"Multiphase Biocatalytic Processes Using Extremophilic Microorganisms for Energy Production"

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Abstract

Bacteria can use a wide variety of alternatives to oxygen as a terminal electron acceptor. This is either because these bacteria are obligate anaerobes, or in the case of many facultative aerobes, alternative acceptors can be used in addition to oxygen. This ability can be utilised to transfer electrons from a variety of substrates to the anode in a microbial fuel cell (MFC), producing an electrical current. There are three methods of electron transfer; direct contact via extensions to the electron transport chain, soluble natural mediators produced by the cells themselves, or artificial mediators added to the cell medium. Of these the former is the most efficient, having energy transfer efficiencies of greater than 90%.

Interest in many cases has centred on bacteria in microbial fuel cells that are capable of reducing metals in the environment, since these organisms predominate in transferring electrons to the metal through direct contact. It is these same types of pathways that are used to transfer electrons to the anode in a microbial fuel cell with the bacteria treating the anode as a metal. Bacterial reduction of a wide variety of transition metals has been found including vanadium, manganese, uranium, iron and chromium. Bacterial species that have been found to be capable of reducing transition metals include Escherichia coli, Pseudomonas putida, Bacillus species and Shewanella oneidensis. These and other bacterial species such as Geobacter are able to transfer electrons to an anode in an MFC. Those bacteria that are capable of reducing iron (III) to iron (II) are generally regarded as having the best potential to transfer electrons to an anode in an MFC, although there are many bacteria capable of iron reduction that cannot produce electricity.

Studies involving microbial fuel cells have focused on a limited number of substrates but a wide variety of organisms. The most common substrate is glucose, although other carbohydrates have been used as well such as organic acids, wastewater and the amino acid glycine.

In this project several types of bacteria were tried in microbial fuel cells (MFC’s)
containing carbon sources that had not been used before, such as glycerol, with a view
to producing electricity, whilst disposing of waste organic compounds. *Dechloromonas*
denitrificans and three species of *rhodococcus* (*R. rhodochrous*, *R. opacus* and *R. ruber*)
were trialled in a variety of MFC’s both bought in and made in house, both mixed and
as pure cultures. Only the organism *D. denitrificans* showed initial signs of current
production which it lost and never regained.

In addition these bacteria were challenged with medium containing a variety of
transition metals including chromium, iron, molybdenum, vanadium and copper.
Different bacteria had different abilities with regard to transition metal reduction.
Whilst *D. denitrificans*, *R. rhodochrous* and *R. opacus* were able to reduce chromate
(VI) to an extent, *R. rhodochrous*, *R. opacus* and *R. ruber* were incapable of iron
reduction. *R. opacus* was capable of vanadium reduction and was possibly able to
reduce copper, neither of which *D. denitrificans* was capable of tolerating. Both *D.
denitrificans* and *R. opacus* were capable of reduction of molybdenum and *D.
denitrificans* could reduce iron.

A mixture of bacterial types were collected from a high iron environment (a slate
quarry). Due to time constraints these bacteria were not formally characterised or
separated as pure cultures. They were apparently capable of tolerating high
concentrations of the above transition metals without much reduction, although one
isolate mixture could reduce iron (III) and chromate (VI). Surprisingly bacterial
mixtures isolated from a high iron environment (a slate quarry) were no more capable of
electricity production than the pure cultures. An initial attempt was made to look at the
mechanisms of transition metal reduction and to see if the bacteria lacked crucial genes
used in electricity production using both *in situ* hybridisation and confocal microscopy
and PCR. Some genes relevant to iron reduction and chromate reduction, but not
current production, were found.
Acknowledgements

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Chapter 1. General introduction

1.1. Volatile organic compounds

1.1.1. Background

The role of volatile organic compounds in the formation of photochemical smog, an environmental problem of international significance, has been recognised for many decades (OCED, 1982). Photochemical smog forms as a result of reactions between volatile organic compounds (VOCs) and nitrogen oxides catalysed by light (Green, 1995). Most photochemical smog forms in temperate and tropical latitudes in the warmer summer months and is regarded as a trans-national problem since pollutants may cross international boundaries. The main effects of this type of pollution are on human health (particularly on the lungs), damage to vegetation and materials. Organic compounds are released into the atmosphere by both natural and man made processes. However, while natural emissions are large they are not thought to be a major part of the photochemical smog formation since they are widely dispersed over the globe (OCED, 1982).

1.1.2. Definition of volatile organic compounds

There are a very large range of compounds that may be classified as volatile organic compounds. The United States Environmental Protection Agency defined a VOC as follows:

“A volatile organic compound (VOC) is any organic compound that, when released into the atmosphere, can remain long enough to participate in photochemical reactions. While there is no clear line of demarcation between volatile and non-volatile organics, the predominant fraction of the VOC burden are compounds which evaporate rapidly at ambient room temperatures. Almost all organics which can be considered VOC have vapour pressures greater than 0.1mm of Hg at standard conditions (20°C and 760mm Hg)” (OCED, 1982).
1.1.3. Sources of VOCs

The main anthropogenic sources of VOCs are from the petrochemical industry, combustion of carbon based fuels, dry cleaning, surface coating, paint manufacture, chemical manufacture and other industrial processes such as degreasing (OCED, 1982). A list of the type of compounds and main sources is given in Table 1.1.

**Table 1.1.** Examples of VOC sources and uses, from (OCED, 1982) and the European Pollutant Emission Register, 2005 http://www.eper.cec.eu.int/eper/pollutant_list.asp#42.

<table>
<thead>
<tr>
<th>Main source(s)</th>
<th>Compound and usages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motor vehicles</td>
<td>Polyaromatic hydrocarbons</td>
</tr>
<tr>
<td>Dry cleaning</td>
<td>Petroleum based (kerosene)/ halogenated hydrocarbons</td>
</tr>
<tr>
<td>Surface coating</td>
<td>Hydrocarbons (aliphatics and aromatics), alcohols, ketones, esters</td>
</tr>
<tr>
<td>Paint manufacture and use</td>
<td>Hydrocarbons (aliphatics and aromatics), alcohols, ketones, esters</td>
</tr>
<tr>
<td>Chemical industry</td>
<td>Hydrocarbons (aliphatics and aromatics), alcohols, ketones, esters, halogenated hydrocarbons</td>
</tr>
<tr>
<td>Industrial</td>
<td>Trichloroethylene used in degreasing</td>
</tr>
<tr>
<td>Petroleum industry</td>
<td>Various aromatic compounds and polyaromatic compounds as well as hydrocarbons</td>
</tr>
</tbody>
</table>

1.1.4. Problems caused by VOCs

Volatile organic compounds can spread over large areas in the atmosphere and can become widely distributed in the environment being found in water and soils as well as the air. VOCs can have considerable longevity in the environment and can cause a number of problems both in the health and environmental fields.
1.1.4.1 Human health

VOCs influence human health in a number of ways. Short term effects of photochemical smog include asthma and eye injuries. Photochemical smog also has a damaging effect on the respiratory tract (Green, 1995; Walker, 1996). Longer term effects can occur due to the toxicity and carcinogenicity of some of these compounds (Hutter and Vandegrift, 1992; OCED, 1982; Walker, 1996).

1.1.4.2. Ozone depletion

At high altitude some VOCs such as halogenated compounds can cause breakdown of the protective ozone layer round the earth. Although many VOCs that are known to have this effect have been phased out under the Montreal protocol, others in use (such as dichloromethane) are thought to to attack the ozone layer (European Pollutant Emission Register, 2005).

1.1.4.3. Environmental contamination

Long term contamination has been reported over numerous sites, requiring extensive and difficult clean-up (Scragg, 2005). Many chemicals have very long half-lives in the environment (Scragg, 2005).

1.1.4.4. Ground level ozone formation

A variety of polyaromatic compounds along with simple aromatics such as benzene can aid the formation of ozone. This is thought to have similar effects to acid rain on plants as well as causing health problems outlined above (OCED, 1982; Green, 1995).

1.1.5. VOCs used in this project

The VOCs and other organic compounds used in this project are shown in Table 1.2 below.
### Table 1.2. Solvents used in the project and their industrial uses. (Allinger et al., 1971; Scragg, 2005; Leethochawalit et al., 2004).

<table>
<thead>
<tr>
<th>VOC/solvent</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>Used as a solvent and intermediate in industrial processes.</td>
</tr>
<tr>
<td>Butanol</td>
<td>Used as a solvent and intermediate in industrial processes.</td>
</tr>
<tr>
<td>2-Propanol (isopropyl alcohol)</td>
<td>Used as a solvent and intermediate in industrial processes.</td>
</tr>
<tr>
<td>1,2,3-Propantriol (glycerol/glycerin)</td>
<td>Glycerol is an enormously versatile compound used in a wide variety of chemical and pharmaceutical products. It is added to foodstuffs, cosmetics and drugs to retain moisture. Glycerol is also used in the manufacture of dyestuffs, explosives and antifreeze. It is also used as a thickening agent and plasticizer. Traditionally it has been made using propylene as a feedstock or from saponification of oils and fats. However, increasingly it is being made as a by product from the manufacture of biodiesel.</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Used as an industrial solvent in large quantities.</td>
</tr>
</tbody>
</table>

#### 1.1.6. VOC reduction and clean-up

A number of different strategies have been adapted to deal with VOCs. These include reduction in use either by reformulation or product improvement, VOC capture (followed by storage and destruction), physical or chemical treatment, filtration using conventional (adsorption filters or wet scrubbing), or biofiltration techniques. Physical and chemical treatment tends to be very energy intensive (Wani et al., 1997; Ratledge and Kristiansen, 2001). Incineration involves the use of large amounts of fuel which is typically natural gas and generates oxides of nitrogen due to the high temperature of combustion (Wani et al., 1997; Ratledge and Kristiansen, 2001). To completely incinerate VOCs at a typical industrial emission concentration of 100ml/m³ requires at least 50l/m³ of methane (Ratledge and Kristiansen, 2001). The nitrogen oxides can be removed, but this adds to the cost of the process, although some incinerators do also
generate electricity. There is also public resistance to the sighting of incinerators.

Other techniques such as wet scrubbing and carbon filter adsorption also have their disadvantages. The first and foremost being that although they remove the VOC at the source of production they still leave a disposal problem (Ratledge and Kristiansen, 2001). Secondly wet scrubbing in particular has low efficiencies with some compounds (Wani et al., 1997). Thirdly, techniques of this type are costly, especially compared to biological methods (Wani et al., 1997). In the case of contaminated sites the above forms of treatment range from the difficult to the impossible due to the fact the contamination is largely found in soil and subsoil. Despite the advantages of biotreatment of organic wastes, the techniques have been slow to catch on. This is possibly due to conservatism within the chemical industry and low profit margins in the field (Ratledge and Kristiansen, 2001).

1.1.7. Biodegradation

Biodegradation uses the natural ability of microorganisms to degrade compounds by their metabolism ultimately to mainly carbon dioxide and water (Paul et al., 2005; Scrugg, 2005; Lovely, 2003; Parales et al., 2002; Hutter and Vandegraff, 1992) and has been of interest since the 1950’s (Alexander, 1994). If carried through to carbon dioxide, water and other inorganic compounds this process is known as mineralization (Prescott et al., 2005; Alexander, 1994). Two methods exist to carry this out, biofiltration and bioremediation. Biofiltration deals with the VOC at the point of production, bioremediation less satisfactorily after the release of the compound(s) into the environment. Potentially both offer an environmentally benign and low energy intensive means of dealing with VOCs and other toxic compounds. Organic chemicals including VOCs differ in their ease of biodegradability, simple aliphatic and aromatic compounds are easily biodegradable (Scrugg, 2005). More recalcitrant compounds include all types of halogenated compounds and some nitrogenated and sulphonated organics (typically explosives or herbicides) (Paul et al., 2005; Ratledge and Kristiansen, 2001), although Freitas Dos Santos and Livingston (1995) achieved 94.5% removal of 1,2-dichloroethane from wastewater streams at a loading of 2g/l, 65% being
converted to carbon dioxide. Bacteria have been isolated that can degrade compounds in industrial use such as oil components, chlorinated aromatics and polyaromatics, although few bacteria are known that will degrade polyaromatics with 5 or more rings (Paul et al., 2005; Kanaly and Harayama, 2000). In general terms for successful biodegradation to be carried out the following criteria must apply (Alexander, 1994);

- The microorganisms must have the necessary metabolic machinery to carry out the process.

- The microorganism must be present at the site of the chemical to be broken down.

- The chemical to be biodegraded must be accessible to the organism to metabolise i.e. not sorbed to an inaccessible surface in the micro-environment or not water soluble.

- Extracellular enzymes involved in the catabolism of the compound must be able to interact with relevant part of the molecule to be degraded.

- If the metabolic process takes place within the cell then the compound of interest must be able to penetrate the cell.

- The environment in which the organism and compound coexist must be conducive to the growth of the organism concerned.

A further advantage of studying biodegradation pathways is that their study may lead to new biosynthetic ones (Parales et al., 2002).

Biofiltration is the use of a mixed consortia of bacteria found in natural materials in a bioreactor (Ottengraf et al., 1986; Wani et al., 1997; Juteau et al., 1999; Roy et al., 2003). Materials used as biofilters include wood, peat, compost or soil (Ottengraf et al., 1986; Alexander, 1994; Wani et al., 1997; Juteau et al., 1999; Ratledge and Kristiansen,
Some workers have attempted to analyse the bacteria involved (Juteau et al., 1999), although in many cases the active consortium is unknown (Bustard et al., 2001). Roy et al. (2003) took compost of municipal waste origin formatting it into balls of a diameter of 20mm or pellets with a diameter of 6.3-10mm which then were used to create biofilters of 15cm diameter and 45cm depth. Water saturated air at a flow rate of 1m$^3$ h$^{-1}$, toluene (up to 500ppm), a nutrient solution and urea (a nitrogen source) was passed through the filters for up to 44 days. Optimal degradation of the toluene was found to be 80-90% after 15-18 days using the smaller pellets although the rate of toluene removal dropped if the nitrogen input was lowered. Examination of the indigenous bacterial content of the compost material from the biofilters using 16S ribosomal RNA analysis revealed the predominant organism present was *Pseudomonas putida* (Roy et al., 2003). Zilli et al. (2000) also examined the removal of toluene in gas stream using sterilised peat mixed with glass beads. A toluene/air mixture was passed through the bottom of the vessel and a mineral salts medium was added batch wise at the top. The bacterium used was *Acinetobacter* sp. The maximum removal rate was 242gm$^{-3}$h$^{-1}$ at an inlet concentration of 4gm$^{-3}$. This gave an efficiency of 23.8%. A 90% efficiency of removal was achieved with a lower load of 113.7gm$^{-3}$h$^{-1}$. Examination of the concentration profiles along the bioreactor indicated the process of toluene breakdown was limited by diffusion at low rates of input and the biological abilities of the organism at high input rates. Despite the lack of nitrogen input this system operated at high efficiency for 10 months (Zilli et al., 2000).

Leethochawalit et al. (2004) grew a mixed consortium of bacteria capable of degrading isopropanol in the gas phase on a matrix of glass sintered beads. The breakdown of both acetone (an intermediate of the isopropanol biodegradation pathway) and isopropanol as the sole carbon sources along with minimal salts medium were studied in a continuous process. Almost total mineralization of the isopropanol and acetone was found at loadings of 380-500gm$^{-3}$h$^{-1}$ (Leethochawalit et al., 2004).

Biofiltration works for a wide variety of compounds and is in commercial use (Alexander, 1994; Wani et al., 1997). However, periodically the biofilter material needs replacing and the use of peat in particular is not regarded as sustainable or
environmentally friendly since peat is a non-renewable resource. Other problems include the large surface areas of plant required, pH control and odours produced by the biofilter materials (Ratledge and Kristiansen, 2001).

Biofiltration is suitable for compounds used and captured during industrial processes and wastewater (Freitas dos Santos and Livingston, 1995), but is of no use for the breakdown of VOCs at existing contaminated industrial sites. For these sites bioremediation using single or mixed consortia of bacteria at the site have been studied as well as removal of the soil and its treatment using landfarming and slurry bioreactors (Scragg, 2005; Ratledge and Kristiansen, 2001). Studies have predominantly centred on two classes of compounds, aromatic hydrocarbons and organochlorines due to their resistance to breakdown, although others have been investigated such as organic sulphur compounds (Coleman et al., 2002; Furukawa, 2003; Ang et al., 2005; Paul et al., 2005; ven der Geize and Dijkstra, 2004). Most biodegradation investigations have used known genera of bacteria, although in some cases species were isolated from the environment that grew on substrates and were then identified. Almost all studies have focused on using the compound to be degraded as the sole carbon and energy source.

*Rhodococcus sp.* have been found to break down a large variety of aromatic compounds, as well as sulphonated, chlorinated compounds, phthalates and steroids (ven der Geize and Dijkstra, 2004; Parales and Haddock, 2004; Larkin et al., 2005; Nakamiya et al., 2005; De Carvalho and da Fonseca, 2005). In one study *Rhodococcus* 11Y sourced from explosive contaminated soil completely mineralised 250μM of the compound RDX in less than 21 hours (Seth-Smith et al., 2002). In this case additional carbon sources (10mM glycerol, 5mM glucose and 5mM succinate) were required. Studies indicated that the ability to degrade this explosive was conferred by a set of genes on a plasmid. These genes could be transferred to other strains unable to metabolise this compound (Seth-Smith et al., 2002). Curragh et al. (1994) studied the breakdown of 1-chlorobutane and 1-chlorohexadecane as the sole carbon sources using *Rhodococcus rhodochrous*. This organism was able to completely remove 1.1mmol of 1-chlorobutane from the growth medium in 35 hours and 5.5mmol 1-chlorohexadecane in approximately 70 hours. In the latter case analysis of the metabolic intermediates
indicated that not all the chlorine was mineralised to chloride, instead being present as organic intermediates. After growth on the above substrates the organism was able to utilise a very wide variety of other aliphatic halogens although the level of breakdown was lower (Curragh et al., 1994).

*Pseudomonas* strains have long been known to be highly versatile micro-organisms capable of degrading a wide variety of naturally occurring and man made organic compounds (Wackett, 2003; Parales and Haddock, 2004). Bacteria of this genus isolated from the contaminated soil of a former gasworks were also found to break down a variety of polyaromatic hydrocarbons with an efficiency of up to 22% for phenanthrene in a mineral salts solution containing 10\( \mu \)g/ml of this compound over 10 days of incubation (Kahng et al., 2002). However, the cells were unable to mineralise benzo[a]pyrene, chrysene or pyrene (Kahng et al., 2002). *Pseudomonas* strains were also found to be capable of breaking down phenols and atrazine (Neumann et al., 2004; Parales and Haddock, 2004). Using phenol as the carbon source at a concentration of 1g/l and atrazine as the nitrogen source at a concentration of 150mg/l *Pseudomonas sp. strain ADP* was able to completely biodegrade both within 30 hours (Neumann et al., 2004). *Pseudomonas* strains were found to degrade aromatic compounds such as naphthalene (as the sole carbon source) added to sterilised soil at 1mg/g soil (Filonov et al., 1999). In this study the moisture content of the soil was found to be critical. Maximum bacterial growth occurred at 40% saturation which was just below the maximum possible moisture holding capacity of the soil (48%) (Filonov et al., 1999).

Bacteria cultured from garden soil and sludge able to biodegrade bis (2-ethylhexyl) phthalate (DEHP) in polyvinyl chloride were identified as *Mycobacterium sp* (Nakamiya et al., 2005). Under optimal growth conditions of 30°C an initial pH of 6.8 and DEHP concentration of 0.1%, 98% of the DEHP was degraded in 21 hours. Coleman et al. (2002) isolated a bacterium able to aerobically breakdown chlorinated organic compounds. The bacteria isolated from a site contaminated with organochlorines were identified using 16S rRNA analysis as having 97.9% identity with *Polaromonas vacuolata* and were able to completely dechlorinate cis-1,2-dichloroethene from an initial concentration of 0.90mM.
Prakash and Gupta, (2000) used sludge found in an anaerobic digester of a municipal wastewater treatment plant to inoculate an upflow anaerobic sludge blanket (UASB) fermentor to biodegrade tetrachloroethylene (TCE). Artificial wastewater containing methanol and acetone was used as an additional carbon source buffered with mineral salts over a 128 day period. Loading the reactor with tetrachloroethylene at a concentration of 5-20 mg/l the bacteria reduced its concentration to below detectable levels. At a loading of 30-50 mg/l TCE the system produced an effluent with a concentration range of 0.1 to 0.45 mg/l TCE. The bacteria involved were identified as *Methanothrix* and *Methanosarcina* (Prakash and Gupta, 2000).

*Buckholderia xenovorans* is capable of breaking down not only a wide variety of polycaromatic hydrocarbons but also polychlorinated hydrocarbons (PCB’s) (Mohammadi and Sylvestre, 2005). Gibson *et al.* (1993) made a study of the ability of this organism to oxidise a wide variety of biphenyls. The biphenyls were added at 0.3% (w/v) in a mineral salts medium with yeast extract. After 24 hours of incubation *B. xenovorans* was able to completely oxidise many of the PCB cogeners studied. This organism shows a clear preference for biphenyls chlorinated at a 2 position on the ring, degrading most of these molecules by 100%. It could only partly oxidise molecules substituted only at the 3 and 4 positions on either rings (Gibson *et al.*, 1993). In 2004 the LB400 strain was reclassified from *Pseudomonas* to *Buckholderia* sp. (Goris *et al.*, 2004).

Members of the genus *Gordonia* are known to degrade alkanes, PAH's benzothiophenes and some pyridine based compounds (Arenskötter *et al.*, 2004). Xue *et al.* (2003) isolated an organism from a water sample taken from an oil well in China. It was able to use as its sole carbon source both liquid paraffin and glycerol, as well as some sugars. Both classical bacterial taxonomy techniques and 16 rRNA analysis identified it as a new member of the *Gordonia* genus and proposed renaming to *Gordonia paraffinivorans* due its ability to metabolise this substrate (Xue *et al.*, 2003). Pyridines offer a potential environmental and human health challenge. Yoon *et al.* (2000) isolated an organism from wastewater that chemotaxonomic characterization and 16S rRNA analysis suggested was a new member of the *Gordonia* genus able to
Isopropyl alcohol, acetone, methanol and higher alcohols can be metabolised by a variety of micro-organisms. Bacteria of the genus *Paracoccus* can metabolise a variety of VOCs (Yamane *et al.*, 1996; Baker *et al.*, 1998; Van Spanning *et al.*, 2000). Many, but not all members of this genus express sets of genes allowing the organism to metabolise alcohols and deal with the toxic aldehyde products of dehydrogenating alcohols (Van Spanning *et al.*, 2000). *Paracoccus denitrificans* as part of these gene sets expresses a methanol dehydrogenase enzyme that allows it to grow on this substrate, the enzyme being linked into its unusual electron transport chain similar to that found in mitochondria (Baker *et al.*, 1998; Richardson, 2000; Prescott *et al.*, 2005). Another enzyme, glutathione dependent formaldehyde dehydrogenase (GD-FALDH), is thought to be partially responsible for metabolising higher alcohols and formaldehyde and some short chain alcohols, functioning as an alcohol dehydrogenase (Van Spanning *et al.*, 2000; Van Ophem *et al.*, 1994). Other types of alcohol dehydrogenase are also present in the organism (Van Ophem *et al.*, 1994). Yamane *et al.* (1996) used the alcohol degrading abilities of *P. denitrificans* with the aim of producing polyhydroxyalkanoates (PHA’s) the building blocks of bioplastics. *P. denitrificans* was cultured in a 5l fermenter with working volumes of 3 litres on single and mixtures of two alcohols at a 0.1% (v/v) under both normal and nitrogen limiting conditions. A mixture of ethanol and n-pentanol was the optimal combination for producing PHA’s with 31% by dried cell weight of these compounds produced (Yamane *et al.*, 1996).

Siller *et al.* (1996) isolated an organism from a former natural gas works. Classical bacterial taxonomy techniques and 16S rRNA analysis identified the organism as a member of the genus *Paracoccus*. The organism could degrade a wide variety of carbon sources anoxically and could degrade acetone in the presence of carbon dioxide, but using acetoacetate without it being present. Acetone was metabolised to PHB’s (Siller *et al.*, 1996). *Rhodococcus erythropolis* has been found to be able to metabolise a wide variety of aliphatic and aromatic alcohols, using them as its sole carbon source (Schenkels and Duine, 2000). Schenkels and Duine (2000) isolated the alcohol dehydrogenase enzyme responsible and sequenced its N-terminal amino acid sequence.
which showed homology to nicotinoprotein alcohol dehydrogenases from other organisms. The isolated alcohol dehydrogenase was able to catalyse oxidation of a large range of primary and secondary alcohols as well as diols. Aldehydes and ketones were reduced in the presence of an alcohol (Schenkels and Duine, 2000). Whilst the alcohol dehydrogenase in *R. erythropolis* could catalyse oxidation of diols, triols such as glycerol were not tried (Schenkels and Duine, 2000). Toyama *et al.* (1995) isolated an organism from soil that could metabolise a wide variety of alcohols as its sole carbon sources. Toyama *et al.* (1995) examined growth using a wide range of alcohols (including glycerol) as the sole carbon and energy source at a concentration of 0.2%. Taxonomic characterization of the organism suggested it was *Pseudomonas putida*. Three different alcohol dehydrogenases were isolated from cells grown on three alcohol substrates which had varying alcohol specificity. Just one of the enzymes was able to utilise glycerol *(Toyama et al., 1995)*. Taxonomic characterization of *Polaromonas vacuolata* showed it can also utilise glycerol indicating this organism may be able to biodegrade this compound *(Irgens et al., 1996)*.

Biodegradation at the contaminated site has an advantage over other methods of remediation in that it offers a low cost and non-destructive method of clean-up *(Ang et al., 2005)*. However, selection, growth of the micro-organism(s) and therefore clean-up can be very slow *(Ang et al., 2005)*. A number of techniques have been investigated to speed these up including the use of surfactants and the addition of inorganic nutrients. These are often in low concentration where required at the site *(Alexander, 1994; Lindstrom et al., 1999)*. The use of genetically modified (GM) organisms has also been investigated to accelerate the biodegradation process *(Paul et al., 2005; Ang et al., 2005)*. Due to concerns about the release into the environment of GM organisms a number of strategies have been developed to cause the bacteria concerned to commit programmed cell death after their task is complete *(Paul et al., 2005)*.

1.1.8. Adaptation of bacteria to VOCs

Bacteria that are able to grow in the presence of and metabolise organic solvents use a variety of techniques to adapt to them. These include alteration of the fatty acids
present in the lipid envelope of the cytoplasmic membrane, formation of vesicles in the cytoplasm and removal of the solvent concerned from the organism using energy dependent pumps (Ramos et al., 2002; Sardessai and Bhosle, 2002). Solvent toxicity to microorganisms is expressed using a log scale of the partition of the solvent concerned between octanol and water known as the logP_{ow} (Ramos et al., 2002). Solvents with a logP_{ow} of less than 3-4 are extremely toxic to microorganisms since they dissolve in the lipid envelope and disrupt its structure (Ramos et al., 1997; Sardessai and Bhosle, 2002; Sardessai and Bhosle, 2004).

Ramos et al. (1997) made a study of the effect of toluene on the growth and metabolism of Pseudomonas putida. Even low concentrations of toluene of 0.3-1% (v/v) had effects on the cell membrane composition increasing the trans to cis fatty acid concentration ratio (Ramos et al., 1997). This mechanism has also been found in Escherichia and Pseudomonas (Aono et al., 1997). This is carried out both by de novo synthesis of new phospholipids and isomerisation of existing ones (Ramos et al., 1997; Aono et al., 1997). It is thought that switching the phospholipid composition in these ways decreases membrane fluidity, which increases with solvent penetration (Ramos et al., 2002; Ramos et al., 1997). Kahng et al. (2002) studied the effects of polyaromatic hydrocarbons (PAH’s) on the lipid composition in the cell walls of Pseudomonas rhodesiae. They found a profound shift in the lipid composition at low levels of PAH concentration in the medium, with the expression of some types of lipids increasing (cis to trans) and others becoming undetectable (Kahng et al., 2002). Phenol also alters the trans to cis fatty acid ratio in a dose dependent manner in Pseudomonas sp. strain ADP, raising it with increasing phenol concentration (Neumann et al., 2004). Modification of the lipid composition can also occur by incorporation of metabolic intermediates into the lipid envelope. Curragh et al. (1994) investigated the breakdown of two aliphatic chlorinated hydrocarbon compounds. They found that chlorinated fatty acids made up 75% of the lipids in cells grown on 1-chlorohexadecane but no chlorinated fatty acids were present in the cells grown on 1-chlorobutane. Breakdown of the longer chain molecule occurs at the non-chlorinated end by action of an oxygenase to form a chlorinated fatty acid (Curragh et al., 1994).
Another method by which bacteria can adapt to solvents is by the use of pumps (Aono et al., 1997). A wide variety of these proteins are found in the plasma membranes (Ramos et al., 2002). Both Escherichia coli and Psuedomonas putida contain a variety of pumps that are capable of dealing with potentially toxic solvents by removing them from the cell (Ramos et al., 2002). Ramos et al. (1997) also discovered using knockout mutants of P. putida that the outer membrane protein OprL is responsible for membrane integrity. Cells lacking the corresponding gene were unable to remove aromatic compounds from their membrane (Ramos et al., 1997). Other methods of adaptation or tolerance include the use of stress response proteins, solvent inactivation proteins and membrane repair proteins (Sardessai and Bhosle, 2002).

The adaptive strategies described above all apply to Gram-negative bacteria that have both a plasma membrane and an outer membrane (Sardessai et al., 2002). As outlined previously Gram-positive bacteria such as Rhodococcus are capable of tolerating VOCs and xenobiotics though they lack a second membrane. No definite proof of the method of adaptation to these types of compounds yet exists. The methods were thought to include at least some of the characteristics found in Gram-negative bacteria (Sardessai et al., 2002). Nielsen et al. (2005) studied the effects of the organic solvents toluene, benzene and p-xylene on Staphylococcus haemolyticus isolated from the gut of an insect that feeds exclusively on oil and asphalt in its larval stage. This bacterium could tolerate a concentration of 100% of the above solvents. Although there was some change in the phospholipid makeup in the organism grown with or without toluene, the fatty acid composition alters in the opposite way to that found in Gram-negative bacteria. In S. haemolyticus the membrane fluidity increased rather than decreased, as would be expected. Thus Gram-positive organisms must employ some other as yet to be determined mechanisms, such as the use of pumps or shock proteins (Sardessai et al., 2002).

There is some evidence that changes in membrane type due to solvent challenge may affect the output of microbial fuel cells. Choi et al. (2003) added ethanol over a range of 0.5%-3% to a microbial fuel cell containing Proteus vulgaris using thionine as an electron mediator (see below) and found a drop in coulombic yields. Examination
revealed that the ratio of saturated to unsaturated fatty acids had increased, leading to the theory that this made the membrane less permeable to the thionine (Choi et al., 2003).

The cell’s ability to tolerate a solvent is not necessarily linked to its ability to metabolise it. Mutant strains of *P. putida* that had their ability to metabolise toluene removed were still able to tolerate it (Ramos et al., 2002). Atrazine had a minimal effect on the *trans* to *cis* fatty acid ratio, unlike phenol in the same study (Neumann et al., 2004).
1.2. Fuel cells

1.2.1. History

The fuel cell was invented by W.R. Grove in the 19th century (Sopian and Daud, 2005). Development has continued since then with an accelerated phase after the second world war during the American space programme (Kordesch, 1989). The cells used in the space programme were based on the work of F.T. Bacon at Cambridge University, England. Modifications of alkaline fuel cells which he had developed, by NASA, involved lowering the pressure and increasing the temperature of operation to increase performance (Kordesch, 1989; Williams, 2004). NASA chose this cell type for development due to its relatively high performance (Williams, 2004).

1.2.2. Definition of a fuel cell

A fuel cell is an electrochemical energy conversion device (Kordesch, 1989; Sopian and Daud, 2005). However, there are a number of differences from other similar devices like batteries. In a fuel cell the “fuel” and oxidant is continuously supplied from an external source (or sources) and must be continuously removed along with any heat generated (Kordesch, 1989). If the fuel or oxidant is cut off or no current is drawn the cell will cease to operate (Williams, 2004). A battery is a closed system in which the chemicals are enclosed within the electrodes. Once these chemicals (and hence the battery) are exhausted it must either be disposed of or recharged using an external energy source. The reactions that take place in a fuel cell are in effect not reversible (Kordesch, 1989). The fuel cell operates as long as fuel and oxidant are provided and thus can be regarded in effect as a continuously cycling battery (Williams, 2004). Fuel cells have long operating lives compared to other forms of energy generation and operate at a higher efficiency (Sopian and Daud, 2005).

The voltage of an individual cell is normally of insufficient size to be useful. Individual cells are therefore usually combined into stacks to increase the power output (Williams, 2004). A number of fuel cells types have been developed which run on a
variety of fuels (Williams, 2004; Sopian and Daud, 2005). The PEM type is described in more detail below since it is the most similar type to microbial fuel cells, but all fuel cells operate on broadly the same principles albeit with different catalysts, fuels and materials of manufacture. Fuels (usually in a gaseous form) are fed continuously to the anode compartment. Oxygen either as air, or in its pure form, to the cathode compartment. An electrochemical reaction takes place at the anode or cathode, depending on the fuel cell type. The reaction produces electrons which flow through a circuit to the cathode as well as anion and cation. The anion or cation (more usually the cation) crosses the semi-permeable membrane between the anode and cathode and combines with oxygen and electrons at the cathode (Haile, 2003; Williams, 2004). The semi-permeable membrane has the function of allowing one of the ion types produced at the anode to cross, but not the other, maintaining a charge differential across the fuel cell device. Below, different types of cell are described. An illustrative cell output (as voltage difference across the cell and current per unit area of anode) and a performance range (as % of fuel input turned into electricity) are given. These vary depending on the exact operating conditions, fuel mix and cell developer and are given graphically in a report as a range of values by Williams (2004).

1.2.3. Polymer Electrolyte Membrane (PEM) Fuel Cells

This type of cell (also known as a proton exchange membrane fuel cell), uses only hydrogen and oxygen or methanol (Kordesch, 1989; Haile, 2003; Smith, 2003). The PEM cell has the advantages that it operates at a relatively low temperature of 80-120°C meaning less energy is needed to get the system up to operating temperature and it can start quickly (Smith, 2003; Williams, 2004). Individual cells are also relatively light. The cell uses the polymer as an electrolyte and electrodes containing a platinum catalyst. The polymer membrane consists of sulphonated polyfluroethylene (usually known by its trade name Nafion) and this along with the catalyst adds to the cost (Kordesch, 1989; Haile, 2003). Another disadvantage of this type of cell is that the platinum is very susceptible to poisoning from carbon monoxide present in the fuel, especially if methane is used a source of hydrogen (Haile, 2003; Bance et al., 2004; Williams, 2004; Biyikoğlu, 2005; Sopian and Daud, 2005). Recently research has been
directed into increasing the operating temperature towards the higher end of the range
given above in an effort to reform the carbon monoxide and hence prolong the anode
life (Williams, 2004). Other research has concentrated on lowering the platinum
loading hence reducing cell cost (Sopian and Daud, 2005).

The PEM fuel cell operates in the following manner. At the anode hydrogen gas is
separated to 2 protons and 2 electrons. The protons cross the semi-permeable
membrane to the cathode to recombine with electrons that have flowed through the
external circuit as well as oxygen. The half reactions and overall cell reaction are
shown below. The overall fuel cell efficiency is 40-50% (Sopian and Daud, 2005). A
PEM cell typically gives a cell voltage output in the order of 0.7V with a current density
of 600 mA/cm² (cell current/anode area) although up to 1.2A/cm² has been achieved
(Williams, 2004; Haile, 2003).

Anode Reaction: \( \text{H}_2 \rightarrow 2\text{H}^+ + 2\text{e}^- \)
Cathode Reaction: \( \frac{1}{2}\text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O} \)
Cell reaction: \( 2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O} \)

1.2.4. Biofuel cells

This type of fuel cell works on the same general principles as above except the fuel
is not acted on by a metal impregnated anode but by an enzyme (Gacesa and Hubble,
1987; Palmore et al., 1998; Ramanavicius et al., 2004; Shukla et al., 2004; Topcagic,
and Minteer, 2005). A number of different fuel types have been utilised, however
research has mainly focused on alcohols although sugars have been used (Gacesa and
Hubble, 1987; Ramanaviciusa et al., 2004; Palmore et al., 1998). The major advantage
of these cells is that they run at ambient temperature.

It is difficult to give half and overall cell reactions for this type of cell since the
anode and cathode setups have varied greatly. Early research (Gacesa and Hubble,
1987) focused on the use of methanol dehydrogenase which oxidised methanol to
formate at the anode with the release of electrons and protons. The protons crossed a
membrane to the cathode to recombine with electrons that have flowed through the circuit between the anode and the cathode, then forming water in conjunction with oxygen. At the time it was not possible for direct transfer of electrons from the dehydrogenase enzyme to the anode. An artificial redox dye was used as a means of electron transfer. This also regenerated the dehydrogenase (Gacesa and Hubble, 1987). Cell reactions are given below.

Anode reaction: \[ \text{CH}_3\text{OH} \rightarrow \text{CH}_2\text{O} + 2\text{H}^+ + 2e^- \]

Cathode reaction: \[ \frac{1}{2}\text{O}_2 + 2\text{H}^+ + 2e^- \rightarrow \text{H}_2\text{O} \]

Cell reaction: \[ \text{CH}_3\text{OH} + \frac{1}{2}\text{O}_2 \rightarrow \text{CH}_2\text{O} + \text{H}_2\text{O} \]

More recent research has focused on removal of the membrane as well as direct and indirect transfer of electrons to the anode from the enzyme.

Using an approach similar to that outlined for the methanol fuel cell above, Palmore et al. (1998) developed a biofuel cell that used three separate enzymes, alcohol, aldehyde and formate dehydrogenase to transfer electrons to the co-factor nicotinamide-adenine dinucleotide (NAD) to form NADH. NADH does not have a high enough electrode potential to transfer electrons directly to the anode. Palmore et al. (1998) searched for an enzyme capable of regenerating NAD. They chose an oxoreductase (diaphorase) from the organism Clostridium kluyveri that was known to oxidise NADH in conjunction with artificial redox dyes acting as an electron acceptor. The 4,4'-bipyridinium salts with known electrode potentials near to NAD/NADH were screened and one of these dyes (benzylviologen) was chosen for use in the cell since it was the only one capable of regenerating NAD and transferring electrons to the anode. The cell design was similar to a PEM cell described above with a semi-permeable membrane of the same type dividing the cathode and anode compartments. The cell gave a current density of 0.68mA/cm² at 0.49V (Palmore et al., 1998).

Topcagic and Minteer, (2005) developed a membrane free biofuel cell with some of the same features as Palmore et al. (1998). At the anode ethanol was consecutively oxidised to acetate by alcohol and aldehyde dehydrogenase. These enzymes transfer the
electrons from the substrate to the anode via NAD/NADH redox pairs and a redox dye (polymethylene green) and they then flow through an external circuit to the cathode. The cathode used the same type of semi-permeable membrane material used in the PEM cell to separate the anode and cathode but chemically modified with bilirubin and bilirubin oxidase and 1mM ruthium bipyridine (Topcagic and Minteer, 2005). The Nafion stabilised the bilirubin oxidase. Due to the fact that both the anode and cathode reactions are catalysed by specific enzymes no semi-permeable membrane was required between the anode and the cathode. The biofuel cell produced a power output of 0.46mW/cm² (Topcagic, and Minteer, 2005).

Ramanavicius et al. (2005) used a radically different approach that involved direct transfer of electrons by an enzyme immobilised on the anode as well as no semi-permeable membrane dividing the anode and the cathode. Alcohol dehydrogenase from Gluconobacter sp. 33 was known to be able to transfer electrons directly to the anode (Ramanavicius et al., 2005) and was immobilised on the anode. Microperoxidase-8 (MP-8) from horse heart and glucose oxidase (GOx) from Aspergillus niger were immobilised on the cathode. Ethanol at the anode was oxidised by the alcohol dehydrogenase. The electrons were transferred to the anode and flowed through an external circuit. At the cathode glucose oxidase catalysed the oxidation of glucose to gluconolactone and hydrogen peroxide. The microperoxidase catalysed the conversion of hydrogen peroxide, electrons from the external circuit and protons produced at the anode to water. The half reactions are shown below

Anode reaction:Ethanol→ Ethanal + 2H⁺ + 2e⁻

Cathode reactions: Glucose + ½O₂ + H₂O→Gluconolactone + H₂O₂  
H₂O₂ + 2H⁺ → 2H₂O - 2e⁻

The output of the cell was low, being in the order of 2.6μA/cm². The cell ran continuously for 48 hours (Ramanavicius et al., 2005).
1.2.5. Microbial fuel cells

1.2.5.1 Introduction

This type of fuel cell uses bacteria to transfer the electrons from the fuel (a substrate that the bacterial cells can utilise) to the anode which replaces the cells’ terminal electron acceptor (Rabaey and Verstraete, 2005). This electron transfer between the anode and the microbial cell can either be by direct contact, release of an extracellular mediator or by an artificial mediator (Rabaey and Verstraete, 2005). Figure 1.1 shows a simple schematic of a microbial fuel cell. Fuel is provided in the form of substrate that can be metabolised by the micro-organisms. Electrons are transferred from the substrate via NAD to the anode producing carbon dioxide in the anode compartment. Protons cross the semi-permeable membrane to the cathode and recombine with electrons that have flowed through the electrical connection between the anode and cathode and oxygen from air present in the cathode compartment.

![Diagram of a microbial fuel cell](image)

**Figure 1.1.** Schematic of a microbial fuel cell. Adapted from Rabaey and Verstraete, (2005).

This fuel cell type has the advantage that it will operate at ambient temperature and will also run on a variety of fuels, described in more detail below (Rabaey and
Verstraete, 2005). MFC’s have also been run continuously for 3-5 years (Habermann et al., 1991; Topcagic and Minteer, 2005).

1.2.5.2. Bacterial energetics

Bacteria gain energy by transferring electrons from a reduced substrate with high potential energy to a terminal electron acceptor with a lower potential energy (Brock and Madigan, 1988; Park and Zieku, 1999; Prescott et al., 2005). Most substances can be either electron acceptors or donors depending on the reaction circumstances (Brock and Madigan, 1988). However, they vary in their tendency to accept electrons (and be reduced), or donate electrons and undergo oxidation. By convention this tendency is related to a standard substance (hydrogen) as half reaction pairs measured in volts and is typically shown as a tower (see figure 1.2) (Brock and Madigan, 1988; Prescott et al., 2005).

**Figure 1.2.** The electron tower. Redox pairs at the top are the strongest reductants and at the bottom of the tower the strongest oxidants. The number of electrons available for transfer is shown in each case. Based on diagrams in Brock and Madigan, 1988; and Prescott et al., 2005.
Compounds at the top of the tower such as glucose have a high reduction potential (redox potential), or potential energy (shown as a negative voltage), expressed as a desire to donate electrons. Compounds at the bottom have a tendency to accept electrons (expressed as a positive voltage) and have a lower potential energy. The bigger the voltage difference between the electron donor and acceptor the greater the free energy (useful energy) that can be generated from the process (Brock and Madigan, 1988; Prescott et al., 2005). Bacteria can derive this potential energy from substances in one of two ways.

The first way energy is obtained is by using respiration. Energy is derived from a substrate by catabolism through the glycolytic pathway, or other pathways that feed into this pathway. This generates two moles of adenosine tri-phosphate (ATP) per mole of glucose in the pathway as far as pyruvate. The glycolytic pathway then feeds into the tricarboxylic cycle. This cycle links the biosynthesis of a wide variety of metabolic intermediates to reduction of NAD, flavin adenine dinucleotide (FAD) and NAD-phosphate (NADP) to their protonated forms (NADH, FADH2 and NADPH) (Brock and Madigan, 1988; Ratledge and Kristiansen, 2001; Prescott et al., 2005). For the process of cellular anabolism (biosynthesis) to proceed the above processes are indirectly coupled to the electron transport chain (Ratledge and Kristiansen, 2001). The electron transport chain (see figure 1.3) consists of a series of mainly protein molecules located in the bacterial plasma membrane (Prescott et al., 2005).
**Fig. 1.3.** The electron transport chain from *Paracoccus denitrificans*. Figure a) shows the aerobic pathway b) shows the anaerobic pathway using nitrate as an electron acceptor. In the aerobic pathway methanol can feed electrons into the electron transport chain. The anaerobic pathway leads to expression of different reductases shown in red that reduce nitrate to nitrogen. Based on a diagram in (Prescott *et al.*, 2005).

The molecules involved in the electron transport chain are mainly, but not entirely, proteins capable of undergoing consecutive linked oxidation and reduction reactions (Brock and Madigan, 1988; Ratledge and Kristiansen, 2001). In aerobic organisms the terminal electron acceptor is oxygen. On the inside of the cell cytoplasmic membrane NADH loses its hydrogen atom. The electron from the hydrogen atom on the NADH is transferred to the electron transport chain and the proton is pumped across to the outside of the membrane. A charge difference develops across the membrane with an acidic exterior and a more basic interior. This generates a proton motive force which allows
useful work to be done across the membrane analogous to a battery (Brock and Madigan, 1988; Richardson, 2000; Ratledge and Kristiansen, 2001; Jormakkaa et al., 2003; Prescott et al., 2005). In the membrane an ATPase (ATP synthase), that spans the membrane pumps protons back into the cytosol using the energy of the proton motive force to generate ATP from adenosine di-phosphate and a phosphate group (Jormakkaa et al., 2003; Prescott et al., 2005). Finally the electrons from the electron transport chain recombine with oxygen on the cytosolic side of the bacterial membrane to form water (Brock and Madigan, 1988; Ratledge and Kristiansen, 2001; Prescott et al., 2005). Oxidative phosphorylation generates 36-38 moles of ATP per mole of glucose whilst regenerating NAD and FAD for use in the tricarboxylic acid cycle (Prescott et al., 2005).

A large number of micro-organisms can generate energy using anaerobic respiration, i.e. they generate a proton motive force similar to that described above. Their terminal electron acceptors can be inorganic compounds such as Fe(III), Mn(IV), nitrate or sulphate as well as organic compounds (Johnson and McGinness, 1991; Lovely, 1993; Richardson, 2000; Kim et al., 2005). Many micro-organisms can switch from oxygen to inorganic electron acceptors and can grow using both (Baker et al., 1998; Richardson, 2000). Anaerobic respiration does not generate as much energy in the form of ATP as aerobic respiration. The electron acceptor is higher up the tower than oxygen. It therefore has a lower affinity than oxygen for electrons from the donor molecule and hence less free energy can be realised from the movement of electrons between the donor and the acceptor (Brock and Madigan, 1988; Prescott et al., 2005).

The second way that organisms can generate energy is by fermentation. During fermentation there is no exogenous electron acceptor, the products of metabolism are used as electron acceptors. These organic substrates are only partially oxidised. Therefore not all their potential energy can be utilised (Brock and Madigan, 1988 Prescott et al., 2005; Scragg, 2005). The electron transport chain is either not present or cannot operate in the absence of an electron acceptor. The TCA cycle and the glycolytic pathway can only partially function since both rely on a steady replenishment of NAD and FAD. The TCA cycle must work in part for the cell to produce 2-
oxoglutarate and oxaloacetate which are essential biosynthetic precursors. However, in order to do so it must find or use another way to regenerate NAD. The NAD regeneration pathways produce a variety of metabolites such as alcohols, hydrogen, organic acids and lactate (Ratledge and Kristiansen, 2001). In fermentation the redox potentials of the donor and acceptor are similar meaning it is a less efficient process and yields only 8% of the energy produced by aerobic respiration (Ratledge and Kristiansen, 2001). The 2 moles of ATP produced by the glycolytic pathway are only ATP that is generated by fermentation (Ratledge and Kristiansen, 2001; Brock and Madigan, 1988).

In a microbial fuel cell the anode, which is an electron sink with a positive potential compared to the cathode, becomes the electron acceptor of the organism (Rabaey and Verstraete, 2005). The anode potential determines the type of metabolism the cells can use. At high anode potential (when it is very positive) the cells will partially utilise their oxidative electron transfer pathway since the difference in redox potentials between the energy source and the anode as terminal electron acceptor is at its highest. At lower anode potentials fermentation or anaerobic respiration becomes the main process due to the lower electrode potential gap between the redox pairs in the bacteria and the anode. A wide variety of bacterial types from aerobic through facultative anaerobes to strict anaerobes can be used (Rabaey and Verstraete, 2005).

If other potential electron acceptors are present in the anode compartment (such as nitrate or sulphate) then the electrons will transfer to them rather than the anode (Rabaey and Verstraete, 2005). This will lower the power output of the cell. Some means must be present for the electrons to be able to pass from the organism to the anode. As can be seen from figure 1.3. the electron transfer from the electron transport chain to oxygen when it is the terminal electron acceptor generally takes place on the cytoplasmic side of the cell membrane. The anode and insoluble transition metals such as iron lie on the outside of the bacterial cell. Electron transfer in these circumstances must take a different route. There are three possible mechanisms which are described in the next section.
1.2.5.3. Electron Transfer Characteristics

1.2.5.3.1. Direct electron transfer

The first method of electron transfer is by direct contact using membrane associated proteins that belong to the electron transfer chain (Kim et al., 2005; Rabaey and Verstraete, 2005). Research interest has centred on bacteria that are capable of reducing metals in the environment. The bacterium Geobacter sulfurreducens was able to reduce iron(III) in aquatic sediments whilst oxidising various organic substrates (Methé et al., 2003; Leang, et al., 2003; Lloyd et al., 2003). A complex of proteins designated FerA was isolated from the outer membrane of this organism which was thought to be involved in electron mediation (Magnuson et al., 2001). A further search of the G. sulfurreducens genome turned up two open reading frames (ORF’s), both described as encoding outer membrane associated c-type polyheme cytochrome proteins sharing a high degree of homology with one another and FerA (Leang, et al., 2003). One of the two proteins (OmcB) was shown using knockout studies to be involved in iron reduction (Leang et al., 2003). Surprisingly despite their similarity the other gene product OmcC is not essential for iron reduction (Leang and Lovley, 2005). Since the isolation of their genes both ferA and omcB are now known to be expressed in other organisms (Millman et al., 2001; Myers and Myers, 2001). In Chlamydia species the OmcB protein does not appear to be expressed on the surface but is present in the periplasm (Millman et al., 2001).

Other related genes omcA and omcF have been isolated, but their products are not thought to be directly involved in reduction of iron (Kim et al., 2005). In Shewanella oneidensis MR-1 it was found that the product of the omcA gene was involved in the reduction of manganese but knockout mutants not expressing this gene could still reduce iron, which suggests other genes are involved (Myers and Myers, 2001). Mutants deficient in the omcF gene were initially found to be unable to grow on iron (III) citrate. The expression of omcB and omcC was removed in these knockout organisms but they recovered due to the expression of another outer membrane cytochrome protein designated OmcS (Kim et al., 2005). It is thought omcF controls
the expression of both \textit{omcB} and \textit{omcC} (Kim \textit{et al.}, 2005).

Interest has also centred on another c-type cytochrome CymA expressed in \textit{Shewanella putrefaciens}. Originally this was thought to be involved directly in the reduction of Mn(IV) and Fe(III). However, it is now thought to be an intermediate in the electron transport pathway (Myers and Myers, 2001). Studies have also suggested a role for another periplasmic protein called PpcA (figure 1.4) in \textit{G. sulfurreducens} which is involved the reduction of U(VI) and humic compounds as well as being an intermediate in the transfer of electrons to iron (III) (Lloyd \textit{et al.}, 2003).

Gorby \textit{et al.} (2006) have been able to show that \textit{S. oneidensis} as well as other types of bacteria produce electrically conductive nanowires in response to growth in oxygen limited medium. Both \textit{omcA} and another gene \textit{mtrC} are required for the organism to be able to express these nanowires which allow the organism to reduce iron (III) to iron (II). It is unclear whether these pili are required for current production in MFC's with mixed findings (Kim \textit{et al.}, 2007; Nevin \textit{et al.}, 2009).

\textbf{Figure 1.4.} Molecular graphic of the 3 dimensional structure of PpcA. Three heme groups involved in its electron transfer function are shown as the mostly grey coloured ball and stick structures, from Pokkuluri, \textit{et al.} (2004).
1.2.5.3.2. Natural mediators

The second means by which electrons can be transferred from NADH to the anode is by the release of naturally occurring soluble mediators (Rabaey and Verstraete, 2005). These pass out of the bacterial cell into the surrounding environment, transferring electrons to a suitable electron acceptor. They are either reversibly reducible and are recycled by the organism or are oxidisable metabolites (Rabaey and Verstraete, 2005). A number of different types have been identified, one being inorganic mediators such as hydrogen or hydrogen sulphide (Katz et al., 2003). Schroder et al. (2004) grew E. coli under anaerobic conditions using a modified platinum impregnated anode coated with polyalanine to catalyse the oxidation of hydrogen gas produced by the organism; in the process transferring electrons to the anode, as well as protecting the platinum from poisoning.

Soluble protein shuttles have been isolated from bacterial media that are capable of transition metal reduction (Seeliger et al., 1998; DiChristina et al., 2001; Mazoch et al., 2004). Other bacteria are capable of using quinones and quinone type compounds to transfer electrons to electron acceptors such as azo dyes (Keck et al., 2002). Certain types of naturally produced antibiotics have been found to be capable of transferring electrons to iron thereby causing reduction (Lies et al., 2005). One class of such naturally occurring compounds are phenazines. These compounds are synthesised by some bacteria including strains of Pseudomonas, Buckholderia and Streptomyces (Mavrodi et al., 1998; Lies et al., 2005). They function as broad spectrum antifungal and antibiotic compounds by their ability to undergo redox reactions with the formation of toxic superoxide radicals but are also capable of reducing iron (Mavrodi et al., 1998). Rabaey et al. (2005) studied the use of phenazines as electron shuttles using a strain of Pseudomonas KRP1 isolated from a microbial fuel cell. This group compared the power output in comparison with E.coli, Enterococcus faecium, Alcaligenes faecalis, Lactobacillus amylovorus and knock out strains of Pseudomonas sp for genes known to be involved in the phenazine synthesis pathways using glucose as a substrate. Phenazines were only produced in Pseudomonas present in microbial fuel cells and the addition of one type (pyocyanin) to knockout strains restored power output to previous
levels. Pyocyanin was recycled up to 11 times (Rabaey et al. 2005). Pyocyanin added to the other strains of bacteria in MFC’s increased the power output except for *E.coli* in which it dropped and *A. faecalis* in which it made no difference (Rabaey et al. 2005). Part of the putative phenazine pathway is shown in figure 1.5 below.

![Pyocyanin Synthesis Pathway](image)

**Figure 1.5.** The proposed pathway for the synthesis of pyocyanin (PCA). The names of the gene products that are part of the metabolic pathway are shown in red at each stage. Redrawn from Mavrodi et al. (1998).

In another study *Geothrix fermentans* was found to produce a soluble but as yet unidentified compound capable of transferring electrons from a variety of organic acids to a graphite anode in a microbial fuel cell (Bond and Lovley, 2005).

1.2.5.3.3. Artificial mediators

A further method of electron transfer from the bacterial cell to the electron acceptor is by the use of artificial mediators, which are typically dyes (Rabaey and Verstraete, 2005). A mediator needs chemical stability in an aqueous bacterial growth medium without being itself metabolised. It also requires a high electrode potential compared to the anode as well as rapid redox reaction times in the organism and at the anode (Katz et al., 2003). Finally the mediator needs to be able to couple to the existing electron transport chain of the organism and to be able to cross the bacterial membrane (Roller et al., 1984; Katz et al., 2003).

Roller et al. (1984) carried out a study on a large range of different redox dyes using a variety of bacteria grown on both nutrient broth and succinate. Roller et al. initially did not measure current output, but rather the rate of dye reduction by the organism
spectrophotometrically expressed as μmol/g dry weight of the organism per second. They predicted that a combination of *Proteus vulgaris* and thionine would yield the highest power output using glucose as a substrate (Roller et al., 1984).

The same group confirmed these findings using a similar combination of bacteria and dyes in a microbial fuel cell obtaining a coulombic yield of 30-60% (Delaney et al., 1984). Allen and Bennetto (1993) investigated the use of thionine in a microbial fuel cell using glucose as a substrate and *P. vulgaris* comparing it to another mediator 2-hydroxy-1,4-naphthoquinone (HNQ). HNQ gave relatively low coulombic efficiencies of electron transfer to the anode (20%). Thionine was found to be less stable but yielded twice the coulombic efficiency of HNQ using glucose as a substrate (Allen and Bennetto 1993). Park and Ziekus (1999) examined the growth of *Actinobacillus succinogenes* on glucose and fumarate in the cathode compartment of an electrochemical cell, using electricity as a source of electrons to enhance the fermentation of glucose and fumarate in this organism. Neutral red was used as the mediator between the electron source and the organism. It was able to replace quinones in the electron transport chain and interact directly in redox reactions with NAD (Park and Ziekus, 1999). Subsequently Park and Ziekus (2000) also examined neutral red, HNQ and thionine as electron mediators in a microbial fuel cell. Although the current yield of 3.5mA was low using neutral red, it was over 10 fold higher than with the other mediators (Park and Ziekus, 2000). Neutral red is chemically stable, non-toxic and is not metabolised by bacterial cells (Park and Ziekus, 2000).

Ieropoulos et al. (2005) compared three types of organisms in three different microbial fuel cell set-ups. The first used *E.coli* and synthetic redox mediators to transfer electrons to the anode, the second used natural mediators in the form of sulphate and sulphide in combination with the organism *Desulfovibrio desulfuricans* and the last cell used *G. sulfurreducens*, which attached to the anode and required no mediator (Ieropoulos et al., 2005). The second type of MFC was most efficient at converting substrate to electricity with a coulombic yield of 64.5%, followed by the third with a yield of 47.4%. The least efficient was the cell using the artificial mediator with a coulombic yield of 28.1% (Ieropoulos et al., 2005).
1.2.5.3.4. Substrates used as sources of electrons

Studies involving microbial fuel cells have focused on a limited number of substrates but a wide variety of organisms (Rabaey and Verstraete, 2005; Du et al., 2007). The most common substrate is glucose, although other carbohydrates have been used (Rabaey and Verstraete, 2005; Du et al., 2007). Chaudhuri and Lovley (2003) were able to convert glucose to carbon dioxide and water in a double chambered glass microbial fuel cell with a Nafion cation exchange membrane separating the anode and cathode compartments. The organism used was *Rhodoferax ferrireducans* which was grown anaerobically on a medium with 2mM glucose as the electron donor. By calculating the number of electrons produced in theory by the glucose and the quantity of glucose consumed in the cell these authors calculated the recovery of electrons as 83% (Chaudhuri and Lovley, 2003).

Niessen et al. (2004) cultured *Clostridium butyricum* or *Clostridium beijerinckii* on starch and molasses or glucose (10 g of each carbohydrate per litre with mineral salts) in anaerobic culture with an anode held at a potential of 200mV. The microbial fuel cell had a modified anode and a Nafion cation exchange membrane separating the anode and cathode. Both types of bacteria produced similar current densities of 1.1 and 1.3mAcm⁻² respectively. Current declined after 5 hours but was rapidly recoverable on replacement of 80% of the medium. The authors utilised *C. beijerinckii* using starch at the same concentration as above in two microbial fuel cells connected in series with the voltage allowed to vary. The maximum power output obtained in this system was 14mW with a current of 30mA and a potential of 473mV.

Other common substrates used have been organic acids such as butyrate, succinate, fumarate, lactate and acetate (Rabaey and Verstraete, 2005; Bond and Lovley, 2005). Acetate was almost fully oxidised to carbon dioxide and water in a microbial fuel cell using *G. sulfurreducens* (Bond and Lovley, 2003). The microbial fuel cell consisted of a glass anode and cathode compartment separated by a Nafion membrane. 5mM acetate was used as the electron donor. In the same way as Chaudhuri and Lovley (2003), Bond and Lovley (2003) calculated the number of electrons harvested as 96.8% and the
microbial fuel cell displayed a maximum output of 0.65mA/cm². Liu et al. (2005) used acetate as a substrate in a microbiological fuel cell using a mixed bacterial consortium of wastewater origin, although this was using a cell with additional electrical input. By additional power input to the cathode they found it was possible to generate hydrogen from the protons and electrons produced by the bacteria. They calculated the coulombic efficiency of the recovery of electrons from the acetate as 60-78% depending on the voltage applied to the cathode (Liu et al., 2005).

