Characterisation and improvement of a microbiological anaerobic bioreactor to remediate acidic and metalliferous titanium processing leachates

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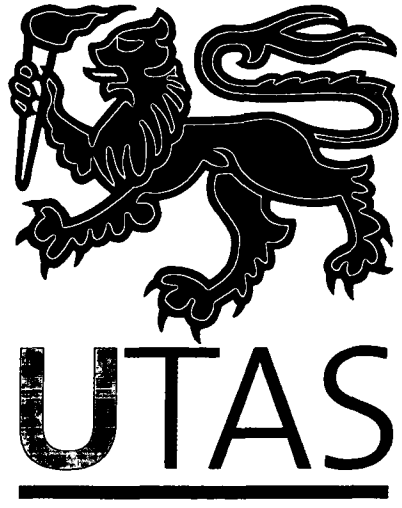
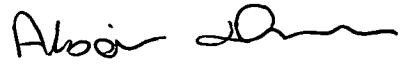


Declaration

This thesis contains no material that has been accepted for the award of any other degree or diploma in any tertiary institution. To the best of my knowledge this thesis does not contain material written or published by another person, except where due reference is made.



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“It is a mistake to think you can solve any major problems just with potatoes.”

Douglas Adams

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Papers and presentations

Dann, A.L., Cooper, R.S. and Bowman, J.P. 2009. Investigation and optimization of a passively operated compost-based system for remediation of acidic, highly iron- and sulfate-rich industrial waste water. *Water Research* 43(8): 2302 - 2316

Dann, A.L. Cooper, R.S. and Bowman, J.P. Characterisation and improvement of a microbiological reduction cell to remediate acidic and metalliferous titanium processing leachates. 2008 ISME Symposium Cairns 17th - 22nd August.

Dann, A.L., Bowman, J.P. and Cooper, R.S. Characterising and testing a novel biological reduction cell to remediate heavy metal and acid-containing mineral processing leachates, Heybridge, Tasmania. The 23rd Annual Conference on Contaminated Soils, Sediment and Water, UMASS, Amherst, October 15 – 18 2007.

Dann, A.L. Microbes with mettle for metal. School of Agricultural Science seminar series, 28th September 2007.

Dann, A.L. Developing and testing a novel biological reduction cell to remediate heavy metal and acid-containing industrial and mine leachates, Heybridge, Tasmania. ASM Annual Microbiology Conference, Gold Coast, July 2 – 6 2006.

Abstract

Wastewaters and leachates from mining and mineral processing are often characterised by low pH and high metal and sulfate concentrations. These can affect water catchment ecosystems and impact food webs deleteriously. In north-west Tasmania a titanium-processing plant operated between 1948 and 1996 on the Blythe River catchment. The sulfate extraction process was used to manufacture titanium dioxide pigment. This produced large quantities of acidic (pH 3 - 5), metalliferous (Fe 800 - 1200mg L^{-1}, Mn 15 - 45mg L^{-1}) and sulfurous (SO_4^{2-} 500 - 1700mg L^{-1}) wastewater. The wastewater was pumped into sludge dams, which leaked into the local catchment, and eventually Bass Strait. This resulted in elevated metal concentrations and a highly visible red plume along the coastline, locally suppressing the marine benthic biota and altering biodiversity.

A novel *in situ* bioremediation system was built in 2001 and managed subsequently by ESD (Environmental Services and Design) Pty. Ltd. The system comprised anaerobic processing sections that incorporated waste agricultural products; including potatoes, spent mushroom compost and straw, to remove the metals and increase the pH. A subsequent artificial wetland system was utilised to reduce effluent BOD (Biological Oxygen Demand) and COD (Chemical Oxygen Demand) to drinking water quality levels.

Using 16S rRNA gene based approaches microbial diversity and community structure was determined over 18 months from different stages of the treatment system. This included untreated sludge dam leachate, pre-treated effluent from an anoxic potato-containing section intended to increase alkalinity and prevent iron precipitation; and effluent from a series of mushroom compost and straw-based

iron-reduction cells. 16S rRNA gene clone libraries revealed a community shift from a mixed iron- and sulfide-oxidising and iron- sulfate-reducing community in the sludge dam leachate to a community dominated by *Acidithiobacillus* spp. and anaerobic fermenters (related to the genera *Bacteroides* and *Paludibacter*) in the potato cell effluent. The reduction cell effluents proved to have higher microbial diversity and greater heterogeneity, including iron-and sulfate reducers, iron-oxidisers, anaerobic fermenters and in one sampled effluent a high proportion of clones clustering with previously uncultured organisms of candidate division OP3. Multivariate statistical analysis of 16S rRNA gene-based TRFLP (Terminal Restriction Fragment Length Polymorphism) data revealed community differences had occurred between treated/post-treated samples and untreated/pre-treated samples. TRFLP analysis also indicated temporal shifts in the bacterial community composition occurred in the reduction cells. Although after 11 months of treatment, microbial communities in three of four reduction cells showed evidence of stabilisation probably due to exhaustion of an available carbon source and layered design of the system. There was no evidence of a seasonal effect on the microbial community.

A series of laboratory-scale microcosm experiments were conducted to evaluate temperature, bicarbonate and various carbon amendments (ethanol, molasses and vegetable oil emulsion) for bioremediation of an acidic, metal- and sulfate-rich titanium processing leachate with the goal of optimising an existing field-based system (described above). In all microcosms pH increased from 4 to 6.5-8 for the length of the experiment due to the high organic matter input but had no effect on other geochemical processes which was similar to the field-scale reduction cells in their first year of operation. The oxidation-reduction potential decreased in all

microcosms but was most stable in the oil emulsion microcosms. Alkalinity production was more substantial in the ethanol, molasses and oil emulsion microcosms (~2500mg L^{-1}) compared to the temperature and bicarbonate microcosms (~600 - 1800mg L^{-1}). The addition of bicarbonate did not increase pH or alkalinity. Iron and sulfate were initially removed but the effect could not be sustained in the unamended and bicarbonate microcosms. Liquid amendments such as ethanol, molasses and vegetable oil emulsion were found to support greater iron removal. However, sulfate removal only reached a maximum of 80% removal and was found to have a lag phase of approximately 80 days, and hence an acclimatisation stage may be needed for enhanced sulfate removal in field-scale bioremediation systems. 16S rRNA gene sequence-based TRFLP profiles revealed all the microcosms had similar bacterial communities but the amended microcosms were more successful in promoting the growth of a select bacterial consortia needed for enhanced sulfate and iron removal. This included a combination of anaerobic fermenters, iron- and sulfate-reducers as well as iron/sulfur/sulfide oxidisers.

Based on the experimental data and the literature a number of recommendations were developed to improve the operational efficiency and longevity of the field-based leachate remediation system.

Chapter 1.

Introduction

Franklin River, Tasmania

Introduction

Microbial sulfur and metal bioremediation

The contamination of water, soil and air by toxic compounds is a serious and escalating problem all over the world due to activities such as processing, surface treatment, mining, burning of fossil fuels and land filling of waste. Metal contaminants persist and accumulate in the food chain; hence, humans are at great risk as we consume many resources. The threat of metal pollutants has encouraged an interest in developing systems for their remediation, removal or neutralisation. Most contaminated sites contain a mixture of pollutants as atomically similar metals, such as iron and manganese, as they usually occur together in rock deposits. Metals, by their very nature cannot be destroyed; however, they can be transformed into less soluble forms by modification of their chemical/physical characteristics. A serious metal-containing pollutant is acid mine drainage. Furthermore, polluted groundwater systems are one of the most difficult environments to remediate.

When Fe^{2+} is oxidised it forms ferric iron that produces a red deposit of iron oxides/hydroxides ($Fe(OH)_3$) and in water a red plume that can pollute waterways and coastal regions; as was the case at Heybridge, Tasmania in the 1980s and 1990s. The idea of passive metal bioremediation is to precipitate the metals, such as iron, as insoluble Fe^{2+} while increasing the pH, removing sulfate anaerobically, and depositing the metals as oxides or carbonates out of the system (Johnson and Hallberg 2005a). Hence, reduction of Fe^{3+} to Fe^{2+} is an important section in a multi-step remediation process and will be the focus of the research. Moreover,

sulfate-reducing bacteria increase the pH by producing sulfides and this induces metals with a low metal-sulfide solubility to precipitate as metal sulfides or metal hydroxides depending on their valency and pH (Drury 1999). Researchers have also found that some iron-reducing bacteria, such as *Shewanella putrefaciens* can utilise aromatic hydrocarbons (Lovley et al. 1994), chlorinated organic compounds such as chlorinated phenols and benzoates (*Geobacter metallireducens*) (Kazumi et al. 1995), heavy metals (*Shewanella alga*, *Shewanella putrefaciens*, *Shewanella oneidensis* , and *Geobacter metallireducens*) (Liu et al. 2002) (Lovley 1995a, 1995b) and uranium (*Shewanella putrefaciens* and *Geobacter* spp.) (Lovley et al. 1991).

Acid mine drainage is produced by oxidation/deposition of pyritic compounds when mine ore is exposed to the air, oxidises and acidifies and ends up in water systems; that is Fe^{2+} oxidises to Fe^{3+} producing the characteristic red deposit. Acid mine drainage affects thousands of kilometres of rivers throughout the world. Researchers have stated the amount of oxidation cannot be by abiotic processes alone, indigenous bacteria can produce acid mine drainage problems as well (Johnson 2003). Conversely passive remediation technology of acid mine drainage relies on the activities of indigenous populations of iron- and sulfate-reducing bacteria (Johnson and Hallberg 2005a).

Treatment of acidic metal-rich waters and acid mine drainage was originally chemical. Most techniques involved the application of chemical precipitators such as carbonate compounds (Patterson et al 1977). Precipitation of heavy metals by lime or limestone is one of the oldest and cheapest methods for removal of metals from acid drainage waters. However, such measures have proven inadequate

(James and Mrost 1965). Research has shown (Dean et al. 1972) that copper (Cu), lead (Pb), cadmium (Cd) and zinc (Zn) will begin to precipitate from dilute solutions at pH values exceeding 5.3, 6.0, 6.7, and 7.0, respectively. However, lime addition was found unsatisfactory for highly pyritic materials, as they soon tend to form clay pans and restrict water movement. Hore-Lacy (1978) observed in one United States case where 2500tonne/ha of lime were added to the tailings at the Bingham Canyon Mine, Utah, that the pH was dropped to a value of 3.5 after 3 months.

The role of sulfate-reducing bacteria in acid mine waters was first studied extensively by Colmer and Hinkle (1947); but Tuttle and colleagues (1969) first published examples of microbial remediation of high acid, metal and sulfur rich waters. Sulfate-reducing bacteria have been widely studied and their metabolism is well known and is indirectly used to precipitate metals. The sulfate is reduced to dissolved sulfide (HS^-) which binds to metals (Me) to form insoluble metal sulfides (MeS) eg.

$HS^- + Me^{2+} \rightarrow MeS + H^+$ (Rabus et al. 2000)

However, iron and metal-reducing bacteria used in a bioremediation context is a recent innovation (Lovley 2000). A lack of information about the factors controlling growth and metabolism of microorganisms in polluted environments often limits their implementation (Lovley 2003).

Ideally, bioremediation strategies would be designed based on knowledge of: the microorganisms that are present in the contaminated area; their metabolic capabilities; and how they respond to changes in environmental conditions (Lovely 2003). Unfortunately, in practice, much of the required information is not

readily available. 16S rRNA clone libraries can inform what bacteria are there as long as there are known sequences in GenBank or other databases and many are unknown in terms of their metabolism as it is difficult to culture them. The indigenous microorganisms living in the contaminated area are the best targets for bioremediation strategies as they are well adapted to survival in the prescence of the existing contaminants and to the local temperature, pH and redox conditions (Johnson and Hallberg 2005a).

Conversely, many treatment studies advocate the introduction of a known bacterium, for example, *Dehalococcoides* sp., used in reductive dechlorination treatments, as its metabolism is known (McKinsey et al. 2004). Furthermore, it was marketed as an almost compulsory addition to all contaminated sites (Suthersan and Horst 2007) without thought of site-specific needs. However, some in the industry still swing towards biostimulation (stimulation of broad native microbial populations) for remediation purposes. The challenges with growing and injecting non-native species into sites and the uncertainty in their ability to overcome the stress of changing environments and competition with the native microbial flora have focused studies on increasing the population of native bacteria through substrate addition (Suthersan and Horst 2007).

Treatment technology for mining and industrial wastewaters

Most of the current technologies are based on the method of raising pH and utilising metal and/or sulfate-reducing microorganisms for treatment of acidic, metal-rich mining and industrial wastewaters (Johnson and Hallberg 2005). These technologies range from basic limestone drains to Permeable Reactive Barriers (PRB) including bioreactors, constructed wetlands and Successive Alkalinity

Producing Systems (SAPS) (Costello 2003) and up flow anaerobic bioreactors (UAB) (Elliott et al. 1998). These technologies have been designed on broad knowledge of the biogeochemical cycles involved including, bacterial metabolism, metal mobility, mineral precipitation, pH and redox potential (Lee and Saunders 2003).

As molecular methods improve and the genomic sequences of these bacteria become available, the genetic and biochemical basis of their activities on metals will be revealed and bioremediation technologies can be vastly improved (Lovley 2003). The advantages of biological approaches include a higher specificity than physical and chemical methods, as well as their suitability to *in situ* methodologies and the potential for improvement by genetic engineering (Valls and de Lorenzo 2002). Although, these technologies may work at the laboratory scale, there are inherent difficulties of reproducing these processes on a large scale due to site-specific hydrology and biogeochemical interactions, and consequently pilot-scale field studies should be carried out before full implementation (Blackburn 1998). Bioreactors, anaerobic wetlands and designed *in situ* systems go some way to alleviate site-specific problems, as they are a form of a controlled environment.

Another technology that has been tested for bioremediation at the laboratory-scale is the fluidised bed reactor (FBR) (Kaksonen et al. 2003). The biomass is retained on an inert carrier material, which is fluidised with water. The advantage of FBRs over packed bed reactors is enhanced mass-transfer of both substrates and toxic products such as H_2S (Kaksonen et al. 2003). For acidic, metal containing wastewaters, the high recycle ratio of substrate to pollutant dilutes the metal

concentrations and the acidity to a manageable level for the bioreactor (Kaksonen et al. 2003). Furthermore, because of intensive mixing, the FBRs are less likely to clog compared to fixed-bed bioreactors (Kaksonen et al. 2004b, 2006). However, FBRs have only been used in field-scale operations for the removal of organic contaminants (Kaksonen et al. 2006).

One of the main problems in bioremediation of acidic waste is raising the pH because pH affects other factors including the solubility of metals and the kinetics of their oxidation and hydrolysis. Drury (1999) found that adding an external source of alkalinity, such as cheese whey, increased the pH, which increased the removal of metals and sulfate. James and Mrost (1965) and Hore-Lacy (1978) found the addition of lime proved unsuccessful on its own. Other commonly used alkaline agents include calcium hydroxide (hydrated lime), soda ash (sodium carbonate), caustic soda (sodium hydroxide) and ammonia (Costello 2003). Combining a bioreactor with addition of an alkalising agent or pre-treatment may prove more effective than either on its own.

Addition of substrates to enhance bioremediation

In situ bioremediation has been found to be more cost effective than containing and shipping contaminated waste for treatment. However, passive *in situ* bioremediation can be slow and affected by climate and rainfall. Hence, *in situ* systems that can be enhanced to minimise these affects are most desirable. In metal-polluted wastewater Fe^{3+} is generally the most abundant electron acceptor for dissimilatory metal-reducing microorganisms in soils and sediments. Most Fe^{3+}-reducing microorganisms can also reduce Mn^{4+} (Lovley 1991). Stimulation of Fe^{3+} and Mn^{4+}-reducing microorganisms in a bioremediation setting can also

help the removal of other contaminants as the Fe^{2+} and Mn^{2+} produced acts as a redox buffer to help prevent reoxidation of reduced metal contaminants (Lovley 1991). Furthermore, some of these organisms may also be capable of reducing other metals as well, such as U^{6+} (Snoeyenbos-West et al. 2000).

Snoeyenbos-West et al. (2000) identified three mechanisms for stimulating iron reduction in sedimentary environments. First, the addition of compounds or organic matter for the bacteria to use as electron donors and carbon sources: for example, acetate, propionate, butyrate, benzoate, ethanol, toluene, H_2 or various composts (Anderson et al. 2003; Lonergan et al. 1997), as well as molasses, cheese whey, ethanol and soybean oil (Sturman 2001; Kaksonen et al. 2004a; Geets et al. 2005; Lindow and Borden 2005a, b), woodchips and paper pulp waste (Hulshof et al. 2006) and fly ash (Gitari et al. 2006). Secondly, the addition of iron chelating compounds. Lovley and Woodward (1996) demonstrated in laboratory studies, in which Nitrilotriacetic acid (NTA) was added, that Fe^{3+} reduction increased. They also showed NTA enhanced reduction of both poorly crystalline and crystalline Fe^{3+} oxide. However, whether this could be applied to an *in situ* bioremediation site is debatable. Large and probably costly amounts would have to be used, and worse the MSDS for Nitilotriacetic acid states it as hazardous and possibly carcinogenic, although, there has been limited investigation of the long-term effects. Lovely and Woodward (1996) speculated that naturally occurring organic acids may also be iron chelators but no one had investigated these properties. Lastly, the addition of electron-shuttling organic compounds such as humic acids or other extracellular quinones could stimulate iron reduction (Lovley et al. 1998; Nevin and Lovley 2000). These compounds

can greatly accelerate iron reduction by alleviating the need for metal reducers to have direct contact to insoluble metals (Lovley 1993).

In summary, additions of a variety of substances can greatly improve bioremediation; however, systems with a flow through set up and involving continual addition of compounds could prove expensive. Organic matter can be added as a solid form, such as compost, which stays stationary in the system, can be topped up periodically, and is relatively cheap if it is a by product of agriculture. Many commercial liquid amendments have been designed, such as HRC® (Hydrogen Release Compound) (Regenesis PL), EOS® (Emulsified Oil Substrate) (EOS Remediation PL) and SRS® (Slow Release Substrate) (Terra Systems Inc.). These products are designed to provide an anaerobic and reducing environment, and were mainly developed for reducing recalcitrant chlorinated compounds; but can be applied to any remediation that requires a reducing environment (Lindow and Borden 2005b). These substances are fast- and slow-release, providing an immediate carbon source and nutrients for bacterial growth in the first few days, as well as slow release degradation products for sustained long-term bacterial respiration (up to 3 years) (EOS Remediation PL).

Many bioremediation systems use cheap cellulosic organic substrates as long-term sources of carbon. Only a small fraction of the organic matter placed in bioremediation systems would be available to iron- and sulfate-reducing bacteria as they typically require simpler molecules (organic acids, alcohols or H_2) for energy (Lovley 2000; Rabus et al. 2000). Anaerobic degradation of complex organic matter to simpler molecules by cellulolytic and fermentative microorganisms is required, and may limit the rate at which substrates become

available for iron- and sulfate-reducers (Hallberg and Johnson 2003). Logan et al. (2005) investigated the interactions between cellulolytic microbes, anaerobic respirers and fermenters, sulfate-reducers and methanogens using a variety of substrates. They found all metabolic activities, except methanogenesis, were limited by cellulose hydrolysis and declined substantially by day 99 in lab-scale permeable reactive barriers. Hulshof and colleagues (2006) found that woodchips did not promote bacterial activity as well as paper pulp waste did. They concluded the woodchips contained an insufficient concentration of labile carbon or nutrients such as phosphorus and nitrogen.

The long-term sustainability of passive treatment systems appears more complicated and less *passive* than previously thought. More research has to be carried out on the depletion rates of different organic substrates to assess their ability to promote sulfate- and iron-reducing bacterial growth. More work mustalso be conducted to understand and differentiate the fundamental biochemical and microbiological reactions that occur in anaerobic bioreactors amended with complex organic substrates (Neculita et al. 2007).

Research in the laboratory and in the field have revealed examples where excess organic matter was present and attempts to keep the bioremediation system anaerobic have worked for the first 4 months and then declined in metal reduction rates (Wendt-Potthoff et al. 2002). They showed Fe^{2+} oxidation rates surpassed Fe^{3+} reduction rates after 5 months and found that adding hay bales to the surface provided an excess of organic carbon and decreased mixing of the fluid beneath them, promoting anoxic conditions. However, at the Heybridge site, hay bales were used and the reduction cells were still not reducing iron adequately. Johnson

and Bridge (2002) also found, in acid environments where aerobic and anaerobic zones are juxtaposed, the cycling of iron between ferrous and ferric is highly dynamic. This may have a serious impact on the overall efficiencies of the systems. Willow and Cohen (2003) found dissolved oxygen had little effect on the Fe^{3+} reduction rate, although, lower pH influents reduced the Fe^{3+} reduction rate but they concluded this was because the pH was inhibiting sulfate-reducing bacteria. However, their experiments were conducted with laboratory simulated acid mine drainage waters. Conversely, Johnson and Bridge (2002) found iron reduction rates were higher in experiments using actual bioreactor samples compared to artificial wastewater. Therefore, using the contaminated leachate/groundwater from the study site in laboratory experiments is probably the best approach, as is conducting the experiment for an extended time to determine long-term reduction rates (Kaksonen et al. 2003).

Improving the efficiency of long-term passive bioremediation

Researchers have experimented with many factors to improve the efficiency of bioreactors and while their specific sites were improved, their methods may not be applicable to other sites (Anderson et al. 2003; Batty and Younger 2004; Bilgin et al. 2005). However, it is possible bioremediation strategies need to be assessed on a site-by-site basis. Gibert et al. (2004) found hydraulic retention time a key factor in the performance of bioremediation systems. Their studies showed high flux rates led to incomplete sulfate reduction and therefore incomplete removal of metals and therefore by extending the residence time from 0.73 days to 2.4 and 9 days increased sulfate removal by 18% and 27% respectively. Johnson and Hallberg (2005) inadvertently found a compost bioreactor that had

not been used for several months worked better than the others that had been used continuously. They concluded the time where no leachate was going into the bioreactor allowed critical microorganisms to proliferate. Furthermore, a retention time as short as 6.5 hours has been found to remove 99.9% of metal contaminants (Kaksonen et al. 2004a).They used a starting retention time of 20 hours and reduced it to 6.5 hours over 160 days with the addition of ethanol in a laboratory-scale fluidised bed reactor.

In situ passive bioremediation systems will always be affected by climatic conditions that will affect their efficiencies, for example, Batty and Younger (2004) found a compost wetland in Durham, UK fluctuated in its metal removal and pH increased during the year. Substrates may be diluted in heavy rainfall in temperate regions where bioremediation is needed and may require different strategies, hence solid organic matter for a carbon source may work better in these regions. Hulshof et al. (2006) predicted long-term metal removal efficiency on a pilot remediation system to last ten years, based on sulfate reduction rates, however, the calculations were based on the first year reduction rates. They did concede that the model represented more the maximum amount of sulfate reduction and metal removal that would potentially occur. Because there are no projects that have operated for more than 5 years, there is a lack of long-term data. Eger (2005) showed from data in both the field and laboratory that the rate of sulfate reduction decreased with time and that after several years' metal reduction was inefficient.

The longevity of passive bioremediation has been reviewed (Eger and Wagner 2003; Sjoblom 2003; Ziemkiewicz et al. 2003). Ziemkiewicz et al (2003) assessed

the projected long-term performance and cost-effectiveness of 137 passive systems in the eastern United States and predicted most would fail or at best perform poorly after approximately 3 years due to exhaustion of degradable carbon sources and required periodical top-ups of organic matter. Kalin et al. (2006) concluded research was needed into rates of bio-mineralization, exchange processes, carbon utilisation and the population dynamics of microbial consortia within bioremediation systems to make long-term predictions.

New technologies are being trialled to address different problems of passive remediation systems. For example, compost-free bioreactors have been designed for treatment of acid mine drainage (EPA. 2006; Tsukamoto and Vasquez 2006). These bioreactors use a continuous liquid carbon source and a rock matrix rather than a compost or woodchip matrix, that collapses over time, is difficult to replace and can clog the system (Tsukamoto and Vasquez 2006). The benefits include better control of biological activity and improved hydraulic conductivity and precipitate flushing. This technology could improve the longevity of bioremediation systems as the matrix does not have to be replaced and the bioreactor does not clog up with precipitates (Tsukamoto and Vasquez 2006).

Site characterisation

The ICI Tioxide titanium-processing plant operated between 1948 and 1996 in Heybridge, located on the north coast of Tasmania on the Blythe River catchment (Figure 1.2 and 1.3). During the plant's operation iron oxide waste was discharged directly into Bass Strait, and later sludge ponds, resulting in elevated metal concentrations and a highly visible red plume along the coastline locally suppressing the marine benthic biota and altering biodiversity (Cooper 2004).

Figure 1.1. Tip Creek before the remediation project, Heybridge, Tasmania. Photo courtesy of Environmental Services and Design, 2005.

A less-recognised impact of the operation was the groundwater contamination and surface runoff, which also entered the Blythe River estuary directly or via Minna Creek. The main sources contributing to the groundwater contamination were an industrial rubbish disposal site and the three large sludge ponds (155,000m^3) within the Minna Creek catchment, including the Tip Creek tributary, (Figure 1.1) which contained metalliferous waste products from the pigment process, primarily iron (12,400t) and manganese as well as smaller quantities of lead, copper and zinc (Cooper 2004). The leachate entered Minna Creek tributary raising the contaminant concentrations, 75ppm Fe, 2ppm Mn and pH 3.5. The creek was effectively lifeless eliminating a valuable habitat for indigenous wildlife including the endemic burrowing crayfish *Parastercoides* (Waight et al 1997). Moreover, the Minna Creek fed into Blythe River carrying the contamination further downstream. In addition, an average of 140m^3/day of

leachate with levels in excess of 1000ppm Fe and 30ppm Mn at a pH 3.5 was entering Bass Strait (Cooper 2004).

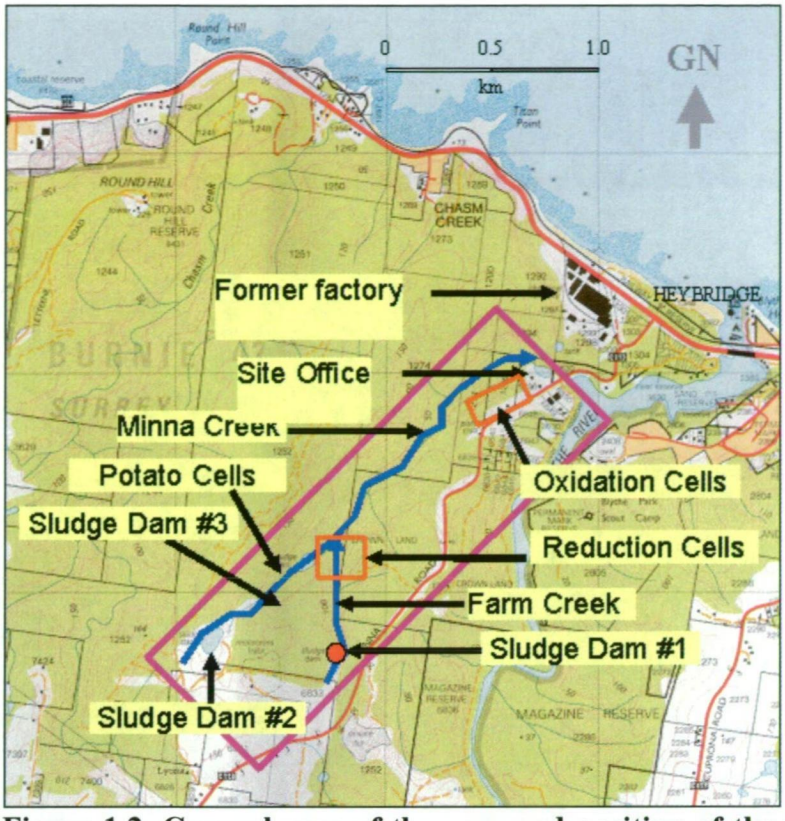


Figure 1.2. General map of the area and position of the remediation project components and former factory site (ES&D, 2005).

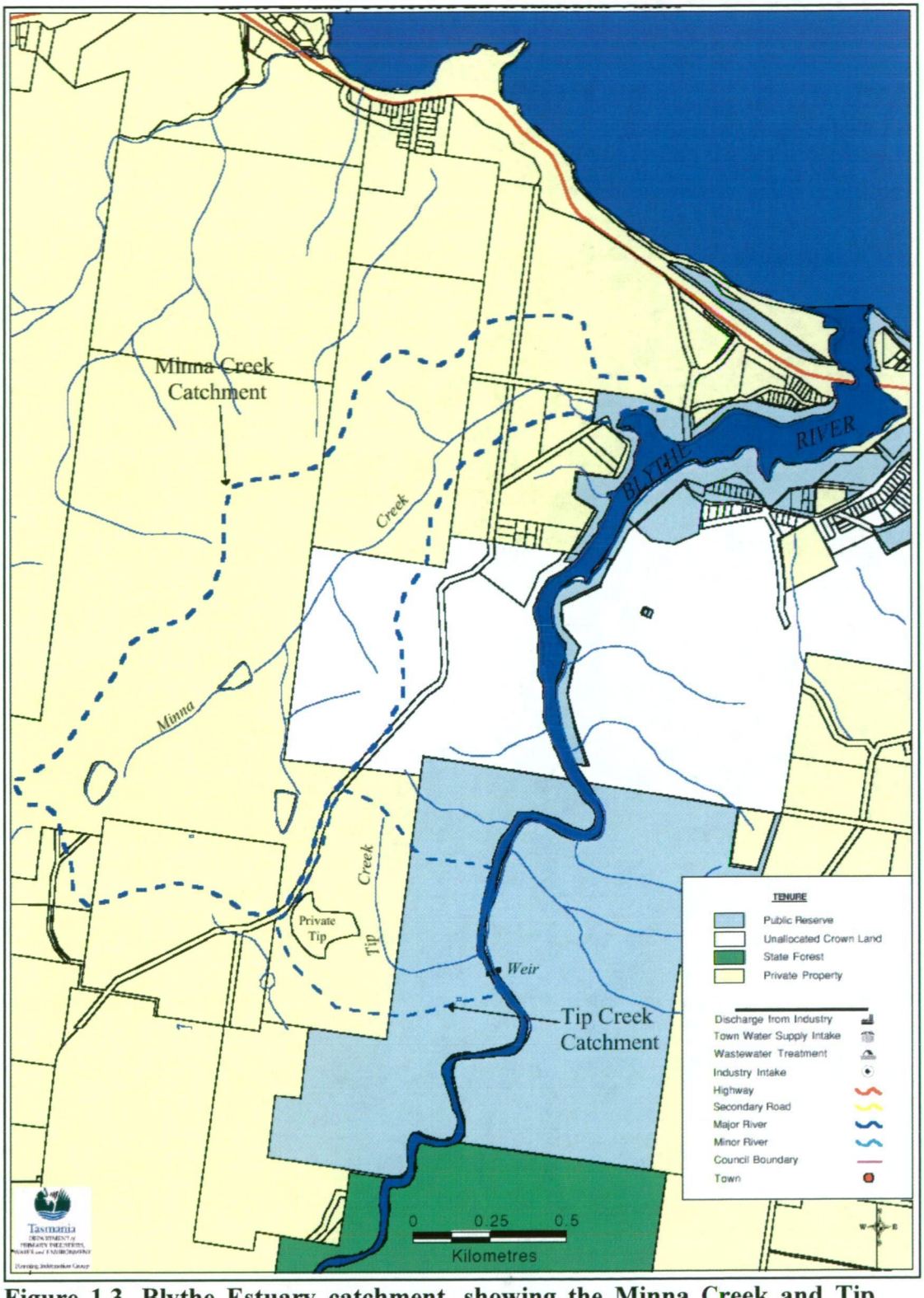


Figure 1.3. Blythe Estuary catchment, showing the Minna Creek and Tip Creek sub-catchments affected by Tioxide operations (ES&D 2005).

Titanium dioxide treatment process

Titanium dioxide pigment can be manufactured by either the sulfate or the chlorine process (Millennium Chemicals 2005). ICI Tioxide used the sulfate process (Figure 1.4) that produces large quantities of waste iron sulfate using ilmenite ($FeTiO_3$) shipped from Western Australia and local sulfuric acid from metal smelting operations (Tioxide Australia 1980). The sulfate process produces a form of pigment called anatase, which is preferred over chloride-derived pigment for use on papers, ceramics and inks. The ore is first dried, ground, and sulfated by agitation with concentrated sulfuric acid in a batch or continuous exothermic digestion reaction. Controlled conditions maximize conversion of TiO_2 to water-soluble titanyl sulfate using the least amount of acid. The resultant dry, green-brown cakes of metal sulfates are dissolved in water or weak acid, and the solution treated to ensure that only ferrous-state iron is present. The clear solution is then further cooled to crystallize coarse ferrous sulfate heptahydrate ($FeS0_4.7H_20$) which is separated from the process and dumped (Millennium Chemicals 2005). Hence, the waste is highly acidic, metal and sulfate rich, and very similar to acid mine drainage except that acid mine drainage typically has iron concentrations of 100 – 500 mg L^{-1} (Ledin and Pedersen 1996) whereas the Heybridge site has 800 - 1200 mg L^{-1}.

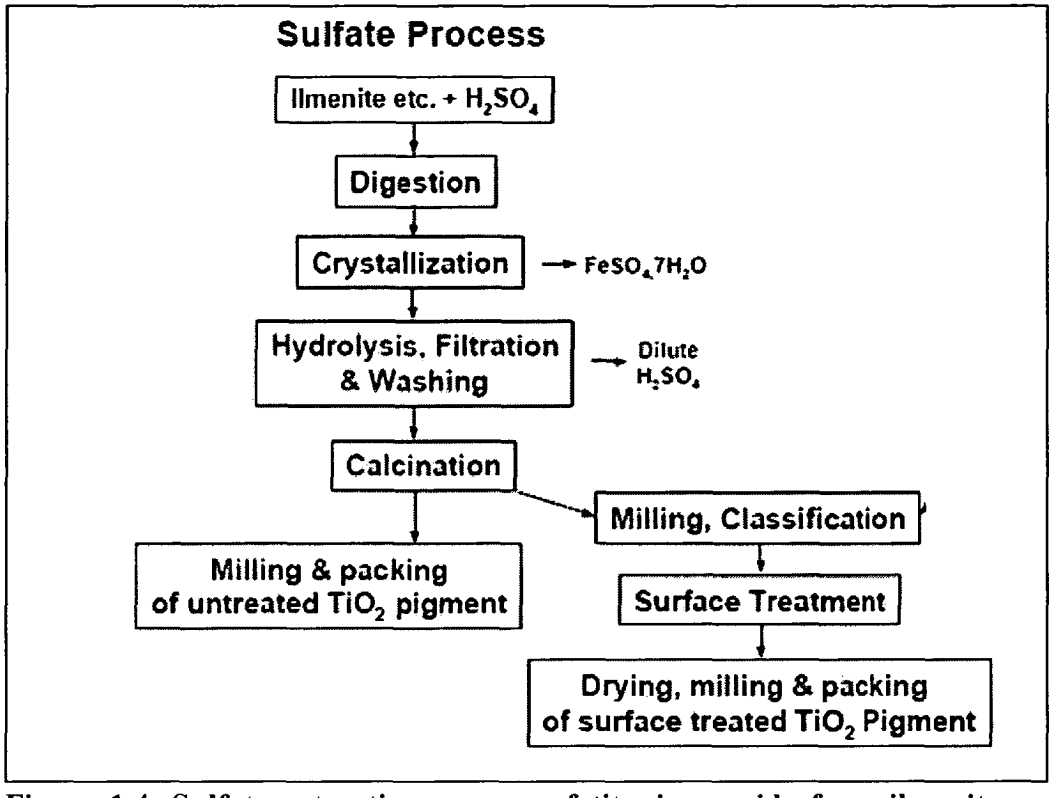


Figure 1.4. Sulfate extraction process of titanium oxide from ilmenite ore (FeTiO3) (Millennium Chemicals PL).

The remediation project overview

After the demolition and disposal of the tioxide pigment plant Echo Remediation (now Environmental Services and Design (ES&D)) was formed to remediate the whole site. The main aims were to contain and treat the leachate on site and minimise further generation of pollutants (Cooper 2004). The long term goal was to establish a passive, low-maintenance, efficient water treatment system *in situ* that could purify leachate generated from the contaminated materials over a thirty year period. The treated water had to be of a standard that could be discharged into the Blythe River, containing specifically 0.3mg L^{-1} and 0.1mg L^{-1} soluble iron and manganese concentration respectively and 250mg L^{-1} sulfate (Australian Drinking Water Guideline ADWG 6).

The remediation began with the industrial waste material being deposited in the sludge dams, which were then clay-capped, uncontaminated surface waters were diverted away from the dams so all leachate was collected at a central site. A combined anaerobic and aerobic wetland system was developed in consultation with Syrinx Environmental PL (Cooper 2004).

Overview of the treatment components

The system has five main components each of which have sub-components. The system has a design flow capacity of $110m^3\ day^{-1}$ of leachate containing up to $95kg\ day^{-1}$ iron. The components are:

- **Collection**: drains at the end of each sludge dam were isolated from the air to diminish precipitation of metals and drained into one central pump. Waste streams were classified on iron content and low content streams were diverted around the pre-treatment into the aeration race.

- **Pre-treatment**: Initial trials showed that iron precipitation made handling of material difficult and blocked pipes. Experiments showed pre-treatment of the leachate using potatoes in an enclosed "cell" prevented precipitation of iron by increasing the pH to approximately 4 – 5 and provided a carbon source for the reduction process.

- **Anaerobic reduction**: An enclosed cell or "compost bioreactor", filled with mushroom compost and covered with straw bales encouraged the growth of sulfate- and metal-reducing bacteria, which reduced the sulfate and metals to their insoluble form.

- **Aeration and settling ponds**: a series of races and settling ponds (Figure 1.2) to rapidly aerate the water to precipitate ferric iron into the settling ponds. The second settling pond is lined with limestone to increase the pH to over 7 and further precipitate ions.

