizing SRB coexist with homoacetogens for acetate and they also out-compete acetoclastic methanogens.
1.2.4 Bacterial Communities in Passive Treatment Systems:

In passive treatment systems with complex organic carbon sources only the low molecular weight water-soluble and labile compounds, such as amino acids, ethanol, sugars and organic acids are available to SRB. Once these have been used up, SRB are dependent on hydrolyzing and fermentative bacteria for sequential breakdown of the more recalcitrant material to simpler organic compounds such as acetate, butyrate, propionate and ethanol (Figure 1.3). Therefore, SRB usually comprise only a fraction of the total microbial community in treatment systems with complex organic carbon sources. Many other microbes are present, most of which are involved with degradation of complex organic matter. For example, an upflow anaerobic bioreactor treating mine drainage with wine waste as the carbon source had a diverse bacterial community composed of bacteria genera such as *Clostridium*, *Citrobacter* and *Cronobacter* in addition to *Desulfovibrio*, which were the most dominant bacteria found when only ethanol was the carbon source (Martins et al., 2011). The former bacteria are gram-negative and ferment carbohydrates such as glucose, sucrose, maltose to lactate, propionate and acetate and make them available to SRB. Similarly, in batch tests performed by Geets et al. (2005), the bacterial community in the batches containing molasses as the organic carbon was more diverse than the community in batches containing acetate or lactate. The bacterial community growing on molasses contained *Cellulomonodaceae* spp., which break down sugars to short chain acids (Geets et al., 2005). The same observations about the diversity of microbial community were made for complex solid organic matrices (Hiibel et al., 2011; Ziganshin et al., 2011). In addition, complex carbon sources had nutritionally versatile SRB species such as *Desulfosarcina variabilis* and *Desulfoarculus baarsii* (Geets et al., 2005; Dar et al., 2007). In general the majority of the bacterial communities growing in complex-carbon systems belong to phyla *Bacteroidetes*, *Firmicutes* and *Spirochaetes*, with a small percentage belonging to the δ-*Proteobacteria* phyla (Hiibel et al., 2011). Schmidtova (2010) found that the overall distribution of bacteria involved in organic matter degradation was similar for different organic materials deployed in the same aqueous
environment. However, the number of SRB and their distribution among various families differed with some organic materials being more effective at sulphate-reduction than others (Schmidtova, 2010).

1.2.5 Molecular Techniques

In the past, characterization of microbial communities in environmental samples was limited to isolation of microorganisms and the ability to culture them. Since only a very small fraction (1%) of microorganisms are culturable (Madigan & Martinko, 2006). Culture-dependent techniques limit the ability to characterize the biodiversity in environmental samples such as soil or contaminated waters that can be a rich source of microorganisms. Modern molecular techniques provide the opportunity to characterize and understand the biodiversity without the need for culturing. These techniques are based on characterization of cellular constituents such as DNA, proteins and fatty acids (Malik et al., 2008). Phospholipids fatty acids (PLFA) is an example of a method that relates the fatty acids to the microorganisms. There are several methods for phylogenetic classification of microbial diversity that are based on the study of the small subunit 16s ribosomal ribonucleic acid (rRNA) conserved gene (Woese & Fox, 1977). Denaturing electrophoresis (DGGE), Terminal-restriction fragment length polymorphism (T-RFLP) and fluorescent in-situ hybridization (FISH) are examples of this category. In addition to being time consuming, lack of sensitivity and at times complexity, these methods have limitations. For instance methods such as FISH and T-RFLP usually should be accompanied with other molecular methods and they do not provide enough information about the microbial diversity if used alone. In some techniques such as PFLA, individual species might have more than one fatty acid and one fatty acid may occur in more than one species. In DGGE it is possible that multiple bands of a simple species occur due to existence of multiple copies of rRNA or different 16S rRNA bands may have identical motilities (Malik et al., 2008). In addition, these methods are more suitable for studying the spatial and temporal changes of the microbial community and in some cases they do not provide enough taxonomic information of the community. Sequence-based analysis is a more complete approach that
produces more detailed phylogenetic information. There are different types of gene sequencing-based analyses, the clone-based approach and next generation high-throughput methods such as Roche 454 pyro-sequencing and Illumina sequencing. In clone-based approaches, a metagenomic library is constructed, which involves DNA isolation and direct cloning of DNA fragments into a host such as *E. coli* or amplification of a specific gene using polymerase chain reaction (PCR) with gene-specific primers and cloning of the amplicons into a suitable vector, which is transformed into a host bacterium (usually also *E. coli*) and screening the resulting transformants. Finally, the specific clones can be selected for sequencing of the environmental DNA gene inserts (Handelsman, 2005). Although in these methods full–length amplicon is obtained, the cost per read is high. Pyro-sequencing is a high-throughput next-generation sequencing approach, which does not require cloning of environmental DNA into host organisms, and can achieve great depths of sequencing (order of thousands of gene fragments or amplicons per sample). In this method, each incorporation of a nucleotide by DNA polymerase results in the release of pyrophosphate, which initiates a series of reactions. As a result of these reactions light is produced with intensity proportional to the number of nucleotides. Roch/454 FLX is a sequencer that uses this pyro-sequencing technology and became commercial in 2004. In this method, the isolated DNA is amplified with PCR using 454 specific adaptors and primers for the gene of interest. Most Roche 454 pyrotag sequencers can only sequence read lengths of ~500bp. Thus only a fragment of the 16S rRNA gene can be sequenced. Commonly particular variable regions of the 16S rRNA gene are targeted, such as the V1, V6 or V8 region. The amplicon library is then mixed with agarose beads whose surfaces are covered with oligonucleotides complementary to the 454-specific adapter sequence and are subject to PCR again. Then the PCR products are sequenced (Mardis, 2008). Bioinformatic methods are required to remove sequences that are a result of noise and do not represent the environmental DNA. The shorter reads obtained with pyrotag sequencing results in less specific phylogeny, and a bias may arise depending on the region sequenced (e.g. V1 or V6). As with any PCR-based approach there are biases associated with preferential amplification of some genes over others. At the time of writing, Illumina-based
sequencing achieved greater depth of sequencing at lower costs, but was limited by read lengths of only 100-150bp.

1.2.6 Selenium

Selenium is in group VI of the periodic table right below sulphur and shares properties with sulphur. It has 4 oxidation states of 0 (Se), -2 (Selenide, Se\(^2\)), +4 (Selenite, SeO\(_3\)) and +6 (Selenate, SeO\(_4\)^{2-}\). Selenate is soluble and the dominant form in neutral to alkaline, highly oxidizing environments, whereas selenite is the product of reduction of selenate by chemical or biological reactions and is present in mildly oxidizing environments. Selenide and selenium are the predominant species in anoxic environments. Selenium is used in different industries such as painting, glass manufacturing, coal combustion and agricultural water. It is especially of a serious problem in the aquatic systems affected by coal-fired power plants. Selenium is one of the most toxic elements to fish and wildlife and has a high potential for bioaccumulation (Outridge et al., 1999). Fish and aquatic habitat take up selenium as organo-Se such as selenomethionine in plants and invertebrates, although uptake of selenate and selenite can also contribute to the selenium in their bodies (Besser et al., 1993). According to Lemly, (1996) Se concentrations higher than 2 µg/L are toxic to fish and water birds (Lemly, 1996). Concentration of selenium in the fresh waters of North America can reach up to 100 µg/L (McDonald & Strosher, 1998) 50 times the toxic concentration of 2 µg/L, which makes it necessary to remove selenium from these waters.

Different techniques have been used for selenium removal such as ferric coagulation, lime softening, ion exchange and reverse osmosis. Biological reduction is another method that has proved efficient and successful (Gusek & Figueroa, 2009).

Different microorganisms have demonstrated the ability to reduce selenate to selenite and selenium. *Entrobacter taylorae* is a selenate-reducing bacterium that was isolated from a rice straw bioreactor (Zahir et al., 2003). This bacterium has been able to remove selenate and reduce it to selenite in a flow-through bioreactor using typric soy agar (TSA) coated sand columns. In that study, selenate was reduced
from 469 µg/L in the influent (river water) to 3.45-6.54 µg/L in the effluent. Seventy percent of this reduction was attributed to the indigenous selenate-removing microorganisms in the river water (Zhang & Frankenberger Jr., 2005). Elemental selenium (Se⁰) is insoluble and the most stable form of selenium. *Bacillus sp. SF-1* is another bacterium that uses selenate as an electron acceptor and reduces selenate to elemental selenium in two steps: First, selenate is reduced to selenite and then selenite is transformed to Se⁰. This bacterium has been very successful in removing high concentration of selenate (41.8 mg/L) to concentrations less than 0.05mg/L in a stirred tank reactor with a retention time of 95.2 hrs (Fujita *et al.*, 2002; Siddique *et al.*, 2007) were able to isolate one Se⁶⁺ reducing bacterium, *Entrobacter hormaechei*, and four Se⁴⁺ reducing bacterium, *Klebsiella pneumonia* (Tar1), *Pseudomonas fluorescenes* (Tar3), *Stenotrophomonas maltophilia* (Tar6) and *Entrobacter amnigenus* (Tar8). In batch experiments using sterilized and non-sterilized sediments inoculated with *Entrobacter hormaechei*, after 2 days Se⁶⁺ was reduced from 2.4 g/L to 0.083 mg/L, 0.587 mg/L and 0.083 mg/L (Siddique *et al.*, 2007). Selenate and selenite can also be removed from waters by sulphate-reducing bacteria. One proposed mechanism is that in circum neutral pH sulphate-reducing bacteria reduce sulphate to sulphide. Sulphide reacts with selenite and as a result precipitation of elemental selenium and sulphur takes place (Hockin & Gadd, 2003). Selenate and selenite can be removed with other mechanisms as well. Figure 1.4 shows a proposed model for the removal of selenium species by SRB.

### 1.2.7 Structure and Degradation of Lignocellulosic Materials

Natural organic materials such as wood chips, straw, hay, leaf compost, waste paper, pulp mill biosolids and many other such materials that are used in passive treatment systems are mostly lignocellulosic materials. The main constituents of these materials are cellulose, hemicelluloses and lignin.

Cellulose is a high molecular weight linear homopolymer of repeated units of cellobiose. Celllobiose is two anhydrous glucose molecules joined together with β-1,4 glycosidic linkages. The long chain
polymers of cellulose are linked to each other by hydrogen and van der Waals bonds. This causes cellulose to be packed in microfibrils. Cellulose has both a crystalline and an amorphous structure. 75% of cellulose is crystalline and less soluble and degradable.

Hemicellulose is a linear and branched heterogeneous polymer, which is typically made up of L-arabinose, D-galactose, D-glucose, D-mannose and D-xylose. Other components such as acetic acid, glucuronic acid and ferulic acid might also be present in hemicelluloses. Hemicellulose can be a homopolymer consisting of only one type of sugar or can be a heteropolymer consisting of two or more sugars. Hemicellulose is different from cellulose by having shorter chains, by a branching of main chain molecules and by being amorphous, which makes it easier to degrade. The main role of hemicelluloses is to bind cellulose and lignin by forming ester bonds with lignin and hydrogen bonds with cellulose.

Lignin is a complicated molecule constructed of phenylpropane units linked in large three dimensional structure. Lignin is closely bound to cellulose and hemicelluloses and gives rigidity and cohesion to the material cell wall. It also forms a barrier against microbial attack (Pérez et al., 2002; Mussatto & Teixeira, 2010).

Cellulose, hemicellulose and lignin form a tertiary structure with cellulose in the core of structure wrapped with hemicelluloses and lignin (Figure 1.5). Anaerobic biodegradation of lignocelluloses is an enzymatic process that takes place as a result of concerted action among various enzymes. Anaerobic bacteria of Clostridiales order found in soils, sediments, termite gut and rumen of ruminant animals contain cellulolytic enzyme complexes called cellulosomes. Other anaerobic bacteria such as Butyrivibrio fibrisolvens, Fibrobacter succinogenes and Ruminococcus flavefaciens also produce cellulase and xylanase enzymes that assist with the biodegradation of lignocelluloses. The multi-enzyme complex of cellulases and hemicellulases can also be found in anaerobic fungi such as Anaeromyces nucruratus, Piromyces sp. and Orpinomyces sp. (Quiroz-Castañeda & Folch-Mallol, 2011).

Some factors that affect degradation of cellulose are pH and surface area. A study by Hu et al. (2005), with ruminal bacteria as inoculum and cellulose as the substrate, showed that cellulose degradation was
most efficient at a pH range of 6-7.5. They suggested that at lower pH the ruminal bacteria were inhibited. They also found out that smaller particle size of cellulose was beneficial to cellulose degradation, since it provided more surface for bacterial attachment (Hu et al., 2005). Palmowski and Müller suggested a model for the connection between reduction of particle size of lignocellulosic materials and their degradation rate. According to their model there will be three consequences as a result of a cut in the size of the materials: 1) Cell rupture 2) particle size reduction and 3) modification in the material structure. The cell rupture results in the release of cell contents and therefore due to the availability of more substrate and nutrients the degradation rate increases. Reduction of the particle size creates higher reaction surface for microorganisms, improves dissolution process from substrate and exposes the surface areas that are otherwise inaccessible with bacteria. These factors all lead to a higher degradation rate. The change in the structure of the materials causes an improvement in the water soakage and therefore improves dissolution of the materials. In addition, more substrate fragments will become available for biodegradation. This model shows the importance of small size of lignocellulosic materials in a biological system (Palmowski & Muller, 2003).

1.3 SUMMARY AND THESIS OUTLINE

Passive biological treatment of mine drainage is a method that is gaining popularity due to its low cost and maintenance; However there is a lack of knowledge regarding design factors, rate of degradation of organic materials and microbial communities in these systems. The objective of this research was to address some of the gaps in knowledge regarding these treatment systems. The research questions that were addressed in this thesis were as follows:
**Question 1:** What start-up and operating conditions are required for a biochemical reactor with a wood, hay and manure mixture to remove selenium and reduce sulphate to less than 100 mg/L? What are the specific challenges with successful start-up of these types of reactors and strategies for overcoming these?

**Question 2:** How does performance of bioreactors with different ratios of organic materials compare during the active and decline phases of operation? Does the ratio of cellulosic to readily available material in the organics affect the start-up, operation and decline of these types of bioreactors?

**Question 3:** How does the composition of organic material change over time in these bioreactors? Is it possible to predict the longevity of these systems based on the change in organic matter composition?

**Question 4:** What specific microorganisms are present in these types of bioreactors? How do these microorganisms change with time and with the properties of organic mixtures? Does the microbial community composition provide information about the performance of these bioreactors?

Chapter 2 addresses the first question in which the hydraulic retention time to achieve successful removal of selenium and sulphate was determined. Initially the ability of sulphate-reducing bacteria to reduce sulphate by oxidizing a mixture of woodchips, hay and manure was investigated through batch studies. In the next step a bioreactor is set up with a mixture of \(40\%_{ww}\) woodchips, \(30\%_{ww}\) hay and \(30\%_{ww}\) manure. The problems associated with the start up and operation of this bioreactor, namely control of pH and oxygen, are addressed and the solutions are presented. The bioreactor was sacrificed after 269 days and the composition of organic materials was measured. In addition the bacterial community in the bioreactor was identified. Two more bioreactors were set up with \(20\%_{ww}\) (\(ww\) stands for wet weight) woodchips, \(50\%_{ww}\) hay and \(30\%_{ww}\) manure. The lessons learnt from the first bioreactor were applied for the start-up and operation of these two bioreactors. The results of the operation of the last two bioreactors
helped to find the hydraulic retention time for removing sulphate to less than 100 mg/L. This HRT was used for the operation of the future bioreactors described in Chapter 3. The bioreactors were sacrificed at two different time points and the composition of organic materials inside them were measured and compared with each other. Also, the microbial community in the bioreactors was identified and compared at the two different time points.

Chapter 3 describes the results of an experiment where the performance of bioreactors containing different ratios of wood and hay are compared and the changes in composition of organic mixture over time were determined. Two series, each containing three bioreactors, were designed and operated with two different mixtures of organic materials. The first series contained 40%\(_{\text{ww}}\) (\(\text{ww}\) stands for wet weight) woodchips, 30%\(_{\text{ww}}\) hay and 30%\(_{\text{ww}}\) manure and the second series contained 20%\(_{\text{ww}}\) (\(\text{ww}\) stands for wet weight) woodchips, 50%\(_{\text{ww}}\) hay and 30%\(_{\text{ww}}\) manure. In each series bioreactors were sacrificed in two different time points. The composition of organic materials were measured and compared at different time points.

Chapter 4 addresses the final research question by examining the changes in the microbial community composition of the bioreactors described in Chapter 3. How the microbial community composition differs depending on the wood to hay ratio and over time is described and correlated with reactor performance. In addition the impact of environmental factors on the distribution of the microbial community is investigated.

Chapter 5 presents the research outcomes and suggestions for the future studies.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Experiment</th>
<th>Successful Organic mixture</th>
<th>HRT(days)</th>
<th>Sulphate removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Zagury <em>et al.</em>, 2006)</td>
<td>Batch</td>
<td>2% Maple woodchips+30% leaf compost+18% Poultry manure</td>
<td>N/A</td>
<td>60 mg/l/day</td>
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<tr>
<td>(Neculita <em>et al.</em>, 2007)</td>
<td>Batch</td>
<td>10% Maple woodchips + 20% Maple sawdust + 10% Poultry manure + 20% Leaf compost</td>
<td>N/A</td>
<td>80-86 mg/l/day</td>
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<tr>
<td>(Bechard <em>et al.</em>, 1994)</td>
<td>Continuous</td>
<td>Alfalfa hay</td>
<td>5</td>
<td>34.20%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fresh alfalfa hay</td>
<td>35</td>
<td>58%</td>
</tr>
<tr>
<td>(Hulshof <em>et al.</em>, 2003)</td>
<td>Continuous</td>
<td>Waste from pulp and paper plant</td>
<td>14</td>
<td>120 mg/l/day (days 0-36) 60 mg/l/day 36-100</td>
</tr>
<tr>
<td>(Waybrant <em>et al.</em>, 1998)</td>
<td>Batch</td>
<td>20% municipal sludge + 10% municipal leaf mulch + 25% hardwood and softwood chips + 20% sheep manure + 25% maple sawdust</td>
<td>N/A</td>
<td>128 mg/l/day</td>
</tr>
<tr>
<td>(Waybrant <em>et al.</em>, 2002)</td>
<td>Continuous</td>
<td>51% composted leaf mulch + 49% sawdust</td>
<td>N/A</td>
<td>74 mg/l/day</td>
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<tr>
<td>(Cocos <em>et al.</em>, 2002)</td>
<td>Batch</td>
<td>3% Maple wood chips + 20% Poultry manure + 30% leaf compost</td>
<td>N/A</td>
<td>84 mg/l/day</td>
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<tr>
<td>(Thompson <em>et al.</em>, 2001)</td>
<td>Continuous</td>
<td>Weathered lodge pole pine</td>
<td>5.1</td>
<td>N/A</td>
</tr>
<tr>
<td>(Christensen <em>et al.</em>, 1996)</td>
<td>Continuous</td>
<td>Cheese whey</td>
<td>N/A</td>
<td>19-27%, 3-4.4 mg/l/day</td>
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<tr>
<td>(Greben <em>et al.</em>, 2007)</td>
<td>Continuous</td>
<td>Grass cuttings</td>
<td>4</td>
<td>156.4 mg/l/day</td>
</tr>
<tr>
<td>(McCaulty <em>et al.</em>, 2009)</td>
<td>Continuous</td>
<td>15 % coarse or fine grained delignified bark compost + 25 % post peel + 30% bark</td>
<td></td>
<td>37.3 mg/l/day</td>
</tr>
</tbody>
</table>
Figure 1.1: Schematic of a passive treatment system with a dry cover

Figure 1.2: Schematic of a passive treatment system with a wet cover
Figure 1.3: Bacterial degradation pathway of lignocellulosic materials

Figure 1.4: A model for removal of selenium by SRB (Hockin & Gadd, 2003)
Figure 1.5: Structure of lignocellulosic materials (Mussatto & Teixeira, 2010)
CHAPTER 2

START-UP AND SUCCESSFUL OPERATION OF BIOCHEMICAL REACTORS FOR
SELENIUM AND SULPHATE REMOVAL

2.1 INTRODUCTION

Mount Polley mine is a copper/gold mine located near the town of Likely in British Columbia, Canada. Figure 2.1 shows a map of the mine with the operation sites and the tailings pond facility. The water in the mine’s tailings storage facility contains approximately 500 mg/L sulphate and 38 µg/L Selenium. The BC Ministry of Environment (BC-MOE) regulated concentrations are 100 mg/L (potential) and 2 µg/L for sulphate and selenium respectively. Therefore, Mount Polley mine needs to treat the tailings pond’s water to meet the BC-MOE’s requirements. To reach this objective a pilot BR was built next to the tailings pond in 2009 (Figure 2.2). The pilot treatment system has a volume of 99,000 m³, which is filled with layers of woodchips, hay and manure and receives the water from the tailings dam. The objective of this project was to recommend to the mine operators suitable organic mixtures and treatment process conditions to use for a bioreactor to successfully remove sulphate and selenium.

Biochemical bioreactors have been used for successful removal of various metals such as iron, nickel, cadmium and copper under sulphate-reducing conditions (Christensen et al., 1996; Waybrant et al., 2002). While active bioreactors using defined carbon sources or complex reactor designs such as the upflow anaerobic sludge bed bioreactor (UASB) have been used to specifically remove sulphate from wastewater (Cline et al., 2003; Li & Baldwin, 2011), passive BRs using complex waste organics have not
yet been proven to be effective for sulphate removal to meet the proposed regulatory guidelines. Although various chemical and active bioreactor methods are used for selenium removal (e.g. http://www.gewater.com/products/abmet-selenium-removal.html), low-cost biochemical reactors based on complex organic material are still in the experimental testing phase for selenium removal. Therefore successful removal of both sulphate and selenium in biochemical bioreactors presented a new challenge that was the objective of the work described in this Chapter.

Hydraulic retention time is an important factor that allows one to determine the size of a BR to remove sulphate down to a target concentration. The HRT of a system is determined by the sulphate removal rate (SRR). The rate of sulphate reduction is determined by the type of carbon source available to them. If carbon source in a bioreactor is a short chain organic acid such as lactate, then SRR is high and HRT needed is short. However if carbon source is a complex organic material with recalcitrant carbon compounds, then SRR is slow and requires a longer HRT to reduce sulphate in the influent to less than a target concentration. Finding the optimum retention time in a BR is necessary to avoid uneconomic designs or insufficient sulphate removal, and was another objective of the experiments described in this Chapter.