Habermann et al. (1991) used a wide variety of substrates including wastewater, starch, cane sugar and crude oil as well as glucose and fructose to grow P. vulgaris, E.coli, Pseudomonas aeruginosa, D. desulfuricans and Pseudomonas fluorescens in a microbial fuel cell. The microbial fuel cell was very stable over a period of years producing up to 50mAcm⁻² using sugar waste. Kim et al. (2007) used alcohols in two different microbial fuel cell configurations using mixed bacterial consortia generating a maximum power density of 40mW/m² and a coulombic efficiency in the range from 42% to 61% using ethanol. Methanol did not result in significant electricity generation (Kim et al., 2007). One group have also achieved microbial growth and current production on cysteine (Logan et al., 2005). Power output was obtained using a mixed bacterial consortium isolated from the seabed for which the 16S rRNA analysis showed the majority of the organisms to be related to Shewanella species (Logan et al., 2004). The power output at 19mW/m² was comparable with microbial fuel cell systems using lactate as a fuel (Logan et al., 2005). However, glucose has produced the highest output of any microbial fuel cell so far at 3.6W/m² (Rabaey et al., 2003).

1.2.5.3.5. Anode types

The vast majority of anodes have been made of carbon, mainly in the form of graphite (Rabaey and Verstraete, 2005) although other types have been tested, mainly involving modified platinum (Schröder et al., 2003; Niessen et al., 2005). The disadvantage of platinum is that it is susceptible to poisoning (Schröder et al., 2003). Habermann et al. (1991) developed a porous graphite anode which was impregnated with small amounts of iron, nickel and cobalt hydroxide. As discussed above this
system produced current densities of approximately 50mA/cm² (Habermann et al., 1991). Impregnation of the anode with manganese may also increase the power output (Chaudhuri and Lovley, 2003). Enlarging the anode raises the efficiency of a chemical fuel cell system, this is also true of microbial fuel cells (Rabaey and Verstraete, 2005; Chaudhuri and Lovley, 2003; Kim et al., 2002).

1.2.5.3.6. Cathode types

Cathode systems have largely been based around ferricyanide chemistry, although the cathode itself has, like the anodes, been platinum or graphite in the main (Logan and Regan 2006; Kim et al., 2007). Logan et al. (2005) used the same mineral salts medium used as the cathode electrolyte to grow the bacteria. Habermann et al. (1991) modified the cathode itself by impregnating it with iron (II) phthalocyanin and vanadium (V) compounds which allowed operation for 2 years. The oxygen electrode is considered preferable to the use of ferricyanide which is regarded as a mediator and is unstable (Logan and Regan 2006; Ieropoulos et al., 2005). Rhoads et al. (2005) operated a microbial fuel cell using Klebsiella pneumoniae grown on glucose in the anode compartment and another bacterium Leptothrix discophora cultured on bacterial culture medium in the cathode compartment which both deposited and recycled manganese as MnO₂ which had been reduced to Mn³⁺ by electrons at the cathode. The average power density was 126.7mW/m² and the cell was able to run continuously for 500 hours (Rhoads et al., 2005)

Liu et al. (2004) manufactured a hollow air cathode which ran through the centre of their cylindrical microbial fuel cell as a tube open to the outside air at its ends. In this type of arrangement as with a PEM fuel cell, the cathode is directly coupled to the permeable membrane allowing oxygen to react in situ at the electrode raising its efficiency (Liu and Logan, 2004; Liu et al., 2004) This type of technology is used in conventional PEM fuel cells (Bultel et al., 2005). Running the cell first on wastewater and only using the bacteria naturally present in the wastewater they obtained a current density of 26mW/m². Passive air exchange gave a higher output than pumping air through the cathode tube (Liu et al., 2004). Rabaey et al. (2005) used a cation exchange
membrane to contain the fuel cell in a cylinder. The cathode consisted of a woven graphite mat wrapped around this through which potassium ferricyanide solution was pumped in a circuit from a reservoir. The anode was made up of graphite granules of diameters of 1.5mm and 5mm connected to the external circuit via a graphite rod contained within the cylinder. Bacteria from an active MFC was used as the inoculum and the system was fed with fuel in the from of acetate/glucose or wastewater through an entry port in the bottom of the cylindrical fuel cell system. The substrate was pumped in through the lower part of the cell and passed out the top at a rate of 720ml/day for up to 60 days. The wastewater gave higher coulombic efficiencies than either glucose or acetate being 96%, 59% and 75% respectively while acetate gave the highest average power output at 52W/m² and wastewater the lowest at 8W/m² (Rabaey et al., 2005).

Liu and Logan (2004) used a different design of single chamber system with the air cathode forming one end of the cylindrical MFC. Again using wastewater they obtained a power density of 146mW/m² which increased to 494 mW/m² with glucose. This type of cathode system also has the advantage of allowing continuous operation whilst ferricyanide would need periodic replacement (Cheng et al., 2005). Cheng et al. (2005) investigated the modification of the cathode; firstly by altering the concentration of platinum in the carbon present, secondly comparing Nafion and PTFE for use as binders for cathode material and thirdly trying cobalttetramethylporphyrin instead of platinum. Nafion worked better than PTFE as a binder in combination with 0.5 mg/cm² platinum and gave a higher power output than cobalttetramethylporphyrin (CoTMPP) based cathodes. However, CoTMPP cathodes yielded a higher power output than carbon alone or the combination of PTFE/0.5 mg/cm² platinum or lower concentrations of platinum with Nafion binder (Cheng et al., 2005).

1.2.5.3.7. The semi-permeable membrane

The semi-permeable membrane used in almost all studies is Nafion, a polymer composed of polyfluoroethane with sulphonic acid groups covalently bound. This membrane is found in PEM and some other chemical fuel cells described above
(Dupont product information, 2002). A number of groups have examined microbial fuel cell systems that have not used a semi-permeable membrane. Jang et al. (2004) used a system in which the cathode was placed on top of the anode. The two compartments were separated by glass wool and beads. The current, although low, was maintained for over a year during continuous operation using enriched sludge as the bacterial source and artificial wastewater containing glutamate and glucose (Jang et al., 2004). Liu and Logan (2004) in their air cathode based system also tried comparing the same MFC with and without a PEM they found higher power outputs without the PEM but lower coulombic efficiency (Liu and Logan, 2004). Oxygen at the cathode is regarded as a disadvantage (Liu et al., 2005; Liu and Logan, 2004). Although the semi-permeable membrane is in theory not permeable to oxygen, in practice some is able to crossover to the anode. This will lower the output of the cell either by becoming the terminal electron acceptor or by disrupting the growth of anaerobic organisms (Gil et al., 2003; Liu et al., 2005; Rabaey and Verstraete, 2005).

1.2.5.3.8. Cell efficiency

Useful work done is defined as Gibbs free energy ($\Delta G$), whose sign is a measure of whether spontaneous change is possible. The thermal energy is defined as the enthalpy ($\Delta H$) or heat of the process (Warn, 1982). The efficiency of a fuel cell is therefore:

$$= \frac{\Delta G}{\Delta H} \quad \text{(Equation 1.1)}$$

(Williams, 2004).

Calculating both the free energy and the enthalpy using thermodynamic tables for the PEM reaction using oxygen and hydrogen gives an absolute maximum efficiency of 237.1/285.8 = 83% (Williams, 2004). Not all the free energy is available as electrical power (Williams, 2004). The remainder of the energy is lost as heat to the cell’s environment. Calculating the efficiency and electrode potential of a microbial fuel cell is more difficult since it depends on the overall efficiency of the biological process taking place. One method of calculating the efficiency is by calculating coulombic
yield. Taking glucose as an example; its oxidation yields 24 electrons per molecule.

\[ C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \]

Taking a coulomb (charge in amps x time in seconds) equal to 96500 for 1 mole of electrons this would be 96500 x 24 for 1 mole of glucose or 2.316C/μmol of glucose. Chaudhuri and Lovley (2003) measured the output of their cell and found it produced an actual output of 742C from the consumption of 389μmol glucose. The theoretical output should be 2.316 x 389 or 900C. This gave an efficiency of 742/900 or 83% (Chaudhuri and Lovley 2003). Knowing the number of electrons involved and the electrode potential of a cell it should be possible to calculate the free energy of the cell’s reaction. The maximum power output for this experiment is not given.

1.2.5.3.6.9. Electrode potential

An MFC will only produce electricity if the overall reaction is thermodynamically favourable (Logan et al., 2006). The maximum useful work done can be defined as;

\[ \Delta G_r = \Delta G^0_r + RT \ln \Pi \]  \hspace{1cm} (Equation 1.2)

where \( G_r \) is the Gibbs free energy for the specific conditions, \( G^0_r \) is the Gibbs free energy (both in Joules) under standard conditions (1 bar atmospheric pressure, 298.15K temperature and 1M concentration of all species). \( R \) is the universal gas constant (8.31447 J mol\(^{-1}\) K\(^{-1}\)), \( T \) is the temperature in Kelvins (K) and \( \Pi \) is the ratio of activities of the products of the reaction divided by the reactants, it has no units. The work done in an MFC can be defined as;

\[ W = E_{\text{emf}}.Q = -\Delta G_r \]  \hspace{1cm} (Equation 1.3)

Where \( Q = n.F \) with \( Q \) as the charge transfer in the reaction in Coloumb (C), \( n \) the number of electrons transferred per reaction mole and \( F \) is Faraday’s constant (9.64853 x 10\(^4\) C/mol) (Logan et al., 2006). Therefore;
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\[ E_{\text{emf}} = -\Delta G_t / nF \]  
\[ \text{(Equation 1.4)} \]

expressed under standard conditions with \( \Pi = 1 \) as;

\[ E_{\text{emf}}^0 = -\Delta G_t^0 / nF \]  
\[ \text{(Equation 1.5)} \]

Substituting 1.4 and 1.5 into 1.2 gives the answer in volts;

\[ E_{\text{emf}} = E_{\text{emf}}^0 - (RT/nF)\ln \Pi \]  
\[ \text{(Equation 1.6)} \]

The total cell potential can be considered as combination of the anode and cathode potentials. The minus sign is because the anode potential is considered a reduction (Logan et al., 2006).

\[ E_{\text{emf}} = E_{\text{cat}} - E_{\text{anode}} \]  
\[ \text{(Equation 1.7)} \]

Equation 1.6 (the Nernst equation) can be used to calculate half reactions for both the anode and cathode. So for acetate being oxidised at the anode the reaction is;

\[ 2\text{HCO}_3^- + 9\text{H}^+ + 8e^- \rightarrow \text{CH}_3\text{COO}^- + 4\text{H}_2\text{O} \]

Giving;

\[ E_{\text{anode}} = E_{\text{anode}}^0 - (RT/8F)\ln([\text{CH}_3\text{COO}^-]/[\text{HCO}_3^-][\text{H}^+]^9) \]  
\[ \text{(Equation 1.8)} \]

Similarly for the cathode if oxygen is the terminal electron acceptor

\[ \text{O}_2 + 4\text{H}^+ + 4 \text{e}^- \rightarrow 2\text{H}_2\text{O} \]

\[ E_{\text{cat}} = E_{\text{cat}}^0 - (RT/4F)\ln([1]/[p\text{O}_2][\text{H}^+]^4) \]  
\[ \text{(Equation 1.9)} \]

Using equations 1.8 and 1.9 an overall theoretical MFC cell voltage can be calculated.
So for example an MFC with an acetate oxidizing anode ($\text{HCO}_3^-$) with the acetate concentration at 5 mM, $\text{CH}_3\text{COO}^-$ at 5 mM, and an oxygen reducing cathode ($p\text{O}_2$) 0.2, all at pH7; has a cell emf of $0.805 - (0.296) = 1.101 \text{ V}$ (Logan et al., 2006). Positive potentials are energetically favourable.

There is one other issue to be considered, that is the redox balance in the MFC. Whilst the above can give an ideal MFC potential, the biochemistry is more complicated in that the transfer of electrons to the respiratory chain is not via acetate but by NADH. The overall reaction for reduction of NAD is:

$$\text{NAD} + 2\text{H}^+ + 2e^- \rightarrow \text{NADH} + \text{H}^+$$

Hence the electrode potential for the half reaction for NAD/NADH under standard conditions with equimolar concentrations of NAD/NADH at pH 7 and $E^{\circ}'' = -0.32\text{V}$ for NADH/NAD is;

$$E^{\circ}'' = E^{\circ} - \frac{RT}{nF}\ln([\text{NAD}]/[\text{NADH}][\text{H}^+]^2) = E^{\circ} - \frac{RT}{nF}\ln([1]/[1'][10^{-7}]^2) = 0.094\text{V}$$

Using the example above for the half reaction for acetate at the anode; $-0.320 - -0.296 = -0.024\text{V}$ This is not thermodynamically favourable. However if the ratio of NADH/NAD is altered to a ratio of $1/10$ using the above relationship;

$$E^{\circ}'' = 0.094\text{V} - \frac{RT}{2F}\ln([0.1]/[1][10^{-7}]^2) = -0.290\text{V}$$

So $-0.290\text{V} - (-0.296\text{V}) = 0.006\text{V}$

This is energetically favourable. Thus the bacteria or bacterium will attempt to balance the ratios of NADH/NAD in an MFC (Logan, 2008).

1.2.5.3.10. Thermodynamics and efficiency of fuel cells

Fuel cells are usually described as being more efficient than other mechanical
energy conversion devices (commonly called heat engines) due to the fact that the energy conversion process is electrochemical and not mechanical. In a conventional heat engine used to generate electricity (for example a diesel generator) the chemical energy of the fuel is converted to heat which then drives a mechanical process to produce electrical energy. Energy is lost with the heat transferred from the engine system not being able to do useful mechanical work and also in a variety of other ways including sound, friction and internal electrical resistance. A fuel cell is not subject to most of these limitations, being a purely chemical process. This in theory should give higher efficiencies than a heat engine. In practice in terms of electrical conversion most chemical fuel cells operate at efficiencies of less than 50%, which is in roughly the same order of magnitude as the maximum theoretical output of a heat engine. Whilst the equations 1.6 and 1.7 above can be used to calculate a theoretical MFC voltage in practice the measured MFC voltage even under open circuit conditions will be lower (Logan et al., 2006). The difference between the two is known as the overvoltage and is the sum of the overpotential (η) at the electrodes and the ohmic losses in the system (R_Ω) multiplied by the current generated (I) (equation 1.10) (Logan et al., 2006).

\[ E_{\text{cell}} = E_{\text{emf}} - (\Sigma \eta_s + \Sigma \eta_c + IR_\Omega) \]

(Equation 1.10)

Inefficiencies in a conventional chemical fuel cell are caused by ions and oxygen crossing between the anode and cathode compartments, slow reaction kinetic effects at the anode and cathode and mass diffusion (Haile, 2003; Bultel et al., 2005). The overpotentials in an MFC include these losses (Logan et al., 2006; Logan, 2008). The measured voltage in MFCs is usually in a linear relationship to the current (equation 1.11). The OCV term includes the overpotential losses above while R_int is the electrical resistance of the system (Logan et al., 2006; Logan, 2008).

\[ E_{\text{cell}} = \text{OCV} - R_{\text{int}} \]

(Equation 1.11)

In practice whilst microbial fuel cells produce a much lower current density than chemical fuel cells, they appear in many cases to be much more efficient in transferring electrons from the fuel to the anode.
1.3. **Thesis aims**

The first aim was to investigate a number of strains of solvent tolerant bacteria to examine their effectiveness at breaking down a number of VOCs and glycerol at a high concentration of these compounds to carbon dioxide and water. Second, if this biodegradation could be carried out in a microbial fuel cell some electricity could be produced as part of the disposal process. Direct transfer of electrons is the most effective means of transfer to the anode in an MFC and this mechanism relies on a series of proteins embedded in the outer cell membrane and periplasm of some bacteria. In nature these enzymes catalyse the reduction of a variety of transition metals, although not all these metals can act as a terminal electron acceptor under anaerobic conditions. Thus study of transition metal reduction in these bacteria was the third area of interest in this project. Finally to attempt to elucidate the pathways used by the bacteria for metal reduction or electron transfer to an anode some tools of molecular biology and proteomics were used. Glycerol was added to the project due to interest at the time in its disposal because of a glut caused by biodiesel production. None of the bacteria had been trialled in MFC’s before or exposed to transition metals used in this project.
Chapter 2. Initial growth studies and media adaptation

2.1. Introduction

The bacteria used in this project were as follows; *Paracoccus denitrificans* (DSM 413), *Polarmonas naphthalenivorans* (DSM 15660), *Dechloromonas denitrificans* (DSM 15892), *Burkholderia xenovorans* (DSM 17367), *Shewanella oneidensis* MR-1, *Shewanella putrefaciens* 200 (NCIB 12577), *Rhodococcus opacus* (DSM 43250), *Rhodococcus ruber* (DSM 7511) and *Rhodococcus rhodochrous* (DSM 6263).

As was outlined in the introduction, *Rhodococcus* species are capable of tolerating and degrading a wide range of VOC’s and other compounds (Schenkels and Duine, 2000; van der Geize and Dijkhuizen, 2004; De Carvalho and da Fonseca, 2005; Larkin et al., 2005; de Carvalho et al., 2009). The genus has had taxonomic deletions and additions since the genus name was revived in 1977 (Bell et al., 1998). Bell et al. (1998) define rhodococci as “aerobic, Gram-positive, non-motile mycolate-containing nocardioform actinomycetes”. Although some of the rhodococci species can cause disease, the main interest has been in the area of environmental biotechnology (Bell et al., 1998; Larkin et al., 2005; De Carvalho and da Fonseca, 2005). As outlined in the bacterial definition rhodococci have cells walls rich in mycolic acid (Bendinger et al., 1993; Bell et al., 1998). This family of compounds are α-branched, β-hydroxylated fatty acids synthetized by the *Corynebacterium-Mycobacterium-Nocardia* group (Bendinger et al., 1993; Sokolovska´ et al., 2003). Rhodococci have mycolic acids containing 34 to 52 carbon atoms (Arenskötter et al., 2004). This is thought to render the cell surface hydrophobic making them more resistant to chemicals, antibiotics and dehydration (Sokolovska´ et al., 2003). Opinion is divided on whether the presence of hydrophobic solvents increases the hydrophobic nature of the cell surface. Nalli et al., (2006) cultured *R. rhodochrous* on hexadecane, 2-ethylhexanol, 2-ethylhexanal, butyric acid, hexanoic acid, octanoic acid, 2-methylhexanoic acid, adipic acid and 2-ethylhexanoic acid with and without di-ethylhexyl phthalate (DEHP) whilst measuring the level of its ultimate breakdown product 2-ethylhexanoic acid (Nalli et al., 2006). When hexadecane was used as the carbon source significant quantities of the 2-ethylhexanoic acid end product were present (Nalli et al., 2006). Nalli et al. (2006)
maintain this was because the hexadecane grown cells became more hydrophobic and could make better contact with the DEHP. As evidence for this they point to floc formation in the hexadecane grown cultures containing DEHP (Nalli et al., 2006). By contrast Sokolovská et al., (2003) found no difference in hydrophobicity using the microbial adhesion to hydrocarbon (MATH) test on Rhodococcus erythropolis cultured on either organic acids or alkanes (Rosenberg et al., 1980; Sokolovská et al., 2003). They did find, however, differences in sensitivity to hydrophobic and hydrophilic dyes and antibiotics, with cells grown on hydrophobic reagents more susceptible to hydrophobic dyes and antibiotics and vice versa (Sokolovská et al., 2003). de Carvalho et al. (2004) observed clump formation in R. erythropolis grown on organic solvents. Rhodococci have been isolated from soil, rocks, groundwater, insect guts, marine sediments, animals and plants (Bell et al., 1998).

D. denitrificans was isolated from the gut of the earthworm Aporrectodea caliginosa found in a garden in Bayreuth, Germany (Horn et al., 2005). D. denitrificans is in the β-proteobacteria class. One of its closest phylogenetic relatives is D. agitata a facultative aerobe with which it shares 97% 16S rRNA gene sequence identity (Horn et al., 2005). D. denitrificans is a Gram-negative motile rod shaped bacterium (Horn et al., 2005).

P. denitrificans is a bacterium usually found in soil or sewage sludge (Van Spanning et al., 2000). It is capable of utilising a range of compounds including alcohols as electron donors or acceptors (Van Spanning et al., 2000).

S. oneidensis was isolated from sediment at the bottom of Lake Oneida, NY, USA (Venkateswaran et al., 1999). It is a Gram-negative facultative anaerobe (Venkateswaran et al., 1999). The range of substrates which it is known to grow on is relatively small (Serres and Riley, 2006). S. oneidensis prefers molecules containing three carbon atoms or less, although it is unable to grow on glycerol (Serres and Riley, 2006).

The bacteria used in this project fall into three distinct groups and the rationale for
the choice of bacteria in each group chosen is different. The first group of bacteria chosen were known to be able to degrade a wide variety of organic substrates (*Burkholderia xenovorans, Rhodococcus rhodochrous, Rhodococcus ruber, Rhodococcus opacus*), but had not as far as could be gathered from the scientific literature been trialled in microbial fuel cells. The second group chosen (*Paracoccus denitrificans, Polarmonas napthalenivorans* and *Dechloromonas denitrificans*) were largely unknown with respect to their abilities to degrade organic substrates and whether they were capable of direct electron transfer to the anode in a microbial fuel cell. There is little literature on these bacteria, but *D. denitrificans* is known to express c-type cytochromes and *P. denitrificans* is able to reduce transition metals (Horn et al., 2005; Mazoch et al., 2004). The last group consisted of *Shewanella oneidensis* MR-1 and *Shewanella putrefaciens* 200. These were chosen as positive controls as bacteria that are known to work in microbial fuel cells.

The aim of the work described in this chapter was to gather information on the basic growth characteristics of the bacteria chosen for this project and adapt them to growth on a minimal salts medium. Some of the adaptation to the minimal salts medium will be described in this chapter, but many of the growth studies were carried out as part of the work described in later chapters. Growth characteristics such as generation time and growth rate are shown in this chapter for bacteria that were used throughout the project, although some others where these characteristics were not established are referred to in later chapters. The reasons why some of the bacteria were not used are described in context in later chapters.

Since all the recommended media (described below) for the bacterial species chosen for this project contained a carbon source and the aim was to grow them on a sole defined carbon source, the bacteria had to be adapted to growth on a minimal salts medium. The media chosen for trials were Brunner's medium (DSMZ medium 457) and medium B (Gerhardt et al, 1981). All the growth studies on medium B were carried out as part of growth studies covered in subsequent chapters.

A number of mathematical models have been developed to describe the growth
cycle of bacteria where the growth cycle consists of a lag phase, exponential phase and maximum cell number realised before the stationary phase (Zwietering et al., 1991; Gibson et al., 1987).

Gibson et al. (1987) describe two models, a modification of the Gompertz curve (equation 2.1) and another logistic (equation 2.2) to describe the growth of *Clostridium botulinum* in pork slurry. These four parameter curves do not directly give length of the lag phase, magnitude of the exponential phase or maximum biomass achieved before the stationary phase, these must be calculated from the four parameters by the formulae described in the equations below. A plot is made of the log_{10} of the population against time.

\[
y = A + C \cdot \exp(-\exp(-B(t-M))) \quad \text{equation} \quad (2.1)
\]

\[
y = A + \frac{C}{1 + \exp(-B(t-M))} \quad \text{equation} \quad (2.2)
\]

The meaning of parameters for both curves is as follows (Gibson et al., 1987):

A The value of the cell count at time equals zero (asymptote at -infinity).
B The relative growth rate at M time.
C The increase in cell count between - infinity and + infinity.
M Time of the maximal growth rate.

Zwietering et al. (1990) compared the growth of *Lactobacillus plantarum* using a number of sigmoidal functions which were modified to ensure the parameters were biologically relevant. These authors developed a modified form of the Gompertz equation shown below in equation 2.3 in which the three parameters of the model represent the lag phase (**λ**), maximum growth rate (**μₐ**), and maximal biomass (**A**) and found it to give the most statistically significant results of a number of different models.
\[ y = A \cdot \exp\left(-\exp\left(\frac{\mu_m}{A} \cdot \left| t - \lambda \right| + 1\right) \right) \quad equation \quad (2.3) \]

A plot is made of the natural log of the relative population against time.

As part of the initial studies of the different bacteria chosen for this project a statistical comparison was made of the regression curves versus the established method of calculating the maximum growth rate \((\mu_m)\), which is to plot the growth data on a log scale against time and calculate the gradient of the straight-line portion.
2.2. Materials and methods

2.2.1. Chemicals

Bacteriological agar was purchased from Difco (UK). Unless otherwise stated, all other chemicals were of the highest purity available and purchased from either Sigma Chemical Company (UK) Fisher (UK), or BDH Laboratory Suppliers (UK).

2.2.2. Bacteria and growth media

Bacteria with the exception of *Rhodococcus opacus*, *Shewanella oneidensis* MR-1, and *Shewanella putrefaciens* 200 were purchased from DSMZ (the German National Resource Centre for Biological Material) and on arrival were stored at +4°C prior to use. Initially bacteria were grown in the media recommended by DSMZ or NICMB, described as follows.

*Shewanella oneidensis* MR-1 and *Shewanella putrefaciens* 200 (both purchased from NICMB) were initially grown in tyrptone soy broth (TSB). This was prepared by dissolving 30g of TSB in 1l of deionised water followed by autoclaving.

*Buckholderia xenovorans* (DSM 17367) and *Rhodococcus rhodochrous* (DSM 6263) were grown in medium 1 which consisted of 5g of peptone and 3g of microbiological meat extract (both supplied by Merck (UK)) per litre at a pH of 7.0.

*Rhodococcus opacus* was donated and therefore its recommended medium was unknown but it was cultured in medium B.

*Paracoccus denitrificans* (DSM 413) was grown on medium 81 which contained the following constituents per litre of deionised water at a pH of 6.8; 2.3g KH₂PO₄, 2.9g Na₂HPO₄·2H₂O, 1.0g NH₄Cl (Acros (UK)), 0.50g MgSO₄·7H₂O, 0.50g NaHCO₃, 0.01g CaCl₂·2H₂O and 5ml of trace element solution SL-6, in addition 0.05g of ferric ammonium citrate was dissolved in 20ml of deionised water and added after being autoclaved separately. The carbon source was 2g of glucose per litre.
SL-6 contained the following constituents; 0.1g of ZnSO$_4$,7H$_2$O, 0.03g of MnCl$_2$,4H$_2$O, 0.3g of H$_3$BO$_3$, 0.2g of CoCl$_2$,6H$_2$O, 0.01g of CuCl$_2$,2H$_2$O, 0.02g NiCl$_2$,6H$_2$O, 0.03g of Na$_2$MoO$_4$,2H$_2$O per litre of deionised water.

*Polaromonas naphthalenivorans* (DSM 15660) and *Dechloromonas denitrificans* (DSM 15892) were grown on medium 830 which consisted of the following components per litre of deionised water; 0.5g of yeast extract, 0.5g of peptone (both supplied by Merck (UK)), 0.5g of casamino acids, 0.5g of glucose, 0.5g of corn starch, 0.3g of sodium pyruvate, 0.3g of K$_2$HPO$_4$ and 0.05g of MgSO$_4$,7H$_2$O. The pH of the medium was adjusted to 7.2 using KH$_2$PO$_4$.

Brunner medium (medium 457) consisted of the following constituents per litre of deionised water; 2.44g of Na$_2$HPO$_4$, 1.52g of KH$_2$PO$_4$, 0.5g of (NH$_4$)$_2$SO$_4$, 0.2g of MgSO$_4$,7H$_2$O and 0.05g of CaCl$_2$,2H$_2$O and 10ml of SL-4 trace element solution all at a pH of 6.9. The carbon source was 2g of glucose per litre. SL-4 trace element solution contained 0.5g of EDTA and 0.2g of FeSO$_4$,7H$_2$O and 100ml of SL-6 per litre of deionised water.

Medium B contained the following constituents per litre of deionised water at pH 7; 0.8g of K$_2$HPO$_4$, 0.2g of KH$_2$PO$_4$, 0.05g of CaSO$_4$,2H$_2$O, 0.5g of MgSO$_4$,7H$_2$O, 0.01g of FeSO$_4$,7H$_2$O and 1g of (NH$_4$)$_2$SO$_4$. In addition the medium was supplemented with 10ml of SL-6 and 2g of glucose as the carbon source. In the case of *Shewanella oneidensis* the carbon source was approximately 22mM lactate.

Agar was added at 15g/l of any of the above media. Autoclaving was carried out at 121°C for 15 minutes for some early studies but this was later reduced to 110°C for 10 minutes.
2.2.3. Enrichment and maintenance of bacterial strains

All bacterial types were aseptically inoculated into their recommended cultures as follows. All the bacteria were supplied as lyophilised solids in sealed glass vials except the *Dechloromonas denitrificans* which was supplied on agar and *Rhodococcus opacus* on a glucose plate. To the lyophilised cultures 1 ml of sterile deionised water was added after the vial seal was broken. After re-suspension 1ml of their recommended medium was added to each of the bacterial types. After mixing 1ml was withdrawn and added to the recommended liquid culture (100ml in a 250-ml Erlenmeyer flask). The remainder of the liquid in the vials was used to inoculate streak plates of the same medium. *Dechloromonas denitrificans* was inoculated into liquid culture by using a microbiological loop. It was added to the agar medium in the same way. The liquid cultures were allowed to grow at 20°C on a KS250 orbital shaker (IKA Werke, Germany) or a Stuart orbital shaker (Stuart, UK) at 150rpm initially and then 160rpm on both unless otherwise stated. No studies were undertaken on the bacteria until more than six generations had passed in any medium. Slope cultures were made after six generations for each culture on its recommended medium and these were stored at +4°C.

2.2.4. Microbial growth measurements

Samples were withdrawn periodically for analysis. Cell growth was monitored spectrophotometrically by measuring the optical density in a 1 cm light path plastic cuvette (Fisher Scientific, UK) at a wavelength of 600nm (OD<sub>600</sub>) using a Lightwave spectrophotometer (Lightwave, Biochrom Ltd, Cambridge UK), and the corresponding total cell number was determined using a haemocytometer slide 0.1mm x 0.0025mm (Superior, Germany) and an Olympus CH-2 microscope.

An optical density reading of a culture sample was taken at 600nm immediately before the count. A coverslip was pushed onto the haemocytometer so that “Newton’s rings” could be seen. If necessary a dilution of the bacteria was made in deionised water but for motile bacteria a 10% or 20% solution of 70% alcohol in deionised water was used. The culture was added to both sides of the haemocytometer and a count was
made of the 50μm squares in the central 25 squares on each side. The average count per
50μm square was calculated and this number was multiplied by 4 x 10^6 to take into
account the volume counted. In addition this number was multiplied by any dilution
factor. The totals for the two sides were averaged. Where possible at least 200 bacteria
were counted per side.

A calibration curve between optical density (OD_{600}) and cell number was
established, yielding the relationships shown in table 2.2. below.

2.2.5. Cell growth studies

For each bacterial type (except *Shewanella oneidensis* and *Rhodococcus opacus*)
four aliquots, each of 100ml of the recommended culture medium for the particular
organism, were made in 250-ml Erlenmeyer flasks. *S. oneidensis* and *R. opacus* growth
studies were carried out in duplicate flasks as part of larger studies. Three were
inoculated with 1ml of identical concentrations of bacterial culture and the remaining
flask was kept sterile as a control whose optical density at 600nm was subtracted from
the average of the other three. The optical density was measured on a Lightwave
spectrophotometer (Biocrom, Cambridge, UK) so that at least three points were
obtained in the lag, exponential and stationary phases. For each growth study one
representative flask was chosen at random to ensure that no contamination existed and
that a monoculture was present. This was carried out using visual examination under
the Olympus CH-2 microscope. Pictures throughout this project were taken using
Canon ixus 70, HP photosmart 735 and Sony DSC-S730 digital cameras.

To calculate $\mu_m$ (the maximum growth rate), $\lambda$ (the lag phase) and $A$ (the maximum
biomass) for the three parameter growth curves, the OD_{600} for each point was divided by
the OD_{600} at time zero. The natural log of this ratio was plotted against time.
Regression curves were plotted (see below) to fit the model. The maximum biomass $A$
was then calculated by back calculating from the natural log by taking the exponential
value of $A$ and multiplying it by the OD_{600} at time zero. This OD_{600} value was then
converted to a cell count using the relationship between the cell count using the
haemocytometer and the turbidity.
The 4 parameter curves described above (Gompertz and logistic) were fitted as custom models using the parameters A, B, C and M. Cell counts converted using the relationship established between haemocytometer cell count and OD$_{600}$ were converted to log$_{10}$ and plotted against time. Using the parameters the maximum growth rate, the lag phase and the maximum biomass were calculated as follows.

For the logistic curve.

\[
\begin{align*}
\text{The maximum growth rate} & = B \times C/4. \\
\text{Lag phase time} & = M-2/B \\
\text{generation time} & = 96 \times \log_{10}(2)/B \times C \\
\text{Maximum cell number} & = A+C
\end{align*}
\]

For the Gompertz curve.

\[
\begin{align*}
\text{The maximum growth rate} & = B \times C/2.718. \\
\text{Lag phase time} & = M-1/B \\
\text{generation time} & = 96 \times \log_{10}(2) \times 2.718/B \times C \\
\text{Maximum cell number} & = A+C
\end{align*}
\]

Units are per hour.

2.2.6. Data analysis and statistics

Where appropriate data were checked for normality in minitab version 11, SPSS v.9 and Rplot v1.4 using the Anderson-Darling test. Results were identical by both pieces of software, so Rplot was used. If the data were normally distributed an F-test was undertaken. On the basis of the result the appropriate homoscedastic or heteroscedastic t-tests were chosen. F-test and t-tests were carried out in openoffice calc. Mann-Whitney U tests were carried out in Rplot v1.4. Paired t-tests were not used.
2.3. Results

2.3.1. Bacterial morphology

The appearance and morphology of the bacteria are shown in table 2.1. An image of *D. denitrificans* growing in medium B is shown in figure 1.2.

Table 2.1. Description of the growth characteristics of the cell types used in this project.

<table>
<thead>
<tr>
<th>Name of bacteria</th>
<th>Description</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. denitrificans</em></td>
<td>White colonies on agar</td>
<td>Small cocci, form large clumps as culture life extends. Culture medium changes from white to grey with age in medium B.</td>
</tr>
<tr>
<td><em>R. rhodochrous</em></td>
<td>Bright red/brown colonies. Colonies or liquid cultures coloured (usually red).</td>
<td>Long rods can be clustered or separate, also seen as a “V” shape with two or more cells forming the “V”.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Colonies colourless on glucose coloured on other substrates in both liquid and solid cultures.</td>
<td>Long rods can be clustered or separate or form strings of cells, also seen as a “V” as above. In liquid culture often coloured, this depends on the carbon source.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>Shiny yellowish green colonies</td>
<td>Motile rods, as they get longer with multiple cells joined together they tend to lose motility.</td>
</tr>
<tr>
<td><em>S. oneidensis</em></td>
<td>Brown in culture and on medium B lactate plates</td>
<td>Motile rods. In liquid culture starts grey changes to brown. When unstirred changes to salmon pink restored to brown immediately on stirring. Brown colonies on plates.</td>
</tr>
<tr>
<td><em>B. xenovorans</em></td>
<td>Yellow-green colonies</td>
<td>Small motile rods.</td>
</tr>
<tr>
<td><em>P. naphthalenivorans</em></td>
<td>White colonies</td>
<td>Long white clustered rods</td>
</tr>
</tbody>
</table>
Figure 2.1. *D. denitrificans* photographed in minimal salts medium.

2.3.2. Cell counts

Satisfactory relationships were established between cell count using a haemocytometer and the turbidity measured at 600nm for four of the bacteria under investigation in this project. A straight line relationship was established between the cell count and turbidity. The regression fit was used to calculate cell counts. The results are shown in table 2.2. below and the regression graphs in the appendix (figure 1).

Table 2.2. The correlation between OD at 600nm and cell count using a haemocytometer.

<table>
<thead>
<tr>
<th>bacteria</th>
<th>Number of observations</th>
<th>Pearson correlation (R)</th>
<th>Significance (p) of correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. denitrificans</em></td>
<td>7</td>
<td>0.986</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>R. rhodochrous</em></td>
<td>10</td>
<td>0.955</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>11</td>
<td>0.968</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><em>S. oneidensis</em></td>
<td>7</td>
<td>0.972</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

2.3.3. Minimal media adaptation

*P. naphthalenivorans* was found to grow poorly on all media tried. It was capable of growth on medium 830 plates, but not in liquid culture. All bacterial types grew on
Brunner medium except *P. naphthalenivorans*, *S. oneidensis* (untested) and *R. opacus* (untested), however in this medium it was difficult to get all the salts to dissolve. *B. xenovorans* was unable to adapt to medium B.

The recommended temperature for *S. oneidensis* was 30°C and the organism had to adapt to growth at 19-20°C. It was also adapted from tryptic soy broth to growth on medium B with lactic acid as the carbon source. Although no turbidity readings at 600nm were taken, by eye the growth rate at first was very slow, taking up to ten days before the turbidity increased enough to require subculture. At generation six growth appeared still very slow so it was decided to delay growth studies. At generation eight growth stalled and very little change in turbidity was seen. The generation eight flask was placed at 23°C in a stirrer incubator and by eye subsequent growth was stronger. Cultures after this were returned to growth at room temperature. From this point forward growth at 19-20°C was increasingly rapid and by generation twelve cells were sub-cultured after 48 hours. Attempts to grow it on other substrates mostly failed, these are covered more fully in the discussion below.

2.3.4. Growth curves and cell growth

Initially OD₆₀₀ values were used to calculate the 3 parameter Gompertz curve. Cell counts were used to calculate μₘ using both the logistic and the Gompertz fits (table 2.3 below). These data were compared with μₘ calculated by the established method and the results are shown in the appendix (Scragg, 2005). Since all the data were normally distributed, a students t-test could be used to check for significant differences between groups. No significant difference was found between μₘ calculated by more traditional means or the three or four parameter regression fits. Figure 1. in the appendix shows a statistical comparison of the four parameter fits with the semi-log method. The three parameter fit also gave no statistical difference. This is not shown, since for reasons outlined in the discussion below it was decided not to use it. The growth curve fits and fitted data are shown in figures 2.2 to 2.6 below.
Table 2.3. Growth characteristics of the main bacteria used in this study on their recommended media. The results for *R. opacus* and *S. oneidensis* are on medium B

<table>
<thead>
<tr>
<th>Culture</th>
<th>Lag phase (hours)</th>
<th>Maximum cell number</th>
<th>Generation time (hours)</th>
<th>Maximum growth rate (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. denitrificans</em></td>
<td>8.45</td>
<td>1.55 x 10⁶</td>
<td>1.52</td>
<td>0.137</td>
</tr>
<tr>
<td>cultured on medium 830.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. rhodochrous</em></td>
<td>11.61</td>
<td>3.54 x 10⁶</td>
<td>2.98</td>
<td>0.101</td>
</tr>
<tr>
<td>cultured on medium 1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>25.48</td>
<td>2.01 x 10⁶</td>
<td>16.98</td>
<td>0.086</td>
</tr>
<tr>
<td>cultured on glucose.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. oneidensis</em></td>
<td>11.86</td>
<td>6.57 x 10⁶</td>
<td>1.58</td>
<td>0.190</td>
</tr>
<tr>
<td>cultured on lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.2. Growth study of *D. denitrificans* cultured on 830 medium. The four plots show the same data in different ways. Plot a) is the 3 parameter modified Gompertz curve (Zwietering *et al.*, 1990). Plots b) and c) show the fit achieved using the Gompertz and logistic curves outlined by Gibson *et al.* (1987). Plot d) is an xy plot of the same data. The error bars show the standard error of the mean for three cultures. All the regression fits had $R^2$ values in excess of 0.986.
Figure 2.3. Medium 1 growth study for *R. rhodochrous*. Four parameter Gompertz fit (a) and xy plot for *R. rhodochrous* (b). The $R^2$ was 0.997 for the regression fit.
Figure 2.4. Gompertz regression fit for *R. opacus* cultured on medium B with glucose as the carbon source. Note this study was the control in the chromate inhibition study covered in the next chapter. The $R^2$ value is 0.993.
Figure 2.5. Regression fit for *S. oneidensis* cultured on lactic acid. Note this study was the control in the chromate inhibition study covered in the next chapter. The R\(^2\) value is 0.998.
Figure 2.6. An xy fit for the *S. oneidensis* data when cultured on lactate. These data were shown in figure 2.5 above. The increase in cell number is so large that it is greater than three logs.
2.3.5. Mixed cultures

Cells were collected from the Blue Lake in Gwynedd, West Wales. This is an outworked slate quarry with remarkably clear but at the same time blue coloured water, due to dissolved copper in the water. The quarry was abandoned due to the first world war and larger quarries surrounding it being more economic. There is a high proportion of iron in the slate which can be clearly be seen in figure 2.7.a.

Sterile clinical swabs were used to inoculate two plates from the water in the lake onto medium B with approximately 1mM iron (III) chloride present and 8.7mM acetic acid as the carbon source at pH 7.2. Colonies of cells could be seen growing on one plate designated BL0001 after 24 hours grown at ambient temperature. The inoculation of the second plate (BL002) failed to produce any colonies so it was re-inoculated from an area of bog by the lake saturated with iron judging by the rust colour of the sediment (figure 2.7.b). This time the swab was placed in the sediment present before a streak plate was made. A third plate (BL003) was also inoculated from another part of the same boggy area in the same way. Again growth (mixed) was visible on the sediment plates after 24 hours at ambient temperature. The plates were used to both inoculate more plates and liquid medium B with acetate as the carbon source supplemented with iron (III) sulphate/chloride. There was no attempt to isolate individual colonies from any of the plates at any stage, the inoculating loop was used to pick up multiple colonies.
Figure 2.7. Blue Lake and Blue Lake cultures. The Blue Lake in Wales where samples were collected for this project is shown in figure 2.7 a) and b). Figure 2.7 c) shows the isolates BL001-3 right to left cultured on medium B with 2mM FeCl₃ and 139mM acetic acid as the carbon source. This colouring was typical and maintained over generations.

BL003 adapted very readily to liquid culture shaken or unshaken, growth being extremely rapid by eye, the other two culture isolates took slightly longer to adapt. No formal growth studies were carried out on these cultures due to time considerations. Under the microscope three distinct cultures could be seen. BL001 consisted of mostly large rod shaped bacteria although large star shaped organisms could be seen (possibly mould). The cells were mostly motile when the culture was young but not when old. BL002 contained a small number of yeast cells and fungal hyphae were possibly seen but was overwhelmingly bacteria (cocci) and these were
non motile. A few motile rods were present. BL003 was again mostly bacteria, rod shaped and overwhelmingly motile although yeast cells were definitely present. The cells were grown on acetate mainly although they could also grow on a mixture of lactate/glycerol in medium B (no studies were made of cells on these media).
2.4. Discussion

A relationship between cell count and turbidity has been established for four out of the seven bacteria used consistently in this project (table 2.2) with a significant correlation. Initial growth studies have been completed for \textit{D. denitrificans} and \textit{R. rhodochrous} on both their recommended media and medium B. \textit{S. oneidensis} MR-1 has no recommended growth medium although lactate is usually used as the carbon source (Serres and Riley, 2006). \textit{R. opacus} growth has been studied on glucose.

Four out of the seven bacteria were successfully adapted into a separate medium. DSMZ recommended media 1 and 830, but they are unsuitable for this project in the long term since they contain unwanted additional carbon sources, as is tryptic soy broth for the same reasons. The most difficult DSMZ medium to prepare was medium 81. It was decided to try Brunner medium, but due to the difficulties with Brunner medium outlined above, a literature search was made for a minimal salts medium that would support growth of \textit{P. denitrificans}. A large number of different media compositions was obtained. Medium B was chosen (Gerhardt \textit{et al.}, 1981). An adaptation of the composition was made by the addition of trace elements as used in medium 81. \textit{B. xenovorans} and \textit{P. naphthalenivorans} showed poor growth on this medium. However, as part of initial chromate studies (described in the next chapter) using chromate free medium B controls suggested \textit{D. denitrificans} reached a higher biomass on medium B and \textit{R. rhodochrous} growth was comparable (see chapter three). \textit{P. denitrificans} growth was lower in that its maximum biomass was approximately halved compared to medium 81 (data not shown). However, it should be noted that \textit{P. denitrificans} achieved almost 10 times as much biomass as \textit{D. denitrificans} and \textit{R. rhodochrous} on its recommended medium.

Attempts were made to get \textit{S. oneidensis} to utilise a variety of substrates including glucose, fructose, glycerol, propan-1,2 and 1,3 diol. Although no OD\textsubscript{600} readings were taken, growth by eye was weak on glucose and non-existent on glycerol, propan-1,2 and 1,3 diol. The cells did appear to grow well on fructose. However, due to a lack of a readily available assay system at Heriot-Watt for fructose, this was not investigated further. Instead the organism was adapted to lactate which is the usual carbon source.
(Serres and Riley, 2006). This is despite *S. oneidensis* MR-1 not having a recognised lactate dehydrogenase gene (Serres and Riley, 2006). *S. oneidensis* is able to outgrow three of the other bacteria (table 2.3) in terms of its growth rate and reaches a much higher biomass than the other three bacteria chosen to go forward, with over three logs of growth when the other bacteria managed just over one. This is remarkable given the carbon source was an organic acid and the media used for the other organisms were less challenging.

The morphology of the organisms has also been described in greater detail than in the literature. *D. denitrificans* when immature gives the impression of being a small motile coccus. However, once the exponential growth is under way the cells are more obviously rod shaped with few being motile (Figure 2.1), although large motile cells and even motile clumps of cells can be seen. *S. oneidensis* are mostly non motile, again the cells tend to be small and motile, not large and motile, although exceptions can be seen. The percentage of motile cells was much lower than for *D. denitrificans*. *B. xenovorans* were mostly motile and of a similar size distribution to *S. oneidensis* and *D. denitrificans*.

The rationale for consideration of an alternative method for calculating the bacterial growth kinetics is the following. First, the method of taking the slope of the line on a log scale is subjective and open to both intentional and unintentional bias due to choosing a start and end point for the exponential phase. Second, when the bacteria are grown on media containing more challenging carbon sources, or transition metals, the growth curve is less often smooth and determining the start and end points of the exponential portion can be a challenge.

Growth curves were plotted for six of the bacteria chosen for this project on their recommended media. Four are shown above in Figures 2.2 to 2.6. In each case the regression fits had very good R$^2$ values.

A comparison of the growth curves shows that in theory all three are suitable. None of them give a significantly different $\mu_m$ than using a semilog method of calculation.
The three parameter Gompertz curve outlined by Zwietering et al. (1990) is easy to fit, usually requiring no initial guesses for the fitting software and giving the three growth parameters of interest directly. The two four parameter curves cited by Gibson et al. (1987) are slightly more challenging to fit, requiring initial guesses for parameters A and M. However, it has been decided to not use the Zwietering model because the meaning of the y-axis is less readily appreciated (figure 2.2a). In succeeding growth studies when the cell count falls from the inoculum level the ratio of relative cell count or OD value at a particular sampling time divided by the OD value at time zero will be negative. The two models outlined by Gibson et al. (1987) avoid this using a straight log_{10} value. This was felt to be more acceptable since transforming data particularly those of large values such as cell counts into logs is quite common. Of these two fits the Gompertz model was chosen rather than the logistic one. This model gives $\mu_{\text{max}}$ values that are closer to the semilog method of calculating them and the curve is suited to asymmetric data as exhibited by cells grown on media with alcohols and chromate present which extend the lag phase. The curves outlined by Gibson et al. (1987) have one disadvantage in that they sometimes overstate the maximum biomass. The reason for this is that the curve is still increasing in the lag phase, rather than having plateaued. This can be seen in figure 2.3a. One reason for this could be that not enough points on the stationary phase were collected. Despite this the advantages of the fit outweigh the disadvantages.

None of these fits are suitable for showing the standard error of the mean. Therefore a line graph will more usually be shown with error bars showing this. However, all the growth characteristics will be calculated using the four parameter Gompertz model as shown in figure 2.4.
Chapter 3. Transition metal reduction and c type cytochromes

3.1. Introduction

Bacteria can use a wide variety of alternatives to oxygen as a terminal electron acceptor. This is either because these bacteria are obligate anaerobes, or in the case of many facultative aerobes in addition to oxygen. One class of alternative electron acceptors are transition metals and indeed reduction of a wide variety of transition metals by bacteria or Archaea has been found including vanadium, molybdenum, manganese, uranium, iron and chromate (Johnson and McGinness, 1991; Lovley, 1993; Erlich, 1997; Vadas et al., 1999; Richardson, 2000; Magnuson et al., 2001; Myers and Myers, 2001; Camargo et al., 2003; Leang, et al., 2003; Lloyd, 2003; Lloyd et al., 2003; Lloyd et al., 2003; Mazoch et al., 2004; Carpentier et al., 2005; Rehder, 2008). Microorganisms that interact with transition metals, either reducing them for detoxification purposes, or using them as terminal electron acceptors to derive energy are thought to have an important role in the geochemistry of soils and aquatic sediments (Lovley, 1993; Erlich, 1997; Lloyd et al., 2003; Carpentier et al., 2005).

Chromate reduction from the (VI) oxidation state to the (III) oxidation state has been studied due to the environmental problems posed by chromate (VI) (Cr (VI)), which is highly poisonous and carcinogenic (Camargo et al., 2003; Gonzalez et al., 2003; Lloyd et al., 2003). A variety of bacteria have been found to be capable of degrading chromate including the bacterial genera of Pseudomonas, Shewanella, Bacillus, Enterobacter, Deinococcus, Desulfovibrio, Rhodobacter, Microbacterium, Burkholderia and Escherichia (Camargo et al., 2003; Gonzalez et al., 2003; Lloyd, 2003; Middleton et al., 2003; Ackerley et al., 2004, Viamajala et al., 2007; Wani et al., 2007; Lloyd et al., 2003). Whilst the bacterial mechanisms involved in the reduction of many transition metals described above involve extensions to the electron transport chain, in the case of chromate the electron transfer mediators are in some cases soluble proteins unattached to membranes (Myers et al., 2000; Park et al., 2000; Gonzalez et al., 2003; Mazoch et al., 2004). Chromate reducing bacteria have been isolated from heavily chromate contaminated sites such as leather tanneries or nuclear plants although in some cases bacteria capable of chromate reduction have been found that have not
been in contact with chromate (VI) (Camargo et al., 2003; Keyhan et al., 2003; Ackerley et al., 2004; Wani et al., 2007). No bacteria have been found that can couple chromate reduction to anaerobic growth (Lovley, 1993). However, a wide variety of bacterial species have been found that can conserve energy through the reduction of other transition metals, the most important being the reduction of iron (III) to iron (II) (Lovley, 1993; Lloyd et al., 2003).

Since the late 1980's bacteria have been isolated that can grow anaerobically utilising iron (III) and Mn (IV) including relatives of Geobacter metallireductans and Shewanella oneidensis as well as Goethrix fermentans and Geovibrio ferrireductans amongst others (Lloyd et al., 2003). No bacteria have been isolated that can completely oxidise sugars to carbon dioxide with coupled anaerobic reduction of iron (III) (Lovley, 1993; Lloyd et al., 2003) and indeed coupled reactions of this type would be thermodynamically unfavourable (Lovley, 1993). Instead organisms such as G. metallireductans rely on other surrounding bacteria metabolising more complicated compounds to fermentation products such as acetate and formate (Lovley, 1993). Those bacteria capable of iron reduction then couple the oxidation of these compounds to the reduction of iron (III) according to the following stoichiometry;

\[
\text{acetate}^- + 8\text{Fe(III)} + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 8\text{Fe(II)} + 9\text{H}^+ \quad \text{Equation 3.1}
\]

\[
\text{formate}^- + 2\text{Fe(III)} + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + 2\text{Fe(II)} + 2\text{H}^+ \quad \text{Equation 3.2}
\]

Some bacteria have been isolated that can utilise xenobiotics and glycerol as carbon and electron sources whilst reducing iron (III) (Johnson and McGinness, 1991; Lovley, 1993).

Electron transport to iron (III) has been localised to both c-type cytochromes and soluble electron shuttles in both G. metallireductans and S. oneidensis (Lloyd et al., 2003). In addition G. metallireductans expresses a membrane bound iron reductase (Lovley, 1993). Desulfovibrio vulgaris has a c3 cytochrome that is a metal reductase that is capable of reducing iron (III) to iron (II) (Lovley, 1993).
Whilst most research studies on transition metal reduction have focused on iron, chromate, manganese, mercury and palladium, other transition metals have also been studied (Lloyd, 2003; Lloyd et al., 2003). Comparatively few studies have been carried out on vanadium (V), molybdenum (Mo) or copper (Cu) (Lloyd, 2003; Lloyd et al., 2003). *S. oneidensis* was found to be capable of reducing V(V) by means of c-type cytochromes (Carpentier et al., 2003). *G. metallireducens* can also reduce vanadium utilising it as a sole electron acceptor (Ortiz-Bernad et al., 2004). In addition species of *Psuedomonas, Micrococcus* and *Desulfovibrio* have been found to reduce vanadium and the ability to reduce it in micro-organisms is thought to be “widespread” (Lloyd, 2003). Molybdenum reduction has been found in *Desulfovibrio desulfuricans, Pseudomonas* strain Dr.Y2, *Serratia marcescens* Dr.Y4, *Staphylococcus aureus* strain Dr.Y11, *Acinetobacter* Dr.Y12, *Thiobacillus ferrooxidans* and *Enterobacter cloacae* (Sugio et al., 1988; Ghani et al., 1993; Tucker et al., 1997; Lloyd, 2003; Lloyd et al., 2003; Shukor et al., 2009). Molybdenum reduction in *T. ferroxidans* is brought about by a sulphur:ferric ion oxidoreductase the reduction of molybdenum being linked to the oxidation of elemental sulphur in this organism (Sugio et al., 1988). In *E. cloacae* the reduction appears to mediated by a b type cytochrome (Ghani et al., 1993). Copper reduction has been found in *T. ferroxidans* and is brought about by the same sulphur:ferric ion oxidoreductase outlined above (Sugio et al., 1990).

The aim of the work described in this chapter was firstly to identify additional bacteria that are capable of reducing chromate or other transition metals. Secondly, to examine if any ability to reduce transition metals could be related to the presence of c-type cytochromes or other mechanisms of electron transfer. This is of particular interest as was outlined in chapter one due to the ability of those bacteria able to reduce transition metals to potentially transfer electrons directly to the anode in an MFC (Lovley, 2006). Chromate was chosen as the first transition metal to test since there is a reliable and sensitive assay for Cr (VI). This involves the formation of a coloured complex between diphenylcarbazide and hexavalent chromium (Mendham et al., 2000; Wani, et al., 2007). The same assay also works for vanadium (Carpentier et al., 2003). Iron, molybdenum and copper were also examined using other assay systems described below.
3.2. Materials and methods

3.2.1. Chemicals

All chemicals were sourced from Fisher UK or Sigma UK. The cells were grown on medium B prepared as outlined in the growth studies section with and without the addition of the various transition metals. Since varying concentrations were used these are described in the results section and each was diluted from the stock solutions whose preparation is described below in cell medium. In the literature the terms for metal compounds are used interchangeably with the pure metals. So for example if the culture medium is prepared with a certain concentration of potassium dichromate the assay results give the concentration as that of Cr (VI) which is what is actually measured by the assay (Ackerley et al., 2004; Wani et al., 2007; McLean and Beveridge, 2001). This convention has been followed here with the exception of molybdenum where for reasons outlined below the assay doesn’t measure the transition metal.

Cell samples taken from the cultures at various time points were assayed for pH and glucose (by FreeStyle Lite™ Blood Glucose Monitoring System) immediately when these measurements were taken. For all other assays the sample tubes were stored at -20°C until required.

2.96g of potassium dichromate was dissolved in 1l deionised water to make a stock solution of 10.1mM.

0.61g NaVO₃ was dissolved in 500ml of deionised water. This produced a stock solution of 10mM.

0.18g iron (III) ferricyanide was dissolved in 25ml of deionised water making a solution with a concentration of 21.8mmol/l. After sterile filtering through a 0.2 micron sterivex filter (Millipore UK), 100µl of this solution was added to 100ml of medium in two flasks in each study twice making a final concentration of 21.86µM added each time. A 10mmol/l iron (III) sulphate solution was prepared by dissolving 0.9996g of
this compound in 250ml of deionised water.

Copper (II) sulphate was prepared by dissolving 625.1mg of CuSO₄·5H₂O in deionised water then making the volume up to 250ml. This was 10.01mmol/l.

Molybdenum was prepared from molybdic acid, the purity was given by the manufacturer as 81-83%. 3.69g was dissolved in deionised water and made up to 250ml volume, this gives a 73.8mmol/l stock solution assuming the purity is 81%.
3.2.2. Cell growth monitoring

Cell growth was monitored by measuring the turbidity at 600nm (OD$_{600}$). The OD$_{600}$ turbidity readings were referenced against deionised water. Cell medium had an OD$_{600}$ measured before cells were added for each culture flask. This value was subtracted from each OD$_{600}$ with cells present for the same flask as the growth study proceeded as a background value. The regression fits determined by the work in chapter two were then used to calculate cell counts using this calculated value. Growth was carried out at room temperature (20°C) unless otherwise stated. Flasks were shaken at 160rpm on a KS250 orbital shaker (IKA Werke, Germany) or a Stuart orbital shaker (Stuart, UK). Unless otherwise stated the growth and transition metal results are the mean of at least two culture measurements. Visual examination of cells was made using a haemocytometer slide 0.1mm x 0.0025 mm (Superior, Germany) and an Olympus CH-2 microscope. All cultures were aerobic. Turbidity measurements were carried out on all the cultures within 5 minutes of sampling.

3.2.3. Assays

3.2.3.1. Glucose assays

Glucose concentrations were measured using the FreeStyle Lite™ Blood Glucose Monitoring System (Abbott, Ireland) except for some cultures involving copper (II) (referred to as the "stick assay" in the text). The range of the meter has upper and lower values of 27.8 and 1.1mmol/l respectively. In this chapter no dilutions were required to bring the readings into the working range. Readings below 1.1mmol/l are returned by the instrument as “lo”, all such readings are reported as 1.1mmol/l.

In copper (II) containing cultures the following glucose assays were carried out in addition. The first was using the Sigma glucose assay kit (Sigma, UK). In this assay glucose is oxidized to gluconic acid at the same time forming hydrogen peroxide by the action of the enzyme glucose oxidase. The hydrogen peroxide reacts with o-dianisidine in the presence of peroxidase to form a coloured product (oxidized o-dianisidine). The
o-dianisidine reacts with sulphuric acid to form a more stable pink coloured product. The intensity of the colour is measured at 540 nm and is proportional to the original glucose concentration.

Glucose Oxidase/Peroxidase Reagent was dissolved in 39.2 ml of deionised water with gentle mixing. The o-dianisidine was reconstituted by adding of 1ml of deionised water to the contents of the vial. It was inverted several times to dissolve it then 0.8ml of this solution was added to the enzyme to make the assay reagent. The glucose standard provided with the kit was diluted in the test tubes used to carry out the assay up to a total volume of 1ml in deionised water. Water was the zero standard. Samples were spun at 4500g for 1 hour at 4°C and required serial dilutions in deionised water. Then in a change to the Sigma schedule 1ml of the reagent was added to the tubes rather than 2ml. The tubes were placed in a waterbath at 37°C for 30 minutes before the addition of 2ml of 6M sulphuric acid per tube. The tubes’ contents were read at 510nm on the Lightwave spectrophotometer (Lightwave, Biochrom, Cambridge, UK) after referencing using water.

The second assay for glucose was using the phenol sulphuric acid method based on the modified method of Taylor (1995). By mixing water, phenol, sulphuric acid and a carbohydrate an exothermic reaction takes place that forms an absorbing compound. Samples were spun at 4500g for 1 hour at 4°C to pellet the cells before the supernatant was used in the assay. The assay was carried out in glass test tubes. 0.2ml of the glucose standard solution, deionised water (the zero standard), or samples were added per tube. 0.8ml of concentrated sulphuric acid was then aliquoted into each tube. After mixing and incubation for 1 minute the tubes were placed in water previously cooled to 4°C. 25μl of 90% phenol in deionised water were added per tube and after 30 minutes the absorbance was read at 480nm.

### 3.2.3.2. Glycerol assays

Glycerol was assayed using the dionex HPLC system. The HPLC system consisted of a reverse phase ODS-L column (250mm x 4.5nm) on a Dionex system with a GS50
pump and ED50 electrochemical detector. The mobile phase was 50 mM perchloric acid (HPLC grade) pH 2.1 adjusted with electrochemical grade sodium hydroxide. The mobile phase was degassed using helium and the rate of the mobile phase was between 0.3 and 0.6ml/min. Cell samples were spun at 4500g for 1 hour and then diluted in deionised water from 2x to 10x into glass chromocol vials. These were then capped and sealed and injected using an Famos autosampler. The data were analysed using the Dionex chromelone software v6.60. The reported glucose and glycerol concentrations are millimoles per litre.

