Development of an Immobilized *Nitrosomonas europaea* Bioreactor for the Production of Methanol from Methane

A Thesis Presented for the Degree of
Master of Engineering in
Chemical and Process Engineering

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Abstract

This research investigates a novel approach to methanol production from methane. The high use of fossil fuels in New Zealand and around the world causes global warming. Using clearer, renewable fuels the problem could potentially be reduced. Biomass energy is energy stored in organic matter such as plants and animals and is one of the options for a cleaner, renewable energy source. A common biofuel is methane that is produced by anaerobic digestion. Although methane is a good fuel, the energy is more accessible if it is converted to methanol. While technology exists to produce methanol from methane, these processes are thermo-chemical and require large scale production to be economic. *Nitrosomonas europaea,* a nitrifying bacterium, has been shown to oxidize methane to methanol (Hyman and Wood 1983). This research investigates the possibility of converting methane into methanol using immobilized *N. europaea* for use in smaller applications.

A trickle bed bioreactor was developed, containing a pure culture of *N. europaea* immobilized in a biofilm on ceramic raschig rings. The reactor had a biomass concentration of 7.82 ± 0.43 g VSS/l. This was between 4 – 15 times higher than other systems aimed at biologically producing methanol. However, the immobilization dramatically affected the methanol production ability of the cells. Methanol was shown to be produced by the immobilized cells with a maximum production activity of 0.12 ± 0.08 mmol/gVSS.hr. This activity was much lower than the typical reported value of 1.0 mmol/g dry weight.hr (Hyman and Wood 1983). The maximum methanol concentration achieved in this system was 0.129 ± 0.102 mM, significantly lower than previous reported values, ranging between 0.6 mM and 2 mM (Chapman, Gostomski, and Thiele 2004). The results also showed that the addition of methane had an effect on the energy gaining metabolism (ammonia oxidation) of the bacteria, reducing the ammonia oxidation capacity by up to 70%. It was concluded, because of the low methanol production activity and the low methanol concentrations produced, that this system was not suitable for a methanol biosynthesis process.

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Contents

1 Introduction

Sustainable development is a philosophy, incorporating social, economic and environmental perspectives. The United Nations' Department of Economic and Social affairs, Division for Sustainable Development defines Sustainable Development as, "Development that meets the need of the present without compromising the ability of the future generation to meet their own needs" (U.N. Department of Economic and Social Affairs 2004).

One of the pressing issues in establishing a sustainable living environment is energy. Throughout last (and this) century, the world has relied heavily on fossil fuels as an energy source. Fossil fuels are deposits of ancient organic remains. Types of fossil fuels include coal, oil and natural gas. There are several problems with the large scale use of these fuels, the most prominent being the emissions when they are combusted. Some fossil fuels when burned produce sulphur dioxide and nitrogen dioxide, albeit minor constituents. However, the large scale of fossil fuel use has produced significant quantities in the atmosphere causing acid rain. The carbon dioxide, nitrogen dioxide and some volatile organic compounds produced are greenhouse gases and are thought to be the major contributors to the increase in global temperature (global warming) (Ministry of Economic Development 2004). An increase in the global temperature could lead to catastrophic problems, such as: melting of the polar ice caps and a subsequent rise in sea levels and altered weather patterns. Figure 1.1 shows the world's heavy reliance on oil, gas and coal compared with other, cleaner energy options.

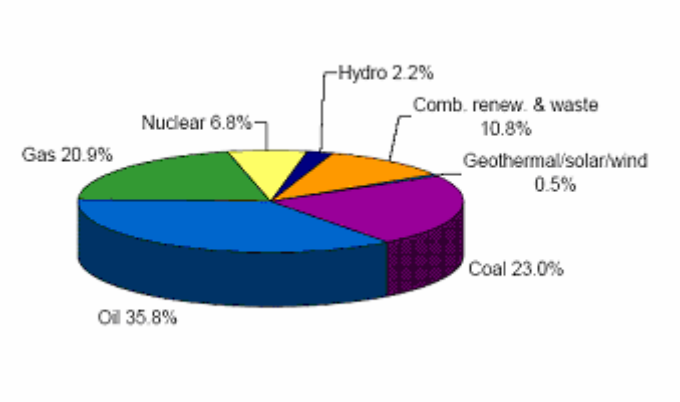

Figure 1.1: Share of total primary energy supplies in 2001 for the world (International Energy Agency 2004)