Effective start-up of a BR is important for achieving successful removal of sulphate and selenium. When starting up a biochemical reactor, in the first step they must be inoculated with a healthy microbial community containing sulphate-reducing bacteria, hydrolyzing bacteria (such as cellulose-degraders) and fermenters. The performance of BR is closely tied to the types of microbes consuming the carbon sources and removing sulphate and selenium. In order to have a well-established microbial community capable of responding to metal and sulphate loads in the influent they should be provided with a suitable environment and carbon source. In the publically available literature scant information is available regarding the start-up procedures used for these treatment systems and the problems associated with attaining treatment goals. Various methods were instituted to improve BR start up in this work.
Providing the right environment such as an optimum pH and exclusion of DO is essential for promoting growth of those microbes critical for metal and sulphate removal, but it is difficult to maintain an environment totally devoid of oxygen in a continuous-flow bioreactor. Thus, dissolved oxygen concentrations were monitored to see if they affected the reactor performance and to determine if design modifications were needed to further eliminate oxygen from the system. Sulphate-reducing bacteria are active over the pH range of 5.5-8, with circum-neutral being optimum. Several factors may contribute to changing pH within a bioreactor, such as the activity of acidogens fermenting sugars into organic acids. To some extent SRB can buffer pH as they produce carbonate, but it is important to monitor pH since adjustment may be required especially during reactor startup as is described in this Chapter.

In this Chapter, design and operation of three continuous flow bioreactors is described. The bioreactors were designed and operated to find the correct start-up and operating conditions, including hydraulic retention time, required for removing sulphate from 500 mg/L to less than 100 mg/L and selenium from 20.3 µL to below 2 µg/L. The changes in organic mixture characteristics over the reactors’ operating period were measured to determine the extent of their biodegradation. Two different ratios of wood, hay and manure were tested. Microbial communities within the bioreactors at the end of their operation were characterized using next generation sequencing technology and correlated with environmental conditions within the bioreactors and their performance. The outcome of this work included recommendations that were made to industry for start up and operation of these types of bioreactor (Mirjafari & Baldwin, 2011; Mirjafari et al., 2012)
2.2 MATERIALS AND METHODS

2.2.1 Selection of Organic Materials and Chemical Analysis

Due to the large costs that are incurred when trucking materials to a mine site, the mining companies prefer to use locally available organic wastes for remediation purposes. The forests in the Mount Polley mine area are an abundant source of spruce, fir and balsam, providing an availability of cellulosic wastes from the logging and milling industries. Grass hay from Beaver Valley Feeds in Williams Lake was another source of agricultural waste available in close proximity to the mine site. Cow manure mixed with bedding material is available from Williams Lake stockyard and is a source of microbial inocula as well as some additional cellulosic materials. Therefore, wood, hay and manure were selected as the organic materials to be used in the BRs. These materials were shipped to our laboratory from Mount Polley mine. Wood pieces (50% fir, 30% balsam, 20% pine and Spruce) were cut to 0.625 cm with a hammer mill. Hay was cut to 1.25 cm with a Wiley mill. Some of the characteristics of these materials, namely total carbon, total organic carbon (TOC), total Kjeldahl nitrogen (TKN), total nitrogen, total phenol and total resin acids were measured by the ALS laboratories (Burnaby, BC). Total carbon and total nitrogen were measured by combusting materials at 900°C. The gases were passed through a reducing agent where the nitrogen oxides are reduced to elemental nitrogen. After removing H₂O from the gases by passing them through magnesium perchlorate the remaining gases (CO₂ and N₂) are separated by gas chromatography and detected by thermal conductivity (Loeppert & Suarez, 1996). Inorganic carbon was determined by weight loss after addition of hydrochloric acid (Nelson & Sommers, 1996). Organic carbon is the difference between total and inorganic carbon. Phenolic acids were determined by Soxhlet extraction of samples using dichloromethane. The extract was solvent exchanged to hexane and derivatized with acetic anhydride and trimethylamine. The final extract was analyzed by gas chromatography (EPA 3540/8270/8041 Soxhlet GCMS). Resin acids were measured by Soxhlet extraction of the solid materials.
with dichloromethane. Then the extract was derivatized and the resulting methylated esters were analyzed by gas chromatography.

Other characteristics, namely dissolved organic carbon (DOC), total soluble proteins, total soluble carbohydrates and neutral-detergent fiber (cellulose, hemicelluloses and lignin) were measured by performing sequential washes with different solvents on the organic solids as described below. In the first step, 2.5 grams of the dry solids were transferred to a 300 ml flask and 200 ml of dH$_2$O was added to them. The flasks were left on a shaker overnight at 200 rpm and room temperature (20°C). At this step all the water soluble materials such as water soluble proteins, sugars and organic acids were dissolved into the water phase. Then the mixture was filtered through 0.45 µm filter papers. The filtrate was analyzed for total carbohydrates, DOC and proteins, using the methods described below. The dried (at 60°C overnight) residue was washed with neutral detergent according to Vansoest et al. (Vansoest et al., 1991). Neutral detergent contained 3.6 g NaOH, 27 g sodium lauryl sulphate, 13.15 g EDTA, 6.13 g sodium borate decahydrate, 4.105 g disodium hydrogen phosphate and 9 ml triethylene glycol in 900 ml de-ionized water. The procedure in the Hall laboratory manual (Hall, 2000) was followed for preparation of the detergent. Washing solids with neutral detergent removed components such as starch, neutral detergent soluble proteins and pectic substances and left neutral detergent fiber (lignin, cellulose and hemicelluloses) in the solid residue. The residue after neutral detergent was used to measure holocellulose, α-cellulose and hemicelluloses following the procedures suggested by Wieder and Starr (Wieder & Starr, 1998). Lignin was measured in the residue from neutral detergent according to Wieder and Starr (1998). Figure 2.3 is the flowchart of the sequential wash experiments. All the chemicals were from ACS grade and from Sigma Aldrich.

pH, ORP and conductivity of the pore water of the bioreactors were measured with a pH/Cond 7200 pH meter (WTW, Weilham, Germany). Dissolved oxygen (DO) was measured with a Symphony SP50D DO meter (VWR). For the water-wash filtrate, total protein (amino acids) was measured with the
Bradford method (Bradford, 1976) and total carbohydrates were measured with phenol-sulfuric acid Dubois assay (Dubois et al., 1956).

Sulphate was measured with the turbidimetric barium sulphate American Water Work Association method 4500-SO$_4^{2-}$ (Eaton et al., 2005). Sulphide was measured with the methylene blue American Water Work Association 4500-S$^2-$ method (Eaton et al., 2005).

2.2.2 Polymerase Chain Reaction (PCR) and Pyro-Sequencing

To study the bacterial community in the bioreactors samples were prepared for pyro-sequencing, which is a next generation sequencing technology (MacLean et al., 2009; Metzker, 2010). After sacrificing each bioreactor the solids samples were transferred to a sterile plastic bag. The pore water samples were centrifuged at 10,000 rpm for 15 min. The residue solids after centrifugation were separated from the supernatant and were transferred to the sterile plastic bags containing the solids from the bioreactors. Then the solid contents in the plastic bags were mixed thoroughly to make it as homogenous as possible. All the operation was done on ice and in the anaerobic fumehood. Then 0.5 grams of the wet solids were weighed and their genomic DNA was extracted using a MoBio Power Soil Isolation Kit (MO BIO Laboratories Incorporation, Carlsbad, CA, USA). Isolated DNA was subjected to polymerase chain reaction (PCR) amplification of the V6 to V8 variable region of the 16S rDNA gene. 12.5 µL of 2xPCR Master Mix (Fermentas Canada Incorporation, Burlington, ON), 10.5 µL of nuclease-free water (Fermentas), 1 µL of genomic DNA (2 ng) and 0.5 µL of FLX Titanium amplicon primers 454T- RA and 454T-FB (20 pmol/ µL) for a 25 µL PCR reaction was used. The primer sequences were 926f (aaactYaaaKgaattgacgg) and 1392r (acgggggtgtgtgtRec) which amplify the V6 to V8 region of 16s rRNA. Primer 454T-RA had a 25 nt A-adaptor (CGTATCGC-CTCCCTCGCGCCATCAG), whereas primer 454T-FB had a 25 nt B-adaptor sequence (CTATGCGCTTGCCAGGCCGGCT- CAG). Each sample contained a specific barcode, which consisted of 10 nucleotides in a row. PCR conditions were 95°C for 3
min; 25 cycles of 95°C; 30 s, 56°C; 45 s, 72°C; 90 s, 72°C; 10 min; final hold at 4°C. The PCR products were verified on a 1% agarose gel. PCR products were sent to the Genome Quebec and McGill University Innovation Centre (Montreal, Canada) for 454 pyrotag sequencing.

2.2.3 DNA Sequence Analysis

For processing and analyzing the pyrotag sequences, scripts included in the Qiime package of commonly used bioinformatic programs was used (Caporaso et al., 2010). Quality control steps were taken to make sure that average quality score was at least 25, there were no mistakes in primer sequence, sequence lengths were between 200 and 1000 bases, there were no sequences with homopolymers longer than 5 nucleotides and there were no ambiguous nucleotides. The pyrotag sequences were clustered into so-called operational taxonomic units (OTUs) based on 97% similarity using cd-hit (Li et al., 2001). The sequences were checked for chimeras using Chimera Slayer and the chimeric sequences were removed from further analysis. Silva version 108 representative set was used as the database for taxonomic assignment of representative sequences for each OTU.

2.2.4 Statistical Methods

The R-Vegan package of software (2.14.2) was used for the detrended correspondence analysis of the microbial community distribution and the relationship among the environmental factors (pH, DO, ORP, sulphate, sulphide), organic matter characteristics (lignin, α-cellulose, hemicelluloses) and microbial community. An OTU-heatmap was built using the heatmap() function in R to compare the OTU representation in different samples.

Means of characteristics of organic mixture (neutral detergent non-soluble, lignin, α-cellulose and hemicellulose) were compared by the Oneway analysis and pairwise Tukey HSD in the statistic software
2.2.5 Batch Experiments

Batch experiments were done to show that mixtures of hay, woodchips and manure are capable of supporting sulphate reduction. Two mixtures were used: Mixture1 with 40%\textsubscript{ww} (\textit{ww} stands for wet weight) woodchips, 30%\textsubscript{ww} hay and 30%\textsubscript{ww} manure and Mixture2 with 70%\textsubscript{ww} woodchips, 15%\textsubscript{ww} hay and 15%\textsubscript{ww} manure. In an anaerobic hood, each mixture was transferred to a 1 L jar. Sulphate solution containing 500 mg/L sulphate was prepared by dissolving sodium sulphate in deionized water. Five hundred millilitres of this solution was added to each jar. Then, the mixtures were inoculated with 100 ml of SRB culture. The jars were filled to the rim with the sulphate solution. They were left in the anaerobic hood and the sulphate concentration in them was monitored every few days. A positive control was prepared by inoculating Postgate B (Atlas, 2004) solution with 100 ml of SRB culture. Negative control contained only 500 mg/L sulphate solution.

2.2.6 Column Experiments

2.2.6.1 Design of the columns

Three Plexi-glass columns (I.D: 11.43 cm, Length: 50.8 cm) were used as upflow passive treatment bioreactors (Figure 2.4). The bottoms of the columns were filled with pebbles and were separated from the top by plexiglass distributors. The perforations in the distributors were $\frac{1}{4}”$ in diameter and were $\frac{1}{2}”$ apart. They were covered with fine meshed cloth. Each column had eight side ports that were used for sampling from different heights of the columns. A mesh cloth was also used to separate the side ports from the materials inside the columns. One quarter inch threaded fittings at the side ports were connected to Tygon tubing, which were kept closed with plastic clamps. The top parts of the columns were filled...
with mixture of hay, woodchips and manure and a bag of sand was placed on top of the mixture to keep it from floating and to filter the effluent before leaving the bioreactor.

Two mixtures of hay, woodchips and cow manure were used in the bioreactors. Mixture1 contained 40\%_{dw} woodchips, 30\%_{dw} hay and 30\%_{dw} cow manure and Mixture2 contained 20\%_{dw} woodchips, 50\%_{dw} hay and 30\%_{dw} cow manure (%_{dw} denotes weight percent based on dry weight). Mixture1 was used in bioreactor 1 (BR1) and Mixture2 in bioreactors 2 and 3 (BR2 and BR3).

2.2.6.2 Inocula

Two sources of bacteria were used for preparing inocula for the bioreactors: sulphate-reducing bacteria culture (SRBC) and cellulose-degrading bacteria culture (CDBC). To prepare and maintain SRB culture in the lab, initially sludge from a UASB reactor that was used for sulphate reduction (Li & Baldwin, 2011) was used as inoculum. Postgate B, a preferred growth medium for SRB (Postgate, 1989), was prepared according to the handbook of microbiological media (Raton, 1993). Some Postgate B was transferred to a 1 L Wheaton bottle. One hundred millilitres of the sludge was transferred to this bottle and it was filled to the rim with the rest of Postgate B. This bottle was kept in a 32ºC incubator. The sulphate concentration in this bottle was monitored every few days until it was reduced to half the initial concentration. At this point, this SRBC was used as the inoculum to prepare another fresh SRB culture. Therefore by regularly transferring the SRB culture into fresh Postgate B medium a healthy SRBC was maintained in the lab.

As the source of cellulose degrading bacteria some cultures containing organic material and water were used. These cultures were initially prepared by adding water to an organic mixture (40\%_{ww} wood chips, 30\%_{ww} hay, 30\%_{ww} manure). These mixtures were incubated at 32ºC. Cellulose activity in these cultures was measure by the method developed with Mewis et al. (Mewis K, Taupp M, Hallam SJ, 2011). Test of cellulose activity after 3 month indicated strong cellulose activity; therefore they were used as the
source for cellulose-degrading bacteria. CDBC were prepared by adding a 25 g/L solution of Luria broth to a mixture of 50% w/w hay, 20% w/w woodchips and 30% w/w manure. Luria Broth contains NaCl, Yeast and Trypton, which is a source of amino acids for the growth of microorganisms (Atlas, 2004). The cultures are incubated at 32°C.

Before start-up of the bioreactors, fresh inocula were prepared in 1 L bottles in an anaerobic hood. To prepare inoculum for bioreactor 1 (BR1), 100 ml of SRBC was added to a mixture of 70% woodchips, 30% hay and 30% manure. To prepare inoculum for bioreactors 2 and 3 (BR2 and BR3), 100 ml of SRBC and 100 ml of CDBC were added to a mixture of 20% woodchips, 50% hay and 30% manure. Bottle 1 (Inoc1) was filled with deoxygenated synthetic mine drainage, STPW1 (w/o selenium, Table 2.1) and was used as the inoculum for BR1. Bottles 2 and 3 (Inoc2 and Inoc3) were filled with deoxygenated STPW2 (w/o selenium, Table 2.1) and were used for inoculation of BR2 and BR3. Table 2.2 shows the composition of influent. Sulphate concentration in each bottle was monitored regularly and when it dropped to less than 50% of the initial concentration the inoculum was transferred to the bioreactors.

2.2.6.3 Packing and operation of bioreactors

1 kg (wet weight) of Mixture1 was presoaked in solution STPW1 (without selenium) and 1 kg of Mixture2 was pre-soaked in Postgate B medium for a few days until the pH of the solution was stable. Then the solids were separated from the liquid phase (STPW1 or Postgate B). The pH of the liquid was adjusted to 7.5 with drop-wise addition of 10N NaOH and it was deoxygenated by bubbling N₂ through it. Solids were mixed with crushed limestone (4% w/w) and were inoculated with 100 ml of Inoc1, Inoc2 or Inoc3 in the anaerobic hood. Then they were transferred to the bioreactors. 1.5-2 liters of deoxygenated solution Postgate B was added to each bioreactor and a pack of sand was placed on top of the organic bed to avoid floatation or loss of organics in the outflow. Table 2.3 summarizes the contents of each bioreactor.
All bioreactors were left in batch mode and sulphate concentration inside them was monitored by taking a sample from the middle sampling port (port 5). When sulphate concentration in the bioreactors dropped to less than half the initial sulphate concentration, continuous flow of synthetic mine water into BR1 and Postgate B into BR2 and BR3 was started. In BR1, STPW1 was pumped at a flow rate of 0.3 ml/min (HRT~4.5 days) for 50 days. Then, due to diminishing sulphate reduction, Postgate B was pumped into it for 6 days, after which the continuous flow stopped and the column was left in batch mode. After a week, pumping of Postgate B resumed at a lower flow rate (HRT~14 days) for 42 days. Finally Postage B was replaced by STPW1 and HRT changed to 28 days.

To start up BR2 and BR3, first Postgate B was pumped into the bioreactors at a very low pump flowrate (HRT~28 days) for approximately two passes (53 days). This was replaced with STPW2, which was used for 48 days before being replaced with STPW3 for the rest of the experiment. All the feed solutions were initially deoxygenated by bubbling N\textsubscript{2} through them and they were fed from tightly closed plastic bags to minimize oxygen infiltration into the bioreactors. The effluent left the bioreactors at port 1 and was collected in a plastic container. The reason for replacing STPW1 by STPW2 was that STPW2 contained nitrogen and phosphorus, which are nutrient elements necessary for bacterial growth and activity. STPW3 contained ferrous iron, which was added in an attempt to precipitate iron sulphide in order to reduce sulphide concentrations in the column.

Samples were taken from the side ports of the bioreactors. They were immediately used for measurement of pH, oxidation reduction potential (ORP), conductivity and dissolved oxygen (DO). Samples taken for sulphate and sulphide measurement were filtered with 0.45 µm syringe filters into 2% zinc acetate (1 ml zinc acetate per 5 ml of sample). Samples taken for other analyses such as dissolved organic carbon (DOC), total soluble carbohydrates and proteins were also filtered with 0.45 µm filters.
2.3 RESULTS

2.3.1 The Batch Tests

Both organic mixtures were able to support sulphate reduction when batch cultures were incubated in an anaerobic chamber at room temperature with no other additives other than inoculum and water with 500 mg/L sulphate (Figure 2.5). In about 15 days sulphate was reduced from 500 mg/L to non-detectable concentrations. Sulphate reduction did take place in the positive control, and there was no sulphate reduction in the negative control. This proves that the SRB added to the organic materials as inoculum were active and sulphate reduction in the organic mixtures was due to SRB growing on carbon sources derived from those organic materials. Thus both mixtures were suitable for passive sulphate reduction, at least in the early stages. Overall rates of sulphate reduction were comparable for both mixtures. Likely these results are due to the availability of electron donors suitable for SRB in the dissolved organic carbon fraction of the organics.

2.3.2 Characterization of Initial Organic Materials

Table 2.4 summarizes chemical characteristics of hay, wood and manure. According to the results, wood has the highest content of organic carbon and C/N ratio and the lowest content of dissolved organic carbon. The very high C/N ratio of wood (228.5) and its low DOC make it unsuitable as an organic carbon source in passive treatment system, when used alone. In addition, wood contains the highest concentration of phenolic acids and resin acids. This is in agreement with the results from other studies (Chang et al., 2000). Wood species are high in resin acids, which in some cases are toxic to bacteria since this helps to protect wood from degradation. Neutral detergent non-soluble materials compose 83.5% of the wood sample. This includes 34.5% lignin, 25.9% α-cellulose and 12.7% hemicellulose. The other 10.4% are other, non-specified, neutral detergent non-soluble materials. The wood sample has the highest
lignin content followed by manure, which has 21.1% lignin. The lignin content of hay is significantly lower and counts for only 4.2%. The reason for the high lignin content of manure is that manure contained woodchips, which are used as bedding material on the farms. Hay is composed of 31.3% and 18.1% of $\alpha$-cellulose and hemicelluloses respectively. These values were highest among the three samples. On the other hand manure contained 11.2% and 9.1% of $\alpha$-cellulose and hemicelluloses, the lowest of the three samples. In addition, hay had the highest percent of DOC. Hay and wood were significantly different in their DOC and lignin content. One objective of this research was to find out the effect of organic mixture on the performance of BR and efficiency of sulphate removal. Preparing two mixtures of these three organic materials with different combinations of hay and wood would make it possible to study the difference in performance of the anaerobic bioreactors with different proportions of easily degradable and recalcitrant materials.

2.3.3 Start-Up of Continuous Flow Passive Treatment Bioreactors

Before starting up the bioreactors, the pH of the water, organics and inoculum mixture was adjusted with NaOH to approximately 7.5. However, during the acclimation period the pH inside the bioreactors decreased to below 6.5, the lower limit for effective sulphate reduction (Figure 2.6). Despite re-adjustment of the pH to neutral by adding more NaOH, it continued to decrease. Likely this was due to organic acids that are produced in the fermentation step (conversion of sugars into organic acids) and acetogenesis (breakdown of organic acids to acetate). Since the starting population of SRB in the bioreactors was small the organic acids could not be consumed as fast as they were produced. Therefore, during start-up of the bioreactors, in order to keep the environment suitable for SRB activity, the pH needed to be adjusted to 7.5-8 by regular addition of NaOH. In BR1 it took 41 days before the pH stabilized at greater than 6.5 and thereafter NaOH addition was needed only sporadically. In BR2 and BR3, pH adjustment was needed for 28 and 53 days, respectively.
Oxidation-reduction potential (ORP) in BR1 initially increased slightly or did not change and only after the introduction of Postgate B it started to drop to values less than -300 mV and fluctuated between -330 mV and -360 mV for the rest of operation. Whereas in BR2 and BR3, ORP dropped to less than -300 mV within 24 days and fluctuated between -300 mV and -400 mV thereafter (Figure 2.7). Oxidation reduction potential measurements confirmed that reducing conditions existed in the bioreactors. Dissolved oxygen (DO) in BR1 was initially high but after 100 days it started to decrease. This was partly due to the inefficient de-oxygenation of the influent feed. Once the method was improved, the level of dissolved oxygen in the bioreactors dropped (Figure 2.8A). In BR2 and BR3 DO was generally lower than BR1. BR2 had a lower oxygen level compared to BR3. DO in BR2 was lower than 0.2 mg/L until day 100, after which it started to increase. In BR3 initially DO was as high as 1.4 mg/L but after 150 days it started to decrease (Figure 2.8B, 2.8C).