3.2.3.3. Microbial adhesion to hydrocarbons (MATH) assay

PUM buffer was prepared as follows; 22.2g of K$_2$HPO$_4$, 7.3g of KH$_2$PO$_4$, 1.8g of urea and 0.2g of MgSO$_4$.7H$_2$O were dissolved in deionised water. The pH was adjusted to 7.1 using H$_2$SO$_4$ and the volume made up to 1l with further deionised water. This buffer was used for all stages of the MATH test. The test was carried out in glass test tubes. These were washed in an Indesit dishwasher on a 70°C wash followed by a rinse on the same cycle. They were dry before use. Cells were harvested in late exponential or stationary phase. An OD$_{600}$ reading was taken immediately. The cells were then spun at 4500g for 10 minutes. The supernatant was decanted off and the cell pellet had 10ml of PUM buffer added. The cells suspension was vortexed briefly and was spun again at 4500g for 10 minutes. This wash step was repeated one more time. The cells were re-suspended in PUM buffer to give an OD$_{600}$ as near to 0.500 as possible. The method outlined by Sokolovská et al. (2003) was used, in that the same test tube was used for both stages of the assay. Cells were first removed from the test tube the OD$_{600}$ read then the cells were returned to the tube and the addition of n-hexadecane made. 1.2ml of cell suspension was aliquoted out followed by 0.2ml of n-hexadecane. The cell suspension with n-hexadecane added was vortexed for 10 seconds and shaken for a further 10 minutes. The aqueous (lower) layer was removed and the OD$_{600}$ re-read. Any removal or return of cells was carried out using disposable 230mm glass pipettes (Volac, UK).
3.2.4.1. Transition metal assays

Cell culture samples were stored at -20°C before assay. Cell culture samples were spun at 4500g for one hour to pellet the cells and the supernatant removed for assay.

3.2.4.1.1. Diphenylcarbazide assay for Cr (VI)

Diphenylcarbazide forms coloured complexes with a number of transition metals used in this project. In the case of chromate (VI) this occurs under acid conditions. Diphenylcarbazide was freshly prepared by dissolving 0.1g of diphenylcarbazide in 50ml acetone. Mendham et al., (2000), state that the acetone should be used at 50% (v/v) to dissolve the diphenylcarbazide, however they do not state what the other 50% of the solvent should be. To reduce excessive acetone use both 25ml of water and 25ml of 95% ethanol were added to 25ml of acetone. Diphenylcarbazide was found to have low solubility in 50:50 acetone/water but dissolved more easily in 50:50 ethanol/acetone than acetone alone. No change was found in the standard curve or QC values in making the solvent composition alterations.

0.2N sulphuric acid was prepared from 3N sulphuric acid stock by making 13.3ml of the 3N stock up to 200ml in distilled water.

The chromate (VI) assay was carried out as follows. Chromate (VI) standards were prepared from the 10.1mM stock dichromate solution. 2ml of stock solution was added to 48ml of distilled water to give a 400μM standard. 1ml of stock solution was added to 49ml of distilled water to give a 200μM standard. 0.5ml of stock solution was added to 49.5ml of distilled water to give a 100μM standard. 0.25ml of stock solution was added to 49.75ml of distilled water to give a 50μM standard. 0.125ml of stock solution was added to 49.875ml of distilled water to give a 25μM standard. 0.5ml of deionised water, standard or sample was added to an eppendorf tube along with 0.5ml of 0.2N sulphuric acid and 0.1ml of diphenylcarbazide. The samples were mixed and placed in a microcuvette and a reading taken at a wavelength of 540nm on the lightwave spectrophotometer (Biochrom, Cambridge, UK). The readings were referenced against a distilled water blank. If dilutions were necessary they were carried out in the cuvettes.
using distilled water. Each sample was assayed in duplicate.

3.2.4.1.2. Diphenylcarbazide assay for vanadium (V)

The same assay for vanadium (V) was carried out in the following way. Vanadium (V) standards were prepared from the 10mM stock NaVO₃ solution. 2ml of stock solution was added to 48ml of distilled water to give a 400µM standard. 1ml of stock solution was added to 49ml of distilled water to give a 200µM standard. 0.5ml of stock solution was added to 49.5ml of distilled water to give a 100µM standard. 0.25ml of stock solution was added to 49.75ml of distilled water to give a 50µM standard. 0.125ml of stock solution was added to 49.875ml of distilled water to give a 25µM standard. 0.5ml of deionised water, standard or sample was added to an eppendorf tube along with 0.5ml of 0.2N sulphuric acid and 0.1ml of diphenylcarbazide. The samples were mixed and placed in a microcuvette and a reading taken at a wavelength of 320nm on a Hach DR/4000U spectrophotometer (Hach, Germany) after 10 minutes. The readings were referenced against a distilled water blank. Each sample was assayed in duplicate.

3.2.4.1.3. Diphenylcarbazide assay for copper (II)

The diphenylcarbazide assay for copper (II) was carried out using the method of Turkington and Tracy, (1958). Copper II standards were prepared by diluting 10.1mM stock copper (II) sulphate pentahydrate over a range of 300-9.3µM. 100ml of 200mM Tris HCl was prepared and the pH adjusted to pH 9.00 using 1M HCl. To each cuvette 0.2ml of water/copper II standard/QC was added followed by 0.25ml of deionised water and 0.25ml of Tris HCl buffer (pH 9.0). Finally 0.1ml of diphenylcarbazide was added and the cuvettes read at 320nm or 495nm on a Hach DR/4000 spectrophotometer (Hach, Germany). The cuvettes were prepared in blocks of 6-8 in number to allow reading within 5 minutes. The diphenylcarbazide assay for copper (II) was carried out at acid pH as follows; 0.5ml of deionised water, standard or sample was added to an eppendorf tube along with 0.5ml of 0.2N sulphuric acid and 0.1ml of diphenylcarbazide. A reading was taken at a wavelength of 320nm on a Hach DR/4000 spectrophotometer.
after 10 minutes. The readings were referenced against a distilled water blank. Each sample was assayed in duplicate.

3.2.4.1.4. Alizarine assay for copper (II)

Alizarine red S forms a water soluble coloured complex with copper (II) (Yazdinejad, 2006). The assay was carried out in the following manner. 0.1457g of alizarine was dissolved in 100ml of deionised water. 0.0333g of copper sulphate pentahydrate was dissolved in 100ml of deionised water. Doubling dilutions were made of this stock standard to create a wide standard range from 1292.1 to 40.4µM. 0.75ml of sample/water or standard was added to a test tube along with 1.25ml of 100mM acetic acid/acetate buffer pH5 and 0.5ml of alizarine stock solution. After 5 minutes the tubes were read at 510nm on the lightwave spectrophotometer. The blank was alizarine/water-buffer and the spectrophotometer was referenced with this solution.

3.2.4.1.5. Cuproin assay for copper (I)

A spectrophotometric assay for Cu(I) using cuproin as outlined by Sugio et al. (1990) was undertaken as follows. The cuproin and the Cu (I) form a purple coloured complex. The assay has to be carried out using a partition between isoamyl alcohol and water since the cuproin is not water soluble. 0.1% (v/v) cuproin was dissolved in isoamyl alcohol (10mg in 10ml). An attempt at preparing CuCl standards was made by trying to dissolve 30.7mg of CuCl in 250ml deionised water to make a 310µM solution (after trying to prepare 1mM and 10mM solutions). While it was not possible to dissolve this standard, 0.5ml of a cell supernatant sample/water was added to 1ml of the cuproin reagent in a test tube. The non aqueous layer was removed into a cuvette and the absorbance read at 546nm after referencing with a water blank. Purple colouration was seen in the sample.

3.2.4.1.6. Bathocuproine disulfonate assay for copper (I)

Copper (I) is extremely unstable in the (I) oxidation state and not very water soluble.
Attempts to dissolve CuCl failed at all three concentrations described in the section above. In addition the colour changed indicating the copper (I) had been oxidised by oxygen in the air to copper (II). Bathocuproine disulfonate (2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid or BCS) forms a coloured complex with copper (I). The copper (I) can be held in the (I) oxidation state by the addition of a reducing agent. Ascorbic acid is used since it is a reasonably strong reducing agent which is non-toxic and doesn’t interfere with the assay. In addition all the assay components are water soluble so no errors due to partitioning between phases would be introduced. The assay used the following methodology. 1g of ascorbic acid was dissolved in 10ml of deionised water. The equivalent of 1g/l of BCS was dissolved in deionised water. The stock Cu(II) standard used in the alizarin red Cu(II) assay was used to prepare a range of copper (II) standards from 646.1 to 2.5\(\mu\)M by doubling dilutions in deionised water. To each cuvette 200\(\mu\)l of sample/standard/QC was added, followed by 200\(\mu\)l of BCS and 350\(\mu\)l of the same 100mM acetate/acetic acid buffer used in the alizarin red assay. To each standard 50\(\mu\)l of the ascorbic acid was added to reduce Cu(II) to copper (I) and after mixing the optical density was read at 484nm. The samples/QC were read at 484nm then 50\(\mu\)l of the ascorbic acid was added and after mixing the optical density was re-read at 484nm on the lightwave spectrophotometer. Taking into account the dilutions, the difference between the initial Cu(I) reading and the total Cu(I) reading after addition of the ascorbic acid to the cuvette was the Cu(II) concentration.

In addition Cu(II) reduction was tested for by impregnating a plate with 300\(\mu\)M of Cu(II) then streaking the organism of interest. 5ml of BCS at the concentration above was then layered onto the plate.

3.2.4.1.7. Ferrozine assay for iron (II)

Fe(III) reduction was determined using the ferrozine assay, this compound forms a purple coloured complex with iron (II). (NH\(_4\))\(_2\)FeSO\(_4\).6H\(_2\)O was used as a standard prepared by weighing 84.4mg of this compound into 250ml of 1M HCl. (NH\(_4\))\(_2\)FeSO\(_4\).6H\(_2\)O was used to prepare the set of iron (II) standards since it is a water soluble compound and is stable in air (Cotton and Wilkinson, 1976). Doubling dilutions
were then made to a bottom standard of 3.97μM Fe(II) and top standard of 507μM Fe(II) in 1M HCl. 50% (v/v) ammonium acetate was prepared by dissolving 50g in 100ml deionised water. Approximately 0.1% (v/v) ferrozine solution was made up by dissolving 0.1529g in 150ml of the 50% (v/v) ammonium acetate. 0.2ml sample/standard/QC was added to a cuvette along with 0.2ml of 1M HCl and 0.4ml of ferrozine solution. The tubes were shaken for 10 minutes and the absorbance read at 562nm after referencing with a 1M HCl blank on the lightwave spectrophotometer. For total Fe(II) each culture was defrosted, vortexed and 0.4ml was aliquoted into an eppendorf tube along with 0.5ml of 1M HCl. The tubes were left 24 hours at room temperature. Then 0.4ml of the extracted Fe(II) solution was added to each cuvette along with 0.4ml of ferrozine solution. Each sample was assayed in duplicate.

In addition Fe(III) reduction was tested for by impregnating a plate with 300μM of Fe(III) sulphate then streaking the organism of interest. 2.5ml of ferrozine at the concentration above was then layered onto the plate.

3.2.4.1.8. Measurement of molybdenum blue

Molybdenum (VI) stock was diluted to make a series of standards from 3.69mM to 0.12mM in deionised water by doubling dilutions. 1g per 10ml of ascorbic acid was dissolved in 0.05M Na₂H₂PO₄ buffer. 250μl of this solution was added to 700μl of each standard in a cuvette. After 30 minutes the absorbance was read at 865nm on the Hach DR/4000U spectrophotometer. The cell samples were read at the same wavelength. Both the cell samples and standards were mixed well before they were read. Scans were carried out on a cell sample and a standard with similar absorbances from 350 to 1000nm with a 2nm interval. The principles of this assay are explained in the discussion.

3.2.5.1. Hydrogen peroxide assay

The National Diagnostics kit (National Diagnostics, USA) was purchased from Fisher Scientific. Hydrogen peroxide for the standards was purchased from a
pharmacy (9%, 30 volume). The standards were prepared by making a 1:100 dilution in deionised water and reading the optical density at 240nm. Using the molar extinction coefficient of 43.6M⁻¹cm⁻¹ the concentration of the stock 1:100 solution was calculated. Standards ranging from 62.17μM to 0.971μM were prepared from the stock in deionised water. To stretch the kit out over many samples, firstly samples were assayed singularly and second the volumes added to each cuvette were downsized, although the ratio of reagent and sample was kept the same as the kit instructions. 20ml of assay reagent was prepared by combining 19.8ml of component A with 0.2ml component B. 75μl of sample or standard was added to 675μl of assay reagent. After 30 minutes of gentle mixing on a shaker at 160rpm the cuvettes were read at 560nm on the lightwave spectrophotometer after blanking against deionised water. The assay works by formation of a complex between xylenol orange and ferric iron, which is produced by the peroxide dependent oxidation of ferrous iron.

3.2.5.2. Quality controls

A quality control (QC) to monitor the performance of the chromate assay was prepared from the supernatant of a D. denitrificans culture that had grown successfully in the presence of chromate. After autoclaving the cells were centrifuged at 3000rpm for 5 minutes and the supernatant decanted and stored at -20°C. On the first few assays in which the QC was used multiple QC’s were run at the both the beginning and end of the assay to establish its value. A QC control chart was plotted. A QC was made from the culture medium of cells of R. opacus cultured in the presence of vanadate, copper and iron. Likewise the cell suspension was sterile filtered through a 0.2μM filter and the supernatant stored at -20°C. Again during the first few assays in which the QC was used multiple QC’s were run at both the beginning and end of the assay to establish its value.

The chromate and copper (I) assays were checked for parallelism as described next; once sufficient QC samples had been run to establish its value, serial dilutions of the QC were made in deionised water. 250μl of neat QC was added to 250μl of water to give a 50% dilution and then 250μl of the 50% dilution QC added to 250μl of water to give
25% of the initial concentration. This dilution was repeated one more time to give a QC at 12.5% of the neat QC concentration. The assay was carried out in the normal way and the two curves were compared for similarity using analysis of variance according the method of Reeve, (2000).

3.2.6. Cytochrome studies

C-type cytochrome detection using visible spectroscopy was initially carried out according to the methods outlined in Holmes et al. (2004) and a personal communication from E. Holmes. The method was then modified using some of the techniques outlined in Sands et al. (1967) and in addition degassing was carried out to attempt to remove oxygen. Haem groups in cytochromes on the cell surface differentially absorb light depending on their oxidation state. By measuring the absorbance in an oxidised state and in the reduced state using a strong reducing agent then taking the difference between the two, a series of peaks on the spectrum can be seen. The wavelengths of the peaks are characteristic for different cytochromes.

The cells were spun at 4500g for at least one hour. The supernatant was decanted and 20mM PIPES (Piperazine-1,4-bis(2-ethanesulfonic acid)) buffer was added to the cell pellet. This buffer had been previously degassed in an ultrasonic bath for approximately 20 minutes. The cells were re-suspended by vortexing and placed in the ultrasonic bath again before reading. Between 3-12mg of sodium hydrosulphite (dithionite) was weighed out into a dry microcuvette. 1ml of cell suspension was added to the cuvette, mixed, capped and read immediately on the lightwave spectrophotometer using scan mode. After a few minutes 3 drops of 30 volume hydrogen peroxide was added and after mixing the scan was repeated. In cells cultured in the presence of copper no hydrogen peroxide was added; the cuvette was scanned then the dithionite was added and the cuvette scanned again.

The scan data were saved to a txt file using the provided lightwave graphic software v1.2. Whole cell spectra are reported as shown by Deeudom et al. (2006) and also difference spectra as outlined by them and others (Deeudom et al., 2006;
Guerrero and Jones, 1997; Sands et al., 1967). In difference spectra the optical density of the oxidised spectrum is subtracted from the optical density of the reduced spectrum.

3.2.7. Native polyacrylamide gel electrophoresis

Gels and four times concentration running buffer were purchased from Pagegel (Pagegel, USA). Tris and Tricine were purchased from Sigma (Sigma, UK). Protein standards were purchased from Sigma UK (sigmoidarker) and “NativeMark” marker (Invitrogen, UK). Stain (Coomassie blue R250) was purchased from Fluka (Fluka, UK). Gels were run in a Sigma CDC minigel electrophoresis unit (Sigma, UK), current was supplied using a Consort E844 power supply (Consort, USA).

Tricine/Tris running buffer was prepared by dissolving 3.56g of tricine and 3.63g of Tris in deionised water then making the volume up to 1L. No pH adjustment was made. Stain solution was prepared by dissolving 0.5g of R250 in 50ml of destain solution. Destain solution was prepared by mixing 400ml of ethanol, 100ml of glacial acetic acid and 600ml of deionised water.

Samples were spun for 1 hour at 4500g at 4°C to pellet the cells. 75µl of cell sample supernatant was mixed with 25µl of 4x running buffer (stored at 4°C). The marker standards were added neat. The page gel was removed from storage at 4°C and from its package. The wells were washed two times with deionised water and then filled with further deionised water. The gel cassette was then placed in the gel running module the other place on the opposite side of the module being taken by an empty casting unit. The screws were tightened up. The inner chamber was filled up so that the running buffer covered the gel loading wells. 44µl of the sample mixture or marker standard was loaded into each well by pipetting them under the deionised water in the wells. The remainder of the running buffer was added to the outer chamber. The gels were run at 200V DC for approximately 1 hour 10 minutes. At the end of this time the gel was removed from the unit, broken open from its case and placed in stain at an initial temperature of 60°C for 5 minutes. The stain was poured
off and it was placed in repeated changes of destain. At the end when destaining was complete the gel was placed back in its case to dry.

3.2.8. Growth studies

3.2.8.1. Initial growth studies

Two batches of medium B were prepared in the normal way, using 2g/l of glucose as the sole carbon source. One batch of medium B was spiked with 30ml of the chromate stock solution giving an apparent final concentration of 300\(\mu\)M Cr (VI) before the volume was made up to 1l with distilled water. Both media types were autoclaved at 121\(^{\circ}\)C for 15 minutes. Four 100ml aliquots of medium were made in 250ml Erlenmeyer flasks. These were as follows; 100ml of medium B control without chromate, a chromate only control and two chromate media for inoculation. 1 ml of cell suspension containing \textit{P. denitrificans} was seeded from cells grown on medium B into the flask containing medium B without chromate and two flasks containing chromate. Samples were taken immediately from all four flasks for OD\(_{600}\) readings and chromate (VI) assay.

A repeat of the above experiment was made using all five bacterial cultures (\textit{P. denitrificans}, \textit{R. rhodochrous}, \textit{D. denitrificans}, \textit{B. xenovorans} and \textit{P. naphthalenivorans}) with the following modifications. An additional control was added; an Erlenmeyer flask without any bacteria added containing medium B to enable the OD\(_{600}\) of the medium B without Cr (VI) to be subtracted from that of the chromate control. One culture flask was grown for each bacterial type. Samples were taken immediately (time zero) and at intervals over 100 hours for growth and Cr (VI) assay.

Fresh medium B was prepared without chromate and with a lower concentration (10ml of Cr (VI) stock, therefore giving a final concentration of 100\(\mu\)M). Again all five cultures were inoculated into a single flask with chromate and a chromate control was maintained without any bacteria. An additional organism \textit{E. coli} (DSM 490) was inoculated from a plate into liquid culture containing the same chromate concentration.
as for the other five organisms. The E. coli was cultured at 37°C. Samples were taken at time zero and at time periods over 60 hours for all the bacterial types.

3.2.8.2. Full growth studies on chromate (VI)

Full growth studies were carried out as follows. Fresh medium B was prepared without chromate, 30ml of Cr (VI) stock was added therefore giving a final concentration of 300μM pre-autoclaving. 100ml of medium B with chromate was aliquoted into 250ml flasks and without chromate present into 250ml flasks as controls. One of the chromate flasks was used as uninoculated control. Cells previously cultured on medium B without chromate were used to inoculate the non-chromate containing flasks. Cells previously cultured on medium B with chromate were used to inoculate the chromate containing flasks, although latterly cells were used that had never been cultured in the presence of chromate. A sample of medium B was used for a background OD600 measurement at the start. Samples were removed immediately and at intervals throughout the culture period. Growth was monitored until the bacteria had reached the end of the exponential phase. Turbidity measurements were carried out on all the cultures within 5 minutes of sampling and Cr (VI) assay on the chromate containing culture samples which were stored at -20°C until assay.

3.2.8.3. Full growth studies on other transition metals

Full growth studies on vanadium (V), molybdenum (VI) or iron (III) were undertaken in the following manner. Flasks were made up in duplicate and autoclaved at 115°C. In addition one flask contained vanadium, molybdenum or iron but no cells. Medium was removed from the flask before the addition of the cells and an equal volume of the transition metal stock solution added after filtering through a 0.2 micron sterivex filter (Millipore UK). A sample of medium B was used for a background OD600 measurement at the start. Samples were removed immediately and throughout the culture period. Growth was monitored until the bacteria had reached the end of the exponential phase.
Two studies were undertaken growing *D. denitrificans* in the presence of ferricyanide. Glycerol was used as the carbon source. Two flasks were used as controls with glycerol and cells in each case. In one growth study all the flasks were shaken as previously, in the other the flasks were unshaken except briefly by hand before samples were taken. The same ferricyanide solution was used in both the shaken and unshaken study.

### 3.2.8.4. Growth inhibition studies

#### 3.2.8.4.1 Chromate growth inhibition studies

Latterly the chromate stock was sterile filtered through a millipore sterivex 0.2 micron filter to avoid autoclaving. Flasks were prepared in duplicate with the following volumes; 100ml, 99ml, 98ml, 97ml and 96ml of medium B with either glucose (2g/l) or lactate (22mM) present. The volumes were made up to 100ml with sterile chromate stock solution. This inhibition study was carried out for *D. denitrificans*, *S. oneidensis MR-1* and *R. opacus*. In addition one study was made for *D. denitrificans* using higher concentrations of chromate. Flasks were prepared in duplicate with the following volumes; 100ml, 98ml, 96ml, 94ml and 90ml of medium B with glucose (2g/l). The volumes were made up to 100ml with sterile chromate stock solution. Growth was monitored until the bacteria had reached the end of the exponential phase. Turbidity measurements were carried out on all the cultures within 5 minutes of sampling and Cr (VI) assay on the culture samples which were stored at -20°C until assay.

#### 3.2.8.4.2. Copper growth inhibition studies

The copper sulphate stock was sterile filtered through a millipore sterivex 0.2 micron filter. Flasks were prepared in duplicate with a volume of 100ml of medium B with 2g/l of glucose as the carbon source. The following volumes of medium were removed and replaced with the same volume of copper sulphate stock; 50μl, 100μl, 200μl, 1000μl and 3000μl. This inhibition study was carried out for *R. opacus*. 
3.2.9. Data analysis and statistics

Where appropriate data were checked for normality in minitab version 11 and latterly Rlplot v1.4 using the Shapiro-Wilk test. If the data were normally distributed an F-test was undertaken. On the basis of the result the appropriate homoscedastic or heteroscedastic t-test was chosen. F-test and t-tests were carried out in openoffice calc with the exception of the statistics for the hydrogen peroxide studies and molybdenum growth studies. Gnumeric was used to calculate F-tests, t-tests and one way analysis of variance (ANOVA) for these studies (McCullough, 2004). Mann-Whitney U tests were carried out in Rlplot v1.4.

Paired t-tests or Wilcoxon matched pair tests were used to investigate any significant differences within groups using a technique used in drug trials (Brady et al., 2004). The t-tests were calculated using gnumeric and Wilcoxon matched pair tests were calculated long hand using the methodology and significance tables (Bailey, 1981).

Non-linear regression for growth and inhibition curves were undertaken in Qtiplot for Linux using a user defined fit. The non-linear logfit model was fitted in gnumeric spreadsheet. Pearson correlations, spearman's rank correlations and their statistical significance were calculated in Rlplot v1.4.

Quality control (QC) charts for assays were plotted by taking the mean QC value and plus and minus once and twice the standard deviation for the QC values for each QC. The inter-assay variation (correlation of variance) was calculated from these data by dividing the standard deviation for the QC values from each different assay by the mean, multiplying by 100 and expressing it as a percentage. The intra-assay variation was calculated in the same way but using QC samples run at the beginning and end of the assay.
3.3. Results

3.3.1. Assay characteristics

3.3.1.1. Assay parallelism and assay variation

The assays passed their parallelism tests, see tables 2 and 3 in the appendix. The QC control charts are also shown, see figures 3 and 4 in the appendix.

3.3.2. Growth studies with chromate

3.3.2.1. Medium B control growth studies

No growth studies using medium B with glucose as the carbon source were made on their own, but as part of other studies involving chromate. Results for *D. denitrificans*, *R. rhodochrous* and *R. opacus* are reported in table 3.1.

3.3.2.2. Initial studies of all bacteria with chromate

As alluded to in the method section above a number of initial studies were carried out on *P. denitrificans*, *P. naphthalenivorans*, *D. denitrificans*, *B. xenovorans*, and *R. rhodochrous*. The first chromate study was made using *P. denitrificans* alone. The behaviour of *P. denitrificans* in medium with and without chromate differed markedly, this was despite the fact that the bacterium is supposed to reduce chromate (Mazoch *et al.*, 2004). Growth in the chromate containing medium was negligible (data not shown). *P. denitrificans* grows well in medium B although this growth is slower and reaches a lower maximum biomass than in medium 81. In the chromate medium containing cells the OD<sub>600</sub> dropped below that of the uninoculated control. Due to the poor nature of the bacterium’s growth the samples were not assayed for chromate (VI) and the growth data are not shown here.

A second study was made this time using all five bacterial types above. Cells grown
on medium B were sub-cultured 1:100 into medium B with 300μM chromate present. Cell growth was compared in medium B with and without chromate and also by a comparison against cell free cultures with medium B with and without chromate by measuring the OD_{600}. Most of the cell types showed no growth on the chromate containing medium (data not shown). *D. denitrificans* and *R. rhodochrous* were the exceptions, although the turbidity dropped for this latter organism below the chromate control without cells before its cell numbers recovered and showed some signs of growth.

All the time zero samples showed a fairly consistent chromate result with the exception of *R. rhodochrous* which was higher. This suggests poor mixing of the culture medium used to grow *R. rhodochrous* when aliquoting it into the flasks. The control showed very little change in Cr (VI) concentrations over the culture time period by the end of the run. Most of the cell types showed no growth on the chromate except *D. denitrificans* and *R. rhodochrous* (data not shown).

Due to the poor growth a repeat of the previous experiment was made using a lower concentration of chromate (in the order of 100μM) with an additional bacterium *E.coli* being tried and a chromate medium without cells used as a blank. The OD_{600} data indicated all the bacterial types had increased or were starting to increase in number by the end of the experiment although only *D. denitrificans* and *R. rhodochrous* had shown any significant increase in cell number shown as OD_{600} in figure 3.1. Similarly only these two organisms showed a modest decrease in chromate (VI) concentration although the other bacteria do appear to be able to tolerate the presence of Cr (VI). The autoclaving of the medium with chromate present reduced the levels by over half compared to the calculated value based on volumes added. The cell number is indicated by OD_{600} since not all bacteria had satisfactory correlations between cell count and optical density established at this point.
Figure 3.1. Initial growth study with chromate using all bacteria in this project. Bacterial turbidity at 600nm is indicated by open squares. Chromate (VI) concentrations are shown by open circles. The chromate control chromate concentration is indicated by the black diamonds with a dashed line. *D. denitrificans* is light blue and *R. rhodochrous* is mauve both with heavier width lines for clarity. The other bacterial types showed a lack of growth and chromate (VI) reduction in marked contrast to *D. denitrificans* and *R. rhodochrous*.

3.3.2.3. Growth studies with medium containing chromate (VI)

Only *D. denitrificans* and *R. rhodochrous* and latterly *R. opacus* showed consistent growth and the ability to reduce Cr (VI). Consequently almost all the work and discussion in the remainder of this chapter will concentrate on these bacteria. Two full growth studies were undertaken as outlined in the materials and methods above for both *D. denitrificans* and *R. rhodochrous* with both chromate free glucose controls and uninoculated chromate medium. These studies were the first studies involving medium B. Table 3.1 shows the characteristics of the cell growth on medium B with and without chromate and other transition metals described later. The growth on medium B for *D. denitrificans* was slower than for the media outlined in chapter 2. table 2.3, but the cells reached a higher biomass. Conversely *R.
*rhodochrous* achieved a faster growth rate but a slightly lower maximum biomass. Both organisms had extended lag phases in medium B compared with their recommended medium.

**Table 3.1.** Growth characteristics of bacteria used for transition metal growth studies including the inhibition studies. Controls (only one of each being shown) were cultured using a carbon source of glucose with the exception of *S. oneidensis MR-1* which used lactate and *D. denitrificans* cultured with iron (III) where the carbon source was glycerol. Note * denotes unshaken cultures with the same concentrations as shaken cultures.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Lag phase (hours)</th>
<th>Maximum cell count (×10⁸)</th>
<th>Generation time (hours)</th>
<th>μmax (hours⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. rhodochrous</em> control</td>
<td>13.27</td>
<td>2.31 x 10⁸</td>
<td>2.34</td>
<td>0.129</td>
</tr>
<tr>
<td><em>R. rhodochrous</em> chromate (205μM)</td>
<td>87.35</td>
<td>6.11 x 10⁷</td>
<td>4.38</td>
<td>0.069</td>
</tr>
<tr>
<td><em>R. rhodochrous</em> chromate (254μM)</td>
<td>64.47</td>
<td>5.39 x 10⁷</td>
<td>10.74</td>
<td>0.028</td>
</tr>
<tr>
<td><em>D. denitrificans</em> control</td>
<td>13.27</td>
<td>1.91 x 10⁸</td>
<td>1.66</td>
<td>0.181</td>
</tr>
<tr>
<td><em>D. denitrificans</em> chromate (209μM)</td>
<td>37.36</td>
<td>2.23 x 10⁸</td>
<td>7.24</td>
<td>0.042</td>
</tr>
<tr>
<td><em>D. denitrificans</em> chromate (195μM)</td>
<td>42.82</td>
<td>1.29 x 10⁸</td>
<td>6.86</td>
<td>0.044</td>
</tr>
<tr>
<td><em>D. denitrificans</em> vanadate (214μM)</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td><em>D. denitrificans</em> vanadate (200μM)</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td><em>D. denitrificans</em> Mo(VI) (2190μM)</td>
<td>19.97</td>
<td>1.48 x 10⁸</td>
<td>2.65</td>
<td>0.114</td>
</tr>
<tr>
<td><em>D. denitrificans</em> iron control (36μM)</td>
<td>25.06</td>
<td>1.48 x 10⁸</td>
<td>6.00</td>
<td>0.050</td>
</tr>
<tr>
<td><em>D. denitrificans</em> iron ferricyanide (57.8μM)</td>
<td>22.61</td>
<td>2.14 x 10⁸</td>
<td>5.92</td>
<td>0.051</td>
</tr>
<tr>
<td><em>D. denitrificans</em> iron control*</td>
<td>36.31</td>
<td>1.02 x 10⁸</td>
<td>23.51</td>
<td>0.013</td>
</tr>
<tr>
<td><em>D. denitrificans</em> iron ferricyanide*</td>
<td>25.81</td>
<td>1.09 x 10⁸</td>
<td>22.52</td>
<td>0.013</td>
</tr>
<tr>
<td><em>S. oneidensis</em> control</td>
<td>11.86</td>
<td>6.57 x 10⁸</td>
<td>1.58</td>
<td>0.190</td>
</tr>
<tr>
<td><em>S. oneidensis</em> chromate (all)</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
<td>no growth</td>
</tr>
<tr>
<td><em>R. opacus</em> control</td>
<td>25.48</td>
<td>2.01 x 10⁸</td>
<td>3.49</td>
<td>0.086</td>
</tr>
<tr>
<td><em>R. opacus</em> chromate (100μM)</td>
<td>25.11</td>
<td>8.09 x 10⁷</td>
<td>9.23</td>
<td>0.033</td>
</tr>
<tr>
<td><em>R. opacus</em> vanadate (214μM)</td>
<td>16.13</td>
<td>2.73 x 10⁸</td>
<td>3.20</td>
<td>0.094</td>
</tr>
<tr>
<td><em>R. opacus</em> copper (10μM)</td>
<td>19.51</td>
<td>1.62 x 10⁸</td>
<td>13.77</td>
<td>0.065</td>
</tr>
<tr>
<td><em>R. opacus</em> copper (333μM)</td>
<td>64.40</td>
<td>3.71 x 10⁸</td>
<td>161.35</td>
<td>0.011</td>
</tr>
<tr>
<td><em>R. opacus</em> Mo(VI) (2190μM)</td>
<td>17.81</td>
<td>4.32 x 10⁷</td>
<td>3.94</td>
<td>0.076</td>
</tr>
<tr>
<td><em>R. opacus</em> iron (300μM)</td>
<td>16.98</td>
<td>2.23 x 10⁸</td>
<td>3.79</td>
<td>0.079</td>
</tr>
</tbody>
</table>
R. rhodochrous was able to reduce the chromate (figure 3.2) marginally, both compared to the control and the initial values of chromate containing cultures according to the chromate assay. There is a considerably longer lag phase compared to the cell growth in medium B chromate free controls and the cell density is an order of magnitude lower. The chromate concentration was determined at 205μM by the assay in both the control and culture flasks. A lower autoclaving temperature of 115°C used rather than 121°C meant the fall in concentration was proportionately lower than usual. Since the results showed only a very modest drop in chromate (VI) levels both compared to the control and the initial starting value, a t-test was carried out. There was no significant difference between the cell free chromate control and the mean cellular chromate value (p>0.1). Despite this lack of significant difference between the control and the cell samples a small reduction in chromate levels could be seen for the last few samples taken in the stationary phase. Thus the experiment was repeated.

Figure 3.2. Growth study for R. rhodochrous comparing growth of cells with and without chromate (VI) present. The left hand axis shows cell number and the right hand axis chromate concentration. The symbol □ represents the cell number for cells with chromate present □ without; ○ chromate concentrations for cell free chromate levels and ○ concentrations of chromate (VI) in the cell cultures. The error bars represent the standard error of the mean for three cultures.

The second growth study is shown below in figure 3.3. The flasks were set-up in
triplicate (254µM chromate) with an additional chromate cell free control (248µM chromate). However, one culture failed to grow and for this reason was excluded from the data shown. The reduction in chromate compared to the cell free chromate flask was larger than the first study. Indeed this time the difference between the chromate control and mean chromate (VI) concentration of the cell cultures was just significant using a t-test (p=0.028).

**Figure 3.3.** Repeat growth study for *R. rhodochrous* comparing the growth of cells with chromate (VI) present. □ represents the cell number for cells with chromate present; ○ chromate concentrations for cell free chromate levels and □ concentrations of chromate (VI) in the cell cultures. The error bars represent the standard error of the mean for duplicate cultures.

The *Rhodococcus* experiments were repeated this time using *D. denitrificans* with the same chromate stock solution volumes added to the flasks (209µM chromate by the assay in the cell culture flasks and 206µM chromate in the control flask). A growth study showed *D. denitrificans* was able to reduce chromate. For the growth study in figure 3.4. all the flasks were set-up in triplicate. However, as with the *Rhodococcus* differential growth rates were seen between flasks, instead of being inhibited completely one chromate culture grew at a faster rate than the other two and for this reason was excluded from the data shown. In this flask the final chromate reduction and maximum cell number was very similar to the other two in the previous study (data not shown).
In the other two cultures the growth rate in chromate medium was lower and the lag phase considerably extended (Figure 3.4), but unlike for *Rhodococcus* the cell density was increased over the glucose controls. There was also a very highly significant difference between the cell free control values and the mean chromate values in the cell samples (p<0.0001) using a t-test. The study was repeated. The findings obtained in the repeat were very similar. A larger reduction in chromate was obtained in the second study, its concentration falling by almost half from an initial concentration of 210µM to 110µM, although the maximum biomass and the growth rate were lower (data not shown). The difference in chromate (VI) levels was very highly significant compared to the chromate cell free control using a Mann-Whitney U test (p<0.001).

![Graph](image)

**Figure 3.4.** First growth study for *D. denitrificans* comparing growth of cells with and without chromate. Chromate (VI) present (right hand axis) and chromate reduction against a cell free control (left hand axis). ▼ represents the cell number for cells with black with chromate present, ▼ for chromate free cell number. Levels of chromate (VI) in the cell cultures ◆ for cultures with chromate ◆ for controls with no cells present. The error bars represent the standard error of the mean for two cultures for Cr (VI) cultures and three for the chromate-free cultures.

In these growth studies the cell morphology was unchanged in chromate cultures.
for both *D. denitrificans* and *R. rhodochrous*. In the case of *D. denitrificans* both long rods and motile cells could be seen with cell size in the normal range for this organism. The cells have been found to be motile when both small and cocci in appearance. As the cells mature they elongate becoming obviously rod shaped. Medium sized motile rods can be seen but the very largest rods appear to lose their ability to move. *R. rhodochrous* had normal sized rods although very large round clumps of cells could be seen.

Rosenberg *et al.* (1980) developed a simple test for microbial adhesion to hydrocarbons (the MATH test) for measuring the adhesion of bacteria to organic non-aqueous solvents and thus their cell surface hydrophobicity. This test is covered in greater detail in chapter four where its use is more relevant. Its use has also been extended to measuring electrostatic cell surface changes (Van der Mei *et al.*, 1993). The test involves the mixing of the bacteria of interest with a non-aqueous hydrocarbon and measuring the optical density of the aqueous portion either before and after treatment or with and without treatment (Rosenberg *et al.*, 1980). The MATH assay affinity data were normally distributed, a t-test showed no statistical difference between cells cultured on glucose only and glucose supplemented with 200μM chromate from two separate sets of cultures for *D. denitrificans*.

Changes could be seen in the medium of both *R. rhodochrous* and *D. denitrificans* when cultured on medium with over 200μM Cr (VI) present. The colour of the medium changed over time to a lighter green and a dark green precipitate appeared in the bottom of these cultures. Such changes were not seen in the cell free control flasks and the change in appearance corresponded to the start of the exponential growth period and beyond. This precipitate was not visible in cultures where autoclaving was not carried out with the chromate *in situ*, which was the case for the repeat growth studies and inhibition studies covered in the next sections below.
3.3.2.4. Growth inhibition studies with chromate (VI) medium

Subsequently several growth studies were undertaken with different levels of chromate ranging from 0-1000μm for *D. denitrificans*. Since the control cell free chromate value was found not to alter greatly over the culture period and since there would not have been room on the shaker for a control flask for each level of chromate, these studies had no cell free control. The results of one growth inhibition study are shown in figure 3.5. At chromate concentrations over 278μM, growth and chromate reduction was negligible. Although there was no cell free control group in the inhibition studies with which to compare the chromate reduction, a technique used in clinical studies is appropriate, that is to use paired tests to look for differences within the particular group (Brady *et al.*, 2004). The data in these tests were divided into two halves, the theory being that any change in transition metal concentration over the cell cycle would be statistically significant. Paired tests cannot use unequal numbers of sample data. In most cases the data sets were even and therefore could be divided in two. In sets of data where there was one too many in one group the first transition metal assay sample result after the culture start was ignored. It was believed this would tend to bias the results against a significant change in concentration. The reduction between one half of the data and the other was statistically significant in the 100μM, 195μM and 278μM cultures (*p*≤0.032) in every case, but for not for 331μM (using one tailed tests).


**Figure 3.5.** Chromate inhibition study for *D. denitrificans*. Graphs a,b,c,d show the effect of increasing levels of chromate on both cell growth and chromate reduction for *D. denitrificans*, ▼ represents the cell number, ○ levels of chromate (VI) in the cell cultures. The error bars represent the standard error of the mean for two cultures. The initial chromate concentrations were 100μM in (a), 195μM in (b), 278μM in (c) and 331μM in (d) as determined by the chromate assay.

The maximum growth rate was determined for cells cultured on each level of chromate and this was plotted against chromate concentration. An attempt was made to fit the data using a variety of kinetic models that have been derived to describe the inhibitory effect of substrates on growth rate. These have been adapted from models for inhibition of enzymes by substrates and are described in greater detail in context in the next chapter. Attempts to fit a variety of models to the data were made using combined data from all the studies with this organism on chromate. The model attributed to Aiba and Shocla was the only one to give a fit (figure 3.6.) in which the model coefficients (one of which is the concentration which was sufficient to affect growth) were in themselves statistically significant. The fact that the coefficients were all statistically significant indicates the model is the correct one for the data.
Chapter 3. Transition metal reduction and c type cytochromes

cconcerned. The Aiba and Shocla model suggests that growth is inhibited by a concentration of chromate above 76.4μM, although the quality of the fit was poor with a degree of scatter.

\[ \text{Figure 3.6.} \] The Aiba and Shocla inhibition model applied to the chromate inhibition data for \textit{D. denitrificans}. The data are a combination of all growth studies on chromate. The R^2 value is 0.864. The K_i is 76.4μM.

A growth inhibition study was undertaken using \textit{R. opacus} on chromate concentrations ranging from 0 to 274μM. The results are shown in figures 3.7. and 3.8 below.
Figure 3.7. Growth inhibition study of *R. opacus* on chromate. Graphs a,b,c and d show the effect of increasing levels of chromate on cell count and Cr (VI) reduction. The □ symbol represents the cell number and ○ Cr (VI) levels. Graph a) 100µM, b) 192µM, c) 237µM and d) 274µM as determined by the diphenylcarbazide assay. The error bars represent the standard error of the mean for two cultures.
Figure 3.8. Glucose and pH measurements in growth inhibition study of *R. opacus* on chromate. Graphs a, b, c and d show the effect of increasing levels of chromate on both cell growth, glucose concentration and pH for this organism. □ represents the cell number, ○ glucose concentrations in the cell cultures and △ pH. Graph a) shows the chromate free control. The initial concentration of chromate as determined by the chromate assay was b) 100μM, c) 192μM, d) 237μM. The 274μM culture where growth was found to be negligible is not shown in this set of charts. The error bars represent the standard error of the mean for two cultures.

The pH and glucose results confirm along with the cell count and chromate assay data that only the cells in the 100μM chromate culture showed any real ability to reduce the chromate (VI), lowering the total concentration of Cr (VI) by about 35%. The 192μM chromate cultures were a little more effective in their ability to reduce chromate (VI) than *R. rhodochrous* at roughly the same concentration, although their cell growth was not as good. As above paired t-tests were used to look for significant changes within the concentration groups. The 100μM and 192μM cultures showed statistically significant falls in chromate concentration between the first half of the time period and
the second half (p=0.002 and p=0.025). All the cultures with chromate went into death phase at the same point in time (76 hours). Again an attempt was made to fit these data to the kinetic models as described above. As before only the Aiba and Shocla model gave a significant fit with a much better R² value than was found with the *D. denitrificans* data (figure 3.9 below).

![Graph](image)

**Figure 3.9.** Inhibition fit using the Aiba and Shocla for *R. opacus* cultured in media containing chromate. Initial chromate (VI) concentrations in media containing glucose are plotted against the maximum growth rate for the cultures, R² = 0.956 and Kᵢ = 38.9µmol/l chromate.

An inhibition study was set up using duplicate flasks with *S. oneidensis MR-1* cultured in the presence of 100µM, 200µM, 300µM and 400µM chromate (VI) plus a chromate free control. The carbon source was 22mM lactate. Only the chromate free control grew, although motile cells were seen in the 100µM chromate culture. Due to these results no assays were carried out on the chromate cultures.

No formal growth studies were carried out on the Blue Lake isolates with chromate but one culture of each was set-up with approximately 100µM of chromate (VI) in medium B with acetate as the carbon source. All three had cells that grew in the presence of chromate. Cells in BL001 grew with a long lag phase (over a week). BL002 and BL003 grew more quickly. The latter two cultures went a yellow green
colour whereas BL001 stayed the original green colour. This suggests some of the cohort of cells in BL002/3 were capable of chromate reduction.

3.3.3. Studies involving other transition metals

3.3.3.1. Vanadium

Since the diphenylcarbazide assay will also measure vanadium (V) levels it was decided to try to grow cells in the presence of this metal. However, growth of <i>D. denitrificans</i> with 214μM vanadium (V) was very poor, the maximum cell density reached was in the order of 1.43 x 10<sup>7</sup> cells per ml (figure 3.10). Consequently no vanadium (V) assay was carried out on the cell samples.

![Graph showing cell number over time](image)

**Figure 3.10.** Growth study of <i>D. denitrificans</i> on vanadium (V). The error bars show the standard error of the mean for three cultures.

Unlike <i>D. denitrificans</i>, <i>R. opacus</i> grew well in the presence of this transition metal at the same initial concentration (figure 3.11). Its growth rate was more rapid than had been found previously with glucose only and its cell density was also higher than with glucose alone. Cells from one flask were examined under the light microscope, no contamination was seen and the cells were of the normal size and
morphology. The vanadium (V) assay was carried out on the cell samples. Some rise in V(V) value was seen in the cell free vanadium control and the *R. opacus* cultures with vanadium present midway through. However, at the end the control value was only slightly higher than its initial value with cells present and within 1μM of the mean value for all the flasks with no cells present pre-inoculation. Whilst there was little change in vanadium (V) concentration in the cell free control the *R. opacus* cultures showed an approximately 50% reduction over the mean V(V) starting value. A statistically significant reduction was seen using a t-test compared to the cell free vanadium control (p=0.0083).

![Graph](image)

**Figure 3.11.** Growth study for *R. opacus* cultured in the presence of an initial concentration of 214μM vanadium (V). In graph a), □ represents the cell number and ◊ levels of vanadium (V) in the cell free medium, and ◊ for cultures with vanadium and cells. In b), □ represents the cell number for cultures in the study with cells and vanadium present, △ pH levels and ○ glucose concentration. The error bars represent the standard error of the mean for two cultures.
No blue colouration was seen to indicate the formation of vanadium (IV). However, approximately 72 hours after the growth study was stopped the culture changed colour from grey to a light sea green precipitate (in both cultures). A sample was taken and examined under the microscope. A precipitate could be seen with cells in between them as shown in figure 3.12. No such colour change was seen in the abiotic control and no precipitate could be seen.

Figure 3.12. Precipitate formation in *R. opacus* cultured in the presence of vanadium (V). The smaller rod like cells can be seen scattered in between the larger crystals.

A culture was grown using glucose as the carbon source with a vanadium concentration of approximately 950µM V(V). Growth, although not measured (the culture was intended for spectroscopic scanning to detect c-type cytochromes), was by eye extremely rapid. Within 24 hours of inoculation the culture colour went from slate grey to a dark green (darker than described above). Over the next few days the culture lightened in colouration comparable to that above.

One flask of each Blue Lake isolate was cultured in medium B with approximately 1000µM V(V) and acetate as the carbon source, again not a formal growth study due to time considerations. The results are shown in figure 3.13. All the cultures grew producing their normal cell suspension colours but with no sign of
any vanadium IV precipitate as seen with the *R. opacus* above.

![Image of flasks](image)

**Figure 3.13.** The Blue Lake isolates cultured on medium containing molybdenum and vanadium. Molybdenum concentration was 2112µM in the front row and vanadium 1000µM in the back row. The flasks are BL001-3 in the order left to right. The cultures were found to be normal in colour with no sign of any coloured precipitates.

### 3.3.3.2. Iron

*D. denitrificans* was also cultured in the presence of iron (III) ferricyanide (21.8µM) added twice using glycerol as a carbon source. In both stirred and unstirred cultures the cell density reached higher levels than the ferricyanide free controls and both the utilisation of glycerol and the fall in pH were larger (figures 3.14, 3.15 and 3.16). In addition the culture medium in the ferricyanide flasks changed colour to a grey blue. In the stirred culture the utilisation of glycerol with and without ferricyanide was compared using a t-test and found to be highly significantly different (*p*=0.004). In the study in which the flasks were not stirred, the difference between the glycerol utilisation in cultures with and without ferricyanide was less marked and was not significantly different using a t-test. It should be noted that the medium B contains iron (II) sulphate at a concentration of 36µM, so it is not entirely iron free.
Figure 3.14. *D. denitrificans* growth study in stirred cultures with and without ferricyanide. 100μl of ferricyanide was added at time zero and again at 27 hours. Cells cultured in the presence of ferricyanide are coloured red ▼ and the controls are black ▼. The carbon source (glycerol) is indicated by the ○ symbol and the same colours. Note there was close agreement for initial glycerol levels in the culture flasks before the cells were added, being 560mM for the control and 583 mM for the ferricyanide cultures. The error bars represent the standard error of the mean for two cultures.
Figure 3.15. *D. denitrificans* growth study in unstirred cultures with and without ferricyanide. 100μl of ferricyanide was added at time zero and again at 28.25 hours. Cultures in the presence of ferricyanide are coloured red ▼ and the controls black ▼. The carbon source (783mM glycerol) for both cultures is indicated by the ○ symbol and the colours the same as the cell counts. The error bars represent the standard error of the mean for two cultures.
Figure 3.16. Growth and pH data for *D. denitrificans* cultures in the ferricyanide studies. a) shaken culture b) unstirred culture, both controls without ferricyanide. The carbon source was glycerol, cell counts are indicated by the ▼ symbol and pH by the △ symbol. The error bars represent the standard error of the mean for two cultures. Growth was much stronger in the shaken culture.

The iron (II) was assayed by the ferrozine assay. The results are shown in figures 3.17 and 3.18. In the stirred cultures there was no statistical difference between the levels of iron (II) in the control and ferricyanide cultures, (*p*<0.35 Mann-Whitney U). In the unstirred cultures the levels of iron (II) were much higher in the ferricyanide cultures, fully reflecting the extra iron added over that already present in the medium B (although iron reduction apparently took place in the controls too). As for the chromate cultures the control and ferricyanide cultures’ Fe(II) data were tested using paired t-tests. All the stirred cultures and the unstirred cultures (both controls and with ferricyanide added) showed significant elevations in iron (II) concentration (*p*=0.01) over the two halves of the cell culture periods.
Figure 3.17. Iron reduction data for *D. denitrificans* growth study in stirred culture with and without ferricyanide. 100μl of ferricyanide was added at time zero and again at 27 hours. Graph a) shows control cells cultured in the absence of ferricyanide b) with ferricyanide. Cells are indicated by (▼) and Fe(II) concentration by Δ. The carbon source was glycerol. The error bars represent the standard error of the mean for two cultures. Both cultures had a basal level of 36μ-M iron sulphate.
Figure 3.18. Iron reduction data for *D. denitrificans* in unsterred cultures with and without ferricyanide. 100μl of ferricyanide was added at time zero and again at 28.25 hours. Graph a) shows control cells cultured in the absence of ferricyanide b) with ferricyanide. Cells are indicated by (▼) and Fe(II) concentration by △. The carbon source was glycerol. The error bars represent the standard error of the mean for two cultures.

*D. denitrificans* was cultured in a single flask with 3166μM iron (III) ferricyanide and both growth and reduction was seen by the colour change (figure 3.38). The cells were examined under the optical microscope, the cells were smaller than normal but a very large number were present. A repeat was made using a flask containing 4073μM iron (III) ferricyanide. Growth was seen but no reduction.

*R. opacus* was cultured in the presence of 200μM iron (III) sulphate with glucose as the carbon source. The iron compound was switched since the ferricyanide can be toxic to bacteria (Kim *et al.*, 2008). The results are shown in figure 3.19. Although the levels of soluble iron (II) rose over the cell cycle (figure 3.19.d), the total iron (II) present in the cultures showed little overall change (figure 3.19.c). The cell free
control levels also showed no change (figure 3.19.d). There was no significant
difference between the levels of iron (II) in the controls and the cell samples
(p=0.327) using a t-test. The cells did not utilise all the glucose nor did the pH fall as
low as in the controls cultured previously.

Both D. denitrificans and R. opacus were streaked onto plates with iron (III)
sulphate at approximately 300μM. Once monocultural growth was seen, ferrozine
reagent was added. The D. denitrificans plate showed an immediate change to the
purple coloured complex. The R. opacus plate took almost 24 hours to change, but
the same purple colouration was seen.

Figure 3.19. Growth study for R. opacus cultured on glucose in the presence of iron
(III). Graph a) shows the cell count (□) versus glucose concentration (○), b) shows
cell number (□) and pH (△), c) the total Fe(II) change over the culture period (●), d)
shows both the soluble Fe(II) concentration (○) and the Fe(II) concentration (x) in the
cell free control. The error bars show the standard error for two cultures.

Hydrogen peroxide can either be an oxidising or reducing agent and thus could be
potentially oxidising or reducing the iron present in the cultures and since *R. opacus* is known to produce this compound the cell samples were assayed using the hydrogen peroxide assay (figure 3.20). A straight-line fit was obtained over the hydrogen peroxide standard range with an $R^2$ value of 0.9983. No overall trend was seen in the iron culture samples. No significant correlation was found between cell growth, total Fe(II) or soluble iron concentration. Comparisons and the background for the hydrogen peroxide assay will be covered below in the context of the copper results and the overall discussion.

**Figure 3.20.** Hydrogen peroxide measurements for *R. opacus* cultured on glucose in the presence of iron (III). Results show the cell count (■) versus hydrogen peroxide concentration (▲). The error bars show the standard error for two cultures.

*R. ruber* was cultured in the presence of approximately 619µM iron (III) ferricyanide. No reduction was seen but some growth occurred (figure 3.38).

The Blue Lake isolates tolerated very high concentrations of iron (III). Cells survived up to 25mM iron (III) sulphate. It was hard to compare growth at this concentration of iron (III) due to its low solubility, but sub-culturing of all these cultures produced viable growing cultures. BL003 cultures went almost black over
time in the presence of 2mM of iron (III) sulphate.

3.3.3.3. Copper

One flask of *R. opacus* and one flask of *D. denitrificans* was cultured in the presence of 200μM copper (II) sulphate. Almost no growth was seen by eye in the case of the latter, however, some growth was seen in the former, albeit with a long lag phase. Cells were passaged from both flasks into medium B with 2g/l glucose and both managed to grow.

A full inhibition growth study was carried out for *R. opacus* with five different concentrations of Cu(II) (10μM, 18.9μM, 89.7μM, 102.3μM and 333.5μM) as determined by the BCS assay. 2g/l glucose was the carbon source in these cultures. Copper (II) inhibited cell growth in that it elongated the lag phase and reduced the maximum growth rate with increasing concentration. However, the more copper (II) present initially, the higher the maximum cell density. The cultures broadly fell into two types of responses to the differing levels of copper (II) (figure 3.21). Cultures containing the two lowest concentrations grew to normal levels of biomass, over only very slightly elongated time periods, with marginally slower maximum growth rates. The cultures with the three highest concentrations of copper also grew but over a very long time period with periodic drops in cell density and much slower maximum growth rates, but they ultimately achieved the highest cell density. These falls in cell density which appeared to indicate the end of exponential phase had been reached were followed by a recovery in cell number and yet more cell growth and can be seen on the graph in figure 3.21 at time periods between 25-80 hours. The cultures with the three highest copper concentrations also had an even lower pH than had been achieved previously and grew over a longer time period at lower pH’s than had been seen before. Each grew over 100 hours at a pH of less than four. All the cultures in this study went an intense pink. The results are shown in figures 3.22 and 3.23.
Figure 3.21. Regression plots using the four parameter gompertz curve for *R. opacus* cultures in the copper (II) inhibition study. Initial concentrations (μM) of copper (II) are shown in the legend. All the R² values were in excess of 0.982. The difference in growth characteristics between the lowest two and highest three initial concentrations can clearly be seen.
**Figure 3.22.** Glucose and pH measurements for the growth inhibition study of *R. opacus* with copper. The graphs show the effect of increasing initial levels of copper (II) on cell growth, pH and glucose concentration. The □ symbol represents the cell number, ○ glucose levels, Δ pH. Graph a) shows the copper free control b) 10μM, c) 18.9μM, d) 89.7μM e) 102.3μM and f) 333.5μM as determined by the BCS assay. The error bars represent the standard error of the mean for two cultures. Note the glucose data are potentially erroneous in d,e and f).

The cultures with the three highest Cu(II) concentrations apparently gave inaccurate results using the glucose stick assay method. In effect the glucose measurements indicated the cultures had used little or even produced glucose over the culture period.
which is unrealistic. Thus figure 3.22 d), e) and f) show glucose stick data only for reasons of completeness. Using the Sigma glucose assay kit gave broadly similar results (data not shown). Both these assays use chemistry based on hydrogen peroxide. The assumption was made that the cells were generating hydrogen peroxide and that the use of the phenol sulphuric acid method would overcome this. This it failed to do showing an even larger apparent increase in glucose levels (data not shown). When 20µl of 30 volume hydrogen peroxide was added to a sample in a cuvette the solution went dark black with an OD >2.5. This could mean that hydrogen peroxide is interfering with the assay.

The diphenylcarbazide and alizarin red assays for Cu(II) failed to give consistent results with the value of the copper (II) concentration apparently increasing markedly over the culture cycle in some assays and showing little change over the growth cycle in others (data not shown). In addition the starting values (pre or time zero) were in most cases inaccurate although some of the diphenylcarbazide assays were near in value to what was expected. The QC values in the diphenylcarbazide assay varied widely and the brown yellow colour was unstable. The alizarin assay cross reacted strongly with iron (II) when this compound was added to a test cuvette. The cuproin assay showed no colour in the standard curve, but a mauve colour was seen for the samples tested, indicating some Cu(I) was present.

Due to the presence of this colour in the cuproin assay which showed some copper (I) was present it was decided to attempt to find a reliable assay for either Cu(I) or Cu(II). The preference was for an assay for copper (I) since a compound that reacts with this oxidation state should not cross react with either copper (II) or iron (II). A literature search on the internet did locate the BCS assay albeit with some difficulty. The methods found in the literature required adaptation (Eaton, 1995; Rapisarda et al., 2002) due to the different sample volumes available. A series of titrations were carried out in cuvettes to optimise the relative ratios and concentrations, the aim being to ensure that the reducing agent was being added in a clear excess so that all the copper (II) present was reduced to copper (I). It is believed this was achieved after a series of experiments for two reasons. Firstly, a straight line curve was obtained over a very
wide concentration range and to a much higher concentration than should have been present in the cultures. Second, the orange coloured complex was stable for at least 24 hours at room temperature. In effect by reading each sample twice with and without ascorbic acid and taking the difference between the results the assay is able to assay for copper in both oxidation states.

In the top two standards a yellow colour was seen on addition of the BCS to the cuvette before the addition of the ascorbic acid. A spectroscopic scan of these cuvettes indicated that no significant absorption took place at 484nm which is the wavelength of interest in the assay (data not shown). The assay was reliable over wide concentration range with very low between and within assay variation as outlined in the appendices (figure 4 and table 3). A test was made for cross reactivity with sodium, iron (II) and copper (II) by adding sodium chloride, iron (II) sulphate and copper (II) sulphate to cuvettes with BCS. None was seen.

The transition metal values obtained using this assay are shown in figure 3.23. Surprisingly there was broad agreement with the general finding for the copper (II) concentration changes found in the other assays, in that the copper (II) levels rose over the growth cycle. Unlike the diphenylcarbazide and alizarin red assays the level was found to be very low at time zero in every culture, although the concentrations in the cell free “pre” samples were in most cases close to those expected. For the lowest copper concentration cultures the Cu(II) level was not measurable since this is at the limits of detection of the assay. The initial concentration reported is that expected from the volume added. Thereafter in every culture the Cu(II) level rises. The same is true of copper (I), its level was low to undetectable in most cultures at the start and then its concentration also rose steadily through the growth cycle. Surprisingly due to its instability in air there were low levels detectable of Cu(I) in the pre-culture samples. However, in every case the level increased over the cell growth cycle beyond this initial value.
Figure 3.23. Copper (I) and copper (II) measurements for the growth inhibition study of *R. opacus* with copper. Graphs show the effect of increasing levels of initial copper (II) concentration on cell growth and copper (I) and copper (II) concentration over the cell growth cycle. The □ symbol represents the cell number, ○ glucose levels, △ pH, △ Cu(I) and ▽ Cu(II). Graph a) shows the copper free control with pH and glucose concentration b) 10μM, c) 18.9μM, d) 89.7μM e) 102.3μM and f) 333.5μM as determined by the BCS assay. The error bars represent the standard error of the mean for two cultures.

Data analysis was carried out to see if there was a significant correlation between the cell number and both the copper (I) and copper (II) increase. The same statistical
methodology was used to analyse cultures of this organism and other organisms in the presence of the other transition metals used in this project. The results are shown in tables 3.2 and 3.3.