- **Aerobic wetlands**: A series of 4-5 clay-lined wetland areas densely planted with a succession of rushes, sedges, aquatic and emergent macrophytes and swamp forest. Phosphorus, nitrogen and residual metals are removed by plant uptake. Water flow is through a series of weirs to promote flow to a final polishing pond, which allows removal of any residual oxidation products.

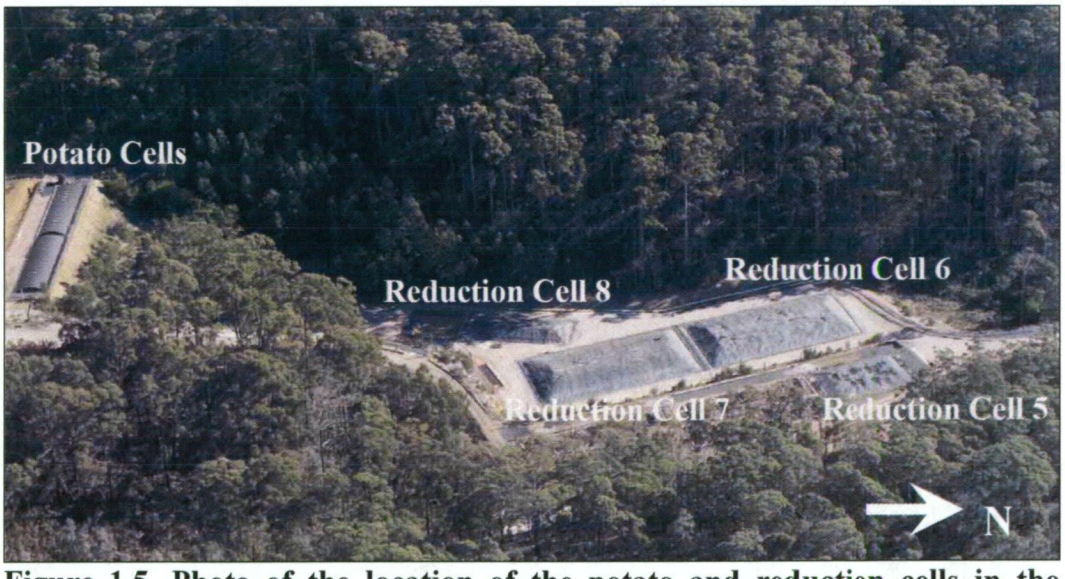


Figure 1.5. Photo of the location of the potato and reduction cells in the Minna Creek Valley, north-west Tasmania. Photo courtesy of ES&D.

Figure 1.6. First aeration race and settling pond after the reduction cells, Heybridge, Tasmania. Photo courtesy of ES&D 2005.

Figure 1.7. Wetland system planted with native plants to remove organic matter and residual metals, Heybridge, Tasmania. Photo courtesy of ES&D, 2005.

Components of the bioremediation system

The sludge dams

The leachate was pumped to the existing ocean outfall between 1996 and 2003 until the downstream water biological treatment system was established. The leachate pumped into this outfall contained 80 kg day^{-1} of Fe, and 10 kg day^{-1} Mn. During the earth works, all materials (140,000 m^3) from the industrial rubbish disposal site in the Tip Creek catchment were removed and placed within the existing sludge ponds prior to clay capping. The three sludge dams were consolidated, clay-capped and fenced. Table 1.1 shows the composition of the three dams on the Heybridge site.

Table 1.1. Sludge dam effluent chemical composition, Heybridge (Cooper, ES&D Technical Report 2004).

Chemical parameter (mg/l)	Sludge Dam 1	Sludge Dam 2	Sludge Dam 3
Acidity	1200	1400	2800-4900
Alkalinity	<0.05	<0.05 - 42	<0.05
Aluminium	29-48	93-220	310-320
Arsenic	nd[a]	<0.001	nd
Cadmium	0.004	0.003-0.095	<0.006
Chromium (III)	nd	0.093	nd
Copper	nd	0.26	nd
Iron	810-980	620-1100	890-1200
Lead	0.018	0.024-0.16	0.19
Manganese	23-44	15-44	26-32
Zinc	nd	1.6	nd
Sulfate	540-810	600-1200	1300-1700
Ammonia	<0.05-1.2	<0.05-1	<0.05-0.9
Total nitrogen	0.11-1.6	0.96-3.4	0.15-1.9
Total phosphate	<0.05-0.14	<0.05-0.13	<0.05-0.1

nd, not determined

The potato cell

Figure 1.8. Potato pre-treatment cell Heybridge remediation site, Tasmania. Photo courtesy of ES&D, 2005.

The collected leachate was found to be unstable and reacted with the air producing a thick heavy iron precipitate, which quickly restricts and blocks the feed lines (Cooper pers. comm.). Pre-treatment with potatoes allowed flow of the leachate to the next stages of the treatment system. The leachate is directed into a treatment cell containing potatoes (Figure 1.7), located on the downstream end of Sludge Dam 3. The purpose of the potato cell is to create anoxic conditions in the leachate stream, and introduce alkalinity into the anoxic water. The creation and maintenance of anoxic conditions in the leachate stream is essential to prevent the precipitation of iron within the potato cell or piping, which would lead to a decrease in the efficiency of overall the metal removal due to coating and/or restriction of the flow of water through the system (Cooper 2004).

A small-scale potato reactor was tested on site at a number of flow and iron loading rates for a period of 1 – 3 months. It was found to enhance the removal of iron in the subsequent reduction cells compared to cells not receiving the pre-treated leachate. There was also a reduction in the amount of hydroxide build-up in the pipe work. Two large-scale potato cells were then constructed such that one can be taken off line for maintenance during the dry summer months without disrupting water treatment (Cooper 2005 pers.comm.).

The potato cell is a new technology that has not been implemented at a large scale at any other site in Australia or the world. The advantage of using potatoes is that they are relatively inexpensive and easily obtainable and provide an alkalinity addition to the water in a relatively passive manner, and prevent pipe clogging (Cooper pers. comm. 2005). The decomposition of potatoes also provides carbohydrate and protein necessary for sulfate and iron reduction to occur in the reduction cell.

The reduction cells

Figure 1.9. Inside reduction cell; mushroom compost and straw bales being added, Heybridge, Tasmania Photo courtesy of ES&D, 2005.

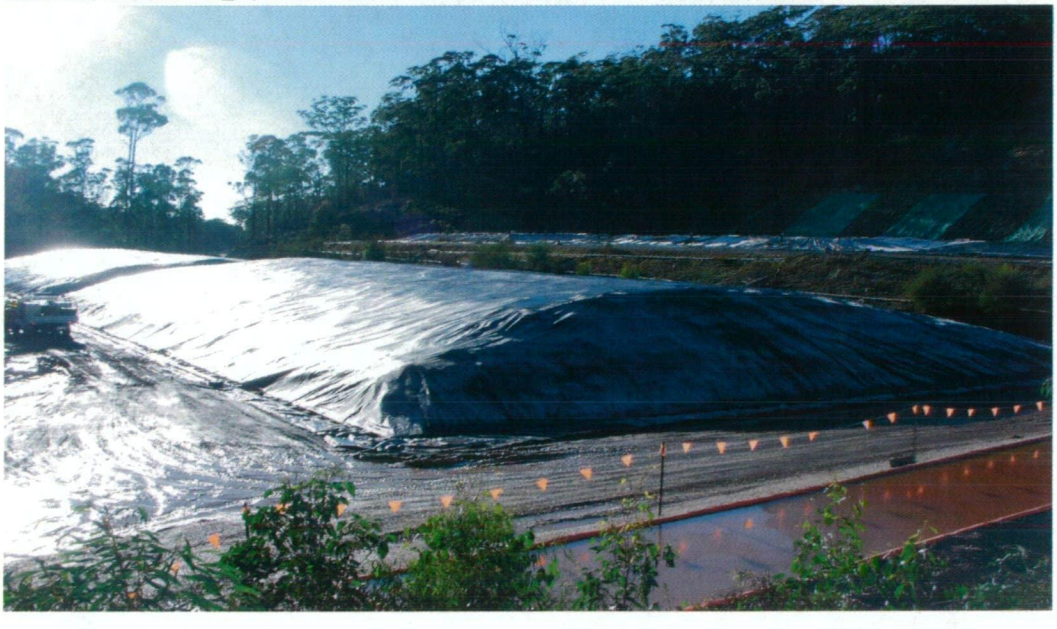


Figure 1.10. Finished reduction cell packed with straw and the black plastic cover, Heybridge, Tasmania. Photo courtesy of ES&D, 2005.

The main function of the reduction cells is to promote microbial sulfate and metal reduction. The cells consist of a base of bluestone overlaid by mushroom compost

and capped with straw bales (Figure 1.8) and then black plastic to create an anaerobic environment (Figure 1.9). This environment encourages sulfate- and metal-reducing bacteria to produce alkalinity and hydrogen sulfide, which precipitates metals from the water as sulfides. Sulfate-reducing bacteria reduce sulfate to sulfide using organic substrates resulting in the removal of metals from solution via three main processes: precipitation as metal sulfides, chemical reduction of metals by the sulfide and raising the pH (Rabus et al. 2000). Iron- or metal-reducing bacteria directly metabolise the metals by dissimilatory or assimilatory reduction (Lovley 2000). The sulfidogenic processes have some advantages over chemical treatment methods, such as better thickening properties of the metal sludge and lower solubility products as compared to hydroxide precipitation (Kaksonen et al. 2004b). Little is known regarding the microbiology of these compost bioreactors and in particular, how the species composition changes over time. How the reduction cells perform in the long term and what has to be done to keep the metal removal rates high is not well understood. ES&D identified low temperatures and fluctuating pH as the main problems with the reduction cells. Furthermore, as the site is in a high rainfall and low temperature valley (Figure 1.10), any improvements should aim to overcome related performance problems.

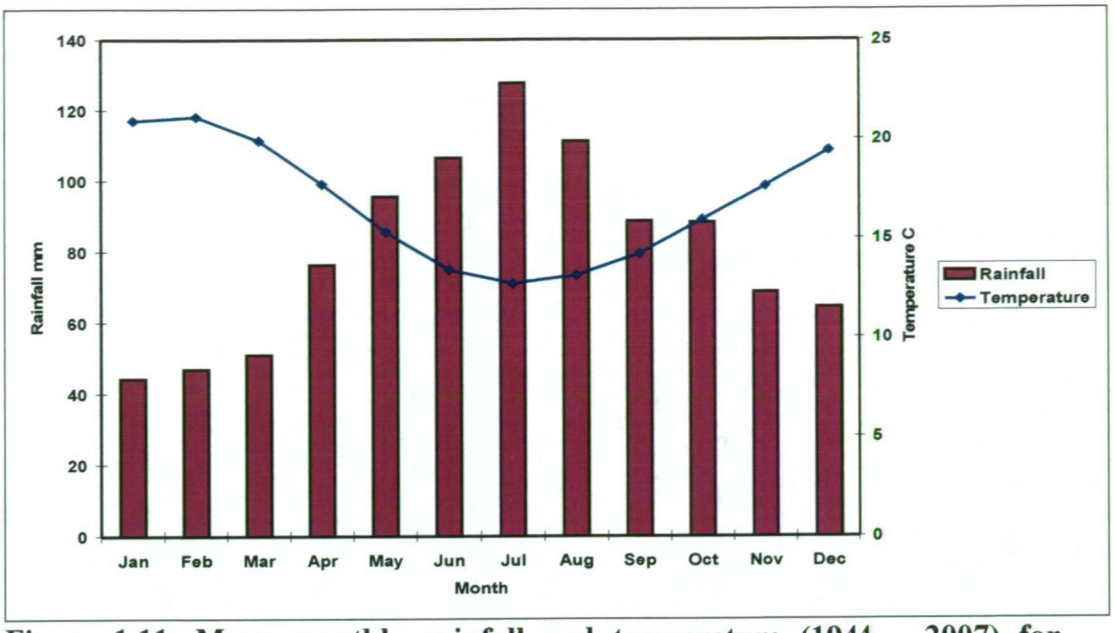


Figure 1.11. Mean monthly rainfall and temperature (1944 – 2007) for Burnie, Tasmania, nearest measuring station to Heybridge (Bureau of Meteorology, 2005).

Physiochemical properties of the Heybridge bioremediation system

pH

Initial measurements showed a near-neutral pH was maintained in the reduction cells in the first year and the potato cells slightly raised the pH by the addition of starch (Figure 1.12).

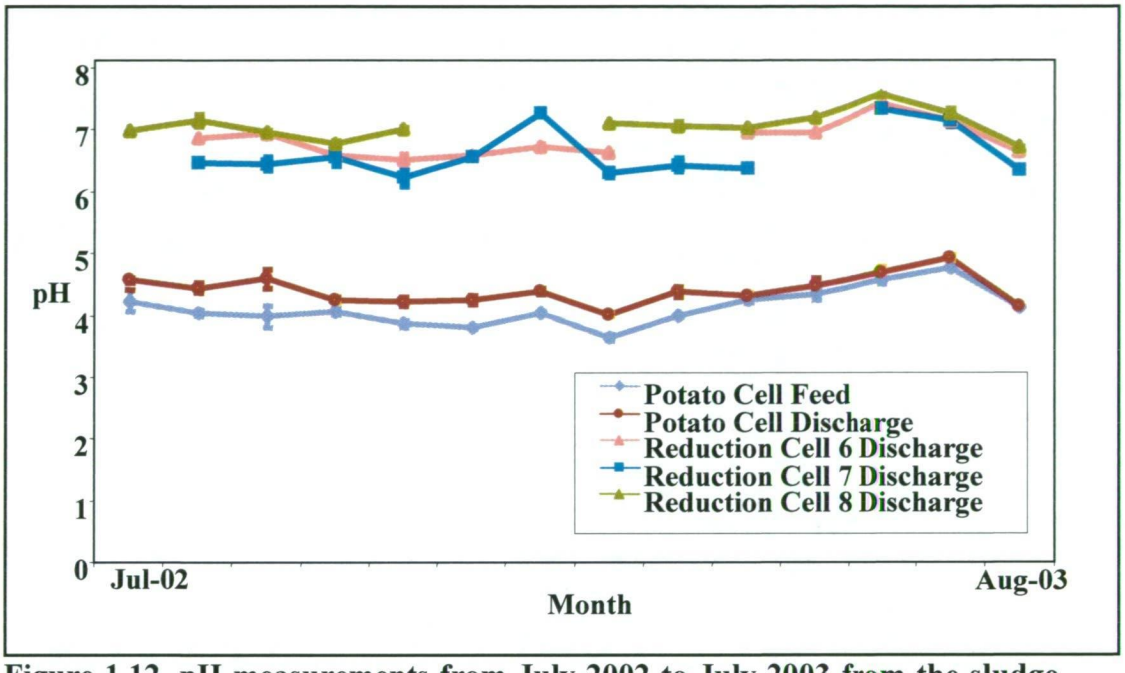


Figure 1.12. pH measurements from July 2002 to July 2003 from the sludge dam (potato cell feed), potato cell discharge and reduction cells 6, 7, and 8 discharges (Data from ES&D Technical Report 2005).

Iron and sulfate removal

Bioremediation cells were effective at removing iron (60-80%), sulfate (70-95%) and some manganese (6-17%) in the first year (Figure 1.13, 1.14). Reduction cells that had leachate pretreated through the potato cell, performed best when temperatures exceed 15°C at the inlet. The critical redox level (–100mV) needed for reduction of sulfate was achieved providing temperature is adequate and loading rate is adequate. The iron loading target was 15g $^{-1}m_3^{-1}$ day^{-1} based on the mushroom compost volume. However, after the first year of operation iron and sulfate removal started to decline. Over the last 4 years, the reduction cells were

running at 20 - 60% iron removal efficiency (Figure 1. 15) and appear to have seasonal variation, where decline in iron removal efficiency is greatest in August to September at the end of winter.

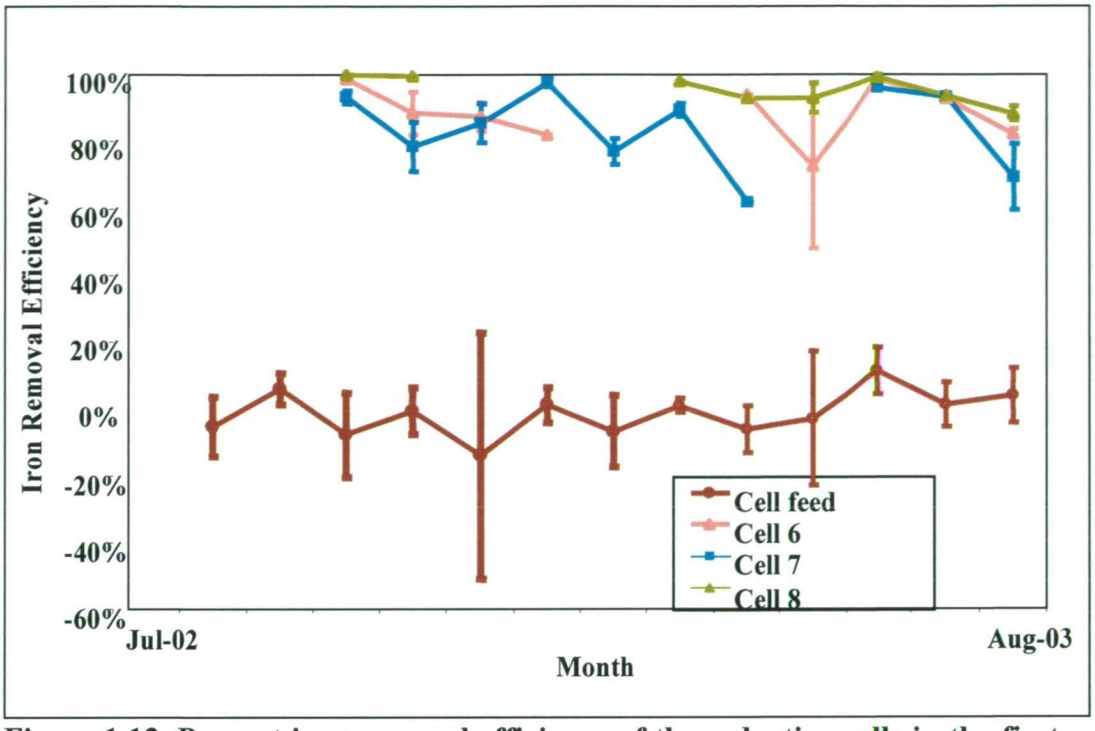


Figure 1.13. Percent iron removal efficiency of the reduction cells in the first year of operation (ES&D 2005).

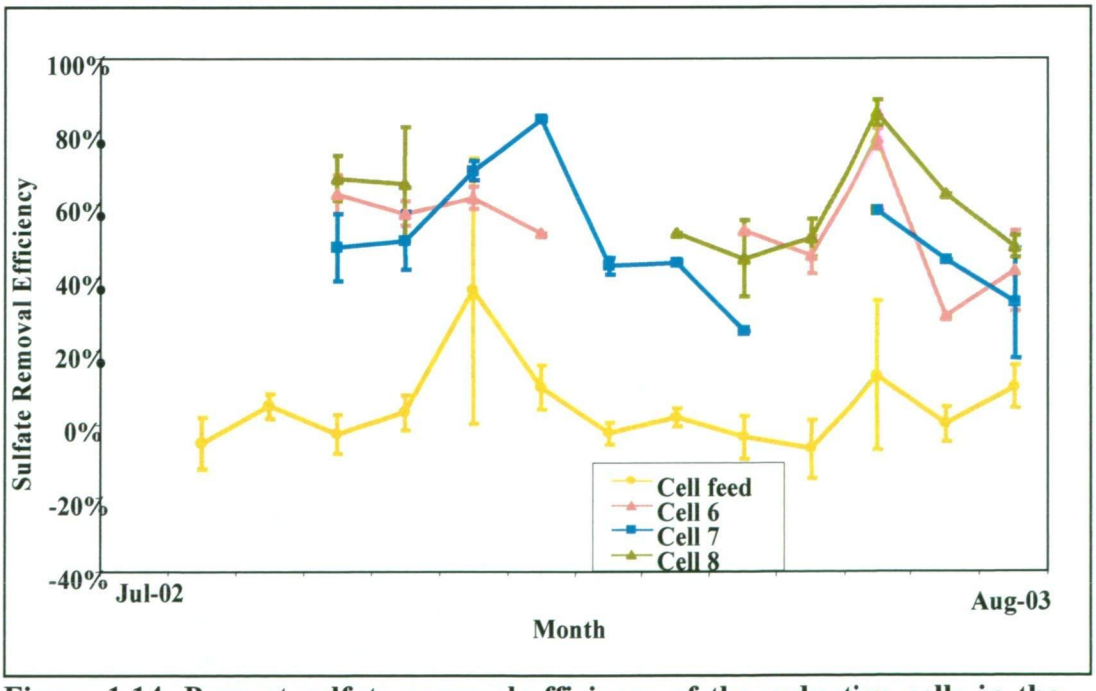


Figure 1.14. Percent sulfate removal efficiency of the reduction cells in the first year of operation (ES&D 2005).

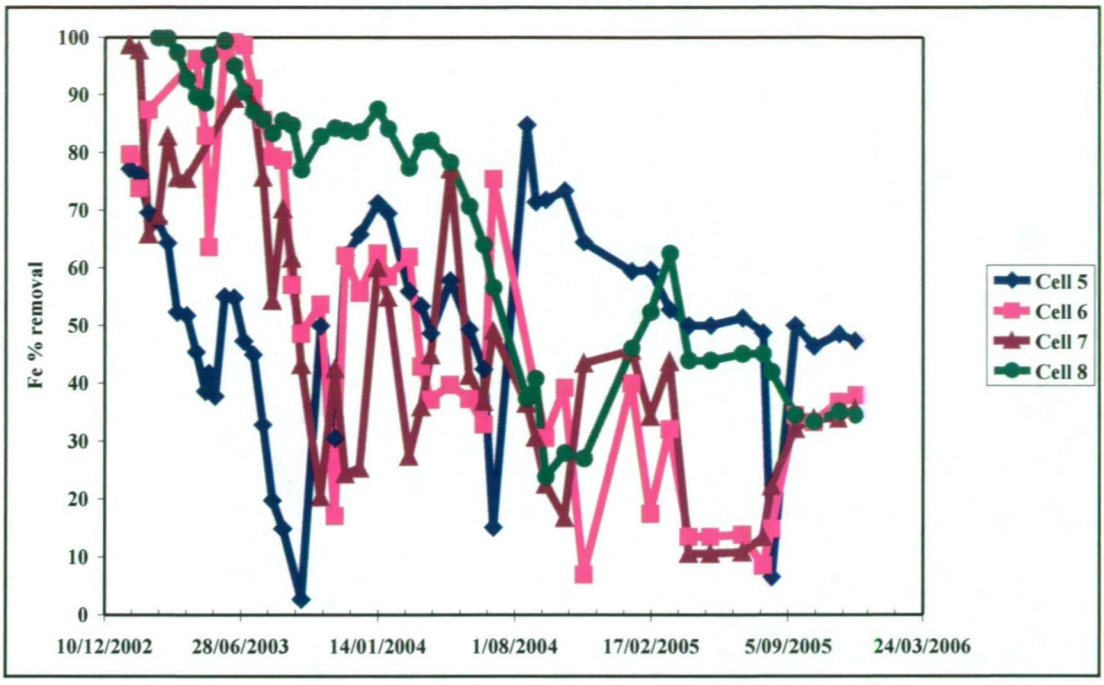


Figure 1.15. Monthly % iron removal efficiency of the reduction cells over time (2003 – 2006), Heybridge, Tasmania. Data from ES&D Technical Report 2005.

Research objectives

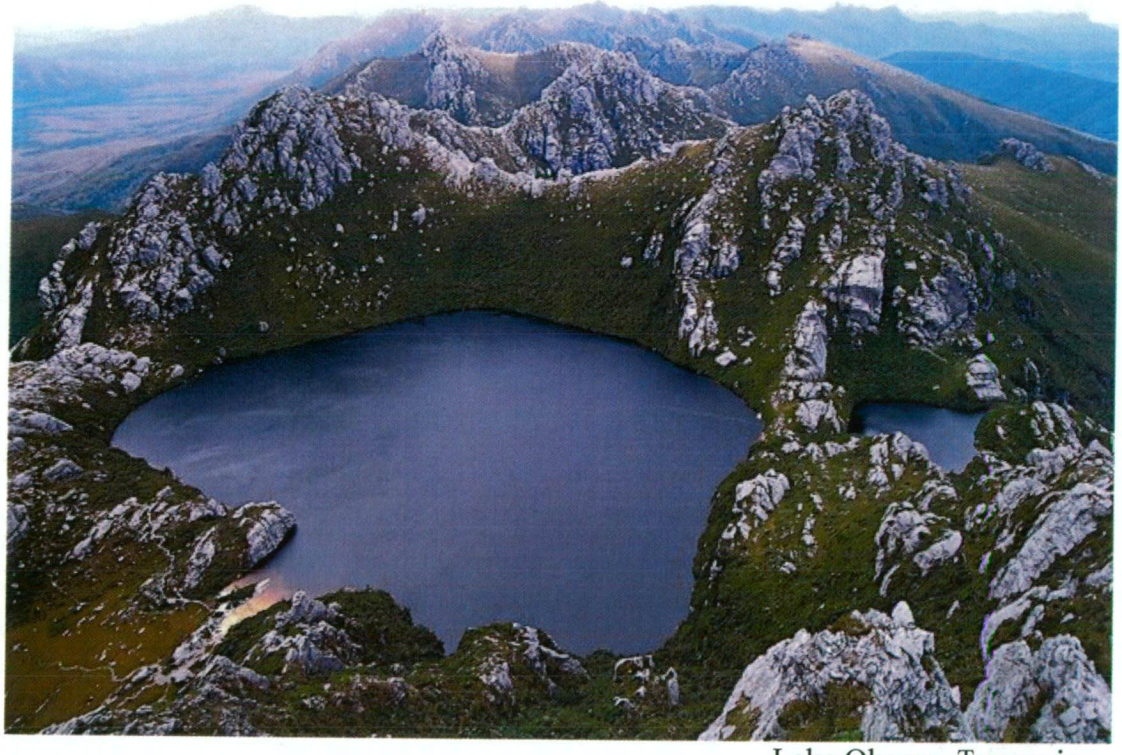
To characterise and optimise a novel remediation strategy for treatment of industrial mildly acidic, metal rich leachate to drinking water quality specifically by;

- 16S rRNA gene clone libraries and terminal restriction fragment length polymorphisms (TRFLP) to identify and monitor bacteria responsible for reducing iron and removing acidity from leachate in the compost system,
- optimise the reduction cell by manipulating the compost parameters as well as trialling cheap, non-toxic carbon sources such as ethanol, molasses, sodium bicarbonate and vegetable oil,
- apply the modifications to pilot scale reduction cells at an industrial site,

- improve the treatment system and permit for a more rapid and economical bioremediation system which can be applied to other contaminated sites in Australia.

Chapter 2.

Literature Review



Lake Oberon, Tasmania

Microbial iron reduction and its role in bioremediation– a review

Microbial iron reduction evolution

Iron is the second most common element on the Earth and the fourth most common in the Earth's crust. It is not surprising that bacteria have evolved to utilise iron and many other metallic elements found in nature in their metabolism. Microbial respiration with ferric iron as the electron acceptor may be the least studied of all the quantitatively important biogeochemical processes, with only sulfate providing a greater potential for the oxidation of organic matter than Fe^{3+} (Lovley 1987). Lovley (1991) further speculated that the reduction of ferric iron might have been the first globally important mechanism for microbial oxidation of organic matter to carbon dioxide.

The discovery that *Geobacter metallireducens* could completely oxidise multi-carbon organic compounds to carbon dioxide coupled to reduction of Fe^{3+} to Fe^{2+} provided a microbial model for this process (Lovley et al. 1987). This process is probably necessary to account for the oxidation of organic matter to carbon dioxide coupled to Fe^{3+} reduction in the banded iron formations. The suggestion that Fe^{3+} reduction may have been the first globally significant process for completely oxidising organic matter back to CO_2 is consistent with the deep-branching phylogenetic clades that Fe^{3+} reduction may have been an early form of respiration (Figure 2.1) (Lovley et al. 1997; Lovley 2002). Vargas et al. (1998) speculated Archaea and Bacteria that are the closely related to the hypothesised last common ancestor could reduce Fe^{3+} to Fe^{2+} and conserve energy for growth.

Iron reduction has also been demonstrated in hyperthermophiles, which have been isolated from several hot microbial ecosystems (Lovley et al. 2000). These populations were found to conserve energy to support growth by oxidising organic matter coupled to Fe^{3+} reduction, and included the archaeon *Pyrobaculum islandicum* and bacterium *Thermotoga maritima*. The finding that all of the extant microorganisms believed to be most closely related to the hypothesised last common ancestor of modern organisms have the ability to couple hydrogen oxidation to Fe^{3+} reduction suggests that the last common ancestor had the ability to use Fe^{3+} as an electron acceptor (Figure 2.1).

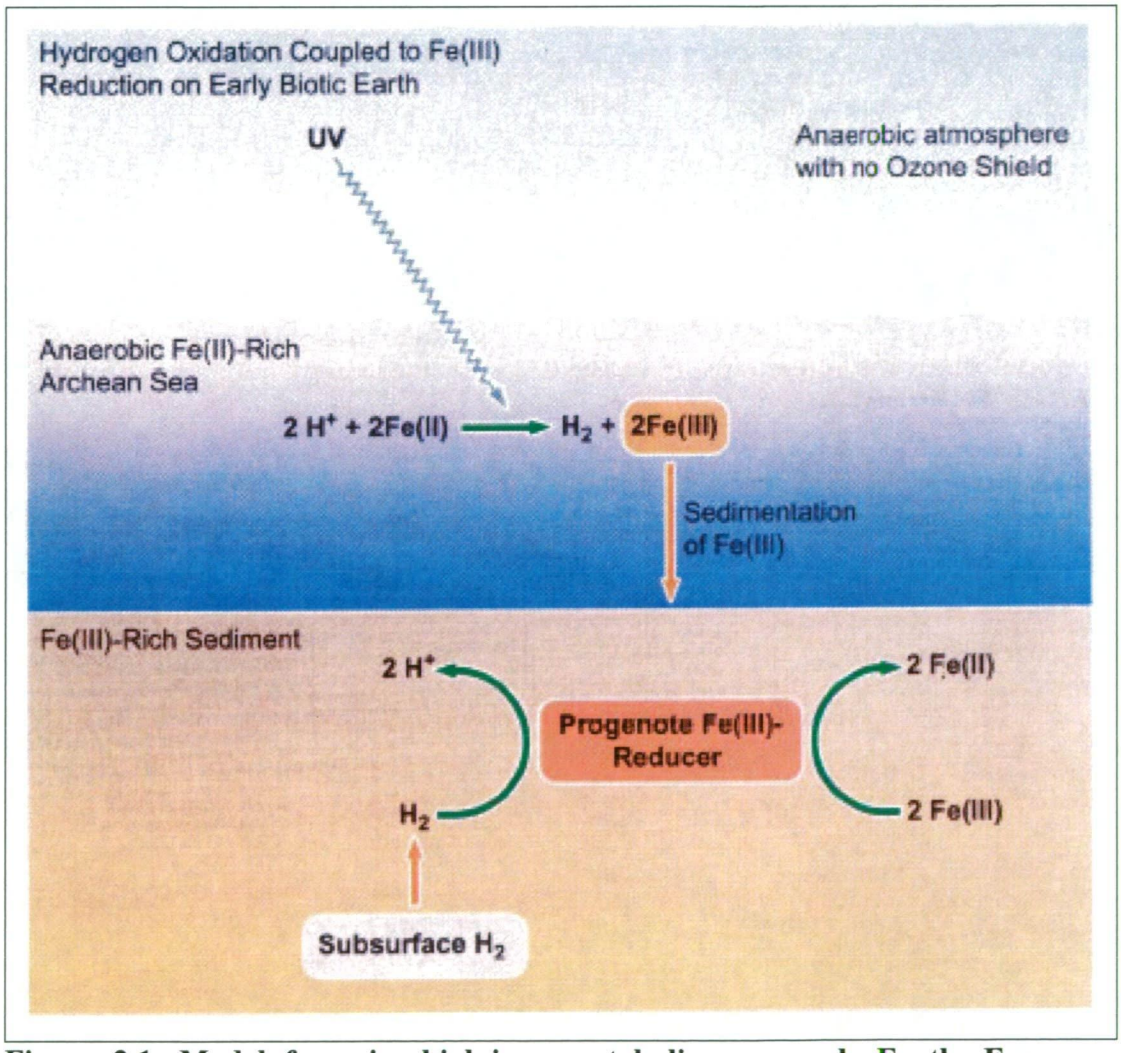


Figure 2.1. Model for microbial iron metabolism on early Earth. From Lovley, 2002.

In many present day sedimentary environments, ferric iron continues to be an important electron sink, even though there is a wide range of other electron acceptors available for organic matter oxidation. Walker (1985) emphasised that under the anaerobic conditions of the first 2 billion years of Earth's history, there was abundant dissolved Fe^{2+} but little dissolved sulfur, oxygen or nitrate. When photosynthesis emerged, a concurrent oxidation of Fe^{2+} to Fe^{3+} occurred as inorganic carbon was reduced to organic carbon.

Microbial biotransformations of metals and metalloid elements are an important step in biogeochemical cycles (Lovley 1993b). Two groups of organisms, dissimilatory and assimilatory (or non-dissimilatory) mainly carry out metal reduction. Dissimilatory reductive processes are carried out under anaerobic conditions, the organisms involved are almost entirely obligate anaerobes, and the metal serves as a terminal electron acceptor for respiration. Non-dissimilatory reductive processes are carried out by aerobic microorganisms, which assimilate metals into cellular components (Lovley 1993b; White and Gadd 1998). However, the dissimilatory iron reducers are the most abundant and include genera such as *Geobacter*, *Shewanella, Desulfuromonas*, *Pelobacter, Ferrimonas, Geovibrio* and *Geothrix.* These organisms have a broad spectrum of other metabolic capabilities as well, for example, many dissimilatory metal reducers can reduce soluble U^{6+} to insoluble U^{4+} (Lovley 2000). Iron-reducing microorganisms have been divided into groups by the processes they use to couple with iron reduction. There are four main groups, fermenters, organic acid oxidisers, aromatic compound oxidisers and hydrogen oxidisers (Lovley 1992).

Organisms also previously thought of as obligate anaerobes and sulfate reducers will reduce iron and other metals under anaerobic and aerobic conditions, and include *Desulfovibrio* spp. and *Thiobacillus* spp. (Rabus et al 2000). In 1993, the mechanisms for electron transport in anaerobic dissimilatory metal reducers were largely unknown as the sites are outside the cell (Lovley 1993a). However, these processes, and their significance to larger biogeochemical cycles were beginning to interest many researchers (Lovley 1995a; White et al. 1995; Roane et al. 1996). At this point however, research had not shown whether the metal-reducing microorganisms available in pure cultures were representative of soil/sediment microbiota. The ability to reduce iron appears to be widespread amongst acidophilic bacteria, presumably because many extremely acidic environments (pH < 3) contained elevated concentrations of soluble iron. The redox potential of the ferrous/ferric iron couple is related to pH and is most positive in extremely acidic environments (Eh = +770mV at pH 2). This value is close to that of the oxygen/water couple (+840mV), implying that ferric iron is an attractive alternative electron acceptor to oxygen in low pH environments (Johnson and Bridge 2002).

Research history of microbial iron reduction

Starkey and Halvorsen (1927), using *Clostridium* sp. and *E. coli,* first determined that microbial metabolism was required for the reduction of Fe^{3+} with glucose. However, they thought Fe^{3+} reduction resulted from the organisms lowering the oxygen concentration and producing fermentation acids, which was considered to chemically shift the Fe^{3+}/Fe^{2+} equilibrium towards Fe^{2+}. Bromfield (1954) provided evidence that reduction of Fe^{3+} was more directly related to the

metabolism of microorganisms. Bromfield (1954) found that aerobically grown cultures of soil bacteria reduced Fe^{3+} when incubated anaerobically. Fe^{3+} was not reduced if chloroform was added, if the cultures were autoclaved, or if the cells were removed by centrifugation. De Castro and Ehrlich (1970) demonstrated that *Bacillus* sp enzymatically reduced Fe^{3+}, which did not occur if the glucose medium was not inoculated with the organism or if glucose was omitted. Furthermore, Fe^{3+} was not reduced when the pH of the uninoculated medium was reduced, demonstrating that a decrease in pH did not induce chemical Fe^{3+} reduction.

Research subsequently concentrated on how microorganisms reduced iron. For example, Munch and Ottow (1983) demonstrated that the reduction of Fe^{3+} during glucose metabolism by *Clostridium butyricum* or *Bacillus polymyxa* was not due to synthesis of fermentation products or a low redox potential. Reduction of Fe_2O_3 was inhibited if the Fe_2O_3 in the culture was enclosed in dialysis tubing, even though pH and redox potential were as low as in cultures where the Fe_2O_3 was not separated. These results indicated a direct contact between the microorganism and Fe^{3+} was required, and the authors suggested that microbial enzymes were necessary to catalyse the reaction.

Ottow and von Klopotek (1969) proposed that two enzymes catalysing Fe^{3+} reduction, and they were linked to electron transport. One proposed pathway was electron transport with nitrate reductase. Experiments revealed reduction of Fe^{3+} was decreased in nitrate reductase negative (nit-) bacteria compared to nitrate reductase positive (nit+) strains. Furthermore, less Fe^{2+} accumulated in cultures of nit+ cultures when nitrate was included in the medium. This was interpreted as a

preference for nitrate reduction (Ottow 1970; Munch and Ottow 1983), but this interpretation was criticised since nitrite is produced from nitrate reduction and nitrite can chemically oxidise Fe^{2+} (Obuekwe et al. 1981). The other proposed pathway was electron transport with ferrireductase, which could reduce Fe^{3+} or Mn^{4+} but not nitrate. Evidence for a ferrireductase pathway that was distinct from nitrate reductase pathway was reduction of Fe^{3+} in organisms such as *Clostridium*, which do not reduce nitrate, and the continued Fe^{3+} reduction in nit- cultures (Ottow and von Klopotek 1969). Once bacterial iron reduction had been proven, research moved into what electron donors could be coupled to iron reduction.