2.3.4 Bioreactors Operation

2.3.4.1 Operation of BR1

Initial operation of BR1 was not successful since once STPW1 was started sulphate concentration at Port 2 steadily increased to around the feed concentration. Since in the acclimation period the bioreactor was in batch mode, concentration of DOC was high and the bacterial community had access to carbon sources. But after starting the flow DOC was washed out of the bioreactor, which deprived SRB from readily available organic carbon. To restore the SRB community, Postgate B medium was pumped into BR1 on 75th day for 5 days. Then the flow stopped and BR1 was left in batch mode for 5 days after which pumping of Postgate B was resumed at a lower pump flow rate (0.1 mL/day) and longer HRT (14 days) to prevent the washout. During this period (42 days) the bioreactor operated successfully since sulphate concentrations were less than 100 mg/L at Port 6 (Figure 2.9). After re-establishing the SRB population, BR1 was fed with STPW1 once again (day 129), but at an even longer residence time of 28 days. Under
these conditions the bioreactor operated successfully reducing sulphate to less than 100 mg/L for approximately another 100 days. Eventually the bioreactor’s performance started to decrease, possibly due to changes in the degradability of the organic materials. More easily degradable component were possibly consumed and the organic material contained more recalcitrant components. Figure 2.10 represents the change in sulphide concentration in different ports of BR1. No trend was observed for the change in sulphide concentration over time. In general, concentration of sulphide was less than 150 mg/L and it was lower in port 2. This is in agreement with the results in Figure 2.11. According to this figure sulphate concentration always decreased from port 8 to port 6. Sulphate concentration continued to decrease; however at times its concentration in port 4 increased and it was always higher in port 2. One explanation for this is that sulphide was oxidized back to sulphate at the top of the bioreactor. Taking samples from the bioreactors allowed a head space to develop above the sand layer from which oxygen could diffuse into the treated effluent resulting in re-oxidation of sulphide back into sulphate.

Each of the side ports represented a different HRT. In BR1, P8, P6 and P4 represented 4, 12 and 20 days of hydraulic retention respectively. According to the results in Figure 2.9, a hydraulic retention time of 12 days was enough to reduce sulphate to less than 100 mg/L.

2.3.4.2 Operation of BR2 and BR3

To enhance the start-up process in BR2 and BR3, these bioreactors were filled with Postgate B medium initially and after the acclimation period further Postgate B was pumped into them for 53 days to help build up the SRB population before adding STPW. Samples were taken from Ports 2, 4, 6 and 8 of the bioreactors. These ports represented 4, 12, 20 and 28 days of hydraulic retention, respectively. In bioreactors 2 and 3 it took between 12-20 days for sulphate concentration to drop to less than 100 mg/L (Figure 2.11).
In general dissolved oxygen in BR3 was higher than BR2 and this has most likely contributed to the less successful performance of BR3. In order to prevent the possibility of sulphide being re-oxidized to sulphate, ferrous iron was added to the influent (replacement of STPW2 with STPW3) on day 132. Precipitation of iron sulphide would reduce the concentration of free sulphide (Figure 2.12) and consequently prevent re-oxidation of sulphide to sulphate. However, even after adding ferrous iron the same phenomenon of sulphate increase towards the top of the bioreactor was observed in BR3. Therefore to completely prevent any head-space from developing, the position of the effluent in BR3 was changed to the top of the bioreactor on day 233 and sample was taken only from the effluent and not from the side ports.

In the beginning of the continuous operation, DOC concentration in BR2 and BR3 was 711 mg/L and 740 mg/L respectively. These concentrations dropped sharply in the first 40-50 days and thereafter they continued to drop in a slower rate and with a sinusoidal trend (Figure 2.13). Carbohydrates were initially 21.85 and 28.94 mg/L in BR2 and BR3 (Figure 2.14) and they were continuously decreasing with a sinusoidal pattern during the course of operation. The periodical increase and decrease in concentrations of DOC and carbohydrates is likely due to the biodegradation of organic materials. When organic materials degrade to smaller and soluble molecules concentration of DOC increases. After these components are consumed with microorganisms their concentration drops. Concentration of proteins in the bioreactors was less than 20 mg/ml all the time.

2.3.5 Characterization of Organic Materials at the Time of Sacrificing of Bioreactors

BR1 operated for a total of 269 days. When the concentration of sulphate started to increase over 100 mg/L this bioreactor was stopped and sacrificed. Figure 2.15 illustrates the changes in the characteristics of the organic material in BR1. As this figure shows the properties of the solid samples
across BR1 were very similar. The NDNS content of the solid samples in P8, P68, P46 and P24 were 0.93±0.01, 0.93±0.01, 0.93±0.0 and 0.94±0.01 g/g-WNS respectively. The α-cellulose corresponding to each of these ports were 0.40±0.03, 0.41±0.03, 0.43±0.03 and 0.43±0.02 g/g-WNS. Hemicelluloses were 0.19±0.03, 0.12±0.10, 0.20±0.05 and 0.19±0.0 g/g-WNS in P8, P68 and P46 and P24. Hemicellulose in P68 was lower than the other ports but the results of Tukey test showed that the difference from other ports was not statistically significant. Lignin was 0.24±0.02, 0.25±0.03, 0.23±0.0 and 0.25±0.07 g/g-WNS in P8 to P24. The results of Tukey test showed that there were no significant differences among the NDNS, lignin, α-cellulose and other NDNS at different ports of BR1 (P>0.05). In Figure 2.15 other holocelluloses and other NDNS in P68 look different from the same characteristics in other ports of BR1. The average values for the other holocelluloses and other NDNS are 0.0073 g/g-WNS and 0.0539 g/g-WNS respectively. The results of Tukey test shows that the difference between these two characteristics in P68 and other ports of BR1 are not statistically significant. The difference observed in Figure 2.15 is due to the large standard deviation. The standard deviations were 0.0065 and 0.0631 for other holocelluloses and other NDNS respectively, which are very large compared to the average values of these two characteristics.

Bioreactors 2 and 3 were sacrificed at two different times. BR2 was sacrificed at its successful sulphate reduction phase, whereas BR3 was sacrificed at its decline phase. Figure 2.16 illustrates the characteristics of the solid (water non-soluble (WNS)) organic material in BR2 and BR3 at time zero and after sacrificing. The initial mixture in BR2 and BR3 contained 0.86±0.04 g-NDNS/g-WNS. The final NDNS in BR2 range from 0.78±0.02 to 0.88±0.02 g/g-WNS, which is not significantly different from the NDNS in the initial sample; However in BR3 NDNS was lower and ranged from 0.66±0.0 to 0.79±0.03 g/g-WNS. Taken together, the NDS contents of solids from BR2 and BR3 were not statistically significantly different from each other, or from the starting material. Only NDS in P67 of BR3 was higher than BR2 (P=0.0217); However in some of the sections between ports, NDNS was statistically significantly different from each other and the starting material (BR0). The significant difference in
NDNS but not in NDS can be justified by comparing the ash content of BR2 and BR3. The results of the Tukey test in these two bioreactors reveals the differences in their ash content. BR3_P56 and BR3_P8 have statistically significantly higher ash content than BR2_P56 and BR2_P8 ($P<0.001$, $P=0.0005$ respectively). Ash content in P56 and P8 of BRT3 are also significantly higher than in BRT0. The ash content of the organic materials increases as they biodegrade. Another source of ash in these bioreactors is the various forms of iron precipitates, for example FeS. A higher FeS is expected in BR3, since it was operating longer than BR2. Assuming all the sulphide generated in the bioreactors is converted to FeS, the total FeS in BR2 will be around 4.05 grams, whereas in BR3 the total FeS generated is 5.40 grams. This can explain the higher ash content of BR3. In all samples lignin was lower than $\alpha$-cellulose, except in BR2-P67 in which lignin was higher than $\alpha$-cellulose (0.33±0.16 g/g-WNS compared to 0.27±0.13 g/g-WNS). This shows that $\alpha$-cellulose was preferentially degraded over lignin. This was expected since lignin is a very recalcitrant component. The percentage of $\alpha$-cellulose in all samples of BR2 was very similar to the $\alpha$-cellulose content of samples of BR3. In BR2 $\alpha$-cellulose ranged from 0.26±0.02 to 0.30±0.02 g/g-WNS. In BR3 it ranged from 0.28±0.13 to 0.30±0.0 g/g-WNS. Hemicellulose had the lowest percentage among the holocelluloses in all samples. Hemicellulose in the initial sample was 0.09±0.02 g/g-WNS. In BR2 it was in the range of 0.06±0.05 to 0.13±0.01 g/g-WNS with the ports 67, 56 and 45 having a lower hemicelluloses. Hemicellulose was higher in samples of BR3 and it was changing in the range of 0.11±0.015 to 0.14±0.01 g/g-WNS.

### 2.3.6 Microbial Community at the Time of Sacrificing

#### 2.3.6.1 BR1

In total 30,700 sequences were identified in BR1. Eighty five percent of the sequences belonged to the domain bacteria, 14.97% to archaea and the rest belonged to the domain eukaryote (Figure 2.17A). Seven main phyla were represented in this bioreactor (Figure 2.17B)
Using R, a heatmap of the OTUs that each contributed to more than 0.5% of all the reads/sequences in the dataset was produced (Figure 2.18). According to this heatmap species from genera such as Vadin-B27 (unclassified environmental group from wastewater sludge), Smithella and Acetivibrio are highly present in P8 and P68, at the entrance of the bioreactor, but their numbers were very lower in P46 and P24, at the exit of the bioreactor. OTUs from genera Treponema, Methanobacterium and Lachnospiraceae, which clustered together, were more present in P46 and P24 of BR1. A different methanogen genus, Methanosaeta was absent in P24 and P46 but was highly represented in P8 and P68 and co-occurred with Fibrobacter, Spirochaetes and Treponema related sequences and those for a third methanogen, Methanosarcina, clustered with each other due to their high presence in the middle of the bioreactor; However, their numbers were very low at the reactor entrance (P8). Other OTUs present in BR1 include two OTUs of iron-reducing bacteria (clone Cl-A12) from genus Bacteroides, which are clustered to Treponema, and Acetovibrio cellulolyticus CD respectively.

As Figure 2.19 shows sequences related to the genera Methanobacterium, Treponema and Lachnospiraceae increase in number across BR1 with the same trend. This confirms the observation in Figure 2.18 that these three genera co-occur and possibly they depend on one another for growth and survival. The co-occurrence of Methanobacterium and Treponema have been observed in previous literature (Dollhopf et al., 2003).

Sulphate-reducing bacteria related sequences composed only 2.84% of all the reads in BR1 with the dominant OTUs closely related to Desulfovibrio idahoensis and Desulfovibrio Sp. S14 pv-2008. Methanogens were more abundant and comprised 13.29% of sequences. The dominant methanogen species most closely related to the bioreactor OTUs were Methanobacterium curvum, Methanosarcina barkeri and an uncultured species of Methanosaeta (Figure 2.20).
2.3.6.2 BR2 and BR3

In total 3594, 31550, 14759 and 5119 sequences were identified in CDB culture (CDBC), BR2, BR3 and SRB culture (SRBC). Only a small number of 97% OTUs were classified in the domain eukaryotes. The majority of 97% OTUs fell into the bacteria domain (Figure 2.21). The 97% OTU table was rarefied to 3341 reads per sample and the most highly represented OTUs were selected as those that contributed to more than 2 percent of all the reads to produce a heatmap (Figure 2.22, Figure 2.23). This heatmap also includes the OTUs present in the SRB and CDB cultures that were used in inoculation of the bioreactors. The nature of OTUs in these cultures are different from those in the bioreactors. Uncultured species classified in the genera of *Atopococcus*, *Sedimentibacter*, *Lactobacillus*, *Ersipelotrichaceae*, *Parbacteoides*, *Desulfobulbus* and *Clostridium Sp. Clone DhR^2/LM-G01* were highly represented in the CDB and SRB cultures. Some of these OTUs persisted in the bioreactors as well.

At least three methanogens are present in both BR2 and BR3 (Figure 2.24). *Methanosarcina mazei* G0, *Methanobacterium curvum* (OTU-1172 and OTU-2398) and *Methanocorpusculum labreanum Z* prevail in BR3, whereas *Methanosarcina barkeri* was more prevalent in BR2. OTUs related to *Terrestrial Miscellaneous GP(TMEG) and Mollicutes* appear together. *Rs-E47 termite group*, TSCOR003-O20 and *Marinitoga Okinawensis* are clustered to each other. Uncultured *Ruminococcaceae* related OTUs prevailed in BR2. Species of this family are common in organic-rich environments where they are fermentative bacteria responsible for breakdown of large macromolecules to smaller more available monomers. *Marinitoga Okinawensis* species are thermophilic chemo-organotrophs that are strict anaerobes and grow on glucose, starch and tryptone (Nunoura et al., 2007). The OTUs that are clustered together since their percentage correlate with each other in all of the samples are likely from microbial species that are metabolically reliant on each other.

The dominant *Methanobacterium* related OTUs in the bioreactors are *Methanobacterium curvum* and *Methanocorpusculum Labreanum Z*. *Methanosarcina* are represented by *Methanosarcina barkeri* and
Methanosarcina mazei Go2. The later one had a much lower presence in the SRB culture (Figure 2.24). There are various species of sulphate-reducing bacteria in the bioreactors and cultures. For instance, Desulfobulbus and Desulfovibrio idahoensis were predominant in the SRB culture. They were present in the bioreactors as well but in substantially lesser proportions.

2.3.7 Environmental Effects on the Microbial Species

In order to find the relationship among various OTUs observed in the bioreactors and also to find out the factors that affect their presence, different statistical tests were done in R-Vegan. Detrended correspondence analysis (DCA) was done to see the clustering of taxonomic groups in different sections of bioreactors BR2 and BR3, and to determine the effect of environmental factors on the distribution of microbial communities. Figures 2.25, 2.26, 2.27 and 2.28 show the effects of the key reactor operating parameters DO, ORP, pH and conductivity as well as properties of the pore water and characteristics of solid materials in the bioreactors on the presence and distribution of the microbes. These figures show that ORP and pH increase in opposite directions. High ORP stimulates growth of species such as uncultured Mollicutes, Methanobacterium curvum, Methanosarcina Mazei Go and Methanocorpusculum labreanum Z. On the other hand Desulfobulbus, Spirochaeta zuelzerea and some OTUs related to Methanobacterium curvum are found in the sites with lower ORP. All the methanogens are present in the sites that are void of oxygen.

Conductivity and pH increase in the opposite direction of ORP and DO. Species such as Marinitoga Okinawensis, Rs-E47 from the termite group and Methanobacterium curvum require a lower pH than Sporobacter, Lachnospiraceae and Mollicutes. DOC, proteins and carbohydrates all had the same effect on the microbial growth and distribution, with DOC and carbohydrates changing more rapidly (Figures 2.27 and 2.28). Marinitoga Okinawensis, Atopococcus., Methanosarcina barkeri and Spirochaetes are examples of species that needed high concentrations of water-soluble materials to grow and survive. On
the other hand *Treponema*, *Parabacteroides* and *Ruminococcaceae* are in samples with more recalcitrant components (NDNS and lignin). *Methanocorpusculum* species are also observed in the sites with less readily available carbon sources. Table 2.5 and Table 2.6 summarize the eigenvalues of each of the environmental factors, solid characteristics and pore water chemistry. As these tables show there is a statistically significant correlation between parameters pH, DO, ORP and conductivity and the composition of the microbial community in the bioreactors.

### 2.4 DISCUSSION

The results indicate that it is possible to use a mixture of hay, woodchips and manure to remove sulphate and selenium from simulated Mount Polley mine tailings pond water to the required regulatory limits. Bioreactor BR2 (containing 40:30:30 of woodchips, hay and manure) had the most successful operation and removed sulphate to lower than 100 mg/L for its entire operating period (230 days) with a HRT as low as 12 days. Sulphate removal rates (SRR) were calculated between the ports P8, P6, P4 and P2 and was highest at the entrance of the bioreactor (130 mg/L.day), which was expected, since sulphate concentrations are higher near the influent and kinetics are a function of sulphate concentration (Kinetics model represented in Chapter 1-Page 5).

The maximum sulphate removal rate measured between the influent and port P8 of BR2 is consistent with other SRRs reported in literature. Sulphate removal rates in a bioreactor, with a similar HRT of 13 days, containing waste from the pulp and paper industry (Hulshof *et al.*, 2003) was 120 mg/L.day during the first 36 days of operation, thereafter decreasing to 60 mg/L.day. Sulphate concentrations in the influent of that bioreactor were much higher, at 4500 mg/L, than in this study. In the same study, SRR in another bioreactor containing woodchips (HRT~14 days) was 80 mg/L in the first 36 days of operation and then decreased to 40 mg/L.day afterwards. Thus, the performance of BR2 was more consistent than
that observed for these other bioreactors possibly due to the combination of hay and wood, or due to the acclimation period on nutrient rich medium that was used when starting up BR2. Greben et al. reported a SRR of 156.4 mg/L.day in a bioreactor using grass cuttings as carbon source with HRT of 4 days and influent sulphate concentration of 1500 mg/L (Greben et al., 2009b). The shorter HRT and higher SRR in this bioreactor was due to the high protein content of the grass, which was more biodegradable and could supply SRB with carbon sources at a faster rate.

First acclimating the bioreactors with a SRB growth medium containing a defined carbon source and other essential nutrients greatly improved their start-up. These nutrients allowed the SRB to grow and increase in number before introducing the influent. The Mount Polley mine tailings pond water does not contain nitrogen, potassium or phosphorus chemical compounds and it appeared as if these nutrients were not being released from the organic material in sufficient amounts. Thus, addition of nutrients N, P and K to the influent was needed. Therefore, it is recommended that bioreactors used for treating these types of mine-affected water should be left to acclimate with a defined carbon source and nutrients for at least the first 20 days before start-up, and that nutrient addition (N, P and K) is required.

During start-up of these bioreactors, it was observed that the sulphate concentration first decreased after the feed entered the bioreactor and then later increased again towards the top. Although the dissolved oxygen concentrations were low throughout the column, it was postulated that sulphide generated from sulphate reduction was re-oxidized back to sulphate. Additionally, as the feed contained low concentrations of cations, there were no sinks for the sulphide, i.e. as in the form of metal sulphide precipitates. Oxygen penetration may have occurred during sampling from the side ports or from a head space at the top of the bioreactor that developed during sampling. This illustrates that successful performance of these types of bioreactors is very sensitive to oxygen infiltration. To prevent re-oxidation of sulphide a sink for its removal is required. To this end it is suggested to add iron to the influent to order
precipitate iron sulphides. This can be done by either using ferrous chloride or scrap iron, whichever is locally available or less costly.

The concentrations of dissolved carbon compounds in the bioreactors increased and decreased periodically (Figures 2.13 and 2.14). This indicated that biodegradation of the recalcitrant organic materials was taking place. But, the composition of the fibrous components such as hemicellulose and α-cellulose in bioreactor BR2 were not significantly different after 250 days of operation from the time-zero sample (Figure 2.16) indicating that biodegradation was slow and theoretically these reactors should be able to run for much longer. Lignin content did not change at all, which was not unexpected due to its recalcitrant nature.