**Table 3.2.** Correlation between cell growth and copper (I) and copper (II) concentrations for *R. opacus*.

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<th>initial copper (II) (μM)</th>
<th>number of samples</th>
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<th>P value</th>
<th>Cu(II) correlation</th>
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</tr>
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<td>14</td>
<td>0.983</td>
<td>&lt;0.0001</td>
<td>0.952</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

In every set of cultures the copper (I) concentration had a statistically significant correlation with cell growth. However, only in the cultures with the three highest initial copper (II) concentrations was there a significant correlation between cell growth and the increase in copper (II) concentration. *R. opacus* also gave significant correlations with the declines in other transition metals (chromate (VI) and vanadium (V)) concentrations over the cell growth cycles (table 3.3)).

As described earlier in the chromate inhibition studies, paired t-tests were used to examine whether a statistically significant change in copper (I) concentration had taken place over the cell cycle. Using this test there was a significant change in concentration in the mean of all culture duplicates except the 89.74μM cultures, although in no case was it highly significant (p>0.01).
Table 3.3. Correlation between cell growth and transition metal reduction. * denotes initial concentration; the same amount was added again during the course of the experiment. The contribution of iron from the iron (II) in the medium B is 36µM.

<table>
<thead>
<tr>
<th>organism</th>
<th>transition metal</th>
<th>concentration (µM)</th>
<th>number</th>
<th>correlation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. opacus</td>
<td>V(V)</td>
<td>214</td>
<td>8</td>
<td>-0.921</td>
<td>0.0012</td>
</tr>
<tr>
<td>R. opacus</td>
<td>Cr (VI)</td>
<td>100</td>
<td>12</td>
<td>-0.904</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>R. opacus</td>
<td>Cr (VI)</td>
<td>192</td>
<td>9</td>
<td>-0.725</td>
<td>0.0271</td>
</tr>
<tr>
<td>R. opacus</td>
<td>Fe (III)</td>
<td>300</td>
<td>8</td>
<td>0.712</td>
<td>0.0476</td>
</tr>
<tr>
<td>R. opacus</td>
<td>Mo(VI)</td>
<td>2190</td>
<td>9</td>
<td>0.539</td>
<td>0.1335</td>
</tr>
<tr>
<td>R. rhodochrous</td>
<td>Cr (VI)</td>
<td>254</td>
<td>12</td>
<td>-0.872</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Cr (VI)</td>
<td>209</td>
<td>15</td>
<td>-0.890</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Mo(VI)</td>
<td>2190</td>
<td>8</td>
<td>0.979</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Fe (III)</td>
<td>0</td>
<td>11</td>
<td>0.985</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>control (shaken)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Fe (III)</td>
<td>21*</td>
<td>11</td>
<td>0.669</td>
<td>0.0243</td>
</tr>
<tr>
<td>ferricyanide (shaken)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Fe (III)</td>
<td>0</td>
<td>9</td>
<td>0.677</td>
<td>0.0450</td>
</tr>
<tr>
<td>(unshaken) control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. denitrificans</td>
<td>Fe (III)</td>
<td>21*</td>
<td>11</td>
<td>0.778</td>
<td>0.0048</td>
</tr>
<tr>
<td>ferricyanide (unshaken)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As previously for chromate, an Aiba and Shocla regression fit was made using the maximum growth rate data obtained in the inhibition study to determine which concentration of copper (II) inhibited cell growth (figure 3.24). The Edwards model gave a good fit ($R^2$=0.96) but the model coefficients were not significant.
**Figure 3.24.** Inhibition modelling for *R. opacus* cultured on glucose with copper (II). Initial copper (II) concentration in each culture is plotted against maximum growth with an inhibition fit using the Aiba and Shocla model, Passos *et al.* (1993), $R^2 = 0.970$, $K_i = 18.9\mu M$ copper (II).

A plate impregnated with copper (II) with glycerol as the carbon source was streaked with *R. opacus*. After several days of growth 2.5ml of BCS was added. The colour change was not immediate but those areas where the BCS was in contact with the cells formed the characteristic orange coloured complex with the BCS.

As outlined above, due to fact that hydrogen peroxide can act as a reducing agent or oxidizing agent and *R. opacus* is known to produce hydrogen peroxide, the cell samples were assayed using the hydrogen peroxide assay (figure 3.25).
Figure 3.25. Growth inhibition study of *R. opacus* with copper showing the effect of increasing initial levels of copper (II) on both cell growth and hydrogen peroxide concentration. The □ symbol represents the cell number and the △ peroxide levels. Graph a) shows the copper free control with pH (Δ) and glucose concentration (○), b) 10μM, c) 18.9μM, d) 89.7μM e) 102.3μM and f) 333.5μM copper (II) as determined by the BCS copper assay. The error bars represent the standard error of the mean for two cultures.

In the cultures containing the four lowest initial copper concentrations a drop was seen in peroxide levels in the cell supernatant over the culture period while for the highest concentration an increase in hydrogen peroxide levels over the culture period.
was found.

No significant correlation was found between cell growth and copper (I) or (II) concentration except for the 333.5μM cultures. For these cultures there was a spearman's rank correlation of R=0.8589, p=0.0015 between cell number and peroxide concentration and pearson's correlations of R=0.7927, p=0.0062 for copper(I) and R=0.7927, p=0.0163 for copper (II) and peroxide concentration. ANOVA revealed no significant differences between hydrogen peroxide levels in any of the five different copper concentration cultures, although the concentrations were higher with the highest initial copper concentrations. There was however, a significant difference between the highest copper concentration culture and the *R. opacus* iron culture which were both cultured on approximately the same concentration of the different transition metals, the peroxide concentration being significantly higher with the iron culture (p=0.0188).

As above a single flask was set-up with approximately 600μM of copper (II) sulphate for each for the Blue Lake isolates. None of the isolates grew very well in copper containing medium. Some modest growth was seen for BL003 although the medium had a light yellow grey colouration rather than the dark brown as seen in figure 2.7 where cells were cultured in the presence of iron, suggesting bacteria that dominated normally were inhibited by the copper. Very slight growth was also seen for BL001, but none for BL002.

3.3.3.4. Molybdenum

*R. opacus* was cultured in duplicate flasks in medium B containing 2190μM of this transition metal. Growth was rapid, almost as fast as the control (table 3.1) however the maximum cell density achieved was low compared to growth with other transition metals, although all of the glucose was utilised. The culture changed from white/grey to slate blue during the culture period. No colouration was seen in the cell free control. The cell cultures were left shaking for approximately another two weeks. Over this time they changed to a brown colour followed by a bright pink colour commonly seen
in these cultures (figure 3.26).

**Figure 3.26.** Image of flasks of *R. opacus* cultured in medium containing 2190μM molybdenum (VI). Flasks 1 and 2 were used in the molybdenum growth study described in this chapter. Flask 3 was sub-cultured from flask 1 used in the growth study. All contained 2190μM molybdenum. The medium in flasks 1 and 2 has changed colouration from grey via blue to brown to pink in colour. The distinctive blue colouration of molybdenum blue can be seen in flask three, although in time it also changed to bright pink.

At 71 hours a sample was examined under the microscope. Normal sized cells were seen with a few large clumps of cells and no contamination. At 168.5 hours all the glucose was utilised (or at least it was at less than 1.1mmol/l) (figure 3.27). The cells were not killed by the molybdenum and could be cultured on.
Figure 3.27. Results for the growth study of *R. opacus* cells cultured using glucose as the carbon source in the presence of molybdenum (VI). Graph a) shows cell count (□), pH (▲) and glucose (..................................). Note (..................................) show the glucose levels in the cell free control. Graph b) shows the cell count and pH (▲). Graph c) shows molybdenum blue results for both the cell cultures (+) and control (+) against cell count. At 186.5 hours both cultures gave a low reading on the glucose stick meter. This is shown as 1.1mmol/l on graph (a). The error bars show the standard error of the mean for two cultures.

The optical density of the cell cultures was measured at 865nm. No significant correlation was found between cell growth and molybdenum blue concentration (table 3.3). There was a significant difference between the concentrations of molybdenum blue in the cell free control and the mean of the cell sample levels, (**p**=0.0342) using a *t*-test. A sample was removed at 50 hours for a wavelength scan, the results of which are described in the next section.

*D. denitrificans* was also cultured in a medium containing this transition metal. It was also found to be capable of growth. A growth study was carried out using the same
methodology as for *R. opacus* with one difference, the media contained only iron sulphate and no ammonium sulphate (see chapter seven). Growth was rapid and the culture grew to a maximum biomass that was almost as high as in the control cultures carried out previously (table 3.3). The pH fell and as the cells entered death phase there was measurable glucose remaining and a strong blue colouration was seen in the culture flasks but not the control (figure 3.28).

Figure 3.28. Image of flasks of *D. denitrificans* cultured in medium containing 2190μM molybdenum (VI). The distinctive blue colouration of molybdenum blue can be seen in the centre and right flask, the left flask is the cell free control.
Figure 3.29. Results for the growth study of *D. denitrificans* cultured using glucose as the carbon source in the presence of molybdenum (VI). Graph a) shows cell count (○), pH (▲) and glucose (●), (●) show the glucose results in the cell free control and (▲) the pH in the control culture. b) shows molybdenum blue levels for both the cell cultures (+) and control (+). The error bars show the standard error of the mean for two cultures.

The cells were assayed in the same way as the samples from the *R. opacus* cultures. The results are shown in figure 3.29. There was a significant difference between the molybdenum blue concentration in the cell free control and the cell cultures using a t-test (p=0.0186) and a highly significant correlation between cell density and molybdenum blue concentration (R=0.9797, p<0.0001).

The Blue Lake isolates were cultured with 300μM of Mo(VI) (one flask of each). Growth was inhibited completely for BL001 (figure 3.13 above). Cell types grew in the other two isolates, with normal culture colours seen. However, no sign of reduction was seen in that no distinctive precipitate was seen in the two cultures that did grow in the presence of this transition metal.
3.3.4. Cytochrome studies

Cells were taken from *R. opacus* in the stationary phase of cultures containing 100μM and 192μM chromate and the cells were examined for the presence of c-type cytochromes. Characteristic peaks could be seen at 549-552nm with a shoulder at 520nm, but not 420nm in the whole cell spectrum and with another peak at 603nm with cells in the 192μM chromate culture reduced spectrum. The oxidised state whole spectrum is fairly featureless as is the reduced spectrum for 100μM chromate. In the difference spectra the same is seen, but the peak around 550nm runs from 549-555nm rather than 552nm with a very small peak at 548nm, in addition there is a peak at 450nm. Figure 3.30 appears to show a peak at 410nm, in actual fact this is an artefact of the plot and not a true peak, 410nm is the shoulder of a large rise in optical density difference between the oxidised and reduced spectra and not a recognisable peak.
Figure 3.30. Spectra for R. opacus cultured in the presence of chromate. Plot a) shows a wavelength scan for R. opacus cultured with glucose in the presence of chromate. The red line shows the optical density for cells in the reduced state cultured in the presence of 192μM Cr (VI), the black line shows the optical density for cells in the oxidised state cultured in the presence of 200μM Cr (VI), the blue line is cells in the reduced state cultured in the presence of 100μM Cr (VI). Some of the wavelengths of interest are shown by arrows. Plot b) shows the optical density difference (difference spectrum), between optical densities oxidised minus reduced for the 200μM Cr (VI) cultures.

Bacterial cells were taken from R. opacus cultures in the stationary phase grown with vanadium (V). Figure 3.31 a) and b) show both the whole cell scan and a difference spectrum. No peaks characteristic of c-type cytochromes were seen.
Figure 3.31. Spectra for *R. opacus* cultured in the presence of vanadium. Plot a) shows a wavelength scan for *R. opacus* cultured on glucose in the presence of V(V). The red line shows the optical density for cells in the reduced state cultured in the presence of 200μM vanadate, the black line shows the optical density for cells in the oxidised state cultured in the presence of 200μM vanadate. Plot b) shows the optical density difference (difference spectrum) oxidised minus reduced.

One flask from each copper concentration culture was sampled and spectroscopic scans carried out to detect the presence of c-type cytochromes. The results are shown in figure 3.32. There is some slight evidence of c-type cytochromes for the highest three concentrations of copper (figure 3.32 inset graph). Very small peaks are seen around 518-520nm and a shoulder at 552nm (arrowed on the inset graph). There is also a peak at approximately 430nm (figure 3.32 ringed) whose expression varies with the initial copper (II) concentration.
Figure 3.32. Difference spectra for *R. opacus* cultures in the presence of five different concentrations of copper (II). The initial concentrations of Cu(II) are shown in the legend in μM. The inset shows the mean optical density difference for the highest three copper concentration cultures. The peaks at approximately 430nm are ringed and the peaks referred to in the text are arrowed.

Scans from *R. opacus* cells grown with iron (III) and Mo(VI) showed no definite signs of the expression of c-type cytochromes (figure 3.33), although a similar peak at 430nm was seen in the molybdenum culture. It is present in the iron cultures, but is so small that it may be noise.
Figure 3.33. Spectroscopic difference scans, oxidised minus reduced for *R. opacus* cells. The results are shown on the same x and y scales for cells grown in the presence of a) iron (III) and b) Mo (VI). The arrow in b) points to a peak at approximately 430nm similar in size and shape to that seen when the same organism was cultured with copper (II). The large peak at approximately 700nm is due to the molybdenum blue colouration.

Spectroscopic scans of *D. denitrificans* cell cultures grown in the presence of molybdenum revealed no obvious peaks due to c-type cytochromes. In cells of this organism grown with chromate (VI) present, distinctive peaks were seen at 409, 412 and 418-420nm (figure 3.34). There is noise on the regions of the spectrum at 520 and 552nm, but there are peaks at approximately these wavelengths. The difference spectra for both these treatments were different to those seen before. The molybdenum difference spectrum altered dramatically with time after the addition of the reducing agent and there was no peak seen in the chromate culture between 350-400nm.
Figure 3.34. Spectroscopic difference scans between oxidised and reduced *D. denitrificans*. Results for cells shown on the same x and y scales cultured in the presence of a) Mo(VI) and b) Cr (VI). Chart a) shows the difference spectra over a time course in minutes after addition of the dithionite. At time zero the same small peak at 430nm (arrowed) can be seen as found in *R. opacus* cultured with copper and molybdenum. b) shows a clear peak at 409-412nm with a peak at 418-420nm (both arrowed).

*D. denitrificans* cells cultured with iron (III) ferricyanide both shaken and unshaken were also scanned. No sign of c-type cytochromes was seen (figure 3.35). However, the same peak at 430nm seen in the cultures of *R. opacus* with copper can clearly be seen in the shaken culture.
Figure 3.35. Spectroscopic difference scans, oxidised minus reduced for *D. denitrificans* cells. Results are shown on the same x and y scales for cells grown in the presence of iron (III). Chart a) shows the difference spectrum in shaken cultures, the same small peak at 430nm can be seen as found in *R. opacus* cultured with copper and molybdenum. b) shows the unshaken cultures.

3.3.5. PAGE studies

Bacteria can potentially reduce transition metals or transfer electrons to anodes using soluble redox shuttles some of which are proteins. An attempt was made to detect any soluble proteins in the cell supernatant. Samples were taken from *R. opacus* and *D. denitrificans* cells cultured in the presence of copper, vanadium and iron and loaded on to a native pagegel and stained with coomassie blue. No proteins were detected (figure 3.36).
Figure 3.36. Native pagegel stained with coomassie blue. Lanes 1-6 represent cultures of cells grown in the presence of iron, copper, vanadium and chromate.
3.4. Discussion

The ability of a number of bacterial types, both Gram-positive and Gram-negative, to reduce chromate (VI) and some other transition metals (molybdenum, vanadium, copper and iron) was investigated. Establishment of culture and assay systems for these transition metals has been achieved. Research has shown that bacteria can transfer electrons from their electron donors to a variety of transition metals using extensions to their electron transport chains (Leang, et al., 2003; Lloyd, 2003), although there are other mechanisms (Gonzalez et al., 2003). In principle bacteria able to transfer electrons to metals in the environment are able to transfer them with high efficiency to an anode in a fuel cell (Bond and Lovley, 2003). The aim of the work in this chapter was to examine whether the bacteria being used are able to directly or indirectly transfer electrons to transition metals. This would give an indication that the bacteria are able to transfer electrons directly to an anode in a microbial fuel cell. Failure would not necessarily preclude the bacteria from working in a microbial fuel cell. There are a variety of different electron transfer mechanisms available and the pathways for metal reduction appear (at least in some cases) specialised to a particular metal (Myers and Myers, 2001; Viamajala et al., 2002).

3.4.1. Chromate reduction

Groups that have investigated the ability of bacteria to reduce chromate are concerned with the ability of micro-organisms to ameliorate the effects of chromate (VI) pollution in soil at industrial sites, as well as to study the cellular mechanisms of transition metal reduction (particularly by c-type cytochromes). Lovley & Philips (1994) localised the reduction of chromate (VI) by the organism *Desulfovibrio vulgaris* to its c3 cytochrome (Lovley & Philips, 1994). Myers et al. (2000) also localised the chromate reduction activity to the cytoplasmic membrane of *Shewanella putrefaciens*.

The bacteria in this study so far fall into three groups regarding their ability to cope with Cr (VI) in their growth medium. *P. naphthalenivorans* seems unable to grow on or reduce chromate to any significant extent, although it should be stated that the growth of this organism was found to be poor on any medium tested. *B. xenovorans, P.*
*denitrificans* and the *E. coli* strain tested appear to be able to tolerate the chromate showing little Cr (VI) reduction, but showed little if any growth, even at levels below 100μM. *B. xenovorans* also does not grow well in medium B. Finally *D. denitrificans R. opacus* and *R. rhodochrous* have shown consistent growth and reduction of the chromate, *D. denitrificans* reaching a higher cell density with chromate than without it in the culture medium.

This growth of *D. denitrificans, R. opacus* and *R. rhodochrous* with chromate had the following characteristics; a long lag phase before any growth took place and incomplete reduction, with maximum Cr (VI) reduction reached before the end of exponential phase where samples were taken beyond this. A green precipitate appeared when bacterial growth got under way accompanied by a drop in the turbidity of the culture medium containing cells below that of the uninoculated control, both occurring when chromate (VI) was added to the medium before autoclaving. Flocs as described by Nalli *et al.* (2006) were also seen in some chromate cultures.

Other groups do not seem to have found the bacteria they used having long lag phases when growing cells on medium containing similar or even higher levels of Cr (VI). Ackerley *et al.* (2004) found that both the wild type *E. coli* AB1157 and its knockout mutant for a flavoprotein nitroreductase that is implicated in Cr (VI) reduction, grew to their maximum biomass in 8 hours with greater than 300μM Cr (VI). Gonzalez *et al.* (2003) again found very rapid growth using both a *Pseudomonas putida* wild type and *P. putida* with a chromate reductase gene cloned in, maximum growth being reached in less than 15 hours. Keyhan *et al.* (2003) also found rapid growth using this organism with considerable chromate reduction after 24 hours of incubation on medium containing up to 30mM Cr (VI). This difference in growth rate might be explained by the difference in medium types used. All these groups were using Luria–Bertani (LB) medium, not a minimal salts medium. Nevertheless the growth times on the medium control without chromate are comparable to that of the LB medium quoted by these groups. It seems likely that the slow growth rate is a function of the *D. denitrificans, R. opacus* and *R. rhodochrous* with genes having to be activated to enable the organism to adapt to Cr (VI). One possible explanation is that the groups above
were all investigating soluble chromate reductases rather than proteins associated with
the cell membrane. The cell membrane associated systems are complicated and involve
more than one protein (Leang, et al., 2003; Methé et al., 2003; Rehder, 2008). Therefore a whole set of genes may need to be activated rather than just one. The exception to this was \textit{R. opacus} cultured in the presence of 100μM Cr (VI). In this culture the lag phase was slightly shorter in time than the control, although the growth rate and maximum biomass was lower. The most comparable lag phase and growth characteristics were found by McLean and Beveridge (2001). Although the lag phase is slightly shorter than was found in this project it was similar, as were the levels of chromate used (McLean and Beveridge, 2001).

Other groups also found more biotransformation than was seen in this study. Keyhan et al. (2003) obtained almost complete reduction of chromate (VI) using \textit{P. putida} up to 400μM and significant reduction proportions at higher concentrations, Gonzalez et al. (2003) using the same organism obtained a far less complete reduction being in the order of 25%. Ackerley et al. (2004) found that \textit{E. coli} reduced the Cr (VI) concentration from over 250μM to between 50μM and 100μM. In every case the authors report that the reduction continued up until the end of the culture measurement period. \textit{D. denitrificans}, \textit{R. opacus} and \textit{R. rhodochrous} largely had stopped reducing Cr (VI) before the end of the exponential growth phase. This could be further evidence that these organisms are accumulating Cr (III). Although chromate in this oxidation state is less poisonous than in the (VI) state, it is still toxic to cells and may inhibit cell growth beyond a certain limit (Cervantes et al., 2001; Gonzalez et al., 2003).

In the \textit{R. opacus} inhibition study both pH and glucose concentrations were measured. The incomplete reduction is not related to the complete utilisation of the glucose or the pH moving outside the growth range for the organism (figure 3.8), since the data show that only in the 100μM chromate culture is the glucose level greatly reduced and even in the chromate free control it is not fully utilised. In the chromate free control it is evidently the low pH, when it falls below 4 that starts to kill the cells. By contrast the pH in the chromate cultures does not change much. These facts and the surprising finding that the cells with chromate present went into death phase at the same
time, indicates that it is the toxicity of the chromate that in the end prevents the cells
growing and therefore complete removal of the chromate (VI). This is presumably the
case for the all the cell types grown on chromate in this project. The levels of chromate
needed to inhibit both *D. denitrificans* and *R. opacus* as determined by the regression
fits of the inhibition models are low being less than 80μM although *D. denitrificans* was
more slightly tolerant than *R. opacus*.

The finding that the OD<sub>600</sub> of the cultures containing bacteria dropped below that of
the uninoculated control was consistent across experiments with chromate (VI)
 autoclaved *in situ* and was thus unrelated to error in sampling. It should be noted that
the OD<sub>600</sub> is increased (approximately doubled) by the addition of chromate compared
with chromate free medium after both have been autoclaved. This is presumably due to
the presence of Cr (III) which is insoluble (Keyhan *et al.*, 2003). Keyhan *et al.* (2003)
found *P. putida* cells became longer and wider up to 150μM, but then further increases
in chromate concentration led to the cells becoming smaller than cells grown without
chromate (Keyhan *et al.*, 2003). *D. denitrificans* was examined under the microscope in
culture medium containing Cr (VI) higher than 200μM and the cells were noticeably
smaller, although Keyhan *et al.* (2003) used much higher concentrations than were used
in this project. This finding could explain part of the drop in OD<sub>600</sub> found in the pre-
exponential phase in these cultures. By contrast *R. rhodochrous* were seen as normal
sized in all cultures examined. However, it would still be expected that a medium
containing some cells would have a higher optical density than cell free medium even if
those cells were smaller, therefore some other additional factor must be at work. Since
the increase in turbidity in medium B with chromate compared to medium B without
chromate is so large and this turbidity is caused by chromate, presumably in the less
soluble (III) oxidation state rather than the (VI) oxidation state (which is soluble), the
most likely explanation is that the bacteria are accumulating Cr (III). Keyhan *et al.*
(2003) and Gonzalez *et al.* (2003) state that Cr (III) being much less soluble is therefore
not bioavailable. However, Garnham and Green (1995) did find that *Anabaena
variabilis* was capable of bioaccumulating Cr(III) which is cationic and interactions
with other bacteria have been reported (Cervantes *et al.*, 2001). There was a simple way
to test this hypothesis, which is to add Cr (VI) after autoclaving the media. In this way
all the chromate will be in the (VI) oxidation state. This assumes no reaction takes place with any iron (II) present in the media. This would appear not be the case since whatever chemical reaction is causing the reduction of Cr (VI) happens during autoclaving and not during the culture period at room temperature where there is little change in the levels in the cell free controls. Adding the chromate after autoclaving stopped this happening, which suggests the chromate (III) hypothesis above is correct. However, related to this was the surprising finding that the chromate (VI) levels fell even at -20°C in samples with cell medium in (although not necessarily cells). This finding was observed over a matter of weeks, which makes it astonishing that very little change was seen at room temperature during what was in most cases comparatively lengthy culture periods. Since this lowering of chromate (VI) levels was seen in aseptic cell free samples it must be due purely to a chemical reaction possibly at the point of thawing. Finally one possible explanation for a drop in chromate (VI) levels over the culture periods with both organisms is that the chromate rather than being reduced is binding to bacterial cells. Unlike Cr (III), which is cationic, Cr (VI) is anionic and is unable to bind to bacterial membranes (Cervantes et al., 2001). In addition a precipitate was seen as the culture periods advanced.

Various groups report different coloured precipitates. Lovley & Philips (1994) report that a white colloidal suspension was seen which was due to Cr (III) hydroxide. Keyhan et al., (2003) state a rusty yellow to yellow precipitate was observed. Although it was not analysed it seems likely the dark green precipitate seen in this study may be due to the formation of trans-[CrCl₃(H₂O₄)Cl₃H₂O formed between the Cr (III) and the chloride ions present in medium B (Cotton and Wilkinson, 1976).

Nalli et al. (2006) saw aggregations of R. rhodochrous cells cultured on hydrophobic organic compounds. These were seen in R. rhodochrous cultures grown on chromate. The view of Nalli et al. (2006) was that these were a reaction to the hydrophobic solvents the cells were cultured on. The fact that these could be seen in the presence of glucose and chromate suggests that this floc formation may be a reaction to stress caused by the chromate. It should be stated that R. rhodochrous and D. denitrificans cells could not be sub-cultured in chromate containing medium to any
great extent. Growth at low concentrations of chromate was seen but after a few generations the cells died. Even passaging cells into chromate free medium rarely worked, although healthy cells could be seen in the chromate medium used.

*S. oneidensis* was found not to be able to grow in the presence of chromate (VI) at all. This finding was totally unexpected, since *S. oneidensis* has the ability to reduce a wide variety of transition metals, including chromate (Viamajala *et al.*, 2002; Brown *et al.*, 2006). No adequate explanation can be advanced for this finding. Live cells were seen in the presence of chromate under the microscope but obviously growth did not take hold.

*R. opacus* would appear to transfer electrons to chromate via extensions to the electron transport chain. The evidence for this is three fold.

First, the lag phase is extended at all levels of chromate tested suggesting a number of genes must be induced (Viamajala *et al.*, 2002). c-type cytochromes are not expressed under normal circumstances (Lovley, 1993).

Second, modest peaks can be seen in the right portions of the spectrum scans for some c-type cytochromes, although the level of expression is low (Figures 3.30 above and 3.37 below). Most data in the literature using spectrum scans presents difference spectra between the oxidised and reduced forms. However, Guerrero & Jones, (1997) and Deedum *et al.* (2005) present both the original scans and the difference data. This is useful to see that the differences between the oxidised and reduced spectra are due to the difference in oxidation state and any shifts that occur. The literature describes a wide variety of absorption peaks ascribable to cytochromes including *c*418, *c*420, *c*421, *c*424, *c*425, *b*426, *b*450, *c*520, *c*522, *b*534, *b*539, *c*548, *c*550-*c*552, *c*554, *b*555, *b*556, *b*558, *b*560, *b*574, *a*590 and *a*603 (Lovley, 1993; Guerrero & Jones, 1997; Roberts *et al.*, 2003; Holmes *et al.*, 2004; Deedum *et al.*, 2005; Horn *et al.*, 2005). Difference spectra peaks can be seen at 450nm, 548nm, 549-555nm, 560nm, 563nm, 578nm, 582nm, 587nm, 603nm and a shoulder is possibly present at 520nm with *R. opacus* cultured on chromate. In addition some peaks are seen at longer wavelengths,
however, there is no reference in the literature to these being relevant to the presence of c-type cytochromes. The peaks at 548nm and 549-555nm are indicative of c-type cytochromes, along with a shoulder at 520nm (Holmes et al., 2004; Guerrero & Jones, 1997). In Enterobacter cloacae it is thought that the c548 cytochrome acts as a "branch point" between oxygen and chromate VI reduction (Lovley, 1993). The 450nm peak is P450 cytochrome and the 603nm peak corresponds to cytochrome a (Guerrero & Jones, 1997; Roberts et al., 2003). However, a cautionary note should be added, no peak is seen at 418-420nm also indicative of c-type cytochromes and all the above peaks are small unlike others in the literature, although in the case of Guerrero & Jones, (1997) it is hard to say how big the peaks were since no y axis scale is given (Guerrero & Jones, 1997; Deedom et al., 2005; Elias et al., 2004). However, in the work described by Carpentier et al. (2005) the peaks are also small. In figure 3.30 above there does appear to be a small peak at around 420nm, however, this is not actually the case, it is actually a shoulder of a small peak from 413-417nm which the plot has made it appear as a peak. This is unlike the other peaks referred to above which although small, are genuine. It should be noted that there does seem to be some confusion in the literature concerning the 420nm peak. Carpentier et al. (2005) regard indication of the presence of a c-type cytochrome in this region as being due to an absorption trough at 420 nm, whereas others regard it as a peak (Guerrero & Jones, 1997, Holmes et al., 2004). In this particular case this discrepancy makes no difference.

In the presence of vanadate at the same molarity the spectrum is totally different to that of the same organism cultured on chromate, which suggests something different biologically is happening with different transition metals. In the presence of vanadium only one peak is seen at 589nm, also as the low optical density difference suggests the oxidised and reduced spectra are almost identical.

D. denitrificans like R. opacus showed definite evidence for c-type cytochromes with a definite and quite significant peak at 418nm being the shoulder of a larger peak at 409-412nm. The spectrum between 500-600nm was very noisy with many small peaks at the appropriate wavelengths of interest described above. The long lag time also lends credence to the up regulation of c-type cytochromes in the reduction of this transition
metal.

Sulphate and organic compounds as well as Fe(II) have been implicated in indirect reduction of chromate by non-enzymatic means and sulphate reducers found to reduce chromate (Lovley and Philips, 1994; Myers et al., 2000; Lloyd et al., 2003). In the case of sulphate, bacteria reduce the sulphate to sulphide which in turn reduces the chromate (VI) (Lovley and Philips, 1994; Myers et al., 2000). However, it has been found that sulphate can also inhibit a chromate reductase enzyme (Park et al., 2000; Lloyd et al., 2003). D. denitrificans can reduce sulphate (Horn et al., 2005). It is unknown whether R. opacus and R. rhodochrous can reduce sulphate. The role of sulphate in indirect reduction of chromate is complex. The reduction has been found to take place under very acidic conditions, at lower pH's than were obtained here (Myers et al., 2000). Sulphate has also been found to have no effect at all on chromate reduction in P. putida even up to a concentration of 1mM (Ishibashi et al., 1990). It seems unlikely that the iron (II) present in the cultures was responsible for the chromate reduction since the iron (II) was added at a much lower concentration than the chromate (VI).

There is good evidence to suggest reduction of chromate was specific and enzymatic (Lovley, 1993). Firstly, there was a long lag phase before growth commenced which suggests some genes had to be expressed. Since the organisms were grown in sulphate containing medium on a regular basis it seems unlikely these enzymes were required for reduction of the sulphate. Second, the sulphate concentration was higher than the chromate, so complete reduction would have been expected of the chromate but only partial reduction was seen. Third, it seems likely that the chromate reduction would have consistently continued after the end of exponential phase, but where measured this generally wasn’t seen. Fourth, for two of the organisms there was a significant inhibition effect with increasing concentration of chromate (Lovley, 1993). This strongly implies the chromate was having some biological effect on the cells. Finally D. denitrificans achieved a higher cell density with chromate than without it in the culture medium. This suggests that the chromate is being used as an additional terminal electron acceptor and the spectroscopic scans also give modest support to this. McLean and Beveridge, (2001) obtained chromate reduction with sulphate in the medium, but
there is also some evidence that sulphide reduction of chromate may not be that important (Lloyd et al., 2003).

3.4.2. Vanadium

*R. opacus* was able to grow in the presence of vanadium (V) and reduce it to a lower oxidation state. Relatively few bacteria have been isolated that can reduce vanadium, though it is believed that the ability to reduce this transition metal is common (Lloyd, 2003; Lloyd et al., 2003). The growth on medium containing this transition metal had opposite characteristics to growth with medium containing chromate. The lag phase was significantly shorter, growth faster and the biomass higher than glucose only controls.

In all flasks in which cells were cultured with vanadium (V) a light green precipitate was seen during the death phase. Ortiz-Bernad et al. (2004) also saw a green precipitate form in cultures, but not in abiotic controls, their analysis suggested that the precipitate was vanadyl phosphate (Ortiz-Bernad et al., 2004). The precipitate found by Ortiz-Bernad et al. was darker than found in the *R. opacus* cultures. Cotton and Wilkinson suggest a complex of V(IV) with acetate is light green (Cotton and Wilkinson, 1976). With approximately 950µM V(V) growth was very rapid and initially a darker green was seen similar to that seen by Ortiz-Bernad et al., (2004) before it became lighter in colour. The reasons for the change in colour from one shade of green to another are unknown.

Unlike chromate the *R. opacus* cells were not poisoned by the vanadium. The entry into death phase is probably initiated by a lack of glucose which was by the beginning of the death phase not at measurable levels. Since the organism had grown to lower pH values on glucose only, reaching a low of 3.69 rather than 3.91 as was the case on culture medium with vanadium present, probably the pH was not responsible for cell death. There is every reason to believe that given more glucose the cells would have cleared the flasks of all vanadium (V). In addition the vanadium does not poison the cells to the extent that they will not grow when sub-cultured which was the case with
chromate. The cells from one of the study flasks were transferred into medium B with glucose only where they grew normally then into vanadate containing medium, then back to glucose only then back into culture with vanadate without any apparent effect.

The spectroscopic scan revealed no evidence at all of c-type cytochromes. As described above this was not the case for the same organism cultured in the presence of chromate (VI), where evidence of some expression of c-type cytochromes could be seen. This is shown most clearly in figure 3.37 for a portion of the spectrum. The lower vanadium plot is almost totally devoid of features, whereas the upper spectrum of cells grown on chromate has a number of peaks clearly visible. Only one peak is visible at 589nm in one of the cultures with vanadate present. The very low optical density difference seen in the lower spectrum is representative of the lack of difference between the oxidised and reduced spectrum.

![Graph showing optical density difference vs wavelength (nm)](image)

**Figure 3.37.** The difference spectra for *R. opacus* cultured in the presence of chromate (VI) and vanadium (V). The spectra are portions of those shown in figures 3.30 and 3.31 above and the major peaks are marked (chromate is black and vanadium red).

Growth in the presence of this transition metal was rapid, even at almost 1mM vanadium (V), this suggests that reduction of this metal takes place at least partially via the same cytochromes used in aerobic respiration. Faster growth was obtained than that found by Ortiz-Bernad *et al.* (2004) using *G. metallireducens* although their cultures
were anaerobic. The periplasmic cytochrome c3 has been found to function in this way, being able to reduce O₂, U(VI), Fe(III) and Cr (VI) (Elías et al., 2004). In some way the vanadium must penetrate the membrane in the cells for this to occur, however being Gram-positive R. opacus does not have an outer membrane or periplasmic space. No soluble reductases have been detected using PAGE and the intracellular nature of the reduction would explain the lack of detectable cytochromes. The faster growth and the ability grow to a higher cell number than cultures without this transition metal being present does suggest that the bacteria are using it as a terminal electron acceptor. Vanadium is however used by bacteria as a co-factor in some enzymes so it is possible this may explain some of the more rapid growth (Rehder, 2008). D. dentrificans would appear to lack the cellular mechanisms to reduce this metal.

3.4.3. Copper

R. opacus was capable of growing in the presence of copper (II). R. opacus grew to a far higher cell density with the elevated levels of copper (II) than with the trace element levels normally found in medium B. The presence of copper at greater than an initial concentration of 17μM apparently affects all the assays tried for glucose. This was thought to be due to the presence of hydrogen peroxide. R. opacus expresses and utilises a class of enzymes called monamine oxidases (Goeke, and Hummel, 2002). These enzymes catalyse the following reaction and one class requires copper (Yagodina et al., 2002).

\[
R\text{-CH}_2\text{NH}_2 + O_2 + H_2O \rightarrow R\text{-CHO} + NH_3 + H_2O_2 \quad \text{Equation 3.3}
\]

The hydrogen peroxide produced is capable of then reacting with Cu(I) to produce a variety of free radicals including the hydroxyl radical

\[
X_{\text{red}} + \text{Cu(II)} \rightarrow X_{\text{ox}} + \text{Cu(I)}
\]

\[
\text{Cu(I)} + H_2O_2 \rightarrow \text{Cu(II)} + \text{OH}^- + \text{HO}^\cdot
\]

\[
\text{HO}^\cdot + \text{DNA} \rightarrow \text{damage} \quad \text{Equation 3.4}
\]
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(reproduced from Macomber et al., 2007)

It is also able to react with Cu(II) in the following reaction to produce Cu(I)

$$\text{Cu(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Cu(I)} + \text{O}_2^- + 2\text{H}^+ \quad \text{Equation 3.5}$$

(from Multhaup et al., 1998)

Cytochrome studies revealed the spectra fall into the two groups as described above for cell growth characteristics. With the two lowest concentrations of copper (II) the difference spectrum slopes upwards from the peak at approximately 430nm to 600nm (figure 3.32). The highest three copper concentration cultures show the opposite. In the lowest two copper concentration cultures on addition of the dithionite the optical density falls compared to the oxidised culture, this means the optical density difference is negative over almost all the spectra above approximately 400nm (figure 3.32). This difference between the oxidised and reduced cultures falls away with increasing initial copper (II) concentration until at 333.5μM the reduced absorbance is higher than the oxidised absorbance, meaning the difference between them is positive in sign. This change is not explained by higher cell number, since the highest cell number was achieved in the 89.7μM initial copper (II) concentration cultures not with 333.5μM (figure 3.32). This change does suggest that the cells are expressing something on their cell surface that allows them to cope with dithionite better when a higher initial concentration of copper (II) was added to the cultures. There is another explanation which is that was the cells are producing progressively higher levels of hydrogen peroxide which will tend to neutralise the dithionite. However, the peroxide assay revealed this was not the case, as there were no statistically significant differences between the hydrogen peroxide levels in the supernatant with increasing initial copper (II) concentration. The final difference between the cultures was differential expression of a peak at approximately 430nm, ringed in figure 3.32. This peak reduces in size until at the highest copper concentration it has almost vanished. This is not a c-type cytochrome since nothing in the literature about peaks for these proteins has been reported at this particular wavelength.
Only for the highest three initial concentrations of copper (II) cultures was any
evidence seen of peaks at the relevant wavelengths for c-type cytochromes (as described
above in the results). The peaks are tiny. In the highest two initial copper (II)
concentration cultures a peak is seen at 548nm, but in the 89.7μM culture tested this
was not seen. Peaks are seen at or around 518nm but 552nm is better described as a
shoulder (figure 3.32). All cultures contained a 420nm peak as a shoulder to the 435nm
peak. Although the peaks are small the difference spectra are very different to some
seen when cultures of the same organism were grown in the microbial fuel cell or in the
presence of vanadium above. In these cultures the difference spectra are essentially
featureless in the spectral region where these cytochrome peaks are seen.

All the cultures reduced copper (II) to copper (I) over the cell cycle. Although some
Cu(I) was present in the culture medium at the start its initial concentration was in every
culture exceeded over the cycle. The initial presence of the Cu(I) was unexpected but is
presumably due to a redox reaction with Fe (II) from the iron (II) sulphate added to the
culture according to the equation;

\[ \text{Fe}^{2+} + \text{Cu}^{2+} \rightarrow \text{Cu}^{+} + \text{Fe}^{3+} \]

Equation 3.6

The reduction during the culture period was partially related to the initial copper
concentration. The cultures with the three lowest concentrations of copper (II)
produced a maximum of less then 8μM Cu(I). The 333.5μM culture reduced to a
maximum concentration of 19.2μM Cu(I) with the 102.3μM culture in between these
two. This reduction of copper (II) to the I oxidation state may be enzymatic or chemical
(Sugio et al., 1989). Although as outlined above there is some modest evidence for the
presence of c-type cytochrome expression in the presence of high concentrations of
copper (II) it is possible that the reactions above (equations 3.3 to 3.5) could play a part
in the relative concentrations of Cu(I)/Cu(II). Hydrogen peroxide can both reduce
copper (II) and oxidise copper (I). The chemical reactions shown in equations 3.4 and
3.5 could cycle the copper between different oxidation states. However, hydrogen
peroxide measurements do not bear this hypothesis out. Hydrogen peroxide levels were
found to be an order of magnitude lower than the copper concentrations and also with
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one exception declined as the cultures proceeded. There was also (except in the case of the highest initial copper concentration) apparently no correlation between either the copper transition state and the hydrogen peroxide concentration. The low hydrogen peroxide levels also imply that the measured glucose levels may be genuine, or at least that the glucose assays were not being interfered with by much lower levels of peroxide in the culture supernatant. In addition the hydrogen peroxide concentrations vary very little with the increasing copper concentrations and in the iron (III) culture were higher, but in this case measured glucose concentrations were plausible over the entire culture period. A few of the culture samples were assayed for peroxide as a cell suspension without the cells having been pelleted; these gave much higher readings, but all were less than 11μM. Whilst the glucose concentrations obtained using the glucose sticks were read with a cell suspension the cells were removed before other assays were performed and the results were broadly similar. One remaining possibility is that the copper interferes with the stick assay. A test using 3mM glucose made up in deionised water with 300μM copper (II) gave a reading of 2.6mM using the stick method. This finding and the fact that all the glucose levels at the start of cultures with the five different concentrations of copper (II) tried were similar and close to that expected suggest the copper is not interfering with this assay. When added at high concentrations, hydrogen peroxide seemed to have an effect on the phenol sulphuric acid assay for glucose, making the mixture darker. The reasons for this are unknown but as outlined above the cell supernatant levels appear to be too low to account for assay inhibition.

In some ways it was surprising to able to detect Cu(I) in aerobically shaken cultures due its instability in the presence of oxygen (Cotton and Wilkinson, 1976). This alone suggests some kind of dynamic chemical and or biochemical process is occurring. The stable orange colour seen on the copper impregnated plate with BCS also alludes to this.

The cultures involving copper were different to all the other cultures cultured with transition metals in that the levels of this transition metal at time zero immediately after the cells were added were also very low or undetectable. In addition the concentrations of the transition metal of interest rise rather than fall over the bacterial growth cycle. To
explain these observations either the cells are binding the copper or internalising it. Since the copper levels drop so rapidly on addition of the cells to the culture medium the former is suggested. Cooksey (1993) reviewed copper resistance in *E.coli* and *Pseudomonas syringae*. These organisms use specialised sets of proteins to bind the copper on the cell surface or periplasmically or to compartmentalise it within the cells (Cooksey, 1993). This is probably not the case here. Whilst growth is slowed with a significantly increased lag phase suggesting genes have to be switched on in response to the copper (possibly to produce c-type cytochromes), whatever binds the copper is expressed on the cell surface constitutively. Copper can be released by washing the cells in 20mM EDTA when binding is associated with the outer membrane (Cooksey, 1993). An attempt was made to wash some time zero samples for several different initial concentrations of copper (II) in 20mM PIPES. This failed to release detectable levels of copper (II) using the BCS assay. However, the use of an EDTA wash worked with the familiar blue colour of copper sulphate being seen almost immediately when the cell pellets were vortexed indicating the cells are binding the copper (II). The cell supernatant was then assayed using the BCS assay. The EDTA in some way interfered with the BCS assay since no colouration was seen, although after overnight incubation the bright orange complex was observed. No significant binding of other transition metals was observed with this organism or others studied.

The second observation which is perhaps more surprising is that the copper (II) levels rise as the cultures start to grow. Again the five sets of cultures fall into two natural groups, at the lowest two initial copper (II) concentrations the copper (II) levels peak roughly midway through the cell growth cycle then fall back. For the cultures with the highest three concentrations of copper although there are some falls over the cycle (possibly these are assay related error) the overall trend is upwards till the cells enter death phase.

There are four possible explanations for this copper increase. The first is that the original cohort of cells used to inoculate the culture die and this releases the copper into the culture. This seems unlikely for the reason that the copper would surely bind to new cells produced in the growth cycle by the same mechanism.
The second possible explanation is that only cells in stationary or death phase (used to inoculate the cultures) bind the copper, while rapidly growing cells don't. As shown in table 3.2 correlations were made to see if there was a statistically significant relationship between cell growth and copper (I)/(II) concentrations. The increase in Cu(I) concentration was significantly correlated with cell growth in all cases and Cu(II) was similarly correlated in all but the two lowest concentration cultures. So the lower copper concentration cultures assay results don't allow this conclusion to be drawn since they grew very rapidly.

Another possible explanation is suggested by the EDTA experiment. That is a compound or compounds produced by the cells gradually displaces the copper. One possibility is organic acids produced by the cells that lower the pH over the growth cycle. The same statistical techniques used above were used to test for a relationship between pH and copper (I)/(II) concentrations (data not shown). The results replicate the cell growth correlations, although the correlation between the increase in copper (I) and decrease in pH was a lot less significant in all cases being only just less than p=0.05 (p=0.0441) in the lowest copper culture. It seems unlikely that acids are exchanging with the copper (II) since there is no significant correlation with acidity in the two cultures with the lowest copper concentrations. In addition the copper release is very rapid in these cultures before much pH change has taken place (figure 3.23). In the three cultures with the highest copper concentrations release is again a rapid process once underway, but its start is delayed to approximately 100 hours in every case (figure 3.23).

The last explanation is that the organism is using copper exporting proteins to transport the copper out of the cells. A search using “copper” of the *Rhodococcus* Rha1 genome produces a number of positive hits for both proteins involved in copper binding and transport (McLeod *et al.*, 2006). The delay in release of copper in cultures with the highest three concentrations could be explained by the need to express the genes for exporting copper. This may explain the long lag phase for these cultures. Possibly for the two lowest copper concentration cultures the transportation proteins that are expressed are unable to deal effectively with higher copper concentrations and the
expression of more effective proteins is required.

*D. denitrificans* was incapable of growing in the presence of copper (II) to any great extent. However, it was not poisoned by it, in that cells were sub-cultured from the copper containing medium into copper free medium and the cells grew normally.

### 3.4.4 Iron

The ferrozine assay was sensitive enough to detect the iron (II) concentration in the control cultures for *D. denitrificans* which contain FeSO₄ at low levels. Iron (II) is unstable at neutral pH being oxidised to Fe(III). The assay results confirm this for the shaken cultures showing a fall in Fe(II) concentrations in the first few hours of culture of *D. denitrificans* with this transition metal. In addition the iron (II) is oxidised by air in acid solutions to Fe(III) (Cotton and Wilkinson, 1976), meaning any iron (II) produced is potentially cycled back to iron (III). The correlations between cell number and the increase in iron (II) concentration were highly significant for both the shaken control and shaken ferricyanide cultures for this organism. Taking all this into account, the results shown for aerobic cultures in figure 3.18 suggest this organism is reducing the Fe(III) to Fe(II). Unexpectedly the concentrations of iron (II) in both the control and the cultures with ferricyanide reached almost exactly the same maximum concentration of 23μM. This confirms that the iron (II) is unstable in aerated cultures at acid pH. Thus adding more iron (III) does not noticeably lead to increased iron (II) in shaken cultures for chemical reasons. There was however a higher maximum biomass for the culture with extra iron (III) added as ferricyanide. This implies that Fe(III) may be acting as an alternative electron acceptor (Lovley, 1993). Ferricyanide has been found to accept electrons from electron transport chains in membrane vesicles of two organisms (Lovley, 1991).

The results for the unshaken cultures are very similar with significant correlations between cell growth and Fe(II) concentrations, although the statistical significance was lower (table 3.5). Again the maximum biomass was higher for the cultures with the iron (III) ferricyanide added although the difference was less pronounced and the cell growth
was much lower in both cases than for either of the shaken culture sets. This time though the higher Fe(III) added resulted in higher Fe(II) than in the pair of control cultures. The iron (II) is more stable than in the shaken cultures for the reason that there is less mixing with oxygen at acid pH. In addition the pH in the unshaken control did not fall as low (figure 3.19). No peaks indicating c-type cytochromes were seen in either set of cultures.

The growth of *R. opacus* on medium with iron (III) added was totally different to that of *D. denitrificans*. The correlation between cell number and the Fe(II) levels was not really significant (table 3.5 above). The iron (II) concentration at the start and the end were very similar, although there was a rise at 48 hours before the levels dropped back. Unlike with *D. denitrificans* there was a cell free control and the iron (II) levels in this flask stayed at or near zero throughout indicating the iron (III) is stable at or near neutral pH. There was a rise in the soluble Fe(II) concentration which correlated very significantly with cell growth (data not shown), however the increase in Fe(II) concentration was very small. No peaks indicating c-type cytochromes were seen in the cultures.

All the above data suggest that this organism is not capable of reducing Fe(III). However, the iron did have an effect on the cells. The lag phase was shorter and maximum biomass achieved was very slightly higher than found in control cultures, while the growth rate was slower and the generation time slightly longer. The glucose carbon source was not utilised fully. This could be due to the fall in pH which reached a mean of 4.0. However, the pH has dropped further than this in other cultures. In the control cultures with glucose as the carbon source the pH dropped as low as a mean of 3.69, although without all the glucose being utilised.

Iron reduction has therefore been seen for one of the bacteria in this project under aerobic conditions. Iron reduction has been detected in bacteria grown aerobically (Johnson and McGinnis, 1991), although it is not necessarily linked to electron transport and is therefore not necessarily growth supportive under anaerobic conditions (Lovley, 1993). One possibility is that non-enzymatic reduction is brought about by the action of
organic acids (Lovley, 1991). However, the experiments described above bely this. In the shaken \textit{D. denitrificans} control cultures the pH fell to below 3.4. There was a very significant correlation between Fe(II) concentration and pH (\(R=-0.836, p=0.005\)) for these cultures. Although the pH in the cultures with ferricyanide was not measured there is no reason to suppose it was greatly different since in all shaken cultures involving this organism the pH fell to less than 4 (this is covered in greater detail in chapter five). In addition the pH in the \textit{R. opacus} cultures fell to less than pH 4 with no dramatic differences between the Fe(II) concentrations at neutral pH in the lag phase and at acidic pH in the death phase. The correlation between Fe(II) and pH was only just significant (\(R=-0.7097, p=0.0486\)) for these cultures. Finally the unshaken \textit{D. denitrificans} control cultures without additional iron (III) added achieved slightly higher Fe(II) concentrations at circumneutral pH than the same batch of medium did when shaken. The correlation between Fe(II) and pH was highly significant (\(R=-0.9187, p<0.001\)) for the unshaken \textit{D. denitrificans} control cultures without additional iron (III) added. Although pH was not monitored in the unshaken ferricyanide cultures an unshaken culture study has been undertaken (see chapter five) and the pH does not fall below 6.5. At this pH organic acids do not reduce Fe(III) (Lovley, 1991). The data taken as whole suggest no obvious causal link between pH and Fe(II) levels in the cultures, possibly because the pH drop even in the shaken cultures is insufficient to reduce the iron (III). Non-enzymatic iron reduction can occur with Mn(IV) (Lovley, 1991), however the levels of manganese are too low in the cultures to have any effect and it is in the II oxidation state.

The lack of a link between ferricyanide reduction and pH was tested. A culture containing \textit{Rhodococcus ruber} had ferricyanide added to it in early exponential phase and growth ceased. The cells were almost poisoned, in that cells taken from this flask did recover but took two weeks to do so, eventually growing to the normal colour for this organism in acetate medium (orange brown). The pH in the failed culture was adjusted from 7.2 to 4.0 using glacial acetic acid and it was left to shake. This pH was chosen since it is roughly the pH that all the cultures of the organisms used in this project consistently achieved. No colour change from yellow ferricyanide was seen (figure 3.38 a)). \textit{D. denitrificans} was cultured with 3166\textmu M ferricyanide. The culture
went confluent apparently losing its yellow colouration and without any blue colouration being seen. Then after another 24 hours the culture colouration changed to a green colour and blue precipitate appeared (figure 3.38 a & b). The culture was centrifuged to remove the cells and ferrocyanide for SDS-gel analysis (see chapter 6), the supernatant was yellow indicating reduction was incomplete.

The work described above strongly suggests the reduction is enzymatic caused by the bacteria and not merely a chemical reduction caused by pH. In addition no colour change was seen for any of three *Rhodococcus* species tried. *R. opacus* could tolerate low concentrations of ferricyanide, but higher concentrations caused growth to cease completely. The use of ferricyanide has two advantages. Firstly, it is soluble, making it more bioavailable than other forms of iron (III), which have a tendency to be insoluble. The pathways in some cases can be distinct although some proteins such as OmcB have been implicated in both soluble and insoluble iron reduction (Holmes *et al.*, 2006). Since this makes it easier for the microorganism to reduce it, it also means that when microorganisms such as the *Rhodococcus* species fail to reduce it then it is a definitive test. Second, the blue colouration makes it easier to see that reduction has occurred and so no assay is required.

**Figure 3.38.** Images of cultures with potassium ferricyanide present. Image a) shows a culture of *R. ruber* in the left hand flask and *D. denitrificans* on the right hand side. b) shows the reduced blue precipitate in the same *D. denitrificans* culture.

A possibility is that iron reduction by *D. denitrificans*, although occurring, is not linked to conserving energy for growth (Lovley, 1991). Both membrane bound and
soluble iron (III) reductases have been found in a variety of organisms (Lovley, 1993) as well as soluble c-type cytochromes (Lovley, 1993). In many organisms such enzymes, although they reduce Fe(III), do not allow it to support growth (Lovley, 1993). No reason for the presence of such proteins is given in the literature. No soluble enzymes have been detected by the native PAGE with the coomassie stain for cultures of any of the of the bacteria used in this project, although this method is relatively insensitive. Horn et al. (2005) state that Fe(III) cannot be used by this organism as an electron acceptor. Whilst definitive proof that this organism can use iron (III) as an electron sink linked to growth has not been obtained, one possible explanation for the above contradiction is the medium composition. The iron (II) sulphate added is oxidised both by autoclaving and shaking to iron (III). It is possible that repeat passaging of the cells in contact with iron (III) has selected out cells that can reduce Fe(III).

Both *R. opacus* and *D. denitrificans* were screened using Fe(III) impregnated plates with liquid ferrozine added. The change in colour with *D. denitrificans* was immediate whereas for *R. opacus* it took many hours. This suggests that *R. opacus* cannot reduce iron (III) and *D. denitrificans* is able to do so (Beliaev and Saffarini, 1998). The colour change with *R. opacus* may be due to the small amount of iron (II) present in the medium B in the agar.

Both iron (II) and iron (III) are regarded as indirectly or directly toxic to cells (Touati, 2000, Channongpol et al., 2002). Fe(II) is capable of forming hydroxyl radicals with hydrogen peroxide in an analogous manner to that described for copper above and shown in equation 3.7. Fe(III) is also found to be toxic in specific circumstances where genes involved in the lipid polysaccharide system were defective in both *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae* (Channongpol et al., 2002).

$$\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \text{HO}^- \quad \text{Equation 3.7}$$

It seems unlikely the toxicity is due to the peroxide since the soluble levels were low compared with the iron (figure 3.21). Iron can be reduced by sulphide compounds
(Lovley, 1991). Since the same medium type was used for both *D. denitrificans* and *R. opacus* and different results were obtained it would not appear that sulphide reduction is implicated.

### 3.4.5. Molybdenum

Both *D. denitrificans* and *R. opacus* were capable of growth on medium containing high concentrations of Mo(VI) and a colouration indicative of the formation of molybdenum blue was seen with cultures of both organisms (Ghani *et al.*, 1993; Lloyd *et al.*, 2003; Lloyd *et al.*, 2003; Shukor *et al.*, 2009). The concentrations tolerated by both *D. denitrificans* and *R. opacus* were similar to those given in the literature for other organisms (Sugio *et al.*, 1988; Ghani *et al.*, 1993). Molybdenum blue is recognised as a complex between Mo(VI) and Mo(V), in this case a complex produced by a combination of bacterial action and the phosphate present in the medium under acidic conditions (Sugio *et al.*, 1988; Lloyd *et al.*, 2003). Comparatively few bacteria have been isolated that are capable of reduction of molybdenum (VI) (Ghani *et al.*, 1993; Lloyd *et al.*, 2003).

A wide variety of assays have been proposed for assaying molybdenum (Elbei and Abou-ela, 1968; Ohashi, *et al.*, 1986; Kamburova and Kostova 2008). Many of these assays are very complicated requiring the use of separation techniques and extraction (Elbei and Abou-ela, 1968; Ohashi, *et al.*, 1986; Kamburova and Kostova 2008). Use of diphenylcarbazide is one method of assaying for molybdenum (Kamburova and Kostova, 2008). However, using exactly the same methodology with this reagent as the chromate version of the assay produced only a very mild mauve colour with the 1.8mM and 3.59mM standards and no standard curve could be obtained. The same batch of diphenylcarbazide was fully functional with a chromate standard. An alternative method is outlined by Sugio *et al.* (1988) and Ghani *et al.* (1993). This is using standard amounts of Mo(VI) reduced by a reducing agent to Mo(V) which forms a blue complex with phosphate, reading the absorbance at a suitable wavelength and producing a standard curve. The blue colouration in the cell cultures is matched to the standards by measuring the optical density at the same wavelength. Sugio *et al.* (1988) suggest a
wavelength of 660nm whilst Ghani et al. (1993) used 710nm. Shukor et al. (2009) suggest neither of these wavelengths give a satisfactory result. In the study no major peaks were revealed at 660 or 710nm using a wavelength scan in either the standards or cell samples. The wavelength (865nm) suggested by Shukor et al. (2009) gave a standard curve for the reduced standards. Comparing the wavelength scans of both a cell sample and a standard with a similar optical density revealed similarity in the scans for both over the wavelength range chosen, this suggests the compounds are the same (Shukor et al., 2009). It is logical that since the colour is blue that it will absorb maximally at the red end of the spectrum.

The growth characteristics with R. opacus cultured in the presence of molybdenum were similar to those found in the previous control growth study without any transition metals present, albeit with a shorter lag phase and a slightly slower growth rate. There were however also some major differences compared with this organism’s growth found previously. The first was all the glucose was utilised. This was only seen before in the transition metal growth studies (where glucose levels were monitored) with vanadium. The second difference was that maximum cell density was comparatively low, very little growth was achieved for the glucose concentration present despite the apparently normal cell morphology. Molybdenum is involved in a wide variety of enzymes within micro-organisms (Ghani et al., 1993) and therefore could promote growth. However, its effects here would appear at first to be mildly inhibitory with slower and lower growth than control cultures. The fact that the glucose levels are undetectable by the end of the growth cycle and the cell density is low suggests that the organism may cope with the toxicity of the molybdenum by means of an ATP dependent pump. The removal of Mo(VI) or Mo(V) or both from within the cells apparently requires much of the energy derived from the glucose to be diverted from cell growth to this task. A search of the Rha1 genome indicates a molybdenum ATP linked pump (McLeod et al., 2006). A search using a copper search term also indicates that Rha1 has copper ATP linked pumps. However, if the glucose measurement and cell growth results are correct then there is no apparent evidence for these proteins in R. opacus for copper. The third and final difference was that the pH (made more acidic initially by the addition of the molybdenum by over one pH unit) did not change greatly over the cell growth cycle.
Growth was definitely not brought to an end by a low pH, nor did the starting pH inhibit the cells from growing (see chapter five).

The molybdenum blue results for *R. opacus* need to be treated with caution. This was the only growth study in which the transition metal being assayed for did not show a significant correlation with cell growth. In addition, although there was a statistical difference between the molybdenum concentration in flasks with cells and the control, it was not highly significant. This latter finding may be explained by a small sample size for the control group as well as the presence of some Mo(V) in the control from the start. In addition the levels of molybdenum blue rose and fell over the cell cycle. Unexpectedly the molybdenum blue concentrations also fell in the death phase. No obvious explanation is available for this rise and fall of the molybdenum blue levels, although it was not a sampling error since it occurred in both cultures at the same time point. An additional check was made to confirm that the change in colour was biologically derived and not some function of the drop in pH over the cell cycle. Mo(VI) standard was mixed with phosphate buffer at pH 5 and left. No colour change was seen in over a week. No evidence was found for c-type cytochromes using the spectroscopic scans for *R. opacus* cultured in the presence of molybdenum, a finding that fits in with the growth data where there was no significant lag phase seen.