Lascelles and Burke (1978) first demonstrated that bacterial iron reduction linked to organic matter oxidation. Their experiments showed that Fe^{3+} citrate was reduced with nicotinamide adenine dinucleotide (NADH) or succinate as electron donors. Rotenone inhibited Fe^{3+} reduction with NADH as the electron donor, indicating a need for a functional primary dehydrogenase for Fe^{3+} reduction. Lascelles et al. (1978) experimented with a variety of electron donors using a membrane preparation of *Staphylococcus aureus;* and showed that the nitrate reductase inhibitor azide did not affect iron reduction. This suggested a component in the electron transport chain other than a nitrate reductase reduced Fe^{3+}.

Arnold et al. (1986) conducted experiments with *Pseudomonas ferrireductans* and suggested the organism contained both a constitutive (an enzyme produced in constant amounts regardless of conditions) and an inducible (enzyme produced in response to conditions) Fe^{3+} reductase. Under anaerobic conditions, cells that had been grown under low oxygen conditions had rates of iron reduction 6-8 fold

higher than the rates in cells grown at higher oxygen concentrations. Furthermore, the addition of an inhibitor (Rotenone, Dicumarol, NaCN or NaN_3) did not seem to affect cells under low oxygen conditions. These results were interpreted as demonstrating the presence of a constitutive Fe^{3+} reductase and a second Fe^{3+} reductase that was induced at low oxygen concentrations. This raised questions as to whether or not bacteria just obtained energy but also increased biomass directly from iron reduction. Later work (Johnson and Bridge 2002) found that two species of the same genus had different enzymatic systems. *Acidiphilium acidophilum* had an inducible iron reductase system while that in *Acidiphilium* SJH it was constitutive.

Balashova and Zavarzin (1980) found that a *Pseudomonas* sp. consumed hydrogen with the reduction of Fe^{3+} with approximately two Fe^{3+} ions reduced per hydrogen ion consumed. After ten days of growth the number of viable cells in media with hydrogen and Fe^{3+} was 4×10^6 cells ml^{-1} compared to media without Fe^{3+}, which was 3×10^3 cells ml^{-1}. Jones et al. (1983) also found an increase in ATP yields in the presence of Fe^{3+} with hydrogen as an electron donor in lake samples but only under certain redox conditions. Further studies reported more or less growth depending on the electron donor available. Jones et al. (1984) found Fe^{3+} reduction did not result in a detectable increase in growth of a glucose-fermenting *Vibrio* sp., although the growth yield of a malate-fermenting *Vibrio* sp. increased nearly 30% when Fe^{3+} was added. Knowledge of the mechanisms for energy generation coupled to Fe^{3+} reduction at this point was in its early stages. Hence, further research explored into different electron donors linked to Fe^{3+} reduction.

Microorganisms involved in bioremediation of metalliferous pollution

In 1987 Dr Derek Lovley and his team at the Environmental Biotechnology Center, University of Massachusetts isolated *Geobacter metallireducens*, a bacterium found to couple iron reduction with oxidation of organic matter (Lovley et al. 1987). However, it was first used for the *in situ* bioremediation of organic contaminants coupled to iron reduction not the bioremediation of metals promoted by organic matter addition (Lovley et al. 1993). Therefore, up until this point most strategies for bioremediation for metal contamination have focused on *ex situ* methods using above ground reactors. However, many instances arose where pumping costs were expensive or other considerations have favoured an *in situ* approach (Anderson and Lovley 1997; Anderson et al. 1998). Lovley et al. (1991) suggested iron-reducing bacteria could reduce soluble uranium (U^{6+}) to insoluble uranium (U^{4+}), the first mention of iron-reducing bacteria being used for bioremediation of recalcitrant molecules.

Many early engineering studies into bioremediation of acid mine drainage and acidic, metal-rich mine wastewaters attributed metal reduction to the action of sulfate-reducing organisms (Czekalla et al. 1985; Gyure et al. 1990; Barnes et al. 1991; Barnes et al. 1992; Dvorak et al. 1992; Scheeren et al. 1992). These bacteria were found to reduce sulfate to sulfide, which bind metals and precipitate as metal sulfides. However, it was more likely a consortium of sulfate-and iron-reducers as well as iron-oxidisers, such as *Acidithiobacillus* spp. as these bacteria were found to be metabolically important (Brock and Gustafson 1976; Ohmura et al. 2002; Duquesne et al. 2003). Furthermore, *Acidithiobacillus* spp. were found to be capable of both oxidation and reduction of iron under varying redox

conditions (Ohmura et al. 2002). Johnson and Bridge (2002) also found *Acidiphilium* spp could reduce Fe^{3+} under microaerophilic conditions; these bacteria are normally obligate aerobes. However, researchers did not clearly understand the mechanisms of microbial metal reduction and how it could be employed for environmental restoration. These versatile bacteria would be the most likely candidates for bioremediation as they do not require strict anaerobic conditions like *Geobacter* spp. and can survive in acidic conditions, which most if not all, mine leachates have. However, the amount of oxygen in the bioremediation system has to be closely monitored, if it becomes too high the acidophiles will start oxidising iron (Johnson and Hallberg 2005).

Despite the environmental significance of dissimilatory Fe^{3+} reduction, little is known about the diversity of microorganisms responsible for this process. Until recently, the only two studied microorganisms were *Geobacter metallireducens (Deltaproteobacteria)* and *Shewanella putrefaciens* (previously *Alteromonas*) *(Gammaproteobacteria)* (Lovley 1991). However, other organisms from these groups have been isolated from a diversity of environments, for example, *Desulfuromonas* spp. and *Geothrix* sp. (Lonergan et al. 1996, 1997; Lovley 2002) (Figure 2.2; Figure 2.3). A greater awareness of the biodiversity and the metabolic capabilities of these microorganisms will have a major impact on bioremediation systems and increase the options available for controlled and directed bioremediation of polluted waters (Johnson et al. 2002).

Lonergan et al. (1996) found a diverse range of bacteria that could reduce iron coupled to the oxidation of organic carbon (Figure 2.2). However, they raised the question whether these organisms all reduced Fe^{3+} via similar electron transport

chains or whether the capacity for electron transport to Fe^{3+} had evolved independently in different groups. For example, earlier studies suggested that more than one *c*-type cytochromes were involved in electron transport of Fe^{3+} in iron-reducing *Gammaproteobacteria* and *Deltaproteobacteria* (Lovley 1995a). However, *Pelobacter* spp. (which lack *c*-type cytochromes) can also grow with Fe^{3+} as the electron acceptor, indicating that *c*-type cytochromes are not necessary for Fe^{3+} reduction (Lovley 1995a). The close phylogenetic relationship between the genera *Pelobacter, Geobacter* and *Desufluromonas* makes it likely that these organisms all have a common mechanism for electron transport to Fe^{3+} and thus the role of *c*-type cytochromes must be further researched. In addition, the discovery that some hyperthermophiles can also reduce iron raised the question of whether mechanisms for dissimilatory iron reduction have been conserved throughout microbial evolution (Lovley 2000). *Pyrobaculum islandicum*, the hyperthermophile most studied, does not contain *c*-type cytochromes (Lovley 2000). Hence, it is unlikely that a single strategy for electron transport to Fe^{3+} is present in all iron reducing microorganisms (Childers and Lovley 2001).

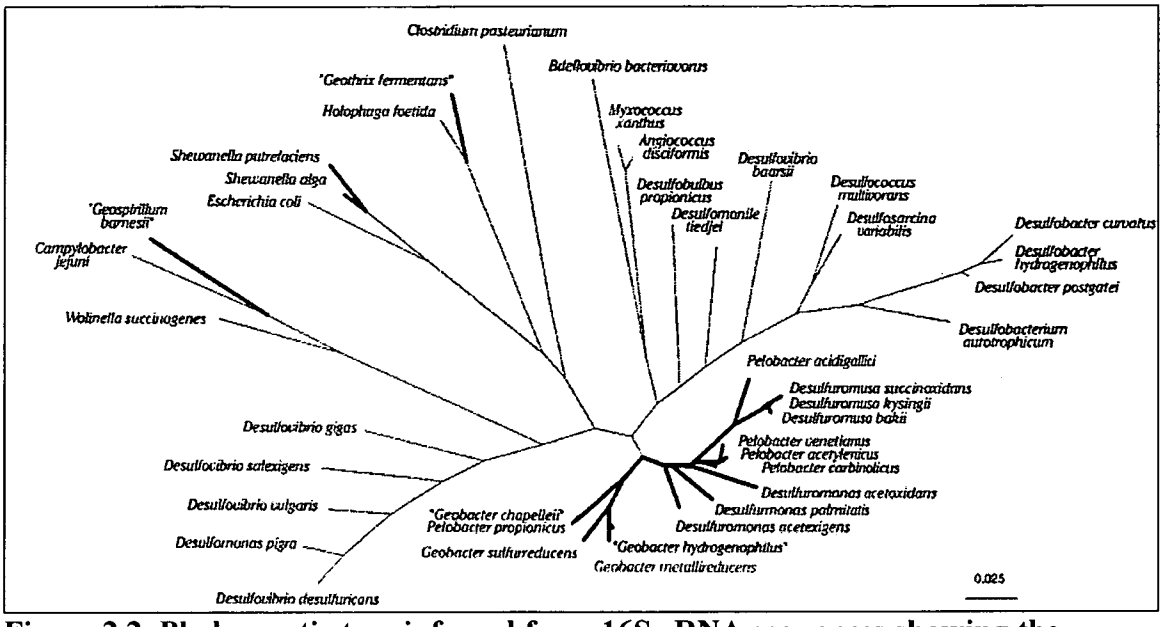


Figure 2.2. Phylogenetic tree inferred from 16S rRNA sequences showing the lineages of Fe^{3+}-reducing bacteria (bold lines). Bar represents a 0.025% sequence difference. From Lonergan et al. 1996.

Studies have demonstrated that ferric iron-reducing microorganisms contain membrane bound electron transport components that are necessary for electron transport to Fe^{3+} oxides, but the protein responsible for the final transfer has not yet been identified (Lovley and Coates 2000) and electron shuttling or chelating between the cell and the iron is probably unlikely in all cases (Lloyd et al. 1999). However, *Shewanella* sp. and *Geobacter metallireducens* have been found to produce pilin-like filaments that function as "nanowires" to transfer electrons outside the cell onto insoluble electron acceptors, such as iron minerals (Leys, et al. 2002; Kus et al. 2005). The filaments, which are only 3-5nm in width, can extend more than 20μm in length. Elucidation of this novel form of electron transfer will provide insight into the mechanisms by which *Geobacter* can contribute to bioremediation of groundwater contaminated with organic and metal contaminants. The ability to mass-produce these long conductive filaments may have application in the development of nanoelectronic devices, sensors, and microbial-based fuel cells (Reguera et al. 2005).

Iron reduction and organic matter metabolism

The ability of some microorganisms to reduce Fe^{3+} has been known since the 19th century. However, it was thought that the known iron-reducers only reduced iron as a trivial side reaction in their metabolism (Lovley 1987). This process was thought to not yield energy to support growth. It was not until the 1980s that Lovley and Phillips (1986) found iron-reducing bacteria were shown to metabolise fermentable substrates to produce energy. The ability of some microorganisms to couple the oxidation of fermentation products to the reduction of ferric iron means that is it possible for a community of microorganisms to completely metabolise organic matter with ferric iron as the sole electron acceptor (Lovley 1987).

Researchers at this stage had been unable to isolate an organism capable of completely oxidising glucose with Fe^{3+} as an electron acceptor. This was puzzling as such a metabolic capability would be competitive in a substrate-limited anaerobic environment over organisms that ferment glucose, as complete oxidation of glucose to carbon dioxide (CO_2) yields more ATP than fermentation (Lovley 1987). However, other fermentation products such as acetate, butyrate, propionate, ethanol and methanol were metabolised with Fe^{3+} reduction in enrichment cultures (Lovley and Phillips 1986). Experiments using sediment samples instead of laboratory cultures also demonstrated Fe^{3+} reduction coupled to fermentation products. Acetate additions were found to stimulate Fe^{3+} reduction in rice paddy soils (Kamura et al. 1963) and freshwater sediments from the Potomac River (Lovley and Phillips 1986). Since fermentation products can be metabolised with Fe^{3+} reduction, the complete anaerobic mineralisation of

fermentable organic matter with Fe^{3+} reduction is possible (Lovley 2000). This potential had not yet been recognised as important in some environments such as metal-rich mine wastes where this metabolism could be exploited for passive, low cost remediation.

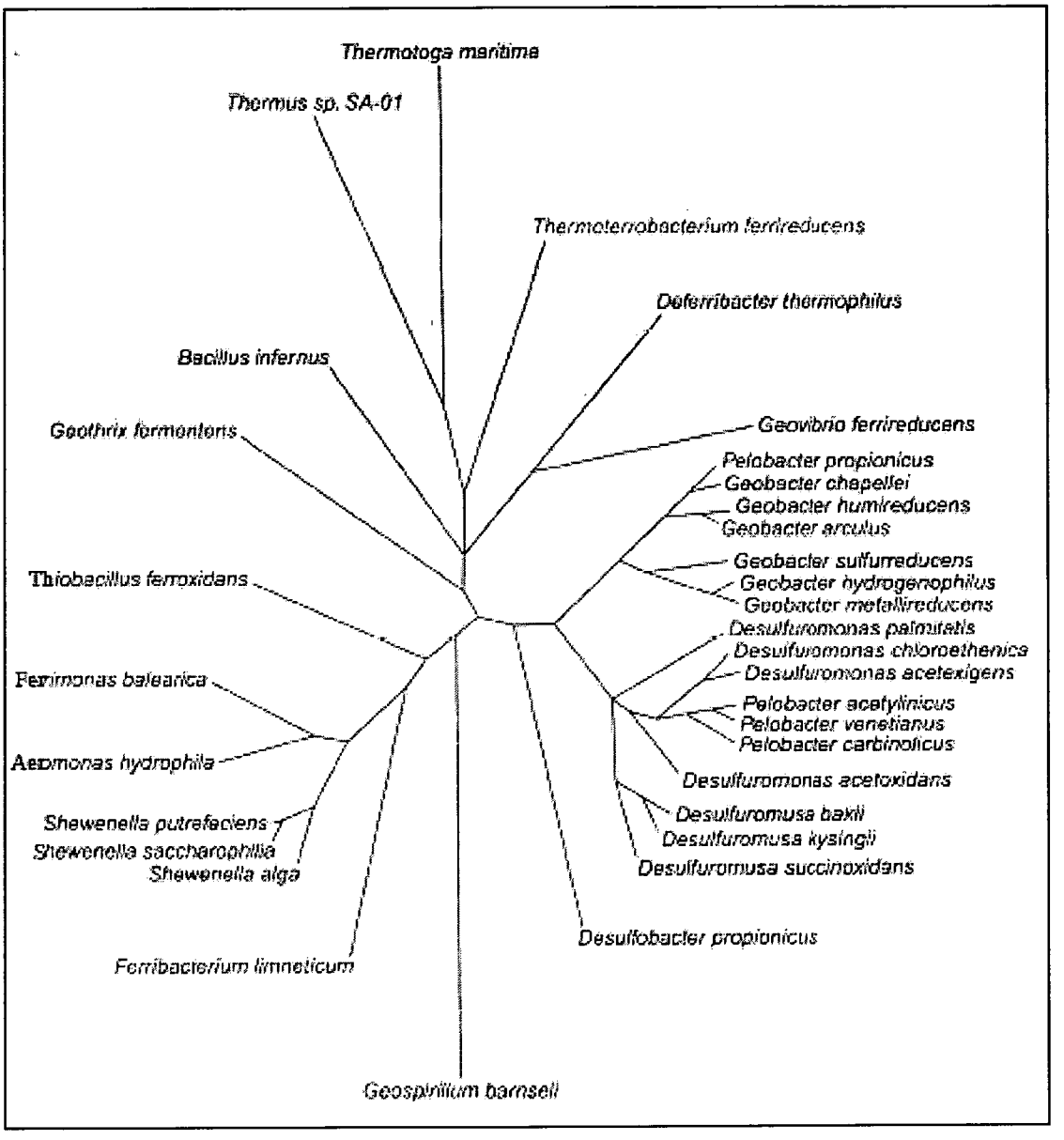


Figure 2.3. Bacteria known to conserve energy to support growth by either oxidising hydrogen or organic compounds with the reduction of Fe^{3+}. From Lovley (2002).

Modern research techniques for determining bacterial communities as applied to acidic, metal-rich wastewaters and acid mine drainage

Molecular biology techniques, using targeted 16S rRNA gene primers on sediment or soils samples, are powerful tools for determining the structure of microbial communities, however are not without their limitations. Lonergan et al. (1996) used these techniques on members of the *Geobacter, Desulfuromonas, Pelobacter* and *Desulfuromusa* branch of the delta subdivision of the *Proteobacteria.* They found that these bacteria were linked physiologically as well as phylogenetically but were diverse enough to be grouped into a single family *Geobacteraceae,* rather than as a single genus as suggested by Devereux et al. (1990). The Lonergan *et al.* (1996) study investigated 16S rRNA gene oligonucleotide probes for *in situ* identification of sulfate-reducing bacteria and found the probes also worked on iron-reducing bacteria such as *Geobacter* sp. and *Pelobacter* sp. as they only had a single base mismatch to the target site of the probe. Although it would be nice to have specific oligonucleotide probes for studying bacterial populations, caution must be applied in using these probes.

The use of 16S rRNA gene-directed probes for studying the distribution of dissimilatory Fe^{3+} reducers is also complicated by the fact that the ability of bacteria to use Fe^{3+} as a terminal electron acceptor is not limited to *Deltaproteobacteria.* Iron reduction has been found in members of *Gammaproteobacteria* including *Shewanella* and *Pseudomonas* (Balashova and Zavarzin 1980; Balashova 1985; MacDonell and Colwell 1985) and *Betaproteobacteria* such as *Ferribacterium limneticum* (Cummings et al. 1999). Cultured iron-reducing bacteria such as *Geothrix fermentans* (Coates et al. 1999) a member of the phylum *Acidobacteria,* and *Geovibrio ferrireducens* (Caccavo et

al. 1996) a member of the phylum *Deferribacteres* have been found to be unrelated to any other iron-reducing bacteria. The wide phylogenetic dispersion of dissimilatory Fe^{3+} reducers suggest that either Fe^{3+} reduction evolved multiple times was a widespread early form of respiration or lateral gene transfer occurred similar to examples in sulfate-reducing bacteria (Meyer and Kuever 2007).

This diversity indicates that using many different 16S rRNA gene-targeted probes would be required to determine the structure of an Fe^{3+}-reducing community and this would not be feasible. Lonergan *et al.* (1996) suggested using 16S rRNA clone libraries and DGGE (Denaturing Gradient Gel Electrophoresis) instead of probes for elucidating the bacterial community composition. However, DGGE is now a common method and no one has come up with a quick and easy diagnostic tool for evaluating iron-reducing bacterial diversity. Techniques such as TRFLP (Terminal Restriction Fragment Length Polymorphism) and ARISA (Automated Ribosomal Intergenic Spacer Analysis) are popular as quick molecular methods for estimating population diversity (Danovaro et al. 2006), especially for industrial applications that do not want expensive and complicated testing methods. TRFLP provides a generalised appraisal of the diversity of bacterial communities by assessing phylogenetic differences of fragments lengths in a given sample (Liu et al. 1997), in contrast, ARISA targets the intergenic 16S-23S internally transcribed spacer sequences (ITS1) which can be variable in length and nucleotide sequence between and within bacterial species. ARISA does not require enzymatic digestion of PCR products and is faster and lower in cost.

Sources of bias in molecular methods

Molecular methods have revolutionised the study of all living things, however, they do not come without their faults. Pitfalls arising from PCR amplification include inhibition of the reaction by co-extracted contaminants, differential amplification (Lueders and Friedrich 2003), formation of PCR artifacts (Suzuki and Giovannoni 1996; Osborne et al. 2005), and biases associated with 16S rRNA sequence variations due to *rrn* operon heterogeneity (Farrelly et al. 1995; Klappenbach et al. 2001). All of these have the potential to influence subsequent measures of the extant microbial diversity (Thies 2007).

Inaccuracies during sizing of TRFs due to differences in purine content of the amplicons have also been reported (Kaplan and Kitts 2003). Despite running the size marker in every lane of the sizing gel, which is meant to increase accuracy in sizing, errors in sizing calls are still common. Marsh (2005) reported that fragments labeled with different fluorophores may have altered mobility in DNA sizing gels or capillaries. Thus, when sample TRFs are compared with the size marker, each labeled with a different fluorophore, base pair lengths are inaccurately calculated. This may lead to miscalls in TRF length of up to 7 base pairs (bp) (Marsh 2005). This would further exacerbate the process of matching TRFs to phylogenetic groups. *In silico* digests of clone libraries from the same DNA extraction would go some way to alleviate this problem.

Despite these limitations TRFLP is still a highly discriminatory, high-throughput method that has been used successfully to characterize microbial communities from a wide range of environments. Any gene for which appropriate primers can be designed can be analysed by this method. The number of publications in which

T-RFLP has been used to characterize microbial communities continues to grow. The technique is robust, highly reproducible, and the ability to resolve a greater number of OTUs, coupled with clone libraries, makes the T-RFLP method an excellent choice for community comparisons, particularly in survey-based studies (Thies 2007).

An alternative solution to overcome these limitations is to use genomic DNA (gDNA) as target that does not need prior amplification. Genomic microarrays are a recently developed, powerful genomic technology and are used to study gene expression in pure cultures, but have been found challenging in their application to environmental samples as many of these samples contain unknown bacteria (Avarre et al. 2007). Chandler et al. (2006) developed a 16S rRNA gene-targeted tuneable bead array for uranium-contaminated sediments undergoing bioremediation. The array used probes targeting bacterial species known to occur in contaminated sites and whose entire 16S rRNA gene sequences were in the GenBank sequence database. The probes were first validated against strains of the targeted bacterial species. This high-throughput method is an effective tool for determining microbial community structure and dynamics. However, the authors are quick to point out the method has limitations in not providing quantitative outcomes and needs further validation against many more bacterial species.

The next logical step is sequencing genomes of the bacteria that are involved in the creation and remediation of acid mine drainage and wastewater. Genome sequencing from environmental samples, or metagenomics, has proven challenging because of the immense diversity of natural samples, with the exception of environments such as the acid mine habitat which have been found

to have relatively low population diversity (Tyson et al. 2004; Abulencia et al. 2006; Ward 2006). Analysis of the functional genes in an environmental sample makes much more sense. Determining which genes are functioning in a sample, especially metabolic genes would provide more insights into the biogeochemical cycles. Microarray technology has proved to be a valuable tool for determining the biogeochemical processes and the bacteria involved (Avarre et al. 2007). Functional gene arrays (FGAs) have been employed in bioremediation studies, for example GeoChip (He et al. 2007). The GeoChip contains 24,000 probes for all the known genes involved in various biogeochemical, ecological and environmental processes, including C, N and S cycling, phosphorus utilisation, organic contaminant degradation and metal resistance and reduction.

Proteomics is an emerging technology that may also be applied to bioremediation. It is the large-scale study of proteins, particularly their structure and functions. Proteins are vital parts of living organisms, as they are the main components of the physiological metabolic pathways of cells. Hence, proteomics may help understand how bacteria react to the presence of toxic substances and provide insights into the mechanisms involved in adaptive responses to chemicals. The identification of the expressed proteins might help to reveal mechanisms of defence, detoxification and adaptation. This information would expand the knowledge of the degradative capability of microorganisms for bioremediation of pollutants by understanding how to modify the regulatory and catabolic genes by providing suitable growth conditions (Zhao and Poh 2008).

Chapter 3.

Microbial Diversity

Russell Falls, Tasmania

Microbial diversity and community dynamics within a novel *in situ* acidic, sulfate- and metal- rich titanium processing leachate treatment system

Abstract

The microbial diversity and dynamics of a novel *in situ* bioremediation system, utilising agricultural waste and composted material was assessed. The system was developed to treat acidic (pH 3 – 5), sulfate (540 – 1700mg L^{-1}) - and iron (800 – 1200mg L^{-1})-rich titanium-processing leachate. Diversity and community structure was determined over 18 months from different stages of the treatment system including untreated sludge dam leachate, pre-treated effluent from an anoxic potato-containing section intended to increase alkalinity and prevent iron precipitation; and effluent from a series of mushroom compost and straw-based iron-reduction cells. 16S rRNA gene clone libraries revealed a community shift from a mixed iron- and sulfate-oxidising and reducing community in the sludge dam leachate to a community dominated by *Acidithiobacillus* spp. and anaerobic fermenters (related to the genera *Bacteroides* and *Paludibacter*) in the potato cell effluent. The reduction cell effluents proved to have higher microbial diversity and greater heterogeneity, including iron-and sulfate reducers, iron-oxidisers, anaerobic fermenters and in one sampled effluent a high proportion of clone clustering within candidate division OP3. Multivariate statistical analysis of 16S rRNA gene-based TRFLP data revealed significant community differences occurred between treated/post-treated samples with untreated/pre-treated samples. TRFLP also indicated temporal shifts in the bacterial community composition occurred in the reduction cells although after 11 months of treatment microbial communities in three of four reduction cells showed evidence of community

stabilisation probably due to exhaustion of an available carbon source and layered design of the system. There was no evidence of any seasonal effect on the microbial community. The GenBank accession numbers for the 16S rRNA gene clones are EU921150 – EU921224 and PopSet identification number is 197203276.

Introduction

The role of sulfate-reducing bacteria in acid mine waters was first studied extensively by Colmer and Hinkle (1947), but Tuttle and colleagues (1969) first published about microbial remediation of high acid, metal and sulfur rich waters. Sulfate-reducing bacteria have been widely studied and their metabolism is well known and is indirectly used to precipitate metals (Rabus et al. 2000). However, there is still a lack of information about the microorganisms involved and factors controlling their growth and metabolism in polluted environments which often limits bioremediation implementation and/or long term remediation strategies (Lovley 2003). Ideally, bioremediation systems would be designed based on knowledge of: the microorganisms that are present in the contaminated area, their metabolic capabilities, how they respond to changes in environmental conditions and whether they can be used to an advantage in remediation.

Molecular biology methods, such as 16S rRNA gene clone libraries and taxonomic specific 16S rRNA gene primers on sediment or soils samples are powerful tools for determining the structure of microbial communities. Furthermore, many of the bacteria found in compost-based bioremediation systems are difficult to culture. Hence, culture-independent techniques are indispensable when characterising the bacterial populations (Alleman et al. 2005;

Geets et al. 2005; Akob et al. 2008). The use of 16S rRNA gene-directed oligonucleotide probes for studying the distribution of dissimilatory Fe^{3+} reducers is complicated by the fact that the ability of bacteria to use Fe^{3+} as a terminal electron acceptor is not limited to one group. Iron reduction not only been found amongst the *Deltaproteobacteria* but also the *Gammaproteobacteria* such as *Shewanella* and *Pseudomonas* (Balashova and Zavarzin 1980; Balashova 1985; MacDonell and Colwell 1985) and more phylogenetically distinct bacteria such as *Geothrix fermentans* (Coates et al. 1999) and *Geovibrio ferrireducens* (Caccavo et al. 1996).

16S rRNA gene-based techniques such as TRFLP are now well-accepted molecular methods for microbial community analysis (Liu et al. 1997). They provide a generalised profile of the microbial community in a complex environmental sample that would otherwise be difficult and laborious to obtain via cultivation or expensive by developing and analysing all the primer target sequences (eg. microarray analysis) or creating and sequencing large 16S rRNA gene clone libraries. TRFLP, in combination with representative 16S rRNA gene clone libraries, can provide a comprehensive microbial diversity profile. TRFLP analysis has been found to be more sensitive to DGGE analysis of complex soil microbial communities because it can produce a higher number of operational taxonomic units (OTUs) and can be analysed overnight using capillary electrophoresis for a larger number of samples (Thies 2007). Furthermore, TRFLP data can be analysed with multivariate statistical methods such as those employed in ecological community structure analysis (Rees et al. 2004; Clarke and Gorley 2006) and thus can provide simple and effective means to interpret data. Several studies have used these techniques in the study of microbial

communities in bioreactors (Johnson and Hallberg, 2005), creosote contaminated soil (Grant et al. 2007), sewage sludge (Macdonald et al. 2007), acid mine drainage (Benner et al. 2000; Bruneel et al. 2006; Nicomrat et al. 2008) and creek sediments (Edmonds et al. 2008).

The ICI Tioxide titanium pigment production plant operated between 1948 and 1996 in Heybridge, located on the north coast of Tasmania on the Blythe River catchment. During its operation iron oxide waste was discharged directly into Bass Strait. The titanium pigment was manufactured by the sulfate process that used large volumes of sulfuric acid to extract titanium from ilmenite ore. This process created large amounts of acidic (pH 3 – 5) metalliferous waste that was separated from the process and stored in clay-lined sludge dams, but which eventually leaked into the water catchment. The leachate is chemically similar to acid mine drainage with high iron (800 - 1200 mg L^{-1}) and sulfate (540 – 1700 mg L^{-1}) concentrations and smaller quantities of manganese lead, copper and zinc (Cooper 2004).

The goal of the remediation project was to establish a passive, low-maintenance, efficient water treatment system *in situ* that could purify leachate generated from the contaminated materials over a thirty year period. A combined anaerobic and aerobic wetland system (Figure 3.1) was developed and operated by Environmental Services and Design PL (ES&D) in order to remove the metals, increase the pH and reduce the biological oxygen demand (BOD) and the chemical oxygen demand) COD. The anaerobic section comprised two "potato cells", a pre-treatment step to keep the metals mobile until the next step, introduce starch as a source of organic carbon for the bacteria, and four anaerobic compost

bioreactors or "reduction cells." The cells consist of a base of bluestone overlaid by mushroom compost and capped with straw bales and then black plastic to create an anaerobic environment (Figure 1.8). This environment encourages sulfate- and metal-reducing bacteria to produce alkalinity and hydrogen sulfide, which precipitates metals from the water as sulfides. However, the reduction cells have slowly decreased in iron removal efficiency from 2002 (Figure 1.11) and were shut down in 2007 (Cooper 2007 pers. comm).

The aim of this study was to determine the bacterial diversity associated processes of this novel *in situ* treatment system using 16S rRNA gene clone libraries and employ TRFLP analysis to track bacterial community changes over time and space within the system.

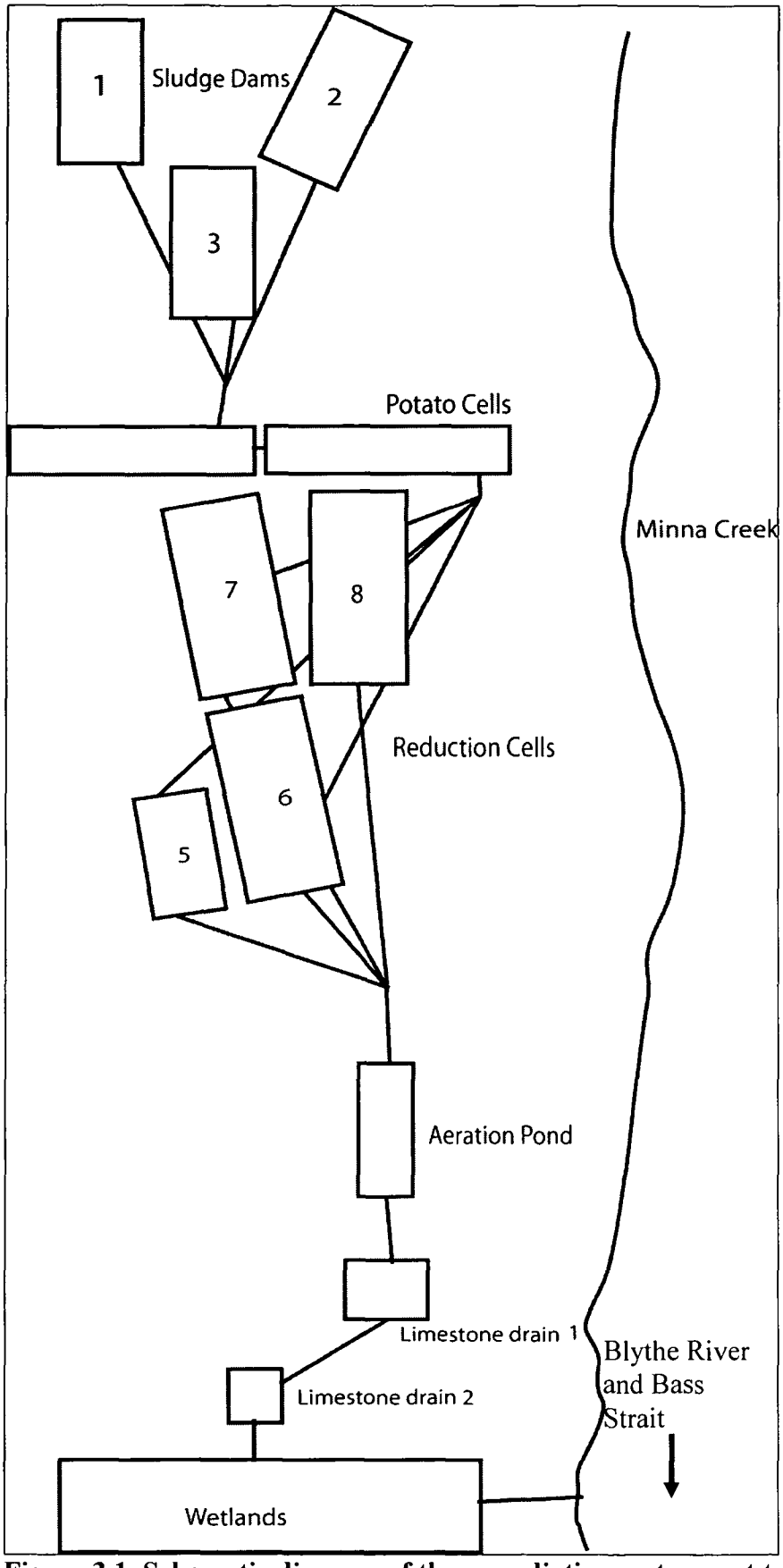


Figure 3.1. Schematic diagram of the remediation system, not to scale.

Materials and Methods

Sampling procedure

Samples were collected from the Minna Creek Valley remediation site north-west Tasmania (41°05.05'S, 145°98.46'E). Triplicate samples were aseptically taken from the potato cell feed (sludge dam leachate) (PCF1-3), reduction cell feed (potato cell) (RCF1-3), reduction cell 7 base water (RC7BW1-3), reduction cell 5 discharge (RC5D1-3) reduction cell 6 discharge (RC6D1-3), reduction cell 7 discharge (RC7D1-3), reduction cell 8 discharge (RC8D1-3) and reduction cell 8 core (RC8C1-3). Triplicate samples were taken from Minna Creek, downstream of the remediation project as a control (SW91 – 3). The discharge pipes were run for 60 seconds to obtain representative samples from the cells and avoid liquid that had been sitting in the pipes. Samples were collected in sterile 50ml Falcon tubes. Larger samples were collected in autoclaved 1L Schott or Nalgene plastic bottles. The samples were stored on ice in a cool bag for transport.

DNA isolation

To establish a clone library from the samples DNA was extracted from PCF1, RCF1, RC7BW1 and RC8C1. The DNA was extracted using the Mobio PowerSoil kit (Mobio Laboratories, Solana Beach California, USA) as stated in the manufacturers' protocol with the exception of the samples were bead-beaten for 10mins at 5000rpm in a Retsch MM300 shaker (Retsch Inc. Newtown Pennsylvania USA). This kit was found to give a higher level of DNA purity and quality than from phenol chloroform extraction or Qiagen DNeasy kit as it contains a humic substance removal step and was developed for difficult environmental samples such as compost, soil and manure. DNA extractions were

examined by agarose gel electrophoresis at 110V for 30mins using 2.0% w/v agarose in 1xTAE buffer (20mM Tris, 20mM glacial acetic acid, 10mM Na_2EDTA, pH 7.0) with 5μg ml^{-1} ethidium bromide as the nucleic acid stain. 5μl of a DNA ladder (Bioline, London, UK) was used as a molecular size marker. The gels were photographed using a Kodak EDAS imaging system and Kodak 1DLE v3.5.4 software (Kodak molecular imaging systems, New Haven, Connecticut, USA).

16S rRNA gene sequencing and cloning

To amplify the 16S rRNA genes a polymerase chain reaction (PCR) was performed using the primer set 519F (5′CAGCMGCCGCGGTAATAC) 1492R (5′-TACGGYTACCTTGTTACGACTT) (Amann et al. 1995). The PCR reactions were performed in duplicate as per the protocol for the Qiagen HotStarTaq Master Mix kit (Qiagen Inc. Valencia, California, USA) using 25μl HotStarTaq master mix, 1μl of 10pmol 519F and 1492R primers each, 5μl of template and filter sterile, autoclaved Milli-Q purified water (MQH_20) to a final volume of 50μl. The PCR amplification program was as recommended in the Qiagen HotStarTaq Master Mix protocol; 15mins, 95°C; 35 cycles of 1min, 94°C; 1min, 52°C; 2mins, 72°C and a final elongation step of 10mins at 72°C and hold at 11°C. The PCRs were conducted on a PTC 200 Peltier Thermal Cycler (MJ Research Inc. Waltam, Massachusetts, USA). PCR products were examined by agarose gel electrophoresis as above.