Sulphate reducing bacteria were only a small fraction of the microbial community with BR2 containing 1.23% and BR3 3.26% of reads related to SRB taxa. These results are in agreement with the results of Hiibel et al. (2011). They found 2-5% SRB in bioreactors containing hay/pine woodchips, and hay/corn stover (Hiibel et al., 2011). In another study by Pruden et al. (2006) they found that SRB comprised a small fraction of the bacterial community of a compost bioreactor (Pruden et al., 2006). According to Logan et al. (2005) sulphate-reducing bacteria rely on fermentative and hydrolyzing bacteria for break down and consumption of the lignocellulosic material, which justifies the higher presence of bacteria other than SRB in lignocellulosic bioreactors. Sulphate reducing bacteria were more prevalent in bioreactor BR3 and correlated with the higher sulphate concentrations found there. Despite there being more SRB in BR3 its performance did not match that of BR2. This is possibly due to higher concentration of dissolved oxygen in BR3, which re-oxidized sulphide back to sulphate. When correlating prevalence of specific SRB genera with organic material properties, Desulfobulbus-related sequences were more represented in sites with higher hemicellulose and lower α-cellulose concentrations. Hemicellulose is more degradable and it is possible that it can provide SRB with carbon sources.
Methanogens were present in all bioreactors, which was unexpected. *Methanocorpusculum*-related OTUs were more prevalent in sites with higher holocellulose and lower hemicellulose, the opposite of that observed for *Desulfobulbus*-related sequences. Thus, methanogens may thrive in places where the more labile organic polymers (such as hemicellulose) are depleted.
Figure 2.1: Map of Mount Polley Mine

(http://www.imperialmetals.com/s/MountPolleyMine.asp)
Figure 2.2: Mount Polley tailings pond and pilot treatment system

(Treatment pond)
Figure 2.3: Flowchart of the sequential wash of the solid organic materials.
Figure 2.4: Diagram of a bioreactor
Table 2.1: Composition of organic inocula for inoculation of bioreactors

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Culture</th>
<th>Organic Mixture</th>
<th>Organic Mixture (g wet weight)</th>
<th>Solution</th>
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<tbody>
<tr>
<td>Inoc1</td>
<td>SRBC</td>
<td>40%&lt;sub&gt;dw&lt;/sub&gt; woodchips+30%&lt;sub&gt;dw&lt;/sub&gt; hay+30%&lt;sub&gt;dw&lt;/sub&gt; cow manure</td>
<td>50</td>
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<td>Inoc2</td>
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<td>STPW2 w/o selenium</td>
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<tr>
<td>Inoc3</td>
<td>CDB, SRBC</td>
<td>40%&lt;sub&gt;dw&lt;/sub&gt; woodchips+30%&lt;sub&gt;dw&lt;/sub&gt; hay+30%&lt;sub&gt;dw&lt;/sub&gt; cow manure</td>
<td>80</td>
<td>STPW2 w/o selenium</td>
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Table 2.2: Composition of influent feed

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<tr>
<th>Composition (g/L)</th>
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<th>STPW2</th>
<th>STPW3</th>
<th>Postgate B</th>
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<td>SO&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;</td>
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<td>0.5</td>
<td>0.6</td>
<td>1.650</td>
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<td>CaSO&lt;sub&gt;4&lt;/sub&gt;.2H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<td>0.483</td>
<td>1.26</td>
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<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0.739</td>
<td>0.253</td>
<td>0.253</td>
<td>-</td>
</tr>
<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>0.094</td>
<td>0.977</td>
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<tr>
<td>FeSO&lt;sub&gt;4&lt;/sub&gt;.7H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<td>0.25</td>
<td></td>
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<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>0.0475</td>
<td>0.5</td>
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</tr>
<tr>
<td>Sodium lactate</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.015</td>
<td>0.015</td>
<td>0.015</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>7.5-8</td>
<td>7.5-8</td>
<td>7.5-8</td>
<td>7.5-8-</td>
</tr>
</tbody>
</table>
### Table 2.3: Contents of each bioreactor

<table>
<thead>
<tr>
<th>Bioreactor</th>
<th>Organic Mixture</th>
<th>Solution</th>
<th>Inoculumn</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR1</td>
<td>Mix 1</td>
<td>STPW1 w/o selenium</td>
<td>Inoc1</td>
</tr>
<tr>
<td>BR2</td>
<td>Mix 2</td>
<td>Postgate B</td>
<td>Inoc2</td>
</tr>
<tr>
<td>BR3</td>
<td>Mix 2</td>
<td>Postgate B</td>
<td>Inoc3</td>
</tr>
</tbody>
</table>
Table 2.4: Characteristics of organic materials

<table>
<thead>
<tr>
<th>Chemical Characteristic</th>
<th>Hay</th>
<th>Wood</th>
<th>Manure</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOC (%)</td>
<td>43.1</td>
<td>48.9</td>
<td>44.9</td>
</tr>
<tr>
<td>DOC (%)</td>
<td>7.46</td>
<td>0.57</td>
<td>2.51</td>
</tr>
<tr>
<td>TKN (%)</td>
<td>1.34</td>
<td>0.214</td>
<td>0.795</td>
</tr>
<tr>
<td>C/N</td>
<td>32.16</td>
<td>228.5</td>
<td>56.48</td>
</tr>
<tr>
<td>Total Nitrogen (%)</td>
<td>1.12</td>
<td>0.29</td>
<td>1.05</td>
</tr>
<tr>
<td>Phenol (mg/kg)</td>
<td>0.125</td>
<td>&lt;0.25</td>
<td>&lt;0.15</td>
</tr>
<tr>
<td>Total resin acids (mg/kg)</td>
<td>&lt;0.6</td>
<td>8920</td>
<td>508</td>
</tr>
<tr>
<td>Neutral Detergent Soluble (%)</td>
<td>7.41±1.2</td>
<td>5.85±0.83</td>
<td>10.79±3.99</td>
</tr>
<tr>
<td>Neutral Detergent Non-Soluble (%)</td>
<td>90.77±1.04</td>
<td>83.46±1.76</td>
<td>60.3±0.99</td>
</tr>
<tr>
<td>α-cellulose (%)</td>
<td>31.31±1.16</td>
<td>25.9±0.87</td>
<td>11.18±1.25</td>
</tr>
<tr>
<td>Hemicellulose (%)</td>
<td>18.12±0.29</td>
<td>12.65±1.052</td>
<td>9.05±1.52</td>
</tr>
<tr>
<td>Lignin(%)</td>
<td>4.2±1.61</td>
<td>34.49±0.8</td>
<td>21.09±0.66</td>
</tr>
<tr>
<td>Protein (mg γ glucin/g)</td>
<td>2.17</td>
<td>N.D</td>
<td>3.72</td>
</tr>
<tr>
<td>Carbohydrates (g-Glucose/g)</td>
<td>47.25</td>
<td>17.85</td>
<td>12.15</td>
</tr>
</tbody>
</table>
Figure 2.5: Results of batch experiments
Figure 2.6: Change of pH profile in A) BR1, B) BR2 and C) BR3
Figure 2.7: Change of ORP profile with time in A) BR1, B) BR2 and C) BR3
Figure 2.8: Change in profile of dissolved oxygen in A) BR1, B) BR2 and C) BR3
Figure 2.9: Sulphate profile in BR1: Phase 1: Day 25, Continuous flow of STPW started; Phase 2: Day 75, PB was pumped; Phase 3: Day 80, BR1 left in batch mode; Phase 4: Day 87, Continuous flow of PB resumed; Phase 5: Day 129, continuous flow of STPW resumed
Figure 2.10: Sulphide profile in BR1: Day 25, Continuous flow of STPW started; Day 75, PB was pumped; Day 80, BR1 left in batch mode; Day 87, Continuous flow of PB resumed; Day 129, continuous flow of STPW resumed
Figure 2.11: Sulphate profile in A) BR2 and B) BR3. Day 79, continuous flow of STPW started
Figure 2.12: Sulphide profile in A) BR2 and B) BR3
A) BR2

Figure 2.13: DOC profile in bioreactors BR2 and BR3

B) BR3
Figure 2.14: Carbohydrates profile in bioreactors BR1 and BR2
Figure 2.15: characteristics of solid organic materials across BR1
Figure 2.16: Change of characteristics of organic material with respect to position in BR2 and BR3
Figure 2.17: Distribution of microbial domains and phyla in BR1
Figure 2.18: Logarithmic heatmap (Log 2) of most highly represented 97% OTUs chosen as those each containing more than 3% of total number of reads in bioreactors BR1. OTU numbers and their Silva 108 assigned taxonomy are given on the right. Sample names are column labels.
Figure 2.19: Relationship among various genera in BR1
Figure 2.20: Logarithmic heatmap (Log 2) of 97% OTUs belonging to methanogens and sulfidogens, each containing more than 0.5% of total number of reads in bioreactor BR1. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 2.21: Distribution of microbial domains in BR2, BR3, CDB and SRB
Figure 2.22: Logarithmic Heatmap (Log 2) of most highly represented 97% OTUs chosen as those each containing 2-5% of total number of reads in bioreactors BR2 and BR3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 2.23: Logarithmic Heatmap (Log 2) of most highly represented 97% OTUs chosen as those each containing more than 5% of total number of reads in bioreactors BR2 and BR3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 2.24: The most highly represented Proteobacteria and Methanogen related OTUs chosen as those each containing more than 1% of total number of reads in BR2, BR3, CDB and SRB (Logarithmic heatmap based on Log 2). OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 2.25: Effect of ORP, pH, DO and conductivity on distribution of microbial community higher than 5% in BR2 and BR3
Figure 2.26: Effect of ORP, pH, DO and Conductivity on distribution of microbial community between 3% and 5% in BR2 and BR3.
Figure 2.27: Effect of DOC, carbohydrates, proteins NDNS, holocellulose, hemicellulose, α-cellulose and lignin on distribution of microbial community higher than 5%
Figure 2.28: Effect of DOC, carbohydrates, proteins NDNS, holocellulose, hemicelluloses, α-cellulose and lignin on distribution of microbial community between 3% and 5% in BR2 and BR3
Table 2.5: Resultant eigenvalues from the Envfit test in R-Vegan relating distribution of microbial community higher than 5% to various environmental factors and solid and liquid characteristic in BR2 and BR3

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r²</th>
<th>Pr(&gt; r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>-0.996795</td>
<td>-0.080001</td>
<td>0.7477</td>
<td>0.040</td>
</tr>
<tr>
<td>ORP</td>
<td>0.996370</td>
<td>0.085124</td>
<td>0.8869</td>
<td>0.018</td>
</tr>
<tr>
<td>DO</td>
<td>0.867651</td>
<td>-0.497173</td>
<td>0.9216</td>
<td>0.010</td>
</tr>
<tr>
<td>Conductivity</td>
<td>-0.371718</td>
<td>0.928346</td>
<td>0.9856</td>
<td>0.001</td>
</tr>
<tr>
<td>DOC</td>
<td>-0.1420174</td>
<td>-0.9898642</td>
<td>0.8967</td>
<td>0.020</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>-0.1896986</td>
<td>-0.9818424</td>
<td>0.6146</td>
<td>0.105</td>
</tr>
<tr>
<td>Proteins</td>
<td>-0.0534039</td>
<td>-0.9985730</td>
<td>0.3552</td>
<td>0.366</td>
</tr>
<tr>
<td>NDNS</td>
<td>-0.7591597</td>
<td>0.6509044</td>
<td>0.5152</td>
<td>0.175</td>
</tr>
<tr>
<td>Holocellulose</td>
<td>0.2610662</td>
<td>0.9653209</td>
<td>0.2710</td>
<td>0.496</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>0.1480140</td>
<td>-0.9889853</td>
<td>0.4520</td>
<td>0.241</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>0.0070196</td>
<td>0.9999754</td>
<td>0.4140</td>
<td>0.295</td>
</tr>
</tbody>
</table>
Table 2.6: Resultant eigenvalues from the ENVfit test in R-Vegan relating distribution of microbial community between 3% and 5% to various environmental factors and solid and liquid characteristic in BR2 and BR3

|                | DCA1     | DCA2     | $r^2$ | Pr(>|$r$|) |
|----------------|----------|----------|-------|----------|
| pH             | -0.47699 | 0.87891  | 0.8188| 0.025    |
| ORP            | 0.81382  | 0.58112  | 0.9148| 0.009    |
| DO             | 0.97483  | -0.22294 | 0.8766| 0.014    |
| Conductivity   | -0.74141 | 0.67105  | 0.8583| 0.007    |
| DOC            | -0.42156 | -0.90680 | 0.3094| 0.295    |
| Carbohydrates  | -0.38124 | -0.92447 | 0.3758| 0.246    |
| Proteins       | -0.15788 | -0.98745 | 0.1193| 0.691    |
| NDNS           | -0.90348 | 0.42862  | 0.3978| 0.289    |
| Holocellulose  | 0.355248 | -0.93477 | 0.1881| 0.512    |
| Hemicellulose  | 0.100589 | -0.99492 | 0.6119| 0.056    |
| $\alpha$-cellulose | 0.001312 | 0.999999 | 0.0769| 0.792    |
3.1 INTRODUCTION

Although passive treatment of mine drainage is becoming more popular, such systems are still regarded as “black boxes” since there is little information about the removal mechanisms and the chemical and biological dynamics occurring in these systems. Most laboratory studies to date on bioreactors with complex carbon sources have been performed over short time periods. Thus, very little has been revealed about how rapidly the organic components are degraded and how this impacts performance of the bioreactor. As the more easily degradable compounds are used up, SRB become reliant on degradation of recalcitrant carbon sources such as cellulose, which is very slow and often a bottle neck in the rate of sulphate reduction (Logan et al., 2005). As a result, the performance of the bioreactor might be impacted with efficiencies decreasing. Therefore, measuring the changes in organic matter characteristics over time will help to predict when the slowdown phase will occur, or how long these systems will potentially last. In this Chapter several bioreactors were run for two different periods of time, one set were sacrificed during their successful period of operation and another set were operated until their performance started to deteriorate, or for as long as laboratory resources permitted. This was done so as to determine the changes in performance, organic matter characteristics and microbial community structure over time, and to compare successfully performing bioreactors with those that were underperforming.
Although it is well known that combinations of different organic wastes work better than using a single source, there is little information on what ratios of labile to recalcitrant components to use. A balance between sufficient SRR and longevity of performance is needed. Thus, mixtures of two different ratios of wood to hay (Mix1 containing 40\%\_dw woodchips, 30\%\_dw hay and 30\%\_dw manure and Mix2 composed of 20\%\_dw woodchips, 50\%\_dw hay and 30\%\_dw manure) were compared in the bioreactors used in the experiment described in this Chapter.

Six continuous-flow bioreactors were operated varying their time of operation and wood to hay ratio at two levels. Two replicates were included; for one of the wood to hay ratios, two duplicate bioreactors were sacrificed at the early time period and for the other wood to hay ratio, two duplicate bioreactors were operated for the longer time period. Reactor performances were monitored over time. Organic matter characteristics were compared for the reactors operating for different time periods and using different wood to hay ratios. Chapter 4 presents the results of the microbial community analysis for this experiment.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Column Experiments

##### 3.2.1.1 Design of the bioreactors

Six Plexi-glass columns (I.D.: 11.43 cm, Length: 50.8 cm) were used as up-flow biochemical bioreactors. The bottoms of the bioreactors were filled with pebbles and were separated from the top by plexiglass distributors for the purpose of evenly distributing the flow. The perforations in the distributors were \(\frac{1}{4}\)" in diameter and were \(\frac{1}{2}\)" apart. The distributors were covered with fine meshed cloth. Each bioreactor had eight side ports that were used for sampling from distances from the influent. A mesh cloth
was also used to separate the side ports from the materials inside the columns. ¼” threaded fittings at the side ports were connected to tygon tubings, which were closed by plastic clamps. The top part of the bioreactors were filled with a mixture of hay, woodchips and manure and a bag of sand was placed on top of the mixture to keep it from floating away and also to filter the effluent before leaving the bioreactor. Two series of bioreactors, each with three bioreactors were used in this experiment. The first series were named BRT1, BRT2 and BRT3 and the second series were named BRTT1, BRTT2 and BRTT3.

A mixture of 40%\textsubscript{dw} woodchips, 30%\textsubscript{dw} hay and 30%\textsubscript{dw} cow manure was used in the BRT bioreactors (Table 3.1). The mixture used in BRTT bioreactors contained 20%\textsubscript{dw} woodchips, 50%\textsubscript{dw} hay and 30%\textsubscript{dw} cow manure (%\textsubscript{dw} denotes weight percent based on dry weight).

3.2.1.2 Inoculum preparation

Two sources of microbes were used for preparing inocula for the bioreactors: a sulphate-reducing bacteria culture (SRBC) and a cellulose-degrading culture (CDBC). To prepare and maintain SRB culture in the lab, initially sludge from a UASB reactor that was used for sulphate reduction (Li & Baldwin, 2011) was used as inoculum. Postgate B, a preferred growth medium for SRB with 1700 mg/L sulphate, was prepared according to the handbook of microbiological media (Atlas, 2004). Some Postgate B was transferred to a 1-L Wheaton bottle. One hundred millilitres of the sludge was transferred to this bottle and it was filled to the rim with the rest of Postgate B medium. The bottle was kept at 32\(^\circ\) C in an incubator. The sulphate concentration in this bottle was monitored every few days until it was reduced to half the initial concentration. At this point, the culture was used as the inoculum to prepare a fresh SRB culture. Therefore by transferring the SRB culture into fresh Postgate B medium a healthy SRBC was maintained in the lab.

As the source of cellulose degrading bacteria, some cultures containing organic material and water were used. These cultures were initially prepared by adding water to an organic mixture (40%\textsubscript{ww} wood
chips, 30%\textsubscript{w/w} hay, 30%\textsubscript{w/w} manure). These mixtures were incubated at 32ºC. Cellulose activity in these cultures was measured by the method developed with Mewis et al. (Mewis K, Taupp M, Hallam SJ, 2011). In this method a 10× stock solution of lysis mix (10% Triton X-100, 100mM ris, 10mM EDTA) was prepared. A solution of 75mg/mL DNP-Cellobioside in DMSO was also prepared. Then DNP-Cellobiose solution was added to the lysis mix to a final concentration of 0.1 mg/mL. This final solution was added to a sample of the cultures and was incubated at 37ºC for 12-16 hours. Then the absorbance was read at 400 nm. Test of cellulose activity after 3 month indicated strong cellulose activity; therefore they were used as the source for cellulose-degrading bacteria. These were used to prepare cellulose degrading bacteria cultures (CDBC) by adding inoculum to a 25g/L solution of Luria broth and a mixture of 50%\textsubscript{w/w} hay, 20%\textsubscript{w/w} woodchips and 30%\textsubscript{w/w} manure. Luria Broth contains NaCl, Yeast and Trypton, which is a source of amino acids for the growth of microorganisms (Atlas, 2004). The cultures were incubated at 32ºC.

Before start-up of the bioreactors, fresh inocula were prepared in 1-L bottles in an anaerobic hood. To prepare these inocula (Org\_inoc1, Org\_inoc2 for the BRT bioreactors and Org\_inoc3, Org\_inoc4 and Org\_inoc5 for the BRTT bioreactors), 100 ml of SRBC and 100 ml of CDBC were added to a mixture of 40% woodchips, 30% hay and 30% manure for the BRT reactors and a mixture of 20% woodchips, 50% hay and 30% manure for BRTT bioreactors. The bottles were filled with deoxygenated synthetic tailings pond water (STPW) (w/o selenium, Table 3.2) and were used for inoculation of the bioreactors. The sulphate concentration in each bottle was monitored regularly and when it dropped to less than 50% of the initial concentration the inocula was used for inoculation of the bioreactors.

Before inoculating the bioreactors, pH, ORP, conductivity and DO in Org\_Inoc1 and Org\_Inoc2 were measured. Samples were taken from each inocula and filtered into 2%\textsubscript{w/v} zinc acetate for measuring sulphate and sulphide concentrations. A liquid sample was taken for measurement of liquid properties such as DOC, proteins and carbohydrates. Also, some sample from the solids was transferred to a sterile
bag and was used for DNA extraction. Org_Inoc1 and Org_Inoc2 were mixed. The pH was adjusted to 8.00 and the mixture was divided into three portions. Each portion was used for inoculation of one BRT bioreactor. Org_Inoc4, Org_Inoc5 and Org_Inoc6 were each used to inoculate one of the BRT bioreactors.

3.2.1.3 Packing and operation of bioreactors

Three eight hundred grams of either a mixture of 40% \text{ww} wood chips, 30% \text{ww} hay and 30% \text{ww} manure (for BRT bioreactors) or 20% \text{ww} wood chips, 50% \text{ww} hay and 30% \text{ww} manure (for BRTT bioreactors) was pre-soaked overnight in 12 liters and 16 liters of Postgate B respectively. Then the solids were separated from the liquid phase. The pH of the liquid was adjusted to 8 with NaOH and it was deoxygenated by bubbling N\textsubscript{2} through it. Solids were mixed with crushed limestone (4\% \text{w}) and were inoculated with 500 ml of Org\textsubscript{Inoc} inocula in the anaerobic hood. Then they were transferred to the bioreactors. 1.5-2 liters of deoxygenated Postgate B was added to each bioreactor and sand packed in a fine meshed cloth was placed on top of the organic bed to avoid floatation or loss of organics in the flow.

All bioreactors were left in batch mode and sulphate concentration inside the column was monitored by taking a sample from the middle sampling port (port 5). When sulphate concentration in the bioreactors dropped to less than half the initial sulphate concentration, continuous flow of Postgate B was started. The batch mode in all bioreactors was forty days. Postgate B was pumped in BRT bioreactors and in BRTT bioreactors for 45 days (HRT~14 days) after which it was replaced by STPW with HRT of 14 days. All the feed solutions were initially deoxygenated by bubbling N\textsubscript{2} through them and they were fed from tightly closed plastic bags to minimize oxygen infiltration into the bioreactors. The effluent left the bioreactors from the top and was collected in a plastic container. Samples were taken from the effluent and were immediately used for measurement of pH, oxidation reduction potential (ORP), conductivity and DO. Samples taken for sulphate and sulphide measurement were filtered with 0.45 \text{µm} syringe filters.
into 2% zinc acetate (1 ml zinc acetate per 5 ml of sample). Samples taken for other analyses such as DOC were also filtered with 0.45 µm filters.

### 3.2.2 Chemical Analysis

Characteristics of organic materials namely dissolved organic carbon (DOC), proteins, carbohydrates and neutral-detergent fiber (cellulose, hemicelluloses and lignin) were measured by performing a sequential wash on the organic solids. Two hundred milliliters of dH₂O was added to 2.5 grams (dry weight) of ground solids in a flask. The flasks were left on a shaker overnight at 200 rpm (at room temperature 20°C). The mixture was then filtered through 0.45 µm filter papers. The filtrate was analyzed for total dissolved carbohydrates, proteins and DOC. Total dissolved protein was measured with the Bradford method (Bradford, 1976) and total dissolved carbohydrates were measured with the phenol-sulfuric acid Dubois assay (Dubois et al., 1956). Dissolved organic carbon was measured using the Shimadzu Total Organic Carbon analyzer (Total Organic Carbon (TOC)-V_cϕH). The dried residue was washed with neutral detergent according to Van Soest et al. (Vansoest et al., 1991). Neutral detergent contained 3.6 g NaOH, 27 g sodium lauryl sulphate, 13.15 g EDTA, 6.13 g sodium borate decahydrate, 4.105 g disodium hydrogen phosphate and 9 ml triethylen glycol in 900 ml de-ionized water. The procedure in Hall laboratory manual (Hall, 2000) was followed for preparation of the detergent. All the chemicals were from ACS grade and from Sigma Aldrich.

pH, ORP and conductivity were measured in the column pore water and bioreactor effluent with a pH/Cond 7200 pH meter (WTW, Weilham, Germany). Dissolved oxygen (DO) was measured with a Symphony SP50D DO meter (VWR). Sulphate was measured with the turbidimetric barium sulphate American Water Work Association method 4500-SO₄²⁻ (Eaton et al., 2005). Sulphide was measured with the methylene blue American Water Work Association 4500-S²⁻ method (Eaton et al., 2005).
The residue after neutral detergent was used to measure holocellulose, α-cellulose and hemicelluloses following the procedures suggested by Wieder and Starr (1998). Lignin was measured in the residue from neutral detergent according to Wieder and Starr (Wieder & Starr, 1998).

### 3.2.3 Statistical Methods

Means of characteristics of the organic material (neutral detergent non-soluble, lignin, α-cellulose and hemicellulose) were compared by the Oneway analysis and pairwise Tukey HSD using the statistic software package JMP 10. The relationship among various liquid and solid phase characteristics was determined by multivariate analysis and preparing a scatter plot matrices in JMP 10.

### 3.3 RESULTS

#### 3.3.1 Start-Up

##### 3.3.1.1 Start-up of BRT (40:30:30 of woodchips:hay:manure) bioreactors

Before starting up the bioreactors, the pH of the water, organics and inoculum mixture was adjusted with NaOH to approximately 8. However, during the acclimation period the pH inside the bioreactors decreased to below 6.5, the lower limit for effective sulphate reduction (Figure 3.1-A). This was likely due to leaching of acidic compounds such as phenolic and resin acids from the organic materials, particularly wood (Venner et al., 2009) into the water. Despite re-adjustment of pH to neutral by adding more NaOH, it continued to decrease. Likely this was due to organic acids that are produced in the fermentation step (conversion of sugars into organic acids) and acetogenesis (breakdown of organic acids to acetate). The starting population of SRB in the bioreactors was small and the organic acids could not be consumed as fast as they were produced. Therefore, during start-up of the bioreactors, in order to keep the
environment suitable for SRB activity, pH needed to be re-adjusted to 7.5-8 by adding NaOH regularly. In all the BRT bioreactors it took 114 days before the pH stabilized at greater than 6.5 and thereafter NaOH addition was needed only sporadically.