Whilst the reduction is believed to be cellular in origin it may be an indirect effect of sulphate (Lloyd *et al.*, 2003). To test this hypothesis *R. opacus* was sub-cultured into culture medium containing only iron sulphate and ammonium chloride instead of ammonium sulphate (see chapter seven) along with approximately 2100µM Mo(VI). The growth characteristics were different, in that this time the culture medium colouration was pink to start with then changed to a blue/brown then finally to mauve. The OD$_{665}$ indicated a high concentration (2800µM molybdenum blue) when read against the standard curve. Whilst this value is too high given that the medium contained only 2100µM of molybdenum it seems unlikely that the pink colour interfered at this wavelength since it would be expected to absorb maximally at the blue end of the spectrum. This result confirms that the reduction is not sulphate linked since the remaining sulphate concentration in the medium is much lower than the
molybdenum concentration.

*Denitrificans* also grew strongly in the presence of this transition metal in low sulphate medium. Its growth characteristics were very different to that of *R. opacus*. The glucose stayed above detectable levels and the pH fell to very much lower levels than that found in the *R. opacus* cultures containing this transition metal. No large swings in molybdenum blue concentration were seen and the level of reduction was much higher than for *R. opacus*. Statistically there was a more highly significant difference between the molybdenum blue levels in the control and cell cultures and a very strong correlation between cell number and growth. In addition the cell density was much higher than that of the *R. opacus* cultures. The differences lead to the conclusion that how this organism interacts with molybdenum does not involve the use of an ATP dependent pump. Low pH was responsible for the end of exponential phase, not a lack of glucose or the transition metal toxicity. Normally *Denitrificans* does not noticeably change colour greatly under different culture conditions. Thus it was surprising to see the change to a blue colouration. No evidence was seen for c-type cytochromes using spectroscopic scans for this organism, a finding that fits in with the growth curve where there was no significant lag phase. The difference spectra altered quite markedly after addition of the reducing agent, something not seen with other transition metals. One possible explanation for this is a chemical interaction with the molybdenum blue complex. Phosphate has been found to inhibit the formation of the molybdenum blue complex, but the phosphate levels were not high enough to cause this to happen here (Shukor et al., 2009).

The Blue Lake bacterial isolates, surprisingly, given the high iron content of the place where they were isolated from seemed to tolerate rather than reduce the transition metals they were exposed to. Although assays were not performed with Blue Lake cultures, three of the transition metals give distinctive coloured precipitates and these weren't seen with Blue Lake cultures except in the case of chromate. Copper was generally toxic to these cells and molybdenum inhibited growth of one isolate completely. This suggests that tolerance of these transition metals is uncommon. The general lack of ability to reduce these and other transition metals tried implies that the
ability to reduce transition metals is infrequent even in bacteria which exist in environments were these metals are found.

3.4.6. Summary of work carried out in this chapter

All the bacteria initially chosen for examination in this project have been screened for growth in the presence of and/or reduction of one or more transition metals. With chromate, initial screening was carried out with *P. denitrificans*, *R. rhodochrous*, *D. denitrificans*, *B. xenovorans* and *P. naphthalenivorans*. Only *R. rhodochrous* and *D. denitrificans*, showed both chromate reduction and growth, with some of the other bacteria at best possibly being able to tolerate chromate. *P. naphthalenivorans* was incapable of growth except on plates so at this point was dropped from the project. During the period when these experiments were carried out *B. xenovorans* lost the ability to grow in medium B and was also dropped.

Full growth studies were then carried out for *R. rhodochrous*, and *D. denitrificans* in duplicate. Although growth varied in some of the culture flasks, statistically significant chromate reduction was seen compared to cell free controls, although in the case of *R. rhodochrous* the reduction was fairly minimal.

Growth inhibition studies were undertaken to see what levels of chromate would stop cell growth completely for both *R. rhodochrous* and *D. denitrificans*. In addition two other bacteria added to this project latterly, *S. oneidensis MR-1* and *R. opacus*, were examined in the same way. *S. oneidensis* was incapable of growth in cultures containing chromate and *R. opacus* grew well with 100µM chromate present but even at this level the toxicity of the chromate (VI) to the organism was evident.

Finally growth was examined using *R. opacus* and *D. denitrificans* in the presence of other transition metals. *R. opacus* was capable of growth in medium with vanadium, copper, molybdenum and iron. Reduction was found with all but iron (table 3.4). *D. denitrificans* was able to grow well in medium with molybdenum and iron present (with reduction). *D. denitrificans* was incapable of growth in medium containing copper or
vanadium (table 3.4).

To summarise *R. opacus*, *R. rhodochrous* and *D. denitrificans* are capable of reducing transition metal(s).

The Blue Lake isolates tolerate rather than reduce the same transition metals tested with the other bacterial types.
### Table 3.4. Brief summary of overall results.

<table>
<thead>
<tr>
<th>organism</th>
<th>transition metal</th>
<th>growth</th>
<th>transition metal reduction</th>
<th>c-type cytochromes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. denitrificans</em></td>
<td>Chromate (VI)</td>
<td>Yes, but cells are poisoned by it. Low levels inhibit growth.</td>
<td>Yes, but % falls with increasing chromate concentration.</td>
<td>Evidence seen in scans, also long lag phase.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>Copper (II)</td>
<td>Almost none.</td>
<td>Not tested, but presumably not.</td>
<td>Not tested.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>Vanadium (V)</td>
<td>Almost none.</td>
<td>Not tested, but presumably not.</td>
<td>Not tested.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>Iron (III)</td>
<td>Yes, growth stronger in higher Fe(III) concentrations.</td>
<td>Yes, especially in unshaken cultures.</td>
<td>None found.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>Molybdenum (VI)</td>
<td>Yes, very strong growth.</td>
<td>Increase in molybdenum blue concentration. Culture went blue.</td>
<td>None found.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Chromate (VI)</td>
<td>Yes, but cells are poisoned by it. Low levels inhibit growth.</td>
<td>Yes, but % falls with increasing chromate concentration.</td>
<td>Evidence seen in scans, also long lag phase.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Copper (II)</td>
<td>Very strong higher biomass achieved in higher concentrations.</td>
<td>Reduction in all cultures to Cu(I).</td>
<td>Possibly found in highest concentration cultures.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Vanadium (V)</td>
<td>Rapid growth, no sign of inhibition at high concentration.</td>
<td>Yes. Dark green cultures.</td>
<td>None found.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Iron (III)</td>
<td>Strong growth but also some inhibition.</td>
<td>No reduction.</td>
<td>None found.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>Molybdenum (VI)</td>
<td>Weak growth but all carbon source utilised.</td>
<td>Some reduction.</td>
<td>None found.</td>
</tr>
</tbody>
</table>
"Multiphase Biocatalytic Processes Using Extremophilic Microorganisms for Energy Production"

Neil Richard Hollow

A dissertation submitted for the degree of Doctor of Philosophy

Volume 2

Heriot-Watt University

School of Engineering and Physical Sciences

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Chapter 1. Alcohol biodegradation

4.1. Introduction

As was outlined in chapter one, a wide variety of bacteria have been found to be capable of biodegrading alcohols (Toyama et al., 1995; Baker et al., 1998; Schenkels and Duine, 2000; Van Spanning et al., 2000). The aim of the work carried out in this chapter was to examine the ability of the bacteria chosen to break down a number of different alcohols before an attempt was made to link this biodegradation to electricity production in the microbial fuel cell.

De Carvalho and da Fonseca, (2005) examined the growth of Rhodococcus erythropolis on a variety of alcohols and alkanes. R. erythropolis was able to grow at concentration up to 2% butanol, 5% pentanol, 15% methanol and 20% ethanol without any adaptation (De Carvalho and da Fonseca, 2005). There has been less interest in the degradation of alcohols than what are seen as more recalcitrant compounds, such as haloalkanes and complex aromatic compounds (Kulakov et al., 1999; Janssen et al., 2005). A number of groups report that alcohols are part of the biodegradation pathway of haloalkanes (Curragh et al., 1994; Poelarends et al., 2000; Janssen et al., 2005). Butanol has been found to be an intermediate in the pathway of the breakdown of 1-chlorobutane by Rhodococcus species (Curragh et al., 1994; Poelarends et al., 2000). Poelarends et al. (2000) isolated Gram-positive haloalkane utilizing bacteria from contaminated sites in Europe, Japan, and the United States. Rhodococcus sp. have large linear plasmids which carry genes that confer on these organisms many of their degradative abilities (Larkin et al., 2005). Examination of the 16S rRNA suggested these bacteria were all Rhodococcus sp and the bacteria shared a highly conserved dhaA gene responsible for encoding haloalkane dehalogenase. This gene was located both on the plasmid pRTL1, as well as on the bacterial chromosome (Poelarends et al., 2000). Kulakova et al. (1997) however had earlier reported the ability to dehalogenate short chain chloroalkanes was lost if the plasmid was excised, or was integrated into the genome. The gene adhA, encoding a dehydrogenase which produces butanal from butanol as part of the dehalogenation pathway is also carried on this plasmid.
(Poelarends et al., 2000). It is unclear as to whether this latter gene is present on the chromosome in addition to the plasmid, although this is possible in this organism (Larkin et al., 2005). Horn et al. (2005) state that methanol, ethanol and propanol are not “growth supportive” for D. denitrificans. P. denitrificans is capable of degrading a wide variety of alcohols (Yamane et al., 1996).

Few groups have looked at the biodegradation of glycerol. Irgens et al. (1996) alluded to its breakdown in the taxonomic characterization of Polaromonas vacuolata. Interest in this area firstly lies in the role of alcohol dehydrogenases and their ability to use glycerol as a substrate. Toyama et al. (2004) state that glycerol dehydrogenases (GLDH) can also be regarded as type I alcohol dehydrogenases, even though their primary substrates are not alcohols, however, these enzymes are also capable of oxidizing several alcohols with high efficiency (Toyama et al., 2004).

The second area of interest in the metabolism of glycerol is the production of one possible metabolic intermediate of its breakdown, propan-1,3-diol (Daniel et al., 1995; Barbirato et al., 1996; Danner and Braun, 1999; Wang et al., 2003). There is an interest the microbial production of propan-1,3-diol as a monomer for the production of polytrimethylene terephthalate (Wang et al., 2003). Citrobacter freundii, Klebsiella pneumoniae, Clostridium butyricum and Enterobacter agglomerans are amongst the organisms that are able to metabolise glycerol (Daniel et al., 1995; Barbirato et al., 1996). Breakdown of glycerol proceeds by a number of possible pathways (figure 4.1). One common pathway proceeds via NAD+ linked glycerol dehydrogenase regenerating NAD+, then via phosphorylation of dihydroxyacetone and the glycolytic pathway (Daniel et al., 1995). The second proceeds via direct phosphorylation of glycerol to glycerol 3-phosphate (Weinhouse and Benziman, 1976). Another common pathway involves the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA) which is then reduced to propan-1,3-diol (Daniel et al., 1995).
Figure 4.1. Some possible breakdown pathways for glycerol.

As described in chapter two, bacteria in batch culture pass through a growth cycle. In this cycle the relationship between the organism’s growth rate and concentration of the substrate it is growing on can be described by the Monod model (Levenspiel, 1980, Okpokwasili and Nweke, 2005). In this model the growth increases to a maximum with increasing substrate concentration, to an upper limit beyond which faster growth is not possible. This model will produce a curve which plateaus at a maximum growth rate \( \mu_{\text{max}} \), at a specific substrate concentration. At high substrate concentrations the maximum growth rate is independent of the concentration of substrate (Okpokwasili and Nweke, 2005). This model whilst holding true at relatively low concentrations of substrate no longer applies at very high concentrations (Levenspiel, 1980; Okpokwasili and Nweke, 2005). There are a number of reasons for this. Firstly, as stated in chapter one substrates such as organic solvents can have detrimental effects on the cell,
compounds can also inhibit cell growth due to ionic effects (Edwards, 1970). Second, by-products or intermediate breakdown products can have toxic effects through disturbing the metabolism of the cell by chemical reactions or inhibition of critical enzymes (Edwards, 1970; Luong, 1987). For both reasons instead of a plateau usually the maximum growth rate plotted against substrate concentration will start low, reach a maximum and fall away as a critical substrate concentration is reached for a particular solvent in a specific cell type. A number of models most of which are derived from the Monod model have been proposed to overcome the limitations of this model taking into account these inhibitory effects and some are shown below.

\[
\mu = \mu_n \frac{S}{K_s + S} \left[ 1 - \frac{S}{S_m} \right]^n \quad \text{equation (4.1)}
\]

\[
\mu = \mu_n \frac{S}{K_s + S} \exp \left[ -\frac{S}{K_i} \right] \quad \text{equation (4.2)}
\]

\[
\mu = \mu_n \frac{S}{K_s + S + \frac{S^2}{K_i}} \quad \text{equation (4.3)}
\]

\[
\mu = \mu_n \left[ \exp \left[ \frac{S}{K_i} \right] - \exp \left[ \frac{S}{K_s} \right] \right] \quad \text{equation (4.4)}
\]

\[
\mu = \mu_n \left[ 1 - \left( \frac{S}{S_m} \right)^n \right] \quad \text{equation (4.5)}
\]

\[
\mu = \mu_n \left[ 1 - \left( \frac{S}{S_m} \right)^n \right] \quad \text{equation (4.6)}
\]

\[
\mu = \mu_n \exp \left( -K_i S \right) \quad \text{equation (4.7)}
\]

\[
\mu = \mu_n \frac{K_i}{K_i + S} \quad \text{equation (4.8)}
\]

Equation 4.1 is the Luong model (Luong, 1987), 4.2 is the Aiba model (Tramšek et al., 2006), 4.3 is the Haldane model (Kumar et al., 2005), 4.4 is the Edwards model (Tramšek et al., 2006), 4.5 is the Levenspiel model (Levenspiel, 1980), 4.6 is another model attributed to Luong (Passos et al., 1993), 4.7 is the Aiba and Shocla model (Passos et al., 1993) and 4.8 is another model attributed to Aiba and Shocla (Passos et
al., 1993). $K_i$ is the concentration of substrate that starts to inhibit growth and $S_m$ is the
concentration of substrate when the growth rate is zero, $n$ is a coefficient determined by
the model fitting software (Passos et al., 2003).

As described in chapter one, Rosenberg et al. (1980) developed a simple test for
measuring the adhesion of bacteria to organic non-aqueous solvents and thus their cell
surface hydrophobicity (Rosenberg et al., 1980; Rosenberg, 1984). The test involves
the mixing of the bacteria of interest with a non-aqueous hydrocarbon and measuring
the optical density of the aqueous portion either before and after treatment or with and
without treatment (Rosenberg et al., 1980; De Carvalho and da Fonseca, 2005). Rosenberget al. (1980) found that Acinetobacter calcoaceticus cells grown on
hexadecane would adhere to this solvent but also others which it could not degrade
(Rosenberg et al., 1980). Therefore cells are partitioned between the aqueous and non-
aqueous layers depending on how hydrophobic their surface is. This led Rosenberg et
al. (1980) to believe the assay could have a role in measuring general cell surface
hydrophobicity (Rosenberg, 1984).

The work described in this chapter is an examination of the growth and morphology
of cells on various organic solvents, both volatile and non-volatile. Experiments were
undertaken to see if the cell surface of some of the bacteria altered with continuous
exposure to glycerol and inhibition studies were carried out to see what level of
glycerol inhibited cell growth. These studies were carried out to determine the types of
VOC’s, solvents or organic compounds that could be used in an MFC without
deleterious effects on cell growth, since in principle using the maximum amount of a
particular carbon source would maximise the power output. In addition there was
concern that organic compounds might have an effect on the power output by altering
the cell surface (Choi et al., 2003).
4.2. Materials and methods

4.2.1. Chemicals

Bacteriological agar was purchased from Difco (UK), unless otherwise stated, all other chemicals were of the highest purity available and purchased from either Sigma Chemical Company (UK) Fisher (UK), Acros (UK), or BDH Laboratory Suppliers (UK). Volatile organic compounds (methanol, ethanol, propan-2-ol (IPA or isopropanol) and butan-1-ol) were obtained from Fisher UK and Sigma UK. Glycerol was obtained from Acros and Sigma UK. Ethanol-1,2-diol was obtained from Acros (UK).

4.2.2. Bacteria

Bacteria were purchased from DSMZ, the German National Resource Centre for Biological Material and on arrival were stored at +4°C prior to use. Initially the bacteria were grown in the media recommended by DSMZ, as described in chapter two. As also described in chapter two, the bacteria were adapted to grow on medium B.

The composition of medium B was identical for culturing cells in the presence of organic compounds. Autoclaving was carried out at 110°C for 10 minutes; glycerol was autoclaved in situ, other compounds were added to the basal medium post autoclaving. The same spectrophotometer was used as that for growth studies in chapter two. Cell counts were calculated as outlined in chapter three.

4.2.3. Adaptation of bacterial strains to organic solvents

*R. rhodochrous*, *P. denitrificans* and *D. denitrificans* were initially passaged from medium B with 2g/l glucose into medium B with 1g/l of glucose only, then into medium B with 1g/l glucose plus the organic compound of interest at 1% (v/v) before finally allowing the cells to grow on medium B and the organic compound. When repeating this work the successive diminution in glucose levels was found to be unnecessary and *R. rhodochrous*, *P. denitrificans* and *D. denitrificans* could be passaged from medium containing 2g/l glucose straight into medium containing 2% (v/v) propan-2-ol, butan-1-ol or 1% glycerol. Cells were adapted to growth on 3% (v/v) of a particular carbon source by repeated passaging. *Rhodococcus opacus* was adapted to growth on glycerol
by another PhD student.

Cultures were grown either in a volume of 100ml in 250-ml Erlenmeyer flasks or 25ml in 100-ml Erlenmeyer flasks. The cultures were allowed to grow at 20°C on a KS250 orbital shaker (IKA Werke, Germany) at 160rpm or a Stuart orbital shaker (Stuart, UK) at the same rate. No growth studies were undertaken on the bacteria until the cultures had been passaged six times in any medium. Where glucose control cultures were used fresh controls were used in each experiment, hence the results obtained vary in each case.

4.2.4. Growth studies

Growth studies used the same experimental methods as described in chapter two except medium B with carbon sources other than glucose was used. Where appropriate a control containing medium B with glucose as the carbon source was also run alongside to provide control cells for the MATH test, the methodology of which is described below. Another control containing the solvent of interest but no bacteria was run at the same time and this was assayed using gas chromatography, as outlined below, to measure the evaporation rate. This control was unnecessary in the case of glycerol which is non-volatile. Cells were inoculated into the above cultures from the same maintenance growth culture of the particular alcohol concerned. A sample was taken immediately before cells were inoculated into the medium; referred to as the “pre” sample below. Inhibition studies were carried out as in chapter three. No cell free controls were used. The organic solvents were added by volume and the concentrations were determined by GC or HPLC and are given in context in the results section below; concentrations are mM (mmol/l).

4.2.5. Gas chromatography

Cell samples were spun at 4500g for 1 hour and the undiluted supernatant injected into the instruments. Ethanol, 1,2 diol and glycerol growth studies were analysed on a Perkin-Elmer 8500 instrument using a carbowax BP20 column. The column length was
15metres and diameter 0.53mm. The mobile phase was nitrogen and its flow rate was
10ml/min, the column temperature followed a gradient from 60-300°C over the 30
minute run. 0.1µl was injected into the instrument. Data were collected and analysed
using Jones chromatography software v2.0.

Isopropanol, methanol and butanol were analysed on a HP5890 instrument using the
same carbowax BP20 column. The mobile phase was nitrogen and its flow rate was
13ml/min, the sample volume injected was 0.1µl split 50:50 after injection. The column
temperature followed a gradient from 60-300°C over the 30 minute run. Data were
collected and analysed using CWS32 software.

4.2.6. High performance liquid chromatography

The HPLC system consisted of a reverse phase ODS-L column (250mm x 4.5mm)
(Capital HPLC, Lothian, UK) on a Dionex system with a GS50 pump and ED50
electrochemical detector. The mobile phase was 50 mM perchloric acid (HPLC grade)
pH 2.1 adjusted to this pH with electrochemical grade sodium hydroxide. The mobile
phase was degassed using helium and the flow rate of the mobile phase was between
0.25 and 0.6ml/min. Cell samples were spun at 4500g for 1 hour and then diluted in
deionised water from 2x to 10x into glass chromocol vials. These were then capped and
sealed and injected using a Famos autosampler. The data were analysed using the
Dionex chromeleon software v6.60.

4.2.7. MATH test

The methodology for this test was described in chapter three in section 3.2.2.3.

4.2.8. Statistics

Parametric statistics (t-tests and F-tests) were carried out in gnumeric 1.8.3. Non-
parametric statistics were undertaken using SPSS 9.0 and Rplot 1.3/1.4. Pearson
correlations and their probabilites were calculated in Rplot 1.4. Growth curves were
fitted and the lag phase, maximum biomass and growth rates calculated as outlined in chapter two.
4.3. Results

4.3.1. Glycerol as the carbon source for D. denitrificans

4.3.1.1. Glycerol growth studies using D. denitrificans

D. denitrificans adapted readily to growth on glycerol. Although it achieved similar levels of biomass as on other media types tried, its growth rate was lower. The calculated maximum growth rates were lower than for cells grown on medium 830 and medium B with glucose. The lag phase is also extended in glycerol grown cells. The cells that were cultured on glucose in the control culture, but were inoculated into this medium from a glycerol culture, grew more slowly than would be expected on this carbon source. Figure 4.2 shows the fitted growth curve for this compound and glycerol concentration (283mM) as determined by GC. There was a highly significant correlation between the drop in glycerol concentration and increase in cell number (Pearson R=−0.8838, p<0.0001).

![Growth curves](image)

**Figure 4.2.** Growth study for D. denitrificans cultured on glycerol and glucose. Graph a) shows growth on glucose and b) on 283mM glycerol as the sole carbon source. Cell number is shown by ▼ and glycerol concentration by ○. The points for glycerol grown cells are a mean of three cultures and the error bars show the standard error of the mean for these three cultures. The glucose concentration was not measured in these cultures.
4.3.2. Glycerol growth inhibition studies using *D. denitrificans*

To determine what level of glycerol inhibited cell growth a study was set up using five different concentrations of glycerol using duplicate cultures (171mM, 392mM, 752mM, 971mM and 2174mM as determined by HPLC). No growth took place at the highest concentration (2174mM) of glycerol. Figure 4.3 shows the results for this study. Table 4.1 shows the growth characteristics for *D. denitrificans* on glycerol from these two studies.

![Glycerol inhibition study graphs](image)

**Figure 4.3.** Glycerol inhibition study on four different initial concentrations of glycerol as determined by HPLC. a) 171mM, b) 392mM, c) 752mM and d) 971mM. Cell number is shown by ▼ and glycerol concentration by ○. The error bars show the standard error of the mean for duplicate cultures. No growth took place at a concentration of 2174mM glycerol.
Table 4.1. Growth characteristics of *D. denitrificans* on glycerol in both the inhibition study and the earlier growth study.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Lag phase (hours)</th>
<th>Maximum cell number</th>
<th>Generation time (hours)</th>
<th>Maximum growth rate (hours⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>283mM glycerol</td>
<td>18.95</td>
<td>1.43 x 10⁶</td>
<td>12.54</td>
<td>0.024</td>
</tr>
<tr>
<td>171mM glycerol</td>
<td>47.15</td>
<td>4.98 x 10⁶</td>
<td>11.98</td>
<td>0.025</td>
</tr>
<tr>
<td>392mM glycerol</td>
<td>32.33</td>
<td>2.95 x 10⁶</td>
<td>11.15</td>
<td>0.027</td>
</tr>
<tr>
<td>752mM glycerol</td>
<td>36.66</td>
<td>2.62 x 10⁶</td>
<td>5.90</td>
<td>0.051</td>
</tr>
<tr>
<td>971mM glycerol</td>
<td>34.22</td>
<td>2.00 x 10⁶</td>
<td>12.62</td>
<td>0.024</td>
</tr>
</tbody>
</table>

An attempt was made to fit the starting concentration (before the addition of cells) to various inhibition models outlined above. None of the models fitted the dataset well; the Luong model (Luong, 1987) was the best, however the $R^2$ value was only 0.67 and the high errors of the coefficients indicated the model was incorrect.

The reduction in glycerol levels is shown in the chart in figure 4.4. This is shown as a % decrease between the last samples taken and the pre samples. The growth rate was faster in the glycerol inhibition study cultures even where the concentration of glycerol was higher and the percentage breakdown of glycerol was larger than it was in the earlier growth study described above. The growth rate in the 752mM culture was the fastest of all the cultures in these experiments.
Figure 4.4. Reduction in concentration of glycerol for *D. denitrificans* cultures. The graph shows data from the growth study and the inhibition study. The standard error bars show the standard error of the mean for either 2 or 3 cultures.

4.3.1.2. Glycerol MATH studies using *D. denitrificans*

The MATH test showed a significant difference was found between the transformed log affinity values for *D. denitrificans* cells grown on glucose and those grown on glycerol for using a t-test (the data were normally distributed). The experiment was repeated and data sets were compared using a non-parametric test (Wilcoxon matched pairs) since they were not normally distributed. This second time the difference was not significant.

4.3.1.3. *D. denitrificans* on other carbon sources

*D. denitrificans* was incapable of growth using methanol, ethanol or butanol as its sole carbon source.
4.3.2. *R. rhodochrous*

This organism was capable of growth on a variety of alcohols. Table 4.2 shows the growth characteristics on each one. The growth was slower and produced less biomass than either medium 1 or medium B using glucose as the carbon source. Examination of the late exponential phase cells cultured on the carbon sources described below using a light microscope indicated the cells were of a normal morphology and size with no large clusters. Compound breakdown was in each case incomplete (see figure 4.12).

4.3.2.1. Ethan-1,2-diol

In the ethan-1,2-diol growth study both the cell free control and the cell cultures showed falling levels of this compound (figure 4.5). There was a statistically significant difference between the concentrations of ethan-1,2-diol in the cell cultures (starting concentration 600mM) and cell free control (starting concentration 608mM) using a t-test (p=0.0125). There was also a very statistically significant spearman's rank correlation between growth and the drop in ethan-1,2-diol concentrations (R=-0.850, p=0.0037).
Figure 4.5. Growth curves for \textit{R. rhodochrous} cultured on 600mM ethan-1,2-diol as the sole carbon source. a) shows the control glucose culture growth. b) shows the cell growth (○) using ethan-1,2-diol as a sole carbon source compared to control cell free ethan-1,2-diol concentration (○) and ethan-1,2-diol in medium with cells (○). The points are a mean of 3 cultures (except for the controls) and the error bars show the standard error of the mean for these 3 cultures.

4.3.2.2. IPA

Unlike the ethan-1,2-diol study the cells in the glucose control grew to a lower maximum cell number than those cells cultured in propan-2-ol (IPA) (figure 4.6). The concentration of IPA in the control was 392 mM and was an average of 366 mM in the cell culture samples at the start of the cultures. There was a significant difference between the levels of IPA in flasks with cells and the cell free control (p=0.014). However, the correlation between cell number and drop in IPA levels was not significant (spearman's rank correlation R=−0.5274, p=0.054).
Figure 4.6. Growth curves for *R. rhodochrous* cultured on 392mM IPA as the sole carbon source. a) shows the glucose control culture growth. b) shows the cell growth (○) using IPA as a sole carbon source compared to control cell free IPA concentration (○) and IPA in medium with cells (○). The points are a mean of three cultures (except for the controls) and the error bars show the standard error of the mean for these three cultures.

4.3.2.3. Methanol

Like the IPA cultures the glucose control grew to a lower maximum cell number than the methanol cultures. Due to a probable pipetting error the starting concentrations of the control and cell culture samples were completely different (figure 4.7), being 244mM in the cell free control at time zero and a mean of 187mM in the cell culture samples. Therefore no statistical comparison of the levels of methanol was possible. The correlation between the fall in methanol concentration and the cell number was just significant by spearman's rank correlation (R=-0.6363, p=0.0479). The fall in methanol concentration due to evaporation of the compound in the control flask was low with a concentration of 231mM at the end of the experiment.
Figure 4.7. Growth curves for *R. rhodochrous* cultured on methanol as the sole carbon source. Graph a) shows the control glucose culture growth. b) shows the cell growth (○) using methanol as a sole carbon source compared to control cell free methanol concentration (●) and methanol in medium with cells (●). The points are a mean of two cultures (except for the controls) and the error bars show the standard error of the mean for these two cultures.

4.3.2.4. Butanol

The control flask with glucose as its carbon source outgrew the butanol grown cells (figure 4.8). Like the methanol study there was a concentration difference for butanol between the control cell free flask and the butanol cultures at time zero (108mM and 97mM respectively). The difference was small and a t-test was applied. Unlike previously with ethan-1,2-diol and IPA there was no significant difference between the control and the butanol cultures in butanol concentration (p>0.05). There was also no significant correlation between the drop in butanol concentration in the flasks with and without cells present (spearman's rank correlation R=−0.5030, p>0.05).
Figure 4.8. Growth curves for *R. rhodochrous* cultured on butanol as the sole carbon source. a) shows the control glucose culture growth. b) shows the cell growth (○) using butanol as a sole carbon source compared to control cell free methanol concentration (●) and methanol in medium with cells present (○). The points are a mean of three cultures (except for the controls) and the error bars show the standard error of the mean for these two cultures.

4.3.2.5. Growth of *R. rhodochrous* on other carbon sources

*R. rhodochrous* grew poorly on glycerol and the samples were not assayed (data not shown). Growth was so poor on ethanol that no formal growth studies were undertaken.
Table 4.2. Growth characteristics of *R. rhodochrous* on various alcohols. Note the butanol study was incomplete in that the cultures had not reached stationary phase.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Lag phase (hours)</th>
<th>Maximum cell number</th>
<th>Generation time (hours)</th>
<th>Maximum growth rate (hours⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>methanol</td>
<td>36.87</td>
<td>5.35 x 10⁷</td>
<td>6.48</td>
<td>0.046</td>
</tr>
<tr>
<td>propan-2-ol</td>
<td>64.48</td>
<td>1.39 x 10⁸</td>
<td>16.09</td>
<td>0.019</td>
</tr>
<tr>
<td>ethan-1,2-diol</td>
<td>45.11</td>
<td>5.43 x 10⁷</td>
<td>10.43</td>
<td>0.029</td>
</tr>
<tr>
<td>butanol</td>
<td>62.99</td>
<td>4.27 x 10⁷</td>
<td>3.35</td>
<td>0.090</td>
</tr>
</tbody>
</table>

4.3.2.6. MATH test results

*R. rhodochrous* cells died too fast to test. The optical density of cells with and without hexadecane present fell in each successive tube as the OD₆₀₀ was measured. Unlike the results for *D. denitrificans* this was systematic error not random error. To test the hypothesis that the cells were disintegrating a sample was examined under the microscope and spun briefly several times. Cell debris could be seen under the microscope and the OD₆₀₀ fell after each spin.

4.3.3. *R. opacus*

4.3.3.1. *R. opacus* using glycerol as its sole carbon source

*R. opacus* was capable of strong growth on glycerol although adaptation from glucose was not as ready as for *D. denitrificans*. An inhibition study on five different concentrations of glycerol was carried out (figure 4.9). The concentrations were 203mM, 283mM, 669mM, 762mM and 1156mM glycerol as determined by HPLC.
Figure 4.9. *R. opacus* glycerol study on four different initial concentrations of glycerol as determined by HPLC. a) glucose control, b) 203mM, c) 283mM and d) 669mM e) 762mM and f) 1156mM glycerol. Cell number is denoted by □ and glycerol concentration by ○. The error bars show the standard error of the mean for duplicate cultures.

As for *D. denitrificans*, the five initial glycerol concentrations in the *R. opacus* inhibition study as determined by HPLC, were fitted using a Luong fit (figure 4.10). The concentration of glycerol required to produce a zero growth rate was 1565mmol/l.
Figure 4.10. Maximum growth rates plotted against initial glycerol concentration for *R. opacus* with an inhibition fit. The regression uses the Luong model as cited by Passos *et al.*, (1993), $R^2 = 0.986$, $S_m = 1565$ mmol/l glycerol.

4.3.3.2. MATH test results

Given that this test failed for *R. rhodochrous*, *R. opacus* was not tested.

4.3.3.3. Other carbon sources

*R. opacus* has been found to grow on a wide variety of solvents and organic compounds including phenol, hexadecane, methanol and kerosene as part of PhD studentship projects at Heriot-Watt. The cells will grow in the presence of chlorinated phenols but cannot use them as their sole carbon source. The cells also grow using acetate as their sole carbon source (chapter seven).
4.3.4. Other bacteria

4.3.4.1. P. denitrificans

P. denitrificans was incapable of growth on glycerol. Studies using this organism were not continued.

4.3.4.2. S. oneidensis

S. oneidensis was incapable of growth on glycerol. Attempts to culture it on closely related compounds failed.
4.4. Discussion

*D. denitrificans* readily adapted to growth on glycerol. Throughout the experiments shown in this chapter as can be seen from table 4.1 and figure 4.4 both the rate of growth and percentage glycerol breakdown using this organism has increased between the two studies. In addition the maximum cell number achievable increased significantly, in the case of inhibition study cultures approximately doubling over earlier experiments. The percentage of glycerol removed also increased dramatically, almost doubling from the first growth study to the inhibition studies which were some months later. One possible reason for these findings is the organism is adapting to the compound using mechanisms outlined in chapter one. The cells were maintained in liquid cultures containing glycerol and had been through many generations using this as the sole carbon source between the studies. In this regard the results of the MATH assay are somewhat curious, in that one test showed a significant difference between cells cultured on glycerol and those cultured on glucose indicating an adaptation of the cell surface to glycerol by increased cell surface hydrophobicity, but the repeat did not. The only explanation that can be advanced in the second case was that the glycerol grown cultures were grown on glucose for three passages before being returned to glycerol as a carbon source. Some of the other possible reasons for this adaptation concerning metabolites will be outlined in the next chapter. Despite this adaptation to glycerol, 2g/l glucose as the sole carbon source still gave a faster growth rate than glycerol, although the maximum biomass was lower. It should be noted, however, 2g/l is a significantly lower quantity of carbon source than the glycerol quantities used above.

Unlike *R. opacus* (covered below), the growth inhibition study for *D. denitrificans* showed an increase in maximum growth rate for the three lowest concentrations of glycerol followed by a rapid fall till zero growth for the very highest concentration (figure 4.11). The probable reason for this is that there are two counterbalancing effects going on. The first is the increasing concentrations of glycerol leading to increased growth rates. The second is an inhibition effect or effects. This could either be enzymatic, in that the glycerol inhibits the metabolic pathway involved in its breakdown.
or effects on the organism's cell membrane or both. Whichever inhibition effect is taking place there is a very sharp cut-off between an increase in maximum growth rate and inhibition leading to a decrease. For the highest concentration of glycerol (2174mmol) although no change could be seen by eye in the turbidity of the culture, under the microscope individual immature motile cells could be seen.

The growth of *D. denitrificans* on glycerol does not follow the Monod model in that clearly growth is not independent of glycerol concentration (Okpokwasili and Nweke, 2005). Growth is more complicated than Monod saturation kinetics (table 4.1). In some way either directly or indirectly cell growth is being inhibited by glycerol. Therefore all the models shown in the introduction to this chapter were tried on the inhibition study data. None of the models fitted. These models appear to work well when the maximum growth rate is decreasing consistently with the increasing potential inhibitor concentration and the shape of the curve is similar to an inverse Michaelis-Menten curve. The case with chromate was special in that it was not the carbon source, so a zero chromate concentration gave a higher maximum growth rate than the lowest concentration of the chromate. In the glycerol inhibition studies there was no glucose control. No models gave a plausible fit due to the increase in maximum growth rate at the 752mM glycerol concentration. As can be seen in table 4.1, unlike the growth rate the maximum biomass does show a consistent fall with increasing glycerol concentration. Therefore the Luong model as given by Passos *et al.* (1993) produced a good fit (figure 4.11) indicating a complete inhibition for growth at a glycerol concentration of 2288mM. Plotting the maximum growth rate data as a scatter graph (4.11 insert) and examining the data by eye 1000mM is the minimum concentration at which growth starts to be affected for *D. denitrificans*. It is at this glycerol concentration that the growth rate is approximately half the maximum that was found for this organism on this carbon source. *D. denitrificans* is also somewhat fastidious and of the carbon sources tried in this project the only one it was capable of using to support growth was glycerol.
**Figure 4.11.** Maximum biomass plotted against initial substrate concentration for *D. denitrificans* cultured on glycerol. The R² value was 0.963 using the Luong model given by Passos *et al.* (1993). The insert shows the same initial glycerol concentrations plotted against the maximum growth rates found for each glycerol concentration.

*R. rhodochrous* was clearly a far more versatile organism than *D. denitrificans*, and it was capable of using a wide variety of compounds as its sole carbon source, although the growth characteristics varied greatly even using the same alcohol. This was true for different flasks within growth studies; in the methanol and butanol growth studies one culture out of three grew far slower than the rest. The growth on isopropanol and methanol was the strongest judging by cell number although the largest percentage breakdown was for ethan-1,2,-diol (figure 4.12). Growth on butanol was particularly poor with no significant correlation between cell growth and reduction in butanol levels. The percentage reduction in alcohols is lower than that of *D. denitrificans* cultured on glycerol. *R. rhodochrous* was unable to grow significantly on glycerol, making culturing the organism on this substrate of no further interest in this project.
The organism was found not to be robust enough to withstand the centrifugation required to carry out the MATH assay despite the successful testing of another strain of *Rhodococcus* by Sokolovská *et al.* (2003). However, some adaptation of the organism to organic solvents would be expected.

![Figure 4.12](image)

**Figure 4.12.** Percentage biodegradation of alcohols for the *R. rhodochrous* cultures. The standard error bars show the standard error of the mean for either two or three cultures.

*R. opacus* is capable of growth on some alcohols, as well as a wide variety of other compounds. The colour of the cells varies depending on the substrate the organism is cultured with (Figure 4.13). The colouration is associated with the cells since when they are centrifuged to a pellet the supernatant is clear and the pellet coloured. The colouration obtained is complicated. It appears to depend on both the solvent and the ionic concentration as well as the stage of the culture. In medium containing glucose, acetate or hexadecane the cells tend to be beige similar to the top culture plate in figure 4.13., but lighter. Cells cultured using methanol initially were the orange colour shown in figure 4.13 (middle culture plate) but with increased adaptation changed to the dark pink colouration shown in figure 4.13 (bottom culture plate) the same colour they grew as on glycerol. In the presence of phenol and chlorinated phenols the cells are very dark.
brown, almost black. In chapter seven when experiments were done in which the ionic concentration of the medium was varied it was found that the colour varied. The colour also changes over the stages of the culture. In the early stages of growth the cells tend to be light grey whatever the carbon source. It is only in the latter stages of exponential phase that the cells change to the colours outlined above. This finding suggests that the colour change may be partly a stress response to a build up of metabolites such as organic acids. However, the finding as described in chapter three that the cells cultured in medium containing copper (II) consistently change to bright pink suggests that the stress response is not only related to the carbon source. *R. rhodochrous* tended to be less varied in colour being red in most cultures, but on hexadecane it was a very pale beige colour. No information regarding the colouration of this genus of bacteria can be located in the literature.
Figure 4.13. Colour variation of *R. opacus* cultured on three different carbon sources. Top plate glucose, middle plate methanol (early generation number on this solvent) and bottom plate glycerol.

*R. opacus*, unlike *R. rhodochrous*, grew very well on glycerol. It proved more difficult to adapt than *D. dentrificans* since having grown for six passages in liquid culture using 1% glycerol as the sole carbon source it then failed to grow. It was finally adapted from a glycerol plate made using the sixth generation culture by inoculating a liquid culture and repeating the above process of liquid culture. In liquid glycerol cultures the organism was found to be pink in colour.
Again, as was found with *D. denitrificans*, on glycerol the growth did not follow a simple saturation model. The inhibition study gave very different results to those for *D. denitrificans* for similar concentrations of glycerol. No increase was seen in the maximum growth rate with increasing concentrations of glycerol, only a decrease. Whether this is due to the effects of the increasing concentration of the carbon source or enzyme inhibition is open to conjecture. The results did allow a model to be fitted indicating that the concentration required to completely inhibit *R. opacus* growth was 1565mM. It also adapted very well to methanol although no growth studies were undertaken.

*S. oneidensis* was found to be incapable of growth on glycerol. This is somewhat surprising, since its genome contains the sequence to encode glycerol kinase (figure 4.1) (Serres and Riley, 2006). Attempts to culture it on closely related compounds such as propan-1,2 and 1,3 diol to see if the organism could feed into the glycerol breakdown pathway via either 2 or 3-hydroxypropanal (see figure 4.1) were unsuccessful. This organism, after apparently adapting to growth on lactic acid, stopped growing completely on all media tried including on typtic soy agar. Its part in this project was over.
Chapter 5. Influence of fermentation products on cell growth

5.1. Introduction

In the previous chapter *D. denitrificans* was shown to be both capable of growth using glycerol as its sole carbon source and also adapting to this compound by achieving faster growth and higher cell numbers. In a microbial fuel cell, particularly if the culture is unstimulated, the build-up of potentially toxic fermentation products could have a negative effect on cell number and therefore power output. As can be seen from the generic metabolic pathway diagram shown in Figure 4.1 a wide variety of fermentation products can be potentially produced. Some of these are acidic such as 2-hydroxypropanoic acid (lactic acid) and some are toxic to cell growth, for example lactic acid, 3-hydroxypropanal (3-HPA) and 2-propanal (Sung et al., 2002; Maris et al., 2004).

Interest in 3-hydroxypropanal has been growing due to its antimicrobial properties, its ability to form polymers directly, or indirectly as the precursor to 3-hydroxypropanoic acid, 2-propanol or acrylic acid which can also be used to form polymers or have other uses (Slininger et al., 1983; Talarico and Dobrogosz, 1989; Sung et al., 2002). 3-HPA production has been studied in a variety of bacteria including *Klebsiella pneumoniae* and proceeds via inducible pathways shown in figure 4.1 (Slininger and Bothast, 1985). After transport into the cell glycerol is broken down aerobically via glycerol kinase to glycerol 3-phosphate or via the formation of dihydroxyacetone by the enzyme glycerol dehydrogenase. Another enzyme (DHA kinase) then links dihydroxyacetone to the glycolytic pathway via its conversion to glyceraldehyde-3-phosphate (Zwaig et al., 1970; Weinhouse and Benziman, 1976; Slininger et al., 1983; Slininger and Bothast, 1985; Barbirato et al., 1996). The pathway of interest in this project is the anaerobic pathway which proceeds via a vitamin B12 coenzyme linked enzyme glycerol dehydratase which dehydrates the glycerol to 3-HPA. This compound either accumulates or proceeds to a further reaction catalysed by the enzyme propan-1,3-diol dehydrogenase to form propan-1,3-diol. This final reaction is linked to the oxidation of NADH and allows the regeneration of NAD⁺ to take place.
It depends on the redox balance in the cell, that is the ratio of NAD+/NADH (Sobolov, and Smiley, 1960; Slininger and Bothast, 1985; Murarka et al., 2008). A redox imbalance can be brought about by starting the cultures growing on glucose as the carbon source first then adding glycerol. This is described in more detail in the discussion. Indeed in Lactobacillus collinoides no production of 3-HPA is observed without both glucose and glycerol being present and it is unable to use glycerol as its sole carbon source (Sauvageot et al., 2000). Interestingly the glycerol dehydratase can be induced under aerobic or anaerobic conditions and higher enzyme activity is present in aerobically grown cells (Slininger and Bothast, 1985).

The literature is unclear as to whether it is the 3-hydroxypropanal itself which is toxic, or the variety of monomeric, hydrated monomeric, and cyclic dimeric isomers the molecule can form under acidic or basic conditions (Sung et al., 2002; Talarico and Dobrogosz, 1989). Barbirato et al. (1996) found that 3-HPA was capable of inhibiting cell growth and therefore propan-1,3-diol formation. Its formation therefore could have an inhibiting effect on cell growth and hence current production in an MFC.

Organic acids are also formed as a result of oxygen transfer limitations with cell build up leading to some fermentation taking place. This leads to drop in pH in the cultures concerned.

In this chapter a series of experiments are outlined in which an attempt was made to look at the influence of pH and 3-hydroxypropanal on D. denitrificans cell growth, the causes of the fall in pH that was found to occur and to see if the cells were capable of propan-1,3-diol formation. This was attempted by culturing cells at the extreme of their pH growth range and using the idea of co-fermentation to skew the redox balance in the cells towards the production of propan-1,3-diol. These factors would have relevance to power output in microbial fuel cells if the cells produced 3-HPA or propan-1,3-diol and couldn’t use them as a carbon source and in addition excessive pH fall could inhibit cell growth if this fall was outside the organism’s normal range.
Chapter 5. Influence of fermentation products on cell growth

5.2. Methods

Many of the pH and propan-1,3-diol growth studies in this section of the project were carried out by Emma Paton as part of an undergraduate research project supervised by the author and her work is acknowledged with thanks. Most of the work in this chapter was undertaken using *D. denitrificans* however some results for *R. opacus* are shown. The concentration of the organic compounds used was as before determined by analysis at the start of the culture run, not by the volume added. These initial concentrations are given in context below in mM (mmol/l).

5.2.1. Cell culture

All chemicals were of the highest quality and were purchased from Fisher or Sigma UK. Cell culture was carried out as in chapter four unless otherwise noted. pH adjustment was made using sulphuric acid or sodium hydroxide. Analysis of glycerol and other metabolites was by HPLC and the Perkin-Elmer 8500 as described previously. A pH meter was used to measure pH unless the use of pH paper (BDH, UK) is indicated.

5.2.2. Propan-1,3-diol production

Cultures were initially grown on glycerol only although a combination of glucose and glycerol was also used (described below). To one culture D-fructose-1,6, bisphosphate (trisodium salt) hydrate was added. 0.24g of this compound was weighed out and dissolved in 3ml of deionised water, sterile filtered and 1ml added to 100ml of medium B containing 49ml/l glycerol, making an expected final concentration of 1.97mM.

5.2.3. 3-hydroxypropanal synthesis

Since it is not possible to purchase 3-hydroxypropanal it had to be synthesised. This was carried out according to Hall and Stern (1950) with some adaptations. The reaction
was carried out in a 150ml round bottomed flask suspended in a waterbath at 50°C placed in a fume hood. Due to the toxicity of 2-propanal nitrile gloves were worn throughout. 33ml of deionised water, 10ml of 3N sulphuric acid and 7.5ml of 2-propanal were added to the flask. The reaction mixture was incubated for 2.5 hours then rapidly cooled to 1°C in a mixture of water and ice. The volume was measured and was found to be 47ml, the colour was a very slightly yellowy brown. Solid calcium carbonate was added to the mixture until the pH was between 6.5 to 7 determined using pH paper. The suspension was spun at 4500g for 1 minute to bring the calcium sulphate formed to the bottom of the tube. The supernatant was then removed and aliquoted into two halves. One half was run down a column packed with silica gel (60-120 mesh) (BDH, UK) previously dried at 90°C for 24 hours. The packed silica gel column was too long to enable the volume of liquid added to elute and some of the second aliquot and some water had to be added to allow sufficient volume to elute to enable its collection. Additional silica gel was added to this portion which was spun as above and the supernatant decanted. Both portions were stored at -20°C, but only the former was used in the cell culture and analysed by HPLC. A sample was prepared for NMR by defrosting, mixing and centrifugation at 4500g for 1 hour followed by filtration thorough a 0.2μm cellulose nitrate filter (Whatman, UK). It was then sent for analysis on a Bruker A400 NMR machine.

Since the exact concentration of 3-HPA was unknown an attempt was made to back calculate its concentration using the HPLC. An HPLC trace from the reaction mixture compared with pure 2-propanal indicated an additional product that could only be 3-HPA (Hall and Stern, 1950). A series of six standards were prepared by doubling dilutions of 2-propanal which were analysed using the HPLC. A straight line fit was obtained with an R² value of 0.97. The calculations were used to determine the efficiency of 3-HPA formation are shown in the appendix.

3.3ml of the 3-HPA solution was added to each of two cultures of D. denitrificans cells with glycerol as the sole carbon source. Two flasks of the same organism were cultured as controls without any 3-HPA present. The controls were analysed using the gas chromatograph for reasons described in context below.
5.2.4. Fractional distillation of cell supernatant

Cell supernatant was combined from two glycerol control cultures spun at 4500g for 1 hour. The supernatant was decanted into a 250 ml Quickfit round bottomed flask and this was sat in a mantle and after anti-bumping granules were added the water cooled fractional distillation apparatus was attached. The temperature was monitored using a thermometer and five fractions collected over a range of temperatures between 55°C and boiling point. All fractions were run on the Perkin-Elmer 8500 instrument on a carbowax BP20 column as described previously then sent for ¹H and ¹³H NMR analysis.

5.2.5. Statistics

Statistical calculations where relevant on data used in this chapter were carried out as described in previous chapters, except data were checked for normality and the Mann-Whitney test carried out in a later version of Gnumeric (1.10.8).
5.3. Results

5.3.1. pH effects

A series of growth studies using *D. denitrificans* were undertaken to study the effects that initial culture medium pH had on cell growth, metabolite production (particularly propan-1,3-diol) and glycerol breakdown (figures 5.1-4 and table 5.1). The aim of the experiments was to examine whether culturing the cells outside their normal pH range could inhibit enzymes in the glycolytic pathway leading to the production of propan-1,3-diol.

In the first experiment cultures were inoculated into medium B at pHs of 6.26, 7.0 and 8.19 containing glycerol at over 1.1M. Strong growth was obtained in the cultures at the two lower pHs but none was seen in the pH 8.19 cultures. The cells inoculated into cultures at pH 7.0 grew at a slightly faster rate and reached a higher cell count than the pH 6.26 cultures (figure 5.1).

A second growth study was made at starting pHs slightly higher than the lower two used above (pH 6.51 and pH 7.47) with approximately the same starting concentrations of glycerol. The cultures started at pH 6.51 reached almost the same cell number as in the pH 6.26 cultures above (figure 5.2).

A repeat of the first experiment was made at initial culture medium pHs of 7.0 and 6.19 (without pH 8.2 cultures) but using a lower glycerol concentration to see what effect this would have. Over the sampling period the cell growth and cell number was higher in the lower pH cultures (figure 5.3).

Cell growth using *D. denitrificans* had found the pH had consistently dropped to less than 4. Two growth studies were carried out using this organism in low pH culture medium (figure 5.4). Medium B is hard to buffer at pH values of less than 4 so the starting pH values were a mean of 2.78. Surprisingly there was some very slight growth at this pH (figure 5.4, d) and the pH fell slightly in both cultures to a mean of 2.75. The
experiment was repeated and this time there was more success in adjusting the culture medium pH to a mean of 3.72. In these cultures more growth was seen (figure 5.4. d) with continuing pH fall.

Whilst some differences were seen in the growth characteristics of the first three studies (figures 5.1-3) there were similarities in other features of the cells’ growth. The starting pH made little difference to the glycerol breakdown, the final sample glycerol concentrations from the different pH cultures in each study were found to be close in value whatever the starting pH. Statistical tests (Mann-Whitney U and in one case a t-test) showed there was no significant difference in glycerol levels in any of the cultures with different starting pH’s in each study. A second common finding was that whatever the starting pH value of the culture the pH values of the cultures converged. Again Mann-Whitney U tests in none of the first three studies showed a significant difference in culture pH values between the initial pHs in each study. This was despite some bias being introduced by the initial differing pH values. In one study (pH 6.51/7.47) the p value was verging on the significant (p=0.054). Although differences in cell count were seen between the sets of cultures in each study, again none showed a significant difference using Mann-Whitney U. Finally no propan-1,3-diol was detected using either the GC or HPLC in any of the above cultures.
Figure 5.1. Growth study of *D. denitrificans* cultured in medium B at pH 6.2 and 7.0. Graph a) shows cell growth and glycerol concentration for cells cultured at pH 6.26. b) shows cell growth and glycerol concentration for cells cultured at pH 7.0. c) pH change and cell growth for pH 6.26. d) pH change and cell growth for pH 7.0. The starting concentration of the glycerol was a mean of 1240mM in the pH 6.26 cultures and 1158mM in the pH 7.0 cultures. Cell number is denoted by ▼, glycerol by ○ and pH by △. The error bars show the SEM of duplicate cultures. Cultures at 8.19 produced no cell growth and data are not shown.
Figure 5.2. Growth study of *D. denitrificans* cultured in medium B at a pH of 6.51 and 7.47. a) shows cell growth and glycerol concentration for cells cultured at pH 6.51. b) shows cell growth and glycerol concentration for cells cultured at pH 7.47. c) pH change and cell growth for pH 6.51. d) pH change and cell growth for pH 7.47. The starting concentration of the glycerol was a mean of 1289mM in the pH 6.51 cultures and 1214mM in the pH 7.47 cultures as determined by HPLC. Cell number is denoted by ▼, glycerol by ○ and pH by △. The error bars show the SEM of duplicate cultures.
Figure 5.3. Repeat growth study of *D. denitrificans* cultured in medium B at an initial pH of 6.19 and pH 7.0. a) shows cell growth and glycerol concentration for cells cultured at pH 6.19. b) shows cell growth and glycerol concentration for cells cultured at pH 7.0. c) pH change and cell growth at pH 6.0. d) pH change and cell growth for pH 7.0. The starting concentration of the glycerol was a mean of 197mM in the pH 6.0 cultures and 181mM in the pH 7.0 cultures as determined by HPLC. Cell number is denoted by ▼, glycerol by ○ and pH by △. The error bars show the SEM of triplicate cultures.
Figure 5.4 *D. denitrificans* cultures grown under a variety of conditions. a) shows cell growth and glycerol concentration for cells cultured without shaking. Cells were cultured on an initial concentration of 724mM glycerol. b) shows cell growth and glycerol concentration for cells cultured at pH below 4 using a starting concentration of 689mM glycerol. c) pH change in the unshaken cultures, the starting pH was 7.4. Cell number is denoted by ▼, glycerol by ○ and pH by △. d) pH change and cell growth for cells cultured in medium at less than pH 4. Cell number is denoted by ▼ in cultures started at pH 3.72 and ▼ in cultures started at pH 2.78, pH by △ in the pH 3.72 cultures and △ in the pH 2.78 cultures. Error bars show the standard error of the mean for two cultures. The error bars show the SEM of duplicate cultures.
Table 5.1. Growth characteristics of *D. denitrificans* in the studies carried out in this chapter.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Lag phase (hours)</th>
<th>Maximum cell number</th>
<th>Generation time (hours)</th>
<th>Maximum growth rate (hours⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH6/7/8 study pH6.26</td>
<td>15.15</td>
<td>2.73 x 10⁸</td>
<td>6.87</td>
<td>0.044</td>
</tr>
<tr>
<td>pH6/7/8 study pH7.0</td>
<td>18.93</td>
<td>5.93 x 10⁸</td>
<td>6.56</td>
<td>0.046</td>
</tr>
<tr>
<td>PH6.51 and 7.47 study pH 6.51</td>
<td>28.06</td>
<td>2.17 x 10⁸</td>
<td>11.4</td>
<td>0.026</td>
</tr>
<tr>
<td>PH6.51 and 7.47 study pH 7.47</td>
<td>32.2</td>
<td>1.10 x 10⁹</td>
<td>6.59</td>
<td>0.046</td>
</tr>
<tr>
<td>growth study pH6.19</td>
<td>17.7</td>
<td>4.07 x 10⁸</td>
<td>2.54</td>
<td>0.119</td>
</tr>
<tr>
<td>growth study pH7.0</td>
<td>18.51</td>
<td>3.71 x 10⁸</td>
<td>4.31</td>
<td>0.070</td>
</tr>
<tr>
<td>Control unshaken</td>
<td>36.31</td>
<td>1.02 x 10⁸</td>
<td>23.51</td>
<td>0.013</td>
</tr>
<tr>
<td>Cell study &lt; pH4</td>
<td>28.43</td>
<td>2.10 x 10⁷</td>
<td>20.81</td>
<td>0.014</td>
</tr>
</tbody>
</table>
It was noticed in the first pH study (figure 5.1 c) that as the cultures entered stationary phase the pH rose. It was decided to examine the \textit{D. denitrificans} cultures after the end of exponential phase to measure pH changes. Long term monitoring was carried out of cells of \textit{D. denitrificans} cultured on approximately 650mM glycerol. Results showed that growth follows a standard microbial growth pattern but growth continues for some time after the end of exponential phase with a rise in pH (figure 5.5). In addition the cells could not only survive a pH fall to as low as 4, but to a certain extent could reverse it.

![Graph showing cell count and pH over time over 400 hours](image)

**Figure 5.5.** Long term growth study of \textit{D. denitrificans}. Error bars show the standard error of the mean for two cultures. Cell number is denoted by ○ and pH by □.

### 5.3.2. Growth of cultures in the presence of 3-HPA

Identical medium was used for both the control cultures and 3-HPA cultures with the exception of the addition of synthesized 3-HPA at a concentration of 0.627mM. No significant growth or reduction in glycerol from a starting concentration of 646mM was seen in the cultures with 3-HPA present (figure 5.6) although a color change took place with the cell culture going a dark brown. The reason for this gradual color change is unknown.
The use of the HPLC was found to be unsatisfactory for the determination of glycerol levels in the control cultures from this experiment. An increase in glycerol was found in the last half of the culture samples. This was thought to be due to retention time of a fermentation product being produced being the same as for glycerol. For this reason the control samples were re-analysed on the Perkin-Elmer GC. This is covered in more detail below.

![Graph](image)

**Figure 5.6.** Growth study of *D. denitrificans* cells cultured with 3-HPA. Cell numbers for the control cultures are denoted by ▼ and cells cultured with the presence of 3-HPA by ▲ and were analysed by HPLC. Likewise ○ shows glycerol levels for the control and ◆ cells cultured with 3-HPA. Error bars show the standard error of the mean for two cultures.

### 5.3.3. Analysis of metabolites

For most studies HPLC was used to analyse the glycerol concentration. Fermentation products can be seen on the HPLC for both *D. denitrificans* and *R. opacus* (figures 5.7 and 5.8). These are typical traces. Other metabolites were seen at time zero. As was mentioned above the control used in the growth study carried out to test the toxicity of 3-HPA was analysed by GC for production of metabolites due to difficulties in separating glycerol and a metabolite produced by the cells (results shown...
in figure 5.6). The glycerol peak area increased over the cell culture cycle when it would be expected to decrease. By running acetic acid and sodium acetate solutions down the HPLC column this additional peak was identified as acetic acid since the retention times matched this unknown peak (12.9min at a flow rate of 0.25ml/min mobile phase). Altering the flow rate of the mobile phase was unable to bring about complete separation of the peaks, hence the use of the GC for analysis of these samples.

Figure 5.7. HPLC analysis of *D. denitrificans* cultures using glycerol as the sole carbon source from the glycerol inhibition study covered in chapter four. a) is a culture at time zero and b) at 101 hours. Peak 1 is an unknown contaminant of the glycerol and peak 2 the glycerol. The ringed zones of the trace show the changes due to production of metabolites over the culture period. The negative peaks are due to the elution of water which is not detected by the electrochemical detector.
Figure 5.8. HPLC analysis of *R. opacus* cultures using glycerol as the sole carbon source from the glycerol inhibition study covered in chapter four. Chart a) is a culture at time zero and b) at 267 hours. Peak 1 is an unknown contaminant of the glycerol and peak 2 the glycerol. The ringed zones of the trace show the changes due to production of metabolites over the culture period. Note the elution rates are different from those shown in figure 5.7 above since the rate of flow of the mobile phase was slower.

NMR analysis was inconclusive due to the impurity of the mixtures, the only metabolite detected being ethanol. An attempt at identification was made by injecting known metabolites to see if the retention times matched those from culture samples. This allowed identification and analysis of ethanol, acetic and propanoic acids. As in chapters three and four, statistics were used to check for significant correlations between cell growth, pH and the acid concentrations. These data are shown in table 5.2 and the growth data in figures 5.9 and 5.10.
Table 5.2. Growth and metabolite production for *D. denitrificans*. Correlations are spearman's rank correlations since the data were not normally distributed. Note the difference in number between analyses involving pH and cell number is due to one sample not having a pH reading measured, all samples collected being analysed by GC.