The PCR products were purified using the Mobio Ultraclean PCR cleanup kit and stored at -20°C. The PCR products were ligated in the Invitrogen pCR 4-TOPO vector (Invitrogen Corp. Carisbad, California, USA). The ligation reaction

included 3µl PCR product, 1µl buffer, 1µl pCR 4-TOPO vector and 1µl sterile purified water to a final volume of 6µl and was incubated at room temperature for 45 to 60mins. Transformation was performed using Invitrogen One-Shot TOP10 chemically competent *E. coli* cells and following the Invitrogen TOPO TA cloning kit protocol. The *E. coli* cells were thawed on ice and 2µl of the ligation reaction was added, mixed gently and incubated on ice for 30min. The mixture was then heat shocked at 42°C for 30secs and put straight back on ice. 250µl of room temperature Invitrogen SOC medium (2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20 mM glucose) was added and the mixture incubated for 1hr at 37°C, shaken at 200rpm. 50µl and 100µl aliquots were spread on pre-warmed Luria agar plates (10g NaCl, 10g Tryptone, 5g Yeast extract, 20g agar L^{-1} MQ H_20) containing 100µg ml^{-1} ampicillin. The plates were incubated at 37°C overnight and 120 well-formed colonies picked. The picked colonies were suspended in 50µl filter-sterilised MQH_20 and stored at -20°C. The plasmid inserts in the suspended colonies was directly amplified in a PCR with the same conditions as the 16S rRNA gene PCR except with plasmid primers M13F (5′-TGTGAAACGACGGCCAGTAGAGTGATCCTGGCTCAG and M13R (5′-CAGGAAACAGCTATGAC).

The PCR products were purified using the Mobio Ultraclean PCR cleanup kit and were then sequenced using the GenomeLab DTCS Quick Start kit and protocol (Beckman Coulter, Fullerton California, USA); 4.0µl DTCS Quick Start Master Mix, 1.0µl of 1.6pmol M13F or M13R, 100ng template DNA and made up to a total volume of 10µl with MQH_2O. The thermocycler program used was as recommended for the kit (96°C for 20secs, 50°C for 20secs, 60°C for 4mins for 30

cycles and then kept at 10°C). The product was then precipitated following the CEQ2000 Sample Plate protocol available online at http://www.beckmancoulter.com/literature/Bioresearch/A-1903A.pdf.

The amplicon inserts were fully sequenced from the forward primer and the reverse primer separately using a Beckman Coulter CEQ8000 Genetic Analysis System. Consensus sequences for each insert were compiled using ChromasPro v1.32 (Technelysium Pty Ltd 2003 – 2005) and BioEdit v7.0.9.0 (Hall 1999) and compared to GenBank database sequences using the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) Basic Local Alignment Tool (BLAST) (Altschul et al. 1997) available online at http://www.ncbi.nlm.nih.gov/BLAST/. Insert sequences ranged from 932 to 996bp in length and were deposited under GenBank accession numbers EU921150 to EU921224.

Phylogenetic analysis of 16S rRNA sequences

The 16S rRNA gene sequences were aligned with sequences from the GenBank database using the BioEdit ClustalW alignment application and checked manually. Phylogenetic trees were created by the Neighbour-Joining algorithm with 1000 bootstraps using the program CLC Free Workbench Version 3.0.3 (Knudsen et al. 2006). 16S rRNA sequences for *Thermotoga maritimum* and *Coprothermobacter platensis* were used as outgroups in the Neighbor-Joining analysis as they are deeply rooted to most bacteria (Bowman, 2005 pers. comm). The null hypothesis that the clone libraries from the sludge dam, potato cell and reduction cells would not be significantly different was tested with the LIBSHUFF v1.22 method (Henriksen. 2004) at http://libshuff.mib.uga.edu. LIBSHUFF uses the Cramer-von

Mises test statistic to answer the question, "are two libraries drawn from the same population and is one a subset of the other?" In order to compare two libraries, the LIBSHUFF analysis determines the coverage of one library (X) by a second library (Y). To accomplish this, each sequence in X is individually compared to all of the sequences in Y, and it is determined whether or not that sequence would be considered unique were it a part of Y. The resulting coverage values from this analysis are referred to as "heterologous coverage values", or "C_{XY}" and the resulting curve of C_{XY} vs D (distance) is called a "heterologous coverage curve", or "$C_{XY}(D)$". The equation for heterologous coverage is, $C_{XY} = 1 - (N_{XY}/n)$ where N_{XY} is the number of sequences in the sample X that are not found in sample Y and n is the number of sequences in X . Similarly to the homologous coverage, C_{XY} will vary based on the value of D selected because N_{XY} will change based on the criterion for what determines a "unique" sequence. The homologous and heterologous coverage curves can then be compared to determine the extent of differences between the two libraries (ΔC). The difference between the two curves may be quantified by the Cramér-von Mises test statistic:

$$\Delta C_{XY} = \sum_{D=0.00}^{0.5} |C_X(D) - C_{XY}(D)|^2$$

where D increases in increments of 0.01.

A Monte Carlo resampling approach is used to infer statistic significance. To perform this resampling, LIBSHUFF shuffles the sequences of the two libraries together and randomly divides them into new libraries containing the same number of sequences as the originals, but with a different randomized distribution of the sequences. These shuffled libraries are then analyzed identically to the

originals and a ΔC value is recorded. The libraries are shuffled an additional 998 times, resulting in a total of 1000 ΔC values; one from the original libraries and 999 from randomly shuffled libraries. When all of the ΔC values are ordered from the highest to the lowest, the rank of the ΔC for the original libraries determines the probability of the two libraries being significantly different. A p-value (probability) for the null hypothesis that the two libraries are sampled from the same population is estimated by $r/1000$, where r denotes the rank of ΔC for the original libraries (Singleton et al. 2001). Clone library percent coverage (C) was calculated by the following equation: $C = (1-(n/N)) \times 100$, where n = number of unique phylotypes and N = total number of phylotypes.

Terminal Restriction Fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis, employing 16S rRNA gene primers 519F and 1492R (sequence as above), was used to determine the changes in the bacterial communities between the sludge dam, potato cells, reduction cells and Minna Creek (Liu et al. 1997). The forward primer 519F was labelled with Beckman Coulter WellRED™ fluorescent dye D3 (green) and 1492R was labelled with Beckman Coulter WellRED™ fluorescent dye D4 (blue). The PCR amplification mixture contained 12.5µl HotStarTaq master mix, 1.0µl of each primer (20µM) and approximately 10ng DNA template and MQH_2O used to make final volume of 25µl. The PCR amplification program was as follows; 94°C, 15mins; 35 cycles of 94°C, 1min; 55°C, 1min; 72°C, 1min with a final elongation of 72°C ,10mins on a PTC 200 Peltier Thermal Cycler (MJ Research Inc.). The PCR products were visualised by agarose gel electrophoresis as above. Four identical PCRs, as above, were run separately and the products pooled and purified using the Mobio Ultraclean PCR cleanup kit and stored at -

20°C. This was to minimise the effects of amplification bias in individual PCRs and more accurately represent the bacterial community and reduce method-associated noise (Clement et al. 1998).

The purified PCR products were digested with 10U of the enzymes *Hae*III, *Hha*I, *Hinf*I, *Msp*I and *Rsa*I (New England Biolabs) the most commonly used 4 base pair cutters found in the literature, in a 96 well plate. Five enzymes were chosen to mitigate the effect of different bacterial groups having similar profiles with the same enzymes. The digests were then desalted and purified using the ethanol precipitation method. To each plate well 1µl 3M Na acetate and 0.5µl of glycogen was added and mixed. 30µl of ice-cold absolute ethanol was added, mixed and the plates were covered with an aluminium sealing mat and incubated at -20°C for 20mins. Plates were spun down in a Sorvall Super T21 centrifuge (Thermo Electron Corp. Waltham, USA) at 4°C for 30mins at 4300 rpm. To remove the ethanol, paper towel was cut to fit the size of the plate and the plate flipped straight over on to the paper and spun gently in a Sigma 2-5 centrifuge (Quantum Scientific, Murarrie, Australia) for 30secs at 300rpm. Two washes comprising 200µl ice-cold 70% ethanol/centrifuged for 5mins. The plate was then air-dried in a laminar flow cabinet. The PCR products and digests were wrapped in aluminium foil to minimise light exposure and loss of fluorescence.

Digested products (1 - 5µl) were mixed with 30µl Sample Loading Solution (Beckman Coulter) and 0.25µl of 600bp DNA size standard (Beckman Coulter) and analysed on the Beckman Coulter CEQ8000 Genetic Analysis System using the Frag-4 method (injection 2.0kV/30secs, run at capillary temperature 50°C/4.8kV for 60 minutes). Results were analysed using the Beckman Coulter

CEQ8000 Genetic Analysis System software. The digests were carried out in duplicate and each run separately on the Beckman Coulter CEQ8000 Genetic Analysis System.

Enrichment sample preparation for scanning electron microscopy

A freshwater medium and 1.5% w/v agar was made according to Lovley (2000). The composition of the medium was: $NaHCO_3$ 2.5g, NH_4Cl 0.25g, $NaH_2PO_4.H_2O$ 0.6g, KCl 0.1g, L^{-1}. The solution was autoclaved and 10ml of vitamin solution and 10ml mineral solution were added after it had cooled. The vitamin solution was made according to Lovley (2000) and composed of: biotin 2.0mg, folic acid 2.0mg, pyridoxine HCl 10.0mg, riboflavin 5.0mg, thiamine 5.0mg, nicotinic acid 5.0mg, pantothenic acid 5.0mg, B-12 0.1mg, p-aminobenzoic acid 5.0mg and thioctic acid 5.0mg L^{-1}. The mineral solution was made according to (Lovley 2000) and composed of: trisodium nitrilotriacetic acid 1.5g, $MgSO_4.6H_2O$ 0.5g, NaCl 1.0g, $FeSO_4.7H_2O$ 0.1g, $CaCl_2.2H_2O$ 0.1g, $CoCl_2.6H_2O$ 0.1g, $ZnCl_2$ 0.13g, $CuSO_4.5H_2O$ 0.01g, $AlK(SO_4)_2.12H_2O$ 0.01g, H_3BO_3 0.01g, Na_2MoO_4 0.025g, $NiCl_2.6H_2O$ 0.024g and $Na_2Wo_4.2H_2O$ 0.025g L^{-1}. Both the vitamin and mineral solutions were filter sterilised.

Poorly crystalline iron oxide was made according to Lovley (2000) for use as the electron acceptor. 32.45g of $FeCl_3.6H_2O$ was weighed into MilliQ double distilled water (MQH_2O) (final concentration 0.4M). This solution was stirred continuously while the pH was adjusted to 7.0 with 10M NaOH solution. The solution was stirred for a further 30mins and the pH checked to make sure it had stabilised. To remove the dissolved chloride the suspension was centrifuged at 5000rpm for 15mins and the pellet resuspended with MQH_2O six times. Solutions

of 100mM acetate, 100mM lactate and 100mM D-glucose were made and autoclaved for use as carbon sources in the enrichments.

All enrichments were set up in MacCartney bottles as follows; 20ml freshwater medium, 4ml carbon source, 6ml of iron oxide and the pH adjusted to 5 – 5.5. The bottles were filled to the top and autoclaved to remove as much oxygen as possible. Two ml of the RCF sample (potato cell effluent) were added to three bottles, each containing one of the three carbon sources. The necks were flamed with a Bunsen burner and sealed with a rubber-sealing lid. The bottles were placed in a black garbage bag inside a black plastic container and incubated at 20°C for 2-3 months.

Enrichment samples were prepared for Scanning Electron Microscopy (SEM) as follows: samples were filtered through sterile polycarbonate 0.2µm filter papers (Poretics, Livermore, USA) in sterile filter housing units (Millipore, Billerica, USA). All steps were conducted through the filter units. The samples were then fixed with 2.5% glutaraldehyde in distilled water (dH_2O) for 30mins and rinsed 3 times in dH_2O. A secondary fixation was carried out by adding 1% osmium tetroxide in dH_2O for 20mins at room temperature and rinsed 5 times with dH_2O. The slides were then put through a gradient of ethanol from 100% to 25% for 5mins to dehydrate. It was found in preceding experiments the more decreasing ethanol concentration steps the fewer bacteria cells collapsed so the steps used were 100%, 75%, 50% and 25% ethanol. Immediately after the final wash the samples were frozen using liquid nitrogen, placed in 50ml Falcon tubes and dried using a freeze dryer (Dynavac, Sydney, Australia) for 24hrs. The samples were then sputter coated with gold (BalTec SCD 050) before visualisation on the

scanning electron microscope (SEM) (FEI Quanta 600 MLA) at the Central Science Laboratory (CSL) University of Tasmania, Hobart (Twin 2008 pers. comm.). To determine precipitate chemical composition the SEM is equipped with Electron Probe Microanalysis (EPMA) instrumentation.

Analysis of TRFLP data

Profile peak data were analysed using the Beckman Coulter CEQ8000 Genetic Analysis System Fragment Analysis software. The lower threshold for peak height was set at 5% of the peak height range and results were filtered for peak areas less than 2000 DFU (dye fluorescent units), peaks less than 60nt (nucleotides) and more than 600nt were eliminated as this is the limitation of the size standard (http://www.beckman.com/literature/Bioresearch/A16039ab.pdf.).

Results from the duplicate runs were combined into a Microsoft Excel 2003 spreadsheet for each enzyme used for each sample. Percent relative peak area was calculated and the peaks that contributed less than 2% of the total peak area were removed to minimise the effect of baseline noise from variable amounts of DNA loaded (Osborne et al. 2006). Forward (fragments from the 5′ end of the sequence) or reverse (fragments from the 3′ end of the sequence) peaks within 0.5 basepairs (bp), which contributed the least to the total peak area, were removed so they were not classed as the same peak. There were no more than two peaks in this category in any one sample and these were removed consistently across duplicates, so did not affect the total profile peak data analysis.

The results were run through T-align (Smith et al. 2005) accessed at http://inismor.ucd.ie/~talign/index.html for comparison with a confidence interval of 0.5bp. Replicate profiles were compared and used to generate a single sample

profile containing only Terminal Restriction Fragments (TRFs) that occurred in all replicate profiles. Subsequently sample profiles representing different communities were compared to produce a list showing whether a TRF was present in a particular sample profile and its relative fluorescence intensity (peak area) to total peak area. A comparison file was generated which listed all TRFs in all consensus profiles. If a TRF was present in a consensus profile of a sample then the average fluorescence intensity is given. If a TRF is absent in a particular consensus profile, a zero value is listed. The TRF size values were rounded up or down to the nearest whole number (<0.49 rounded down, > 0.5 rounded up).

The results for all the enzymes for each sample were then combined and analysed in PRIMER v6 (Clarke and Gorley 2006). A similarity matrix using the Bray-Curtis coefficient was calculated. The Bray-Curtis coefficient was chosen because of its ability to deal with data sets containing many zeros, and a zero value is selected when two samples have no TRFs in common. The inclusion or exclusion of TRFs jointly absent in two samples does not change the similarity value, and inclusion or exclusion of further samples does not change the similarity between other sample comparisons (Clarke and Warwick 2001).

The similarity matrix was analysed using MDS (Multi-Dimensional Scaling). MDS ordination uses an iterative algorithm that involves a "goodness of fit" estimate, (i.e. stress). Stress value greater than 0.2 indicates the plot is close to random, stress less than 0.2 indicates a useful 2 dimensional picture, and if less than 0.1 corresponds to an ideal ordination with no prospect of misinterpretation (Clarke and Warwick 2001). Therefore, an interpretation of a MDS plot is straightforward; points that are close together represent samples that are similar in

community assemblage, and points far apart correspond to different values of the data variables. The ordinations in this analysis were all computed using 50 and 100 random restarts and there was no difference in the minimum stress, 50 random restarts were sufficient.

Analysis of similarities (ANOSIM) was used to test the statistical significance between samples over time, site and treatment factors. ANOSIM tests the null hypothesis that similarities between and within groups will be the same. The test statistic is R, the difference of ranked similarities *between* samples/sites to ranked similarities among replicates *within* samples/sites. Global R is *between* all replicates of all samples and the R statistic is between the replicates of two samples. R approaches 1 if all replicates within samples/sites are more similar to each other than any replicates from different samples/sites. The significance level (p) is a measure of the probability the observed R is by chance in 1000 permutations. Dispersion indices (MVDISP) were also calculated in PRIMER v6 to examine variation between treatment and time heterogeneity.

The dispersion index (IMD) compares the average rank among one set of samples with the average range derived from another, ignoring between treatment similarities in the similarity matrix. A large dispersion index (maximum = 1) means all similarities in one sample are less than any similarities in another and the converse is the case for dispersion index = -1. This gives a description of relative multivariate variability, meaning multiple samples from a highly "unstable" environment will have more population variability than samples from a "stable" environment.

Percent similarity contributions of TRFs within treatments were calculated with SIMPER, and compared to the associated clone library produced from the same samples with the same primer set and to the restriction maps of clones digested *in silico* with the same enzymes used in the TRFLP analysis. The *in silico* digests, with these enzymes, were performed using the ISPaR (In Silico PCR and Restriction) program on the MiCA (Microbial Community Analysis) web site http://mica.ibest.uidaho.edu (Shyu et al. 2007).

Results and Discussion

Clone library analysis

16S rRNA clone libraries were constructed for samples from the sludge dam (PCF) (44 clones), potato cell (RCF) (36 clones) and reduction cell 7 effluent (RC7BW) (43 clones) and reduction cell 8 core (RC8C) (36 clones). The percentages of clones assigned to major bacterial taxonomic groups are summarised in Figure 3.2. Total number of clones for each section of the treatment system, the number of unique clones or operational taxonomic units (OTUs) and percent coverage for each clone library is summarised in Table 3.1. Representative phylogenetic groups inferred from the 16S rRNA gene clone libraries, the number of clones, percent of the total, closest match within a phylogenetic group and % similarity for each section of the treatment system sampled are summarised in Table 3.2. No members of the *Archaea* were detected. Neighbor-joining phylogenetic trees were constructed for the major bacterial groups (Figures 3.3 – 3.10).

Sludge dam

The majority of clones from the sludge dam leachate were similar to beta- (18%), delta-(18%) and gammaproteobacterial (22%) iron- and sulfur-oxidising bacteria, such as *Gallionella* spp. and "*Ferribacter*" sp. (Figure 3.4), *Acidithiobacillus* spp. (Figure 3.5) as well a sulfur-respiring *Desulfurella* spp., a member of the class *Deltaproteobacteria* (Figure 3.6). Nearly half the clones (4%) were similar to anaerobic, carbohydrate fermenters of the phylum *Bacteroidetes* (Figure 3.8) related to the genus *Bacteroides* spp. and acid-tolerant, anaerobic fermenter *Paludibacter propionicigenes* (Ueki et al. 2006) (Figure 3.8). The sludge dam is clay-lined and capped. Hence, the fermenters may have access to organic matter attached to the clay and stratification in oxygen levels may be occurring. Clone library percent coverage was 73%.

Potato cell

In the potato cell, 33% of the clones matched *Deltaproteobacteria* (mainly *Desulfurella* spp.) and 55% of the clones matched *Bacteroidetes* (mainly clones related to *Bacteroides* spp. and *P. propionicigenes*). None of the *Desulfurella* type strains characterised to date is known to be capable of growth in the acidic (pH 4 - 5) conditions present in potato cell (Miroshnichenko et al. 1998). Thus, the microorganisms yielding the 16S rRNA gene sequences recovered from the potato cell effluent may represent the first acidophilic taxon within the *Desulfurella* genus. Furthmore, similar sequences have been found in acid sulfate springs in Yellowstone National Park (Boyd 2007). *Betaproteobacteria* were not detected. 11% of the clones matched *Acidithiobacillus* spp. a similar proportion to those from the sludge dam (16%) leachate. The presence of potato starch and a

largely anoxic environment may have allowed the fermentative bacteria to out-compete the *Betaproteobacteria*. Clone library percent coverage was 67%.

Reduction cells

The reduction cell effluent (RC7W) and core samples (RC8C), exhibited a much more diverse bacterial community to each other, possibly due to the presence of the high levels of organic matter in the core sample from reduction cell 8 that were not present in the effluent sample from reduction cell 7.

Core sample clones clustered with beta- and deltaproteobacterial iron-reducers such as *Ferribacterium limnieticum*, *Geobacter* spp. and *Anaeromyxobacter*, where members of the latter genus have been found to use Fe^{3+} as an electron acceptor (Cardenas et al. 2008). Potential sulfate-reducers present were mainly related to members of the genus *Desulfotalea*, one clone was 99% related to denitrifying bacterium *Acidovorax delafieldii*. A core clone was found to be 94% related to the gammaproteobacterium *Dokdonella koreensis,* associated with aerobic soil samples (Yoon et al. 2006) and eight clones grouped with an epsilonproteobacterium clone found in rice rhizospheres (unpublished) (Figure 3.6). One clone was 95% related to the alphaproteobacterial species *Acidisphaera rubrifaciens,* an acid-tolerant, aerobic phototrophic bacterium (Figure 3.3). The core samples also contained clones which matched to *Firmicutes* and comprised mainly anaerobic heterotrophs some of which are related to bacteria found in compost (Weon et al. 2007) (Figure 3.9) and one clone was related to the genus *Desulfotomaculum* a known sulfate reducer (Stackenbrandt et al. 1997).

Reduction cell 7 effluent sample clones clustered in the classes *Beta-*, and *Deltaproteobacteria*, and included presumed iron-and sulfur-oxidisers such as

Thiomonas spp. and *Gallionella ferruginea*. A clone 93% related to the genus *Desulfobacterium* may also be involved in sulfate reduction. A high proportion of clones (35%), particularly from reduction cell 7 effluent, grouped among uncultured representatives from the candidate division OP3 and Termite Group 1 (Figure 3.10), which have been previously detected in contaminated aquifers, sulfidic hot springs or bioreactors treating chlorinated solvents (Hugenholtz et al. 1998).

Both effluent and core samples have clones that matched to phyla *Bacteroidetes*. Effluent sample clones clustered with anaerobic fermenters such as *Bacteroides*, *Porphyromonas* and *Dysgonomonas* (Figure 3.8); the core sample clones clustered with the genus *Cytophaga*, which is known to include anaerobic cellulose degrading bacteria (Gherna and Woese 1992). Core sample clones clustered with candidate division OP10, soil and contaminated aquifer clones. *Acidithiobacillus* spp. and *Desulfurella* spp. common in the potato cell effluent were not detected in the reduction cell effluent clone libraries possibly due to higher pH and competition with organic matter fermenters. Clone library percent coverage for reduction cell 7 and 8 was 40% and 28% respectively.

LIBSHUFF comparisons of the clone libraries are summarised in Table 3.3. The ΔC values, indicating clone library divergence were highest between the potato cell and the reduction cell effluent samples. In heterologous Libshuff comparisons the potato cell effluent library was very similar to the sludge dam leachate library ($\Delta C = 0.026$, $p>0.05$) indicating the potato cell community is a subset of the source leachate community. The clone library comparisons were otherwise significantly different between the samples ($p<0.001$), indicating different

communities exist in different stages of the remediation system, especially in the downstream reduction cells.

Clone libraries from the different stages of a novel *in situ* bioremediation system treating acidic, metal- and sulfate-rich leachate illustrate the complex nature of microbial communities. Further analysis of clone libraries of core samples or from a range of depths within these sites may have illuminated this diversity even more. Blothe et al. (2008) found, in sediments impacted by acid mine drainage, a gradual change in microbial community from the acidic, oxic and ferric iron-rich upper sediments to the mildly acidic, anoxic ferrous iron-rich lower sediments. Furthermore, an in-depth study of > 2000 16S rRNA gene clones from acid-impacted lakes (Percent et al. 2008) found diversity changes were strongly correlated to depth, hydraulic retention time, dissolved inorganic carbon and metal concentration. Changes in bacterial community in complex environmental samples over site and time can be tracked with simpler methods such as TRFLP instead of creating clone libraries at each sampling point, which are much slower and more expensive.

Table 3.1. The number of clones found from each section of the treatment system, Heybridge, Tasmania, percent number of unique operational taxonomic units (OTUs) and percent clone library coverage. (= (1-(# unique clones/# total clones)x100).

Phylogenetic match	Sludge Dam	Potato Cell	Reduction Cell 7 effluent	Reduction Cell 8 Core
Alphaproteobacteria	0	0	0	1
Betaproteobacteria	7	0	2	4
Gammaproteobacteria	9	4	0	1
Deltaproteobacteria	8	12	3	3
Epsilonproteobacteria	0	0	0	8
Bacteroidetes	20	20	8	4
Firmicutes	0	0	0	11
Other	0	0	30	4
Total # clones	44	36	43	36
# unique OTUs	12	12	26	26
% Coverage	72.7	66.7	39.5	27.8

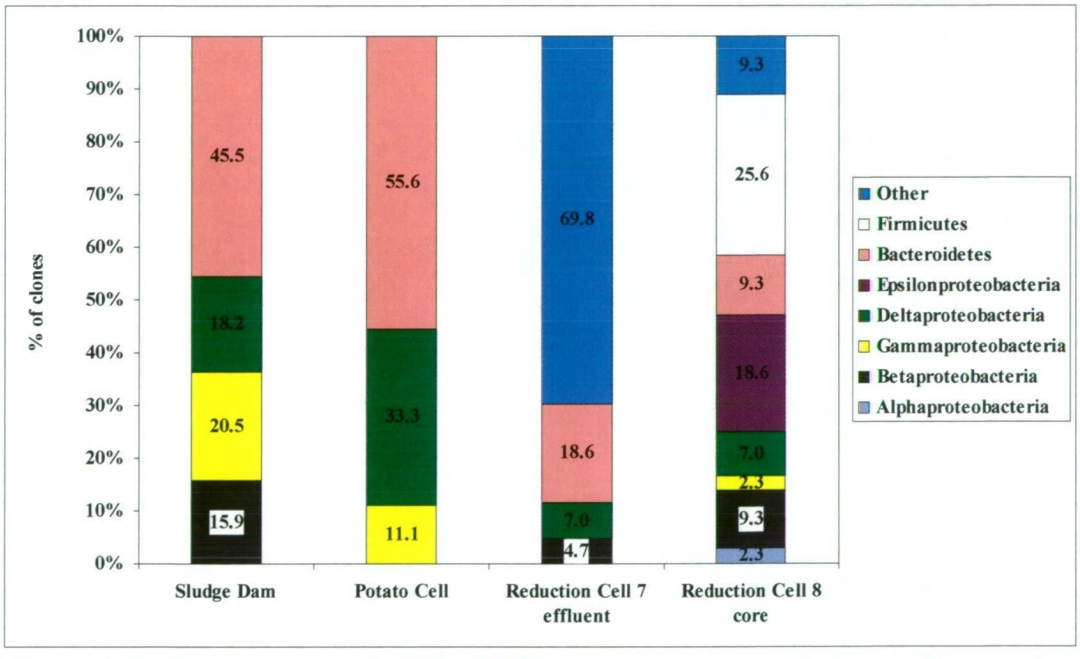


Figure 3.2. Percentages of 16S rRNA gene sequence phylogenetic match cloned from Sludge Dam, Potato Cell and Reduction Cell 7 and 8 samples.

Table 3.2. Representative phylogenetic groups inferred from the 16S rRNA gene clone libraries, the number of clones, percent of the total, closest match within a phylogenetic group and % similarity for each section of the treatment system at Heybridge, Tasmania.

Sample site	# of clones	% of total clones	Representative clone	Phylogenetic group	Closest match	% similarity
Sludge Dam	2	4.5	PCF18	*Betaproteobacteria*	*Gallionella ferruginea* (iron oxidation)	97
	4	9.1	PCF50	*Betaproteobacteria*	*Ferrovum myxofaciens* (iron oxidation)	98
	9	20.5	PCF61	*Gammaproteobacteria*	*Acidithiobacillus ferrooxidans* (sulfate/iron oxidation and iron reduction)	99
	8	18.2	PCF22	*Deltaproteobacteria*	*Desulfurella* spp. (sulfur respiration)	98
	10	22.7	PCF33	*Bacteroidetes*	*Bacteroides* spp. (complex carbohydrate fermentation)	92
	10	22.7	PCF36	*Bacteroidetes*	*Paludibacter propionicigenes* (complex carbohydrate fermentation)	92
Potato Cell	4	11.1	RCF30	*Gammaproteobacteria*	*Acidithiobacillus ferrooxidans* (sulfate/iron oxidation and iron reduction)	99
	12	33.3	RCF39	*Deltaproteobacteria*	*Desulfurella* spp. (sulfur respiration)	98
	20	55.6	RCF91	*Bacteroidetes*	*Paludibacter propionicigenes* (complex carbohydrate fermentation)	92
Reduction Cell 7 Effluent	1	2.3	RC7BW49	*Deltaproteobacteria*	*Desulfobacterium indolicum* (sulfate reduction/indole degradation)	93
	2	4.7	RC7BW28	*Deltaproteobacteria*	*Syntrophus* spp. (fatty acid utilisation)	95
	6	14.0	RC7BW93	*Bacteroidetes*	*Bacteroides* spp. (complex carbohydrate fermentation)	92
	16	37.2	RC7BW50	Candidate division OP3	Uncultured bacterium TANB84 (dechlorinating community)	93
Reduction Cell 8 core	1	2.8	RC8C34	*Alphaproteobacteria*	*Acidisphaera rubrifaciens* (acidophile)	95
	3	8.3	RC8C16	*Betaproteobacteria*	*Ferribacterium limneticum* (iron reduction)	98
	1	2.8	RC8C20	*Deltaproteobacteria*	*Geobacter* spp. (iron reduction)	91
	8	22.2	RC8C8	*Epsilonproteobacteria*	Uncultured bacterium SRRT05 (rhizosphere)	99
	4	11.1	RC8C64	*Bacteroidetes*	*Cytophaga fermentans* (complex carbohydrate fermentation)	97
	3	8.3	RC8C17	*Firmicutes*	*Clostridium* spp. (fermentation)	93
	2	5.6	RC8C57	*Firmicutes*	*"Ureibacillus rudaensis"* (Compost/soil organism)	98
	1	2.8	RC8C67	*Firmicutes*	*Virgilbacillus halophilus* (soil organism)	93
	3	8.3	RC8C18	Candidate division OP10	Uncultured bacterium HDBW-WB58 (contaminated aquifer)	94

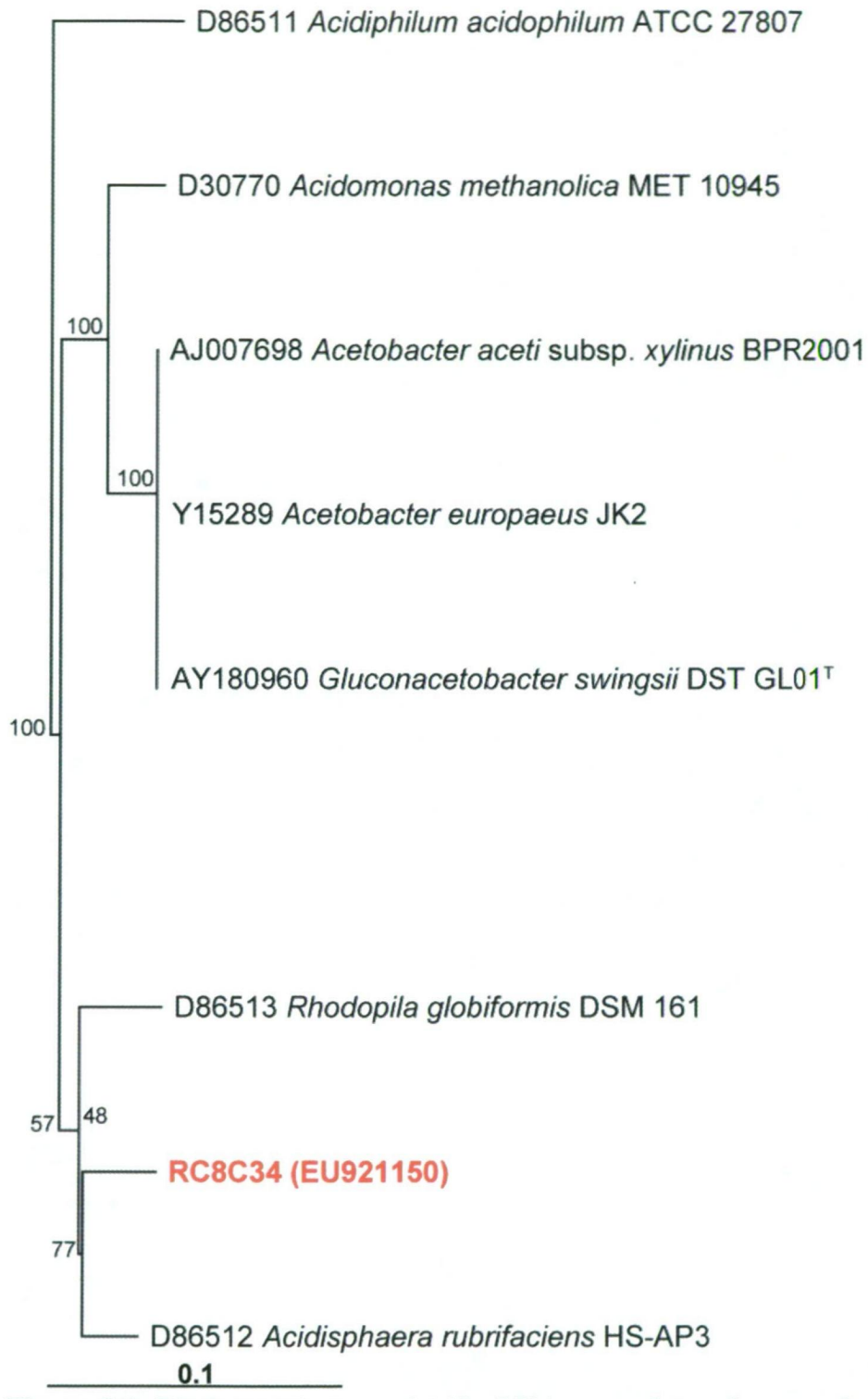


Figure 3.3. Phylogenetic tree of 16S rRNA gene clones from environmental samples from bioremediation system, Heybridge, Tasmania, affiliated with class *Alphaproteobacteria* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = reduction Cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

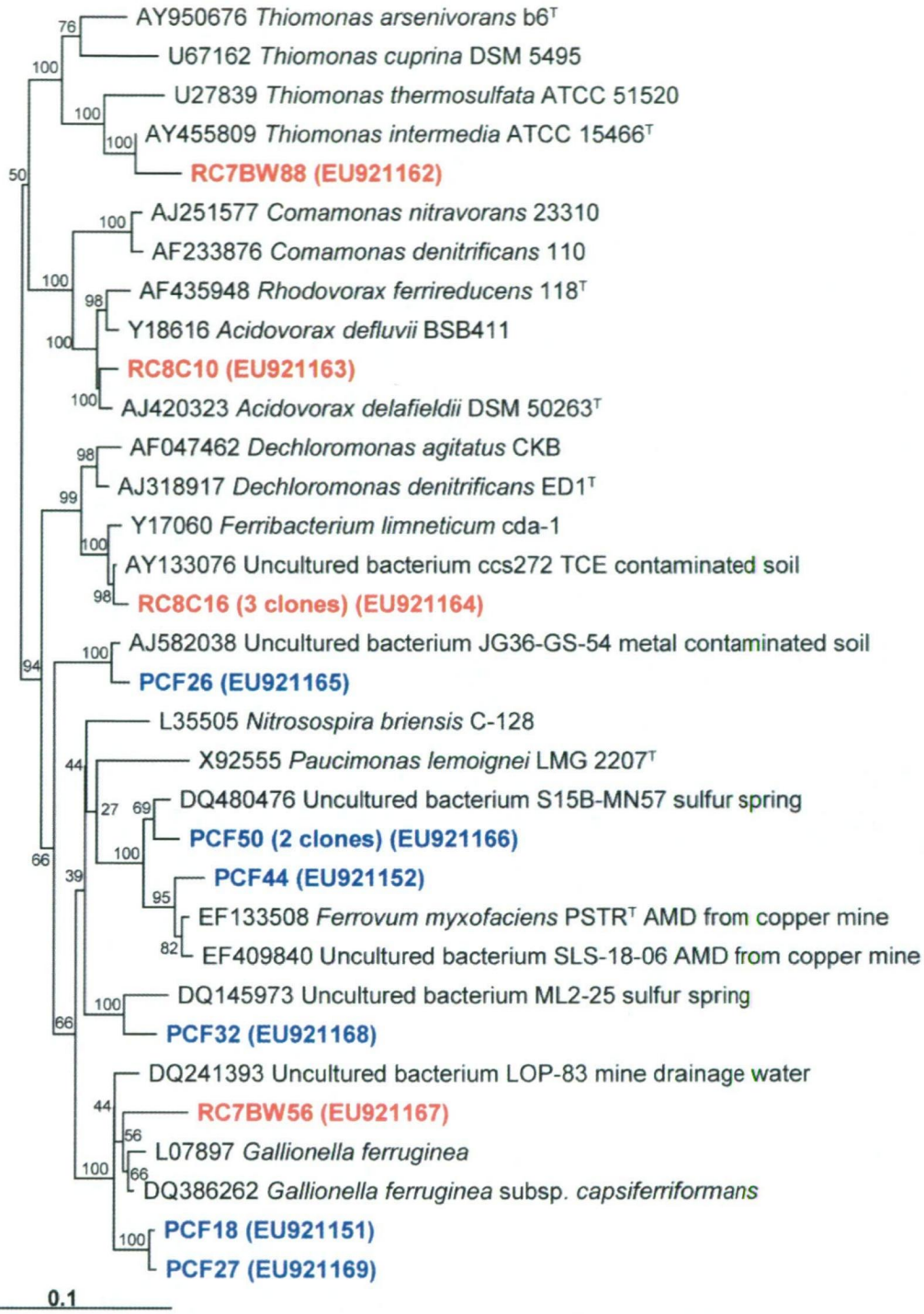


Figure 3.4. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania affiliated with class *Betaproteobacteria* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell, blue type = Sludge dam. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

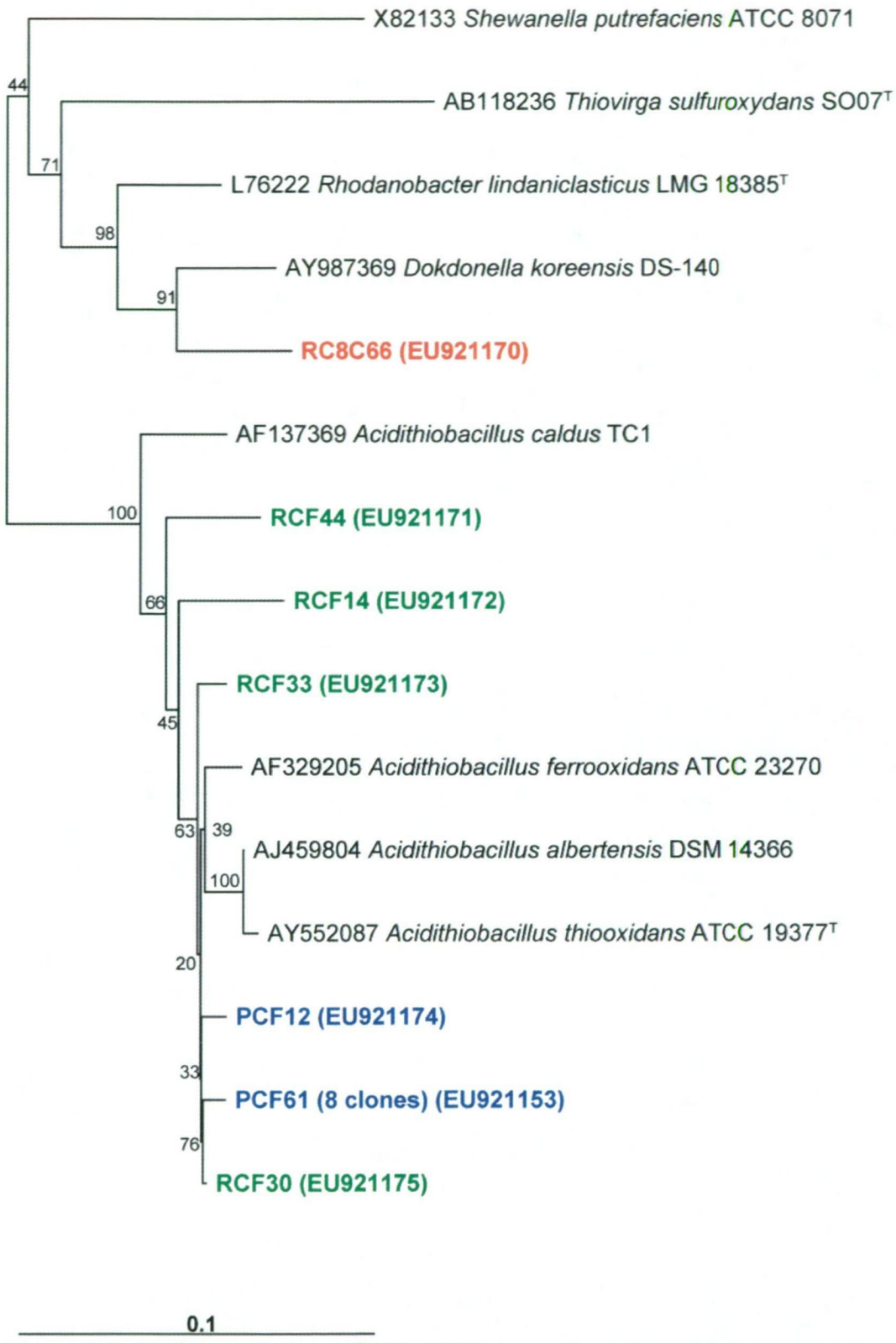


Figure 3.5. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania affiliated with class Gammaproteobacteria using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell, blue type = Sludge dam., green = Potato cell. Outgroups used were Coprothermobacter platensis and Thermotoga maritima (not included).