3.3.1.2 **Start-up of BRTT (20:50:30 of woodchips:hay:manure) bioreactors**

During the acclimation period the pH inside the BRTT bioreactors was well below 6.5. (Figure 3.9-A). pH was lower than 6.5 in BRTT1 and BRTT2 during the first 159 days of operation. In BRTT3 it took 193 days to measure pH values higher than 6.5. These results are in agreement with the result from the BRT bioreactors.

ORP in all three BRTT bioreactors was below -100 mv. In BRTT1 ORP dropped to below -300 mv in 130 days. In BRTT2 after 59 days ORP dropped to below -300 days but it raised to over -200 mv on day 103. After 144 days it dropped to below -300 mV again and fluctuated between -300 mv and -370 mv until the last day of operation. BRTT3 reached to ORP values lower than -300 mv in 144 days and the ORP stayed below -300 mv for the rest of its operation period (Figure 3.9-B).

Dissolved oxygen in all three bioreactors was below 1 mg/L all the time, except on day 130 that it increased to 1.8 mg/L in BRTT2 (Figure 3.9-C).

3.3.2 **Operation of Bioreactors**

3.3.2.1 **Operation of BRT (40:30:30 of woodchips:hay:manure) bioreactors**

The previous experiments with the duplicate bioreactors (Chapter 2) showed that HRT of 14 days was enough to reduce sulphate to less than 100 mg/L. Therefore in the current experiment, the HRT in the three bioreactors was 14 days. In BRT1 (Bioreactor sacrificed earlier), ORP in the Postgate B phase (with the continuous flow of PB) was between -250 mV and -300 mV and after introduction of STPW it dropped to -350 mV. In BRT2 and BRT3 (Bioreactors sacrificed later), ORP was always below -300 mV.
except on day 374 that the ORP jumped to -280 mV in BRT3 (Figure 3.1-B). Dissolved oxygen (DO) in the bioreactors was always below 1 mg/L during the operation except on day 374 that it jumped to 5.7 mg/L and 4.7 mg/L in BRT2 and BRT3 respectively (Figure 3.1-C).

Conductivity of the bioreactors was very high right after the introduction of STPW (12.67-13.14 mS/cm) but it dropped sharply to 4 in 129 days and it was fluctuating between 1.45-2.6 mS/cm for the rest of operation (Figure 3.1-D).

All three bioreactors removed sulphate from 600 mg/L in STPW to concentrations well below 100 mg/L in the 200 days after their start up (Figure 3.2). BRT1 was sacrificed after 159 days from the start of the bioreactor but BRT2 and BRT3 continued their operation until day 430. In these two bioreactors the effluent sulphate increased over 100 mg/L and fluctuated between 100-200 mg/L after day 230. This trend continued in BRT3 until the last day of operation; However in BRT2, after day 270 sulphate dropped to lower than 100 mg/L again and it stayed below 100 mg/L for the rest of operation (Figure 3.2). Sulphide concentration in the bioreactors was initially 14 mg/L (Figure 3.3). In BRT1 it gradually increased to 55.60 mg/L. In BRT2, sulphide increased with a steep slope to 252.15 in day 91 but it decreased very quickly to 24 mg/L on day 105 and was below 60 mg/L until day 271. After that it started to increase and fluctuated between 36-130.5 mg/L. Sulphide in BRT3 was always below 70 mg/L. Comparing the change in sulphate concentration with the change in sulphide concentration shows that in general these two parameters were changing in the opposite directions. When sulphate increased, sulphide decreased and vice versa.

At the beginning of the STPW phase (start of the continuous flow of STPW) DOC was 2825.83 mg/L, 2043.33 mg/L and 2570 mg/L in BRT1, BRT2 and BRT3 respectively but it decreased sharply until day 129 and after that it continued to change at a slower rate. DOC was changing with a sinusoidal trend until day 271, after which DOC did not change considerably (Figure 3.4). The same trend was observed for the total dissolved carbohydrates. Initially there were 39.65, 23.06 and 30.90 mg-glucose/L of carbohydrates
in BRT1, BRT2 and BRT3. These concentrations dropped with time following a sinusoidal trend (Figure 3.4). Concentration of total dissolved proteins in all the bioreactors was less than 20 mg/ml all the time.

Selenium was removed from initial concentration of 20.3 µg/L to less than 0.2 µg/L in the effluent (Figure 3.5).

### 3.3.2.2 Operation of BRTT (50:20:30 of woodchips:hay:manure) bioreactors

All three bioreactors removed sulphate from 600 mg/L to lower than 100 mg/L all over their operation life (Figure 3.10). BRTT1 and BRTT2 were sacrificed after 159 days and BRTT3 continued its operation until day 430. Sulphide concentration in BRTT1 and BRTT2 was always below 15 mg/L; However in BRTT3 sulphide was fluctuating all the time between concentrations as low as 1 mg/L and as high as 95 mg/L (Figure 3.11).

In the beginning of the STPW phase, DOC was 4000 mg/L, 5700 mg/L and 5300 mg/L in BRTT1, BRTT2 and BRTT3 respectively. DOC decreased to about 300 mg/L in BRTT1 and BRTT2 until day 144 after which it increased to 1400 mg/L on day 159. In BRTT3 DOC dropped down to about 650 mg/L in 30 days. Then it jumped to 1700 mg/L on day 130, after it started to decrease continuously until the last day of operation (Figure 3.12). The sinusoidal trend observed in the BRT (40:30:30 of woodchips:hay:manure) bioreactors was not observed in BRTT3 (50:20:30 of woodchips:hay:manure). The carbohydrates in BRTT1 and BRTT2 were about 200 mg-Glucose/L at the time the STPW started to flow through the bioreactors and they dropped very rapidly to about 53 mg/L on day 159. BRTT3 started with a lower carbohydrate concentration (71 mg-Glucose/L) and it continuously decreased with a sinusoidal pattern during its life time (3.12). Proteins were present only in negligible concentrations in all the BRTT bioreactors. Selenium was removed from 17 µg/L to concentrations as low as 0.1 µg/L (Figure 3.13).
3.3.3 Organic Matter Degradation

3.3.3.1 Degradation of organic materials in BRT (40:30:30 of woodchips:hay:manure) bioreactors

BRT1 operated for 159 days. During this time it was in its successful operation phase and was removing sulphate very effectively to concentrations well below 100 mg/L. Therefore it was sacrificed to capture the microbial community in the successful operational phase and to measure the change in the composition of organic material. Bioreactors BRT2 and BRT3 were sacrificed at day 455. Efficiency of sulphate removal in BRT2 started to decline on day 229. BRT3 continued removing sulphate to less than 100 mg/L until day 423. On day 455 sulphate removal efficiency had declined in BRT3 as well.

At the time of sacrificing, the contents between each two ports were analyzed both for the characteristics of the solids and the quality of pore water. Figure 3.6 illustrates the changes in the ash, neutral detergent soluble and non-soluble content of the organic material in the three bioreactors. The NDNS content of the Time-Zero sample (BRT0) was 0.89±0.00 g/g-WNS. The average NDNS contents of all samples from BRT1 (bioreactor sacrificed earlier), BRT2 and BRT3 (bioreactors sacrificed later) were statistically significantly lower than BRT0. BRT1-P8, BRT1-P78, BRT1-P67 and BRT1-P56 were higher in NDNS than the same ports in either BRT2 or BRT3. The ash content in the bioreactors was changing as well, with BRT0 having the lowest ash, as opposed to BRT2 and BRT3, which had the highest ash contents. Some of the ash contents of the solids between ports of BRT1, BRT2 and BRT3 were statistically significantly different from the ash content of BRT0. For instance P8, P78, P67 and P45 of BRT1 had higher ash contents than BRT0 (P=0.0041, P<0.000, P<0.0001 and P<0.0001 respectively). Ports P56, P67 and P8 of BRT2 and BRT3 were also higher than BRT0 in ash content (P<0.0001). Comparing the ash contents of BRT1 with BRT2 and BRT3 shows that only BRT2-P8, BRT2-P56, BRT2-P67 and BRT3-P56 are statistically significantly different (higher) from their counterpart.
samples in BRT1 \( (P<0.0001, \ P=0.0028, \ P=0.001 \ \text{and} \ P=0.0229) \). In addition, the ash content in the higher ports (P45 and P34 and P23) of all bioreactors was lower than the ash content in the lower ports near the influent.

NDNS in the initial sample contained 0.55 ± 0.01 g/g-OM of holocellulose and 0.20±0.00 g/g-OM of lignin and 0.17±0.02 g/Other NDNS/g-OM (Figure 3.7). Holocellulose was composed of 0.35±0.01 g/g-OM \( \alpha \)-cellulose and 0.17±0.02 g/g-OM hemicellulose, and 0.1376 g/g-OM other holocelluloses such as pectin. The samples in the lower sections of BRT1 (P8, P78, P67), BRT1_P45, and all samples in BRT2 and BRT3 were statistically significantly lower in holocellulose than BRT0. However only BRT2 and BRT3 had a statistically significantly lower \( \alpha \)-cellulose than BRT0. The \( \alpha \)-cellulose content of BRT1 was comparable with BRT0. Hemicelluloses in BRT1 are comparable to the hemicellulose content of BRT0. Only BRT1_P45 had a statistically significantly lower hemicellulose than BRT0 \( (P<0.0001) \). All the samples of BRT2 and BRT3 have significantly lower hemicellulose than BRT0 except BRT3_P45 and BRT3_P34. The OM in the longer running columns, BRT2 and BRT3, contained proportionally lesser amounts of hemicellulose than was found in the shorter duration column BRT1.

BRT3_P34 and BRT3_P45 had significantly higher amount of lignin than BRT0 \( (P=0.0005 \ \text{and} \ P=0.0012) \). The lignin content of other samples of BRT1, BRT2 and BRT3 were not different from that in BRT0. Results of Tukey test confirms that there is not a statistically significantly difference between BRT1 and BRT0. Only a few samples in BRT2 and BRT3 had a lower lignin content than BRT0.

3.3.3.2 Degradation of organic materials in BRTT (50:20:30 of woodchips:hay:manure) bioreactors

Bioreactors BRTT1 and BRTT2 were both successfully removing sulphate to 15 mg/L until day 159. The two bioreactors were sacrificed on day 159 in order to be able to compare their performance with bioreactor BRT1. Bioreactor BRTT3 operated until day 430 and it was removing sulphate to less than 100 mg/L. Although BRTT3 was not in its decline phase yet, it was sacrificed on day 430 to make comparison
with bioreactors BRT2 and BRT3 easier. At the time of sacrificing, the contents between each two ports were analyzed both for the characteristics of the solids and the quality of pore water.

Figure 3.14 illustrates the changes in the ash, neutral detergent soluble and non-soluble content of the organic material in the three bioreactors. The results show that the ash content in P78 and P8 of BRTT3 were statistically significantly higher than the ash content in BRTT0 ($P=0.0002$ and $P=0.0199$ respectively). Ports P8 through P45 of BRTT3 had a statistically significantly higher Ash than the same ports of BRTT1 ($P<0.0001$). The ash content of ports P8, P67 and P56 of BRTT3 were significantly different from the ash content of the same ports in BRTT2 ($P<0.0001$, $P=0.0003$, $P=0.0027$). The NDNS content of the Time-Zero mixture of organic materials used in these bioreactors (BRTT0) was $0.65\pm0.00$ g/g-WNS ($0.96\pm0.02$ g/g-OM). This value increased significantly in all BRTT1, BRTT2 and BRTT3. BRTT3-P56 and BRTT3-P8 had statistically significantly lower NDNS than the same ports in BRTT2 ($P=0.0002$ and $P=0.0233$). The NDNS in BRTT1 and BRTT2 were not significantly different.

NDNS in the Time-Zero sample contained $0.62\pm0.02$ g/g-OM of holocellulose and $0.23\pm0.04$ g/g-OM of lignin (Figure 3.15). Holocellulose was composed of $0.38\pm0.01$ g/g-OM $\alpha$-cellulose and $0.20\pm0.01$ g/g-OM hemicellulose. The rest were other holocelluloses such as pectin. Holocellulose in BRTT1, BRTT2 and BRTT3 were comparable. Only BRTT1-P56 had a significantly higher holocellulose than BRTT2-P56 ($P=0.0032$). $\alpha$-cellulose in P8, P67 and P56 of BRTT1 were significantly lower than BRTT0 ($P=0.0006$, $P=0.0052$, $P=0.0063$). Only P45 of BRTT3 had significantly lower $\alpha$-cellulose than the same section of BRTT2 ($P=0.0093$). All samples in BRTT3 had lower $\alpha$-cellulose than BRTT1 with P45 and P78 being significantly lower than BRTT1 ($P=0.0002$, $P=0.0093$). All samples in BRTT3 had a significantly lower $\alpha$-cellulose than BRTT0 ($P<0.0001$). Hemicellulose values in BRTT1, BRTT2 and BRTT3 were statistically significantly different from BRTT0; BRTT3_P78 and BRTT3_P45 ($P=0.0009$, $P=0.0049$) had a statistically significantly lower hemicellulose than BRTT1-P78 and BRTT1_P45; However hemicellulose in BRTT3 were comparable to BRTT2. Lignin content had not changed
significantly in the BRTT bioreactors. Lignin in all the three bioreactors was higher than in BRTT0. Ports P8, P78, P56 and P45 in BRTT3 had a lower lignin than the same ports in BRTT1 ($P=0.0033$, $P=0.0016$, $P=0.0016$, $P=0.0094$). Ports P45 and P56 in BRTT3 had a lower lignin than the same port in BRTT2 ($P=0.0081$, $P<0.0001$).

### 3.3.4 Comparison of Composition of Organic Materials in BRT and BRTT Bioreactors

Figure 3.17 compares the ash, neutral detergent soluble and neutral detergent non-soluble contents of BRT1, BRTT1 and BRTT2, which were sacrificed after 159 days (Young bioreactors). The ash content of BRTT0, the initial sample from BRTT1 and BRTT2 was significantly higher than the ash content of the initial sample in BRT1 (BRT0); However the ash content in the samples of the BRT1, BRTT1 and BRTT2 after 159 days were comparable. One of the factors that contributed to ash in the bioreactors was formation of iron sulphide. In BRT1, average sulphide concentration over 159 days was 88 mg/L. In BRTT1 and BRTT2 the average sulphide concentrations were 22 and 14 mg/L respectively. Lower sulphide concentrations in BRTT1 and BRTT2 could result in lower iron sulphide formation and therefore the ash generated in some ports of BRTT1 and BRTT2 were substantially lower than in BRT1.

NDS in BRTT0 was substantially higher than BRT0 (Figure 3.17). This shows that the organic mixture in the BRTT bioreactors was less recalcitrant than the organic mixture in BRT bioreactors. After 159 days NDS in BRT1 was slightly more than NDS in BRT0, which means that recalcitrant materials were degraded down to more easily degradable materials. In BRTT1 and BRTT2 NDS after 159 days was lower than BRTT0. The reason could be that recalcitrant components in BRTT bioreactors were not degrading, or the rate of consumption of NDS in these bioreactors was higher than the rate of degradation of recalcitrant components. This trend was observed after 455 days when BRT2 and BRT3 were sacrificed. NDS in the two bioreactors was still lower than in BRTT3 (Figure 3.18).
Figure 3.19 compares various components of organic mixtures in the BRT and BRTT bioreactors. According to this figure NDNS in most sections of BRT1 were comparable with NDNS in BRTT1 and BRTT2. BRT1_P45 had a significantly higher NDNS than BRTT1_P45 and BRTT2_P45 ($P<0.0001$). Also, BRT1_P56 had a significantly higher NDNS than BRTT2_P56 ($P<0.0001$). The difference in the NDNS content of the BRT and BRTT bioreactors becomes more significant in the older bioreactors BRT2, BRT3 and BRTT3. As Figure 3.19 shows, ports P45, P56, P67 and P8 of BRT2 and BRT3 all have a significantly lower NDNS than BRTT3_P45, BRTT3_P56, BRTT3_P67 and BRTT3_P8 ($P<0.0001$).

Holocellulose in ports P56 and P8 of BRT1 were significantly higher than the same sections of BRTT2 ($P=0.0011$ and $P=0.0062$ respectively); However holocellulose in P56 and P8 of BRT2 and BRT3 were significantly lower than the same sections of BRTT3. The young and old bioreactors had comparable content of $\alpha$-cellulose. Hemicellulose in BRT1_P56 and BRTT2_P56, BRT1-P67 and BRTT2_P67 were significantly different ($P<0.0001$, $P=0.0458$), with higher $\alpha$-celluloses measured in BRT1. The same trend was observed in the older bioreactors with Hemicelluloses in P56 and P8 of BRTT3 being significantly lower than in BRT2 and BRT3. In all BRT and BRTT bioreactors holocellulose, $\alpha$-cellulose and hemicelluloses decreased over time.

### 3.4 DISCUSSION

All bioreactors that were sacrificed early performed similarly despite their wood to hay ratio. They had similar overall sulphate removal rates (41 mg/L.day) until the last day of their operation. In the early time phase dissolved organic carbon in the hay-rich bioreactors was 20 to 50 percent more than in the wood-rich bioreactors. This was expected since the results of characterization assays on individual organic materials showed a significantly higher DOC in hay (Chapter 2-Table 2.4). Therefore hay-rich bioreactors
contained more easily degradable and labile components and a higher sulphate removal rate was expected in them. The similar sulphate reduction rate in bioreactors with either wood to hay ratio indicates that in the early time periods and in the presence of high concentrations of labile components rate of sulphate removal was zero order and independent of concentration of labile components.

In the longer running bioreactors, sulphate reduction of bioreactors with higher wood to hay ratio suddenly declined (37 mg/L.day) after 230 days. The sulphate concentration was especially high in one of the wood-rich bioreactors and was fluctuating all the time. At the same time, sulphide concentration in this bioreactor was lower. It is possible that oxygen was penetrating into this bioreactor, which resulted in re-oxidation of hydrogen sulphide to sulphate and therefore high sulphate concentrations. This is in spite of low dissolved oxygen concentrations measured in this bioreactor. It is likely that the penetrated oxygen has been consumed in the oxidation reaction. The bioreactor with higher hay to wood ratio continued to operate at the same SRR over its entire period of operation.

Pulles and Heath (2009) observed lag phases of 90-150 day duration in their bioreactors containing woodchips, grass cuttings, manure, chicken litter and sewage sludge, followed by a high performance phase of up to 240 days followed by a crash phase (sudden decrease in performance). During the crash phase sulphate reduction still took place although at a significantly lower rate (Pulles & Heath, 2009). In a bioreactor containing 61 percent leaf mulch and sewage sludge and 39 percent sawdust and sewage sludge sulphate removal rate decreased from 67 mg/L.day to 38 mg/L.day after 18 pore volumes (Waybrant et al., 2002). If the HRT was 15 days this would equate to 270 days. Thus all these studies are consistent in their observation of similar time periods at which presumably, the readily available material runs out and performance drops. This shows that successful reactor performance can be extended by adding more hay. Hay is high in protein, carbohydrates and nitrogen (Table 2.4-Chapter 2-Page 56), which makes it a suitable carbon source for sulphate-reducing bacteria.
The fiber contents (α-cellulose, hemicellulose and lignin) of organic materials in the lower parts (P8, P78, P67 and P56) of all the bioreactors were higher than the upper parts (P34 and P23) and were decreasing over time. This shows that biodegradation in the upper ports was not as intense and effective as it was in the lower ports of the bioreactors. One reason that could explain this observation is that sulphate concentration in P4 of all the bioreactors was lower than the concentration in lower ports. This could make SRB less efficient in reducing sulphate. As a result, the dissolved organic carbon in P4 was not consumed as much and it was accumulated. The increase in the concentration of DOC stopped other bacteria (hydrolyzing and fermenters) from degrading the organic materials (Davey & O'toole, 2000).

The time-zero wood-rich bioreactors contained 160 grams α-cellulose. This value decreased to 45 grams in long-running bioreactors. Therefore 70 percent of α-cellulose was degraded in these bioreactors after 455 days. In the time-zero hay-rich bioreactors α-cellulose was 110 grams, which decreased to 27 grams in the long-running bioreactor, which is equal to degradation of 77 percent of α-cellulose after 435 days.

Hemicellulose in the time-zero wood-rich bioreactors was 75 grams, which decreased to about 22 grams in the long-running bioreactors (71 percent biodegradation). The time-zero hay-rich bioreactors contained 57 grams hemicellulose. In the long-running bioreactor hemicellulose ranged 8 grams. This is equal to 86 percent biodegradation.

The results show that α-cellulose was degrading more in the hay-rich bioreactors. This was expected because lignin is known to be a barrier to degradation of α-cellulose (reference). The time-zero organic mixture in the bioreactors containing more hay had a substantially lower lignin content than the wood-rich bioreactors, which makes this organic mixture more easily degradable.

The ash content of the time-zero organic mixtures were 12.5 and 135 grams in the wood-rich and hay-rich bioreactors respectively. In the long-running wood-rich bioreactors ash content ranged 115 to 177
The ash content of the long-running hay-rich bioreactor was 154 grams. Since ash is not biodegradable it was not expected to see any changes in the ash content of the bioreactors over time. Therefore the extra ash measured in the bioreactors is coming from other sources. In these bioreactors hydrogen sulphide was generated as a result of metabolic activities of sulphate-reducing bacteria. Hydrogen sulphide likely reacted with iron in the synthetic tailings pond water and precipitated as iron sulphide, which contributed to ash in the bioreactors. Assuming that fifty percent of hydrogen sulphide dissociated to sulphide at neutral pH, the amount of hydrogen sulphide precipitated in the wood-rich and hay-rich bioreactors were 7 and 8 grams respectively; However the difference in the ash content of the bioreactors from time-zero to their last day of operation was more. It is possible that other types of precipitation reactions were taking place in the bioreactors, which contributed to ash. For instance, in these bioreactors iron can precipitate as iron oxyhydroxides (Johnson & Hallberg, 2005; Zagury et al., 2006; Martins et al., 2009).