<table>
<thead>
<tr>
<th>test</th>
<th>number</th>
<th>correlation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH/propanolic acid</td>
<td>9</td>
<td>-0.6524</td>
<td>0.0568</td>
</tr>
<tr>
<td>pH/acetic acid</td>
<td>9</td>
<td>-0.8219</td>
<td>0.0066</td>
</tr>
<tr>
<td>pH/ethanol</td>
<td>9</td>
<td>-0.8812</td>
<td>0.0017</td>
</tr>
<tr>
<td>Cell number/pH</td>
<td>9</td>
<td>-0.9117</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cell number/acetic acid</td>
<td>10</td>
<td>0.8018</td>
<td>0.0053</td>
</tr>
<tr>
<td>Cell number/ethanol</td>
<td>10</td>
<td>0.8994</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Cell number/propanolic acid</td>
<td>10</td>
<td>0.7164</td>
<td>0.0197</td>
</tr>
<tr>
<td>ethanol/acetic acid</td>
<td>10</td>
<td>0.8711</td>
<td>0.0010</td>
</tr>
</tbody>
</table>

Almost all the data had statistically significant correlations between cell number and pH, pH and metabolite concentration and cell number and metabolite concentration. The exception was the pH/propanolic acid correlation, while the cell number and propanolic acid correlation was also only just statistically significant. While most of these correlations were not surprising such as an association between pH and acetic acid, they do suggest a causal link between the pH, metabolite concentration and cell number and in the case of acetic acid and ethanol that the metabolic pathways may be linked. Production of both organic acids matched cell growth very closely (figure 5.9) showing no increase until exponential growth started then increasing in concentration very rapidly at this point. Ethanol was different, the GC results suggesting an almost straightline increase over the culture period without any obvious relationship to the stage of the growth cycle. All three metabolites went on increasing in concentration in the stationary phase of growth. Whilst the ethanol concentration dropped slightly a few times during the culture period, the acetic acid showed a number of large decreases at various stages of the cell growth cycle (figure 5.10).
Figure 5.9. *D. denitrificans* control cultures from the 3-HPA study analysed for fermentation products by GC. a) shows cell growth and glycerol concentration for cells cultured without shaking. b) shows cell growth and acetic acid concentration. c) shows cell growth and propanoic acid concentration. d) shows cell growth and ethanol concentration. Cell numbers are shown in black (▼) and metabolite concentrations in colour. Error bars show the standard error of the mean for two cultures. The starting concentration of glycerol was 646mM in the control as determined by HPLC (figure 5.6 above) and 652mM in the control as determined by GC.
Figure 5.10. *D. denitrificans* control cultures from the 3-HPA study analysed for fermentation products by GC. pH (∆), acetic (○) and propanoic acid (◇) concentrations are shown. The error bars show the SEM for duplicate cultures.

5.3.4. Propan-1,3-diol production

A series of experiments were carried out to attempt to make *D. denitrificans* and *R. opacus* produce this metabolite using glycerol and glucose simultaneously as the carbon sources by causing an imbalance in the ratio of NAD⁺/NADH. Both carbon sources were present at the start, but additional glycerol was added as the culture time proceeded (firstly some of the same medium without glucose then medium B at a high pH to attempt to control the pH). The results of one of these experiments are shown in figures 5.11 and figure 5.12. The initial mean concentration of glycerol was 92mM in the *R. opacus* cultures and 60mM in the *D. denitrificans* cultures before the cells were added. The glucose concentration was 13.1mM. At 26 hours 2ml of cell supernatant was removed from each flask which contained 100ml of culture at the start of the experiment for sampling and 3ml of fresh medium B with 657mM glycerol added. At 48.5 hours 2ml of cell supernatant was removed and 5ml of medium B containing 657mM glycerol at a pH of 9.44 was added. At 51 hours the last addition was repeated but 5ml from each flask was removed first. At 123 hours 2ml samples were taken and another 3ml of medium B at a pH of 9.44 added and at 148.75 hours 3ml samples were taken and 3ml of the high pH medium added. The results indicate that the glucose is
used up rapidly first despite the cells having been adapted to growth on glycerol. Large variations in pH don't apparently have much effect on the cell growth. No evidence of propan-1,3-diol production was found using the HPLC or GC (in which some individual samples were run to check the HPLC results) for any of these experiments.

**Figure 5.11.** Glucose and pH data for the co-fermentation experiment carried out using *D. denitrificans* and *R. opacus*. Graph a) shows data for *D. denitrificans* and b) data for *R. opacus*. Cell number is shown by (○) for *D. denitrificans* and (□) for *R. opacus*, glucose concentration is shown by (○) and pH by (▲). The dashed arrows show the time points where fresh glycerol medium was added. The error bars show the standard error of the mean for two cultures.
Figure 5.12. Glycerol and cell count data for the co-fermentation experiment carried out using *D. denitrificans* and *R. opacus*. Cell number is shown by (○) for *D. denitrificans* in graph a) and in graph b) by (□) for *R. opacus*, glycerol concentration is shown by (●). The dashed arrows show the time points where fresh glycerol medium was added. The error bars show the standard error of the mean for two cultures.

5.3.5. Effect of fructose 1,6, bisphosphate

Fructose 1,6, bisphosphate is known to inhibit glycerol kinase (figure 4.1). The *D. denitrificans* culture was noticeably orange after the addition of the solution (this compound was orange coloured). Cell growth was rapid and after 24 hours the orange colour had disappeared and the culture was a grey colour. Analysis of the culture medium using HPLC didn't suggest any 1,3-propanediol was produced.
5.4. Discussion

In this chapter a variety of studies are described on *D. denitrificans* and some on *R. opacus* to attempt to determine the identity and any inhibition of growth by metabolites produced by fermentation as well as attempted production of one (propan-1,3, diol). In chapter three a significant fall in pH was found for both *D. denitrificans* and *R. opacus* when these organisms were cultured on both glucose and glycerol. Additional peaks were seen on the HPLC traces as the cell growth cycle progressed for both organisms (figure 5.7 and 5.8). Since the pH falls the logical deduction is that these peaks are at least in part organic acids.

Due to the complexity of the metabolites produced and the masking effect of high concentrations of glycerol attempts to identify fermentation products by NMR were inconclusive, with the exception of ethanol. In addition, attempts to purify metabolites by fractional distillation also failed since enough glycerol was evaporated below boiling point to prevent identification of any other compounds present. Using the gas chromatograph to attempt to identify compounds by retention time on the column did yield some results, confirming the production of ethanol and two organic acids propanoic and acetic acid. Low concentrations these compounds were found at the start of the culture period, either from the stock cell culture used as an inoculum or they were impurities present in the glycerol or both. The HPLC system was not capable of resolving the glycerol peak from at least one metabolite, probably acetic acid.

The data in table 5.2. above show that there are significant correlations between cell number and the production by *D. denitrificans* of metabolites identified, but not between propanoic acid and pH. There is also a significant correlation between pH and ethanol production and production of ethanol/acetic acid. The correlation between cell growth and the metabolites produced is unsurprising, more so is the lack of correlation between pH and propanoic acid. This suggests that propanoic acid is not responsible for the fall in pH as does the modest significance of the correlation between cell growth and propanoic acid production. Propanoic acid also didn’t show any major swings in concentration unlike acetic acid, which suggests that *D. denitrificans* is unable to
metabolise this acid. Propanoic acid was not tried as a sole carbon source for this organism but *D. denitrificans* is able to metabolise this compound (Horn et al., 2005). The metabolite data and some of the pH data do suggest that this bacterium can to an extent control the medium pH by using acteic acid (and possibly other organic acids) as carbon source even when the main carbon source provided is not exhausted. The pKa values of both propanoic and acetic acids are insufficiently high to fully account for the pH's at the concentrations present (the calculations are shown in the appendices). However, the acetic acid does give a significant correlation with pH, although possibly this is a reflection mathematically of the greater change in acetic acid concentration over the growth cycle. The data strongly suggest another acidic compound (or compounds) is largely responsible for the low pH found during these cultures. For reasons outlined elsewhere in this discussion it was not possible to identify this compound or compounds. Other peaks can be seen on the GC trace, more than were obtained using HPLC (figure 5.13). They are, however, very small compared to the glycerol peak although the organic acid standards also gave relatively small peaks on the BP20 GC column. *R. opacus* samples run using the same column also gave similar minor peaks (data not shown). All three metabolites identified went on increasing during stationary phase, however, this is less surprising since the high cell number in this phase of cell growth would potentially lead to oxygen transfer limitations.
Figure 5.13. GC trace from *D. denitrificans* cultured with glycerol as the carbon source in the initial glycerol growth study. The largest peak is glycerol. The peak at less than one minute is probably gas from the sample injection process. A number of very small peaks can be seen before and after the glycerol peak.

The production of ethanol by *D. denitrificans* in one way was surprising since the organism is incapable of using it as a carbon source (chapter four). However, the metabolic pathways for production of ethanol and acetic acid are frequently linked in micro-organisms (Prescott *et al.*, 2005), the high significance of the ethanol/acetic acid correlation as well as the presence of both compounds suggests that this is the case here.

The acidity of the culture medium also affects growth. A whole series of studies were carried out by E. Paton primarily to attempt to limit the glycolytic pathway. Culturing both *D. denitrificans* and *R. opacus* outside their physiological pH range can potentially cause the organism to ferment and produce propan-1,3-diol (Barbirato and Bories, 1997). These pH data are also useful for the main focus of this project. With one exception growth was faster and the maximum biomass higher for *D. denitrificans* cells started at higher pH. This suggests there is a lower pH range at which high acidity inhibits enzymes in critical metabolic pathways. The further above this the more
growth is possible before this lower pH limit is met. However, the cells would not grow when inoculated at a normal starting density at pH 8.19. A surprising finding was that when cells were in exponential growth they would grow at very high pH. In an attempt to keep the cell culture medium between 7.5 and 8.0 medium B at alkaline pH with glycerol was added to the growth medium (see below). Due to the poor pH control of medium B the pH exceeded pH 8 in one flask. Strong cell growth continued and eventually the pH fell. This was true for both *D. denitrificans* and *R. opacus*. One possible explanation is that the cells are growing at a maximum rate when the alkali was added and a sub population of the cells are able to adapt to this pH change. Since there are more cells present than at inoculation there are more cells that can cope with the pH change. This finding is also borne out by the long term study where the pH rises from a low of approximately 4 to just below 5. When GC was carried out on a sample of this culture glycerol was still present (data not shown). The data imply that at that point in the cycle *D. denitrificans* is not primarily metabolising glycerol but organic acids and by utilising these it raises the pH enough to continue growth. *D. denitrificans* also managed some increase in cell number when the starting culture medium pH was less than 4. In general *D. denitrificans* would grow outside the physiological pH range (6.1-8.3) at the lower end, but not at as high a pH value when sub-cultured into fresh medium (Horn *et al.*, 2005). The organism has adapted to low levels of pH but not higher pH values. The probable reason for this is that the cells have been frequently overgrown in stock cultures, in addition the medium B for the early parts of this project was not buffered to a specific pH. In general the pH of unadjusted medium B is approximately 6.8. This slightly lower pH than the organism’s optimum (7.0) meant that the cells have adapted to growth outside the higher pH range of the organism. The slight growth at less than a pH of 4 shows that in an unstirred microbial fuel cell some growth of the organism could still take place and that *D. denitrificans* might even be able to raise the pH by metabolising some acidic products of such growth.

It has been known for some years that certain strains of *Citrobacter/Klebsiella/Clostridium* can ferment glycerol to propan-1,3-diol (Forsberg, 1987; Yazdani and Gonzalez, 2007). More recently it has been discovered that this compound can also under certain instances be produced under aerobic conditions
E. Paton made extensive attempts to produce propan-1,3-diol using *D. denitrificans* and *R. opacus* from glycerol as the sole carbon source and then using glucose and glycerol as the carbon sources under the direction of N. Hollow. A number of different strategies were attempted to overcome the limitations of aerobic only culture conditions. The cell supernatant was analysed mainly by HPLC with some samples being analysed by GC for glycerol, propan-1,3-diol, 3-hydroxypropanal (3-HPA) and propan-1,2-diol.

The first strategy was to use *D. denitrificans* cultured on high concentrations of glycerol alone. The cells were grown aerobically or by limiting the oxygen transfer by putting parafilm over the foam bung or growing the cells in plastic centrifuge tubes. Analysis of the supernatant revealed no obvious peaks that matched any of the above metabolites (data not shown). The GC did however pick up a small peak that matched exactly the retention time of 2-propanol (data not shown). It is possible this was actually 1-propanol which is a metabolic end product of the production of propan-1,2-diol (figure 4.1). It would be expected that the isomeric compounds would have a very similar if not identical retention time although 1-propanol was not tested on the column. The hope that growing very high cell numbers would force some fermentation to take place under aerobic conditions was not fulfilled. In some cultures a large fall in the initial glycerol concentration was seen (figures 5.2 and 5.12 a). This fall was not seen in all cultures, but could be due to the kind of interactions with the cell membrane covered in chapter four.

The next strategy attempted was to switch off the glycolytic pathway by growing the cells outside their normal physiological pH range. This is the origin of the pH studies shown in figures 5.1-5.4 covered above. As discussed above *D. denitrificans* was found to grow surprisingly well at acid pH, with some growth as low as pH 3.6 but not at pH's much above pH 8. *R. opacus* has since been tested at an initial medium pH of 6.1 and was found to grow reasonably well at this pH (see chapter three). However, again none of the metabolites of interest were detected in any quantity. The problem with this method is that it is a non-specific way of achieving the aim and whilst it is possible that
the glycolytic enzymes are pH sensitive it just as likely that glycerol dehydratase and 1,3,-propanediol dehydrogenase are pH sensitive also. Indeed this was the case in chemostat cultures using *Clostridium butylicum* where lowering the pH to 4.9 reduced the 1,3,-propanediol yield (Forsberg, 1987).

Another possible strategy is the use of fructose 1,6 bisphosphate to inhibit glycerol kinase. As outlined in chapter four there are three possible routes for the metabolic breakdown of glycerol (figure 4.1). One possible enzymatic route is via glycerol kinase and then the glycolytic pathway or via DHA and another kinase to form glyceraldehyde-3-phosphate. It has been found that both these kinase enzymes can be competitively inhibited by fructose 1,6 bisphosphate, with as little as 1.7mM required (Zwaig *et al.*, 1970; Weinhouse and Benziman, 1976). In theory blocking the link to the glycolytic pathway should mean that the organism would grow via fermentation producing 1,2,-propanediol or 1,3,-propanediol Attempts were made to culture *D. denitrificans* in the presence of glycerol and 1.97mM fructose 1,6 bisphosphate. This compound was bright orange and enough was added to noticeably colour the cell supernatant. The colour disappeared over the culture period and none of the secondary metabolites of interest were detected. One possible explanation for the lack of success is the presence of fructose 1,6 bisphosphatase. This enzyme catalyses the dephosphorylation of fructose 1,6 bisphosphate to yield fructose-6-phosphate and a phosphate group (Fraenkel and Horecker, 1965). A second reason could be that not quite enough of this compound was added. The vial Acros dispatched contained an orange solid and the name was given as D fructose 1,6 bisphosphate trisodium salt hydrate. It was not realised at the time that this should be a white crystalline powder, not orange. Acros had mislabelled the vial which contained D fructose 1,6 bisphosphate trisodium salt octahydrate. Since the molecular weight of this compound is higher less than 1.97mM was mistakenly added.

The last strategy tried was co-fermentation (figures 5.11 and 5.12). This method involves using a redox imbalance brought about by using a sugar and glycerol as joint carbon sources (Sobolov, and Smiley, 1960; Xiu *et al.*, 2007). Both the aerobic and anaerobic methods of production in many cases rely on the idea of co-fermentation. Talarico *et al.* (1988) found that adding glucose to the fermentation raised levels of 1,3,
propandiol and lowered the levels of 3-HPA. The theory is that culturing the cells on a carbohydrate such as glucose leads to the excess production of NADH through the glycolytic pathway and the TCA cycle (figure 4.1), this NADH is then recycled to NAD$^+$ at least partially through the secondary metabolic pathway used to make propan-1,3-diol from glycerol (Veiga-da-Cunha and Foster, 1992). E. Paton undertook a number of co-fermentation sets of cultures using *R. opacus* and *D. denitrificans* one of which is shown above in figures 5.11 and 5.12. Again no obvious 1,3-propandiol peaks were seen on the HPLC or GC traces. Despite being adapted to and sub-cultured from glycerol the cells utilised the glucose first in both cultures.

A number of very different approaches to production of 1,3-propandiol from glycerol have failed. In addition to some reasons outlined above the instability of the 3-hydroxypropanal intermediate (also known as reuterin) could be a factor (Sung *et al.*, 2003). The production of this metabolite is of interest in its own right for a variety of industrial uses (Slininger *et al.*, 1983; Talarico *et al.*, 1988; Sung *et al.*, 2003). 3-hydroxypropanal can form a variety of isomers and monomeric, hydrated monomeric, and cyclic dimeric forms at both acid and alkaline pH (Sung *et al.*, 2003). In addition it can be converted to acrylic acid, 2-propenal (acrolein) and 3-hydroxypropionic acid (Slininger *et al.*, 1983). 2-propenal spontaneously forms a polymer which is yellow in colour and can be converted to acrylic acid (Slininger *et al.*, 1983; Sauvageot *et al.*, 2000). (Yamashiata, 1996). In addition 2-propenal is extremely volatile so some would be lost by evaporation.

There is some evidence that this is why no meaningful quantities of 1,3-propandiol have been detected. Both with increasing adaptation to glycerol and particularly on growth with glucose and glycerol the *D. denitrificans* cultures have been seen to have a yellow colour. This yellow colour was first seen in cultures contaminated with yeast where it was even more noticeable. However, almost all cultures were found to have the colouration as the organism adapted to the glycerol and on the basis of checks for contamination by eye under the microscope or by plating out the colour was seen when no yeast was present. There is also some evidence from the gas chromatograph. Traces of the 3-HPA synthesised were overlaid on a sample from a co-fermentation using *D.*
denitrificans. The 3-HPA had a large number of peaks presumably due to its many isomers as well as unreacted 2-propanal. Many but not all of these peaks were replicated in the culture sample although they were tiny in comparison (data not shown). Whilst a yellow colouration was seen with D. denitrificans, R. opacus cultures grown on glycerol are pink/red so it is not possible to comment on the colouration of these cultures beyond this. The 3-HPA added to the D. denitrificans culture was extremely toxic to the cells, a concentration of 58.9mM almost totally stopped growth. The generally accepted level to stop microbial growth in vitro is 1mM (Schaefer et al., 2010). The 3-HPA is thought to be toxic due to the reaction of the aldehyde group on the 3-HPA with thiol groups on proteins in the cells (Schaefer et al., 2010). Whilst it is possible that 2-propanal present either unreacted from the chemical synthesis or from dehydration of the 3-HPA may be causing the toxicity, work suggests this is not the case (Schaefer et al., 2010). If the cells do make appreciable quantities of 3-HPA then its toxicity would inhibit growth and hence current production in an MFC, however, the D. denitrificans cells are relatively resistant to this compound since a little growth was seen at a much higher concentration than 1mM (Schaefer et al., 2010).

The final possibility is that neither organism has the genes required to operate this pathway. It is noticeable that neither organism could use propan-1,2-diol or propan-1,3-diol as their carbon sources. There is nothing in the literature on the breakdown of these compounds but the assumption must be that the same pathways used to make them would operate in reverse. On the basis of the work carried out in this chapter any 3-HPA, propan-1,2-diol or propan-1,3-diol produced would be inhibitory to the cells therefore reducing the power output in an MFC. However, pH gradients produced may be less inhibiting to growth particularly if the cell number in the culture is high.

It was hoped to use GC-MS to answer many of the outstanding questions above related to production of metabolites, however this was not possible for technical reasons due to the interference of the salts in the medium.
Chapter 6. Studies of cellular metabolism at the molecular level

6.1. Introduction

As was described in chapters one and three not all bacteria possess the ability to directly or indirectly transfer electrons to an anode in a microbial fuel cell without an exogenous mediator (Lovley, 2006; Logan and Regan, 2006). Only those bacteria that carry and express genes for c-type cytochromes or can produce natural redox shuttles can transfer electrons to the anode in an MFC. In MFC’s electron transfer is found in micro-organisms that are able to reduce transition metals (particularly iron) (Park et al., 2001; Kim et al., 2002; Logan, 2008; Zuo et al., 2008). Species of the bacteria Geobacteraceae have been found to be capable of transition metal reduction and directly transferring their electrons to the anode in a microbial fuel cell, along with the bacterium Shewanella putrefaciens and a number of other organisms (Holmes et al., 2004). Geobacter sulfurreducens is the most studied organism utilised in microbial fuel cells since it gives high current densities and reduces iron (III) (Kim et al., 2008). The genomes of both G. sulfurreducens and S. putrefaciens have now been sequenced (Fredrickson and Romine, 2005). This has led to new insights into the biology of these bacteria but also provide tools for comparative genomics (Fredrickson and Romine, 2005).

Whilst the great majority of electron transfer to transition metals is by direct contact through c-type cytochromes associated with the cell membrane, it has been postulated that proteins could also act as shuttles (Seeliger et al., 1998; DiChristina et al., 2001; Mazoch et al., 2004). Seeliger et al. (1998) isolated a c-type cytochrome with a molecular weight of 9.57Kda which was present in the bacterial medium, the periplasmic space and the cell membrane. The expression of this protein would be a way for this organism to reduce insoluble forms of iron (Seeliger et al., 1998).

Many groups have examined the numbers and types of cells present on the anodes in MFCs, visualising the bacteria using fluorescent in situ hybridisation (FISH), live dead cell staining or electron microscopy (Bond and Lovley, 2003; Liu and Logan, 2004;
Kim et al., 2006; Reguera et al., 2006; Mohan et al., 2007; Milliken and May, 2007; Scott and Murano, 2007; You et al., 2007; Lanthier et al., 2005; Zuo et al., 2008; Nevin et al., 2009; Yi et al., 2009).

The aim of the work in this chapter is to search for specific genes from Geobacter and Shewanella spp. and other species of bacteria that may have been conserved in evolution that are involved in electron transfer. It was hoped that the results whether positive or negative could explain some of the characteristics of the bacteria chosen for this project in medium containing transition metals or in microbial fuel cells. By using PCR and FISH it was hoped to identify some of these genes of interest. For some genes both FISH and PCR were used or in one case another gene involved in making the electron shuttle. This meant several different oligonucleotide sequences were used to probe for the same gene.

In addition live/dead studies were carried out using a Baclight kit (Invitrogen, UK). Bacteria with intact membranes stain fluorescent green using SYTO 9, whereas bacteria with damaged membranes stain fluorescent red using propidium iodide which binds non-specifically to DNA. Binding by the dyes to the background is very low and it remains virtually non-fluorescent. Live/dead staining was carried out for a number of reasons. It was a relatively simple way to learn about the technique of confocal microscopy and it allowed the relative numbers of dead and live cells to seen. Live/dead staining also provided a non-specific counter-stain to the specific FISH probe stains. The dead stain was chosen because the bacterial DNA would be more available to hybridise with the probe sequence since the cell membrane is compromised, in addition the fluorescence wavelength did not interfere with any of the dyes bound to the probes.

For all the fluorescent studies the bacterial cells were fixed with formaldehyde. Whilst killing any cells that are alive the fixation preserves the cells and stops any further degradation, allowing storage of the cells at -20°C until use.

Finally an attempt was made using polyacrylamide gels to look at differential expression of proteins in the cell medium for bacterial cells cultured in medium with
transition metals present to attempt to detect any protein redox shuttles produced by the cells. Any identified proteins of interest would have been analysed using matrix-assisted laser ionization desorption mass spectroscopy (MALDI) to allow identification of the protein type and a more accurate estimate of molecular weight to be made.
6.2. Materials and methods

As previously chemicals were purchased from Sigma or Fisher (UK). The microbial fuel cell studies used are described in chapter seven. The anode material is of two different types and is more fully described in chapter seven.

6.2.1. Live/dead cell studies

Anode material was cut into approximately 1cm squares either immediately on removal from the microbial fuel cell or after storage at -20°C after fixation (see below). In initial studies 1.9μl of SYTO 9 and an equal volume of propidium iodide were added to 3ml of medium B and the anode material in a 5ml bijou. After mixing the cells were visualised under a Leica DMIRE2 confocal microscope (for details see below). Images were averaged by the Leica confocal software up to sixteen times an image.

6.2.2. Fluorescent in situ hybridisation (FISH)

6.2.2.1. Choice of sequences

Initially three probes were purchased from Alta Biosciences (Birmingham, UK). Two of these were chosen by taking random sequences one from the Rhodococcus Rha1 genome (glycerol kinase gene) and another from a human antibody kappa sequence located using Pubmed (McLeod et al., 2006). The third was a RT-PCR primer sequence against the PCAR2984 cytochrome c family protein (Haveman et al., 2006). All three were compared by BLAST against the Rha1 genome (McLeod et al., 2006). All the probes were labelled with the fluorescent dye 3-[O-(N-carboxy-(di-O-pivaloyl-fluorescein)] (FAM) at the 5' end, 5'-AAGCACGGGCTCCTCTGGACCCAGG-3' (glycerol kinase), 5'-ATGAAAATGCTTGGGTGCT-3' (PCAR2984, c-type cytochrome) and 5'-GTGAATCTTTGCAAAAAAAAAAAAAAAAAAA-3' (human kappa antibody sequence). The latter sequence was chosen as a negative control that should not bind to the bacterial DNA and indeed no matched sequences were found in the Rha1 genome using BLAST (McLeod et al., 2006). The probes were re-suspended in 1ml of sterile
deionised water, aliquoted into sterile eppendorf microtubes and stored at -20°C.

A further seven oligonucleotides were purchased from Alta Biosciences (Birmingham, UK) for three-colour staining. The sequences were chosen by locating the entire gene sequence by searches on Pubmed or KEGG, then entering the entire sequence into the on-line version of primer3. Four of these probes were labelled with 6-carboxy-1,4-dichloro-2’,7’-dichlorofluorescein (TET) at the 5’ end and were as follows; \textit{E. coli} strain K-12 glycerol dehydrogenase 5’-TTGACCGCTATCTGCTGTTG-3’, OmcB 5’-CGCTTTCATGCCGTTACT-3’, \textit{S. oneidensis} MR-1 OmcA 5’-CGGTCAAGCTTCTGTTTTCTCC-3’ and phenazine operon PhzE from \textit{P. aeruginosa} PA7 5’-AACAGGTCAAGCTGGAAGAA-3’. The remaining three probes were labelled at the 5’ end using 3-(3’,6’-dipivaloylfluorescein-2-yl) (Gig harbor green) and were as follows; \textit{P. putida} MK1 chromate reductase (ChrR) 5’-TGTACAACGAGGACGTCGAG-3’, PpcA Pcar_1628 a c-type cytochrome from \textit{Pelobacter carbinolicus} 5’-AACGGAAGGACGACATTCCG-3’ (Haveman et al., 2006), and \textit{S. oneidensis} MR-1 CymA 5’-CATTGTTTCTGCTGGTTTTT-3’. The probes were re-suspended in 1ml of sterile deionised water aliquoted into sterile eppendorf microtubes and stored at -20°C.

\subsection*{6.2.2.2. FISH method}

FISH was undertaken based on the methods outlined by Richter et al. (2007), Lanthier et al. (2002) and Lanthier et al. (2005). The anode material was fixed in paraformaldehyde 4% (wt/vol) in PBS (pH 7.2) for 1 hour at 20°C or 90% ethanol (v/v) at 80°C for 4 seconds. The electrodes were then washed in PBS for 5 minutes (this step was omitted in some studies with no discernible effect). The anode material was stored at 20°C in PBS buffer-50% ethanol until hybridization, or treated using the remainder of the protocol immediately.

The samples were dehydrated in a series of 50, 80, and 95% (v/v) ethanol solutions for 5 minutes each and were then incubated in 10ml of acetylation solution (100mM triethanolamine, 0.25% acetic anhydride (v/v), 0.09% NaCl (w/v); pH 7.2) for 10
minutes. The electrodes were washed gently with deionised water and then cut into 1cm squares and placed in separate tubes (in either sterile eppendorfs or 5ml bijous). Hybridization was carried out in hybridization buffer solution which consisted of 400 to 1000µl of 30% deionized formamide (v/v), 0.9M NaCl (w/v), 0.02M Tris-HCl, 0.01% sodium dodecyl sulphate (w/v) at a final pH 7.2 containing 25ng per µl of the labelled oligonucleotides. The tubes were incubated at 46°C for 3 hours in a Hybaid “shake and bake” hybridization oven (Hybaid, USA). The tubes were wrapped in foil to protect them from the light during this incubation and at all subsequent stages until the microscopy. The electrodes were washed once for 20 minutes at 48°C in 1-2ml of washing buffer per tube which consisted of 0.04M NaCl; 0.02M Tris-HCl, 0.01% sodium dodecyl sulfate (w/v) and 0.005M EDTA at a pH of 7.2. They were transferred using tweezers washed in 70% ethanol each time to fresh tubes in the hybridization oven. A final wash took place with 1ml of deionised water per tube (in some studies 1.9µl of propidium iodide was added per tube at this stage). The anodes were stored in the dark until examination under the confocal microscope, where immediately before use they were mounted on clean slides.

The cells were scanned using a Leica DMIRE2 confocal microscope fitted with argon and helium lasers and a krypton-argon dual laser. Excitation wavelengths were set at 476, 488, 496, 514 and 543nm. Detection was set at 500, 525, 537, 560 and 645nm. The wavelengths used at any one time depended on the dye combinations being utilised. The acquisition software was Leica confocal software version 2.61. The autogain feature in the Leica confocal microscope software was used to reduce the background and differentiate between cells and background. Three-colour staining was carried out as above but two compatible probes with suitable detection wavelengths were added at 25ng/µl and the propidium iodide or SYTO 9 was added after hybridisation and washing. Images were saved by the microscopy software in tiff image format. Unfortunately many of the photos reproduced do not do justice to the observations made. There was a clear loss of quality between the confocal microscope and the exported tiff files and then a bigger loss of image quality on printing. It should be stated at this point as is alluded to in the results section that no conclusions were drawn on only one set of images. Multiple magnifications were used in almost every
case often with different groups of cells on different sections of the anode. In each case the group of cells and magnification chosen have been to show the clearest results (either positive or negative for a particular gene). The PCR was used as a confirmatory test for many of the genes.

6.2.3. Gel electrophoresis on concentrated samples

6.2.3.1. Sample concentration

Samples were concentrated using Centricon YM-3 centrifugal filter devices which have a molecular weight cut-off of 3kDa. The chosen samples were centrifuged at 4500g for 1 hour to produce a stable pellet. The supernatant was removed by pipetting and placed in a Centricon YM-3 centrifugal filter device. The centrifugal filter devices can hold a maximum volume of 2ml. The starting volume varied in each case. In some cases two flask samples from the same time period were combined since after sample had been removed for a variety of transition metal assays described in chapter three the total volume in each sample tube was too low to approach this volume. Where relevant, which samples were combined is outlined in the results section below. A known volume was added to each filter device, one for each supernatant. The retentate vial was placed on the tube as a cap. The tubes were spun at a maximum of 4500g until the liquid level was judged by eye to be just above the white filter membrane (the minimum to prevent the sample going dry). This took a different time period depending on the sample and its volume. Then 50μl of PBS was added, the supernatant was then pipetted out measuring the volume at the same time and stored at -20°C until use.

An alternative method of sample concentration was also tried. 100ml of 10% trichloroacetic acid was prepared in acetone and stored overnight at -20°C. 20ml of cell supernatant from cells cultured with 3166μM ferricyanide (chapter three) was added to this acetone/trichloroacetic acid mixture immediately on its removal from the freezer. This was mixed and stored at -20°C for approximately 96 hours. The suspension was then centrifuged at 50,000g in an Avanti J-26 XP centrifuge for 20 minutes. The supernatant was gently tipped off and replaced with approximately 10ml of ice cold
acetone. The centrifugation step was repeated and then the (invisible) pellet was redissolved in 200µl of PBS with gentle swirling and was stored at -20°C. The concentration is therefore 20000/200 = 100x.
6.2.3.2. Non reducing gels (native gels)

The method used is as described in chapter three.

6.2.3.3. Reducing gels

Reducing gels were purchased from AMS Biotechnology (Oxford, UK) and run on the minigel system used for the native gels. Sigma molecular weight markers (Sigma, UK) were used as standards. The lyophilised standards were re-suspended in 100μl of deionised water and then diluted five fold in 20% v/v glycerol/deionised water. They were not boiled. These were stored on ice whilst the samples were prepared.

The samples for the 4-20% gradient gel were prepared as follows. The final volume was 40μl. The reducing agent (2-mercaptoethanol) was diluted twenty fold into the sample buffer provided by pagegel. The volume of sample was up to 30μl remaining after assay. 10μl of the sample buffer/reducing agent was added to this and if necessary enough deionised water to make the volume up to 40μl. The samples were then boiled for three minutes at 99°C in sealed micro sample tubes and immediately cooled by placing them on ice. The gel was removed from its packaging and locked into the gel rack in the gel apparatus and running buffer added as before. All the tubes were briefly centrifuged in an eppendorf centrifuge before loading onto the gel. To wells one and two, 3μl of the standards were added, followed by 33μl of each of the samples in wells 3-11 and finally in well twelve 5μl of the standard. The voltage was set at 175V, the current at 80mA and the gel was run for one hour and twenty minutes. As the front reached nearly the bottom of the gel the power was switched off and the gel removed from its casing. Staining was carried out in destain solution (40% v/v ethanol, 10% acetic acid, 50% deionised water) at 60°C with the addition of 0.2% w/v coomassie blue. Destaining was carried out in the same solution without the coomassie blue before being photographed.

The methodology for the 16% gel was similar, except the gel was stained for haem groups first before being stained for proteins.
6.2.3.4. Haem staining

This was carried out using methodology adapted from Francis and Becker (1984), using the 4-20% gradient gel. 20ml of 500mM citric acid was prepared by dissolving 2.1g of this chemical in 15ml of deionised water then adjusting the pH using 2M NaOH to 3.4. 5mg of O-dianisidine was dissolved in 4.5ml of deionised water. The gel was destained for 30 minutes in gel destain followed by two changes of deionised water. The O-dianisidine and citrate buffer was mixed together and added to the gel in its tank along with 20μl of 30 volume hydrogen peroxide. This was left overnight at +4°C. The gel was then stained using the ferrozine reagent after two washes with deionised water.

A repeat was made for the 16% SDS gel as follows. Approximately 12.5% trichloroacetic acid (TCA) was prepared by dissolving 6.39g in 50ml deionised water. The gel was not stained using coomassie blue at this stage but instead was placed in the TCA for 30 minutes. The gel was then washed twice in deionised water, being left in the second wash overnight at +4°C. Approximately 500mM of tri-sodium citrate was preprepared by dissolving 1.49g in 10ml of deionised water. The pH was adjusted to pH 4.46 using citric acid powder. Two vials of dimethylbenzadene (DMB) were dissolved in 9ml of deionised water. 2ml of the citrate solution was added to 9ml of the DMB and 20μl of 30 volume hydrogen peroxide poured over the gel in the tank and left to shake.

6.2.3.5. Ferrozine staining of gels

A solution of 50mM PIPES was prepared by dissolving 1.68g of PIPES in 80ml of deionised water. 0.0487g of ferrozine (approximately 1mM when made up to 100ml total volume) was weighed out and dissolved in the above after 0.082g FeCl₃ was added and then the pH adjusted to 6.97 using 2M NaOH. 70.9mg of NADH (Sigma , UK) (reduced form) was added. The volume was made up to 100ml with deionised water and the reaction mixture was added to the gel tank which was left overnight at +4°C. Both gels above stained for haem staining were stained using ferrozine.
6.2.4. PCR

Materials for PCR were kindly provided by Dr. P. Morris with the exception of the primers which were purchased from Sigma-genosys (UK), the Taq polymerase purchased from Sigma (UK) and mastermix from Qiagen (UK). The primers were designed using primer3 v1.10 and are shown in table 6.1.

Table 6.1. Details of the primers used in this project.

<table>
<thead>
<tr>
<th>gene</th>
<th>primer name</th>
<th>forward (5’-3’)</th>
<th>reverse (3’-5’)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K12 motility basal-body rod protein</td>
<td>flgE</td>
<td>CCAGCCAGACACTGAATCAA</td>
<td>CGCGAGGTGTAATCATTCTT</td>
<td>64.0/63.6</td>
</tr>
<tr>
<td>iron reductase from S. putrefaciens</td>
<td>cymA</td>
<td>GGCAATCCACACAGGTCTTT</td>
<td>CATTCGCTATCCTCCGGTGT</td>
<td>63.9/63.8</td>
</tr>
<tr>
<td>cytochrome iron reductase from G. sulfurreducens</td>
<td>omcS</td>
<td>CACGGGAAGTTACGTCCTTT</td>
<td>ATAGGAACCGCTAGGACT</td>
<td>63.8/63.8</td>
</tr>
<tr>
<td>cytochrome from G. sulfurreducens</td>
<td>ferA</td>
<td>ACGAGTTCCAGACCAACCACC</td>
<td>GCCAGTATCGTCCCCAGTTGT</td>
<td>64.1/63.9</td>
</tr>
<tr>
<td>cytochrome c family protein from G. sulfurreducens</td>
<td>omcE</td>
<td>CTCTGACAGACGACATGAATTA</td>
<td>GGGGTAGTACAGTCAGAT</td>
<td>64.0/63.9</td>
</tr>
<tr>
<td>cytochrome c family protein from G. sulfurreducens</td>
<td>omcF</td>
<td>GTCCACCCGGACAAAGACC</td>
<td>GAGCACTCCGGACCTTTCA</td>
<td>64.4/64.0</td>
</tr>
<tr>
<td>cytochrome c family protein from G. sulfurreducens</td>
<td>omcT</td>
<td>AAACGACTACTGCGACTCCGACTA</td>
<td>GCAGCAGCTCGTTCACTCCAGTTC</td>
<td>64.2/63.8</td>
</tr>
<tr>
<td>iron reductase from S. onedensis MR-1</td>
<td>mitB</td>
<td>GAGTCGCGTAACCAACCAGAT</td>
<td>CCTGTGGAGACCTATGTT</td>
<td>63.8/63.9</td>
</tr>
<tr>
<td>iron reductase from S. putrefaciens MR-1</td>
<td>mitD</td>
<td>TCTGCATGAGCTGCCAATAAC</td>
<td>CAGACATTTGCTCGTCATT</td>
<td>64.0/63.9</td>
</tr>
<tr>
<td>iron reductase from S. onedensis MR-1</td>
<td>mitC*</td>
<td>CAGACACCGACACAGACATT</td>
<td>TTTCCAACCTACAGCTGTGGG</td>
<td>64.2/64.1</td>
</tr>
<tr>
<td>iron reductase from S. onedensis MR-1</td>
<td>mitA</td>
<td>GCCAATACCTTTCAGGATG</td>
<td>ATAGCTCTGCGCAGATGGATG</td>
<td>63.9/63.9</td>
</tr>
<tr>
<td>Pseudomonas phenazine pathway gene</td>
<td>phzD</td>
<td>ACTGGCTGCTGACAAAGTG</td>
<td>GCCCTGAGTCTGCGTGGAGTA</td>
<td>64.3/65.0</td>
</tr>
<tr>
<td>Electron transfer in biofilms (G. sulfurreducens)</td>
<td>omcZ</td>
<td>GGCAGTACATCAGGACTA</td>
<td>GGCAGTATGTCGAGAGATG</td>
<td>63.8/63.9</td>
</tr>
</tbody>
</table>

* also known as omcB.

Each primer was dissolved in the appropriate volume of sterile deionised water to a
make stock solution of 100μM in sterile eppendorf tubes. A 10x dilution of each was
made in sterile deionised water in sterile eppendorf tubes, this was the working stock
solution used in the PCR experiments. PCR was carried out as set out in table 6.2
below. The bacteria were prepared for use by centrifugation of 50-100μl of cell
suspension from maintained culture stocks for 20 minutes at 13400rpm in the eppendorf
centrifuge. The supernatant was discarded and the pellet re-suspended in 10-20μl of 1x
PCR buffer. The resulting cell suspension was boiled for 11-12 minutes and
immediately stored on ice until 2μl was added to the PCR tubes. The tubes were placed
in the Applied Biosystems 2700 thermal cycler and after the temperature had reached
94°C the program was paused and 0.5μl of Taq polymerase (Sigma, UK or Mastermix,
Qiagen, UK) was added per tube. Alternatively using mastermix a simplified hot start
was used in which the cycler was brought up to 94°C and then the tubes were placed in
the machine. The PCR cycle used was as follows; 94°C for 5 minutes at the start, then a
repeated cycle of 94°C for 30 seconds, annealing at 62°C for 30 seconds followed by
extension at 72°C for 30 seconds. This cycle was repeated 30 times, followed at the end
by a final temperature of 72°C for 7 minutes. The mastermix instructions recommended
an annealing temperature 5°C below the melting temperature. This did not give
stronger bands so the temperature was returned to 62°C. The mastermix was out of date
and gave weak bands with an annealing temperature of either 59°C or 62°C, so a
volume of 0.5μl Sigma Taq was added per tube. For each gene amplification a control
was run without bacteria in which 2μl of sterile deionised water was added rather than
cell suspension.
Table 6.2. PCR experiment mix.

<table>
<thead>
<tr>
<th>Component</th>
<th>Sigma Taq per tube</th>
<th>Mastermix Taq per tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (10X)</td>
<td>5µl</td>
<td>included (25µl total)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>included in buffer</td>
<td>included (25µl total)</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
<td>8µl</td>
<td>included (25µl total)</td>
</tr>
<tr>
<td>Taq</td>
<td>0.5µl</td>
<td>included (25µl total)</td>
</tr>
<tr>
<td>Primer A (forward)</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>Primer B (reverse)</td>
<td>1µl</td>
<td>1µl</td>
</tr>
<tr>
<td>Template (bacteria)</td>
<td>2µl</td>
<td>2µl</td>
</tr>
<tr>
<td>Deionised sterile water</td>
<td>To 50µl total tube volume</td>
<td>23µl in controls 21µl in samples</td>
</tr>
</tbody>
</table>

Gels were prepared by dissolving agarose at 1.25% (w/v) in 25ml-75ml of 0.5x TBE buffer. After the agarose had been dissolved by heating the suspension in a microwave oven until it boiled, 1µl of ethidium bromide at 10mg/ml was added and the agarose solution poured into the gel casting apparatus.

10µl of loading dye (Fermentas, UK) was added to every tube. The gel was placed beneath the 0.5X TBE buffer in the running apparatus and 4µl of 100bp DNA ladder (Fermentas (UK)) or 2µl hyperladder IV (Bioline, UK) was added to well one in each row followed by 10µl of each sample. The gel was run at 100V for 30 minutes in a Runone electrophoresis apparatus before being visualised under UV light and the gel photographed using grabit software or a Canon ixus digital camera. *E. coli* K12 (provided by Dr P. Morris) was used as a positive control for the *flgF* gene.
6.3. Results

6.3.1. Live/dead stain

The live/dead stain was undertaken first before oligonucleotides probes were purchased to see if there were any cells on the surface of the anode and if so their relative abundance, as well as to gain experience of the confocal microscope.

A clear difference was seen between the different types of anode materials used in this project. The cheaper NCBE anode material is not apparently woven but is a series of interlocking straight fibres with a very open structure (figure 6.1 (a)). The more expensive E-tek cloth has a woven structure and is more dense (figure 6.1 (b)).

![Images](a), (b)

**Figure 6.1.** Different anode materials visualised optically under the confocal microscope. a) NCBE material, b) E-tek anode cloth, the edge of the cloth can be seen on the left hand side.

*R. opacus* and *D. denitrificans* cells were found in much higher quantities on the E-tek anode material (figures 6.2 and 6.3). In addition *R. opacus* and *D. denitrificans* were found to grow in a different pattern on the E-tek anodic material (figures 6.2 and 6.3). *D. denitrificans* cells are seen clustered on the anode material (figure 6.2), following straight line fibres. By contrast *R. opacus* follows the curved weave (figure 6.3). In the case of *D. denitrificans* cells the tunnelling feature of the software was used to take pictures in layers through the anode material and the pattern of growth of this organism.
was consistent throughout (data not shown).

Figure 6.2. *D. denitrificans* on the surface of the anode cloth material (E-tek). Image a) shows an optical image of the anode material with an arrow pointing to one of a number of clusters of cells shown attached to the straight anode fibre, b) shows dead cells stained with propidium idodide visualised as red, c) shows the same portion of anode stained to show live cells with SYTO 9 coloured green. Finally d) shows a cluster of cells at a higher magnification stained with SYTO 9, the arrow indicates individual cells seen as rods.
Figure 6.3. *R. opacus* on the surface of the anode cloth material (E-tek). Image a) shows live cells with SYTO 9 coloured green, b) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red, c) shows cells clearly following the weave of the anode material stained using SYTO 9. Finally d) shows an optical image of the anode material. Note some straight fibres can be seen.

6.3.2. FISH

6.3.2.1. Initial studies

Initial studies using the three probes 5'-AAGCACGGCCTCCTGACCACCG-3' (glycerol kinase) 5'-ATGAAAAATGCCTTTGGATGCT-3' (PCAR2984) and the human kappa antibody control were undertaken using cells cultured on the NCBE anode material in the microbial fuel cell. Very low power output was obtained and there were apparently fewer cells for both organisms than on the E-tek cloth. In addition the fibres in the cloth appear to be fluorescent. Working at increased magnification indicated that
cells were not in general growing along the fibres (for either organism). Clusters of cells can be seen attached to the fibres with both organisms in figures 6.4-6.6.

However, despite these findings clear differences between the fluorescent probe treatments can be seen. In the case of *R. opacus* no cells were seen stained positive for glycerol kinase, but cells (a large number of clumped cells can be seen in figure 6.4 e) and f)) were seen stained specifically for PCAR2984, the e-type cytochrome sequence. No stained cells were detected using the human antibody sequence (Figure 6.5). In figure 6.4 a) a fluorescent sphere can be seen (also just in figure 6.4 b); this is not a group of cells, being too regular. What it was is unknown. The cells on this anode material also grow in a similar manner to *D. denitrificans* on the E-tek material. No non-specific binding was seen in the anode material exposed to cells and not to probes (figure 6.5).
Figure 6.4. *R. opacus* on the surface of the anode cloth material (NCBE) stained with the three initial gene probes. Image a) shows a group of cells stained with probe for glycerol kinase at 320 x magnification, b) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red at 320x magnification, c) shows cells stained using the probe for PCAR2984, (c-type cytochrome) at 270 x magnification. d) the same portion of anode stained to show the dead cells stained with propidium iodide at 270 x magnification. e) and f) show the anode stained as c) and d) at 10 x magnification.
Figure 6.5. *R. opacus* on the surface of the anode cloth material (NCBE) stained with the negative control. Image a) shows the cells stained with the probe 5'-GTGAATCTTTGCAAAAAAAAAAAAAAAAAAAA-3' (human kappa antibody sequence) coloured green, b) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red, c) control anode with cells but no fluorescent probes. Finally d) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red. All images being at 10 x magnification.

*D. denitrificans* grew on the NCBE anode material in a very similar way to *R. opacus*, with clusters of cells attached to fibres. The results for the three initial probes were identical to those with *R. opacus* (figure 6.6). The fibres show fluorescence with the FAM probe but no clusters of cells can be seen stained with the probe for glycerol kinase or the human antibody kappa sequence, figure 6.6 a) and c). Clusters of dead cells can be seen on the anode material in 6.6 b) and d) which makes the fibres appear thicker. In figure 6.6 c) what appear to be organised structures can be seen. These are droplets of liquid and could be seen as such on the optical channel (not shown).
**Figure 6.6.** *D. denitrificans* on the surface of the anode cloth material (NCBE) stained with the positive control. Image a) shows staining with the probe 5'-AAGCACGCGCTTCGACCACCG-3' (glycerol kinase), b) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red, c) anode stained with 5'-GTGAATCTTTGCAAAAAAAAABAAA-3' (human kappa antibody sequence) and d) the same portion of anode showing the dead cells stained with propidium iodide. All at the same magnification (10 x).

Positive staining for PCAR2984, a c-type cytochrome can be seen in figure 6.7. The staining matches a clump of cells seen attached to the anode (figure 6.7. c). Although non-specific staining can be seen along the fibre the cells are seen as a non-regular clump.
Figure 6.7. *D. denitrificans* cells on the surface of the NCBE anode stained for PCAR2984. a) shows cells stained using the fluorescent DNA probe 5'-ATGAAAAATGCTTTTGATGCT-3' (PCAR2984, a c-type cytochrome). b) the same portion of anode shows the dead cells stained with propidium iodide. c) the same portion of the anode visualised optically. The clump of cells seen in a) and b) is indicated by the arrow. All images are at 180 x magnification.

The second control anode material without fluorescent DNA probes did appear to fluoresce at the wavelength used to visualise the FAM labelled probes. A large cluster of what appears to be cells can be seen in figure 6.8. The reasons for this are unknown. Possibly this was due to inadequate washing during the staining process. Whilst some non-specific fluorescence was seen with some probes, particularly of the fibres, this was easily identified and a positive result for staining was only considered when the dead cell staining matched the particular specific probe staining.
Figure 6.8. *D. denitrificans* on the surface of the anode cloth material (NCBE) at 110 x magnification. Image a) control anode with cells but no fluorescent probes. b) the same portion of anode shows the dead cells stained with propidium iodide visualised as red.
6.3.2.2. Three-colour staining

6.3.2.2.1. *D. denitrificans*

As with the two-colour staining the cells did not follow the weave but grew along the straight fibres. Lower numbers of cells could be seen on the E-tek material than found previously. Like FAM, TET also binds to the fibres in some experiments making interpretation difficult in these cases. Surprisingly since this organism can metabolise glycerol the FISH gave negative results for both the glycerol dehydrogenase and the glycerol kinase genes (Figure 6.9 a) and b).

![Images of staining results](image)

**Figure 6.9.** *D. denitrificans* on the surface of the anode cloth material (E-tek) stained with probes for genes involved in glycerol metabolism. Image a) staining with probe for (glycerol kinase), b) glycerol dehydrogenase c) the same portion of anode stained to show the dead cells stained with propidium iodide visualised as red d) the same portion of anode shown optically. All at the same magnification (167 x).
The cells stained negatively for *omcB* (figure 6.10) and *omcA* (figure 6.11). There was no overlap between the specific probe staining and the non-specific dead cell stain, the fluorescence is seen on the fibres.

**Figure 6.10.** *D. denitrificans* on the surface of the anode cloth material (E-tek) stained with the probe for *omcB*. Image a) anode stained with probe *omcB* 5'-CGCTTTTCATGCGGTATACT-3. b) the same portion of anode stained with propidium iodide visualised as red. Individual rod shaped cells can be seen superimposed on the fibres. Both at approximately 300x magnification.

**Figure 6.11.** *D. denitrificans* on the surface of the anode cloth material (E-tek) stained with the probe for *omcA*. Image a) anode stained with probe *omcA* 5'-CGGTCAGCTTCTGTTCCTCC-3. b) the same portion of anode stained with propidium iodide visualised as red, individual rod shaped cells can be seen. Both are at approximately 300 x magnification.
Negative results were obtained with cells stained with probes for the phenazine \textit{phzE} gene from \textit{P. aeruginosa} PA7 and the \textit{P. putida} MK1 chromate reductase gene \textit{(chrR)} (figure 6.12). Despite the fluorescent dyes binding to the anode fibres, in both samples no fluorescence overlap of individual cells could be made with the dead stain.

\textbf{Figure 6.12.} \textit{D. denitrificans} on the surface of the anode cloth material (E-tek) stained with probes for the genes \textit{phzE} and \textit{chrR}. Image a) anode stained with probe for the phenazine \textit{phzE} gene from \textit{P. aeruginosa} PA7 5'-AACAGGTCGGAGTGAAGAA-3', image b) cells stained with 5'-TGTACAACGGAGGACGTCGAG-3' \textit{P. putida} MK1 chromate reductase, c) dead stain, individual rod like cells can be seen attached to the anode. These images are taken without the microscope’s autogain feature hence the overexposure. Note, the large red shape in the centre of the picture is not cells, it appears to be a crystal. The cells are seen as red rods in the bottom half of c). The magnification is 320 x.

The fluorescent \textit{in situ} hybridisation using glycerol kinase was repeated against the green harbour probe for PpcA and the Pcar_1628 c-type cytochrome to check for non specific binding to the fibres. Both probes gave a positive result with cells clearly visible (figure 6.13) showing this result was genuine and not an artefact of non-specific binding.
Figure 6.13. *D. denitrificans* staining with probes for the genes for glycerol kinase and the cytochromes PpcA and Pcar_1628. a) staining with the probe for glycerol kinase shows a cluster of cells, image b) cells stained with PpcA Pcar_1628 c-type cytochrome 5'-AACGGTAAGGACGACATTCG-3, image c) the same portion of anode shows the dead cells stained with propidium iodide visualised as red. All at the same magnification (320 x).

Weak positive signals were seen with a combination of probes for PCAR2984 c-type cytochrome and CymA (figure 6.14 below).

Figure 6.14. *D. denitrificans* staining with probes for the genes for PCAR2984 and cymA. a) staining with 5'-ATGAAAAATGCTTTGATGCT-3' (PCAR2984, c-type cytochrome). b) cells stained with CymA 5'-CATTTCTTGCTGTTTT-TT-3'. c) the same portion of anode shows the dead cells stained with propidium iodide visualised as red. Weak fluorescence was seen in the cell clusters circled. All at the same magnification(195 x).
6.3.2.2.2. *R. opacus*

*R. opacus* showed weakly positive results for glycerol kinase (figure 6.15). The fibres exhibit florescence at the detection wavelength for SYTO 9 (figure 6.15. a). However, this is not responsible for the fluorescence at the FAM detection wavelength since the images show the same area of anode material and the fluorescence shown by the fibre doesn't match that seen in figure 6.15. c). In addition some overlap is seen with the dead cells (figure 6.15 b)). The glycerol dehydrogenase probe was definitely negative (figure 6.15 d)).

![Images](a) (b) (c) (d)

**Figure 6.15.** *R. opacus* stained with probes for genes involved in glycerol metabolism. a) Anode fibres exhibit fluorescence at the detection wavelength for SYTO 9. b) dead cells stained with propidium iodide. c) cells stained with the probe for glycerol kinase. d) the same portion of the anode stained with the probe for glycerol dehydrogenase. All at 117 x magnification.

Very slight overlap is seen with the dead cells with the probe for PCAR2984 (figure
6.16 a and b)), however cells can be seen clustered along the anode fibre. A high degree of overlap was found between the dead cells and the probe for \textit{omcB} (figure 6.16 c)). This was repeated for \textit{omcB} and the same result was obtained (not shown).

\textbf{Figure 6.16.} \textit{R. opacus} stained with probes for the genes PCAR2984 and \textit{omcB}. a) Dead cells stained with propidium iodide. b) cells stained positively with PCAR2984, a c-type cytochrome, c) cells stained with the probe for \textit{omcB}, d) the same portion of the anode visualised on the optical channel, some of the fibres can just be seen. Magnification 136 x.

\textit{R. opacus} gave negative results for \textit{omcA} and the negative control human kappa antibody sequence (data not shown), as well as \textit{ppcA} and \textit{cymA} (figure 6.17).
Figure 6.17. *R. opacus* cells stained with probes for the gene *cymA* and human kappa antibody negative control. a) *R. opacus* cells stained with propidium iodide dead stain, b) cells stained with *cymA* 5'-CATGGTTTCTGCTGTTT-3', c) human kappa antibody negative control, d) the same portion of anode shown optically. All are at the same magnification (127 x).

Fluorescent cells were seen for *R. opacus* stained with the *phzE* gene probe. However, no match could be seen with the dead cell stain (figure 6.18 a) and b)). A negative result was obtained using the probe for the chromate reductase gene (data not shown).
Figure 6.18. *R. opacus* cells stained with probes for the *phzE* gene. a) *R. opacus* cells stained with propidium iodide dead stain, b) the same portion of anode shown stained for *phzE*. Both at the same magnification (320 x).

In staining repeated for PCAR2984 what appears to be cells can be seen clustered along the fibre (figure 6.19 c). Some slight overlap of fluorescence between the PCAR2984 probe and the *omeB* probe could be seen and also between the dead cell stain and the *omeB* probe fluorescence, although the staining was very weak.

Figure 6.19. *R. opacus* dead cells stained with probes for the genes *omeB* and PCAR2984. a) *R. opacus* dead cells stained with propidium iodide, b) cells clustered on the outside of the anode stained for *omeB*, c) cells stained with PCAR2984, a c-type cytochrome. All at the same magnification (223 x).
6.3.2.2.3. *R. ruber* and *R. rhodochrous*

Some studies were carried out on *R. ruber* and *R. rhodochrous*. *R. ruber* cells were found to follow the weave as seen for *R. opacus* previously (figure 6.20).

![Image](image.png)

**Figure 6.20.** *R. ruber* cells stained with the dead cell stain propidium iodide. Cells can be seen following the curve of the weave, magnification 38 x.

*R. ruber* appeared to stain positively for both the *omcB* and *cymA* genes. Some overlap in the staining with dead cells was seen circled in figure 6.21 although the dead cell staining is very weak. Another set of images was obtained which again showed overlapping dead cell and specific fluorescence for these two probes, this time the dead cells stained stronger than the specific probe stains (images not shown).
Figure 6.21. *R. ruber* cells stained with probes for the genes *omcB* and *cymA*. a) *R. ruber* cells stained with the *omcB* oligonucleotide stain, b) cells stained with *cymA*, c) dead cells stained with propidium iodide, the circle shows the cells that have stained in all three, d) live stain channel non-specific autofluorescence of the same region. All at the same magnification (225 x).

*R. ruber* gave negative results using the probe for the *omcA* gene. The *omcA* staining seems to run along the centre of the fibre and there is almost no overlap with the dead cells (figure 6.22). Whilst the fluorescence looks like cells, some overlap with the dead cells would be expected. The chromate reductase gene staining did overlap with much of the the dead cell fluorescence, but again was weak.
Figure 6.22. *R. ruber* cells stained with probes for the genes *omcA* and *chr*. a) *R. Ruber* cells stained with propidium iodide, b) *omcA* stained fibres. c) Cells stained with fluorescent probe for chromate reductase. All at the same magnification (166 x).

*R. rhodochrous* was probed for *omcB*. A lot of the fibres appeared to stain positively. The fluorescence was probably due to the fibres since none of the fluorescence for the *omcB* gene matched that of the dead cells. The dead cells stained the outside of the fibres and the *omcB* probe the centre. However, some cells were found without any apparent fibre staining (figure 6.23). These gave no fluorescence using the *omcB* specific probe. Weak positive staining was seen for *cymA*.

Figure 6.23. *R. rhodochrous* cells stained with probes for the genes *omcA* and *cymA*. a) *R. rhodochrous* dead cells stained with propidium iodide, b) the same portion of anode stained for *omcB*, c) the same clump of cells stained positively for *cymA*. All are at the same magnification (180 x).

Likewise the *R. rhodochrous* cells did not stain for *omcA* and very weak staining
was seen with the probe for chromate reductase (figure 6.24).

Figure 6.24. *R. rhodochrous* stained with probes for the genes *omcA* and *chrR*. a) *R. rhodochrous* dead cells stained with propidium iodide, b) the same portion of anode stained for *omcA*. c) the same clump of cells stained for chromate reductase, very weak staining can be seen circled. All at the same magnification (172 x).
6.3.2.2.4 BL002

Cells in the BL002 isolate stained positively for *omcA* and *omcB* (figure 6.25). Cells were also seen in that were positively stained using the dead stain but that did not stain for these genes using the specific probes (data not shown).

![Images](image1.png)

**Figure 6.25.** BL002 cells stained with probes for the genes *omcA* and *omcB*. a) BL002 cells stained with propidium iodide and b) with the probe for *omcA*, both at 320x magnification, c) stained for dead cells using propidium iodide, d) for *omcB*, both these are at 163 x magnification.

BL002 cells stained weakly for the *chrR* and the *phzE* genes (figure 6.26 below). In this set of images multiple individual cells can be seen attached to the anode fibres that stain as dead.
Figure 6.26. BL002 cells stained with probes for the \( \text{phzE} \) and \( \text{chrR} \) genes. Image a) BL002 cells visualised on the fibres of the anode, b) the same cells stained with propidium iodide. c) stained using the probe for \( \text{phzE} \), d) stained using the probe for \( \text{chrR} \), all are at 73 x magnification.

Cells in BL002 stained positively for the \( \text{ppcA} \), \( \text{cymA} \), PCAR2984 (figure 6.27) and glycerol kinase genes (not shown). Unknown types of cells can be seen in figure 6.27. f). These latter cells stained positively for both PCAR2984 and \( \text{cymA} \) (the corresponding dead cell image and PCAR2984 are not shown). The cells are unlike droplets of dye being too irregular.
Figure 6.27. BL002 cells stained with propidium iodide and the probes for cymA, ppcA and PCAR2984 genes. Image a) shows PI stained cells, (b) ppcA (both at 160 x magnification), c) shows another group of cells stained with propidium iodide, d) PCAR2984, e) cymA, c), d) and e) at 162 x magnification. Image f) shows a group of possible Archaea or mold cells stained with a probe for the cymA gene at a magnification of 187 x.