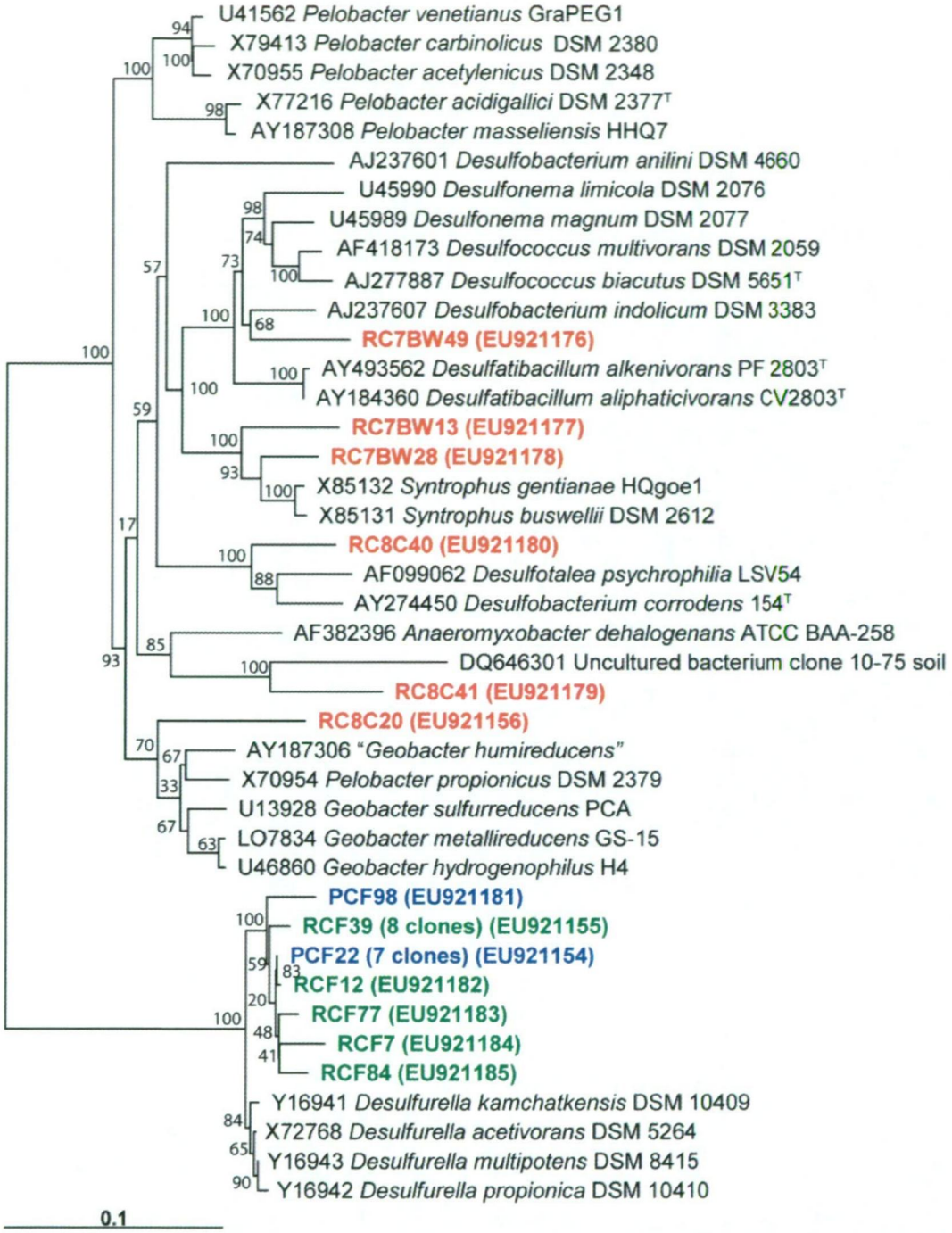


Figure 3.6. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania affiliated with class *Deltaproteobacteria* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell, blue type = Sludge dam., green = Potato cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

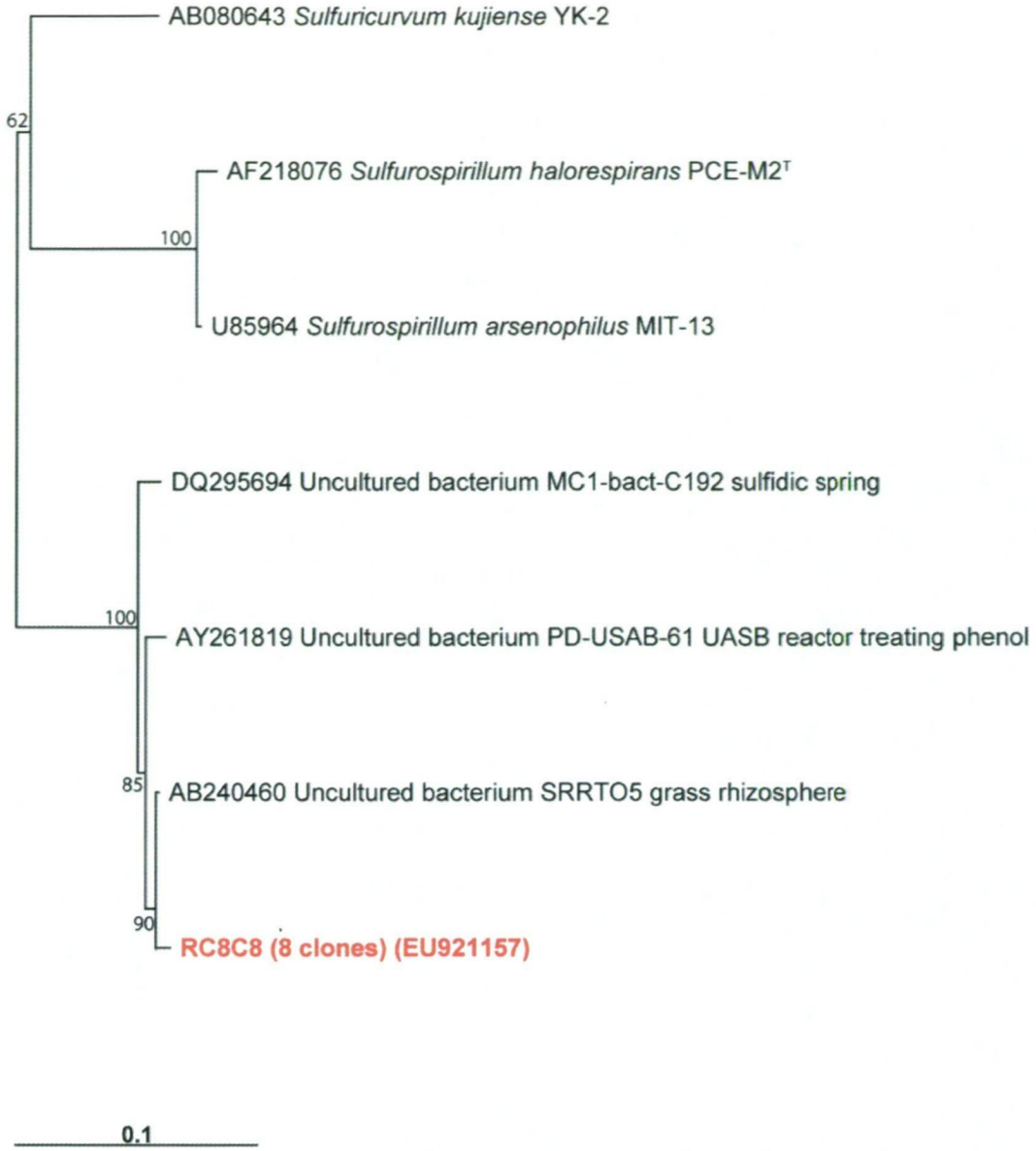


Figure 3.7. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania, affiliated with class *Epsilonproteobacteria* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

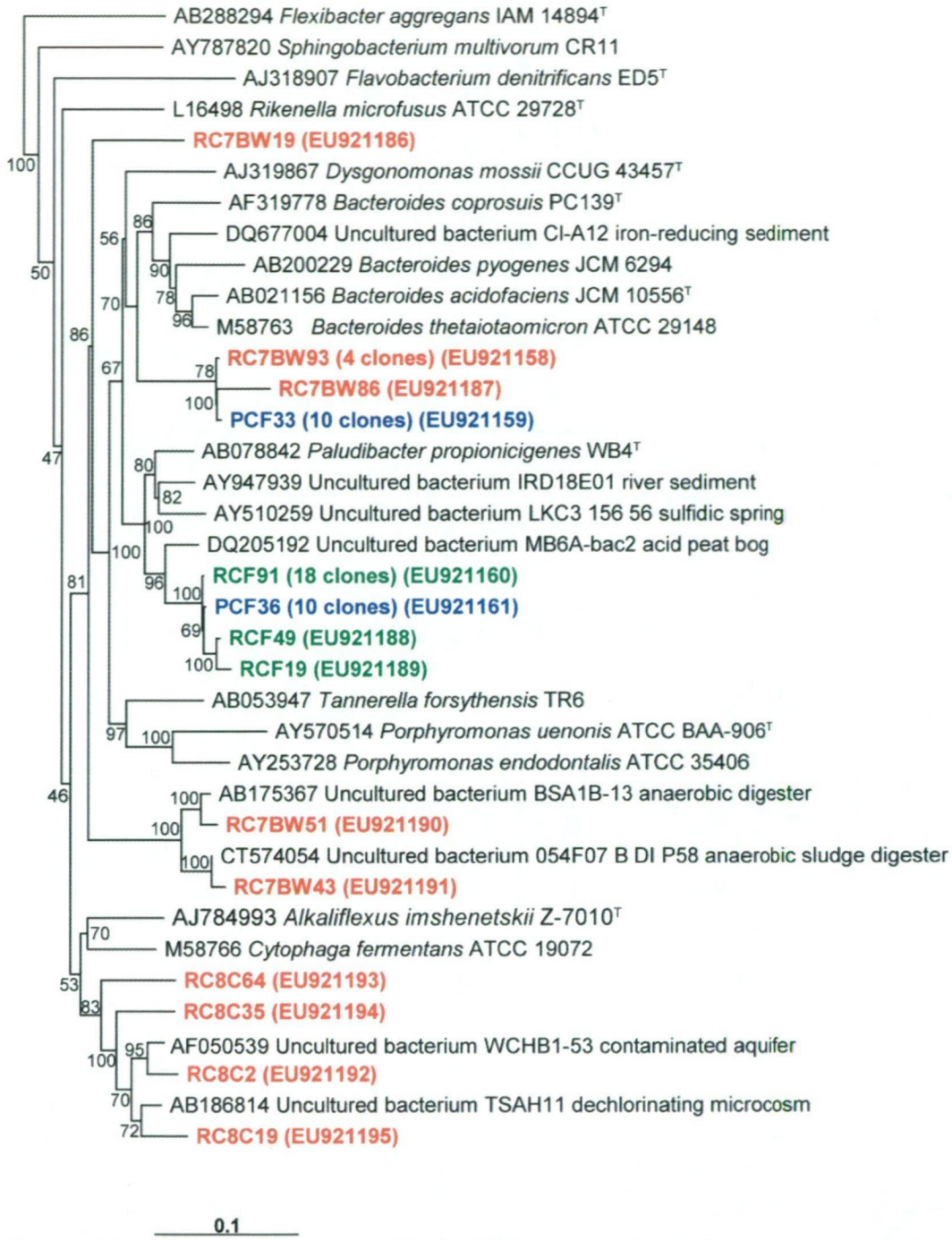


Figure 3.8. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania, affiliated with phylum *Bacteroidetes* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell, blue type = Sludge dam, green = Potato cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

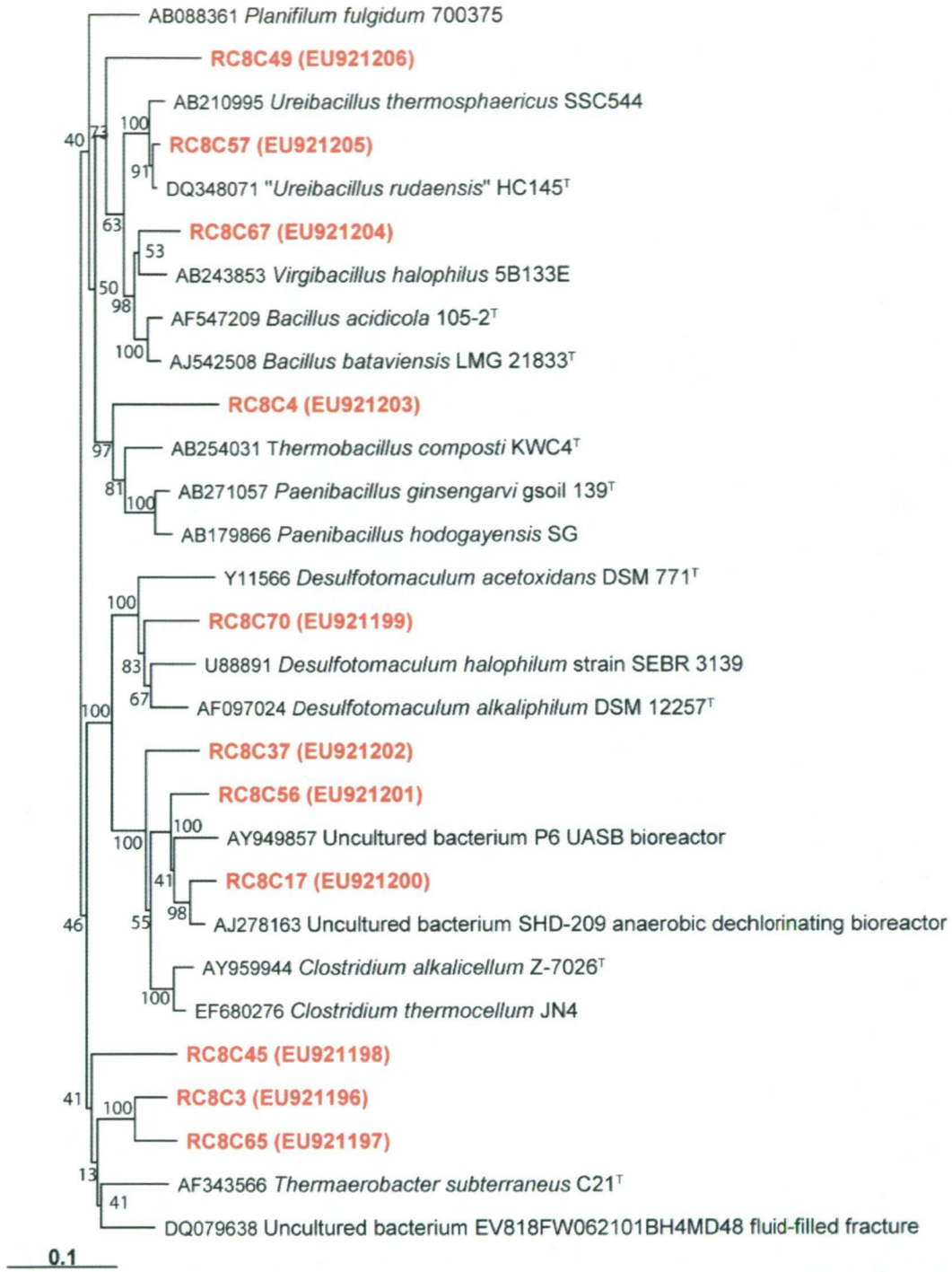


Figure 3.9. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania ,affiliated with phylum *Firmicutes* using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

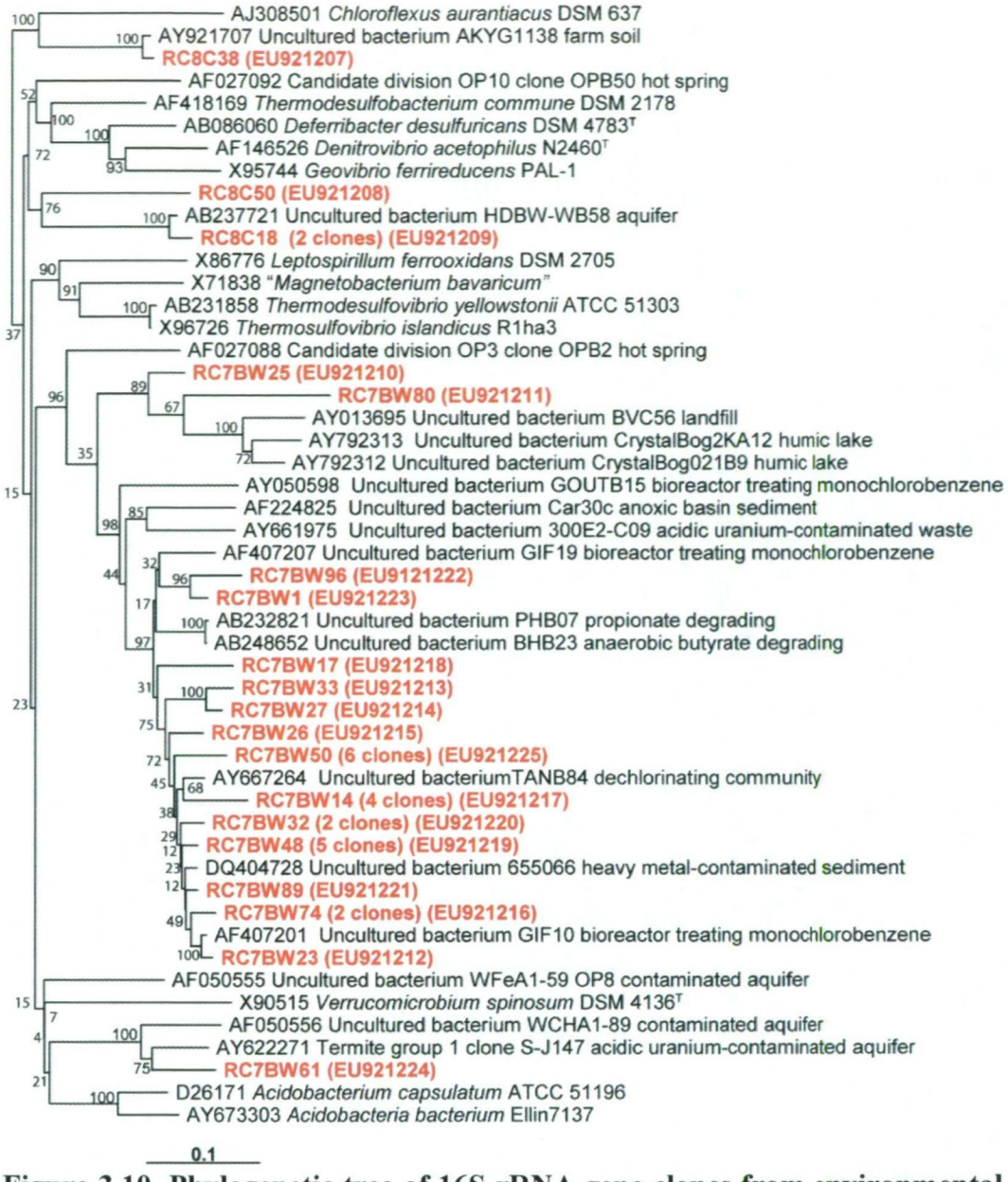


Figure 3.10. Phylogenetic tree of 16S rRNA gene clones from environmental samples from a bioremediation system, Heybridge, Tasmania, affiliated with Candidate Division OP3 using the Neighbor-Joining algorithm in BioEdit v7.09. Red type = Reduction cell. Outgroups used were *Coprothermobacter platensis* and *Thermotoga maritima* (not included).

Table 3.3. LIBSHUFF comparisons of the sludge dam, potato cell and reduction cells clone libraries. ΔC = difference between the clone libraries and *p* = probability of the difference being significant. Bonferroni corrected $\alpha = 0.0085$

Comparison	ΔC	*p*
Sludge Dam/Potato Cell (X/Y)	1.710	0.001
Potato Cell/Sludge Dam (Y/X)	0.026	0.097
Sludge Dam/Reduction Cell (X/Y)	6.318	0.001
Reduction Cell/Sludge Dam Y/X)	7.645	0.001
Potato Cell/Reduction Cell (X/Y)	11.877	0.001
Reduction Cell/Potato Cell (Y/X)	8.859	0.001

TRFLP analysis of treatment and site variability of bacterial communities

A total of 128 TRFs (Sludge Dam = 37, Potato Cell = 36, Reduction Cells – 74, Minna Creek = 43) were used for further analyses. The sludge dam, potato cell, reduction cells and Minna Creek were divided into groups by the level of treatment for statistical analysis. Hence, samples will be classed as, sludge dam = untreated, potato cell = pre-treatment, reduction cells = treated and Minna Creek = post-treatment. The ANOSIM pairwise comparisons for the sites are summarised in Table 3.4 and the MDS plot of pairwise comparisons between sites illustrated in Figure 3.11. To better illustrate differences between the reduction cells a MDS plot was also created that included reduction cells and Minna Creek cluster data (Figure 3.12). The Global R statistic was 0.26 with a $p<0.01$, hence there are some significant differences between TRFLP profiles from the different stages of the treatment system. Reduction cell 5 effluent compared to effluent from reduction cells 7 and 8, the sludge dam and the potato cell effluent, was the most significantly different. Reduction cell 6, 7 and 8 effluent samples were not significantly different. The potato cell effluent and sludge dam leachate were the most significantly different from all other sites and were found to be not significantly different from each other.

Analysis of similarity (ANOSIM) pairwise comparison between treatment levels (Table 3.5) showed that there were differences indicated by a global R statistic of 0.242 ($p<0.03$). However, some of the permuted R values were negative. Chapman and Underwood (1999) point out some situations in which negative R values may occur in practice, as for example, when the community is species-poor and individuals have a heavily clustered spatial distribution, so that

variability within a group is more extreme than between groups. This example supports the notion that the bioremediation system is highly heterogeneous within sites with respect to microbial community composition. The MDS plotted ANOSIM TRFLP peak area data and the corresponding ANOSIM pairwise comparisons (Figure 3.13) showed a large community shift from the untreated sludge dam leachate to Minna Creek water samples (post-treatment), for example the loss of *Betaproteobacteria* and the increase in compost-associated bacteria such as *Firmicutes* and uncultured groups. However, the reduction cell (treated) communities were not significantly different to Minna Creek. The potato cell (pre-treated) community was not significantly different to the sludge dam, as they both contain large proportions of *Bacteroidetes*, *Deltaproteobacteria* and *Gammaproteobacteria,* supporting the clone library analysis.

The dispersion indices (IMD) between treatments (Table 3.5) show that all the community similarities within the sludge dam leachate samples and potato cell effluent samples are different to the Minna Creek samples (IMD = 1.0). This indicates that the sludge dam and potato cell communities are less variable than the other sections of the system. However, the IMD within Mina Creek communities and the reduction cell communities are close to one (0.977) indicating the reduction cells communities are more heterogenous. Furthermore, the reduction cell communities are more diverse than the sludge dam (0.27) and potato cell (0.206) communities are. Warwick and Clarke (1993) noted that in a variety of environmental impact studies, the variability among samples from impacted areas was much greater than that from control areas. To date, dispersion analysis has not been widely used on microbial data; however, Rees et al. (2004) noted that it might be useful in contamination/pollution studies involving

microbial populations. The reduction cells are probably the most "disturbed" because of the constant influx of leachate forming a complex environment with compost and straw, whereas, the sludge dam and potato cell, are by comparison more homogenous environments. The potato cell only has a single source of organic matter. Minna Creek community also has low variability, likely due to it being a stable, less disturbed environment.

Similarity percentage (SIMPER) analysis was used to determine the TRFs that contributed to the top 60% to 90% average similarity within samples between treatment sites (Table 3.6). The sludge dam leachate TRFs matched *Acidthiobacillus* spp. (~16.5%), and *Desulfurella* spp. (~15%) of which were in similar proportions in the clone libraries. Other TRF matches not found in the clone library include *Desulfosporosinus* sp. (16%) (a mesophilic sulfate reducer) and *Thermoanaerobacter* sp. (15.5%) (a thermophilic and anaerobic genus found to reduce iron and sulfate and ferment sugars). The potato cell effluent also had TRFs matching the clone library, with members of the genera *Acidithiobacillus* (5.5%), *Paludibacter* (15%) *Bacteroides* (13%) and *Desulfurella* (5%). The potato cell effluent also had matches to *Acidocella sp., Desulfosporosinus* sp and *Dechloromonas* sp. The change in percent similarities between community profiles from the different sites of the remediation system (Table 3.5) are supported by the change in TRFs contributing to the similarity (Table 3.6). This indicates a shift in community composition from a few dominant species in the sludge dam and potato cell to a more diverse community in the reduction cells and Minna Creek. The reduction cell profiles had the highest number of TRFs contributing to the similarity between samples indicating it was the most diverse which is supported by the clone library. It also appears the TRFLP profiles for the

reduction cells covered more of the diversity than the clone libraries. A relative measure of diversity based on number of TRFs found in each site sample; also indicate an increase in diversity from the sludge dam to the reduction cells (Figure 3.14).

The reduction cells most likely have a high degree of heterogeneity in bacterial communities because of complex organic matter and layered design, with possible aerobic, microaerophilic and anaerobic environments being present simultaneously. Clone libraries and TRFLP profile analysis indicate three to four metabolic bacterial groups were found in the reduction cells i.e. iron and sulfate reduction, complex carbohydrate fermentation and organic acid utilisation. These microbial metabolic processes have been previously reported in similar systems (Bechard et al. 1994; Logan et al. 2005). The four reduction cells appeared to have similar microbial communities, which are different to the other sections of the remediation system. The broader comparison of treatment level better illustrates the progressive change in bacterial communities from untreated to post-treatment. The bacterial diversity changes are also reflected in the significant differences between the clone libraries and the increasing number of TRFs found in samples from each successive treatment stage.

A possible influence on the decline of the reduction cell efficiency is most likely the exhaustion of the organic matter in the mushroom compost over time and the straw unable to break down to simpler molecules fast enough for the bacteria to use. Logan et al. (2005) found sulfate-reducing bacteria showed growth limitations through all but the early establishment phase of a permeable reactive barrier filled with wood shaving, manure and limestone rock. The sulfate- and

iron-reducing bacteria cannot directly oxidise complex organic carbon compounds, relying on a complex community of anaerobic fermenters for substrates. Furthermore, many of the phylogenetic matches from the TRFLP analysis are related to acidophiles, indicating the pH has probably dropped in the reductions cells, which was indicated as a problem contributing to their decline (Cooper 2005 pers. comm.). For a long-term solution, for the remediation of acid mine drainage and mineral processing wastes would have to take into account the interactions of different bacterial metabolic groups. Neculita et al. (2007) concluded emphasis on bacteria other than sulfate- and iron-reducers is required so that anaerobic degradation of complex organic matter is not the limiting factor for these bacteria to grow.

Another influence, which may be responsible for the decline in iron and sulfate removal efficiency, is the design of the reduction cells; the layers of compost and straw do not provide a uniform environment. Biogeochemical stratification would occur quickly and availability of organic matter would not be consistent throughout the cell. Bioreactors where the filler substrate is uniform and is porous, and with a high degree of surface area, such as a mixture of sand/gravel, woodchips and compost, have performed better than compost-based systems (Tsukamoto et al. 2004). Gagliano (2004) also found the major concentrations of metals, sulfate and bicarbonate at the interface between compost and the overlaying clay, indicating the metabolic activities of target sulfate- and iron-reducing bacteria occur in a thin zone of optimal conditions indicating thick layers of compost were not utilised by a large proportion of the target bacteria.

Table 3.4. Analysis of similarity (ANOSIM) pairwise comparisons computed from the Bray-Curtis similarity matrix of TRFLP relative peak abundance data from treatment site samples and % average similarity (SIMPER) analysis between site samples calculated in PRIMER v6. Bold = significant differences.

Group Comparisons	R Statistic	*p*	% Average Similarity
RC5, Minna Creek	**0.352**	**<0.143**	34.03
RC5, Potato Cell	**0.556**	**0.029**	25.14
RC5, RC6	0.063	0.338	30.95
RC5, RC7	**0.321**	**0.043**	33.02
RC5, RC8	**0.321**	**0.033**	27.47
RC5, Sludge Dam	**0.648**	**0.029**	22.99
RC6, Minna Creek	0.074	0.31	33.13
RC6, Potato Cell	**0.451**	**0.012**	21.35
RC6, RC7	0.128	0.119	33.15
RC6, RC8	-0.05	0.641	32.54
RC6, Sludge Dam	**0.512**	**0.012**	23.33
RC7, Minna Creek	0.296	0.107	38.29
RC7, Potato Cell	**0.636**	**0.012**	30.18
RC7, RC8	0.039	0.32	35.67
RC7, Sludge Dam	**0.753**	**0.012**	26.79
RC8, Minna Creek	-0.247	0.893	38.94
RC8, Potato Cell	**0.352**	**0.048**	25.81
RC8, Sludge Dam	**0.438**	**0.036**	25.9
Potato Cell, Minna Creek	**0.704**	**<0.1**	29.82
Sludge Dam, Minna Creek	**1**	**<0.1**	23.12
Sludge Dam, Potato Cell	-0.111	0.8	38.33

Figure 3.11. MDS (Mutli-dimensional Scaling) plot of the ANOSIM pairwise comparisons (Table 3.3) on relative peak abundance data from treatment site samples, calculated in PRIMER v6. RC = reduction cell.

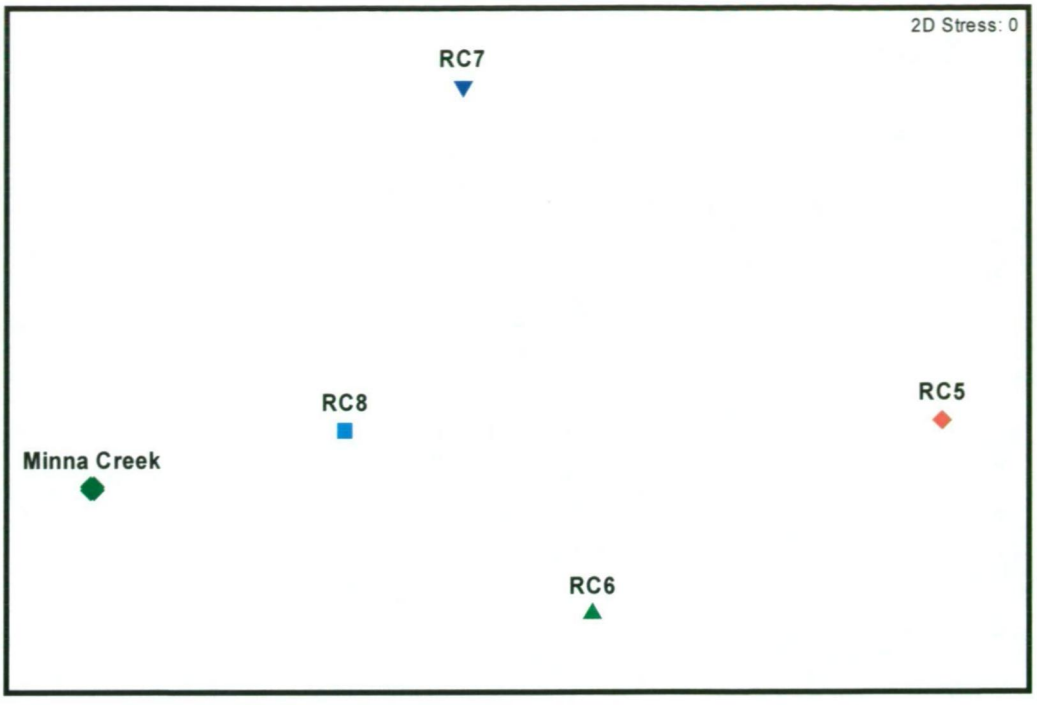


Figure 3.12. MDS plot subset from Figure 3.12 of the reduction cells (RC) and Minna Creek ANOSIM pairwise comparisons (Table 3.3) on relative peak abundance data, calculated in PRIMER v6.

Table 3.5. Analysis of similarity (ANOSIM) pairwise comparisons of treatment level from the Bray-Curtis similarity matrix of TRFLP relative peak abundance data and % average similarity by SIMPER analysis and dispersion indices (MVDISP) calculated in PRIMER v6. Bold is significant difference.

Groups	R Statistic	*p*	% Average similarity	IMD
treated, pre-treated	**0.408**	**0.017**	25.66	0.206
treated, untreated	**0.474**	**0.007**	24.91	0.27
treated, post treatment	-0.151	0.781	36.29	0.977
pre-treated, untreated	-0.111	0.8	38.33	0.111
pre-treated, post treatment	**0.704**	**<0.1**	29.82	1
untreated, post treatment	**1**	**<0.1**	23.12	1

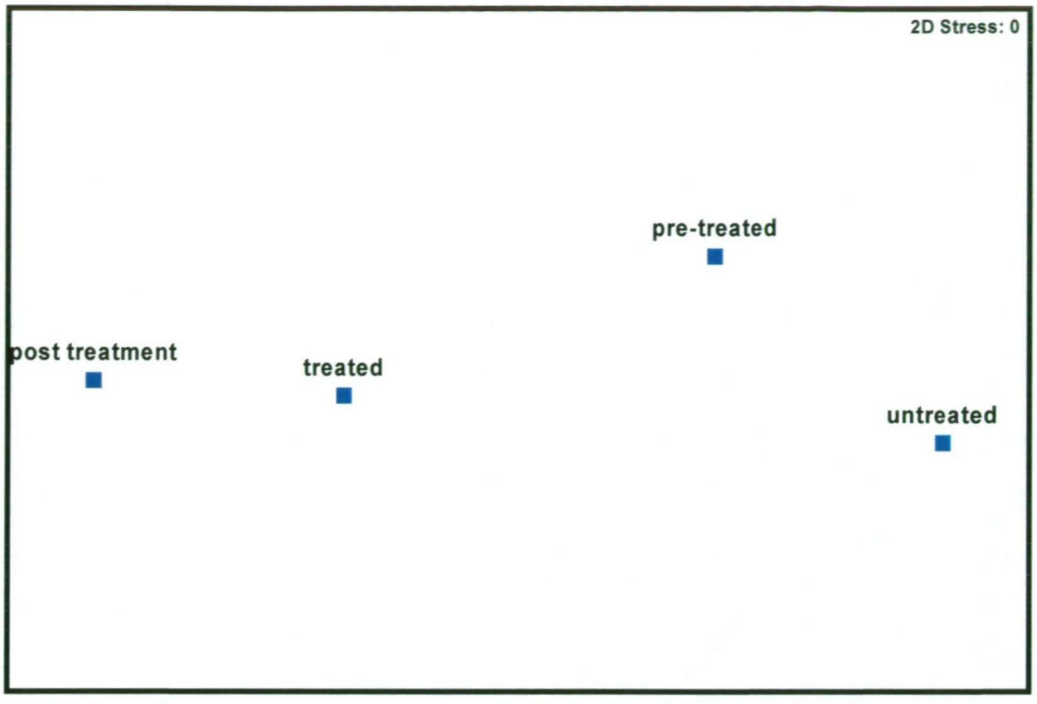


Figure 3.13. MDS plot of the ANOSIM pairwise comparisons of TRFLP peak abundance data from site samples divided into treatment stage of the bioremediation system, calculated in PRIMER v6. Untreated = sludge dam, pre-treated = potato cells, treated = reduction cells combined and post treatment = Minna Creek.