Lignin in the wood-rich bioreactors was 88 grams initially. In one of the long-running bioreactors lignin had decreased to 55 grams, which is about 62% of the initial lignin. In the second long-running bioreactor lignin had increased to 93 grams. There is a substantial difference between the lignin content of the two long-running bioreactors. Since lignin cannot be generated, the high lignin content in one of the long-running bioreactors could be attributed to the experimental errors. In the hay-rich bioreactors lignin at time-zero was 2.4 grams, which had decreased to 1.75 grams (73 percent of initial lignin) in the long-running bioreactor. Lignin has a very complex molecule structure due to the cross linkages of phenyl propane units (Fengel & Wegener, 1989), which makes it very hard to break and biodegrade. In addition, biodegradation of lignin is an oxidative reaction and it can be limited in the anaerobic environments due to the fact that extracellular enzymes need oxygen to produce $\text{H}_2\text{O}_2$, which breaks down lignin. Therefore biodegradation of lignin in this study was unexpected. Ko et al. (2009) also observed lignin biodegradation in sulphate-reducing environment (Ko et al, 2011). They suggested that it is possible that hydrolysis of cellulose generates $\text{H}_2\text{O}_2$, which then is used for break down of lignin. Also, the woodchips
used in our experiments was partially degraded, which makes it easier for lignin to biodegrade in anaerobic environments (Thompson et al., 2001).

In the experiment by Wang et al. the degree of biodegradation of various wood species (2 by 5 cm) over 1400 days was measured in 8-L lab landfill reactors (Wang et al., 2011). The degree of degradation of cellulose, hemicelluloses and lignin varied by wood species. Red Oak, a hardwood, had the highest loss of cellulose and hemicelluloses (15% and 16% respectively). There was no lignin loss in Red Oak. In contrast, concentration of lignin had decreased in Eucalyptus and Spruce by 12% and 17% respectively. The length of the study by Wang et al. (2011) was 4.3 times longer than our experiment; However in our experiment after 455 days 70 and 80 percent of $\alpha$-cellulose and hemicellulose were degraded. The higher rate of degradation in our study could be due to the smaller size of particles (0.5 by 0.5 cm). Small particle size provides microorganisms with a higher surface area to attach and attack and makes the biodegradation more effective.

The hay-rich bioreactors were more effective for sulphate reduction and at the same time more biodegradation occurred in these bioreactors. If biodegradation continues with the same rate, it takes about 590 days for these bioreactors to run out of $\alpha$-cellulose; However, degradation of lignin was taking place more slowly and it takes 1430 days for lignin to degrade completely. Degradation of lignin was a slow process in these bioreactors. As a result, it is possible that sulphate removal efficiency drops in these bioreactors while lignin becomes their only carbon source.

In these experiments bioreactors BRT2 and BRT3 were running as duplicates and it was expected to have the same results for the pore water chemistry and organic material composition. Although these two bioreactors were behaving similarly, the results of the chemical analysis and organic matter composition were not exactly the same. As an example, sulphate concentration in BRT3 bioreactor was higher than sulphate concentration in BRT2. The same difference was also observed in the composition of organic materials. Neutral detergent non-solubles (NDNS) in most ports of BRT2 were higher than in BRT3.
Despite all the effort that was made to start-up the bioreactors with the same initial organic mixture and environmental factors (pH, dissolved oxygen,...) it is possible that the bioreactors started off with slightly different organic mixtures or the microbial community in the inocula were slightly different and these small differences led to more pronounced differences in the chemistry of pore water and the organic matter composition. In addition, it is likely that during the operation of these bioreactors small problems, such as penetration of oxygen, happened that interfered with their performance and led to different results.

In summary, the results of the experiments in this chapter helped to answer research questions 2 and 3 (Chapter 1-Page 19). The results of sulphate analysis in the pore waters of the bioreactors (Figure 3.2, Page 105 and Figure 3.10, Page 113) showed that the composition of organic materials in the bioreactors impacts their performance. The hay-rich bioreactors (BRTT bioreactors) with a higher ratio of cellulosic to readily available material performed better regarding removing sulphate to lower than 100 mg/L and were more reliable. These bioreactors did not enter crash phase throughout their life time. In contrast wood-rich bioreactors entered their crash phase after 200 days and were behaving erratically. These bioreactors were not reliable with regards to sustaining removal of sulphate to lower than 100 mg/L.

The results of this chapter also showed that recalcitrant components, namely α-cellulose and hemicellulose were getting decomposed, although with different rates. Rate of decomposition of α-cellulose was slow. Hemicellulose was getting biodegraded with a slow rate in the wood-rich bioreactors (BRT bioreactors). In contrast, hemicellulose in the hay-rich bioreactors (BRTT bioreactors) was getting decomposed faster. Lignin did not change significantly in neither wood-rich nor the young hay-rich bioreactors, which was expected since it is a recalcitrant component. The rate of decomposition of hemicellulose and α-cellulose can help to estimate the longevity of passive biochemical reactors.
Table 3.1: Experimental design of the bioreactors

<table>
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<th>Bioreactor</th>
<th>Composition of organic material</th>
<th>Inoculumn</th>
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</thead>
<tbody>
<tr>
<td>BRT1</td>
<td>40% dw woodchips + 30% dw hay + 30% dw cow manure</td>
<td>Org_Inoc1</td>
</tr>
<tr>
<td>BRT2</td>
<td></td>
<td>Org_Inoc2</td>
</tr>
<tr>
<td>BRT3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRTT1</td>
<td>20% dw woodchips + 50% dw hay + 30% dw cow manure</td>
<td>Org_Inoc3</td>
</tr>
<tr>
<td>BRTT2</td>
<td></td>
<td>Org_Inoc4</td>
</tr>
<tr>
<td>BRTT3</td>
<td></td>
<td>Org_Inoc5</td>
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Table 3.2: Composition of influent feed

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</thead>
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</tr>
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</tr>
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<td>MgSO$_4$</td>
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<td>FeSO$_4.7$H$_2$O</td>
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<tr>
<td>NH$_4$Cl</td>
<td>0.026</td>
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<tr>
<td>Sodium lactate</td>
<td>-</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.015</td>
</tr>
<tr>
<td>pH</td>
<td>7.5-8</td>
</tr>
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</table>
Figure 3.1: Change of A) pH B) ORP C) DO and D) Conductivity in BRT1, BRT2 and BRT3. Start of pumping of PB: Day 38; Start of pumping of STPW: Day 83
Figure 3.2: Change of Sulphate in BRT1, BRT2 and BRT3. Start of pumping of PB:
Day 38; Start of pumping of STPW: Day 83

Figure 3.3: Sulphide change in BRT1, BRT2 and BRT3
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Figure 3.5: Selenium concentration in BRT1, BRT2 and BRT3 bioreactors
Figure 3.6: The ash, Neutral Detergent Soluble and Non-Soluble Contents of BRT1, BRT2 and BRT3. NDNS and NDS are calculated as a percent of the water non-soluble materials with ash.
Figure 3.7: Comparison of NDF in BRT1, BRT2 and BRT3
Figure 3.8: Spearman correlation among chemical properties of pore water and solid characteristics of organic mixture in BRT bioreactors
Figure 3.9: Change of A) pH B) ORP C) DO and D) Conductivity in BRTT1, BRTT2 and BRTT3. Start of pumping of PB: Day 38; Start of pumping of SMD: Day 83
Figure 3.10: Sulphate change in BRTT1, BRTT2 and BRTT3

Figure 3.11: Sulphide change in BRTT1, BRTT2 and BRTT3
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Figure 3.19: Comparison of neutral detergent fiber in BRT and BRTT bioreactors
Figure 3.19 continued
CHAPTER 4

COMPARISON OF MICROBIAL COMMUNITY IN BIOREACTORS WITH TWO DIFFERENT WOOD TO HAY RATIOS

4.1 INTRODUCTION

In a passive bioreactor, treatment takes place as a result of synergistic relationships among various microbial groups. The organic materials are composed of various components with different degrees of biodegradability. The nature of the microbial community growing on the organic materials varies depending on the types of organic materials used and their characteristics (Hiibel et al., 2011; Ziganshin et al., 2011). The water-soluble components are easier to biodegraded and have a higher rate of biodegradation and therefore these components are consumed by microbes first. As these are consumed, the microbial community becomes deprived of the more available and easily degradable components. As a result the nature of the microbial community changes in order to accommodate bacteria that are able to biodegraded the more recalcitrant components. Knowledge about the types of microorganisms that grow in passive bioreactors and their correlation with the characteristics of the organic materials could help in choosing the right inoculum and/or nutrient amendments for these systems to avoid the slowdown phase of the bacterial degradation. In addition, information about the dynamics of microbial community and the impact on the performance of bioreactors is useful for troubleshooting of the bioreactors.

There is scant information regarding the microbial community in the passive bioreactors. In all the previous studies (Woese & Fox, 1977; Handelsman, 2005; Malik et al., 2008) molecular techniques such as denaturing electric gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism (T-
RFLP) and fluorescent in-situ hybridization (FISH) have been used for characterizing the microbial community. None of these studies have used pyro-sequencing, which is a next generation sequencing technique. In addition, the impact of type of inocula on the microbial community in the bioreactors has never been studied. Moreover, the correlation among the microbial community and the individual components of the organic materials have not been delved into.

In this research, six bioreactors were set up with two different wood:hay:manure mixtures: Mix1 contained 40\textsuperscript{\%}_dw woodchips, 30\textsuperscript{\%}_dw hay and 30\textsuperscript{\%}_dw manure. Mix2 was composed of 20\textsuperscript{\%}_dw woodchips, 50\textsuperscript{\%}_dw hay and 30\textsuperscript{\%}_dw manure. The bioreactors were sacrificed at two different times and the solid matrix inside them was used to characterize the microbial community and to measure the change in the properties of the organic materials. The DNA extracted from the solid contents was sequenced with the pyro-sequencing technique. DNA sequences were analyzed using Qiime pipelines. The impact of environmental factors as well as components of organic on the distribution of microbial community was studied using the Envfit script in R-Vegan. Finally the microbial community in the bioreactors with two different organic mixtures were compared.

4.2 MATERIALS AND METHODS

4.2.1 Polymerase Chain Reaction (PCR) and Pyro-Sequencing

To study the microbial community in the bioreactors, samples were prepared for pyro-sequencing. Genomic DNA of the solid samples after sacrificing was extracted using a MoBio Power Soil Isolation Kit (MO BIO Laboratories Incorporation, Carlsbad, CA, USA). Isolated DNA was subjected to polymerase chain reaction (PCR) amplification of the V6 to V8 variable region of the 16S rDNA gene. 12.5 µL of 2xPCR Master Mix (Fermentas Canada Incorporation, Burlington, ON), 10.5 µL of nuclease-
free water (Fermentas), 1 µL of genomic DNA (2 ng), and 0.5 µL of FLX Titanium amplicon primers 454T-RA and 454T-FB (20 pmol/µL) for a 25 µL PCR reaction was used. The primer sequences were 926f (aaactYaaaKgaattgacgg) and 1392r (acgggcgtgtgtRc), which amplify the V6 to V8 variable region of 16s rRNA. Primer 454T-RA had a 25 nt A-adaptor (CGTATCGC-CTCCCTCGCGCCATCAG), whereas primer 454T-FB had a 25 nt B-adaptor sequence (CTATGCGCCTTGCCAGCCGCT-CAG). Each sample contained a specific barcode, which consisted of 10 nucleotides in a row. PCR conditions were 95ºC for 3 min; 25 cycles of 95ºC; 30 s, 56ºC; 45 s, 72ºC; 90 s, 72ºC; 10 min; final hold at 4ºC. The PCR products were verified on a 1% agarose gel. PCR products were sent to the Macmaster University (Hamilton, Canada) for 454 pyrotag sequencing.

4.2.2 DNA Sequence Analysis

For processing and analyzing the pyrotag sequences, scripts included in the Qiime 1.5 package of commonly used bioinformatic programs was used (Caporaso et al., 2010). The pyrotag sequences were clustered using cd-hit (Li et al., 2001) into so-called operational taxonomic units (OTUs) based on 97% similarity. The sequences were checked for chimeras (using ChimeraSlayer) and the chimeric sequences were removed from further analysis. The 108-silva reference set was used as the database for taxonomic classification of representative sequences from the OTUs.

4.2.3 Statistical Methods

R-Vegan software (2.14.2) was used for the detrended correspondence analysis of the microbial community distribution and the relationship among the environmental factors (pH, DO, ORP, sulphate, sulphide), organic matter characteristics (lignin, α-cellulose, hemicelluloses) and microbial community. An OTU-heatmap was built using the heatmap package in R to show the OTU abundance in different samples.
4.3 RESULTS

4.3.1 Microbial Community

4.3.1.1 Microbial community in BRT (40:30:30 of woodchips:hay:manure) bioreactors, Time_Zero sample, inocula and cultures

The first step in analyzing the DNA sequences was to produce an OTU heatmap using the pipeline in Qiime. The resultant OTU heatmap was rarefied to depth of 5226. Therefore the samples that had an OTU count lower than 5190 were filtered from the OTU table. In total 20642, 25559 and 25639 reads were identified in the solid samples of BRT1, BRT2 and BRT3 respectively. These reads were taxonomically classified as 7% Archaea, 92% Bacteria and 1% Eukaryota in BRT1, 24% Archaea, 72% Bacteria and 4% Eukaryota in BRT2 and 33% Archaea, 64% Bacteria and 3% Eukaryota in BRT3. In the domain Archaea, the majority of the OTUs belonged to the phyla Euryarchaeota (100% in BRT1, 99.94% in BRT2 and 99.98% in BRT3). The dominant phyla in the domain bacteria belonged to Bacteroidetes, Firmicutes and Spirochaetes (Figure 4.1). Figures 4.2 and 4.3 present the OTU heatmaps for those OTUs representing higher than 20 percent of all reads, and those OTUs between 10 and 20 percent. In the BRT reactor, the most highly represented OTUs that account for 80% of all reads obtained from sequencing surprisingly were constrained to only a few taxonomic groups. According to these two Figures in the cellulose-degrading cultures CDBC1 and CDBC2, genera Bacteroides and Lactobacillus and families Pseudomonodaceae and Ruminococcaceae are the dominant. The same OTUs from these genera were also present in the organic mixture inocula Org_Inoc1 and Org_Inoc2 with the genus Bacteroides being the most dominant. In addition, genus Arenibacter had a higher presence in the Org_Inoc1 and Org_Inoc2 than in the CDB cultures. Some OTUs such as those affiliated with Pseudomonas Stutzeri and Lysinibacillus did not exist in the cellulose degrading cultures CDBC1 and CDBC2, but they were highly present in Org_Inoc1 and Org_Inoc2. After inoculation of the column organic materials with inocula
Org_Inoc1 and Org_Inoc2, a sample was taken before transferring the inoculated mixture into the columns (called Time_Zero mixtures). As Figure 4.3 shows OTUs affiliated with *Pseudomonas Stutzeri*, *Pantoea Sp ATY74, Pantoea Ananatis, Pseudomonodaceae, Stenotrophomonas* and *Lysinibacillus* had a strong presence in the Time_Zero samples; However most of these were absent from the final bioreactor samples upon sacrificing. OTUs affiliated with the family *Ruminococcaceae* that were highly represented in the cellulose-degrading cultures, were less predominant in the Time_Zero samples, while the Bacteroides-related OTUs were still highly represented.

Some of the OTUs are clustered together and form blocks on the heatmaps. For instance, *Spirochaeta Sp. Buddy* and three *Parabacteroides* related OTUs are clustered together with *Porphyromonadaceae* and a *Methanocorpusculum Labreanum Z*. They all form a block that is present in all the pore water and solid samples from the bioreactors but absent from the Time_Zero samples. The trend in *Methanocorpusculum*-related OTUs differs from the trends for the other OTUs in this block in that the *Methanocorpusculum* and *Lachnospiraceae* related OTUs have a higher presence in the solids samples (taken at the end of the run) while the other OTUs have a higher presence in the liquid samples (taken part way through the run). Except for the OTUs belonging to the families *Porphyromonadaceae* and *Lachnospiraceae* other OTUs (such as OTUs 3380, 1170 and 1371) in this block were present in neither the CDB cultures nor the inocula. A second block of OTUs consisted of two OTUs affiliated with the genus *Bacteroides* and the iron-reducing clone Cl-A12 that were clustered together. These OTUs were present in all the samples including the cellulose-degrading cultures and inocula; however they appear to predominate the liquid samples more. All the OTUs in the solid samples from BRT1 are less well represented in the solid samples from BRT2 and BRT3. This indicates that the microbial community changes over the lifetime of the bioreactors. Right below the second block is another block that consists of several OTUs classified as *Bacteroidetes* clustered with *Comamonadaceae*. These OTUs are present in the Org_Inoc samples as well as the liquid samples but they are only highly present in the solid samples from BRT1 and are sporadically present in the solid samples from BRT2 and BRT3. In contrast, two large blocks of OTUs
are highly present in the solid samples of BRT2 and BRT3 (Figure 4.3). These OTUs mainly belong to *Mollicutes* and methanogens such as *Methanoregula*, *Rs_E47 termite group*, *Methanobacterium Curvum* and *Methanosacrina Mazei Go*. Only a few of these OTUs, for instance the three later bacteria were present in the solid samples of BRT1 in lighter shades.

Proteobacteria-related OTUs comprised only 3.58%, 5.30% and 6.17% of the total reads in BRT1, BRT2 and BRT3 respectively. Figure 4.4 presents the highly represented Proteobacteria-related OTUs (i.e. those with reads each containing more than 2% of the total number of reads in the BRT samples). The first observation was that although Proteobacteria OTUs were highly present in the CDACs, the inocula and the Time_Zero samples, only a few OTUs such as those classified as *Pseudomonodaceae*, *Stenotrophomonas* and *Comamonadaceae* were still present in the solid samples. Three OTUs classified as *Desulfovibrio sp.*., *Desulfovibrio Idahoensis* and *Smithella sp.* clustered together and were more highly represented in the older reactors BRT2 and BRT3. Of these three OTUs, only one related to an uncultured *Desulfovibrio sp.* clone was present in the BRT1 solid samples; This phylogenetic study suggests that the majority of SRB in the BRT reactors belonged to the genus *Desulfovibrio*, although other genera such as *Desulfococcus*, *Desulfobulbus* and *Desulfobacterium* were also represented.

### 4.3.1.2 Microbial community in BRTT (20:50:30 of woodchips:hay:manure) bioreactors, Time_Zero sample, inocula and cultures

Pyrotag sequences were processed and analyzed in Qiime. Sequences that were more than 95% similar were clustered into an OTU and an OTU table was generated. Then the OTU table was rarefied with a depth of 4965. In total 14824, 14600 and 20222 OTUs were identified in the solid samples of BRTT1, BRTT2 and BRTT3 respectively. In BRTT1 there were 18.49% Archaea, 81.16% Bacteria and 0.35% Eukaryota; In BRTT2 there were 13.76% Archaea, 86.13% Bacteria and 0.11% Eukaryota and in BRTT3 20.59% Archaea, 79.31% Bacteria and 0.09% Eukaryota were present. In the domain Archaea, the majority of the OTUs belonged to the phyla Euryarchaeota (99.89% in BRTT1, 100% in BRTT2 and
The dominant phyla in the domain bacteria belonged to *Bacteroidetes, Firmicutes* and *Spirochaetes* (Figure 4.11). The rarefied OTU table was used to produce OTU heatmaps in R-Vegan 1.14.

OTUs higher than 20 percent and OTUs between 10 and 20 percent are shown in Figures 4.12 and 4.13 respectively. The first observation in these heatmaps is the type of species in the organic inocula (Org-Inoc 1, Org-Inoc-2 and Org-Inoc-3) and sulphate-reducing bacteria cultures (SRBC1 and SRBC2) are totally different from the species in the bioreactors. Also, solid samples contain different species than the liquid samples of the bioreactors. SRBC cultures contain large numbers of Iron-Reducing enrichment clone Cl-A12 (OTU-285), *Tumebacillus* (OTU-5777), *Coriobacteriaceae* (OTU-3549 and OTU-7364) and *Bacteroides Sp. 1_1_6* (OTU-5490). None of these species are present in the bioreactors. Some of these species are also present in the organic inocula. In addition, the organic inocula contain a *Terrestrial miscellaneous GP (TMEG)* species (OTU 179). Bioreactors have more diverse species than the cultures. Three OTUs of *Iron-Reducing enrichment clone Cl-A12* (OTUs 285, 499, 246) are amongst the highest present OTUs in the solid samples along with *Bacteroides* (OTUs 1687, 9602, 356) and *Ruminococcaceae* (OTU 493). Various species of methanogens are present in the solid phase of the bioreactors. For instance a *Methanosarcina mazei Go* (OTUs 173) coexists with a *Parabacteroides* and another OTU of *Methanosarcina Mazei Go* (OTU 439) is clustered with a *Spirochaeta*. There are two *Methanocorpusculum* OTUs, one (OTU 340) clustered with a *Lachnospiraceae* (OTU 658). A *Methanoregula* (OTU 341) is clustered with a *TSCOR003-O20* (OTU 292). In addition to methanogens, there are three OTUs of *Spirochaetes zuelzerea* (OTUs 227, 444, 2690), one *Spirochaeta sp. buddy* (OTU 480) and a bacteria from phyla *Spirochaetes* in the solid phase. In the liquid samples, bacteria from genera *Anaerofilum* (OTU 1876), *Bacteroides* (OTUs 9627 and 3648), *Roseburia* (OTU 417), family *Lachnospiraceae* (OTU 759), as well as species *Marinitoga Okinawensis* (OTU 278) are observed.

Proteobacteria compose only 1.17%, 1.19% and 7.35% of the OTU in BRTT1, BRTT2 and BRTT3 respectively. Figure 4.14 represents the Proteobacteria higher than 2% in the BRTT bioreactors. This
heatmap shows that the liquid samples from the bioreactors had the highest numbers of Proteobacteria. OTUs from genus *Entrobacter* (OTUs 9852, 4866 and 10090) had the highest number in the liquid samples, followed by OTUs of *Rheudopseudomonas Sp. CZ-1* (OTU 9974 and 10055) and an OTU belonging to genus *Acinetobacter* (OTU 8092). Proteobacteria were only sporadically present in the solid samples of BRTT1 and BRTT2; However BRTT3 contained various species of SRB, for instance *Desulfobulbus* (OTUs 306 and 351), *Desulfovibrio Idahoensis* (OTU 573), *Desulfovibrio Sp. X2* (OTUs 783, 794) and *Smithella* (OTUs 804, 638). An interesting observation is that neither SRBC_1 nor SRBC_2 contained sulphate-reducing bacteria. This is despite the fact that these two cultures were maintained in Postgate B and they were products of several transfers of SRB cultures in the lab. Also, the Time_Zero samples contained ample numbers of various species and genera of Proteobacteria. However, none of the OTUs were present in the organic cultures (Org_inoc1, Org_inoc2 and Org_inoc3) and SRB cultures (SRBC_1 and SRBC_2).