Live/dead staining showed very large clusters of cells attached to the anode; one
such cluster can be seen in figure 6.28. Rod shaped cells can clearly be seen attached on the anode as a cluster on the right hand side of the anode fibre. Individual cells can be seen on the left hand side of the fibre, these have also stained as live and dead.

![Image](image_url)

**Figure 6.28.** Cells from the mixed culture BL002 stained with live/dead stain. Image a) shows a cluster of cells on the optical channel, b) SYTO 9 stained cells and c) propidium iodide stained cells, all are at 168 x magnification.

### 6.3.2.2.5 BL003

Cells in this isolate stained positively for glycerol dehydrogenase and with less intensity for glycerol kinase (data not shown). The cells stained positive for PCAR2984 and *omcB* (figure 6.29). The very bright staining seen in figure 6.29 b) appears to show cells stained positively for PCAR2984 growing along the anode, although no overlap is seen with the dead cells. However, if they are positively stained they are a different set of cells from those seen in figure 6.29 a) and c).
Figure 6.29. BL003 cells stained with probes for the genes for $omeB$ and PCAR2984. Image a) shows dead cells stained with propidium iodide. Image b) cells stained for PCAR2984 c-type cytochrome. Image c) shows cells stained with the probe for $omeB$. Image d) shows the same group of cells seen in e) and f) visualised through the optical channel. Images e) and f) show cells stained for PCAR2984 and $omeB$ respectively. Images a), b) and c) are at 158 x magnification, d), e) and f) are at 242 x magnification.

As well as for $omeB$ positive staining was seen for cells on the anode with the probe for $omeA$ (figure 6.30). Many of the cells that have stained are cocci and not rod shaped.
Figure 6.30. BL003 cells stained with propidium iodide and the probe for the omcA gene. a) BL003 cells stained with propidium iodide b) cells stained with the probe for omcA. Both at are 91 x magnification. There is considerable overlap between the dead cell staining and omcA probe staining, but not all the dead cells stain positively.

Cells from BL003 did not stain positively for either the phenazine operon phzE gene or the chromate reductase gene chrR (data not shown). By contrast the cells did stain positively for ppcA (figure 6.31 below).

Figure 6.31. BL003 cells stained for the ppcA gene. a) stained with propidium iodide and b) with the probe for ppcA, both are at 143 x magnification.

Figure 6.32. shows a variety of cell types stained with probes for PCAR2984 and cymA. A clump of cells can be seen optically which stains positively for both cytochromes. In addition a yeast cell or algal cell can be seen, this also stains positively
for both. What is thought to be a rod shaped cell can also be seen, this does not stain positively for PCAR2984, but some staining can be seen with *cymA*.

![Figure 6.32](image)

**Figure 6.32.** BL003 three-colour staining with probes for the genes PCAR2984 and *cymA*. a) BL003 shows cells visualised using the optical channel. The yellow arrow shows the rod shaped bacterium, the turquoise arrow shows what appears to be a yeast or algal cell and the white arrow shows a clump of cells attached to the anode. b) shows the same cells stained with propidium iodide. c) shows cells stained for PCAR2984 and d) cells stained for *cymA*. The rod shaped bacterium does not stain for either of these genes. The magnification is 85 x.

One section of anode was stained separately with live/dead stain. Cells were seen both growing along the anode fibres and in clusters on the anode weave (figure 6.33 below).
Figure 6.33. BL003 cells stained with live/dead stain (live SYTO 9, green and dead propidium iodide, red) on the same two sections of anode. Images a) and b) show a cluster of cells at 39 x magnification, Images c) and d) show cells growing along the anode at a magnification of 73 x.

6.3.3. Summary of FISH results

The FISH results broadly confirm the growth characteristics of the bacteria in the presence of transition metals and in microbial fuel cells. So for example *D. denitrificans* gave negative results for genes involved in vanadium reduction such as *omcB* and *cymA* but tested positive for some genes involved in iron reduction such as PCAR2984 and *ppcA*. The *Rhodococcus* strains tested negative for most of the genes involved in iron reduction with the possible exception of *omcB* whose protein product can reduce vanadium, which *R. opacus* is able to do. The *chrR* gene was missing in all the organisms tested except *R. rhodochrous* although this organism, *R. opacus* and *D. denitrificans* could reduce chromate, as was the *phzE* gene in all three of these organisms. The Blue Lake consortia examined tested positive for most of the genes including *phze* and *chrR*.
6.3.4. Gel electrophoresis

6.3.4.1. Non reducing gel

In chapter three an attempt to look for differential expression of proteins in the cell medium of *R. opacus* and *D. denitrificans* supernatant failed. Protein concentration in the supernatant is at levels below the sensitivity of coomassie blue to detect. A native gel run as before but using concentrated samples this time detected faint bands (figure 6.34). Bands were obtained in samples cultured with chromate (VI) at 20 hours (lane 3) and iron (III) ferricyanide at 94.5 hours (lane 4) (the samples from both flasks in both cases being combined for that sample time). The band in lane 3 was at an identical position to that seen in lane 4 but after further destaining was lost as a discrete band. The band in lane 4 has a notional molecular weight of 750kDa using the regression fit seen in figure 6.34. This is very likely to be aggregated proteins rather than a single band of protein with this molecular weight since native gels cannot really give an accurate estimate of molecular weight. Thus the experiments was repeated under reducing conditions.
Figure 6.34. Native page gel using concentrated cell supernatant samples. Lanes 1, 2 and 12 are molecular weight standards. Lane 3 is *D. denitrificans* combined cultured with chromate (VI) (18.7x concentration). Lane 4 combined cultured with Fe(III) (11.2x concentration). Lane 5 *R. opacus* cultured with chromate (VI) (13.1x concentration). Lane 6 *R. opacus* cultured with V(V) (17.9x concentration). Lane 7 *R. opacus* cultured with Cu(II) (24.2x concentration). Lane 8 *R. opacus* cultured with Fe(III) (20x concentration). Lane 9 *R. opacus* cultured with Cu(II) (24.2x concentration). Lane 10 *R. opacus* cultured with Mo(VI) (16.2x concentration). Lane 11 *D. denitrificans* cultured with Mo(VI). The molecular weight of the markers in kDa are shown to the left of the gel. The superimposed graph shows the Rf value fitted against log molecular weight. The R² value of the second order polynomial fit was 0.992.
6.3.4.2. Reducing gel

A number of bands were seen on the 4-20% gel (figure 6.35). *D. denitrificans* samples showed more than one band which are apparently up-regulated with culture time, several of which are arrowed in figure 6.35 below. In addition similar bands were seen in cells cultured in the presence of molybdenum and from the MFC. Unfortunately the bands for the standards did not appear so there was no way of calculating the molecular weight.

![Reduced SDS 4-20% gradient page gel using concentrated cell supernatant samples. Lanes 1, 2 and 12 are molecular weight standards. Lane 3 is *D. denitrificans* cell sample cultured with chromate (VI) as in figure 6.34. Lane 4 *D. denitrificans* cultured with Fe(III) t=0 hours (concentration 12.5x). Lane 5 *D. denitrificans* with iron (III) t=4 (concentration 16.7x). Lane 6 as 5 t=72 (concentration 15.4x). Lane 7 as 6 t=103.5 (concentration 13.3x). Lane 8 *R. opacus* cultured with Fe(III). Lane 9 *R. opacus* cultured with Cu(II). Lane 10 *D. denitrificans* culture from the MFC (15.4x concentration). Lane 11 *D. denitrificans* cultured with Mo(VI).](image-url)
Some of the samples were used in a repeat 16% SDS gel (which is optimised for separation of low molecular weight proteins) using markers donated by Dr P. Morris (figure 6.36 below). No bands were seen in the sample lanes concentrated using TCA/acetone. As previously in the other lanes a number of bands could be seen. It should be noted that the band in lane 7 is weaker since there was insufficient sample left to load at the same volume as the others from that group. Since marker bands were visible the molecular weight of some of the bands of interest (arrowed) that seemed to be the same as those in figure 6.35 above were calculated from the regression fit on the $R_f$ against log molecular weight. These were in descending order of size; 19,080, 17,285, 9,932 and 5,603 Daltons.
**Figure 6.36.** SDS PAGE gel electrophoresis. Lanes 1 and 2 contain identical loadings of the gel marker standards. Lane 3 is *D. denitrificans* cultured with medium containing ferricyanide and concentrated by the TCA/acetone method. Lane 4 *D. denitrificans* cultured with Fe(III) t=0 hours (concentration 12.5x). Lane 5 as lane 4 t=4 (concentration 16.7x). Lane 6 as lane 4 t=45.5 (concentration 13.3x). Lane 7 as lane 4 t=72 (concentration 15.4x). Lane 8 as lane 4 t=103.5 (concentration 13.3x). Lane 9 as lane 3. The regression fit for the R$_f$ values with equation and quality of fit used to calculate the standard curve and therefore the approximate molecular weights of the unknowns is shown. The circle is the surface underneath the gel when it was photographed.

**6.3.4.3. Haem and ferrozine staining**

No bands were seen that stained using the DMB in either attempt. There was no staining seen using the ferrozine reagent on the gel.
6.3.5. PCR

*E. coli* K12 gave a positive result with the *E. coli* K12 flagellum gene *flgF* showing the PCR system was functioning (figure 6.38). None of the cell types tried gave a positive result with *ferA* (iron reductase from *G. sulfurreducens*) or *phzD* (phenazine operon D) gene from *P. putida*. *S. putrefaciens* however gave a single band of approximately the correct size with *mtrA* (219bp) (figure 6.37 below).

![Figure 6.37](image)

*Figure 6.37.* Agarose gel showing the PCR results for three genes *ferA*, *phzD* and *mtrA*. The lane marked M shows the 100bp ladder, lane 1 control, lane 2 *R. opacus*, lane 3 *R. ruber*, lane 4 *D. denitrificans*, lane 5 BL001, lane 6 BL002, lane 7 BL003 and lane 8 *S. putrefaciens*. The loading wells are marked.

No positive bands were seen after PCR using primers for *mtrB*, *mtrC*, *mtrD*, *omcE*, *omcF*, *omcS*, *omcT* and *omcZ* with DNA derived from *R. opacus*, *R. ruber*, *D. denitrificans*. BL001, BL002, BL003, *S. putrefaciens* and *S. onedensis*. Despite the motility of certain of the cells examined no positive results were seen for *flgF* gene amplification for any of the bacterial types. A number of weak bands were seen in
BL002 for the *flgF* and *cymA* genes, however on repeat these were not seen (figure 6.38).

**Figure 6.38.** Agarose gel showing the PCR results for the genes *flgF* and *cymA*. The lane marked M shows the 100bp ladder, lane 1 control, lane 2 *R. opacus*, lane 3 *R. ruber*, lane 4 *D. denitrificans*, lane 5 BL001, lane 6 BL002, lane 7 BL003 and lane 8 *S. putrefaciens*. Wells 1-4 in the third row are loaded with samples from *S. onedensis* and *E.coli* K12 *flgF* gene amplification and *S. onedensis* and *E.coli* K12 *cymA* gene amplification. The arrow shows the weak band seen for *E.coli flgF*. The loading wells are marked.
6.4. Discussion

Microscopy studies with and without probes revealed useful information about the relative numbers of bacteria on the anode and the manner in which they grew. Clear physical differences can be seen in the two main types of anode used in this project, the anode material from NCBE being straight fibres and the E-tek material being a weave but with some straight fibres. This difference possibly affects how cells grow on the anode material, certainly more cells are seen on the E-tek material. However on both types of anode material the number of cells seen was very low which was not surprising given the low power output of the microbial fuel cells. The possible reasons for this low power output will be discussed in the next chapter. In addition even when the voltage output has peaked many cells stain as living rather than dead.

The fluorescent dye used for some of these genes (TET) seems to bind non-specifically to the anode fibres in some images, as the images show what appear to be cells clustered along the fibre. However, when compared to the dead cell stain in some cases they are in the centre of the fibre whereas the dead cells appear to be on the outside. There would not be an apparent good biological reason for dead cells to be on the outside of the fibre and live cells to be on the inside if anything the opposite would be expected due to nutrient depletion. Where no overlap can be found with the fluorescence from the dead cells the results have been taken to be negative. It should be stated that this has not happened in every case and overlapping has been found with some probes. In addition the apparent non-specific binding is not uniform within a sample or between samples. The other stain, Gig harbor green, gives very weak fluorescence. This does not seem to be non-specific binding since it does not occur in every case i.e. many results were unequivocally negative. This was particularly true of the "Blue Lake" cells.

The fluorescent in situ hybridisation has identified a number of genes of interest in this thesis as being present or absent in the bacteria used. Of particular interest were *omcA* and *omcB*. These genes encode c-type polyheme cytochromes that have been implicated in reduction of iron, vanadium, manganese and transfer of electrons to the
anodes in microbial fuel cells (Myers and Myers, 2001; Myers and Myers, 2002; Leang, et al., 2003; Myers et al., 2004; Gorby et al., 2006; Bretschger et al., 2007; Nevin et al., 2009). \textit{D. denitrificans} cells seem to lack these two genes as confirmed by the PCR, (\textit{mtrC} is also known as \textit{omcB}) (Bretschger et al., 2007). Manganese reduction has not been tested in this organism, but as was described in chapter three there is strong evidence that \textit{D. denitrificans} can reduce iron and also that vanadium is toxic to the cells. Thus both the presence or absence of these genes does not totally explain some of this organism’s capabilities, at least concerning transition metal reduction.

Another probe tested which gave very weak fluorescence was \textit{cymA}. This gene encodes a tetraheme c-type cytochrome that can reduce iron (III), fumarate and nitrate (Myers and Myers, 1997; Myers and Myers, 2000). Its expression appears linked to \textit{omcA} and \textit{omcB} (Gao et al., 2008). \textit{D. denitrificans} is able to utilise fumarate and nitrate as terminal electron acceptors (Horn et al., 2005). Data in chapter three suggest it can also reduce iron (III). PCR was used to probe for the presence of this gene due to interpretation problems using the FISH and the importance of this gene, at least in iron reduction. No bands were obtained, for the gene sequence tested. One possible explanation for the weak fluorescence of the \textit{cymA} sequence coupled to the absence of \textit{omcA} or \textit{omcB} is that the probe may be hybridising with a different gene that shares some sequence homology with \textit{cymA}.

The \textit{D. denitrificans} genome apparently also lacks one other gene with relevance to microbial fuel cells. This is \textit{phzE} a gene that encodes for one enzyme in the pyocyanin pathway (figure 1.5., chapter one) (Mavrodi et al., 1998). Phenazines such as pyocyanin are produced by a limited range of bacteria including \textit{Pseudomonas} species although other bacteria can produce antibiotics with reduct abilities including \textit{Burkholderia}, \textit{Brevibacterium}, and \textit{Streptomyces} species (Mavrodi et al., 1998; Hernandez et al., 2004; Khan et al., 2005). Phenazines have been found to facilitate the transfer of electrons to the anode in an MFC (Rabaey et al., 2005). Again primers were purchased for a different gene in the same family from the same organism (\textit{phzD}) due to some of the problems interpreting some of the FISH results for \textit{phzE}. PCR confirms the negative FISH result for \textit{D. denitrificans}.
The bacterium also tested negative for chromate reductase. The protein expressed by this gene is believed to be localised to the periplasmic space (Park et al., 2000). However, this enzyme probably does not allow chromate (VI) to be the sole electron acceptor (Park et al., 2000). Compared to Pseudomonas putida a different chromate reductase gene must be involved in reduction of this transition metal in this organism.

Fluorescence was also obtained with D. denitrificans with probes directed against two c-type cytochromes found in Pelobacter carbinolicus (Haveman et al., 2006). One gene (ppcA) encodes a member of a family of cytochromes found in Geobacteraceae, its function is to link other cytochromes and an outer membrane iron reductase (Lloyd et al., 2003; Haveman et al., 2006). The other gene PCAR2984 encodes an outer membrane diheme c-type cytochrome c also found in Geobacteraceae. It is suggested that this cytochrome may also have a role in iron and uranium reduction (Haveman et al., 2006). Whilst the staining for ppcA was consistent, only weak fluorescence was seen in the case of the latter cytochrome. The presence of these genes in Pelobacter carbinolicus apparently does not allow electrons to transfer to the anode in microbial fuel cells (Richter et al., 2007).

The most surprising R. opacus result from the fluorescent in situ hybridisation was that the organism seemed to lack the omcA gene, but possibly not omcB. The assumption was that R. opacus could not reduce iron (III) and so these genes would both be absent. In addition the organism is Gram-positive and therefore has no outer membrane that these proteins are associated with, although Gram-positive organisms have been found capable of both electricity production and iron reduction (Park et al., 2001; Leang, et al., 2003; Sone et al., 2003). Both the omcA and omcB genes have been linked to reduction of iron although there is some evidence that their role may not be vital in S. putrefaciens MR-1 (Myers and Myers, 2001; Myers and Myers, 2002; Leang, et al., 2003; Myers et al., 2004; Gorby et al., 2006; Bretschger et al., 2007). The results so far from studies with S. putrefaciens MR-1 suggest that these two genes are partially linked. The omcB gene is just downstream of omcA in S. putrefaciens MR-1 but they are not transcribed together, however, mutants lacking expression of omcB mis-localise omcA (Myers and Myers, 2001; Myers and Myers, 2002). In addition omcB is involved...
in reduction of vanadium (V) (Myers et al., 2004). This would explain the vanadium
growth data obtained for R. opacus and D. denitrificans and also the results obtained for
both organisms concerning cymA which is also linked to the reduction of vanadium
(Myers et al., 2004). This gene is apparently not present in D. denitrificans which is
unable grow with vanadium (V) present in the medium. It is also absent in R. opacus
that grows both faster in the presence of vanadium and to a higher cell density without
this gene. In addition as alluded to earlier in chapter three the organism is obviously
expressing the gene(s) used constitutively due to the speed at which growth takes place
(unlike growth with chromate (VI) where there is a considerable lag). Using Blastn for
the omcB hybridisation sequence against the Rhodococcus Rha1 genome gave no hits
with a cut-off value of less than 10 (McLeod et al., 2006). Similarly the sequence gave
the same type of results for S. putrefaciens CN-32. At a cut-off value of 10 both
organisms gave some sequences that encode unrelated proteins. The primers for the
PCR for this gene also give some hits for Blasts against both the Rha1 and S.
putrefaciens CN-32 genomes, in the case of Rha1 proteins unrelated to cytochromes.
Taken together the Blast and hybridisation data suggest the probe for omcB may be
partly hybridising to another gene sequence.

For other genes R. opacus gave results consistent with its inability to reduce Fe (III),
as ppcA and cymA were not found. The results for glycerol kinase are contradictory. It
may be that the choice of probe was sub optimal. Like D. denitrificans, it also gave
negative results for both phzE and the chromate reductase (chrR) sequence. The phzE
result was confirmed by the PCR for another gene from the same pathway, phzD.

The other two strains of Rhodococcus gave broadly similar results for the omcA,
omicB, cymA and chrR sequences compared to R. opacus. The main difference is that
both genomes or plasmids of R. ruber and R. rhodochrous apparently contain cymA.
This was a surprise since neither tolerates iron (III) ferricyanide well. R. ruber could
grow in very low concentrations but no precipitate of ferricyanate could be seen. R.
ruber does not contain the chrR gene for chromate reduction whilst R. rhodochrous
apparently does, although the fluorescence was weak. R. ruber apparently matches R.
opacus in its genetic profile for omcA and omcB, but R. rhodochrous more logically
(according to the literature quoted above) has neither gene in its genome. \textit{R. ruber} was tested using PCR which did not confirm the \textit{cym}A result.

The cultures isolated from the Blue Lake gave some surprising results. BL003 was positive for the genes that degrade glycerol. Bacteria such as \textit{Shewanella} carry these genes whether or not they can use glycerol as a carbon source. What was more interesting was they clearly tested positively for genes corresponding to \textit{omc}A, \textit{omc}B, PCAR2984, \textit{cym}A and \textit{ppc}A, but not \textit{chr}R or \textit{phz}E. This was surprising since up until the point that they were tried in the microbial fuel cell they would not reduce iron (III) ferricyanide although BL003 would tolerate it. They also tolerated vanadium (V) although didn't appear to reduce it since no precipitate was seen. Some types of cells in BL002 and BL003 were able to tolerate chromate (VI). The FISH data are robust for these isolates since dead cells were seen that did not hybridise with the probes tried when other clumps of dead cells did, in the same cultures. Moreover the fluorescence was stronger than for the single cell cultures, giving less doubt that the fluorescence result was false. This strongly suggests that the hybridisation system was working and that binding was specific. Thus there is a contradiction with the \textit{cym}A, \textit{phz}E and \textit{omc}B (\textit{mtr}C) gene PCR results. No bands were obtained for these or related genes for any of the isolates. Again either the primers don't work or the probe is picking up a another gene or one that is slightly different. Possibly there is not enough DNA template from the bacteria that stained positively to amplify, given that the cultures were mixed. BL001 cells were not tested using FISH due to time considerations.

The PCR has been alluded to in parts of the discussion above. Compared to the FISH the same or related genes were used in the PCR. A number of other genes of interest were also searched for using PCR. These included the genes \textit{mtr}A, \textit{mtr}B and \textit{mtr}C which encode haem cytochromes known to be present together in the outer membrane in \textit{S. oneidensis} MR-1 and are linked together in a seven gene cluster (Carpentier \textit{et al.}, 2005; Ross \textit{et al.}, 2007; Beliaev and Saffarini, 1998). As was mentioned above the \textit{omc}A and \textit{mtr}C genes are known to be closely associated and their gene products are known to be involved in dissimilarity metal reduction (Shi \textit{et al.}, 2006; Ross \textit{et al.}, 2007). The proteins encoded by \textit{mtr}A and \textit{mtr}B are thought to be
involved in both iron and manganese reduction and possibly, in the case of \textit{mtrA} vanadium reduction (Pitts et al., 2003; Rehder et al., 2007; Beliaev and Saffarini, 1998). Knockouts in \textit{S. oneidensis} MR-1 suggested that these three genes are involved in current production in MFC's, since mutants produced only 20\% of the current with these genes (and \textit{cymA}) absent (Bretschger et al., 2007). The cytochrome MtrD is not apparently implicated in current production in MFC's but is thought to be involved in iron and to an extent vanadium reduction (Bretschger et al., 2007; Rehder et al., 2007). None of these genes were present in any of the cell types tried using PCR except \textit{S. putrefaciens} in which the \textit{mtrA} gene was apparently present. It is very likely that the other three genes are present in \textit{S. putrefaciens} but the gene sequences may vary between \textit{S. putrefaciens} and \textit{S. oneidensis} MR-1 from where the primers were derived. The \textit{S. putrefaciens} strain CN-32 has been sequenced (Copeland et al., 2009). Blasting against this sequence using all the \textit{mtr} gene primers chosen found that only \textit{mtrA} has complete DNA sequence identity and the primers for the other three genes would therefore not work. These three were tried using \textit{S. oneidensis} MR-1. As was outlined in chapters two and three this organism had died completely. However, there was a slope culture with colonies still present. These were scraped off aseptically and the cells concentrated as described. No bands were seen for these three genes with this organism despite the fact the fact the \textit{S. oneidensis} MR-1 genome was the source of the primers. One possible explanation is that there was insufficient DNA sequence to amplify. No smears could be seen on the gels when DNA from this organism was used, unlike others tried.

The remaining genes designated “\textit{omic}” were also probed for using PCR. \textit{omicE} is involved in reduction of insoluble iron in \textit{G. sulfurreducens} (Mehta et al., 2005). \textit{omicF} is thought to control the expression of other genes related to electricity production including \textit{omicB}, \textit{omicS} and \textit{omicE} (Kim et al., 2008). Knockouts lacking this gene do not produce as much current (Kim et al., 2008). \textit{omicS} is also implicated in reduction of insoluble iron (Mehta et al., 2005). Deletion of both \textit{omicE} and \textit{omicS} does not prevent reduction of soluble iron (Mehta et al., 2005). \textit{omicE} is thought to have a role in current production in MFC's, its expression in \textit{G. sulfurreducens} being slightly higher on electrodes than on medium containing iron (III) citrate (Holmes et al., 2006). Deletion
of the \textit{omcS} gene inhibited current production in this organism (Holmes \textit{et al.}, 2006). The expression of \textit{omcS} and \textit{omcT} was upregulated in \textit{G. sulfurreducens} during growth on the anode comapred with iron (III) citrate, both genes are thought to be co-transcribed (Holmes \textit{et al.}, 2006). The exact function of \textit{omcT} at present is unknown. Recent analyses of \textit{G. sulfurreducens} biofilms in MFC’s suggest that \textit{omcS} and \textit{omcT} are less important than was previously thought, being down regulated in relatively thick anode biofilms, while \textit{omcB} and \textit{omcE} were up regulated and another gene (\textit{omcZ}) was found to be necessary for current production in biofilms (Nevin \textit{et al.}, 2009). It was surprising that none of these five genes was found in any of the organisms tested especially since three cultures were mixed and extracted from a high iron environment. This implies that the ability to reduce iron and other transition metals or transfer electrons to the anode in an MFC is markedly less common than would appear to be the case from the literature.

The native page gel produced a high molecular weight band of approximately 750kda weakly expressed in \textit{D. denitrificans} for cells grown in the presence of chromate (VI) and iron (III). This seems high for a protein possibly exported into the supernatant and a literature search failed to find any bacterial proteins of this molecular weight. The most likely explanation was that the bands were low molecular weight proteins aggregated together. Running the same samples under reducing conditions showed this was the case. In addition there was an alteration in expression over the iron (III) culture time course. Whilst most of the bands were thought to be internal proteins externalised due to cell death and subsequent lysis four bands (arrowed) were thought to be worthy of further examination. They are of the same order of size as found by Seeliger \textit{et al.} (1998). A faint band was just visible to the naked eye using the GIMP imaging software at time zero, by four hours it is clearly visible and also at 72 hours. Its expression declined at 103.5 hours. This is possibly due to the \textit{D. denitrificans} metabolising the proteins in the supernatant, there is diminution of all the visible bands at this time point. This was confirmed by the repeat gel. No definite evidence was seen for the presence of haem groups in the separated proteins on the gel although the haem can disassociate and could have been lost. It was not possible to investigate these bands further since the MALDI instrument was out of order during the remainder of the lab based part of the
Chapter 6. Studies of the cellular metabolism at the molecular level

project and beyond.
Table 6.3. Summary of results for this chapter. The proteins whose genes were probed for are listed, as are their putative functions and the results using the organisms tried in this project.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>OmcA 83-kDa 10 haem</td>
<td>Iron, vanadium, manganese reduction and electron transfer in MFC’s</td>
<td>Absent in <em>R. opacus</em> and <em>D. denitrificans</em>. Present in BL002 and BL003.</td>
</tr>
<tr>
<td>OmcB (mtrC) 10 haem 73Kda</td>
<td>Iron, vanadium, manganese reduction and electron transfer in MFC’s</td>
<td>Possibly present in <em>R. opacus</em> absent in <em>D. denitrificans</em>. Present in BL002 and BL003.</td>
</tr>
<tr>
<td>CymA 21-kDa tetraheme</td>
<td>Iron, fumarate, nitrate and vanadium reduction.</td>
<td>Weak, probably absent in <em>D. denitrificans</em> and <em>R. opacus</em>. Apparently present in <em>R. ruber, R. rhodochrous</em> and also BL002 and BL003 cells. Not confirmed by PCR.</td>
</tr>
<tr>
<td>PhzE and PhzD</td>
<td>Antibiotic synthesis, electron transfer in MFC’s</td>
<td>Absent in <em>D. denitrificans</em> and <em>R. opacus</em> and BL003. Present in BL002.</td>
</tr>
<tr>
<td>ChrR</td>
<td>Chromate VI reduction</td>
<td>Absent in <em>D. denitrificans</em>, <em>R. opacus</em> and BL003. Present in <em>R. rhodochrous</em> and BL002</td>
</tr>
<tr>
<td>PCAR2984 2 haem groups?</td>
<td>Iron and uranium reduction</td>
<td>Present in <em>D. denitrificans</em> but not <em>R. opacus</em> or BL003. Present in BL003.</td>
</tr>
<tr>
<td>PpcA periplasmic 9.6Kda cytochrome</td>
<td>Iron reduction</td>
<td>Present in <em>D. denitrificans</em> but not <em>R. opacus</em>. Present in BL002 and BL003.</td>
</tr>
<tr>
<td>OmcE 30Kda cytochrome</td>
<td>Iron reduction</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>OmcF 9.4Kda cytochrome</td>
<td>Controls expression of <em>omcB, omcS</em> and <em>omcE</em> genes.</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>OmcS 50Kda cytochrome</td>
<td>Iron reduction (insoluble)</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>OmcT</td>
<td>Function unknown.</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>OmcZ</td>
<td>Current production in biofilms</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>MtrA</td>
<td>Iron and manganese reduction also vanadium?</td>
<td>Absent in bacteria tested except <em>S. putrefaciens</em>.</td>
</tr>
<tr>
<td>MtrB</td>
<td>Iron and manganese reduction</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>MtrD</td>
<td>Iron and vanadium reduction</td>
<td>Absent in bacteria tested.</td>
</tr>
<tr>
<td>FerA 89 Kda</td>
<td>Iron reduction</td>
<td>Absent in bacteria tested.</td>
</tr>
</tbody>
</table>
Chapter 7. Microbial fuel cell studies

7.1. Introduction

Reduction of metals in the environment by microbiological means has been of academic interest for some years and is believed to be a very important part of the geochemical cycle (Lovely, 1993; Lloyd et al., 2003). The ability of micro-organisms to couple the oxidation of some compounds to reduction of organic or inorganic compounds has been found to be widespread in both the archaea and bacteria families (Erlich, 1997; Phung et al., 2004; Rabaey and Verstraete, 2005; Logan and Regan, 2006; Kim et al., 2007; Du et al., 2007). The ability of micro-organisms to transfer electrons to the anode in a fuel cell has been known for almost 100 years (Shukla et al., 2004). In 1910 M.C. Potter at the University of Durham discovered that organisms could produce current (Shukla et al., 2004; Du et al., 2007). His work was repeated at Cambridge in the 1930's and in the 1960's NASA took an interest in microbial fuel cells with a view to using them in manned space flight. Photovoltaics were thought to be more practical so interest waned until after the oil shocks of the 1970's (Shukla et al., 2004).

When enrichment cultures using sludge have been used in an MFC, examination of the genetic profile of the organisms on the anode have found representatives of a wide variety of bacterial genera, both anaerobic and aerobic present including Pseudomonas, Lactobacillus and Clostridium (Park et al., 2001; Pham et al., 2003; Du et al., 2007). In some studies the majority of bacteria found on the anode have been in the proteobacteria family with representatives from some or all its five sub-divisions (Kim et al., 2006; Kim et al., 2007; Logan, 2008; Boroles et al., 2009; Fedorovich et al., 2009). One study has suggested the majority are firmicutes and another found entirely Shewenella species. Other studies found the anode population to be entirely γ-proteobacteria (Park et al., 2008; Rabaey et al., 2004; Hou et al., 2009). Or that Bacteroidetes were the most abundant phylotype (Kim et al., 2006).
Both mixed consortia of bacteria and single organisms have been used in microbial fuel cells. It has been recognised that mixed bacterial consortia give higher power outputs in MFC's, although there is one instance of a pure culture giving a comparable power output (Ringeisen et al., 2006; Rabaey, 2006). Single organism studies are useful for examination of the mechanism of electron transfer, genetics and growth characteristics of particular cell types (Logan et al., 2006; Choi et al., 2007; Kim et al., 2008; Lanthier et al., 2008). Several species of Geobacteraceae have been found to be capable of directly transferring their electrons to the anode in a microbial fuel cell, along with Shewanella putrefaciens and a number of other organisms (Holmes et al., 2004). Of these organisms, Geobacter sulfurreducens is the most studied organism utilised in microbial fuel cells since it gives high current densities and reduces iron (III) (Kim et al., 2008). Many of the species used in microbial fuel cells are anaerobic, although facultative anaerobes can be used and there is some evidence that G. sulfurreducens is a facultative anaerobe (Park et al., 2001; Lin et al., 2004; Lovley, 2006). Whilst keeping the oxygen levels to a minimum in an MFC is obviously useful since oxygen is an alternative electron acceptor, facultative anaerobes have been successfully utilised in fuel cells in one instance giving higher power output in the presence of oxygen (Mohan et al., 2008).

One of the aims of this project has been to seek to use facultatively anaerobic bacteria and substrates not used in MFC's before. Bacteria were used in microbial fuel cells under mildly aerobic conditions. This chapter will seek to link the ability to reduce transition metals by the bacteria chosen to the production of electricity in microbial fuel cells using some of the organic compounds chosen as fuel.
7.2. Materials and methods

All chemicals were sourced from Fisher UK or Sigma UK. Cell growth was monitored by measuring the turbidity at 600nm (OD$_{600}$). The OD$_{600}$ turbidity readings were referenced as described previously. Growth was carried out at room temperature. Visual examination of cells was made using a haemocytometer slide 0.1mm x 0.0025 mm (Superior, Germany) and an Olympus CH-2 microscope. Cell culture and growth studies in flasks and use of HPLC for measuring glycerol concentrations were undertaken as described in chapters two, three, four and five and any exceptions are covered individually. Glucose was measured using the glucose sticks as outlined in chapter three. Spectroscopic scans were also carried out as outlined in chapter three. Bacterial stock cultures used to inoculate microbial fuel cell trials wherever possible were in exponential or stationary phase. Dilutions into fresh medium in the MFC were in the range 1:3 to 1:10.

7.2.1. Fuel cells

A number of fuel cells were used in these studies. These are demarcated by their relative size and shape rather than the the type of membrane used (if any), as these were varied in any case.

7.2.1.1. Miniature fuel cell

This was purchased from National Centre for Biotechnology Education at the University of Reading. The kits provided both cathode exchange membranes, gaskets and also carbon fibre electrodes which were used in other fuel cells. Figure 7.1 below shows the various MFCs in operation. Some adaptations were made in house over time and are described below.
Figure 7.1. Microbial fuel cells used in this project. a) miniature fuel cell purchased from NCBE b) medium MFC made in house to same design as a). c) broken large MFC. d) medium cylindrical MFC also made in house, fitted with reference electrode. e) same set up for anaerobic growth study.
7.2.1.2. Medium sized fuel cell

This was made in house roughly to the specifications of the miniature MFC with swagelok fittings to allow electrical connections and sampling, but was bigger in size.

7.2.1.3. Medium cylindrical fuel cell

This was made in house from cylindrical perspex with swagelok fittings to allow electrical connections and sampling. This cell was modular and was used with both semi-permeable membranes and air cathodes in a variety of different configurations.

7.2.1.4. Very large fuel cell

This was made in house from perspex. Unfortunately this cell split on first use when it came into contact with 70% ethanol.

7.2.1.5. Cathode/anion exchange membranes

Cathode exchange membranes were purchased from VWR and NCBE. In addition VWR donated a set of anion exchange membranes. Additional cathode exchange membranes (CEM) (Nafion 424) were purchased from Sigma (UK).

7.2.1.6. Anode/cathode material

NCBE provided carbon cloth material with the miniature MFC's. This was used in many of the experiments for both anode and cathode. In addition cloth material was purchased from E-tek (USA). The anode material consisted of plain carbon cloth with no wet proofing. For the cathode this was impregnated with platinum at a loading of 0.5mg/cm² using 10% platinum.
7.2.1.7. **Ultrafiltration membranes**

An ultrafiltration membrane was purchased from Spectrum labs (USA).

7.2.1.8. **Air cathodes**

An air cathode was purchased from Electro-Chem-Technic (UK). This material was cut to size before use and a wire carefully inserted between the two materials by peeling the very edge of the two component materials apart.

Another was made *in house* as follows. Circuitworks conductive expoxy glue (purchased from Onecall Farnells, UK) was prepared by mixing parts A and B on a piece of cardboard in roughly equal volumes. This was used to bond the NCBE anode cloth to the NCBE cathode exchange membrane. A large square of these materials was bonded together with three wires sandwiched at different points around the square in between the two materials. The materials were placed under books to hold them together until the expoxy had dried. The resulting air cathodes were prepared by cutting up the sheet into three parts and cutting them to the size required to fit across the medium cylindrical fuel cell.

7.2.1.9. **Microammeters**

EL-USB-4 USB microammeters were purchased from Lascar Electronics (UK).

7.2.1.10. **Other**

Crocodile leads were provided with the multimeter and microammeters, additional lead and crocodile clips along with resistors were purchased from Maplin, UK or provided by electrical engineering at Heriot-Watt University.
7.2.2. General methodology

7.2.2.1. Assembly

The cells had neoprene gaskets which were, in the case of the miniature cell, provided. All other gaskets were made in house. The gaskets were greased with silicone grease. Anode and cathode material (if required) was cut to size and placed in each compartment. Material was placed between the anode and cathode and the ion exchange membrane or air cathode to stop electrical shorting via this route. Usually this was a j-cloth but in some initial runs in the miniature fuel cell a plastic gauze was used. On initial runs using the miniature fuel cell the material was passed out of the holes and made contact using crocodiles clips. On the other fuel cells tried and on the miniature one after in house adaptation, the cloth material was screwed into a bolt fitting. Contact was checked using a multimeter by measuring resistance and the MFC was assembled using bolts which were tightened before leak testing using water. The medium sized cylindrical MFC was modular and could be assembled in a variety of different configurations using an air cathode or different types of membranes with different distances between the anode and cathode. These will be noted in the context of experiments described below.

7.2.2.2. Sterilisation

The rectangular cells were sterilised using 70% ethanol. After the irreversible breakage of the largest fuel cell of a cylindrical type, other disinfectants were tried. In some instances 10% v/v 30% hydrogen peroxide was added with the alcohol. 4M sodium hydroxide was used in cells fitted with air cathodes and hydrogen peroxide used in the medium sized cell.

7.2.2.3. Current and voltage measurement

Voltage was measured using a Fluke 8842 multimeter. Current was measured using EL-USB-4 USB microammmeters. Control and data downloading was carried out using
software provided by Lascar. The microammeter was set-up before connection using its own dedicated software on a PC. This allows the time and date and starting time and reading interval to be set. The instrument had a resistance of 110Ω. For most studies this was used as the only load in series with the MFC unless otherwise stated.

7.2.2.4. Reference electrode

The reference electrode was a Mettler Inlab 301 (Ag/AgCl electrode) purchased from Fisher Scientific UK. An attempt was made to use the reference electrode in conjunction with the miniature MFC by making a connection between the electrode placed in a 50ml syringe housing and the MFC. This leaked and it was also impossible to remove airlocks in the tubing in between the two. A cylindrical perspex housing was made in house with a gasket fitting that could be tightened at the top around the upper part of the reference electrode and a swagelok fitting that connected it to one of the ports on the cylindrical medium MFC.

When used, the reference electrode holder was screwed onto the uppermost port on the medium MFC and sterilised and washed at the same time as described below. The electrode holder was removed and wrapped in a sterile alcohol wipe. The MFC was filled with cell suspension right to the top of the swagelok fitting. The electrode holder was then screwed onto the top of the MFC and filled with further cell suspension to a level that would cover the bottom of the electrode. The electrode was wiped with an alcohol wipe then placed in the fitting which was then tightened up.

At several points during the microbial fuel cell run the load was disconnected and the open circuit voltage measured between the reference electrode and the anode by making a connection using a single piece of wire.

7.2.2.5. Catholyte preparation

0.1M sodium phosphate/0.1M potassium ferricyanide was prepared by dissolving 0.1224g of Na$_2$HPO$_4$.2H$_2$O, 0.0987g of NaH$_2$PO$_4$.2H$_2$O and 0.49g of K$_3$[Fe(CN)$_6$] in
15ml of deionised water. The pH was adjusted to 7 ± 0.1 using 1M sodium hydroxide.

7.2.3. Usage

7.2.3.1. Miniature MFC

The miniature MFC had two filling holes on each compartment. Initially in studies using the small MFC the holes were open on the anode side. Sterile foam bungs cut up were used to fill the holes. The miniature MFC was modified *in house*. The holes were drilled out with threads so they could be capped and made airtight and an extra set of securing bolts was added in the middle between the top and bottom bolts to stop bowing out of the two compartment sides. Connections were made to resistance loads as described below. Sterilisation was carried out using 70% ethanol (v/v). Between three to five washes of the anode compartment were carried out and three washes of the cathode compartment in the miniature MFC when alcohol had been used to sterilise it. Growth was carried out in a class II laminar flow cabinet at ambient room temperature without shaking or stirring. Once the voltage showed a consistent fall the runs were stopped and the microbial fuel cells either dismantled, or washed with tap water and stored at +4°C. Samples were removed before washing or dismantling for cell counts and assays. Individual experiments are described within the context of the results section below due to the number and complexity of the experiments tried. The volume in the miniature MFC varied but was typically between 5-8ml.

7.2.3.2. Medium cylindrical MFC

The MFC was assembled as shown in figure 7.1 d) above in its two chamber configuration and sterilisation was carried out. In the medium MFC, 4M NaOH was used to sterilise the anode side or the sole compartment for at least one hour. Then 20-30ml of 1M HCl and approximately 500ml of deionised water was used to bring the pH back to neutrality. The pH of the wash as it was poured out of the cell was checked with pH paper. The MFC anode compartment was filled with cell culture, preferably in early exponential phase, and the cathode side with either cell medium without a carbon source
or catholyte described above. Alcohol wipes were used to sterilise filling holes before medium was added. In most runs using this MFC small glass beads (5mm) were present throughout. This reduced the volume of liquid to approximately 50-100ml.

In trials using air cathodes the MFC had only one compartment and the air cathode was sandwiched between the perspex that made up the anode compartment and a perspex end piece with a hole in it.

This MFC was also used to culture cells anaerobically. Sterilisation and set-up was carried out as above (figure 7.1 e) but a nitrogen line was used to pass this gas over the cell suspension surface. Two 0.2 micron air filters were used, nitrogen being passed in through one and out through another both attached to the two ports on the MFC. The rate of flow of the gas was controlled by a flow meter (Coleparmer, UK). The MFC was sterilised in the normal way. The filters attached to short lengths of silicon tubing were autoclaved with the open end of the tubing wrapped in foil. The ends of the tubing were unwrapped immediately before use and attached to the ports on the MFC in the class II flow hood. Further lengths of silicon tubing were used to attach the air filters to the gas supply and to carry the gas to water for measuring the flow rate. This was carried out by inverting a measuring cylinder with water in it, holding it upside down under water, pushing the silicon outlet tube into it and timing the displacement of a known volume of water. Cells were added and the MFC left under no load overnight in every case so that the open circuit voltage (OCV) could be established. In one case the cell was used with an air cathode (manufactured in house).

7.2.3.3. Calculation of internal resistance

The internal resistance of the microbial fuel cells was calculated using the methods outlined by Logan (2008). The microbial fuel cells were put under a variety of external resistance loads ranging from 5.9Ω to 3.29MΩ and the voltage measured at these loads. The current for each load was calculated using Ohm's law. The current data were used to plot the polarisation curve current (mA per cm² of anode material) versus voltage, or if this was not capable of a linear fit over a portion, a plot of the power density curve
was made of current (mA/cm²) versus power output (mW/m²). By fitting a quadratic regression, differentiating it and solving for x in the equation, the current at the maximum point on the curve (the maximum power output of the MFC y-axis) was obtained. Substituting this x value in the quadratic formula the maximum power output y was determined. The power was converted to power production from the system by dividing by the anode’s surface area converting cm to metres and converting mW to W and cancelling out the units of area in the calculation. The assumption made is $R_{\text{int}}$ is equal to $R_{\text{ext}}$ at maximum power output (Logan, 2008). Using the the current at the maximum point on the curve and the maximum power output, $R_{\text{ext}}$ and hence $R_{\text{int}}$ is calculated. Full working is shown in the appendix. For any run the maximum voltage was used to calculate the maximum power unless otherwise stated. Power and current are reported as described (Rabeey and Verstrate, 2005; Logan, 2008).

7.2.3.4. Conductivity measurements

These were carried out on a Hanna H18033 conductivity meter. The meter was calibrated immediately before use with a 0.1M KCl solution which has a conductivity of 12.89mho/cm. The electrode was washed in deionised water and dried with tissue paper before use.

7.2.3.5. Coloumbic efficiency

This was not calculated due to the low voltages obtained in the MFC runs.

7.2.3.6. SEM imaging

This was carried out by M. Millar using samples of anode material fixed as described in chapter six and stored at -20°C until use. The samples were mounted onto aluminium stubs and then gold sputtered. The Hitachi S2700 Scanning Electron microscope was run at an accelerating voltage of 10kV.
7.3. Results

7.3.1. Initial experiments

7.3.1.1. Miniature MFC

These studies were carried out using glycerol at 49ml/l in medium B as the feedstock in the miniature MFC with *D. denitrificans*. Cathode exchange membranes and anode material provided by NCBE were used to separate the cells and catholyte and provide an electrical connection from the MFC to the outside. A series of two initial experiments were made without any cells but with medium and ferricyanide to measure background battery effect. No current was detected, current readings being taken every five minutes.

Two trials were made with cells in the MFC. In both runs current was detected using the micro-ammeter (figure 7.2), current readings being taken every five minutes in the first run and every minute in the second. It was later discovered that the circuit had been connected incorrectly and that the resistor had been place in parallel with the micro-ammeter when it should been placed in series (figure 7.3). A sample was taken for a streak plate from the second run (figure 7.2. b)) and no sign of contamination was seen.

The first trial voltage and current showed a highly significant correlation by Spearman's rank correlation (R=-0.925, p<0.0001). However in the second trial run the voltage and current could not be correlated by Spearman's rank correlation due to the increased size of the data set as a result of the increased sampling rate. The size of the data set were beyond the size that the software could perform this test on.
Figure 7.2. Results of two initial runs in the miniature MFC using *D. denitrificans* where current was detected using the multimeter. The □ symbol denotes voltage and the ▽ symbol denotes current. a) is the first trial and b) the second.

Figure 7.3. Circuit diagrams used. The incorrect circuit set-up used on the first few initial runs is shown by a). b) shows the correct circuit digram used from then on forward. The multimeter was set to read voltage. Note the micro-ammeter has a resistance of 110Ω.

Attempts to repeat the above immediately failed using a second identical MFC, since catholyte leaked across the membrane and changed to a blue colour on the anode.
side. Small motile cells typical of *D. denitrificans* were seen in catholyte samples. Rips could be seen in the CEM and these were photographed under the microscope (figure 7.4).

![Image](image.jpg)

**Figure 7.4.** A tear in CEM purchased from NCBE. It can be seen centre field.

A variety of different trials were undertaken with Nafion 424, anion exchange membranes and an ultrafiltration membrane using 49ml/l glycerol or 2g/l glucose as the carbon source with the same organism. No current was detected using the microammeter and lower maximum power outputs were obtained. In parallel a series of experiments was carried out to look at some issues that might have caused this lowered power output. Some of these are covered in chapter three and chapter five (the effect of metabolites, of not mixing the cell suspension and the toxicity of ferricyanide). The remainder such as effect of the anode material and of varying medium ion composition are covered later in this chapter.

### 7.3.1.2. Effect of anode material on the cells

One possible reason for low power output was that the bacteria used might find the anode material toxic. Duplicate *D. denitrificans* cultures using glycerol as the carbon source (unshaken) were grown with and without NCBE anode material. The starting concentration of the control was 724mM glycerol and there was 811mM glycerol in the cultures with the electrode material present (figure 7.5). The cultures in the anode-free sample grew to a higher cell density on average than the cultures with anode material.
present. However, the glycerol concentrations in the cultures with anode material present were lower at the end of the culture period than the cultures without. Neither of these results were significantly different using a t-test.

![Graph](image1.png)

**Figure 7.5.** Growth data for unshaken *D. denitrificans* cells with and without NCBE anode material. Graph a) control without and b) with anode material present in the flasks. Glycerol concentration in mM is shown in red and cell number in black. The error bars show the standard error of the mean for duplicate cultures for cell count and glycerol concentration determined by HPLC.

### 7.3.2. Miniature MFC advanced studies

#### 7.3.2.1. Internal resistance

The internal resistance of the miniature MFC was calculated by putting the cell under external resistance loads. The resulting voltages obtained were used to calculate the internal resistance as outlined in the appendix. The internal resistance data were used to calculate the maximum power output as described in the appendix for these
trials and others using the same organisms in the miniature fuel cell. The results of these and all the internal resistance calculations are shown in table 7.1 and table 7.2 below. A polarisation and power curve for *D. denitrificans* is shown (figure 7.6).

**Table 7.1.** Internal resistance and power data for MFC trials in which the internal resistance was measured. The carbon source used was acetic acid with the exception of the trial using *R. opacus* with the anion exchange membrane (AEM) which used 2g/l glucose all in medium B. Varying concentrations of acetic acid were used.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MFC Set-up</th>
<th>( R_{\text{int}} (\Omega) )</th>
<th>OCV (V)</th>
<th>( P_{\text{max from OCV}} ) ( \text{mW/m}^2 )</th>
<th>( P_{\text{max under load}} ) ( \text{mW/m}^2 )</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. denitrificans</em></td>
<td>CEM in miniature MFC</td>
<td>41170</td>
<td>384</td>
<td>( 4.25 \times 10^{-3} ) ( 3.74 \times 10^{-8} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>CEM in miniature MFC</td>
<td>84513 Not taken</td>
<td>-</td>
<td></td>
<td>6.90 \times 10^{-11}</td>
<td>Resistance calculated from polarisation curve.</td>
</tr>
<tr>
<td>Blue Lake mix</td>
<td>CEM in miniature MFC</td>
<td>195505</td>
<td>372</td>
<td>1.89 \times 10^{-4} 2.35 \times 10^{-11}</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. putrefaciens</em></td>
<td>CEM in miniature MFC</td>
<td>3333</td>
<td>225</td>
<td>0.51</td>
<td>1.73 \times 10^{-4}</td>
<td></td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>AEM in miniature MFC</td>
<td>10128</td>
<td>170</td>
<td>1.68 \times 10^{-2} 4.44 \times 10^{-7}</td>
<td>0.2M phosphate medium</td>
<td></td>
</tr>
<tr>
<td><em>S. putrefaciens</em></td>
<td>CEM in medium MFC</td>
<td>3996</td>
<td>742</td>
<td>1.03</td>
<td>3.36 \times 10^{-5}</td>
<td>Anaerobic</td>
</tr>
</tbody>
</table>
Table 7.2. Power output results for some other trials using the internal resistance data from table 7.1. The carbon sources used were acetic acid (concentrations of which varied) and glycerol, the growth medium, medium B.

<table>
<thead>
<tr>
<th>Organism</th>
<th>MFC Set-up</th>
<th>$R_\text{in}$ (Ω)</th>
<th>OCV (V)</th>
<th>$P_{\text{max from OCV}}$ (mW/m²)</th>
<th>$P_{\text{max under load}}$ (mW/m²)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture</td>
<td>CEM in miniature MFC</td>
<td>41170</td>
<td>51</td>
<td>$9.59 \times 10^{-5}$</td>
<td>$1.26 \times 10^{-7}$</td>
<td><em>R. opacus/R. Ruber/R. rhodochrous</em> and <em>D. denitrificans</em>.</td>
</tr>
<tr>
<td><em>R. opacus</em></td>
<td>CEM in miniature MFC</td>
<td>84513</td>
<td>75</td>
<td>$3.79 \times 10^{-5}$</td>
<td>$1.73 \times 10^{-8}$</td>
<td>Used in FISH studies.</td>
</tr>
<tr>
<td><em>D. denitrificans</em></td>
<td>CEM in miniature MFC</td>
<td>41170</td>
<td>354</td>
<td>$3.37 \times 10^{-3}$</td>
<td>$3.89 \times 10^{-7}$</td>
<td>The carbon source was 670mM glycerol.</td>
</tr>
</tbody>
</table>
Figure 7.6. Polarisation and power curve plotted for \textit{D. denitrificans} cultured on 670mM glycerol in medium B. The data presented here were used to calculate the internal resistance, the workings of which are shown in the appendix. The polarisation curve is voltage plotted against current density and the power curve voltage plotted against power density. In the polarisation curve the three different numbers show the different regions of power loss, 1) activation, 2) ohmic and 3) concentration. The (●) symbol shows the points on the polarisation curve curve and the (■) symbol points on the power curve. The slope if linear is $R_{\text{int}}$. The $R^2$ value for region two was 0.99.

7.3.2.2. Medium composition

The medium composition was altered in two different ways. The first variation was to lower the sulphate concentration. The second was to alter the ionic concentration to affect conductivity and therefore resistance.
7.3.2.3. Sulphate concentration

Sulphate and nitrate are potential alternative electron acceptors which could compete with the anode for electrons (Rabaey and Verstraete, 2005). Medium B contains no nitrate but original composition contains a high quantity of sulphates (see chapter two). Midway through the project the sulphates were exchanged for chloride salts with the exception of the iron (II) sulphate which was at trace levels. No formal growth studies were undertaken. *D. denitrificans* was unaffected by the change but *R. opacus* would not grow for more than 3-4 generations in the low sulphate medium. Due to this concern some sulphate was added back (calcium sulphate as per medium B composition). This seemed to provide enough sulphur for *R. opacus*. In general cells were maintained in normal sulphur strength medium and inoculated into low sulphate medium only for an MFC trial. In general terms however the change did not seem to make any difference as far as power output was concerned. Certainly the use of normal medium B had no effect on the transition metal reduction which was almost all carried out in this medium.

7.3.2.4. Conductivity

A batch of medium B was made up and the conductivity monitored as it was prepared to see which component(s) had the largest effect. The results for this are shown in table 7.3.
Table 7.3. Composition of normal strength medium B and its effect on conductivity.

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight in grams</th>
<th>mmol/l</th>
<th>μS/cm x 1000 (cumulative total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL-6 trace elements</td>
<td>5ml</td>
<td>Not calculated</td>
<td>0.00</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.8</td>
<td>4.6</td>
<td>1.09</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2</td>
<td>14.7</td>
<td>1.23</td>
</tr>
<tr>
<td>CaSO₄.2H₂O</td>
<td>0.05</td>
<td>2.9</td>
<td>1.24</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.5</td>
<td>20.3</td>
<td>1.52</td>
</tr>
<tr>
<td>FeSO₄.2H₂O</td>
<td>0.03</td>
<td>0.1</td>
<td>1.53</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.03</td>
<td>7.8</td>
<td>3.48</td>
</tr>
<tr>
<td>pH adjustment</td>
<td>-</td>
<td>-</td>
<td>3.36</td>
</tr>
</tbody>
</table>

The medium had a total conductivity of 3660μS/cm which gave a calculated resistance of 273 ohm/cm (full calculations are shown in the appendix). Ammonium sulphate had the largest single effect on conductivity followed by di-potassium phosphate. The other components individually had comparatively minor effects.

Next 0.1M and 0.2M sodium phosphate medium B were prepared (medium B with the potassium salts exchanged for their sodium equivalents). In both cases the conductivity was far higher and the single biggest contributor by far was the di-sodium phosphate (data not shown). The 0.2M phosphate medium was divided in two and used to grow cells with glucose and glycerol as the carbon sources. The addition of the carbon sources lowered the conductivity from 11750μS/cm to 10390μS/cm for the addition of 25ml/l glycerol and 11620μS/cm for 1.22g of glucose per 500ml of medium with resistances of 96 and 86ohm/cm repetitively. The conductivity of the 0.1M phosphate medium was 7420μS/cm (134ohm/cm) (without a carbon source).

Finally low ionic strength medium B was prepared (table 7.4). The 1l of medium was divided in two and glycerol added to one half and glucose to the other for fuel cell trials.
Table 7.4. Composition of low ionic strength medium B and its effect on conductivity. On the addition of 24.55g of glycerol the conductivity was 0.97\(\mu\)S/cm. The addition of 2g of glucose lowered the conductivity to 1.05\(\mu\)S/cm (952380ohm/cm).

<table>
<thead>
<tr>
<th>Component</th>
<th>Weight in grams</th>
<th>pH</th>
<th>(\mu)S/cm x 1000 (cumulative total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised water</td>
<td>-</td>
<td>5.86</td>
<td>0.00</td>
</tr>
<tr>
<td>SL-6 trace elements</td>
<td>5ml</td>
<td>5.63</td>
<td>0.00</td>
</tr>
<tr>
<td>(K_2)HPO_4</td>
<td>0.36</td>
<td>9.21</td>
<td>0.56</td>
</tr>
<tr>
<td>KH_2PO_4</td>
<td>0.11</td>
<td>7.8</td>
<td>0.65</td>
</tr>
<tr>
<td>CaSO_4.2H_2O</td>
<td>0.05</td>
<td>7.73</td>
<td>0.65</td>
</tr>
<tr>
<td>MgSO_4.7H_2O</td>
<td>0.25</td>
<td>7.53</td>
<td>0.89</td>
</tr>
<tr>
<td>(NH_4)_2SO_4</td>
<td>0.2</td>
<td>7.52</td>
<td>1.23</td>
</tr>
<tr>
<td>FeSO_4.2H_2O</td>
<td>0.03</td>
<td>7.49</td>
<td>1.24</td>
</tr>
<tr>
<td>pH adjustment</td>
<td>-</td>
<td>7.26</td>
<td>1.26 then 1.18</td>
</tr>
</tbody>
</table>

No major effect from any of the above was seen in any of the fuel cell runs in terms of increased voltage and therefore power output. The change from potassium to sodium did not seem to have any effect on cell growth although again no formal growth studies were carried out on the cells in this medium.

7.3.2.5. Other carbon sources

As described in chapter four *D. denitrificans* is a fastidious organism that will not grow on most substrates of interest in this project. Glucose was tried as a carbon source on a number of occasions using this organism in the miniature fuel cell at a concentration of 2g/l (4g/l in a few cases). No current was detected and the voltage was very low (data not shown).

However, samples were taken for cytochrome studies (carried out as described in chapter three). The cytochrome assays revealed mixed results with cells cultured using
glucose as the carbon source. In two MFC cultures no sign of c-type cytochromes could be seen (one of these is shown in figure 7.7). In the third, definite evidence of c-type cytochromes was seen (figure 7.8), with major differences between the reduced and oxidised spectra and peaks at 418nm and 552nm. The supernatant glucose concentration was 10.6mmol/l, down from 13.1mmol/l at the start which indicated some modest growth in this culture.
Figure 7.7. Wavelength scans for *D. denitrificans* cultured on glucose in the miniature MFC. Plot a) The red line shows the optical density for cells in the reduced state, the black line shows the optical density for cells in the oxidised state. Plot b) shows the optical density difference (difference spectrum), reduced minus oxidised.
**Figure 7.8.** Repeat wavelength scans for D. denitrificans cultured on glucose in the miniature MFC. Plot a) The red line shows the optical density for cells in the reduced state, the black line shows the optical density for cells in the oxidised state. Plot b) shows the optical density difference (difference spectrum), reduced minus oxidised.

*D. denitrificans* was also tried in the miniature MFC using 35mM acetic acid as the carbon source in medium B (low sulphate). Whilst no current was detected using the micro-ammeter the trial produced the highest voltage output obtained in two years. In addition the anode material was fixed and used for fluorescent *in situ* hybridisation studies as described in chapter six. For these reasons this trial will be described in more detail than others. The cell consisted of E-tek anode (18cm²) and cathode cloth material (24cm²) and a Nafion 424 CEM. The external resistance was provided by the micro-ammeter (110Ω). The anode side was sealed by the use of threaded caps.

The cell number in suspension increased from $1.83 \times 10^7$ to $3.57 \times 10^7$ per ml indicating modest growth. The maximum power density obtained is shown in table
7.2 and the voltage data are shown in figure 7.9.

![Graph showing voltage over time]

**Figure 7.9.** Voltages obtained with time for *D. denitrificans* in the miniature MFC. The carbon source was 35mM acetic acid in medium B (low sulphate). The external load was 110Ω.

Cells in suspension were harvested and tested for c-type cytochromes. The results are shown in figure 7.10. Surprisingly given the higher voltage than the glucose runs (maximum voltage achieved was 3.8mV compared with less than 0.5mV) there was no evidence of c-type cytochromes.
Figure 7.10. Wavelength scans for *D. denitrificans* cultured on 35mM acetic acid in the miniature MFC. Plot a) The red line shows the optical density for cells in the reduced state, the black line shows the optical density for cells in the oxidised state. Plot b) shows the optical density difference (difference spectrum), reduced minus oxidised.

Cells were seen on the anode using the dead stain (figure 7.11) and specific FISH stains (chapter six).
Figure 7.11. *D. denitrificans* cells clustered on the anode visualised using the propidium iodide dead stain. This picture was taken of the anode from the cell culture in the miniature MFC using low sulphate medium B and 35mM acetate as the carbon source at 243x magnification.