In an effort to encourage the use of cleaner energy sources and reduce the global emissions of greenhouse gases, an international agreement, the Kyoto Protocol, was established in 1997. New Zealand committed to this agreement on 19 December 2002 (New Zealand Climate Change Office). The document requires New Zealand to reduce its carbon dioxide equivalent emissions to the levels released in 1990, a reduction of 401.7 x 10^6 tonnes carbon dioxide equivalent (t CO_2e) (Ministry for the Environment 2005). This reduction will be implemented during the period 2008-2012. The Protocol uses carbon credits to compare greenhouse emissions and carbon dioxide sinks. If, for example, a country has more sinks than emissions it would have spare carbon credits, which it could sell to countries in the opposite situation. Recent predictions have estimated New Zealand's emissions (including reduction generated by sinks) to be above its target with an excess somewhere in the range of 11.3 x 10^6 to 62.6 x 10^6 tonnes carbon dioxide equivalent (t CO_2e) (Ministry for the Environment 2005). As well as the detrimental environmental effect, New Zealand could be forced to buy a significant number of carbon credits at great cost.

The world's amount of crude oil and natural gas are suspected by some as being limited. The proven amount of oil reserves and natural gas according to the World Energy Council website (2004) is 1.05 x 10^{12} barrels at the end of 1999. According to the same source at the end of 1999 the rate of consumption by the world was 71 x 10^6 barrels/day. At the current rate of production the fossil fuels could be produced for 40 years and gas for 55 years (World Energy Council 2004). Energy demand is also increasing at an average rate of 1.9 % per year (world average) (Ministry of Economic Development 2004) indicating the end of a once plentiful energy supply As the total amount of oil reserves is depleted, extraction becomes more difficult, limiting the rate of production of fossil fuels. Oil production appears to be likely to peak in the first part of this century. The peak in oil production combined with the increase in demand will mean an end to cheap and easily available oil (Ministry of Economic Development 2004). Supply of natural gas in New Zealand is also of concern. The supply from the Maui gas field, which over recent decades has contributed up to 80% of the natural gas in New Zealand, is also declining (Ministry of Economic Development 2004). Therefore, it is not only the environmental problems associated with large scale use of fossil fuels but also the supply of these fuels that raises concerns for developing a sustainable living environment.

New Zealand, like the rest of the world, relies heavily on fossil fuels. New Zealand used approximately 533 pJ of energy per year, in 2003 (Ministry for the Environment 2005). Almost half (263 pJ) of the energy used in New Zealand is in the transport sector which relies almost totally on fossil fuels (Ministry of Economic Development 2004). Transport is therefore one of the most significant greenhouse gas emitting sectors and accounts for 45 % of New Zealand's carbon dioxide emissions. The other sectors are residential and industrial (52 pJ and 220 pJ respectively).

In order to solve these environmental and supply problems, other cleaner and less vulnerable energy solutions are required. Renewable energy is energy that is essentially inexhaustible (sustainable) due to its short life cycle. These renewable fuels are synonymous with clean energy alternatives. Using renewable energy sources reduces the emission of greenhouse gases associated with the use of fossil fuels. It should be noted that fossil fuels are also renewable, but the time it takes for these fuels to be produced is extremely long and therefore can be exhausted.