### 4.3.2 Environmental Effects on the Microbial Species

#### 4.3.2.1 Effect of environmental factors on the microbial community in BRT (40:30:30 of woodchips:hay:manure) bioreactors.

In order to find the relationship among the various OTUs observed in the BRT bioreactors and also to find out the environmental factors that affect their presence, different statistical tests were performed using the vegan package in R. Detrended correspondence analysis (DCA) was done to compare the microbial communities in the culture, inocula and bioreactor samples. The effect of environmental factors on distribution of the microbial communities was also tested using the envfit function in R (vegan). Figures 4.5 and 4.6 show the effect of DO, ORP, pH and conductivity on the presence and distribution of the microbes.
The first observation was that the taxonomic groups more strongly associated with BRT1 were very different from those associated with bioreactors BRT2 and BRT3. The microbial communities of samples within bioreactors clustered closely with each other. Of the measured environmental variables, conductivity had the largest effect on microbial community composition. The taxonomic groups *Bacteroides* (OTUs 3388, 123, 277 and 49), *Lachnospiraceae* (OTUs 1727), *Comamonadaceae* (OTU 1127) and *Ruminococcaceae* (OTU 1886) were found in samples with higher conductivities, such as the ones obtained from reactor BRT1. Whereas in samples with lower conductivities, from reactors BRT2 and BRT3, methanogen and planctomycete-related OTUs were more predominant. BRT1 had a higher DOC compared to BRT2 and BRT3. In addition to sulphate and sulphide, various components in DOC such as organic acids, fatty acids and alcohols contribute to conductivity.

For effective operation of the bioreactor (i.e. sulphate reduction from 600 to 100 mg/L), pH and ORP are also important parameters and they were expected to influence the microbial community composition. In the higher pH samples, predominantly represented were RS_E47_termite group (OUT 1515), *Bacteroidetes* (OTU 123), *Lachnospiraceae* (OUT 1727) related OTUs as well as various methanogens such as *Methanocorpusculum Labreanum Z* (OUT 1371), *Methanosarcina Mazei Go* (OTUs 4063), and *Methanobacterium Curvum* (OUT 74). Methanogens were the dominant group of microbes observed in the low-ORP sites, such as various samples of BRT2 and BRT3. Highly anaerobic conditions (none or close to zero dissolved oxygen) selected for different genera of methanogens as well as OTUs classified as *Mollicutes* (OTUs 983), *Rs_E47_termite group* (OTU 529) and *Porphyromonadaceae* (OTU 2796); however other microbial groups such as *Spirochaeta sp. Buddy* (OUT 311), *Parabacteroides* (OUT 1975), and *TSCOR003 O20* (OUT 910) were found where some oxygen was present. DOC, water-soluble carbohydrates and water-soluble proteins also impacted the type of microbial community in the bioreactors. Methanogens were more present in sites with higher concentrations of carbohydrates and proteins (Figure 4.7 and 4.8). At the time of sacrificing, all the pore water samples from BRT3 contained a high concentration of carbohydrates compared to the samples from BRT2 and BRT1. In Figures 4.7 and
4.8 it can be seen that carbohydrates are increasing in the direction of the BRT3 samples. Higher soluble protein concentrations were correlated with other OTUs classified as *Pseudomonas Stutzeri* (OUT 1745), *Stenotrophomonas* (OTU 812), *Bacteroidetes* (OTU 123) and *Pantoea Sp. ATY 74* (OTU 1184). Figures 4.9 and 4.10 indicate that OTUs classified as *Ruminococcaceae* (OTU 329), *Spirochaeta Sp. Buddy* (OTU 311) and the *Rs. E47 termite group* (OTU 1515) were all found in the samples from older bioreactors BRT2 and BRT3 that had lower fractions of NDNS, α-cellulose and hemicelluloses. These are all hydrolyzing and fermenting bacteria that biodegrade recalcitrant materials such as holocelluloses.

4.3.2.2 **Effect of environmental factors on the microbial community in BRTT (20:50:30 of woodchips:hay:manure) bioreactors.**

Figures 4.15 and 4.16 show the effect of DO, ORP, pH and conductivity on the presence and distribution of the microbes contributing to higher than 20 percent and between 10 and 20 percent of OTUs in the BRTT bioreactors. BRTT1 and BRTT2 are closer to one another and more distant from BRTT3. pH and DO were changing in completely opposite directions. In general pH in BRTT1 was lower than BRTT2 and closer to BRTT3. Species such as *Methanosarcina mazei G01* (OTUs 479, 173), and genera *Lachnospiraceae* (OTU 759) are seen in the sites with higher pH and lower DO (BRTT2). This is expected since methanogens are strict anaerobes and can’t tolerate oxygen. In addition, this shows that methanogens prefer pH closer to 7. On the other hand, species such as *Spirochaeta Zuelzerea* (OTUs 227, 2690, 319) and *Entrobacter* (OTU 9852) are present in zones with higher DO, which implies that these species can tolerate some oxygen and can survive in pH lower than 7. ORP changes with a small rate among the sites and the lowest ORP were measured in BRTT3. Conductivity had the most substantial change amongst various samples and bioreactors, with the conductivity in BRTT1 and BRTT2 being the highest. Microbial species such as methanogens are present in the sites with the highest conductivity (5-8.25 mS/cm), whereas bacteria such as *Spirochaeta zuelzerea* (OTUs 2690, 319 and 227) and *Coriobacteriaceae* (OTU 3549) are observed in the site with low conductivity (1.3-1.4 mS/cm).
DOC, water-soluble carbohydrates and water-soluble proteins also impacted the type of microbial community in the bioreactors. DOC was substantially higher in BRTT1 and BRTT2. The results of the Envfit test shows that methanogens grow better in sites with higher concentrations of DOC. Carbohydrates were more effective in promoting growth and activity of *Fibrobacter* (OTU 593), *Bacteroides* (OTU 9602 and 1687) and *Entrobacter* (OTUs 10090 and 9852). *Spirochaeta zuelzerea* (OTU 2690, 227 and 319) can metabolize on more recalcitrant materials (Figures 4.17 and 4.18). This result is also confirmed in Figures 4.19 and 4.20. These species are all observed in sites with higher NDNS. Lignin has the least rate of change among the bioreactors and is slightly higher in BRTT1. Among the holocellulose constituents, hemicelluloses has the fastest change with higher hemicelluloses occurring in BRTT1 and the lowest in BRTT3. This confirms the higher degree of degradability of hemicelluloses than α-cellulose.

### 4.4 COMPARISON OF MICROBIAL COMMUNITY IN BRT (40:30:30 OF WOODCHIPS:HAY:MANURE) AND BRTT (50:20:30 OF WOODCHIPS:HAY:MANURE) BIOREACTORS

Figure 4.21 demonstrates the heatmap of microbial community in the young BRT and BRTT bioreactors that were sacrificed after 159 days. The population of some species of methanogens were higher in BRTT1 and BRTT2. For instance, *Methanosarcina mazi G01* (OTUs 143 and 3740) and *Methanocorpusculum labreanum Z* (OTUs 369 and 247) were more plentiful in the BRTT bioreactors. Bacteria belonging to the phyla *Fibrobacter* (OTUs 494, 325 and 871) are highly represented in BRTT1 and BRTT2; However their population is very small in BRT1. The population of bacteria belonging to the phyla *Spirochaetes* were substantially higher in the BRTT bioreactors. Species *Spirochaeta zuelzerea* (OTUs 366, 198 and 375) and *Spirochaeta sp. buddy* (OTU 212) and a bacteria belonging to the family *Spirochaetes* (OTU 361) are all present in higher numbers in BRTT1 and BRTT2. In contrast bacteria
belonging to the phyla Bacteroidetes have higher numbers in BRT1. For instance bacteria from genus Bacteroides (OTUs 1647, 686 and 180) are more abundant in BRT1. Some Ruminococcaceae, for instance OTU-1900, OTU-2960 are plentiful in BRTT1 and BRTT2; However they are only present in small numbers in BRT1. Mollicutes (OTUs 3002, 408) are only present in BRT1.

The microbial community in the older bioreactors BRT2, BRT3 and BRTT3 are more similar (Figures 4.23 and 4.24). Most of the species present in BRT1 are also present in BRTT1 and BRTT2 and their number of reads is similar. There are a few exceptions. Bacteria belonging to genus Treponema (OTUs 192 and 926) are more abundant in BRTT3. Some methanogens, namely Methanoregula (OTU 208), Methanosarcina barkeri (OTU 476) and Methanopaherula palustris (OTU 757) are also more abundant in BRTT3.

4.5 DISCUSSION

The majority of bacteria in the bioreactors were classified in the phyla Firmicutes, Bacteroidetes and Spirochaetes. Organic mixtures in the bioreactor are composed of various components, namely cellulose, hemicelluloses, lignin, sugars and proteins. These components are degraded and consumed by different groups of microbes. Hydrolyzing bacteria are responsible for breaking down recalcitrant components such as cellulose and lignin to smaller molecules. These bacteria mainly belong to phyla Spirochetes and Firmicutes (Burrell et al., 2004; Dröge et al., 2006; Fernando et al., 2010). Ruminococcaceae, Spirochaeta zuelzerea, Spirochete Sp. buddy, Treponema and Rs E47 Termite group are examples of species belonging to the phyla Spirochaetes. On the other hand, bacteria belonging to phylum Bacteroidetes are able to break simpler molecules such as sugar and amino acids down to organic acids, alcohols and H₂. Examples of bacteria belonging to this group are Parabacteroidetes and Mollicutes (Keceli & Miles, 2002).
One important outcome of this study was the significance of type of inoculum used in the passive bioreactors. The results of pyro-sequencing in the bioreactors showed that the microbial communities that developed in the bioreactors after start up became more diverse than those in the sulphate-reducing and cellulase active starting cultures and the bioreactor inocula. In particular OTUs related to *Parabacteroides*, *Rs E47 termite group* and *Spirochaeta Zuelzerea* were more prevalent in the bioreactors than in the inocula. In contrast, OTUs related to *Lactobacillus*, *Pantoea* and *Coriobacteriaceae* were prevalent in the inocula and cultures but did not persist in the bioreactors. This shift in the bacterial community was due to the change in the organic acids, fatty acids and polysaccharides. Batch cultures were abundant in dissolved organic carbon, including sugars, proteins and carbohydrates and were predominant with the bacterial communities that were able to use dissolved organic components; whereas in the bioreactors most of the dissolved organic materials were washed out after start of the continuous flow. Therefore bacteria that were able to degrade less available components such as starches and cellulose grew and became prevalent in order to degrade the recalcitrant components and provide sulphate-reducing bacteria with available carbon. Therefore the right inocula must include bacteria that are able to degrade recalcitrant components, as well as sulphate-reducing bacteria.

Methanogens were highly represented in the bioreactors and they were more predominant in bioreactors that were sacrificed later. This was unexpected since it was believed that SRB out-compete methanogens when there is ample sulphate. Species from genera *Methanobacterium*, *Methanoregula*, *Methanocorpusculum* and *Methanosarcina* were predominant in the bioreactors. Methanobacterium curvum belong to family Methanobacterium and use H$_2$ as substrate. Methanocorpusculum and Methanoregula consume alcohols and formate in addition to H$_2$ for methane production. It is possible that in the metabolic pathways of bacteria present in the bioreactors H$_2$ have been produced, which has encouraged growth and activity of methanogens to consume hydrogen.
Sulphate reducing bacteria comprised a small fraction of the microbial community. This is in agreement with the results of Hiibel et al. (2011). They found 2-5% SRB and 25-30% fermentative bacteria in bioreactors containing hay/pine woodchips, and hay/corn stover (Hiibel et al., 2011). Similarly in a study of microbial community in a series of teabags containing silage, compost and molasses Schmidtova and Baldwin (2010) found that Bacteoidetes and Firmicutes were the dominant phyla (50-60% and 20% respectively). In another study by Pruden et al. (2007) they found that sulphate-reducing bacteria comprised a small fraction of the bacterial community of a compost bioreactor (Pruden et al., 2007). According to Logan et al. (2005) sulphate-reducing bacteria rely on fermentative and hydrolyzing bacteria for break down and consumption of the lignocellulosic materials, which justifies the higher presence of bacteria other than SRB in lignocellulosic bioreactors (Logan et al., 2005). In a study of the microbial community in the cow rumen, they found out that the majority of the bacteria belonged to the phylum Firmicutes, Bacteroides and Spirochaetes (Fernando et al., 2010).

Some OTUs that were observed in the pore water samples taken from the bioreactors before introduction of synthetic tailings pond water and Postgate B were not represented in the solid samples. For instance, sulphate-reducing bacteria and methanogens were not observed in the pore water samples; However, Methanocorpusculum labreanum Z was an exception and was represented in the pore water samples as well as solid samples. Wang et al. (2011) in experiments conducted on a two-stage anaerobic bioreactor hydrolyzing grass silage observed different microbial population in the pore water samples and the solid samples (Wang et al., 2011). Planktonic OTUs in the pore water are those that do not form a granule or are loosely attached to the solids. On the other hand, microbes such as methanogens and sulphate-reducing bacteria that are well known for forming granules are only present in the solid samples (Hulshoff Pol et al., 1983; Omil et al., 1997a; Gonzalez-Gil et al., 2001). Some OTUs related to genera Bacteroides and Parabacteroides as well as OTUs related to Iron-reducing clone Cl A12 species were highly represented in the pore water samples as well as solid samples from the bioreactors sacrificed earlier, but were not represented in the solid samples of the bioreactors that were sacrificed later. Pore
water samples were taken at earlier times during the bioreactors’ operation. Therefore some of the differences among the bacterial communities in these samples and solid samples could be associated to the operation time.

The microbial community in the bioreactors shifted over time. In the older bioreactors methonegens became more prevalent. In the two older wood-rich bioreactors Firmicutes and Spirochaetes were more predominant than Bacteroidetes. In the old hay-rich bioreactor, Bacteroidetes did not change significantly; However Firmicutes became less prevalent and Spirochaetes was more predominant. This was expected, since in the older bioreactors concentration of dissolved organic carbon had decreased, which leads to diminishing of Bacteroidetes that were feeding on the labile components. At the same time $\alpha$-cellulose and hemicelluloses had decreased as well, which shows that bacteria feeding on recalcitrant components were active in the bioreactors. These results emphasis the need for using inocula containing Spirochaetes and Firmicutes for inoculation of passive bioreactors, which can improve performance of bioreactors by continuously providing bacteria with labile components and increasing rate of sulphate removal.

Environmental factors such as pH, oxidation-reduction potential and dissolved oxygen impact growth and metabolic activities of microorganisms in the bioreactors. This is also true about the characteristics of the organic mixtures. The decorespondence graphs and Envfit () test are used to statistically correlate distribution of microbial community to the environmental factors and organic matter characteristics. To the author’s knowledge these statistical methods have not been used for the study of passive bioreactors previously. These information are specially useful in understanding the mechanism of treatment and troubleshooting the bioreactors. For instance, in the wood-rich bioreactors OUT-1184 appears in sites with high lignin and hemicelluloses and protein (Figures 4.8 and 4.10). Therefore this OUT is possibly involved in degradation of hemicelluloses to soluble proteins. In the hay-rich bioreactors, OUT-1150 is possibly involved in degradation of $\alpha$-cellulose to glucose (Figures 4.15 and 4.17).
Some microbes in these systems co-occurred with other microbes. Methanogens formed clusters with bacteria from phyla Spirochetes. For example *Methanoregula* was clustered with *Treponema*, *Methaonsphaerula Palustris* was clustered with *Spirochetes* and *Methanosarcina mazei* Go co-occurred with *Spirochaeta*. This co-occurrence is due to the metabolic reliance of methanogens on *Spirochaetes*. In addition to co-occurrence of methanogens and *Spirochaetes*, other bacteria were clustered to each other as well. Species from phyla *Fibrobacter* and family *Ruminococcaceae* are known to degrade cellulose to smaller molecules such as formate, hydrogen and acetate (Flint *et al*., 2008). Bacteria from this family were clustered to *Roseburia*, which are known to break down pectins to butyrate (Duncan *et al*., 2012). Bacteria belonging to family *Fibrobacter*, co-occurred with *Acetanaerobacterium*, which are able to ferment mono, di- and oligosaccharides (Chen & Dong, 2004). *Spirochaeta Zuelzerea* and *Planctomycetes* also formed clusters in the bioreactor samples.

Many of the OTUs in the wood-rich (40:30:30 of woodchips:hay:manure) and hay-rich (50:20:30 of woodchips:hay:manure) bioreactors were closely related to those found in other systems with similar environmental conditions. For instance bacteria from family *Lachnospiraceae* were found in bioreactors treating wastewater containing lactate and sulphate (Zhao *et al*., 2008). OTU-329 and OTU-1886 both classified within the *Ruminococcaceae* family in the wood-rich bioreactors were both 96% similar to *Clostridium leptum*, an anaerobic butyrate-producing bacteria found in a bioreactor hydrolyzing grass silage (Wang *et al*., 2011). Grass silage is an organic material similar to hay except that it has undergone lactic acid fermentation. One *Bacteroides* related OTU (1321) was particularly predominant in the wood-rich bioreactors as well as the starting cultures and inoculum. This was 99% similar to the *Bacteroides Sp. 253c* found in the microbial community of gas pipelines (Zhu *et al*., 2003). Possibly presence of these *Bacteroides* in gas pipelines was to breakdown the complex carbohydrates such as ethane, propane and butane to simple organic acids to be used by SRB, which are known to be present if there is sulphur in the gas. In the same study several species of *Pseudomonas*, e.g. *Pseudomonas Stutzeri* were found. OTUs closely related to this species were also present in the cellulose-degrading cultures and organic inocula as
well as the time-zero samples, but they did not persist in the bioreactors. *Pseudomonas* species are often found in contaminated aquatic environments rich in soluble carbohydrates such as gasoline and solvents. They produce exopolysaccharides, which facilitate their attachment to other bacteria thereby forming a biofilm (Leahy et al., 1996; Page, 1996). Environmental clones closely related to OTU 1673 (taxonomically classified as uncultured *Planctomycete*), which were present in large numbers in the solid samples of old wood-rich bioreactors were found in a CSTR bioreactor that was treating an acetate-containing wastewater in low dilution rate of 0.025 d\(^{-1}\) (Shigematsu et al., 2003) as well as in an anaerobic bioreactor that was treating a wastewater containing acetate and ethanol (Siggins et al., 2012). It is possible that in the wood-rich bioreactors acetate and/or ethanol were produced as a result of metabolic activities of some bacteria present in them. OTU-289, *Methanocorpusculum Labreanum Z* in the hay-rich bioreactors was 100% and 99% similar to the *Methanocorpusculum* species found in a bioreactor digesting cattle manure and maize straw (Ziganshin et al., 2012). The samples from the middle layer of a compost bin containing swine manure and rice straw contained species 98% similar to OTU-417 (*Roseburia*) in the liquid sample of hay-rich bioreactors (Guo et al., 2007). Although compost is an aerobic process, the middle layers contain anaerobic zones that are similar to the bioreactor in this study and finding the same microorganisms in the two systems is expected.

A *Parabacteroides* related OTU, number 821, in the wood-rich bioreactors were closely related to *Porphyromonas Cangingivalis* species which are anaerobic cells that produce acetate, propionate, isobutyrate, butyrate and isovalerate by fermenting cooked meat (Collins et al., 1994). Another *Parabacteroides* related OTU, number 1170, in these bioreactors was similar to a bacterium found in the fungus garden microbiom (Suen et al., 2010). The genomic and physiological characteristics of this microbiom show that it has a high biomass-degrading capacity, which is similar to the bovine rumen. In the same study, they found *Pantoea* as the dominant genera, which had sequences to code for enzymes known to be involved in plant polymer degradation, including cellulases, β-galactosidases, chitinases, α-xylosidases and α-mannosidases (Collins et al., 1994). *Pantoea* sp. ATY74 was highly represented in the
cellulose-degrading cultures and inocula of wood-rich bioreactors. It is likely that Pantoea sp ATY74 in the cultures and inocula was involved in degrading hemicelluloses. Also, it is possible that Parabacteroides related OTUs in the bioreactors produced organic acids such as acetate, propionate and butyrate during their metabolic activity.

OUT-4549 (Coriobacteriaceae) in hay-rich (50:20:30 of woodchips:hay:manure) bioreactors was similar to an RFLP type found in the gut of termite Coptotermus formosanus. It was affiliated with the family Bacteriaceae. This termite has the ability to penetrate a variety of wood species (Shinzato et al., 2005). A consortium of bacteria was found in the gut of this termite with bacteria belonging to different phyla such as Bacteroidetes, Clostridiales, Bacilalles, Spirochaetes, Actinobacteria and Proteobacteria. Degradation and digestion of wood in the gut of these termites take place as a result of a symbiotic relationship among these different groups of bacteria. The similar anaerobic environment in the hay-rich bioreactors coupled with presence of wood chips in them makes them a suitable environment for the growth and activity of microorganisms similar to the termite gut. Species 99% similar to the Spirochaetes Zuelzerea (OUT-227) in these bioreactors was found in fuel cells that were using rice paddy field soil and cellulose as carbon source (Ishii et al., 2008).

The young wood-rich bioreactor (sacrificed early) was predominated with bacteria belonging to phyla Bacteroidetes. In contrast the two young hay-rich bioreactors were predominated by bacteria from phyla Firmicutes and Spirochaetes. Despite the difference in their bacterial community, all the young bioreactors performed similarly and had the same sulphate removal rate. Later on during the bioreactors’ operation the bacterial community in the two old wood-rich bioreactors became similar to the bacterial community in the old hay-rich bioreactor; However in spite of their similar microbial community, the sulphate removal rate in the old wood-rich bioreactors was lower than the old hay-rich bioreactors, which removed sulphate with the same rate as the two young hay-rich bioreactors. It is possible that wood-rich bioreactors that were sacrificed later would perform the same as old hay-rich bioreactors if bacteria from
Phyla Spirochaetes and Firmicutes were more represented in them initially. This is a very important conclusion confirming the importance of using the proper inocula containing bacteria that are able to degrade recalcitrant components.