7.3.2.6. Other organisms

*R. opacus* was used in the miniature MFC using both 2g/l glucose and 2% (v/v) methanol as the carbon source. No current was detected and the voltages obtained were low (data not shown). However, the glucose was completely utilised, being depleted to less than 1.1mmol/l.

A study using acetate (35mM) was carried out in the miniature fuel cell using *R. opacus*. As for the acetate study using *D. denitrificans* the trial was the most successful using this organism for a long time, the cells on the anode were fixed and studied using fluorescent *in situ* hybridisation. The voltage data are shown in figure 7.12, reaching a high of 1.6mV.
Figure 7.12. Acetate trial using *R. opacus* in the miniature fuel cell. The external resistance of the cell was 110Ω. The concentration of acetate used in medium B was 35mM.

Studies using *R. ruber* and *R. rhodochrous* were also carried out using 44mM and 52mM acetate as the carbon source in the miniature fuel cell. The voltages achieved using these organisms were very low (<1mV) but cells were seen on the anode (chapter six and figure 7.14 below).

Mixed cultures obtained from the Blue Lake (BL002 and BL003) were trialled in the miniature MFC. Slightly better voltages were obtained than BL001-3 all mixed but the voltages obtained were very low (<1mV) using 70mM acetate as the carbon source. Figures 7.14 and 7.15 below show images of the cells on the anode. Catholyte crossed the Nafion membrane and blue colouration was seen on the anode side with BL002. This was due to a damaged CEM membrane caused by hydrogen peroxide used in the sterilisation process.

A single culture of *S. putrefaciens* was also tried using 35mM acetate. Blue colouration was seen on the anode side as with BL002 indicating leakage of the catholyte. The *S. putrefaciens* culture produced the highest power output found in the
microbial fuel studies with the exception of the two initial \textit{D. denitrificans} trials.

7.3.2.7. Other membranes

Other membrane types were tried in the miniature MFC using both \textit{D. denitrificans} and \textit{R. opacus} with glucose at 2g/l and glycerol at various concentrations as the carbon sources. None of the results in terms of voltage output were comparable to the trials described above. Although membrane integrity was achieved with the anion exchange membrane the ultrafiltration membrane allowed catholyte to cross (seen by a colour change to yellow in the anode compartment) and cells to cross (seen in an anode sample under the microscope). The anion exchange membrane gave a higher output under load using \textit{R. opacus} than the CEM did using the same organism and the internal resistance was lower (table 7.1). However, the power output was still very low.

7.3.2.8. Mixed cultures

Three trials using mixed cultures were tried. In one trial cells were collected from sludge around an outside area used for oil chemical engineering research at Heriot-Watt University. The sludge was inoculated into medium B with 49ml/l glycerol as the carbon source. This mixture of cells were inoculated into the medium square MFC but the cells failed to thrive in this medium and no current was detected with minimal voltages in this cell.

In another trial a mixture of \textit{D. denitrificans}, \textit{R. opacus}, \textit{R. ruber} and \textit{R. rhodochrous} was created by mixing an equal volume of cells in normal sulphate medium B with 86mM acetate as the carbon source. After three generations the cells were placed in the miniature MFC with acetate as the carbon source in low sulphate medium B. Again no current was detected, but the voltage was slightly higher (1.85mV) than had been found with the organisms alone with the exception of \textit{D. denitrificans}. Power outputs were lower than with \textit{D. denitrificans} using the internal resistance of this organism to make the power output calculations (table 7.2).
In the third trial an attempt was made to use the oligotrophs collected as detailed in chapter two. 1ml of BL001, BL002 and BL003 culture suspension was diluted into 9ml of low sulphate acetate medium and trialled in the miniature MFC using 70mM acetate as the carbon source. The power output is shown in table 7.1.

7.3.2.9. Anode/cathode materials

Changing from the NCBE material to the E-tek material made no major difference to voltage output or current detection. In fact the best results were obtained with the cheaper NCBE material which has a higher resistance. Resistance measurements using the Fluke multimeter and electrode material over a one centimetre distance established the resistance of the NCBE anode cloth as 33Ω and the E-tek material as 21Ω (Logan, 2008).

7.3.2.10. Open circuit voltage

The open circuit voltage (OCV) was measured using the same multimeter as above without a load at the beginning of the run. For the miniature MFC the OCV varied greatly from +28mV to over +400mV. It was almost never steady before connection of the load, usually falling but sometimes rising. Higher OCV's did not seem to produce higher voltages once the load was attached.

7.3.3. Medium fuel cell

This fuel cell was tried a few times but it was found hard to maintain a seal across the large surface area between the two compartments. One trial involved an attempt at a mixed culture from sludge collected at Heriot-Watt University described above. The culture did not adapt well to glycerol, so was a failure.

7.3.4. Medium cylindrical fuel cell

The medium cylindrical MFC was tried in a variety of combinations of anode material, medium ion exchange membranes, bacteria, air cathodes, carbon sources
and anode/cathode spacings. In every case a lower voltage was achieved than runs in the miniature MFC and no current was detected using the microammeter except in a couple of runs where some brief battery effect was seen. Some of these trials are described below.

7.3.4.1. Anode/cathode material

Varying the type of anode or cathode material or their sizes made no discernible difference. As in the miniature MFC, using an anion exchange membrane did not seem to give a higher voltage output.

7.3.4.2. Performance

In only one trial in the medium cylindrical fuel cell was the voltage in any way comparable with the miniature fuel cell (shown in figure 7.13). In this trial 4g/l glucose was used as the carbon source in conjunction with *R. opacus* and the air cathode that was purchased rather than made *in house*.

![Graph](image)

**Figure 7.13.** Voltage output using the medium fuel cell with *R. opacus* cultured on 2g/l glucose using the air cathode. This air cathode was purchased.
7.3.4.3. Anode and cathode spacing

The modular nature of this MFC did allow studies on cathode and anode spacing to be made. These studies suggested that pinning the cathode and the anode between the gaskets and a J-cloth on either side of the ion exchange membrane so that the distance was minimised did increase the cell voltage very slightly. The distance was reduced from several centimetres to 1 centimetre. This was the established procedure for operation of this MFC when used with ion exchange membranes.

7.3.4.4. Reference electrode studies

A number of studies were carried out using bacteria and the reference electrode in the medium cylindrical MFC. *D. denitrificans* consistently gave voltage differences between the reference and anode below -300mV using a variety of carbon sources, the lowest being -467mV using 68mM glycerol. The only exception was when the OCV was measured across the anode and *in house* air cathode when the voltage rose from +14.4mV over two hours to +106mV, then when left overnight steadied at +138mV.

*R. opacus* gave a minimum voltage of -220mV (glycerol medium B). When re-measured 24 hours later the voltage was -217mV and then rose to +180mV over the course of some hours.

In almost every case the voltage rose rapidly from the point when the connection was made between the reference and the anode. The exception to that is the trial where the lowest voltage of -467mV was obtained. When the OCV between the reference and anode was rechecked 24 hours later the voltage difference remained reasonably steady at -340mV. It should be stated that the maximum potential difference across the MFC in this run was less than 1mV.
7.3.4.5. Use of air cathodes

The modular nature of this MFC allowed not only different air cathodes to be used but also different sized air cathodes. Varying the size between 5cm and 1.5cm diameter made no great difference to voltage output. Note that was the effective area due to the size of the opening at the end of the MFC allowing full contact with the air. The actual size was slightly larger than this so it would stay in place and maintain a seal. Following these initial studies in general however, the smaller air cathode size was used to save material and reduce the internal resistance of the cell.

A difference was found between the bought-in air cathode and the in house air cathode in terms of OCV. For the purchased air cathode the OCV was negative but would start rising towards zero either before cells were added or before a load was placed across the cell contacts. The voltage would then rise towards zero and higher over some hours with cells present. The in house air cathode had a positive voltage to start with.

7.3.4.6. Anaerobic cultures

BL001, BL002, D. denitrificans, and S. putrefaciens were tried in the medium MFC under a nitrogen stream. In the case of BL002 the system used an air cathode and 70mM acetate as the carbon source. D. denitrificans was trialled twice once using 49ml/l glycerol and once using 4g/l glucose. S. putrefaciens gave the highest voltage using 70mM acetate as the carbon source and the OCV was also the highest, at 742mV approximately double that found for the other bacteria tried anaerobically. The internal resistance was measured as described above but was still extremely high (7993Ω). The maximum power output under load and the theoretical maximum power using the OCV are shown in table 7.1.
7.3.4.7. OCV

Like the miniature MFC this varied greatly from a positive voltage of less than 100mV to 742mV. No obvious correlation was seen between the starting OCV voltage and subsequent performance.

7.3.5. SEM images

Images were taken using the scanning electron microscope of anode material collected for the confocal microscopy studies and stored at -20°C, with the exception of *S. putrefaciens* grown under anaerobic conditions which was treated as described in chapter six for confocal staining and microscopy but not used for this purpose due a shortage of time (figures 7.14 and 7.15 below). The *Rhodococcus* strains can be seen as rods growing from a single cell at the centre. What appears to be large clusters of cells can be seen with the other bacteria. For BL003 there are very few cells to be seen but there do seem to be fungal hyphae present. *S. putrefaciens* grown under anaerobic conditions seems to show fewer individual cells, but what appear to be some large clusters are visible.
Figure 7.14. SEM images, the magnification and organism are shown on the image. The scale bar is shown as dots with a value. All the anode material is E-tek cloth.
Figure 7.15. More SEM images of the cells and an image of the weave used in this project. The scale bar is shown as dots with a value.
7.4. Discussion

In this project single and mixed bacterial types not used before in microbial fuel cell experiments have been tried in a variety of microbial fuel cell configurations. *D. denitrificans* did appear at first to be capable of transferring its electrons to the anode in an MFC without the addition of an exogenous mediator. Current was detected by use of the micro-ammeter on two occasions using this organism. Whilst the micro-ammeter was incorrectly set up being in parallel with the 500 ohm load rather than in series, it still detected current through what in effect was an external load of 90.4 ohms. Beyond those two trials the experiments have not been repeatable, in that current has not been directly detected using the micro-ammeter. The voltages obtained have been lower, with a gradual apparent downward trend in the maximum voltage achieved by the system under load. In the subsequent experiments when the same concentration of glycerol was used the voltage was comparable to those when current was detected, then the voltage dropped to a maximum of 2-3mV in the next few studies, then just over a millivolt and then less than a millivolt for many following studies.

There are a number of ways that are used to report current and power output for microbial fuel cells (Logan and Regan, 2006; Rabaey and Verstraete, 2005; Logan *et al.*, 2006; Logan, 2008). In this project the reported power outputs have been normalised to anode area, being reported both as the maximum theoretical power output using the open circuit voltage (OCV) and using the maximum recorded power output calculated using the maximum recorded voltage under an external load in each run. Both the internal resistance (where calculated) and the OCVs have also been reported since these are frequently referred to in the literature. When the maximum power of the systems used in this project have been calculated from the external and internal loads and cell EMF, the power outputs per square metre in general don't compare favourably with those in the literature (Rabaey and Verstraete, 2005; Logan and Regan, 2006; Rabaey *et al.*, 2006; Chang *et al.*, 2006; Kim *et al.*, 2007). The exception in this project was *S. putrefaciens* (purchased as a positive control) grown under aerobic conditions which possibly gave higher power outputs than reported for this organism in the literature as well as the highest power output in this project (Rabaey & Verstraete, 2005). The power
output was also higher than that of *Erwinia dissolvens* (although this set-up was not mediatorless) (Rabaey & Verstraete, 2005). It is unclear whether the $P_{\text{max}}$ values referred to for *S. putrefaciens* are theoretical power values based on the open circuit voltage or under an external load (Rabaey & Verstraete, 2005; Kim et al., 2002). Even if the results given by Kim et al. (2002) are under load the results are comparable; $3.2 \times 10^4$ mW/m$^2$ compared with $1.73 \times 10^4$ mW/m$^2$ in this project. The open circuit voltages found in this project do fall within the ranges outlined in the literature as do some of the current densities normalised for anode area (Shukla et al., 2004). However, in these systems the electron transfer is by mediators and not by direct cell contact on the anode surface (Shukla et al., 2004).

Providing the reaction in the MFC is thermodynamically favourable, using the Nernst equation (equation 1.6 in chapter one) a theoretical upper voltage output (the cell EMF) can be established for an MFC (Logan et al. 2006). In practice even under open circuit conditions where the external resistance is infinity and no current is being drawn, there are internal losses in the MFC meaning the open circuit voltage is lower (Logan et al. 2006). As was alluded to in chapter one the power loss is made up of a number of different contributing factors. These include internal resistance losses due to the medium, anode, cathode, membrane or wiring (ohmic losses). Ohmic losses are the most important (Logan, 2008). Another cause of loss is activation losses due to the requirement for the activation energy needed for redox reactions. These are largest at low currents. Metabolic losses can occur if the anode potential is unsuitable, ideally it should be as negative as possible since the energy gain for the bacteria is higher, but if its too low then fermentation takes place lowering energy production. Finally concentration losses can occur due to concentration gradients in various parts of the system being formed as components are utilised. These mainly occur at high current densities due to limited mass transfer to electrodes, especially protons to the anode. The activation, ohmic and concentration losses in power can be described by a polarisation curve which is voltage plotted against current density (Logan et al. 2006; Logan, 2008). The polarisation curve in figure 7.6 clearly shows these distinct regions of losses. Both the steep slope found in the straightline fit in the ohmic region of loss and the symmetrical power curve suggest a high internal resistance in the MFC (Logan et al.
2006). The internal resistances reported in the literature vary greatly from a few ohms to thousands of ohms (Ieropoulos et al. 2005; Logan, 2008). In this project the internal resistances were uniformly high (table 7.1) and higher than the range reported in the literature.

A very wide ranging series of experiments were undertaken to investigate the inability to repeat apparent early success with *D. denitrificans* and account for the high internal resistance and low power outputs with this and other organisms tried, both using the MFC and molecular biology. One possibility is that the instrumentation was at fault and that the micro-ammeter was giving false readings when current was directly detected. This can be eliminated as a reason, since the same instrument has been used repeatedly since. It has failed to detect any but the most brief amounts of current mostly at the beginning of runs (battery effect) or when a loose connection has occurred across the load midway through an experiment leading to a temporary battery effect. Three of these instruments have been purchased and the only problem found with them has been their interaction with the software used to set them up and retrieve the data. This led to one being replaced under warranty.

Another possibility was that a loose connection during the two runs where current was detected led to a repeated battery effect. This has been seen in this project and occurred when the leads connecting the crocodile clips to the USB adaptor supplied by Laskar worked their way loose from the screw threads on the USB adaptor. However, this would not appear to be the case here. When this phenomenon has been seen previously the swings in voltage have been both more rapid and far higher in value as the voltage rises towards the type of values found in the OCV then falls back to the very low level seen in the MFC run. The graphs shown in this chapter strongly suggest what would be expected when a biological effect is occurring, in that the voltage rises steadily, peaks then falls steadily. No huge swings in voltage were seen and at the same time for levels of current were always measurable except at the start of the second successful trial. In that trial the current then rose from undetectable, steadied for nearly 10 hours with no change seen before a fall occurred. Due to Ohm's law a statistically highly significant correlation would be expected between the voltage and current. Since
they were measured with separate instrumentation this was checked and was found to be the case. All the above suggests a real biological effect.

Microbial fuel cells with bacteria that are capable of transferring electrons directly to an anode give negative voltages against a reference electrode (Kim et al., 2002; Liu and Logan, 2004; Zhang et al., 2006; Fedorovich et al., 2009). The voltages that are reported in the literature vary as do the values when electron transfer can be considered to be taking place between the bacteria and the anode (Kim et al., 2002; Liu and Logan, 2004; Zhang et al., 2006). The cut-off voltage used in this project was -300mV (personal communication Viatcheslav Fedorovich). Values are almost always reported as a single figure but Zhang et al. (2006) did track the voltage between the anode and reference electrode with time. Whilst the voltages against the reference electrode using *R. opacus* and *D. denitrificans* were not read on a regular timed basis for the period the reference electrode was connected to the MFC, as seen by Zhang et al. (2006) the voltage rose towards zero in all cases. However, growing *D. denitrificans* under a nitrogen stream using two different carbon sources failed to give a voltage at which the organism could be considered to be transferring electrons efficiently to the anode.

Alongside these studies a whole series of experiments have been undertaken to look at the MFC system architecture and the effect of components involved in the systems on the cells. Ionic strength of the culture medium in an MFC has been to found have apparently contradictory effects in the literature. Liu et al. (2008) found that increasing the ionic strength using an increase of 200mM in the NaCl concentration increased the power density by 25% between two different otherwise identical media. Fan et al. (2008) also found a consistent increase in power density with increasing phosphate buffer molarity. They claimed to obtain an even higher power density using 200mM bicarbonate compared to the highest phosphate buffer solution (which was also 200mM), although this paper was criticised for not measuring the partial pressure of carbon dioxide in the headspace and availability of the bicarbonate buffer (Fan et al., 2007; Ahn, 2008). In addition Fan et al. (2008) found that lowering the phosphate concentration from 200 to 50mM increased the proportion of the internal resistance due to the medium from 47.3 to 78.2%. Min et al. (2008) also found adding 100mM
phosphate to wastewater led to a power density that was four times higher than wastewater without any phosphate. Feng et al. (2008) found adding either 50mM or 200mM PBS to brewery wastewater increased the power densities by 136-158%. Jang et al. (2004) obtained a significant increase in current by adding 1M NaCl. However, Gil et al. (2003) established that increasing the ionic concentration using salt made very little difference to the maximum value of the current, but salt or water only gave a low current output. The conductivity changes made to the medium here had little effect. It is also unlikely that the medium itself was acting as an electron sink. None of the metals were at either a high enough concentration to compete with the anode or in most cases the right oxidation state. Sulphate can act as an alternative electron sink but its near total removal from the culture medium made no difference to the results. Its reduction to sulphide could in any case produce a mediator capable of transferring electrons to the anode (Ieropoulos et al., 2005). There was no nitrate (another potential electron sink) present in the medium (Rabaey and Verstraete, 2005).

A remote possibility is that the anode material itself may be toxic to the bacterial cells. Whilst there are no reports of this in the literature, it was decided to compare growth with an without anode material present. The cultures without the anode material grew to a higher cell density than with material present. However, this is more likely to be related to oxygen transfer than toxicity of the material since the maximum growth rate in the culture with the anode material was almost the same (0.011hours⁻¹ compared with 0.012hours⁻¹) and the maximum cell density achieved was about 12% lower.

Another concern is the higher concentration of other cations in the anode compartment, compared to protons which can transfer across the CEM to the cathode compartment. CEM’s are not proton specific but allow the passage of other cations (Rozendal et al., 2006). This lowers the pH on the anode side raising the anode potential and potentially killing the micro-organisms. In addition the raised pH in the cathode compartment lowers the cathode potential (Rozendal et al., 2006). The ions also bind to the sulphonate groups in the membrane inhibiting the transfer of protons (Kim et al., 2007). Studies in chapter five suggest D. denitrificans can survive, grow and even raise the pH of medium it is in by metabolising acetic acid, so potential pH
alterations in the microbial fuel cell are probably not a barrier to current production.

Other researchers have found electrode spacing to be more critical than than the type of membrane or medium used (Kim et al., 2007). The operation of MFC’s with CEM’s is further complicated by the fact that these membranes work better at acid pH than at alkaline pH and fewer of the other competing ions are carried across the CEM (Biffinger et al., 2008). The ionic concentration was found to have little obvious effect on power density in this project. Whilst individual components of the medium were identified that had the greatest effect on conductivity, composition changes certainly did not restore the power outputs for D. denitrificans to the levels obtained in the first few experiments with glycerol. The resistance of the medium was quite high (273ohm/cm) but lowering or raising it had no obvious effects on power output. In early runs the pH of the medium B was not adjusted and as has been mentioned above is generally in the region of 6.8, although there was one trial using medium at a pH of 6.48. In later runs the pH was adjusted to 7±0.1. Where measured the pH during runs dropped to between pH 6-7 although there were runs where the pH was lower (pH 5.3-5.5) in the miniature fuel cell using D. denitrificans. This was after mixing the cell suspension, so the pH gradient would have been higher. There is no indication that the starting pH had any effect at all and in the cultures below pH 6 small motile cells could be seen indicating the presence of live healthy cells. There is an additional reason to believe the CEM membrane was not responsible for the lack of power obtained, that is that single chamber air cathode systems were tried without any more success.

Kim et al. (2007) found that anion exchange membranes gave the highest power output and coulombic efficiency compared with other types of membranes in two chamber systems. This was also confirmed by another group (Sleutels et al., 2009). A number of different membranes were tried in both the medium and miniature fuel cells. The anion exchange membrane did seem to have a marginally higher performance in the trial carried out. This was probably due its lower internal resistance. The miniature MFC had a much higher internal resistance when fitted with cation rather than anion exchange membranes using R. opacus but ultimately the slightly increased performance did not fundamentally alter the MFC’s performance with this organism. Ultrafiltration membranes had been tried by Kim et al. (2007), although they gave the lowest voltages
and power outputs. The ultrafiltration membrane did not seem to have the tensile strength to cope with tension required when tightening up the bolts on the MFC and cells clearly transferred across it. Although tears could be seen in the CEM materials, the main problem was found to be a lack of grease on the gaskets. Adding fresh grease each time the cells were dismantled solved the problem in that catholyte was not then seen in the anode compartment.

Almost all the studies made using all the organisms were aerobic, for reasons of practicality; the department does not have anaerobic growth facilities apart from access to a nitrogen line. In principle oxygen should be rapidly metabolised by the cells with carbon dioxide filling the head-space, although oxygen can still diffuse from the cathode chamber that must be oxygenated (Liu and Logan, 2004; Biffinger et al., 2008). Although generally MFC’s in the literature are reported as being operated under anaerobic conditions there are some instances where aerobic conditions have been used. Generally these have been found to have lower power outputs where facultative anaerobes such as Shewanella have been used (Kim et al., 2002; Ringeisen et al., 2007). In two cases bacteria collected from anaerobic sewage sludge were used (Mohan et al., 2007; Oh et al., 2009). These gave contradictory results. Mohan et al. (2007) found increased power output under aerobic conditions, Oh et al. (2007) found the opposite, in that after oxygen was removed power output was restored, again using mixed cultures in sewage sludge. The system design in these two cases was different. Mohan et al. (2007) used a single chamber MFC with an air cathode, while Oh et al. (2007) constructed a conventional two chamber MFC with a cathode exchange membrane separating the two chambers.

Surprisingly the results for S. putrefaciens grown under aerobic conditions in this project were much better than under anaerobic conditions, at least under load (table 7.1). There are a number of possible explanations for this finding. It might be that until the oxygen in the headspace gas is used up and replaced by carbon dioxide the bacteria outgrow anaerobic cultures of the same organism, giving a higher number of bacteria in the MFC which switch to anaerobic respiration using the anode and hence a higher power output. It is possible that the bacteria might be sharing part of the oxidative
electron transfer pathway with an anaerobic respiration pathway, meaning the presence of oxygen is less detrimental to power output. This explanation seems unlikely since it does not fit in with what has been found, that this strain of *Shewanella* is incapable of producing current under aerobic conditions (Kim *et al.*, 2002). The final and most likely explanation is that of the difference in system architecture. The anaerobic culture was carried out in the larger MFC. This suggests in some way the system architecture in this larger MFC was defective. The only major difference was that of distance between the anode and cathode which was higher in the medium MFC. In the miniature MFC anode spacing was very hard to control or measure and the electrodes were simply suspended in the anode and cathode chambers, which were pinned directly together. In the medium MFC although the two chambers were pinned together there was a gap (barrel) between them which meant the closest possible distance was 1cm while in the case of the miniature MFC the distance between the electrodes was in the order of 2-3mm. It seems unlikely that the presence of oxygen made any difference with the other bacteria tried. Firstly, the best results using *D. denitrificans* were found using the miniature MFC with its openings on the anode side very permeable to the air. In the initial experiments these were sealed using small sterilised pieces of foam bung. Latterly the workshop at Heriot-Watt University machined threads into the holes so they could be sealed using steel closures, trapping CO₂ produced by metabolism. This made no difference to the power output, nor did the use of the larger MFC that always had threaded screw caps that could be sealed. On some occasions opening the caps on both the MFC’s used in this project would release gas which implies CO₂ build-up, in addition in some trials using the miniature MFC the liquid level on the cathode side would rise, presumably due to the gas on the anode side pushing the CEM towards the cathode side. Lastly, both two chamber and one chamber systems were tried under a nitrogen stream and whilst the stream was difficult to control it seems likely at the flow rates tried that the chamber was anaerobic. Air cathodes can transfer oxygen into the MFC chamber, and two j-cloths was used to stop this (Fan *et al.*, 2007).

Liu and Logan, (2004) found higher output in systems using air cathodes. This is apparently due to the absence of a proton exchange membrane. The contributions made by the resistances of the membrane, the electrolytes and the cathode lead to higher
internal resistances and hence lower power output in two chamber MFC's (Fan et al., 2008). In addition the air cathode is more efficient at transfer of oxygen at the cathode exchange membrane (Fan et al., 2008). Again in this project this was not found to be the case, the highest voltages being obtained in conventional two chamber systems. The air cathode purchased had a very high resistance and it may be that the in house air cathode was defective, possibly the glue did not allow the electrons, protons and the oxygen to meet.

Cheng et al. (2006) and Jang et al. (2004) investigated spacing. In general reducing the spacing between either the electrodes or the anode and the air cathode reduced ohmic resistance and increased power output (Cheng et al., 2006; Logan, 2008). If the electrodes are too close then the power output will fall despite lower resistance (Logan, 2008). However, Cheng et al. (2006) and Jang et al. (2004) obtained slightly more complicated results than this. Jang et al. (2004) found that decreasing the distance in a two chamber MFC gave a higher voltage but lower power density. Cheng et al. (2006) obtained a lower power density when the distance was reduced from 2cm to 1cm in a single chamber MFC. Altering the spacing made no major difference to the power outputs in this project although it was generally kept as low as possible by pinning the anode and cathode material between the j-cloth and the perspex on each side. Logic suggests this is the correct course of action due to the resistance of the medium (Logan, 2008).

Lower voltages and absolutely no current were obtained with the other bacteria tried in this project. In the case of the Rhodococcus species this is not surprising since they are not capable of reducing iron and there is clear link between iron reduction and the ability to directly transfer electrons to the anode in an MFC (Chaudhuri and Lovley, 2003; Pham et al., 2003; Back et al., 2004; Holmes et al., 2004; Holmes et al., 2006; Lovley, 2006). In addition Rhodococcus species are Gram-positive with no outer membrane meaning the biology of the mechanisms used in direct transfer of electrons must be different from those found in Gram-negative bacteria. However, this does not preclude these organisms from current production in microbial fuel cells. Gram-positive organisms have been found to work in microbial fuel cells either by indirect
transfer of electrons (via excreted redox shuttles) or direct transfer (Milliken and May, 2007; Liu et al., 2009; Marshall and May, 2009; Logan & Regan, 2006). One organism, C. butyricum strain EG3, has been reported able to reduce soluble iron (Milliken and May, 2007). Clearly the Rhodococcus strains tried here, although related to the Corny bacterium used by Liu et al. (2009), are not capable of indirect transfer either. The voltages measured against the reference electrode confirmed these findings.

It was much more disappointing that three separate isolates from a high iron environment were incapable of transferring electrons directly or indirectly via natural mediators to the anode. A specific attempt was made to select bacteria that could both grow on acetate (since it is a non-fermentable fuel) and tolerate iron for the reasons outlined above (Lee et al., 2003; Choo et al., 2006; Lovley, 2006). Other groups have isolated bacterial consortia or individual bacteria from sewage sludge, starch wastewater or even yoghurt or manure (Gil et al., 2003; Lee et al., 2003; Cheng et al., 2006; Kim et al., 2007; You et al., 2007; Scott and Murano, 2007). Some electrochemically active bacteria have been isolated from soil, seawater and freshwater (Rabaey, 2006; Kim et al., 2007; Hou et al., 2009). No other groups have taken the approach taken in this project of attempting to enrich bacteria from an iron rich environment, perhaps because iron is seen as ubiquitous in ecosystems and bacteria play a vital role in the cycling of iron between its different oxidation states (Lovley, 1993; Lloyd et al., 2003). Phylogenetic analyses of bacteria on anodes in MFC's have found diverse groups of bacteria from all taxa (Logan and Regan, 2006; Choo et al., 2006; Kim et al., 2007; Kim et al., 2007; Hou et al., 2009). This makes it more surprising that after selecting bacteria using the enrichment method outlined in chapter two and having collected a mixture of 3-4 different bacteria per isolate, there was no success as far as current production was concerned. Even under anaerobic conditions no appreciable voltage was obtained.

The number of bacteria seen on the anode material was by eye an order of magnitude lower than others have found in the literature. Most SEM or confocal images show or describe a completely confluent biofilm (Bond and Lovley, 2003; Liu and Logan, 2004; Reguera et al., 2006; Milliken and May, 2007; You et al., 2007;
Lanthier et al., 2008; Zuo et al., 2008; Nevin et al., 2009). The exceptions are the studies of Mohan et al. (2007); Kim et al. (2006) and Scott and Murano, (2007) which have relatively few bacteria present. Whether there are few bacteria present in this project because they are not capable of electron transfer or due to some problem with the system architecture is unknown. In any case a low number of bacteria present on the anode does not seem to preclude power production. Ren et al. (2011) followed the time-course of six identical MFCs inoculated with the same wastewater sludge over six weeks. Over this time the MFC’s anodes were analysed including with confocal microscopy using live/dead staining. Some current and power production was seen at three days when the images look comparable to those obtained with this project. The power density reached a maximum at two weeks when the image shows the anode surface being very far from confluent (Ren et al., 2011). Finally the electron micrograph images of cells on the anode show no sign of nanowires (Reguera et al., 2006; Gorby et al., 2005). These are very distinctive (Gorby et al., 2005).

Kim et al. (2002) found the coulombic yield rose on increasing the initial bacterial concentration used to inoculate the MFC, as did Park and Zeikus (2002). Increased temperature has also been found to raise power outputs (Min et al., 2008; Feng et al., 2008). These variations were not examined in this project, but would have been had consistent positive results been obtained.
Chapter 8. Conclusions and further work

8.1. Conclusions

In this project a number of bacteria have been found to be able to reduce transition metals. No known strain of *Dechloromonas* or *Rhodococcus* has been reported in the literature that can reduce many of the transition metals tried in this research. The exceptions are chromate and iron. Reports of chromate resistance and reduction have been found in other strains of *Rhodococcus* (Trivedi *et al.*, 2007; Henne *et al.*, 2009). *Dechloromonas aromatica* RCB has had its complete genome sequenced and some genes seem to be involved in chromate resistance, if not actual reduction. Iron reduction has been reported in a strain of *Dechloromonas* (Kim *et al.*, 2006)

In the microbial fuel cell, *D. denitrificans* apparently produced small amounts of measurable current. However, the results were not reproducible. Various strains of *Dechloromonas* have been found on the anodes in MFC's as part of mixed consortia (Choo *et al.*, 2006; Kim *et al.*, 2006; Kim *et al.*, 2007). They may be only indirectly involved in electron transfer by breaking down substrates to metabolic by-products that can be utilised by the other cells actually involved in electron transfer (Lee *et al.*, 2003; Kim *et al.*, 2007). In addition the reference electrode gave a low enough potential difference between it and the anode to suggest this organism was transferring electrons to the anode. *D. denitrificans* can also reduce soluble iron (III) to iron (II) unlike *R. opacus* which is incapable of iron reduction. Other strains tried in this project including *R. opacus* gave no indication of any ability for direct or indirect transfer of electrons to an anode. There was insufficient potential difference between the reference electrode and the anode with *R. opacus* to indicate any ability to transfer electrons. *Rhodococcus* strains are Gram-positive bacteria unlike *Dechloromonas* which are Gram-negative; in principle, therefore, they cannot have the outer membrane proteins required for electron transfer, although direct transfer of electrons to an anode by Gram-positive organisms is possible (Park *et al.*, 2001; Marshall and May, 2009).

It seems likely that the bacteria chosen were incapable of electron transfer to the anode in an MFC, however, there is some evidence from the studies carried out in the
project that the MFC itself might be defective. As has been mentioned previously the anode/reference potential difference was sufficiently negative with *D. denitrificans* to suggest electrons were transferring to the anode, a finding not replicated with *R. opacus*. In addition *S. putrefaciens* gave a very high OCV under anaerobic conditions and possibly higher power outputs than have been reported in the literature using this organism. While it is unknown whether this strain can transfer electrons to the anode in an MFC, this genus is capable of electron transfer in an MFC (Park and Zeikus, 2002; Kim *et al.*, 2002). A variety of *Shewanella* strains were tried by Bretschger (2008) including *S. putrefaciens* with positive results. The internal resistance was found to be very high in both the MFC's used with all the bacteria tested. The polarisation curve plotted for *D. denitrificans* and shown in the appendix has three distinct regions (Logan, 2008). Very small drops are seen in the activation energy and mass transfer regions of the curve and a steep gradient implying large losses (high internal resistance) in the ohmic region of the curve. This suggests either the bacteria have no ability to transfer electrons or there is something at fault with the system set-up. The polarisation curves plotted for other bacteria were all similar (data not shown). There is no definitive way with the work that was done in this project to differentiate between the ohmic losses due to the bacteria simply not being able to transfer electrons to the anode and electrical resistance losses due to defective system architecture. The fact that *S. putrefaciens* gave some power suggests it is the latter, as it also had the lowest internal resistance.

There is one remaining possible explanation for the poor results with the bacteria chosen and that is the attachment of the cells to the anode. Both the fluorescent studies and the SEM images confirmed that there were very few bacteria on the anodes compared with most other studies (Bond and Lovley, 2003; Liu and Logan, 2004; Kim *et al.*, 2006; Reguera *et al.*, 2006; Mohan *et al.*, 2007; Milliken and May, 2007; Scott and Murano, 2007; You *et al.*, 2007; Lanthier *et al.*, 2005; Zuo *et al.*, 2008; Nevin *et al.*, 2009; Yi *et al.*, 2009). This may be simply because the bacteria are not capable of transferring electrons for reasons described earlier and don't grow well; however, it may be due to a lack of ability of the cells to attach to the anode, although in examples above where there was little colonisation the MFC’s still worked (Kim *et al.*, 2006; Mohan *et al.*, 2007; Scott and Murano, 2007). There is very little information in the literature on
attachment of cells to an anode and the subsequent formation of a biofilm (Logan, 2008). However, there must be a mechanism for cell attachment to anode materials and if so that implies some cells cannot manage to attach (Biffinger et al., 2007; Logan, 2008). Bacteria need to condition any surface they attach to and this is facilitated by secretion of a polymer (glycocalyx) which protects them and occurs under low nutrient concentrations (Biffinger et al., 2007). Biffinger et al. (2007) studied the power from a biofilm (only using S. oneidensis DSP10) and found it lower than with the planktonic cells present. Bond and Lovley, (2003) found the opposite in that the planktonic cells of G. sulfurreducens were not responsible for the power output. This fits in with characteristics of this organism which whilst motile needs to attach at least temporarily to Fe(III) to be able to reduce it (Bond and Lovley, 2003). Yi et al. (2009) isolated a strain of G. sulfurreducens from an MFC with enhanced power production characteristics compared to the wild type cells used it to inoculate the MFC. The new strain was more likely to attach to graphite or glass than the original strain, but formed thinner biofilms with a lower number of c-type cytochromes (Yi et al., 2009).

Whilst there is little in the literature about attachment to the anode in microbial fuel cells there is however some research on attachment to insoluble transition metals in the environment (Burns et al., 2010). By introducing knock-out mutations in S. oneidensis, attachment of this organism to insoluble iron (III) was severely impaired but reduction of iron (III) was not (Burns et al., 2010). Attachment took place via a versatile class of enzymes called serine proteases. These enzymes have a variety of functions from adhesins, to maturation of cell surface toxins, or protein breakdown (Burns et al., 2010), and are separate from the c-type cytochromes involved in electron transfer. What would be interesting to know is whether the outer membrane c-type cytochromes responsible for electron transfer can also act as attachment factors, which would make biological sense. There is some evidence that this is indeed the case (Lower et al., 2008; Burns et al., 2010). Lower et al. (2008) used a phage display library to screen polypeptides for binding to haematite. They found that the sequence that gave the best simulated binding was found in a protein encoded by a DNA sequence adjacent to the omcA and mtrC genes, in addition a similar sequence was found in these proteins in S. oneidensis (Lower et al., 2008). It is known that these proteins can bind to FeO3 (Lower et al.,
2008). Adhesion of the cells to the iron is via van de Waal’s or electrostatic charge (Burns et al., 2010; Lower et al., 2008). The carbon electrodes would presumably be uncharged so the attachment must be via hydrophobic interaction.

*D. denitrificans* is mainly planktonic when the cell cultures are young, but as the culture matures almost all the cells become non-motile, possibly due to nutrient exhaustion (Biffinger et al., 2007). Biffinger et al. (2007) found that limiting the nutrient concentration at least at first helped the biofilm formation, however Park and Zeikus (2002) found that increasing the concentration of lactate up to 250mM led to a higher power output. Both groups were using *Shewanella* species. Varying concentrations of glycerol were used in this project without any effect on power production.

Planktonic cells have predominantly been found to work in MFC's, but probably require extra-cellular shuttles (Lanthier et al., 2008; Zuo et al, 2008). It is unknown whether *D. denitrificans* has any such shuttles, although testing for one type (phenazines) was negative. Although planktonic cells work in MFCs, the power output seems better in systems where they attach (Bond and Lovley, 2003; Biffinger et al., 2007). Flagella can also help cells attach (Yi et al., 2009). In early trials the anode material by chance was primed with cell debris, which improves attachment, since the same anode material was reused a number of times with an alcohol wash for sterilisation purposes, which is unlikely to remove the cell debris (Biffinger et al., 2007). *Pseudomonas* that do not produce mucoid slime are not able to bind to hexadecane and *Streptococcus mutans* was found to lose its ability to stick with repeated sub-culturing (Rosenberg, 1984). de Carvalho et al. (2009) found changes in *Rhodococcus erythropolis* cell surface hydrophobicity and charge depending on what substrate the cells were grown on.

Potentially the planktonic *D. denitrificans* cells are failing to attach for some reason as they mature and some change in the cell surface morphology with repeated sub culturing led to the loss of the ability to attach seen in the early trials, as described previously in chapter one (Choi et al., 2003). Tests on this organism (chapter four) to
see if its cell surface was more adapted to organic solvents were contradictory. A stock slope of a glycerol (less adapted) culture which had been stored at +4°C for some months was tried in the miniature MFC, but still showed the negative MFC results seen with more extensively glycerol adapted cultures. As can be seen in the SEM pictures in chapter seven the *D. denitrificans* cells did not seem to attach in large numbers. Whilst the organism lacks the *omcA* and *omcB* genes, there are clearly multiple methods of attachment and electron transfer available (Myers and Myers, 2001; Viamajala *et al.*, 2002; Lanthier *et al.*, 2008; Lower *et al.*, 2008; Yi *et al.*, 2009; Burns *et al.*, 2010). The SEM images show other cell types attached more readily than *D. denitrificans*, particularly the non motile *Rhodococcus* species, although more *D. denitrificans* cells are seen present on the confocal images of the anode. Some limited motility of *R. opacus* has been seen as part of this and another PhD project. A very small proportion of *R. opacus* cells do seem to be motile. This limited motility doesn't help this organism in an MFC however.

What was more surprising due to the importance of iron reduction to electron transfer in MFC's was that the bacterial consortia isolated from the high iron environment of the slate quarry were incapable of transferring electrons to the anode in a microbial fuel cell. There are precedents for this in the literature. Johnson, and McGinnis (1991) found approximately only 40% of cell types isolated from a high iron environment were capable of reducing iron (III). The lack of power production in the MFC does agree with the findings on transition metal reduction using these isolates. This strongly suggested tolerance rather than reduction of high concentrations of almost all the transition metals tested. The possible exception for transition metal reduction is chromate, where two of the three Blue Lake consortia did apparently reduce it. Some cells in the BL002 consortium were able to reduce soluble iron (III) ferricyanide at very low concentrations. A blue colouration was seen on the anode side of the miniature MFC when it was run after sterilising the compartments with approximately 3% (v/v) hydrogen peroxide in 70% (v/v) ethanol which evidently damaged the Nafion 424 CEM. However, at higher concentrations in flasks growth took place without the formation of any blue precipitate indicative of iron (II) ferrocyanide (chapter three). This implies that the cyanide is in some way inhibiting the metal reduction pathway,
which would suggest that soluble iron reduction involves the electron transport system (Myers et al., 2000; Lovley, 2003; Chang et al., 2006). The same happened with D. denitrificans although the concentrations required of ferricyanide were much higher before inhibition of reduction took place.

All the bacteria tried seemed to be missing crucial genes required for indirect or direct electron transfer (chapter six). Whilst a limited set of PCR primers were tried, a sufficient number of related genes were targeted to give an indication of their presence or absence, even allowing for the degeneracy in the genetic code. S. putrefaciens did test positive for one of the mtr genes from S. onедensis MR-1 suggesting this approach may have some validity. The FISH data are in agreement with the PCR data in almost every case with the exception of some of the genes in the Blue Lake consortia. The staining was often stronger in these mixed consortia and specific staining was seen that aligned with the dead cell staining as well as some showing no match. This strongly suggests that the hybridisation staining was specific and cells stained with specific probes were genuine. The gene cymA was found in the Blue Lake consortia tested by FISH. This gene is strongly associated with omcA and omcB and indeed these genes were present as well (Carpentier et al., 2005; Gao et al., 2008). Whilst genes for nanowires such as pilA were not tested for there is no sign of these distinctive bacterial appendages using SEM (Gorby et al., 2006).

It would appear that the ability to transfer electrons to the anode in a microbial fuel cell is both more complicated and less common than an overall study of the literature would suggest. Whilst bacteria capable of iron and manganese reduction are linked with the ability to transfer electrons to anodes in MFC’s, strains that have lost the ability to reduce iron are still capable of electron transfer to the anode. In addition bacteria isolated from mixed consortia on anodes that can reduce iron cannot transfer electrons to the anode in an MFC (Bretschger et al., 2007; Richter et al., 2007; Zuo et al., 2008). The results from S. oneidensis MR-1 suggest that different genes are involved in electron transfer to different insoluble electron acceptors with a high degree of genetic redundancy (Bretschger et al., 2007). The work in this project would appear to support this in the organisms studied. The scientific view as to whether genes such as omcB,
are critical to electron transfer in MFC’s has changed several times (Kim et al., 2008; Holmes et al., 2006; Nevin et al., 2009). Added to this new genes such as omcZ are being discovered with apparent relevance to direct electron transfer (Nevin et al., 2009). Single cell types have been found to work in MFC's although mixed consortia are more common (Fedorovich et al., 2009). One possibility for the failure of the Blue Lake consortia compared with consortia of cells used from waste-water and sewage sludge by other workers is that the number of different bacteria in waste-water is much higher to start with. Therefore there is a far higher chance that some bacteria present will have the ability to transfer electrons to the anode in an MFC.

The project was unable to link the biodegradation of VOC’s to energy production for a number of reasons. Firstly, one of the main bacteria used (D. denitrificans) would not grow on the volatile alcohols concerned. Second, bacteria of the other main bacterial genus (R. opacus) used that would grow on the alcohols seems even worse at electron transfer. Lastly the cylindrical perspex was damaged by the alcohols chosen, making the MFC made from this material unusable with these solvents. Kim et al. (2007) found little electricity production using the one VOC tried in this project (methanol).

8.2. Future work

There is much work to be done on the Blue Lake isolates which were collected in the final year of this project. At least some of the bacteria in these mixed consortia have some genes of relevance. It would have been good to have made pure cultures and tried them in the MFC including using organic solvents such as VOC's since they were found to grow well on lactate and glycerol. It would also be of interest to identify the species of bacteria.

Further genetic work is required. The number of primers used needs to be expanded for each gene and new genes are being discovered with apparent relevance on a regular basis.

Electrical impedance spectroscopy would have provided more information about the
internal resistance of the cell and whether the largest contribution was ohmic, kinetic, or transport limited (Fan et al., 2007).
Chapter 2. Initial growth studies

Figure 1. Relationship established between cell count and turbidity for four of the bacteria used in this project. Graph a) shows the data for *D. denitrificans*, b) for *R. rhodochrous*, c) for *R. opacus* and d) *S. oneidensis*. The graphs show the equations for the straightline fit, the $R^2$ value and the Pearson correlation coefficient.
Table 1. Maximum growth rate data for bacterial growth studies used in a statistical comparison of methods of calculating them. Note most of these growth studies are on experiments carried out in chapters 2, 3 and 4. Some were unsuccessful attempts at chromate growth studies where only the cultures without chromate present grew, these are not referred to in the text but the growth data were used in this comparison. The results of this comparison are shown in figure 2.

<table>
<thead>
<tr>
<th>Experiment number description and location</th>
<th>&quot;semi-log method&quot;</th>
<th>four parameter gompertz</th>
<th>Logistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. denitrificans cultured on medium 830 (pg 55)</td>
<td>0.271</td>
<td>0.137</td>
<td>0.135</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium 1 (pg 55)</td>
<td>0.207</td>
<td>0.101</td>
<td>0.369</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B only (pg 55 &amp; 100)</td>
<td>0.212</td>
<td>0.129</td>
<td>0.109</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B with Cr(VI) (pg 92)</td>
<td>0.119</td>
<td>0.069</td>
<td>0.062</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B with Cr(VI) 93</td>
<td>0.019</td>
<td>0.017</td>
<td>0.016</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B only (pg 94)</td>
<td>0.217</td>
<td>0.181</td>
<td>0.136</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B with Cr(VI) (pg 94)</td>
<td>0.072</td>
<td>0.042</td>
<td>0.040</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B with Cr(VI) (pg 94)</td>
<td>0.022</td>
<td>0.076</td>
<td>0.052</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B only</td>
<td>0.086</td>
<td>0.085</td>
<td>0.094</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B only</td>
<td>0.067</td>
<td>0.066</td>
<td>0.045</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B without Cr(VI)</td>
<td>0.100</td>
<td>0.043</td>
<td>0.050</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B without Cr(VI)</td>
<td>0.121</td>
<td>0.071</td>
<td>0.073</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B with glucose (pg 173)</td>
<td>0.124</td>
<td>0.053</td>
<td>0.010</td>
</tr>
<tr>
<td>D. denitrificans cultured on medium B with glycerol (pg 173)</td>
<td>0.039</td>
<td>0.024</td>
<td>0.025</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B with glucose (pg 177)</td>
<td>0.089</td>
<td>0.251</td>
<td>0.057</td>
</tr>
<tr>
<td>R. rhodochrous cultured on medium B with ethan-1,2-diol (pg )</td>
<td>0.060</td>
<td>0.029</td>
<td>0.033</td>
</tr>
</tbody>
</table>
Figure 2. Statistical comparison of the established semi-log method and the 3 parameter gompertz regression method of calculating μmax.
Chapter 3. Transition metal reduction and c type cytochromes

Initially a quadratic relationship was established between the chromate standards and the absorbance at 540nm. However, the relationship was linear to above the 200μM standard and use of this was found to be unnecessary with culture samples. A linear fit was found with an $R^2$ value in excess of 0.99 with the top standard at 200μM. Samples having an optical density above that of the top standard were diluted to bring them into the standard curve range.

The vanadium assay was carried out over the same range and was found to have a good linear fit with an $R^2$ value in excess of 0.98.

Copper (II) standards did not give a straight-line fit in either Cu(II) assay. Instead they were fitted with a non-linear logfit model. This gave an $R^2$ value in excess of 0.96 in the diphenylcarbazide assay and 0.98 in the alizarin red assay. The copper (I) assay using BCS gave very significant linear fit $R^2>0.99$ over a very wide range of standard concentrations. The ferrozine assay for iron (II) gave a linear fit with an $R^2$ value typically in excess of 0.99. The molybdenum blue standards gave a straight-line fit with an $R^2$ value in excess of 0.9813.

Studies using the diphenylcarbazide assay

Chromate (VI) gives a purple-mauve coloured complex with diphenylcarbazide. This fades very gradually. Both vanadium and copper gave very light yellow-brown solutions. Copper diphenylcarbazide assay solutions also have a dark brown precipitate and the colour formed was unstable.

No QC’s were found to be outside two standard deviations of the mean for the chromate assay. Inter-assay variation was 8.3% and 14.8% for two different sets of quality controls. Intra-assay assay variation was less than 10% in all assays. A surprising finding was that the value of the QC dropped with increased storage time, even at -20°C the value of the QC dropped over a period of weeks. It was unrelated to
cell activity since an attempt was made to stop any cellular effect by filtering the samples through a 0.2 micron filter. In addition cell free samples stored for a month gave low results compared to the value expected. Thus a fresh QC had to be prepared and samples analysed reasonably quickly. In figure 3) the decline in value can be seen, before this decline the inter assay variation was less than 5% for 7 assays. QC values for the copper (II) by diphenylcarbazine assay varied widely which is one reason why it was abandoned.

Figure 3. Quality control charts for the chromate diphenylcarbazine assay. Graphs a) and b) show different QC batches derived from cell cultures. b) shows the drop off in QC values due to chemical change. The inter-assay variation was calculated at 8.3% for a) and 14.8% for b), intra-assay variation was <10% in both cases. The blue line shows the mean chromate (VI) QC value, the dark dashed line is two standard deviations from the mean value and the lighter grey line one standard deviation.

The assay passed its parallelism test for chromate (VI). The results for two tests of quality controls are shown in table 2. The QC dilution curve failed its statistical test on non-similarity, it is therefore behaving in a similar manner to the standard curve (Reeve, 2000).
**Table 2.** Analysis of variance results for the Cr(VI) parallelism test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Critical F value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-similarity</td>
<td>3</td>
<td>0.0422</td>
<td>0.0141</td>
<td>6.8</td>
<td>215.7</td>
<td>0.2724</td>
</tr>
<tr>
<td>Residual</td>
<td>1</td>
<td>0.0021</td>
<td>0.0021</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For diphenylcarbide assays involving other transition metals the assumption was made that the chemistry in the assay was similar for other transition metals, so that a pass would be replicated. In addition no dilution of samples was made to bring them into range of the assay for other metals. Since very few assays were run for the copper or vanadium diphenylcarbide assay, no attempt at parallelism or creating a QC control chart was made. However, both the intra-assay and inter-assay variation were greater than 10% for the V(V) assay. This is possibly a function of the low absorbance values at 320nm.

**Copper (II) assay by alizarin**

The alizarin assay for copper (II) gave a plum coloured complex, however it also cross reacted with iron (II). For this reason the assay was abandoned.

**Copper (I) assay**

Copper (I) gives a bright orange coloured complex with BCS. The intra-assay variation was less than 5% and the inter-assay variation for the total copper (I) was 3.5% (figure 4). More assays were run using this assay than any other except for chromate (VI) so the assay was checked for parallelism as above and passed (table 3.2).
**Figure 4.** Quality control charts for the copper (I) BCS assay. Graphs a) and b) show QC batches derived from cell cultures. b) shows the total Cu(I) after addition of the reducing agent. The blue line shows the mean copper (I) QC value, the dark dashed line is two standard deviations from the mean value and the lighter grey line one standard deviation.

**Table 3.** Analysis of variance results for the copper (I) assay parallelism test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Critical F value</th>
<th>probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-similarity</td>
<td>3</td>
<td>0.037</td>
<td>0.012</td>
<td>0.603</td>
<td>9.28</td>
<td>0.656</td>
</tr>
<tr>
<td>Residual</td>
<td>3</td>
<td>0.062</td>
<td>0.021</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Iron (II) assay**

Iron (II) gives a bright purple coloured complex with ferrozine reagent. Both the intra-assay and inter assay variation were greater than 10%.
Chapter five. The influence of secondary metabolites on cell growth

Number of moles of 2-propenal and 3-HPA.

density of 2-propenal = 0.839 g/ml

Molecular weight of 2-propenal = 56.06 g

therefore the 7.5ml of 2-propenal by weight is 7.5ml x 0.839g/ml = 6.2925g

no. of moles of 2-propenal = 6.2925/56.06 = 0.1122

The purity is 95% so;

= 0.1122 x 95/100 = 0.1066 moles

in the reaction mixture

Since this reaction mixture had a total volume of 33+10+7.5 or 50.5ml this is equivalent to 0.1066 x 1000/50.5 = 2.1106 moles/litre 2-propenal. At the end of the reaction period the volume had reduced to 47ml.

A series of standards were prepared from 0.444M to 7.106M of the 2-propenal. The 2-propenal peak was identified at a retention time of 11.4 minutes at a flow rate of 11.4ml/minute. The 3-HPA and two of the standard traces are shown in figure 5.
Figure 5. HPLC traces for two 2-propenal standards and synthesised 3-HPA sample. Trace a) shows the 0.88M 2-propenal standard, b) the 7.106M standard and c) the 3-HPA sample.
Figure 6. The standard curve for the 2-propanal assayed by HPLC. The R² value for the fit was 0.967. The equation of line was used to determine the 2-propanal concentration left in the 3-HPA sample is shown.

A mean peak area of 1.0982 gave a concentration of unreacted 2-propanal left in the 3-HPA sample as 0.270M. The stoichiometry of reaction is 1:1 (Hall and Stern, 1950) so assuming no evaporation losses the 3-HPA concentration is

\[ \text{concentration} = 2.1106 - 0.270 = 1.846 \text{M}. \]

3.3ml was added to each culture flask so the concentration was \( \frac{3.3}{1000} \times 1.846 \text{M} \)

\[ = 6.09 \text{mM} \]

In the total volume 103.3ml this is \( 6.09 \text{mM} \times 1000/103.3 \) = 58.9mM

Calculations of pH due to the release of ethanoic acid in the bacterial medium.

The highest concentration of ethanoic acid over the culture period as determined by GC was used, this was 42.2mmol/l
Appendix

Acid dissociation constant for ethanoic acid

\[ K_a = 1.8 \times 10^{-5} \]

The dissociation of ethanoic acid is and the concentrations of each component are;

\[ \text{CH}_3\text{COOH} \leftrightarrow \text{CH}_3\text{COO}^- + \text{H}^+ \]

(0.0422-x) \hspace{1cm} x \hspace{1cm} x \text{ all in mol/dm}^3

\[ K_a = [\text{CH}_3\text{COOH}][\text{H}^+]/[\text{CH}_3\text{COO}^-] \]

or

\[ 1.8 \times 10^{-5} = x^2/(0.0422-x) \]

solving the root of the quadratic equation gives H+ and using the formula

\[ \text{pK}_a = -\log_{10}[\text{H}^+] \]

\[ \text{pH} = 2.16 \]

Using exactly the same method for propanoic acid concentration of 12.1mmol/l and a dissociation constant of 1.3 \times 10^{-5} gives a pH of 1.86.

Chapter 7. Microbial fuel cell studies

Calculation of internal resistance of the microbial fuel cell.

This was carried out using a method outlined in Microbial Fuel Cells by Bruce E. Logan using resistance loads between 110\(\Omega\) and 3290110\(\Omega\). The polarisation curves were plotted as outlined by Logan using the data shown in table 4 below.
Table 4. The raw data for *D. denitrificans* cultured in the miniature MFC on glycerol.

<table>
<thead>
<tr>
<th>load</th>
<th>mV</th>
<th>current</th>
<th>mA/cm²</th>
<th>power (W)</th>
<th>mW/m²</th>
<th>volts</th>
</tr>
</thead>
<tbody>
<tr>
<td>110</td>
<td>1.14</td>
<td>0.0000104</td>
<td>0.0004836</td>
<td>0.000000012</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
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<td>9.41</td>
<td>0.0000094</td>
<td>0.0004377</td>
<td>0.000000088</td>
<td>0.04</td>
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</tr>
<tr>
<td>10128</td>
<td>82.18</td>
<td>0.0000081</td>
<td>0.0003792</td>
<td>0.000000667</td>
<td>0.31</td>
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<tr>
<td>50910</td>
<td>253</td>
<td>0.0000050</td>
<td>0.0002322</td>
<td>0.000001257</td>
<td>0.59</td>
<td>0.25</td>
</tr>
<tr>
<td>99910</td>
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<td>0.000000943</td>
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<tr>
<td>996110</td>
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<td>0.0000052</td>
<td>0.000000040</td>
<td>0.02</td>
<td>0.36</td>
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</table>

**Figure 5.** Polarisation and power curve for *D. denitrificans* in the miniature MFC. The data in black show the polarisation curve (left axis) and in red the power curve (right axis). Note the OCV voltage was added into the data for plotting purposes. The $R^2$ value for the polynomial curve is 0.953.

Two methods were used to calculate the internal resistance from these data. The first method took the slope from the middle of the three slopes shown in figure 5 above. First using the polarisation data.
Appendix

Slope = -1180.89 (the sign is ignored) and the anode area = 2.8 x 4 x 2 = 22.4 cm² (doubled since there are two sides exposed to the organism)

\[
\text{internal resistance} \quad = \quad \Delta E/\Delta I
\]

Therefore internal resistance \( = 1180.9 \text{ (V/mA/cm²)} \times \left( \frac{1}{22.4} \text{ (cm²)} \right) \times 1000 \text{ (mA/A)} \)
\[
= 52718.3 \Omega
\]

Second using the power curve data. The current density at the highest point on the curve was determined by fitting a second order polynomial to the entire data set. By differentiating this equation with respect to \( x \), the current density was obtained at that point, where the slope of the curve is zero.

\[
Y = -0.00208901 + 4552x + -9.69097x \times 10^6x^2
\]

\[
dy/dx = 4552 - 19381940x
\]

\[
0 = 4552 - 19381940x
\]

\[
x = 0.0002349 \text{ mA/cm}^2
\]

Using this value in the quadratic expression \( y \) was calculated, that is the peak power density.

\[
= 0.5324 \text{ mW/m}^2
\]

Changing this from total output from the system.

\[
\text{Power} = \text{Power}_{\text{anode}} \times \text{area}_{\text{anode}}
\]

\[
= 0.5324 \text{ mW/m}^2 \times 22.4 \text{ cm}^2 \times (\text{W/10}^3 \text{ mW}) \times (\text{m}^2/10^4 \text{ cm}^2)
\]

\[
= 1.14 \times 10^{-6} \text{ W}
\]
Appendix

\[ R_{\text{int}} = P/I^2 \]

\[ R_{\text{int}} = 1.14 \times 10^{-6} \, \text{W} \left[ \frac{1}{0.0002349 \, \text{cm}^2/\text{mA}} \times (1/22.4 \, 1/\text{cm}^2) \times (10^3 \, \text{mA}/\text{A}) \right]^2 \]

\[ R_{\text{int}} = 41170\Omega \]

All the other internal resistances were calculated using these methods.

**Calculation of power outputs**

\[ P = IE_{\text{emf}} \quad \text{1)} \]

Where \( E_{\text{emf}} \) is the cell emf under an external load \( R_{\text{ext}} \). By measuring the voltage and using the relationship,

\[ I = \frac{E_{\text{emf}}}{R_{\text{ext}}} \quad \text{2)} \]

and substituting 2) into the first equation 1) for power

\[ P = \frac{E_{\text{emf}}^2}{R_{\text{ext}}} \quad \text{3)} \]

The power output related to the total power in the system is a function of the internal resistance, the internal resistance \( R_{\text{int}} \) can be considered as being in series with the external resistance. Hence 3) becomes;

\[ P = \frac{E_{\text{emf}}^2}{(R_{\text{ext}} + R_{\text{int}})} \quad \text{4)} \]

This equation 4) gives the maximum power theoretically possible.

The maximum power under load is given by the following expression

\[ \left( \frac{E_{\text{emf}}}{R_{\text{ext}} + R_{\text{int}}} \right) \left( R_{\text{ext}} / (R_{\text{ext}} + R_{\text{int}}) \right) \]

\[ = \frac{E_{\text{emf}}^2 \cdot R_{\text{ext}}}{(R_{\text{ext}} + R_{\text{int}})^2} \quad \text{5)} \]
substituting OCV for cell emf in 5) gives the most useful relationship for maximum power in an MFC (Logan, 2008).

\[ = \frac{OCV^2_{\text{emf}} R_{\text{ext}}}{(R_{\text{ext}} + R_{\text{int}})^2} \quad 6) \]

The values obtained using equations 5) and 6) are normalised to anode area by dividing by the anode area then multiplied by 1000 to put the units in mW and by another 10,000 to convert from cm² to m².

**Medium conductivity**

Calculated as follows;

3660μS/cm is 3660 x 10⁻⁶ Siemens

\[ = 0.00366 \text{ Siemens} \]

The resistance is the reciprocal of this.

\[ = 1/0.00366 = 273 \Omega. \]
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