Table 3.6. TRFs contributing to 60 - 90% similarity within site samples, calculated in SIMPER analysis of the TRFLP peak abundance data, in the reduction cells (treated), potato cell (pre-treated), sludge dam (untreated) and Minna Creek (post treatment) and their possible phylogenetic matches. TRF = size (basepairs) + enzyme + forward/reverse.

	Untreated	Pre-treated	Treated	Post-treatment	
TRF	Contrib%	Contrib%	Contrib%	Contrib%	Phylogenetic match
130 msp R	0	4.7	8.61	5.31	*Acidovorax* sp./*Ferrovum myxofaciens*
124 hae R	5.04	15.12	8.1	5.83	*Paludibacter propionicigenes*
119 rsa R	0	4.44	6.44	6.67	*Dechloromonas* sp.
127 hae R	6.29	5.46	5.5	6.13	*Acidithiobacillus ferrooxidans*
121 rsa R	0	0	4.78	6.04	*Acidocella* sp./*Desulfitobacterium* sp./*Desulfovibrio* sp.
80 rsa R	0	5.04	3.97	1.49	*Bacteroidetes* clone AY792299 humic lake
171 hinf R	0	0	3.69	1.74	*Desulfomicrobium* sp.
132 msp R	0	0	3.64	4.97	*Acidiphilium* sp.
94 msp F	0	0	3.48	0	*Syntrophus* spp.
153 hinf R	16.49	0	3.22	0	*Acidithiobacillus* spp.
80 hha F	15.24	0	2.97	0	*Desufurella* spp.
73 hha R	0	0	2.69	4.65	*Nitrospira* sp./*Sulfitobacter* sp
77 hha R	0	0	2.59	4.4	*Paludibacter propionicigenes*
98 hae F	0	0	2.34	0	*Desulfuromonas* sp.
152 hha R	0	13.07	2.06	5.11	*Bacteroides* sp./*Porphyromonas* sp.
116 hae F	0	0	1.82	0	*Bacteroides* sp./*Deferribacter* sp.
80 hae R	0	0	1.76	1.7	*Rhodobacter* sp./*Sulfitobacter* sp.
201 msp F	0	0	1.68	0	*Acidosphaera rubrifaciens*
75 rsa F	0	5.08	1.67	0	*Acidosphaera rubrifaciens*
306 hae R	0	0	1.22	0	*Ferribacterium limneticum*
91 hinf R	0	4.91	1.2	0	*Gallionella ferruginea*
65 rsa R	0	0	1.19	0	*Bacteroides* sp.
368 rsa F	0	0	1.14	1.47	*Desulfovibrio* sp.
69 hha R	0	0	1.04	1.77	*Acidiphilium* sp./*Acidocella* sp./*Sulfitobacter* sp.
75 hha F	0	0	1.01	0	Candidate division OP8 bacterium
71 hha R	0	0	0.96	4.14	*Nitrospira* sp.
80 hinf R	15.59	0	0.8	0	*Thermoanaerobacter* sp.
71 hae R	0	0	0.59	0	*Geovibrio ferrireducens*
80 msp R	15.89	14.73	0	0	*Desulfosporosinus* sp.
82 hinf R	0	5.58	0	0	*Bacteroides* sp.
70 hae F	0	5.54	0	0	*Bacteroidetes*
156 hinf R	16.58	4.66	0	0	*Desufurella spp.*
Total % similarity	**91.12**	**88.33**	**80.16**	**61.42**	

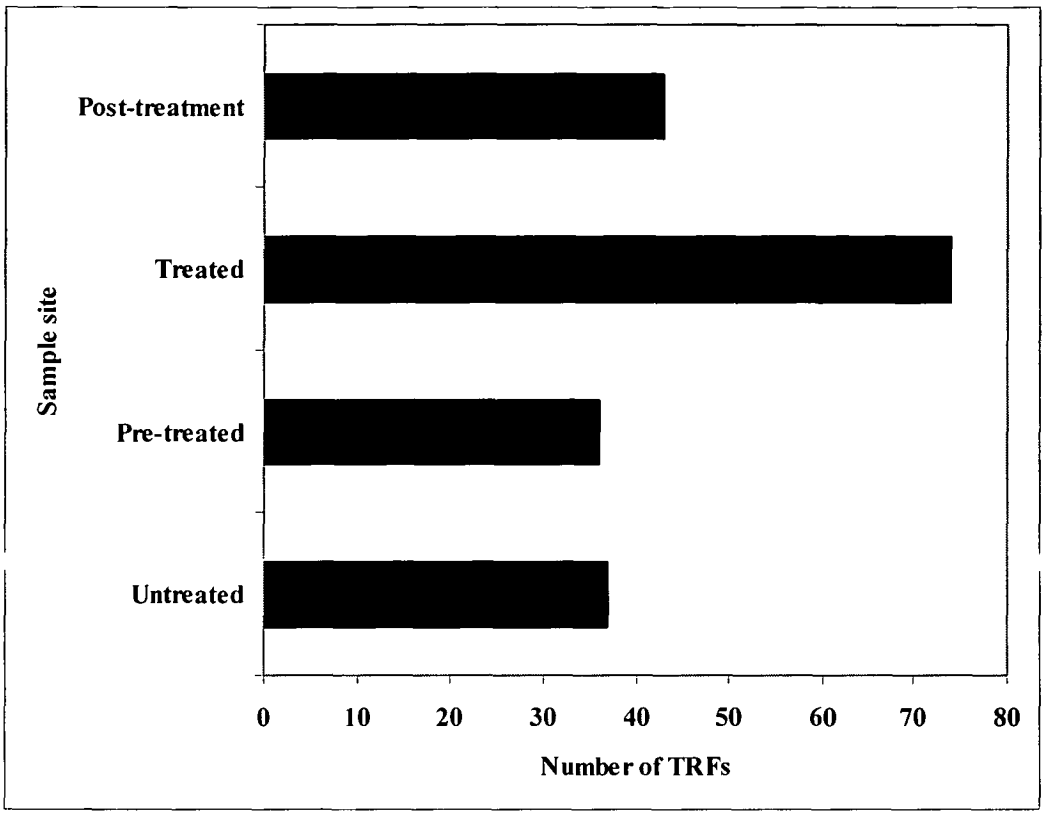


Figure 3.14. Graph of the number of TRFs found in the samples from the different treatment stages of the bioremediation system, Heybridge, Tasmania. Untreated = sludge dam, pre-treated = potato cells, treated = reduction cells combined and post treatment = Minna Creek.

TRFLP analysis of temporal variability of bacterial communities

A typical TRFLP chromatogram demonstrates changes occurring in the microbial community in samples over 18 months (May 2005, April 2006 and November 2006) (Figure 3.15). The global R statistic from the ANOSIM analysis of site samples grouped by time of sampling was 0.545 (p<0.01) demonstrating that the overall differences in bacterial communities between sites over time were statistically significant. The pairwise comparisons (ANOSIM) and percent average similarity calculated with SIMPER, between all reduction cells at the three time points (May 2005, April 2006, November 2006) are summarised in Table 3.7. The MDS plot of the ANOSIM pairwise comparisons between these points shows substantial variability also existed within the treatment stages during the survey period (Figure 3.16).

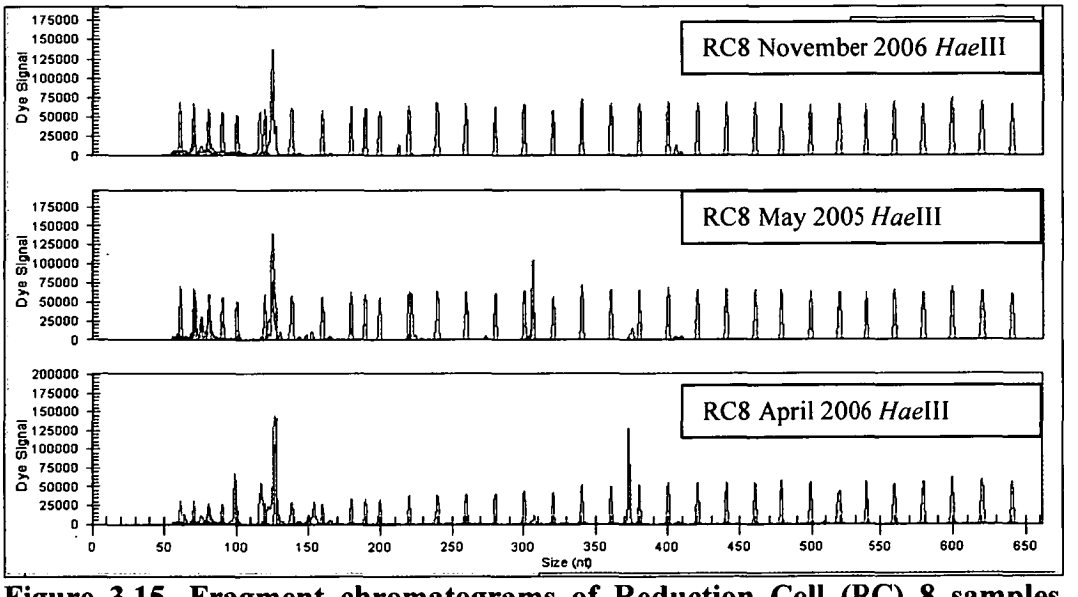


Figure 3.15. Fragment chromatograms of Reduction Cell (RC) 8 samples from May 2005, April 2006 and November 2006 with the restriction enzyme *Hae*III. Red peaks = size standard, blue peaks = reverse fragments, green peaks = forward peaks.

Reduction cell 5 effluent sample bacterial community appears to shift the most in similarity between April and November (R statisitic = 1) (RC5 was not operational in 2005) and is most different in bacterial communities to the other reduction cell samples over time. Reduction cell 5 is smaller and more exposed to sunlight and the delay in operation may be the cause of the difference. However, the iron removal efficiency for this cell also declined over time indicating it would eventually become similar to the other reduction cells (Cooper 2007, pers. comm.).

The TRFLP peak data show the similarity in the bacterial community of reduction cell 6 and 8 did not change over the three time points (Figure 3.16). The percent average similarity in TRFLP profiles decreased slightly over time for reduction cell 6 (31% for May 2005/April 2006 to 27% for April 2006/November 2006) and increased slightly over time for reduction cell 8 (28% to 32%). The iron removal efficiency for these reduction cells declined from 44% to <10% from 2002 to 2006 and the cells were shut down in 2007. The Reduction cell 7 bacterial community appeared to stabilise over time as the ANOSIM analysis of the TRFLP profiles from May 2005 and April 2006 were significantly different (R = 0.5, $p<0.33$, 28% similarity). However, the April 2006 and November 2006 TRFLP profiles were not significantly different and the percent average similarity increased to 45%. Hence, as the reduction cells declined in efficiency the bacterial population in reduction cells 6, 7 and 8 became similar to one another and plateau.

ANOSIM analysis of the TRFLP peak area data from all reduction cells over all time points (Global R = 0.359, $p<0.001$) indicate an overall stabilisation in the

bacterial community from May 2005 to November 2006. The ANOSIM analysis showed that the samples became less significantly different over time (R=0.272 to 0.206, $p<0.02$; Table 3.8, Figure 3.17). Furthermore, the bacterial community from all the reduction cells appeared to become more similar to that of Minna Creek from May 2005 to April 2006 and then shift away again in November 2006 (R = 0.241 – 0.108 – 0.234, $p<0.25$). Dispersion indices (Table 3.8) indicate most of the similarities in April 2006 were lower than any of the similarities in the other time samples. Furthermore, the MDS plot of the ANOSIM pairwise comparisons (Figure 3.16) showed reduction cell 5 (April 2006) appears to be the most different in bacterial community from all the other sites, which probably contributed to this overall difference for April 2006. Reduction cell 5 is different in size and position to the other cells and its operation started later than the other reduction cells.

SIMPER analysis was used to calculate the TRFs that contributed to >80% average similarity within samples of the reduction cells between time points and the possible phylogenetic inference determined from *in silico* restriction digests of 16S rRNA gene clones (Table 3.9). The phylogenetic groups give an indication that the bacterial community is similar between reduction cell samples over time, as the same TRFs occurred in all reduction cell time profiles such as 124haeR (*Paludibacter propionicigenes*). However, the percent similarity contribution (SIMPER) of these common groups are different between the time points which may indicate the diversity does not change significantly but the relative abundances of these common groups do. The relative diversity, based on the number of TRFs tallied for each reduction cell at each time point, indicate bacterial diversity increased from May 2005 to November 2006 for reduction

cells 7 and 8 but is probably not statistically significant (Figure 3.18). The higher percent contributions (SIMPER) of TRFs in May 2005 indicate matches to iron- and sulfate-reducers were more prevalent, whereas in November 2006 the higher TRF percent contributions match anaerobic fermenters indicating a shift in bacterial communities over time. However, this cannot be correlated to absolute quantitative changes in community structure. There was no evidence of seasonal variation, as May and April are similar seasonally; the bacterial communities were still significantly different.

Though the system may have a high degree of heterogeneity, some bacterial clone matches appeared to be ubiquitous across sites and over time, including *Acidithiobacillus* spp. and *Paludibacter propionicigenes*. The genus *Acidithiobacillus* is capable of switching metabolic processes from iron- and sulfur-oxidation in aerobic environments to sulfate- and iron-reduction in anaerobic environments (Brock and Gustafson 1976; Johnson and Bridge, 2002; Ohmura et al. 2002) and is commonly encountered in acid mine drainage (Duquesne et al. 2003; Nicomrat, et al. 2008). *P. propionicigenes* an acidophile, has been found to utilise various sugars (Ueki et al. 2006), and a high proportion of clones similar to members of this genus were found in the sludge dam and potato cell effluent samples. Propionate-producing bacteria might be beneficial as propionate can be utilised by sulfate- and iron-reducing bacteria as an electron donor; however, the fermenters may compete with other bacteria for carbon sources (Lovley 2000).

Exacerbating the lack of accessible organic matter are probably the low annual temperatures of the local climate; the reduction cells are sited in a shaded valley

where mean summer temperatures do not reach much above 20°C. Once the temperature inside the reduction cells drop, it would be hard to increase again without expensive artificial heating. Temperature also affects organic substrate decomposition, and oxygen and hydrogen sulfide solubility. Potential amendments and improved system design to promote growth of indigenous microorganisms still have to overcome low temperatures for bioremediation in temperate, alpine, and high latitude areas.

Hot composting is ideal for raising temperatures. However, if left passive, it does not last for long periods of time. However, column experiments (Tsukamoto et al. 2004) have shown that if the bacteria have been allowed to acclimatise, iron and sulfate reduction were not affected by lower temperatures. Furthermore, low temperatures negatively affect the methanogens, which compete with SRB and IRB for organic substrates, so lower temperatures may benefit these systems. In a similar bioremediation system Gagliano (2004) found no consistent variation in metal and sulfate removal with sampling date of compost wetlands in southern Ohio (temperature range: 17 - 27°C in summer to ˜5 - 4°C in winter) the major correlation was with wetland stratigraphy. Temperature decreases may magnify other factors already affecting bioreactor efficiency.

Table 3.7. ANOSIM pairwise comparison analysis and SIMPER % average similarity of the Bray-Curtis similarity matrix for reduction cells over time.

Groups	R Statistic	*p*	% Average similarity
RC5April 06, RC5November 06	1	0.333	30.3
RC5April 06, RC6April 06	0.75	0.333	26.46
RC5April 06, RC6November 06	0.75	0.333	28.22
RC5April 06, RC7April 06	0.25	0.667	27.5
RC5April 06, RC7November 06	1	0.333	35.11
RC5April 06, RC8April 06	1	0.333	22.92
RC5April 06, RC8November 06	1	0.333	31.42
RC5November 06, RC6November 06	1	0.333	22.88
RC5November 06, RC7November 06	1	0.333	33.53
RC5November 06, RC8November 06	1	0.333	27.54
RC6April 06, RC5November 06	0.25	0.667	36.11
RC6April 06, RC6November 06	0.25	0.333	27.36
RC6April 06, RC7April 06	-0.5	1	36.28
RC6April 06, RC7November 06	0.5	0.333	32.4
RC6April 06, RC8April 06	-0.25	1	44.29
RC6April 06, RC8November 06	0.5	0.333	27.87
RC6May 05, RC5April 06	1	0.333	29.31
RC6May 05, RC5November 06	0.75	0.333	42.71
RC6May 05, RC6April 06	0.25	0.667	30.95
RC6May 05, RC6November 06	0.75	0.333	25.74
RC6May 05, RC7April 06	0.75	0.333	26.76
RC6May 05, RC7May 05	1	0.333	38.03
RC6May 05, RC7November 06	1	0.333	40.62
RC6May 05, RC8April 06	1	0.333	22.9
RC6May 05, RC8May 05	0.5	0.333	35.25
RC6May 05, RC8November 06	1	0.333	34.31
RC6November 06, RC7November 06	0.5	0.333	44.64
RC6November 06, RC8November 06	-0.25	1	44.57
RC7April 06, RC5November 06	1	0.333	26.42
RC7April 06, RC6November 06	0	1	28.1
RC7April 06, RC7November 06	0	1	44.8
RC7April 06, RC8April 06	-0.25	1	35.97
RC7April 06, RC8November 06	0.25	0.333	35.01
RC7May 05, RC5April 06	0.75	0.333	38.73
RC7May 05, RC5November 06	0.5	0.667	36.83
RC7May 05, RC6April 06	0.5	0.333	26.68
RC7May 05, RC6November 06	1	0.333	24.84
RC7May 05, RC7April 06	0.5	0.333	28.08
RC7May 05, RC7November 06	1	0.333	38.28
RC7May 05, RC8April 06	1	0.333	26.13
RC7May 05, RC8May 05	0.25	0.667	37.68
RC7May 05, RC8November 06	1	0.333	34.61
RC7November 06, RC8November 06	0	1	55.91
RC8April 06, RC5November 06	1	0.333	19.56
RC8April 06, RC6November 06	0.5	0.333	30.17
RC8April 06, RC7November 06	0.75	0.333	36.9
RC8April 06, RC8November 06	0.75	0.333	32.49
RC8May 05, RC5April 06	0.75	0.333	27.29
RC8May 05, RC5November 06	0.5	0.333	36.1
RC8May 05, RC6April 06	0	0.667	28.79
RC8May 05, RC6November 06	1	0.333	24.72
RC8May 05, RC7April 06	0.5	0.333	25.66
RC8May 05, RC7November 06	0.75	0.333	33.18
RC8May 05, RC8April 06	0.75	0.333	28.05
RC8May 05, RC8November 06	0.5	0.333	29.92

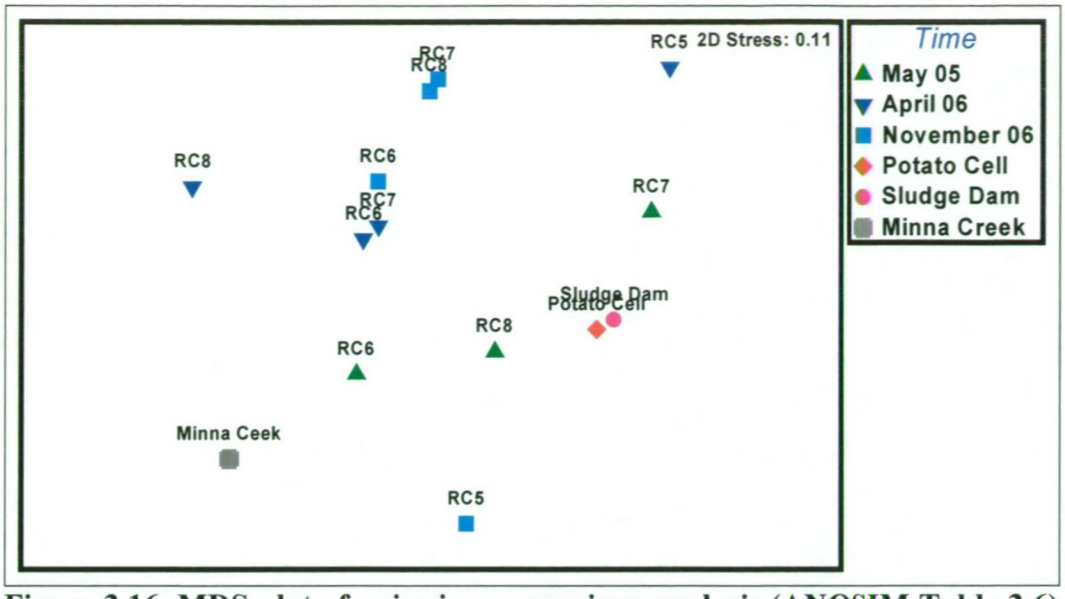


Figure 3.16. MDS plot of pairwise comparison analysis (ANOSIM Table 3.6) of TRFLP peak area data between sites of the bioremediation system over time of sampling. RC = reduction cell and reduction cell number.

Table 3.8. ANOSIM pairwise comparison analysis and SIMPER % average similarity and dispersion indices (MVDISP) of the Bray-Curtis similarity matrix for reduction cell samples combined over time compared to the other treatment levels. Bold is significant difference.

Groups	R Statistic	*p*	% Average similarity	IMD
May 05, April 06	**0.272**	**0.018**	28.28	-0.481
May 05, November 06	**0.309**	**0.015**	33.49	0.043
May 05, Potato Cell	**0.543**	**0.024**	30.88	-0.111
May 05, Sludge Dam	**0.691**	**0.012**	30.49	-0.289
May 05, Minna Creek	0.241	0.119	38.78	1
April 06,November 06	**0.206**	**0.011**	31.39	0.403
April 06,Potato Cell	**0.669**	**0.006**	20.34	0.333
April 06, Sludge Dam	**0.661**	**0.006**	19.77	0.405
April 06, Minna Creek	0.108	0.248	33.47	0.952
November 06, Potato Cell	**0.591**	**0.012**	27.08	-0.214
November 06, Sludge Dam	**0.661**	**0.006**	25.87	-0.19
November 06, Minna Creek	0.234	0.133	37.23	0.857

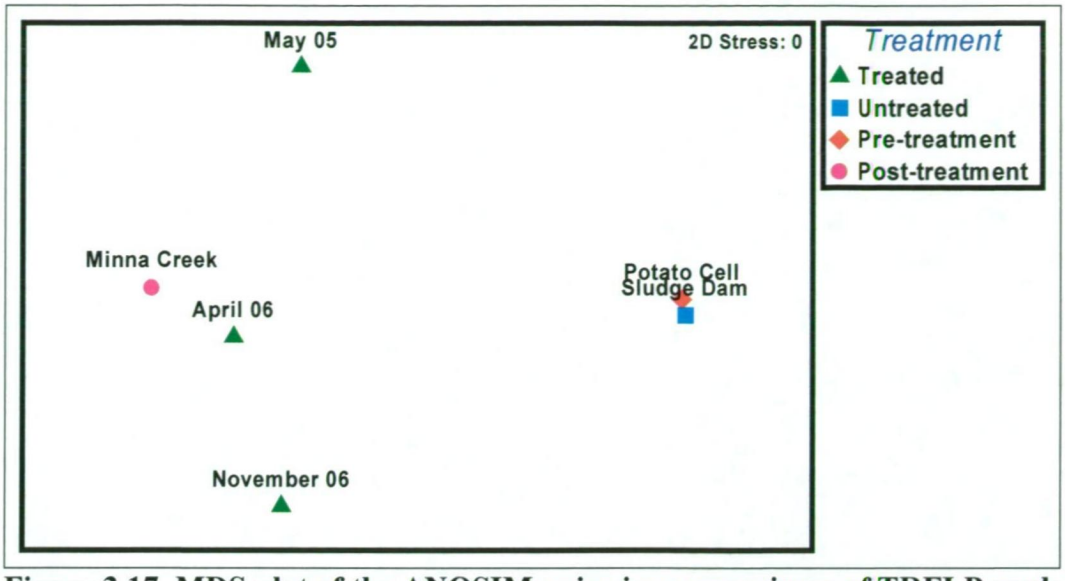


Figure 3.17. MDS plot of the ANOSIM pairwise comparisons of TRFLP peak area data from reduction cells (treated) combined over time compared to the sludge dam (untreated), potato cell (Pre-treatment) and Minna Creek (Post-treatment) samples.

Table 3.9. TRFs contributing to >80% similarity, calculated in SIMPER analysis (PRIMER v6) of the TRFLP peak abundance data of the reduction cells over time and their phylogenetic inference, TRF = size (base pairs) + enzyme + forward/reverse. The five TRFs contributing the highest % similarity for each time point are bold.

	May-05	Apr-06	Nov-06	
TRF	Contrib%	Contrib%	Contrib%	Phylogenetic match
306 hae R	**10.43**	0	0	*Ferribacterium limneticum*
124 hae R	**8.25**	2.56	**11.86**	*Paludibacter propionicigenes*
130 msp R	**7.63**	**11.41**	4.44	*Acidovorax* sp./*Ferrovum myxofaciens*
119 rsa R	**7.56**	4.49	5.03	*Dechloromonas* sp.
80 hha R	**6.58**	0	2.92	*Firmicutes*
152 hha R	6.35	0	1.65	*Bacteroides* sp./*Porphyromonas* sp.
128 hae R	6.34	2.77	**5.27**	*Acidithiobacillus ferrooxidans*
120 rsa R	4.11	4.18	0	*Acidovorax* sp./*Desulfotomaculum* sp.
75 rsa F	4.04	1	0	*Acidosphaera rubrifaciens*
121 rsa R	3.8	2.69	**5.35**	*Acidocella* sp./*Desulfitobacterium* sp./*Desulfovibrio* sp.
400 hha R	3.67	0	0	*Sulfurospirillum* sp.
75 hha F	3.57	0	0	Candidate division OP8 bacterium
69 hha R	2.34	1.14	0	*Acidiphilium* sp./*Acidocella* sp./*Sulfitobacter* sp.
94 msp F	2.17	1.23	5.26	*Syntrophus* spp.
132 msp R	2.11	3.15	3.47	*Acidiphilium* sp.
80 hae R	1.61	0	1.6	*Rhodobacter* sp./*Sulfitobacter* sp.
71 hha R	0.91	3.05	0	*Nitrospira* sp.
77 hha R	0.78	2.24	3.04	*Paludibacter propionicigenes*
171 hinf R	0.69	**6.66**	2.47	*Desulfomicrobium* sp.
201 msp F	0	**7.96**	0	*Acidosphaera rubrifaciens*
153 hinf F	0	**5.2**	2.49	*Firmicutes/Ferribacterium* sp./*Dechloromonas sp.*
98 hae F	0	**4.76**	2.08	*Desulfuromonas* sp.
368 rsa F	0	4.61	0	*Desulfovibrio* sp.
372 hae R	0	4.42	0	*Acidovorax* sp./*Comamonas* sp./*Rhodovorax* sp.
73 hha R	0	4.36	1.81	*Nitrospira* sp./*Sulfitobacter* sp
80 rsa R	0	3.47	**6.41**	*Bacteroidetes* clone AY792299 humic lake
91 hinf R	0	2.42	0	*Gallionella ferruginea*
65 rsa R	0	2.41	1.52	*Bacteroides* sp.
116 hae F	0	0	**5.29**	*Bacteroides* sp./*Deferribacter* sp.
137 hha R	0	0	3.44	*Bacteroides* sp.
88 rsa R	0	0	3.42	*Desulfobacterium* sp.
110 rsa F	0	0	3.09	Termite Group 1
163 hinf R	0	0	2.49	*Syntrophus* spp.
Total % Similarity	**82.94**	**86.18**	**84.4**	

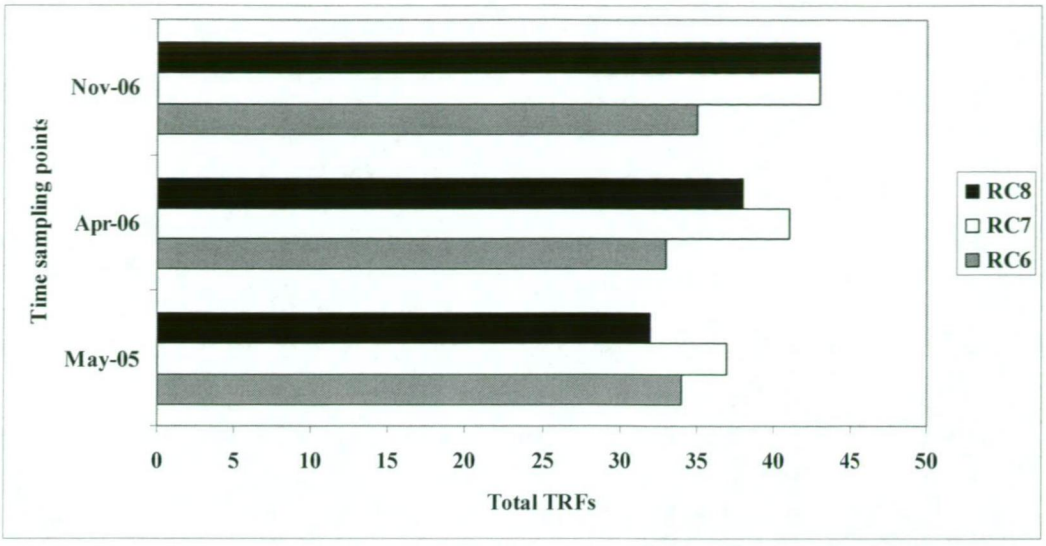


Figure 3.18. Graph of the total number of TRFs for the reduction cells from the different time point samples of the bioremediation system, Heybridge, Tasmania. RC = reduction cell. Reduction cell 5 was not included.

Scanning electron microscopy and electron probe microanalysis

The reduction cell enrichment broths prepared for scanning electron microscopy photography (Figure 3.19a-e) show a mixed culture of short and long rods, vibrio-shaped as well as cells with similar morphology to the twisted and stalked bacteria *Gallionella ferruginea* (Figure 3.19b). Similar cells to the polygonal-shaped cells (Figure 3.19b) have previously been found in diesel sludge (Vrdoljak et al. 2005) and their 16S rRNA gene sequence matched with *Paenibacillus* sp., which a reduction cell clone (RC8C4, EU921203) grouped. The electron probe analyses revealed the precipitates present on the SEM samples were iron oxyhydroxides and iron oxyhydroxysulfates, and have morphology similar to goethite and schwertmannite. Cells were either disassociated with the precipitates (Figure 3.19b) or associated with the precipitates (Figure 3.19c-e).

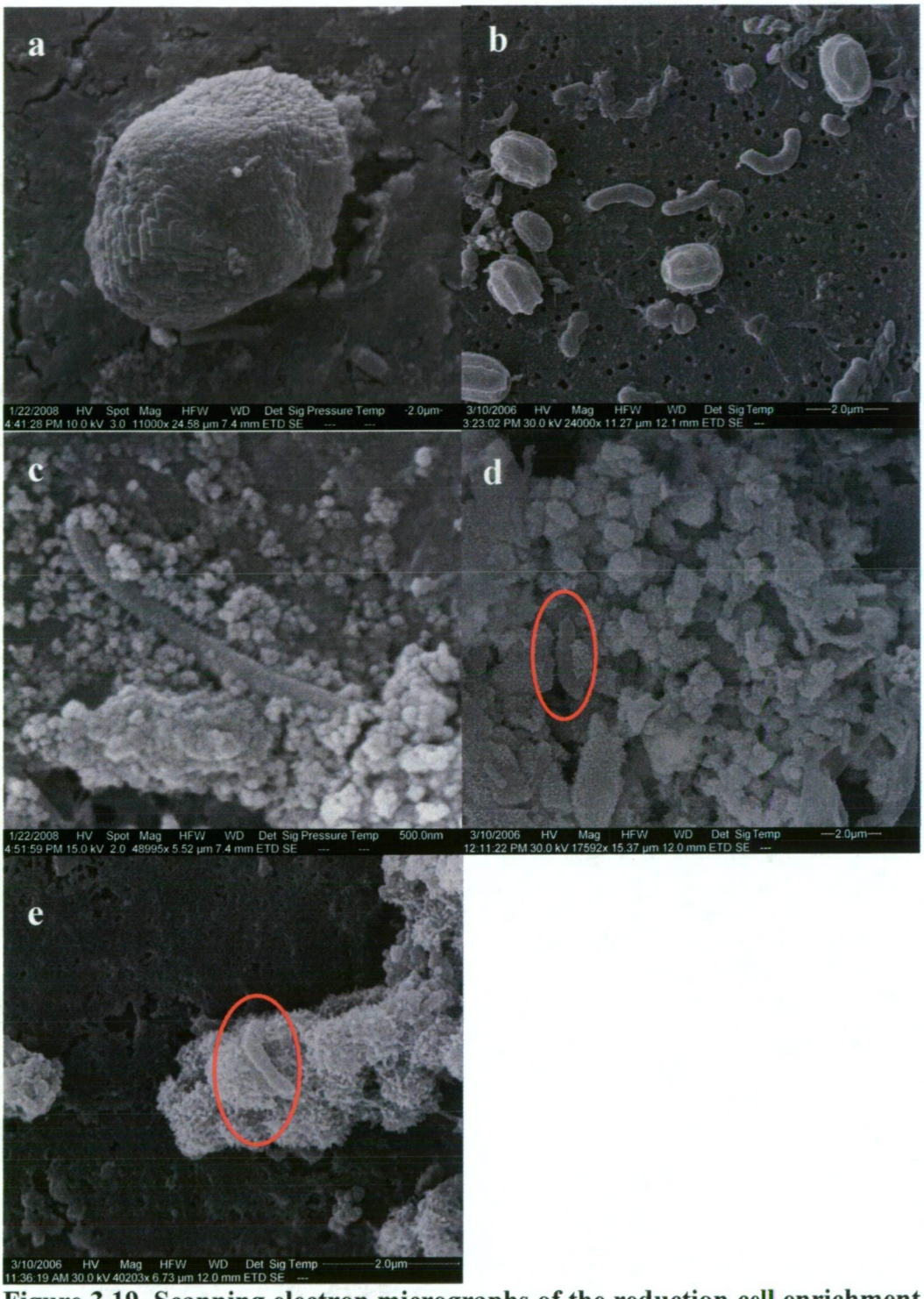


Figure 3.19. Scanning electron micrographs of the reduction cell enrichment. (a) - Iron oxyhydroxide precipitate, disassociated cells, (b) - twisted and stalked similar to *Gallionella* spp. and polygonal-shaped cells similar to *Paenibacillus* spp., (c-e) - rod-shaped cells (indicated by red circles) associated with the precipitates.

Conclusion

The 16S rRNA gene clone libraries and TRFLP analysis has shown the microbial communities in a novel and passive bioremediation system can be very complex and heterogeneous within and between treatment stages. Microbial community composition was observed to change over time as which may have been influenced by organic matter availability due to system design, pH decrease, seasonal temperatures, rainfall, leachate flow rates and hydraulic retention time. This complex nature will have effects in the design and management of these systems, keeping the system homogeneous with respect to organic matter addition, acclimation period and delivery of the fluid to be remediated. Frequent monitoring of changes in the microbial community, using methods like TRFLP, as well as the chemical analysis and alternative organic biomass amendments could improve management and efficiency of these systems. Laboratory-scale microcosms or pilot studies set up to determine the best amendments for increasing the efficiency of the reduction cells and their possible applications to other remediation systems would be beneficial.

Chapter 4.

Microcosm Experiments

Cradle Mountain, Tasmania

Laboratory-scale experimental microcosm assessment of microbiological remediation of acidic, metal-rich industrial waste water using a compost-based reduction system

Abstract

A series of laboratory-scale microcosm experiments were conducted to evaluate the influence of temperature, bicarbonate and various carbon amendments (ethanol, molasses and vegetable oil emulsion) on bioremediation of an acidic, metal- and sulfate-rich titanium processing leachate. In all microcosms, pH increased from 4 to 6.5-8 over the length of the experiment due to the organic matter input but this had no affect on other geochemical processes measured. The oxidation-reduction potential (ORP) decreased in all microcosms (700mV to -100mV) but was the most stable in the oil emulsion amended microcosms. Alkalinity production was more substantial in the ethanol, molasses and oil emulsion amended microcosms (~2500mg L^{-1}) compared to the different temperature and bicarbonate amended microcosms (~600 – 1800mg L-1). The weekly addition of 15 mg L^{-1} bicarbonate did not increase pH or alkalinity. Iron and sulfate were initially removed in all microcosms but removal could not be sustained in the low temperature and bicarbonate microcosms. The ethanol, molasses and vegetable oil emulsion amendments were found to sustain 99% iron removal for the length of the experiment. In all the microcosms sulfate removal only reached a maximum of 80% removal and was found to have a lag phase of approximately 80 days, and hence an acclimatisation stage may be needed for enhanced sulfate removal. 16S rRNA gene sequence based on terminal restriction fragment length polymorphism (TRFLP) profiles revealed that all the microcosms had similar bacterial communities but the amended microcosms were more

successful than the unamended microcosms in promoting the growth of a select bacterial consortia needed for enhanced sulfate and iron removal. Major populations detected included a combination of anaerobic fermenters, iron- and sulfate-reducers as well as iron/sulfur/sulfide oxidising bacteria.

Introduction

Mining and mineral processing wastewaters and leachates are often characterised by low pH and high concentration of metals and sulfate. These can be highly toxic to organisms in affected waterways and catchments and traditionally these have been neutralised with lime and other alkalising agents (Costello 2003). Passive and semi-passive bioremediation systems are attractive technologies as they can be cost effective and can incorporate both site remediation and overall ecosystem reclamation by using indigenous microbiota and flora (Johnson and Hallberg, 2005; Whitehead et al. 2005). Furthermore, they allow supplementation with simple and cost effective carbon sources such as molasses, cheese whey, ethanol and soybean oil (Sturman 2001; Kaksonen et al. 2004b; Geets et al. 2005; Lindow and Borden 2005a; 2005b), woodchips, paper pulp waste (Hulshof et al. 2006) and fly ash (Gitari et al. 2006).