The old wood-rich bioreactors (BRT2 and BRT3) were running as duplicates; however, their microbial community were not exactly the same as it was expected (Figure 4.12 and Figure 4.13). We can expect identical results in the duplicate-running bioreactors only when all the start-up and operating conditions are identical. The inocula (Org_inoc2 and Org_inoc3) had similar microbial communities, although different microbes were not represented the same in them. For instance, Corriobacteraceae (OTUs 3549 and 6589 in Figure ) were not represented the same. In addition, there were microbes such as OTUs 147 and 292 that were identified in Org_Inoc3 but were absent in Org_Inoc2 (Figure 4.13). These dissimilarities in the inocula have led to a dissimilarities in the bacterial community of the bioreactors during their operation.

In summary, the experiments in this chapter helped to identify the microbial community that grows in the passive biochemical reactors. The results of pyrosequencing showed that the type of microbes that grow in these bioreactors depends on the type of the organic materials and mixtures that are used in them. In the young bioreactors there was a sharp difference between the microbial communities that were identified in the bioreactors running on the two different mixtures (Figure 4.21). The reason was that the composition of organic materials with regards to the concentration of DOC, carbohydrates, α-cellulose and hemicellulose were different. The difference in the composition of organic materials faded in the old bioreactors (Figure 4.22) as a result of decomposition of α-cellulose and hemicellulose as well as consumption of DOC with microorganisms. Therefore, the microbial community became more similar in the old bioreactors with the two different mixtures.
Figure 4.1: Percentage of various phyla and bacteria in BRT1, BRT2 and BRT3
Figure 4.2: Logarithmic (Log 2) Heatmap of 95% OTUs chosen as those each containing more than 20% of total number of reads in bioreactors BRT1, BRT2 and BRT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 4.3: Logarithmic (Log 2) Heatmap of 95% OTUs chosen as those each containing 10%-20% of total number of reads in bioreactors BRT1, BRT2 and BRT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 4.4: Logarithmic (Log 2) Heatmap of Proteobacteria and Methanogens representing higher than 2% of total number of reads in bioreactors BRT1, BRT2 and BRT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Table 4.1: Resultant eigenvalues from the Envfit test on pH, DO, ORP and conductivity in R (Vegan)-Higher 20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r²</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.68713</td>
<td>-0.72653</td>
<td>0.3685</td>
<td>0.097</td>
</tr>
<tr>
<td>ORP</td>
<td>0.23432</td>
<td>0.97216</td>
<td>0.2802</td>
<td>0.168</td>
</tr>
<tr>
<td>DO</td>
<td>-0.34077</td>
<td>0.94015</td>
<td>0.2472</td>
<td>0.222</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.99331</td>
<td>-0.11547</td>
<td>0.9876</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 4.5: Impact of pH, ORP, DO and conductivity on the distribution of microbial community higher than 20% in BRT1, BRT2 and BRT3. The length of the vectors indicates the rate of change of the characteristics. The direction of the vector indicates the direction in which each characteristics increases.
Table 4.2: Resultant eigenvalues from the Envfit test on pH, DO, ORP and conductivity in R (Vegan)-10%-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r2</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.8677142</td>
<td>0.4970634</td>
<td>0.3755</td>
<td>0.071</td>
</tr>
<tr>
<td>ORP</td>
<td>0.0010443</td>
<td>0.9999995</td>
<td>0.6285</td>
<td>0.006</td>
</tr>
<tr>
<td>DO</td>
<td>0.2621381</td>
<td>0.9650304</td>
<td>0.3264</td>
<td>0.102</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.9900087</td>
<td>0.1410062</td>
<td>0.9822</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Figure 4.6: Impact of pH, ORP, DO and conductivity on the distribution of microbial community between 10%-20% in BRT1, BRT2 and BRT3. Vector length indicates characteristics rate of change. Vector direction shows characteristics increase.
Figure 4.7: Impact of DOC, carbohydrates and proteins on the distribution of microbial community higher than 20% in BRT1, BRT2 and BRT3. Vector length indicates characteristics rate of change. Vector direction shows characteristics increase.

Table 4.3: Resultant eigenvalues from the Envfit test on DOC, carbohydrates and proteins in R (Vegan)-Higher 20%
Table 4.4: Resultant eigenvalues from the Envfit test on DOC, carbohydrates and proteins in R

(Vegan)-10%-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r2</th>
<th>Pr(&gt;\r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC</td>
<td>0.962225</td>
<td>-0.272257</td>
<td>0.8312</td>
<td>0.001</td>
</tr>
<tr>
<td>Protein</td>
<td>0.398955</td>
<td>-0.916971</td>
<td>0.4449</td>
<td>0.042</td>
</tr>
<tr>
<td>Carbs</td>
<td>-0.044944</td>
<td>-0.998990</td>
<td>0.6634</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Figure 4.8: Impact of DOC, carbohydrates and proteins on distribution of microbial community representing 10%-20% in BRT1, BRT2 and BRT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.
Figure 4.9: Impact of NDF on the distribution of microbial community higher than 20% in BRT1, BRT2 and BRT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.5: Resultant eigenvalues from the Envfit test on NDF in R (Vegan)-Higher 20%
Figure 4.10: Impact of NDF on the distribution of microbial community representing 10%-20% in BRT1, BRT2 and BRT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.6: Resultant eigenvalues from the Envt test on NDF in R (Vegan)-10%-20%

|          | DCA1  | DCA2  | r2    | Pr(>|r|) |
|----------|-------|-------|-------|----------|
| NDNS     | 0.97677 | -0.21430 | 0.4849 | 0.040    |
| Holocellulose | 0.96682 | -0.25546 | 0.5733 | 0.017    |
| X..cellulose | 0.98632 | -0.16487 | 0.6147 | 0.011    |
| Hemicellulose | 0.92992 | -0.36777 | 0.7954 | 0.001    |
| Lignin   | 0.41048 | -0.91187 | 0.1040 | 0.524    |
Figure 4.11: Percentage of various phyla and bacteria in BRTT1, BRTT2 and BRTT3
Figure 4.12: Logarithmic (Log 2) Heatmap of 95% OTUs chosen as those each containing more than 20% of total number of reads in bioreactors BRTT1 and BRTT2 and BRTT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right. Sample names are column labels.
Figure 4.13 Logarithmic (Log 2) Heatmap of 95% OTUs chosen as those each containing 10%–20% of total number of reads in bioreactors BRTT1 and BRTT2 and BRTT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right. Sample names are column labels.
Figure 4.14: Logarithmic (Log 2) Heatmap of Proteobacteria and Methanogens representing higher than 2% of total number of reads in BRTT1, BRTT2 and BRTT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right. Sample names are column labels.
Figure 4.15: Impact of pH, ORP, DO and conductivity on the distribution of microbial community higher than 20% in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.7: Resultant eigenvalues from the Envfit test on environmental factors in R-Vegan-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r2</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.992490</td>
<td>0.122325</td>
<td>0.0084</td>
<td>0.978</td>
</tr>
<tr>
<td>ORP</td>
<td>0.069120</td>
<td>0.997608</td>
<td>0.1375</td>
<td>0.621</td>
</tr>
<tr>
<td>DO</td>
<td>0.513324</td>
<td>0.858195</td>
<td>0.0830</td>
<td>0.727</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.998405</td>
<td>-0.056466</td>
<td>0.6971</td>
<td>0.030</td>
</tr>
</tbody>
</table>
Figure 4.16: Impact of pH, ORP, DO and conductivity on the distribution of microbial community representing 10%-20% of total reads in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.8: Resultant eigenvalues from the Envfit test on environmental factors in R (Vegan)-10%-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r²</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>0.992490</td>
<td>0.122325</td>
<td>0.0084</td>
<td>0.975</td>
</tr>
<tr>
<td>ORP</td>
<td>0.069120</td>
<td>0.997608</td>
<td>0.1375</td>
<td>0.656</td>
</tr>
<tr>
<td>DO</td>
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<td>0.858195</td>
<td>0.0830</td>
<td>0.719</td>
</tr>
<tr>
<td>Conductivity</td>
<td>0.998405</td>
<td>-0.056466</td>
<td>0.6971</td>
<td>0.024</td>
</tr>
</tbody>
</table>
Figure 4.17: Impact of DOC, proteins and carbohydrates on the distribution of microbial community higher than 20% in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.9: Resultant eigenvalues from the Envfit test on pore water chemistry in R (Vegan)-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r2</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC</td>
<td>0.87330</td>
<td>0.48718</td>
<td>0.8525</td>
<td>0.004 **</td>
</tr>
<tr>
<td>Protein</td>
<td>0.93376</td>
<td>0.35790</td>
<td>0.9071</td>
<td>0.001 ***</td>
</tr>
<tr>
<td>Carbs</td>
<td>0.99809</td>
<td>0.06184</td>
<td>0.8181</td>
<td>0.005</td>
</tr>
</tbody>
</table>
Figure 4.18: Impact of DOC, carbohydrates, proteins on the distribution of microbial community between 10%-20% in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.10: Resultant eigenvalues from the Envfit test on pore water chemistry in R (Vegan)-10%-20%

<table>
<thead>
<tr>
<th></th>
<th>DCA1</th>
<th>DCA2</th>
<th>r2</th>
<th>Pr(&gt;r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOC</td>
<td>0.87330</td>
<td>0.48718</td>
<td>0.8525</td>
<td>0.003</td>
</tr>
<tr>
<td>Protein</td>
<td>0.93376</td>
<td>0.35790</td>
<td>0.9071</td>
<td>0.001</td>
</tr>
<tr>
<td>Carbs</td>
<td>0.99809</td>
<td>0.06184</td>
<td>0.8181</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Figure 4.19: Impact of NDF on distribution of microbial community higher than 20% in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.
Figure 4.20: Impact of NDF on distribution of microbial community between 10%-20% in BRTT1, BRTT2 and BRTT3. Vector length shows characteristics rate of change. Vector direction shows characteristics increase.

Table 4.11: Resultant eigenvalues from the Envfit test on NDF in R (Vegan)-10-20%

|           | DCA1  | DCA2  | r²    | Pr(>|r|) |
|-----------|-------|-------|-------|----------|
| NDNS      | 0.59636 | -0.80272 | 0.6595 | 0.038    |
| Holocellulose | 0.73943 | -0.67323 | 0.8702 | 0.001    |
| X..cellulose | 0.69471 | -0.71929 | 0.6967 | 0.021    |
| Hemicellulose | 0.95437 | -0.29861 | 0.8304 | 0.001    |
| Lignin    | 0.14556 | -0.98935 | 0.1838 | 0.551    |
Figure 4.21: Logarithmic (Log 2) Heatmap of microbial community higher than 10% in the young bioreactors BRT1, BRTT1 and BRTT2. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 4.22: Logarithmic (Log 2) Heatmap of microbial community representing 5%-10% of total reads in young bioreactors BRT1, BRTT1 and BRTT2. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 4.23: Logarithmic (Log 2) Heatmap of microbial community representing higher than 10% of total number of reads in old bioreactors BRT2, BRT3 and BRTT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right hand side. Sample names are column labels.
Figure 4.24: Logarithmic (Log 2) Heatmap of microbial community representing 5% - 10% of total number of reads in old bioreactors BRT2, BRT3 and BRTT3. OTU numbers and their Silva 108 assigned taxonomy are given on the right. Sample names are column labels.
CHAPTER 5

CONCLUSIONS, RECOMMENDATIONS AND FUTURE WORK

5.1 CONCLUSION

Passive treatment of mine drainage is gaining popularity due to their low cost and maintenance. These systems are being used in mine sites in the past 20 years but they are still like black boxes. Design of these systems is based on trial and error. Although there are numerous studies (Bechard et al., 1994; Prasad et al., 1999; Zagury et al., 2006; Martins et al., 2009) regarding the type of complex organic materials that can be used in these systems, the rate of degradation of these materials is still unknown. In addition, there is a lack of understanding regarding the microbial communities that are active in these systems. The research presented in this thesis have addressed the gap of knowledge in these areas. The experiments were designed to address the research questions that were raised in Chapter 1 (Pages 18 and 19).

Answer to research question 1:

Question 1: What start-up and operating conditions are required for a biochemical reactor with a wood, hay and manure mixture to remove selenium and reduce sulphate to less than 100 mg/L? What are the specific challenges with successful start-up of these types of reactors and strategies for overcoming these?

The experimental set-up in Chapter 2 was used to address the first research question. Monitoring and operation of the three bioreactors in this chapter resulted in valuable outcomes regarding start-up of
passive bioreactors. The following represents a list of that need to be met for a successful start-up and operation of these bioreactors:

1. The results of the experiments in Chapter 2 suggest that maintaining an anaerobic environment in the bioreactors is crucial. Penetration of oxygen into the bioreactors resulted in oxidation of sulphide back to sulphate. This phenomenon happened in the pilot anaerobic bioreactor in Mount Polley mine as well, with the effluent sulphate concentration increasing by time. This pilot bioreactor was covered with three meters of water on the top. It is likely that this depth of water was not enough to avoid oxygen penetrating into the bioreactor. De-aerating the influent mine drainage is one step that can be taken to keep the bioreactors void of oxygen. Although in an industrial scale this method may not be feasible. Another way of achieving an anaerobic environment is by covering the bioreactors with a hard cover such as soil or water. A cover of soil is more effective, since the oxygen can penetrate into the bioreactor through the water column. Also, adding a source of iron to scavenge sulphide will prevent its re-oxidation to sulphate.

2. Another environmental factor that affects the performance of SRB is pH. Sulphate-reducing bacteria perform best in a pH range of 6.5 to 7 (Odom & Singleton, 2011). Therefore it is essential to monitor pH of the bioreactor and adjust it to neutral range by adding sodium hydroxide to the influent. This is of utmost importance in the start-up phase to grow a strong SRB community.

3. It is also essential to inoculate bioreactors with healthy inocula containing both sulphate-reducing bacteria and cellulose-degrading bacteria. The inocula must contain at least 10 percent of the volume of the bioreactors.

4. Sulphate concentration in different ports of these bioreactors was monitored regularly. Each port represented a different hydraulic retention time. According to the results a retention time of 12-20 days was enough to reduce sulphate to less than 100 mg/L.
**Answer to research question 2:**

**Question 2:** How does performance of bioreactors with different ratios of organic materials compare during the active and decline phases of operation? Does the ratio of cellulosic to readily available material in the organics affect the start-up, operation and decline of these types of bioreactors?

The results of the experiments in Chapter 3 showed that performance of bioreactors was affected by the ratio of wood to hay. The hay-rich bioreactors removed sulphate to lower than 100 mg/L successfully throughout the life time of the bioreactors and had a more reliable and sustainable performance. In contrast, in the wood-rich bioreactors efficiency of sulphate removal dropped after 200 days and thereafter sulphate concentration was fluctuating.

**Answer to research question 3:**

**Question 3:** How does the composition of organic material change over time in these bioreactors? Is it possible to predict the longevity of these systems based on the change in organic matter composition?

Measurement of ash and neutral detergent fiber namely $\alpha$-cellulose, hemicellulose and lignin in wood-rich and hay-rich bioreactors at two different time points suggest that biodegradation of organic materials in these bioreactors was taking place. In the young wood-rich and hay-rich bioreactors $\alpha$-cellulose did not change substantially in the first 159 days but after 455 days it significantly decreased from the initial concentration before start-up of the bioreactors. This suggests that it takes longer than 160 days to measure a significant degradation of $\alpha$-cellulose. Hemicellulose did not change substantially in the young wood-rich bioreactors, which shows that degradation of hemicellulose in this mixture is slow. In the hay-rich bioreactors hemicellulose was significantly lower even in the young bioreactors. This shows that biodegradation of hemicellulose in this mixture was faster than the wood-rich bioreactors. This information is of value in choosing the type of organic materials for the passive treatment systems. To the author's knowledge this research is the only work that has studied the change in the composition of
organic materials in the passive treatment systems. A mixture that can sustain sulphate reduction over a long time period and results in effluent sulphate concentrations below 100 mg/L is desirable. Although in the time scale of industrial operations 455 days is considered short and data over a longer period is required to find suitability of potential organic materials for a passive bioreactor. However the rate of decomposition of organic materials can be used as a predictor to the longevity of the biochemical reactors.

**Answer to research question 4:**

**Question 4:** What specific microorganisms are present in these types of bioreactors? How do these microorganisms change with time and with the properties of organic mixtures? Does the microbial community composition provide information about the performance of these bioreactors?

The dominant phyla identified in hay-rich and wood-rich bioreactors were *Bacteroidetes*, *Spirochaetes* and *Firmicutes*, with the last two phyla being more dominant. This is in agreement with the results from other researches (Hiibel et al., 2011). Sulphate-reducing bacteria composed only a small fraction of the microbial community in the bioreactors. The microbial community in the young wood-rich bioreactors containing was distinct from the community in the hay-rich bioreactors. However in the older bioreactors the microbial communities in the bioreactors with two different mixtures were similar. This suggests that the factor time played an important role in the growth and distribution of microbial community. Different groups of bacteria require different environments to grow and metabolize. Bacteria such as *RS-E47 termite*, *Mollicutes* and *Ruminococcaceae* grow better on recalcitrant components, whereas others such as methanogens require readily available components. *Mollicutes* and methanogens require an anaerobic environment but *Ruminococcaceae* can tolerate some oxygen. The results of the experiments in Chapter 4 showed that there was a correlation between the type of bacteria growing in these bioreactors and the composition of organic materials in them. In the young bioreactors the contents of recalcitrant
components as well as the concentration of readily available materials (DOC) were different. This resulted in growth and development of a different microbial community in the hay-rich and wood-rich bioreactors.

The knowledge about the type of bacteria in the passive bioreactors, their environmental requirements and their metabolic is necessary for troubleshooting the bioreactors. This work has shed light on some of these information. But further research is needed to fully understand the kinetics and mechanism of treatment in the passive systems and to understand the relationship among groups of bacteria.

5.2 Recommendations to the practitioners

Passive biochemical reactors were used for treatment of mine drainage but their design and operation were based on trial and error due to lack of information and knowledge in the publications. The results of experiments in this research are valuable for the mining industry and practitioners.

Based on the results of this thesis, the organic mixture that is used in the biochemical reactors should be rich in dissolved organic carbon to provide microorganisms with enough energy for a long time period. At the same time, they should be low in lignin but contain enough degradable recalcitrant components, namely α-cellulose and hemicellulose. This will help the sustainability of degradation of these components since their biodegradation will continually provide the microbial community with energy and food source. In our experiments, hay was a suitable organic source and it is likely that using an organic mixture containing more hay would add to the longevity of a passive bioreactors. But at the same time we must keep in mind that the mixture used in the bioreactors must have enough porosity and hydraulic permeability that allows tailings pond water to pass through them with ease. A low porosity would lead to short-circuiting and drop in the sulphate-removal efficiency. Although organic materials such as woodchips are recalcitrant but having them in the mixture can add to the porosity and provide some structural support and biofilms can attach to their surface.
In the start-up phase of these bioreactors it is very important to have a strong bacterial community before continuous flow of mine drainage in them. In our experiments we added Postgate B to the bioreactors and left them in batch phase. The batch phase gave time to sulphate-reducing bacteria to feed on Postgate B and grow a strong community. Using Postgate B is not practical in real size treatment systems. But it is recommended to add a simple carbon source such as ethanol and lactate and leave the system in batch phase for a month. In addition, adding nutrients such as nitrogen, phosphorous and potassium are necessary to support bacterial growth and activity, especially if the organic materials used in these bioreactors are nitrogen limited.

The continuous flow of mine drainage must be started with a low flowrate and be increased stepwise. This prevents wash out of bacteria from the bioreactor. During the operation of the bioreactor it is crucial to monitor the pH regularly and adjust it to circum-neutral.

Care must be taken to prevent diffusion of oxygen into the bioreactors. Presence of oxygen in these bioreactors not only impacts biological activity of bacteria such as sulphate-reducing bacteria, it can oxidize hydrogen sulphide back to sulphate, which reduces the efficiency of sulphate removal.

Also, it is recommended that the mining companies monitor the microbial community in their treatment system. The microbial community and their change can be used for troubleshooting of the bioreactors. In addition it can be used as an indicator of availability of degradable organic carbon in the bioreactors.

**5.2 FUTURE WORK**

More work is required to understand the passive biological treatment systems fully. The followings are some suggestions to cover more areas related to these systems:
1. To learn more about the microbial community in these systems it is suggested to characterize the functional genes of the bacteria growing in the bioreactors. Although pyrosequencing was an easy and quick method for obtaining 16S rRNA sequences present in our bioreactors, many of these sequences could not be matched with any previously characterized microorganisms. Therefore it was not known what the actual metabolic potential was of all the microbes in the treatment system. In addition, functional genes across different species are not necessarily conserved. Therefore more work needs to be done on enriching and isolating microbes from these environments in order to functionally characterize them. So it is recommended to use metagenomics to identify the functional genes.

2. For the future research it is also recommended to find the active pathways that take place in the biochemical reactors. This can be done by identifying the intermediate and final products in the pore waters using HPLC and other chemical analysis. This helps to understand the biodegradation mechanism in these biological systems.

3. Finding the kinetics of the biological activities and reactions in the biochemical reactors is of special value since it helps to design the treatment systems. This can be done by using the data of sulphate concentration, DOC, carbohydrates, as well as the concentration of various organic acids.

4. During the course of our experiments, we realized that the sequential washes used for measurement of $\alpha$-cellulose, hemicellulose and lignin were hard to perform accurately. Also, it is possible that specific factors were interfering with the results. For instance, accumulation of microbes in the bioreactors would contribute to the organic matter, and formation of precipitates would contribute to the ash content. were associated with overestimation of some of the components, especially lignin. Therefore it is recommended to find more accurate and easier to perform methods to measure the extent of biodegradation. For instance
5. To measure how much of the organic matter is used up in the bioreactor a mass balance needs to be performed. One way of doing this would be to measure the mass reduction of organic materials by weighing the initial organic mixture as well as the organic mixture at the end of each time period. In addition, in order to find out the sources that are contributing to the ash content of the old bioreactors it is recommended to identify the composition of ash in the bioreactors. This will help to distinguish between ash formed due precipitate formation from the ash content of the original organic material. This information will help to estimate the rate at which the organic matter degrades.
REFERENCES


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