Sulfate- and iron-reducing bacteria (SRB and IRB) are known for their capacity to precipitate or reduce metals to metal sulfides or metal oxides/hydroxides (Lovley 2000). SRB reduce sulfate to hydrogen sulfide with organic electron donors or hydrogen. Hydrogen sulfide precipitates metals as metal sulfides and oxidation of organic matter produces alkalinity (Rabus et al. 2000). IRB use ferric iron (Fe^{3+}) as a terminal electron acceptor in anaerobic respiration. The ferric iron is reduced to ferrous iron (Fe^{2+}), which is insoluble at low pH, and organic matter is oxidised

to CO_2 (Lovley 2000). The activity of these bacteria has been exploited for the removal of metals from contaminated wastewater, acid mine drainage or contaminated groundwater in on-site bioreactors and reactive barriers (Sierra-Alvarez et al. 2006; Da Silva et al. 2007; Hiibel et al. 2008; Rowe and Johnson 2008; Touze et al. 2008).

Before active implementation of *in situ* bioremediation systems their feasibility, efficiency and optimisation are best investigated via batch or continual flow-through experimental systems; for example, laboratory-scale microcosms can be used for this purpose (Geets et al. 2005; Geets et al. 2006). Since successful passive *in situ* bioremediation relies on the management of indigenous microbial populations, the leachate and material from the site of interest should be used with these. To optimise metal reduction processes, an insight into the bacterial community is also useful as this information can be used to determine what is the best strategy to increase the growth of target organisms. Efficiencies obtained in laboratory bioreactors are generally better than in pilot or full-scale bioreactors, none of which have remained operational for more than four years (present study site included) (Neculita and Zagury, 2008). However, careful selection of a suitable carbon source is of great importance to ensure high performance and longevity, and rigorous and methodical testing of these must occur in the laboratory.

This study involves analysis of iron reduction and sulfate removal from pre-treated leachates obtained from a remediation scheme developed for reclamation of a titanium processing plant site that operated for more than 50 years on the Tasmanian north-west coast. The titanium was extracted using sulfuric acid and

this process created acidic, metal-rich wastewater that was pumped into holding dams. After the plant was closed down, the dams leaked into the local river catchment and coastal waters (see Chapter 1 for site characterisation). Batch-type microcosm experiments were conducted to determine factors that would improve the efficiency of microbial sulfate- and iron-reduction in pre-treated titanium processing leachates. Factors included temperature, alkalinity addition and electron-donors such as ethanol, molasses and vegetable oil.

Materials and Methods

Pre-treated leachate samples were collected from the ES&D Heybridge remediation site near Burnie, north-west Tasmania (41°05.05'S, 145°98.46'E). To collect enough material for microcosm experiments, leachate was collected in a plastic 50L brewers drum fitted with an outlet at the base. Mushroom compost and straw were also collected from the remediation site in bags.

Microcosm experiments

To determine if amendments could improve the iron and sulfate removal efficiency of the Heybridge site as the design currently stands, laboratory-scale microcosms were set up with the same basic design using untreated mushroom compost, straw, bluestone and potato cell effluent. Five treatments were trialled in the microcosms; temperature (10, 20 and 25°C); recirculating and non-recirculating bicarbonate additions; ethanol; molasses; and vegetable oil emulsion addition. Because of space restrictions, the temperature microcosm experiments were set up using 3L glass containers with a plastic outlet in a stopper inserted into the base (Figure 4.1a-c). The other microcosm experiments were set up in 30L brewing barrels, which had an outlet at the base for sampling and were

incubated at 20°C in a temperature-controlled room (Figure 4.1d). All microcosms had a 5cm bluestone gravel layer, and then were half filled with mushroom compost followed by straw, analogous to the Heybridge field site reduction cells (Chapter 3). 150ml of potato cell effluent (designated RCF in Chapter 3) was added to the 3L microcosms per day and 1500ml was added to the 30L microcosms per day, equivalent to the volumetric rate of potato cell effluent added to the Heybridge site reduction cells per day. Amendments were chosen based on literature reports and their ready availability, cost and lack of toxicity, in keeping with the policy of the operation at Heybridge. Geets et al. (2005) tested six carbon sources and found them to enhance the efficiency of metal reduction from best to worst in the following order; HRC® (Hydrogen Release Compound) > molasses > methanol > lactate > ethanol > acetate. Consequently, molasses and ethanol were chosen for utilisation in this study. Sodium bicarbonate was also identified by ES&D as a possible amendment as there were problems with keeping the pH high enough for iron- and sulfate-reduction (Cooper pers. comm). Edible oil emulsion amendments were also examined. These oil emulsions have been used for enhanced anaerobic bioremediation of chlorinated solvents (Anon 2007; Borden 2003, 2006) and evaluated for anaerobic bioremediation of acid mine drainage in laboratory column experiments (Lindow and Borden 2005a; 2005b). Furthermore, selection of locally available substrates is preferred because they are more cost effective than commercial additives and mixtures of substrates seem to perform better than a single source (Neculita and Zagury 2008). Therefore, the continued use of the mushroom compost and straw as a substrate was required. Two controls were set up under the same conditions as the temperature microcosms; a sterile control (sterile compost and influent,

autoclaved for 30 mins at 121°C) and a compost control (compost and sterile influent) incubated at 20°C. The other microcosms were set up as follows:

- **Bicarbonate microcosms:** 100ml of a 5g L^{-1} solution of sodium bicarbonate (Sigma Aldrich) was added to both recirculating and non-recirculating microcosms twice a week to get a final concentration of 15mg L^{-1}. However, when the recirculating microcosm was sampled, half of the discharge was kept and mixed with the same volume of leachate and added back into the microcosm. The sodium bicarbonate was added separate since when mixed with the leachate the iron precipitated,

- **Ethanol microcosm:** 100ml of 15% ethanol was added once a week for a final concentration of 0.5mg L^{-1} (Kaksonen et al. 2004b).

- **Molasses microcosm:** 40ml of a 200g L^{-1} solution of molasses was added once a week to a final concentration of 200mg L^{-1} (Sturman 2001).

- **Vegetable oil emulsion microcosms:** Oil in water emulsion was made using 5g L^{-1} soy lecithin as the emulsifier. 200ml of water, 400ml edible vegetable oil and 400ml of the lecithin solution were blended in a kitchen blender (Sunbeam Co Ltd, Botany, NSW) for 5mins, 100ml of this was added to the initial 10L of leachate, mixed again, and added to the microcosm. The microcosm was allowed to sit for 5 days; after which the oil droplets adhere to the soil particles. After 56 days, molasses was also added to the vegetable oil emulsion (100mg L^{-1}) before being added to the microcosm (Lindow and Borden 2005a; 2005b).

Measurement of pH and Oxidation Reduction Potential (ORP)

The microcosm effluent pH was measured with an Orion 250A pH meter (Thermo Fisher Scientific, Waltham, Massachusetts). The pH meter was calibrated with pH 4.0 and pH 7.0 calibration solutions as specified in the manufacturer's protocol. The effluent ORP was measured using a Hanna ORP electrode (HI3230B) with a Hanna Microcomputer (HI9025C) (Hanna Instruments, Ann Arbor, Michigan). The ORP probe was calibrated and treated as specified in the manufacturer's protocol. In short, the probe was tested with a standard redox solution (HI7020) (200 – 275mV) and pre-treated with an oxidising solution (HI7091) and reducing solution (HI7092). Samples were measured for ORP as soon as they were taken to minimise the effect of oxygen introduction during the sampling process.

Measurement of bicarbonate (HCO_3^-) alkalinity production

Microcosm effluent alkalinity was measured as bicarbonate (HCO_3^-) following the bicarbonate alkalinity acidimetric titration protocol in the Australian Standard for Waters (AS3550.3-1992). 10ml to 25ml of sample was titrated with 0.02M HCl to an endpoint of pH 4.5 using a Metrohm 702 SM Tritrino autotitrator (MEP Instruments, Herisau, Switzerland). The alkalinity was calculated from the following equation:

$$\text{Alkalinity (mg L}^{-1}\text{ CaCO}_3) = \frac{V_1 \times C \times 50040}{V}$$

Where V_1 = volume of HCl to pH 4.5 (ml), C = molarity of HCl, V = volume of test sample (ml). To convert mg L^{-1} $CaCO_3$ into L^{-1} HCO_3^- results were multiplied by 1.219. Samples were analysed in duplicate.

Measurement of iron removal using the ferrozine/Fe^{2+} method

The method for the measurement of microcosm effluent Fe^{2+} was that used by Lovley and Phillips (1986) and Viollier et al. (2000). Fe^{2+} was determined by adding 1ml or 1g of sample to 5ml of 0.5M HCl, and incubated at room temperature for 1 hour. 1ml of the sample/HCl was then added to 5ml of ferrozine (1g L^{-1}) in 50mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid) buffer, mixed for 15sec and incubated at room temperature for 30min. The mixture was passed through a 0.2µm filter (Advantec MFS Inc. Dublin, USA) and the absorbance (562nm) was determined using a GBC UV/VIS 916 spectrophotometer (GBC Scientific Equipment, Dandenong, Australia). Fe^{2+} standards (0, 10, 50, 100 250 and 500 mg L^{-1}) were prepared from ferrous ethylenediammonium sulfate and a standard curve was plotted to determine the equation for calculating Fe^{2+} concentrations. A trial was carried out to determine the minimum amount of ferrozine/HEPES buffer needed to form the ferrozine-iron complex to be accurately measured on the spectrophotometer. This was done by adding the sample/HCl mix to different volumes of ferrozine/HEPES buffer (5, 10 and 20ml); 5ml was found to be sufficient. Samples and standards were measured in duplicate.

Measurement of SO_4^{2-} anion removal

Microcosm effluent sulfate (SO_4^{2-}) was measured following the turbidimetric method of Rayment (1992), which involves precipitation of SO_4^{2-} in the sample by $BaCl_2$ in the presence of HCl. The precipitation of $BaSO_4$ was performed under precise conditions in order to produce suspended crystals of uniform size.

The turbidity or absorbance of the suspension is then measured spectrophotometrically and compared with known standards.

Each sample was filtered through a 0.2μm filter to remove debris. 1ml of sample was added to 0.5ml of "conditioning reagent" (75g NaCl in 300ml of deionised water(dH_2O), 30ml of 10M HCl, 100ml of 95% ethanol and 50ml glycerol) and mixed. This mix was made up to 5ml with 200μl of "seed solution" and dH_2O and left to precipitate for 1h, and the absorbance measured at 420nm on the GBC UV/VIS 916 spectrophotometer. The "seed solution" consisted of 23g BaCl, 4ml K_2SO_4 solution (0.544g in 1L dH_2O), 46ml dH_2O, heated to dissolve the BaCl; then left to precipitate for 1h after which the supernatant being retained and the residues discarded. Another 4ml of K_2SO_4 solution was added to the supernatant and the above steps repeated three times. The solution was then diluted to 50ml with dH_2O and stored away from light. A stock sulfate standard (300mg $SO_4^{2-}S$) was prepared by dissolving 1.6306g K_2SO_4 in 1L dH_2O stored in the dark. Working sulfate standards of 0, 1.5, 6.0, 15, 30, 60 and 90 mg SO_4^{2-} were prepared from the stock sulfate standard and their absorbance measured at 420nm and a standard curve was plotted to determine the equation for calculating sulfate concentrations. Samples and standards were measured in duplicate.

Terminal Restriction Fragment Length Polymorphism analysis

The TRFLP analysis on the microcosm samples was the same protocol used in Chapter 3, page 55 and 59.

Results and Discussion

Microcosm observations

The temperature and control microcosms set up in glass jars (Figure 4.1a-c) illustrate the gradation of iron deposition with red ferric hydroxide deposited at the water/air interface in the straw, while the black ferrous silfide deposited in the mushroom compost. The other microcosms, set up in the beer barrels, also had the same layering of iron deposition. A black material was occasionally found in the effluent when the microcosms were sampled. This resulted in higher recorded concentrations of iron in the effluent, indicating an occasional flushing out of iron precipitates.

Figure 4.1. Photos of microcosms set up at 10°C (a), 20°C(b), 25°C(c) in glass jars with a tap at the bottom (not in view) and the ethanol (right) and molasses (left) microcosms (d) in brewing barrels incubated at 20°C and all filled with mushroom compost, straw and leachate from the Heybridge remediation site.

pH and oxidation reduction potential (ORP)

The pH for the controls and all the temperature and carbon amendment microcosms (Figure 4.2a - e) increased from 4 to 6.5 – 8 within the first 2 to 6 days and stayed between 6.5 to 7.5 for the remainder of the experiments similar to the first year of operation of the reduction cells (Figure 1.12). The leachate pH remained at pH 3.5 to 4.5. Temperature or treatment did not affect the pH value, the high organic matter kept it at near neutral and the pH appeared to have no effect on the other measured biogeochemical processes.

The effluent ORP (Figure 4.3) dropped from initial 763mV to between 350 to 120mV after 130 days (the ORP for the first 129 days of the controls, temperature and bicarbonate microcosms were not measured due to a faulty probe). However, for the ethanol, molasses and oil emulsion supplemented microcosms, which showed a similar pattern in regards to a change in pH to the other microcosms, the ORP dropped within the first 4 days. The ethanol and molasses supplemented microcosms (Figure 4.3d) initially dropped to approx. 350mV and then slowly dropped over the next 200 days to around 150mV and stayed at that ORP value. The 10°C and 20°C oil emulsion microcosms (Figure 4.3e) both dropped to -48mV in the first 3 days and remained between -50 and -100mV for 180 days. The sterile control ORP (Figure 4.3a) did decrease but with a large fluctuation of around 100mV at approximately 250 days from the start. This result suggests temperature and bicarbonate addition had no effect on maintaining oxidising or reducing conditions as these microcosms had a similar result to the sterile control. The ORP of the ethanol, molasses and vegetable oil emulsion microcosms stayed

low and relatively stable for the duration of the experiment. Furthermore, temperature had no effect on the redox conditions in the vegetable oil emulsion microcosms, as the ORP in the 10°C oil emulsion microcosm was similar to that of the 20 ° C oil emulsion microcosm.

Generally SRB and IRB require an anoxic and reduced environment with a redox potential lower than -100mV (Postgate 1984; Lovley 1991). However, measurements of ORP at outlets may not reflect the ORP values inside the microcosms, so some studies have included multiple replicate microcosms and sacrificed a replicate at various times to measure internal physio-chemical characteristics (Logan et al. 2005). Hence, at the end of the microcosm experiments ORP values were taken from the centre of the mushroom compost (Table 4.1). This illustrated the ORP was much lower inside the microcosms especially the carbon amended microcosms, low enough for iron and sulfate reduction. Furthermore, where the ORP values have been found to be higher inside full-scale bioreactors it was thought to be due to concurrent formation of anoxic microenvironments, microaerophilic and aerobic environments (Lyew and Sheppard 1997).

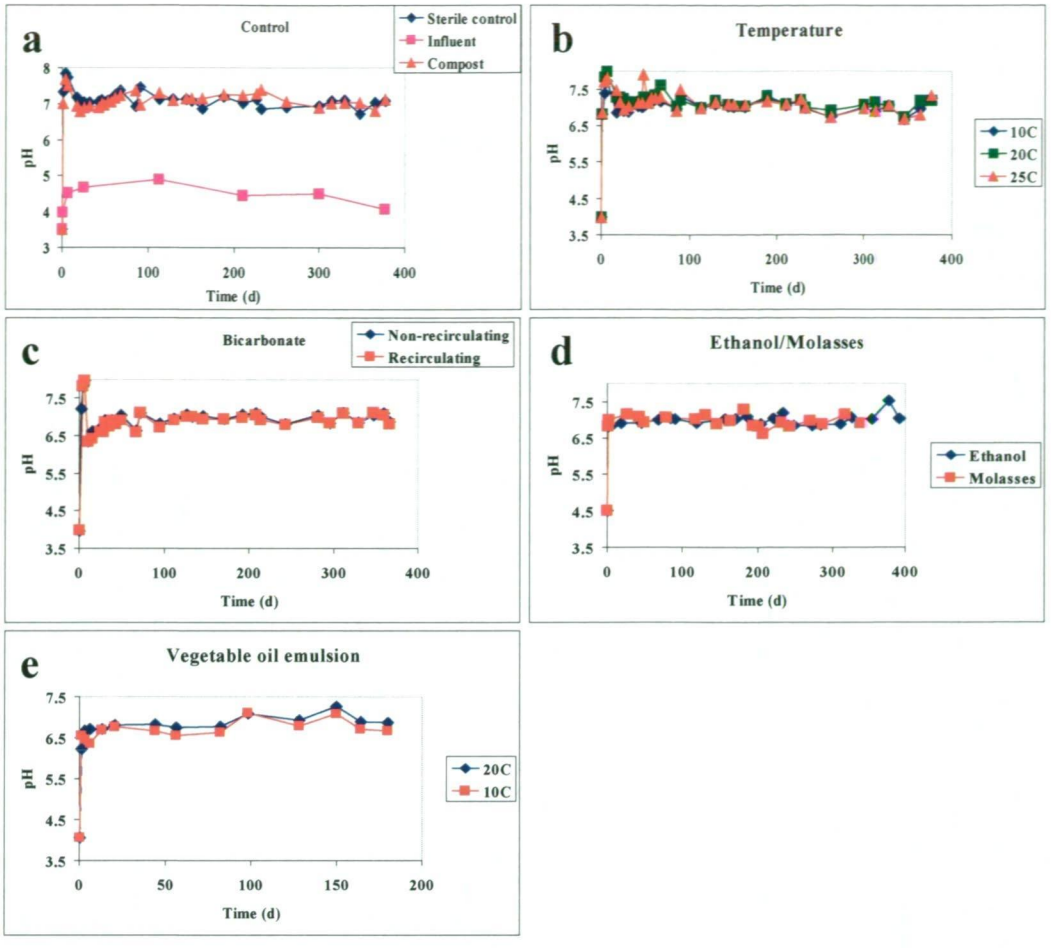


Figure 4.2a-e. pH of the microcosm effluents over time compared to the influent (leachate) (pink line). % relative standard deviation (SD) < 1%.

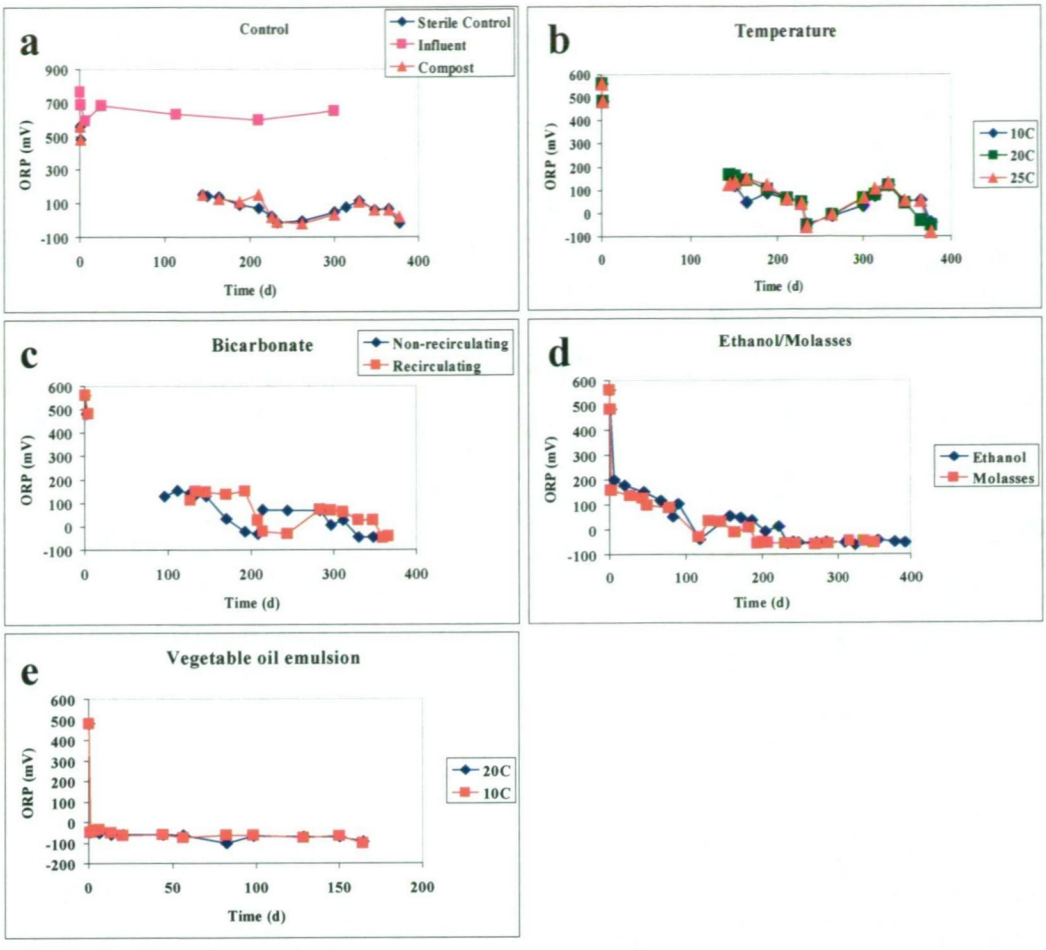


Figure 4.3a-e. Oxidation reduction potential (ORP) measurements for microcosm effluents over time compared to the influent (leachate) (pink line). % relative standard deviation SD < 5%.

Table 4.1. ORP measurement of the centre of the microcosm at the end of the experiment.

Microcosm	ORP (mV)
Sterile Control	-21
Compost	-22.8
10C	-40
20C	-63
25C	-81
Bicarbonate recirculating	-108.2
Bicarbonate non-recirculating	-216
Ethanol	-130.8
Molasses	-213
OE10	-226
OE20	-234

Alkalinity production

Influent alkalinity was not detectable hence; any production is most likely to be from HCO_3^- production from microbial sulfate- and iron-reduction and oxidation of organic matter by:

$SO_4^{2-} + CH_3COOH + 2H^+ \rightarrow HS^- + 2HCO_3^- + 3H^+$ (Rabus et al. 2000).

Substantial alkalinity production (~ 2500mg L^{-1}) occurred in the molasses, ethanol and vegetable oil emulsion microcosms (Figure 4.4d, e) compared to the sterile and compost control microcosms (~ 600mg L^{-1}) indicating microbial metabolic activities were greater in the amended microcosms and excess organic acid production was not a problem. This alkalinity production corresponds to the increased iron and sulfate removal in the amended microcosms compared to the control and temperature microcosms.

The temperature-based microcosms all had an initial increase in alkalinity production but then this decreased. Alkalinity in the vegetable oil emulsion microcosms appeared to continue to increase after 180 days and was not affected by temperature. The ethanol microcosm appeared to drop in alkalinity production after 272 days. Even with bicarbonate added the bicarbonate amended microcosms (Figure 4.4c) did not have the highest alkalinity production (1800mg L^{-1}), suggesting the addition of bicarbonate to buffer the system is not required and endogenous bicarbonate production is sufficient.

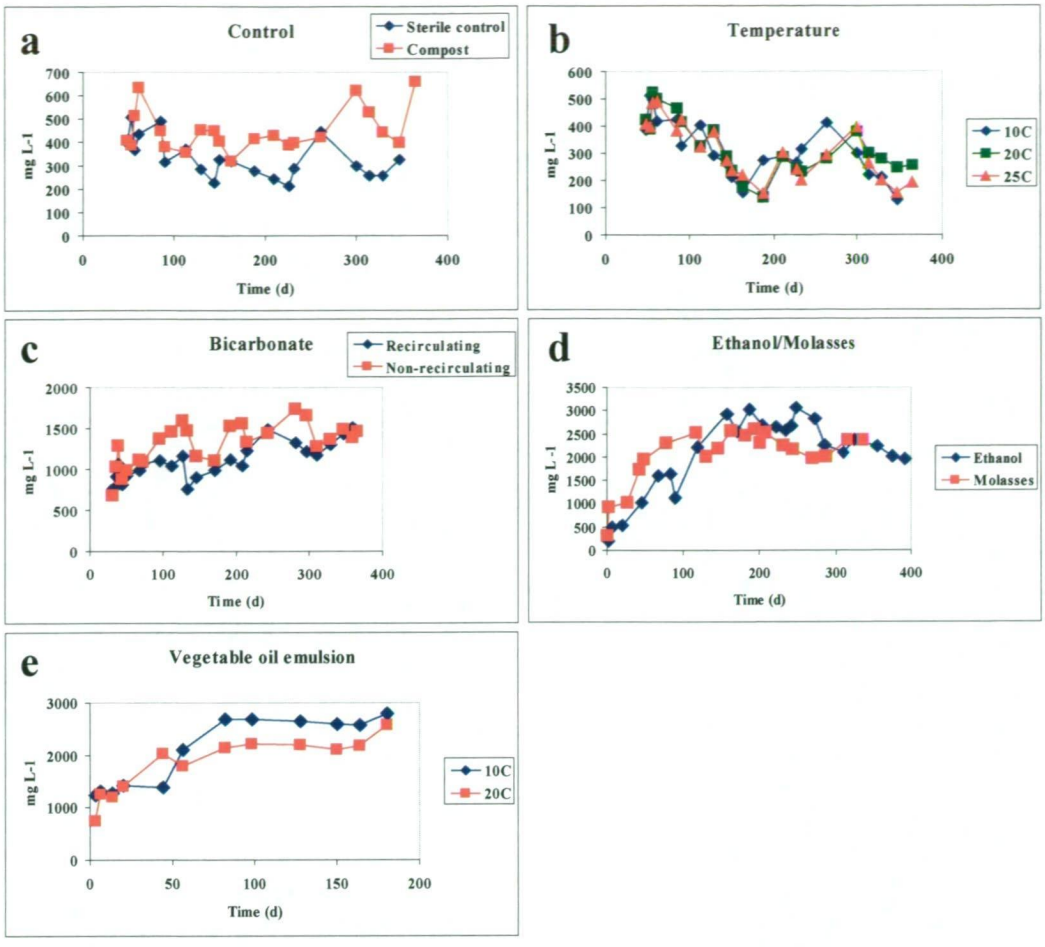


Figure 4.4. Alkalinity production levels (Bicarbonate) in microcosm effluents over time. The influent alkalinity was not detected. % relative standard deviation (SD) < 5%.

Iron removal

All the microcosms showed a similar pattern of iron removal (Figure 4.5). There was an initial increase followed by a slight decline and then an increase again. This is probably because, initially iron adsorbed to the compost, there was a lag and then the biomass levels of sulfate- and iron-reducing reducing bacteria increased. After approximately 100 days, the more complex carbon sources have probably begun to break down, allowing more carbon to be utilised by both iron- and sulfate-reducing bacteria. The reduction cells also fluctuated between 80 to 100% iron removals for the first year of operation (Figure 1.13) but some of the microcosms were not as stable.

The sterile control (sterile compost and sterile leachate) (Figure 4.5a) had some degree of iron removal > 90% ferrous Fe^{2+} was retained in the microcosm in the first 50 days. After this, the iron removal cycled between 30 - 80% with a decline over time and the Fe^{2+} levels in the effluent increased. The organic matter appears to bind some of the iron, which is retained in the microcosm. Autoclaving probably broke down the compost. However, iron removal appears unstable in the control and after some accumulation was iron was released from the microcosm. The compost control (compost and sterile leachate) also removed iron between 60 – 90% of initial levels, with the exception of the sample from day 150.

All the temperature-based microcosms (Figure 4.5b-e) retain >90% of the ferrous iron in the first 50 days with cycling between 30 - 80% for the remaining time. The 10°C microcosm had the largest range of fluctuation in iron retention. The higher temperature microcosms had some iron retention stability, the 25 ° C microcosm levelled to 60% iron removal, and the concentration in the effluent

remained constant. This indicates higher temperature promoted microbial iron reduction or indirect precipitation from sulfate reduction with the oxidation of organic matter, which levels out.

The bicarbonate microcosms (Figure 4.5f-g) also retained >90% of the ferrous iron for the first 50 days and cycled between 50 – 80% iron retained in the microcosm. These microcosms also appeared unstable in iron retention, with the non-recirculated bicarbonate microcosm being less stable than the recirculating microcosm. The addition of bicarbonate appeared not to promote stable iron precipitation. The large decreases in iron retention coincided with black precipitates in the effluent samples indicating iron was periodically washed out.

The ethanol microcosm (Figure 4.5h) retained between 55 – 70% of Fe^{2+} in the first 200 days, increased to 99%, and maintained low Fe^{2+} concentrations in the effluent for the remaining time. The possible explanations for this could be ethanol was incompletely oxidised to acetate and hydrogen coupled to sulfate (Equation 1) and iron (Equation 2) reduction The former is then coupled to sulfate (Equation 3) and iron (Equation 4) reduction and oxidised to CO_2 producing bicarbonate, hydrogen sulfide and metal sulfides (Equation 5) as follows:

$$2CH_3CH_2OH + SO_4^{2-} \longrightarrow 2CH_3COO^- + HS^- + H^+ + 2H_2O \quad (1)$$

$$2CH_3CH_2OH + 12Fe^{3+} + 5H_2O \longrightarrow 2HCO_3^- + 12Fe^{2+} + 14H^+ \quad (2)$$

$$CH_3COO^- + SO_4^{2-} \longrightarrow 2HCO_3^- + HS^- \quad (3)$$

$$CH_3COO^- + 8Fe^{3+} + 4H_2O \longrightarrow 2HCO_3^- + 8Fe^{2+} + 9H^+ \quad (4)$$

$$HS^- + Fe^{2+} \longrightarrow FeS_2 + H^+ \quad (5)$$

However, acetate oxidation is a slower process than the other processes (Kaksonen et al. 2003) so a delay in increased iron reduction could be expected as sulfides are produced and simpler carbon molecules accumulate.

The redox potential for the ethanol microcosm also took approximately 200 days to reach equilibrium; this may have been required for increased iron reduction. In addition, acetate and hydrogen are widely used by sulfate- and iron-reducers. Hence, the by-products of ethanol utilisation may support a higher diversity of sulfate- and iron-reducing bacteria and continue to retain iron and buffer the redox and pH conditions (Dar et al. 2007).

The molasses microcosm (Figure 4.5i) retained 50 to 99% iron over 366 days. Many iron-reducers can use complex sugars as electron donors (Lovley, 2000) but not sulfate-reducers, as they prefer simple organic acids (Rabus et al. 2000). This may explain why the iron reduction increased and the sulfate reduction decreased in the first 50 days of the molasses microcosm experiment. However, sulfate retention increased to ~70% at day 78 (Figure 4.6i) and the iron retention declined to 50% as the sulfate removal rate levelled out the iron removal rate started to increase again to >80%. This may indicate that even though sulfate reduction indirectly precipitates iron, iron-reducers play a large role in the removal of iron. Furthermore, in effluent samples from days 164 and 194 black precipitates were observed. These may have been iron sulfides since an increase in the concentrations of Fe^{2+} and SO_4^{2-} were observed in both these days effluent samples indicating iron precipitates may occasionally become mobile.

The vegetable oil emulsion microcosms began later than the other microcosms and were not run for as long. However, both the 10°C and 20°C oil emulsion

microcosm retained up to 80% iron in the first 80 day, s and this increased to 99% for the remainder of the experiment (Figure 4.5j and k). At day 82 molasses was added to the oil emulsion, at half the concentration added to the molasses microcosm (100mg L^{-1}) which may account for the increase in iron retention after this time. Iron reducers use complex sugars as electron donors in dissimilatory iron reduction and as the oil breaks down into low molecular weight fatty acids and hydrogen (Borden 2006). This produces a reduced environment very quickly as observed in the redox potential for both oil emulsion microcosms, -48mV after three days (Figure 4.3e).

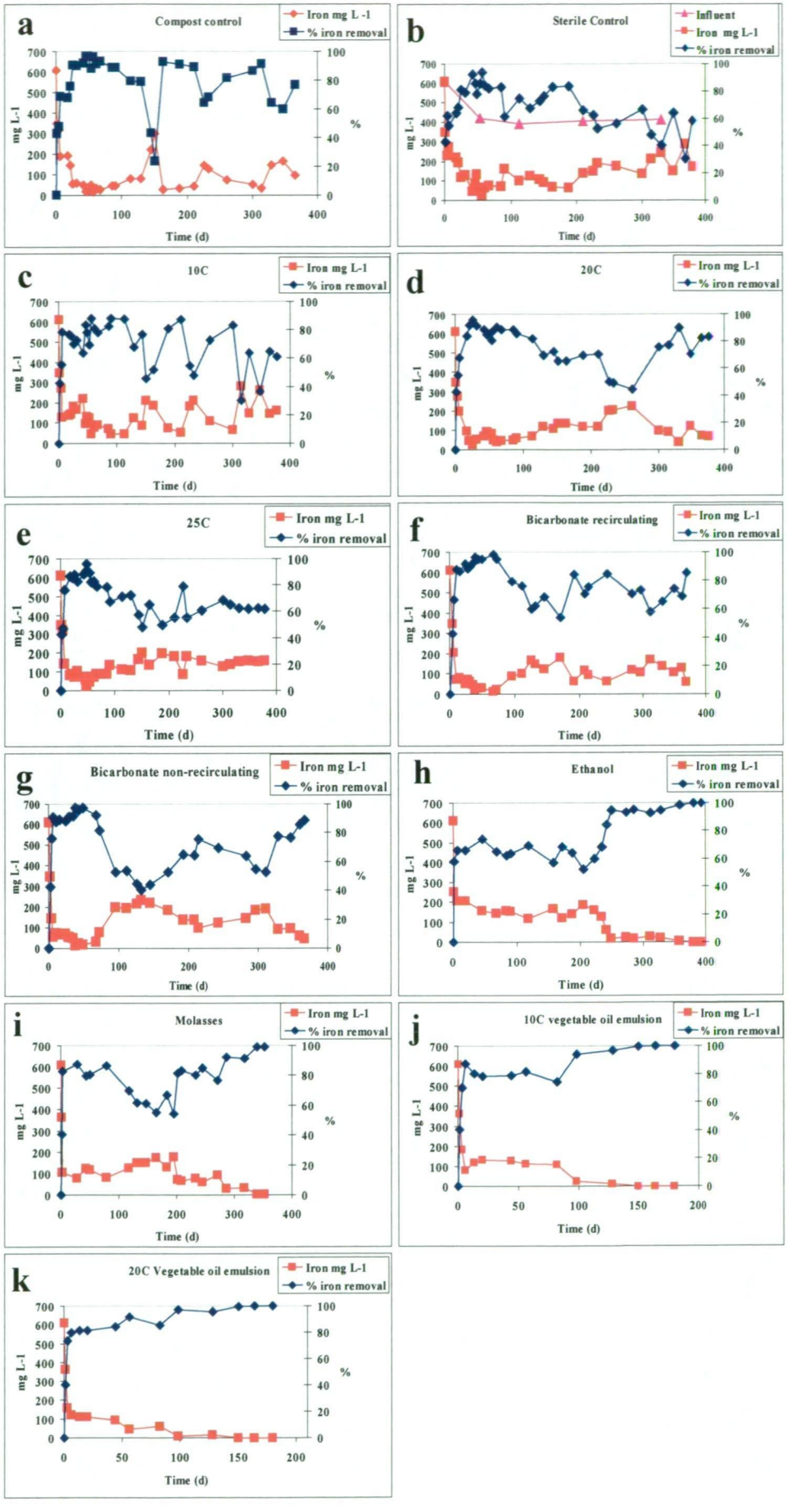

a
Compost control
Iron mg L -1
% iron removal
mg L-1
%
Time (d)
b
Sterile Control
Influent
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
c
10C
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
d
20C
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
e
25C
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
f
Bicarbonate recirculating
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
g
Bicarbonate non-recirculating
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
h
Ethanol
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
i
Molasses
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
j
10C vegetable oil emulsion
Iron mg L-1
% iron removal
mg L-1
%
Time (d)
k
20C Vegetable oil emulsion
Iron mg L-1
% iron removal
mg L-1
%
Time (d)

Figure 4.5. Iron removal over time, (measured as Fe^{2+}) percent difference from total in the influents (blue line) and iron mg L^{-1} per day (red line) in microcosm effluents. % relative standard deviation (SD) < 0.8%. Control microcosms (a,b), temperature microcosms (c-e), bicarbonate amended microcosms (f,g), liquid carbon amended microcosms (h-k). Time was measured in days (d).

Sulfate removal

The sterile and compost control, temperature and bicarbonate microcosms (Figure 4.5a-g) all behaved similarly and cycled between 10 - 80% sulfate removal but after 365 days removal decreased to ~60%. Hence, changing temperature and bicarbonate addition did not enhance sulfate reduction. The concentration of sulfate in the microcosm influent did become lower over time possibly due to H_2S forming in the feed container.

The ethanol microcosm (Figure 4.6h) only removed 10 – 40% sulfate in the first 100 days, possibly due to the redox potential not being low enough during this time (200 – 100mV) as the ORP for sulfate reduction (-1 to -300mV) is lower than that for iron reduction (0 to -100mV). Competitive exclusion by iron-reducing bacteria is also a possibility, as they have been found to out compete sulfate-reducers for electron donors (Lovley 2000). After 100 days, the sulfate reduction increased to 60 to 80% for the remaining time, which coincides with the microcosm being left static unintentionally for 29 days. This may indicate that for improved sulfate removal bioreactors need to be filled and left static for a time for the organic matter to break down into simpler molecules for terminal electron donors and the microorganism community to accumulate.

The molasses microcosm (Figure 4.6i) had low sulfate removal (10 – 20%) in the first 50 days, but when this microcosm was also left static at the same time as the ethanol microcosm, sulfate removal rates increased to 50 to 80% for the remaining time. This may be due to the same reasons as the ethanol microcosm as this microcosm ORP dropped at a similar time. Neculita and Zagury (2008) also

observed a lag period of about 80 days before sulfate reduction occurred and speculated it was because the higher initial ORP values retarded the start of sulfate reduction.

The vegetable oil emulsion microcosms (Figure 4.6j-k) were filled with the compost, straw and amended leachate and left for a week before being tested and this is reflected in the immediate increase in iron and sulfate removal rates. Sulfate removal was up to 60% for both the 10°C and 20°C incubated microcosms initially and was sustained at 60 to 80% removal for the remaining time. The oil is slowly broken down to hydrogen and organic acids to complement the compost present in the microcosms and these compounds can be used by a variety of sulfate reducers (Borden and Rodriguez 2006). However, the maximum sulfate removal achieved was 80%. This is the same as the sulfate removal efficiency of the full-scale reduction cells (Figure 1.14). If the microcosms were run for a longer time sulfate removal may have been higher as the removal rate had increased at the last sampling time point. The slow increase in sulfate-reducing bacteria biomass is also indicated in the gradual increase of bicarbonate production. Geets et al. (2006) also found sulfate reduction took eight weeks to reach 80% removal and furthermore, the metal and sulfate removal rates fluctuated similarly to what was seen in the present study and speculated it was fluctuations in sulfate-reducing bacteria (SRB) activity.

Hence, various carbon sources could be added to the mushroom compost, straw to improve the sulfate reduction. Neculita and Zagury (2008) found with amendments with four different grades of carbon sources (maple wood chips, maple saw dust, composted poultry manure and leaf compost) sulfate

concentrations decreased more than in amendments with three carbon sources (without the woodchips). The ethanol, molasses and vegetable oil emulsion microcosms did not experience as many or as severe fluctuations in sulfate removal as the temperature or bicarbonate microcosms, so these amendments may provide a more steady release of readily accessible terminal electron donors for SRB. Measurement of dissolved organic carbon degradation rates, correlated to sulfate and iron reduction may have been useful. O'Reilly and Colleran (2006) found increasing the chemical oxygen demand (COD)/SO_4^{2-} ratio was detrimental to sulfate reduction as the increased organic compounds increased the growth of methanogens, syntrophs and acetogens that can compete with sulfate-reducers for carbon sources. Dar et al. (2008) also found that at high lactate/sulfate ratios that acetogens and methanogens were the dominant microbial communities. In addition, the compounds produced from sulfate and iron reduction can be deleterious to the bacterial population.

The leachates at the Heybridge site have generally higher concentrations of metals and sulfate than acid mine drainage sites. Most studies work with less than half the concentration of metals and sulfate found at Heybridge; 50mg L^{-1} and 1000mg L^{-1} (Logan et al. 2005) , 310 to 380mg L^{-1} and 1100 to 2000mg L^{-1} (Tsukamoto et al. 2004), 80mg L^{-1} and 250mg L^{-1} (Johnson and Hallberg 2005) respectively. High concentration of metals and sulfide have been found to be toxic and their build up in passive *in situ* bioremediation systems can be detrimental to the longevity of these systems (Utgikar et al. 2002; Kaksonen et al. 2004a; Neculita et al. 2007). Since separate unit processes for sulfate reduction and metal precipitation increase costs and space utilisation, a combined process is more desirable. Measurement of dissolved sulfide may have determined which

treatment is most prone to sulfide toxicity; Kaksonen et al. (2004a) found an ethanol amended fluidised-bed reactor was more prone to it than one amended with acetate. Recent compost–free bioreactor design has included a flushing capacity and the precipitates are moved away from the main bioreactor chamber (Tsukamoto and Vasquez 2006). Furthermore, compost-free bioreactors do not have problems with compost depleting and/or clogging the system as they use a matrix with large pore spaces (rocks) for the precipitates to move through to the base and not cause any toxicity to the sulfate- and iron-reducing bacteria.

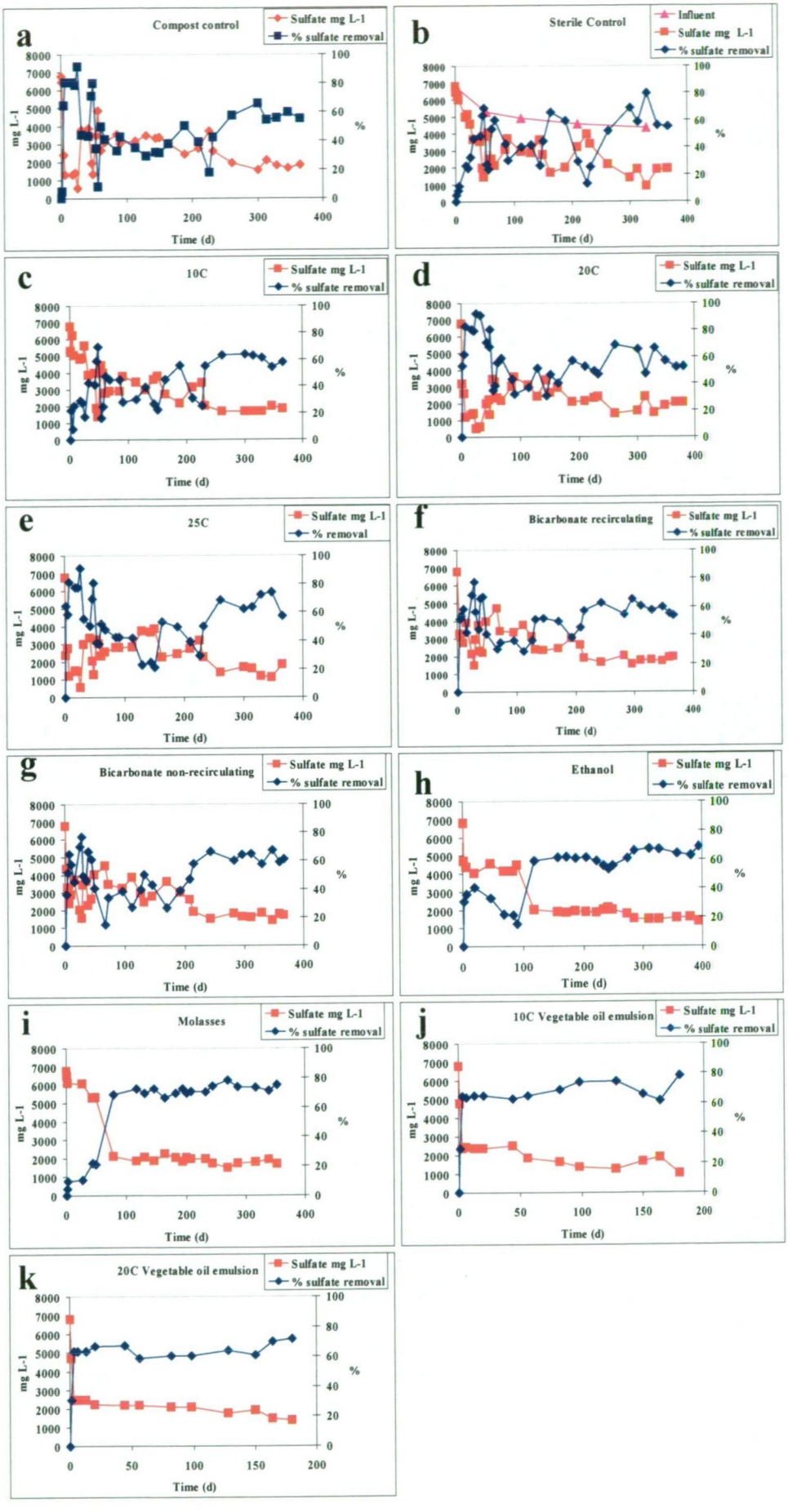

a
Compost control
Sulfate mg L-1
% sulfate removal
b
Sterile Control
Influent
Sulfate mg L-1
% sulfate removal
c
10C
Sulfate mg L-1
% sulfate removal
d
20C
Sulfate mg L-1
% sulfate removal
e
25C
Sulfate mg L-1
% removal
f
Bicarbonate recirculating
Sulfate mg L-1
% sulfate removal
g
Bicarbonate non-recirculating
Sulfate mg L-1
% sulfate removal
h
Ethanol
Sulfate mg L-1
% sulfate removal
i
Molasses
Sulfate mg L-1
% sulfate removal
j
10C Vegetable oil emulsion
Sulfate mg L-1
% sulfate removal
k
20C Vegetable oil emulsion
Sulfate mg L-1
% sulfate removal
mg L-1
%
Time (d)

Figure 4.6. Sulfate removal over time, percent difference from total (blue) in the influents and sulfate mg L^{-1} per day (red) in microcosm effluents. %relative standard deviation (SD) < 0.8%. Control microcosms (a,b), temperature microcosms (c-e), bicarbonate amended microcosms (f,g), liquid carbon amended microcosms (h-k). Time is measured in days (d).

TRFLP analysis of microbial communities in the microcosm experiments

A combined 192 TRFs were used for analysis of microcosm microbial communities with TRFLP profiles obtained approximately every 60 days of the experiments. The ANOSIM pairwise comparisons and SIMPER (percent average similarity) data are summarised in Table 4.2., the global R = 0.045, (p<0.14), that is, analysis of similarity between all samples. This indicates there is no significant difference between communities for the different treatments, which is to be expected as all the microcosms have the same starting material. In the microcosms, the high amount of organic matter coupled with biogeochemical stratification could cause a heterogeneous microbial community structure, similar to the field-scale reduction cells. Furthermore, the R statistic is an absolute measure of ranked differences between and within groups and is not affected by the number of replicates, whereas p is. The TRFLP profiles have low replicates but are derived from five restriction enzymes so there will be more focus on the R statistic in this analysis and percent average similarity calculated in SIMPER. A MDS plot of the pairwise comparisons between treatments shows no significant grouping and all treatments appear different to each other (Figure 4.7). The MDS plots based on treatments calculated from the Bray-Curtis similarity matrix show clearer relationships (Figure 4.8) as indicated by the circles, which include all samples for a particular treatment.

A similarity profile (SIMPROF) was used to test for evidence of real group structure (i.e. was not random) between samples and to test if the similarity profile of the TRFLP peak area data did not arise by chance using 1000

permutations. The real similarity profile π = 2.57, (p<0.001) did not come from the null hypothesis distribution of π. That is, the clustering of the samples is random. Hence, the groupings of the samples on the MDS plots are most probably true.

The similarities in TRFLP peak area profiles over time appear to become higher with increasing temperature (Figure 4.8a). This indicates that the higher temperature (25°C) incubation selected for a few dominant bacterial groups, which remained stable over time. The 25°C microcosm community had higher similarity to the compost and influent (22% and 29% respectively) than the other temperature microcosms. Hence, the microbial community composition in all the temperature microcosms is probably the same, but some bacterial groups are selected for at higher temperatures.

There was no significant difference between the recirculating and non-recirculating bicarbonate microcosm TRFLP peak area profiles (Figure 4.8b). Both of these had higher percent average similarities with the influent (25% and 24%) than with the compost (21 and 20%). However, the non-recirculating bicarbonate microcosm is significantly different to that of the influent (R = 0.369, p<0.05) and compost (R = 0.226, p<0.02) than the recirculating microcosm.

The ethanol and molasses microcosm TRFLP peak area profiles (Figure 4.8c) had some compositional overlap (28% similar), and the ethanol community grouped more closely to that of the compost (R = 0.161, p<0.09,) than the influent (R = 0.407, p<0.03). However, the molasses microcosm was not significantly different to that of the influent (28% similar) but was different to that of the compost (R = 0.222, p<0.04), (22% similar).

The vegetable oil emulsion microcosm communities had less variability in their TRFLP profiles over time (Figure 4.8d) than the other microcosms, indicating a less diverse and stable microbial community. The community seems somewhat different with respect to temperature that of 20 °C is more similar to the influent (35%) and that of 10 ° C more similar to the compost (24%). However, there is some overlap, and the SIMPER analysis revealed they had 31% similarity to each other and five of the dominant peaks in common.

Results from the SIMPER analysis reveal the major peaks responsible for the community differences among the treatments (Table 4.3). Seven TRFLP peaks contributed 40 to 70% of the total similarity in all treatment TRFLP profiles. These peaks were most similar to anaerobic fermenters from the phylum *Bacteroidetes,* such as *Paludibacter propionicigenes* and *Bacteroides* spp., which were found in the Heybridge remediation system stages (Chapter 3). These bacteria appear to be important in anaerobic degradation of complex organic carbon compounds to simpler molecules. This could limit the rate at which substrates become available to sulfate- and iron-reducing bacteria and enhancing their biomass may be important as sulfate- and iron-reducing bacteria biomass (Neculita et al. 2007). More research is required to understand and differentiate biogeochemical cycles in anaerobic bioreactors fed with complex organic substrates.

The other dominant peaks, as inferred from *in silico* digests of phylotypes from Chapter 3, match the iron- and sulfate-reducers *Ferribacterium limneticum (Betaproteobacteria), Desulfotomaculum* spp. *and Desulfosporosinus* spp. (*Firmicutes*), *Desulfomicrobium* spp. (*Deltaproteobacteria*). *Acidithiobacillus*

ferrooxidans (*Gammaproteobacteria*) which are capable of both iron/sulfur/sulfide oxidation and iron/sulfate reduction (Ohmura et al. 2002). This indicates all the microcosms had similar communities but the amended microcosms were more successful in promoting the growth of a stable bacterial consortia needed for enhanced sulfate and iron removal over time.

The total number of TRFs found in each treatment indicated the relative diversity of the microcosm TRFLP profiles (Figure 4.9). The ethanol, molasses and oil emulsion microcosms had a lower diversity and probably selected for the few bacteria that enhanced iron and sulfate removal, which included anaerobic fermenters, sulfate/iron reducers and sulfur/sulfide/iron oxidisers. However, the TRFLP peak areas cannot be used for quantifying the absolute bacterial community. Quantitative methods such as qPCR or FISH probing of functionally important genes such as *dsrAB* and *aprA* (Ben-Dov et al. 2007), *mtr*ABC (Fredrickson and Zachara 2008) and quantitative gene expression correlated to rates of sulfate and iron reduction (Chin et al. 2004, 2008) would have improved knowledge of the levels of metabolic important bacteria and if they differed between communities.

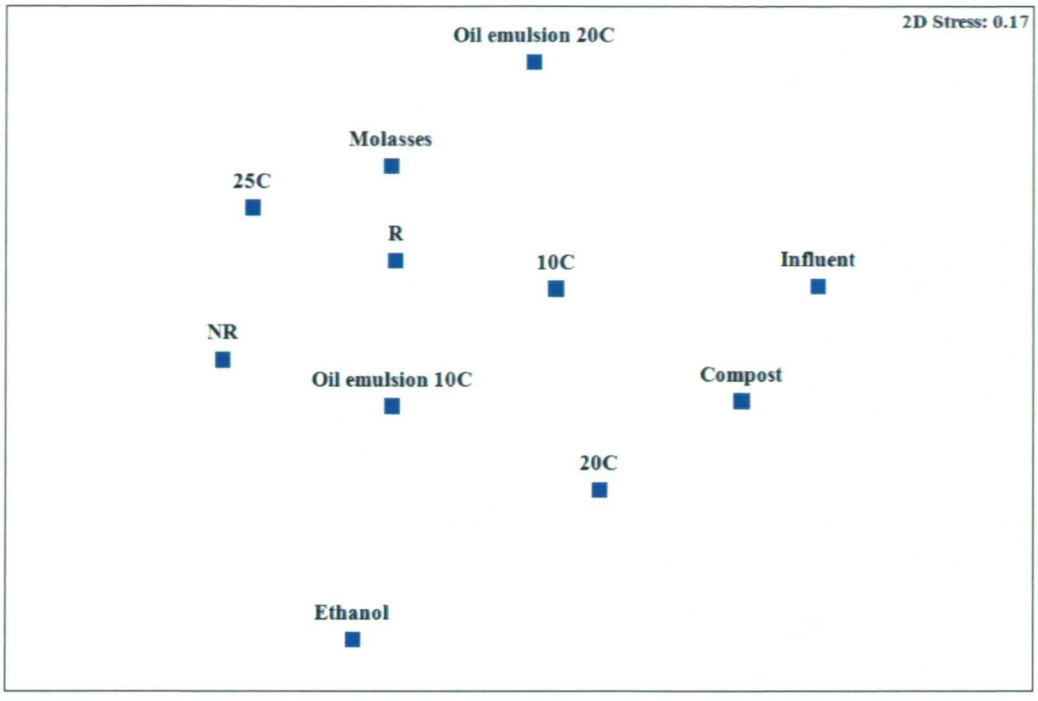


Figure 4.7. MDS plot of ANOSIM pairwise comparisons of combined microcosm TRFLP peak area analysis. R = recirculating bicarbonate, NR = non-recirculating bicarbonate, C = degrees Celsius.

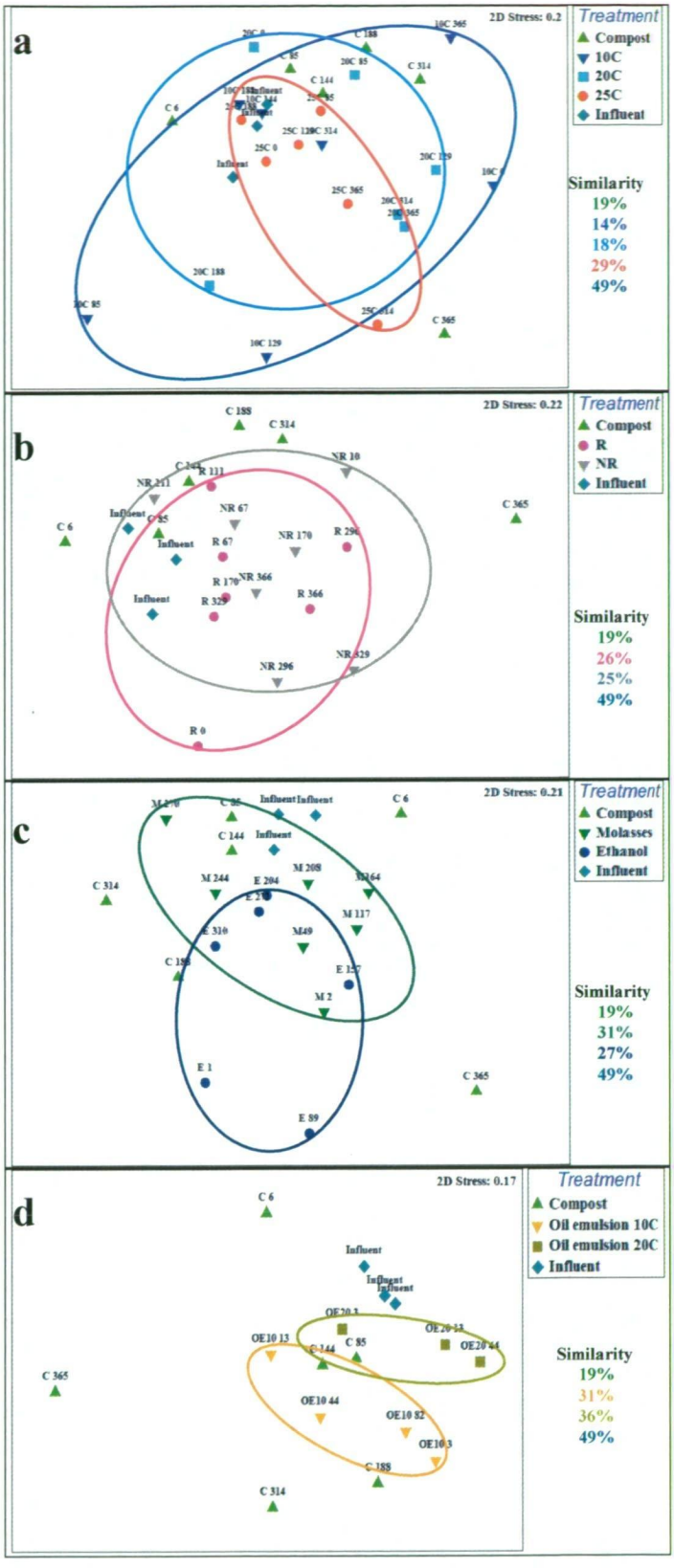


Figure 4.8. MDS plots of microcosm TRFLP peak area similarity matrix (Bray-Curtis) samples over time with % average similarity and representative % similarity clusters within treatment samples (SIMPER) for a; temperature, b; bicarbonate, c; ethanol and molasses and d; vegetable oil emulsion microcosms. (label numbers indicate day the sample was taken).

Table 4.2. ANOSIM pairwise tests between microcosm TRFLP peak area data and % average similarity (SIMPER). R = recirculating bicarbonate, NR = non-recirculating bicarbonate, C = degrees Celsius, bold = significant.

Groups	R Statistic	*p*	% average similarity
Compost, 10C	-0.012	0.542	16.77
Compost, 20C	-0.002	0.474	17.91
Compost, 25C	**0.137**	0.104	22.29
Compost, R	**0.16**	**0.052**	20.76
Compost, NR	**0.226**	**0.019**	20.4
Compost, Molasses	**0.222**	**0.036**	21.59
Compost, Ethanol	**0.161**	**0.084**	20.36
Compost, Oil emulsion 10C	0.075	0.305	23.5
Compost, Oil emulsion 20C	0.08	0.381	20.87
Compost, Influent	-0.093	0.607	25.25
10C, 20C	-0.116	0.899	17.39
10C, 25C	-0.041	0.679	21.15
10C, R	-0.104	0.922	20.59
10C, NR	0.023	0.391	20
10C, Molasses	-0.014	0.555	21.72
10C, Ethanol	0.062	0.201	18.02
10C, Oil emulsion 10C	-0.19	0.9	21.93
10C, Oil emulsion 20C	-0.262	0.883	22.92
10C, Influent	-0.171	0.808	21.28
20C, 25C	0.035	0.323	23.01
20C, R	0.044	0.297	19.38
20C, NR	**0.126**	0.168	20.34
20C, Molasses	**0.181**	**0.082**	19.79
20C, Ethanol	0.095	0.223	19.41
20C, Oil emulsion 10C	0.008	0.438	21.46
20C, Oil emulsion 20C	0.062	0.357	19.09
20C, Influent	**0.105**	0.31	16.97
25C, R	0.008	0.429	27.75
25C, NR	-0.017	0.526	28.04
25C, Molasses	-0.066	0.735	30.19
25C, Ethanol	**0.15**	**0.097**	25.93
25C, Oil emulsion 10C	0.091	0.229	28.76
25C, Oil emulsion 20C	**0.167**	0.202	31.4
25C, Influent	**0.284**	0.107	29.05
R, NR	-0.18	0.978	28.45
R, Molasses	-0.126	0.915	28.77
R, Ethanol	0.028	0.32	26.3
R, Oil emulsion 10C	-0.175	0.897	29.69
R, Oil emulsion 20C	-0.056	0.542	29.1
R, Influent	0.087	0.308	25.3
NR, Molasses	-0.016	0.54	27.87
NR, Ethanol	0.016	0.424	27.15
NR, Oil emulsion 10C	-0.172	0.848	29.42
NR, Oil emulsion 20C	**0.115**	0.258	27.45
NR, Influent	**0.369**	**0.042**	24.11
Molasses, Ethanol	**0.111**	0.137	27.52
Molasses, Oil emulsion 10C	-0.119	0.779	32.83
Molasses, Oil emulsion 20C	0	0.508	30.74
Molasses, Influent	0.095	0.35	28.38
Ethanol, Oil emulsion 10C	0.087	0.31	28.95
Ethanol, Oil emulsion 20C	**0.389**	**0.036**	25.47
Ethanol, Influent	**0.407**	**0.024**	23.63
Oil emulsion 10C, Oil emulsion 20C	**0.111**	0.4	31.43
Oil emulsion 10C, Influent	**0.667**	**0.057**	24.42
Oil emulsion 20C, Influent	**0.556**	0.1	34.63

Table 4.3. TRFs contributing to >80% similarity, calculated in SIMPER analysis of the TRFLP peak abundance data, of different treatments and their phylogenetic matches. TRF identity = size (base pairs) + enzyme + forward/reverse. R = recirculating, NR = non-recirculating. The five TRFs contributing the highest % similarity for each microcosm are bold.

	Compost	10C	20C	25C	Bicarb R	Bicarb NR	Molasses	Ethanol	OE10C	OE20C	Influent	
TRF	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Contrib%	Phylogenetic match
(124 hae R)	**16.73**	**16.47**	**16.55**	**19.09**	**11.19**	**26.1**	**27.08**	**26.34**	**31.65**	**27.01**	**11.66**	*Paludibacter propionicigenes*
(119 rsa R)	**12.83**	**11.16**	**5.46**	**8.62**	**8.73**	**12.52**	**12.83**	**8.04**	2.86	**15.49**	**7.09**	*Ferribacterium limneticum*
(152 hinf R)	**12.61**	2.46	**14.17**	2.25	1.87	0	3.96	0	**10.8**	0	5.6	*Desulfotomaculum* spp.
(152 hha R)	**9.98**	0	0	2.27	7.33	0	0	4.89	**6.67**	4.79	**7.75**	*Bacteroides/Porphyromonas* spp.
(129 msp R)	**8.1**	**11.58**	0	**15.02**	**10.72**	**8.66**	**14.24**	3.82	0	0	**14.2**	*Acidovorax/Ferrovum myxofaciens* spp.
(148 hinf F)	5.5	**7.35**	**17.3**	2.54	2.86	2.49	0	0	2.75	0	0	*Bacillus/Clostridium* spp.
(72 hae R)	3.43	0	0	0	0	0	0	0	0	0	0	*Desulfobacterium* spp.
(122 msp R)	3.03	0	0	0	0	0	0	0	0	0	0	*Porphyromonas/Bacteroides* spp.
(152 hae R)	2.42	0	0	0	0	0	0	0	0	0	0	*Porphyromonas/Bacteroides* spp.
(125 msp R)	1.86	0	0	0	0	0	0	0	4.37	0	0	*Gammaproteobacteria*
(148 hha F)	1.33	0	2.56	0	**7.35**	3.68	2.31	0	0	0	0	Rumen bacteria clones
(73 hha R)	1.3	3.28	1.75	0	0	0	0	1.5	**6.26**	0	0	*Desulfosporosinus* spp.
(80 msp R)	0	**7.94**	4.63	3.47	2.28	1.79	0	0	0	**6.23**	**18.54**	*Desulfosporosinus* spp.
(154 hinf R)	0	6.83	0	0	3.36	3.25	2.42	0	2.52	0	0	*Desulfurella/Desulfobacterium* spp.
(77 hha F)	0	4.78	0	0	1.93	0	1.93	0	3.95	0	0	OP8 Candidate division
(80 hinf R)	0	4.01	**7.23**	4.89	1.4	0	0	0	0	5.31	0	*Thermoanaerobacter* spp.
(127 hae R)	0	3.87	0	2.87	6.19	**8.21**	6.67	**14.62**	3.26	0	5.38	*Acidithiobacillus ferrooxidans*
(80 hha F)	0	1.65	0	**8.32**	0	0	0	0	0	5.31	3.49	*Desulfurella* spp.
(371 rsa F)	0	0	4.21	0	0	0	0	**6.01**	0	0	0	*Bacteroides* spp.
(85 rsa R)	0	0	4.14	0	1.25	0	2.77	0	0	0	0	*Acidithiobacillus* spp.
(110 rsa R)	0	0	3.3	0	0	1.42	0	0	0	0	0	*Bacteroides* spp.
(150 hinf F)	0	0	2.12	0	0	1.47	0	4.7	0	0	0	*Firmicutes/Dechloromonas* spp.
(74 rsa F)	0	0	1.66	0	0	2.54	0	0	0	0	0	*Alphaproteobacteria*/OP3 Candidate division
(80 hae R)	0	0	0	**5.66**	0	0	**6.92**	0	0	**12.81**	6.05	*Sulfitobacter* spp.
(221 hae F)	0	0	0	3.49	0	0	0	0	0	0	0	*Deltaproteobacteria*
(171 hinf R)	0	0	0	2.48	0	0	**8.32**	0	5.14	0	0	OP11 Candidate division
(306 hae R)	0	0	0	2.34	0	0	0	0	0	0	0	*Betaproteobacteria*
(121 rsa R)	0	0	0	0	**10.59**	**7.72**	0	4.25	**12.18**	**13.44**	0	*Desulfomicrobium/Desulfovibrio* spp.
(75 msp R)	0	0	0	0	4.56	0	0	1.69	0	0	0	*Alphaproteobacteria*
(368 rsa F)	0	0	0	0	0	0	0	**6.63**	0	0	0	*Desulfovibrio* spp./OP3 Candidate division
(409 hha R)	0	0	0	0	0	0	0	3.48	0	0	0	*Desulfurella* spp.
(122 hha R)	0	0	0	0	0	0	0	0	0	0	5.24	*Paludibacter propionicigenes*
% similarity	**79.12**	**81.38**	**85.08**	**83.31**	**81.61**	**79.85**	**89.45**	**85.97**	**92.41**	**90.39**	**85**	

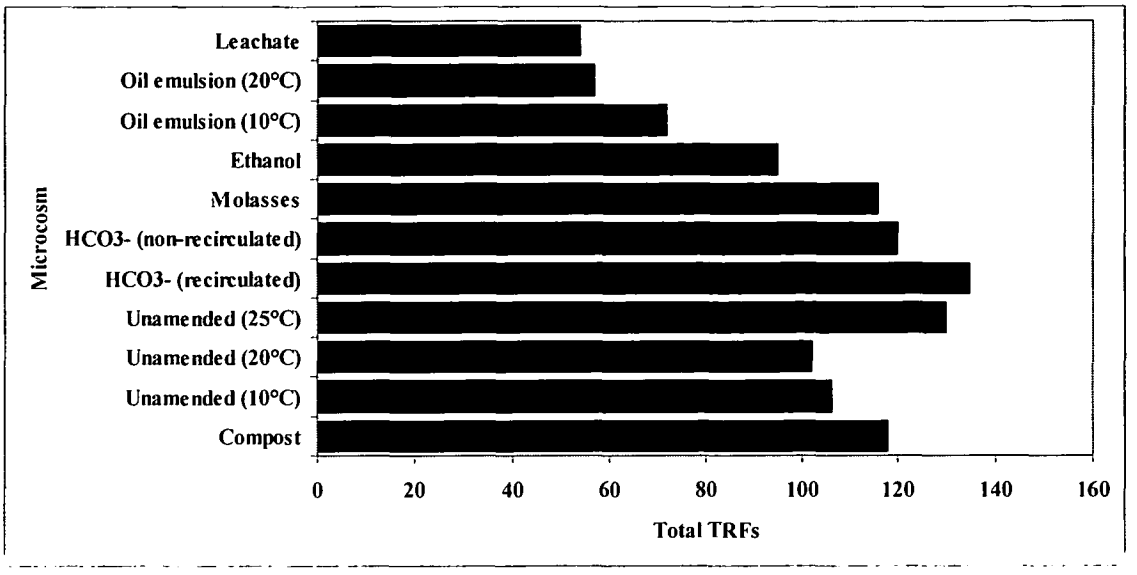


Figure 4.9. Graph of total TRFs for each treatment as an indication of microbial diversity.

Conclusion

The laboratory-scale microcosm experiments showed that liquid amendments to the microcosms, especially vegetable oil emulsion, sustained pH and redox conditions, alkalinity production and iron and sulfate removal for the length of the experiment. Coupled to this TRFLP analysis indicated, in the amended microcosms, a stable, low diverse microbial community was supported over time. This community was not affected by temperature and would readily survive the temperatures at the field site. This microbial community included carbohydrate fermenters, iron-and sulfate-reducers, combining a variety of metabolic processes that were required for anaerobic bioremediation of the leachate.

Temperature increases and bicarbonate addition (with the potato starch already added via the influent) was not adequate in providing a suitable environment for the above-mentioned microbial community. This was demonstrated by these microcosms having erratic and fluctuating iron and sulfate removal as well as a diverse and changing microbial community over the length of the experiment. This was similar to the performance of the field-scale reduction cells in the first year of operation.

The ethanol and molasses provided adequate nutrients for bacteria involved in carbohydrate fermentation and iron and sulfate removal but not to the extent of the oil emulsion amended microcosms. Hence, as the reduction cells design is presently the addition of vegetable oil emulsion would improve the metal and sulfate removal. However, re-design and pilot-scale cells to test which combination of design and amendment would work best should be considered.

Chapter 5.

General Discussion and Conclusions

Bass Strait, Tasmania

General Discussion and Conclusion

The current thesis focused on the microbial community of a novel, passive, *in situ* bioremediation system, Heybridge, Tasmania. This system was set up to remediation acidic, sulfate- and metal-rich wastewaters produced from titanium dioxide processing. The levels of microbial diversity and the community dynamics these types of bioremediation systems based on anaerobic reduction is important to be able to their influence on biogeochemical processes involved and how this affects efficient pollutant removal.

The first part of this study investigated the microbial community of various stages of the bioremediation system including, the sludge dams holding the leachate, a novel pre-treatment section (potato cells) and compost-based anaerobic bioreactors (reduction cells). The Heybridge system was found to have a complex microbial community performing a variety of metabolic processes including iron and sulfate reduction and iron and sulfide/sulfur oxidation as well as fermentation of carbohydrates. A large percentage of the phylotypes found were uncultured clones of which there is no knowledge of their metabolism. Both the 16S rRNA clone libraries and TRFLP analysis illustrated very complex bacterial communities and dynamics within and between sites as well as over time.

The anaerobic bioreactors (reduction cells) have decreased in efficiency of metal removal over time, and so the second part of this study was to set up laboratory-scale microcosms to determine if temperature, bicarbonate addition and liquid amendments would be improve the efficacy of the system. The unamended microcosms showed a similar general trend to the reduction cells in their first year of operation, that is, sustained near-neutral pH but erratic sulfate and iron removal.

The microcosms contained a similar microbial community to the reduction cells however, a less diverse and stable microbial community, such as found in the oil emulsion microcosms, appears to support sustained alkalinity production, redox conditions, sulfate and iron removal.

The anaerobic bioreactors, which are filled with mushroom compost and straw, were designed to encourage the growth of sulfate-reducing bacteria to indirectly precipitate the metals by sulfide and alkalinity production. The clone library analysis and TRFLP data showed that the bacteria involved were a combination of sulfate reducers, sulfide oxidisers and iron reducers and oxidisers and carbohydrate fermenters. The decline in efficiency of these bioreactors may have been because the mushroom compost supported bacterial growth for a number of years before becoming exhausted of carbon and electron donors and the straw was not being degraded into simpler molecules fast enough for the sulfate and iron reducers and iron and sulfide oxidisers to utilise. The decline was first thought to be because of low temperature and a decrease in pH (Cooper 2005 pers. comm.) as they are sited in a valley that has mean annual temperatures of 10 – 20°C. However, there appeared to be no climatic affect on microbial communities.

The literature indicates the compost bioreactors' efficiency may be improved by making the fill more homogeneous. Such as a coarse mixture of woodchips, pulp waste or straw that has been mulched or turned into silage, as well as a periodic dosing of a liquid amendment of readily metabolised carbon compounds such as ethanol or molasses. Vegetable oil emulsions have proved invaluable with remediation systems that require highly reducing conditions (Lindow and Borden, 2005a; 2005b). In the present study vegetable oil emulsion proved to be the best

at improving iron and sulfate reduction even at low temperatures (10°C) and at the current design. So having three different carbon sources based on their degradation rate, (fast, intermediate and slow) would probably extend the life of the passive *in situ* bioreactors. Neculita and Zagury (2008) used amendments with three and four different grades of carbon sources and they found that sulfate removal significantly increased with the four amendments. Hence, in the current experiment, as well as mushroom compost, straw and a liquid carbon source, addition of another medium to slow degrading carbon source like small woodchips may improve the sulfate reduction. Furthermore, Neculita et al. (2007) argued that providing means for improving cellulose hydrolysis was important in enhancing conditions for sulfate- and iron-reducing bacteria.

Tsukamoto and Vasquez (2006) tested a compost-free bioreactor, based on a rock matrix and a liquid carbon source and proposed this would be better than a compost or woodchip matrix, which is consumed by bacteria, eventually collapses over time, is also difficult to replace and can clog the system. The benefits include better control of biological activity and improved hydraulic conductivity and precipitate flushing. This technology could improve the longevity of bioremediation systems as the matrix and carbon source do not have to be replaced and do not clog up with precipitates.

Sulfate removal, and to a lesser extent, iron removal, appeared to have a lag phase, of around 80 days, which has been found in other similar studies (Geets et al. 2006; Neculita and Zagury 2008). At the start up stage of these bioremediation systems, it may be beneficial to fill the bioreactors with contaminated leachate/sludge and delay the commencement of flow for several months to allow

the bacterial biomass to accumulate, and then slowly increase the inflow rate so as not to shock or wash out the bacterial biomass. The Wheal Jane bioremediation system, which is similar to Heybridge, was shut down for 10 months after which the metal removal levels came back up to their initial high values (Hallberg and Johnson 2003; Johnson and Hallberg 2005). Furthermore, Geets et al. (2006) found after 6 months of shutting down a laboratory-scale microcosm, which failed to remove sulfate and heavy metals, removal rates increased to the levels at the start of the experiment. They also intentionally disrupted another microcosm, which resulted in stagnation, and after restoration and substrate amendment found the removal rates increased. This may correspond to the breakdown of complex organic carbon compounds into simpler molecules required for SRB. Some complex organic compounds take up to 180 days to break down (Marschner and Kalbitz, 2003).

Recommendations

- To improve the uniform availability of substrate for sulfate- and iron-reducing and fermentative bacteria, removing the straw bales, mulching, and mixing with woodchips and coarse sand/rocks is recommended.

- To increase porosity/hydraulic conductivity for improved flows as well as precipitate removal to include coarse, non-reactive sand/rocks at approximately 20%w/w of the total volume of reduction cell volume, in with the straw mix. Design considerations of flow direction and space available at the base for precipitate retention eg a large rock base layer.

- To provide a slow to medium degrading carbon source for bacteria such as wood pulp waste/small wood chips/saw dust at approximately 10 to 20%w/w to increase the long-term metal and sulfate removal efficiency of the reduction cells.

- To provide a fast-degrading liquid carbon source for bacterial biomass levels to increase initially, such as ethanol/molasses/vegetable oil emulsion to the mushroom compost or replace the mushroom compost, as liquid can be added externally and never become exhausted.

- After filling the reduction cells with compost/straw/sand/wood waste mix and potato cell effluent, allow the cells to sit for 1 to 3 months for the bacterial community to increase and become stable. Possibly add lactate substrate initially to increase microbial biomass and switch to a more cost effective alternative for later amendments.

- After the initial static phase slowly increase flow regimes to the reduction cells so as not to flush out the bacterial biomass.

- It may be practical to keep the potato cells as they provide some carbohydrates and protein for the bacteria as well as keeping the metals soluble for delivery to the reduction cells.

- Employ methods such as TRFLP to track the changes in microbial diversity.

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