Many of these renewable energy alternatives are directly or indirectly related to energy from the sun. Major renewable energy options are hydro, geothermal, solar, photovoltaic, wind, ocean and biomass energy (Carless 1993). Various types of these renewable energy sources are commercially available and are currently used. Of New Zealand's total energy use, approximately 140 pJ are from renewable resources (Ministry of Economic Development 2004). The most predominantly used type is hydropower which accounts for approximately 84 pJ and 60% of New Zealand's electricity generation (Ministry of Economic Development 2004). There is significant room for improvement and there are substantial renewable resources to achieve this. These resources include more hydropower, wind, geothermal and biomass energy (Ministry of Economic Development 2004).

This research involves the development of process technology for the production of a renewable energy source, biomass energy. Biomass energy is energy stored in organic materials such as plants and animals. Examples of biomass energy sources include: wood; vegetative, sugar, oil, and starch crops; municipal and process wastes; animal products and wastes; and algae. Biomass energy can be used directly or converted into biologically derived fuels (biofuels) such as ethanol, methanol, methane (biogas) or biodiesel. Biomass energy offers two major advantages in the reduction of greenhouse gas emissions. Firstly, biomass is

an energy substitute for fossil fuels; and secondly the release of carbon dioxide during combustion of the biomass is offset by the sequestration of the carbon dioxide by plants during growth. Another advantage of biofuels over other renewable energy sources is that many can be used directly in internal combustion engines. Biomass also has lower sulphur content in it than some fossil fuels. Therefore the combustion of biomass fuels does not contribute to acid rain to the same extent. However particulate matter emission is still significant high (Golob and Brus 1993).

An important biomass energy source (biofuel) is biogas. Biogas is a mixture of gases: the major species are methane and carbon dioxide. The process that produces biogas is called anaerobic digestion and is the biological degradation of organic material in an oxygen free environment (anaerobic environment). The composition of the gas is usually in the range of 45-80% methane depending on composition and origin of the waste (Wheatley 1990). Anaerobic digestion occurs in three stages. The stages are: hydrolysis, acidogenesis and methanogenesis. Figure 1.2 shows the order, general groups of substrate and products of the process. The potential energy from biogas from organic waste sources in New Zealand is in the order of 1.4 pJ (Efficiency and Conservation Authority 2005).

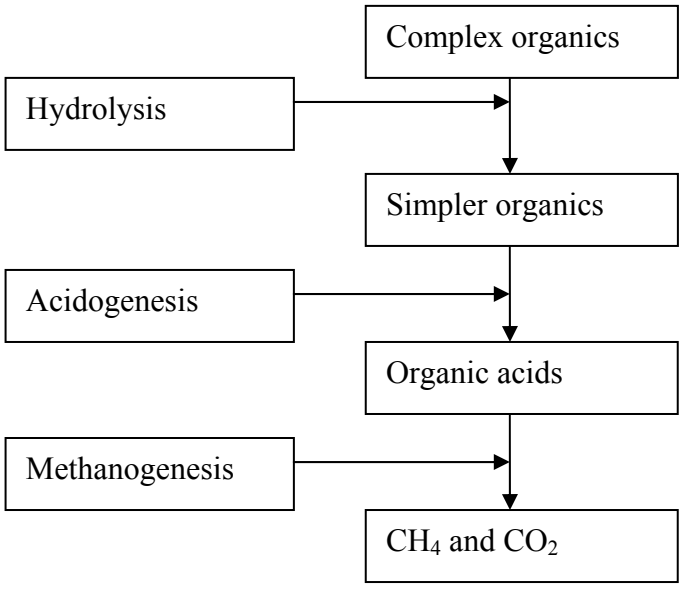

Figure 1.2: Schematic diagram of the major biological processes of anaerobic digestion.

Methane is a good fuel and biogas being a renewable fuel has environmental advantages. However, being gas it has some disadvantages when compared to liquid fuels (Section 2.1). By converting the biogas into a liquid, the fuel would be more versatile. Therefore, the

overall project is to develop a process converting organic waste biologically into a liquid fuel. The process would utilize anaerobic digestion for conversion of organic waste into methane and biomass (for use as a fertilizer). Following this, the methane can either be used directly as a fuel or converted into a liquid fuel (methanol) (Fig. 1.3)

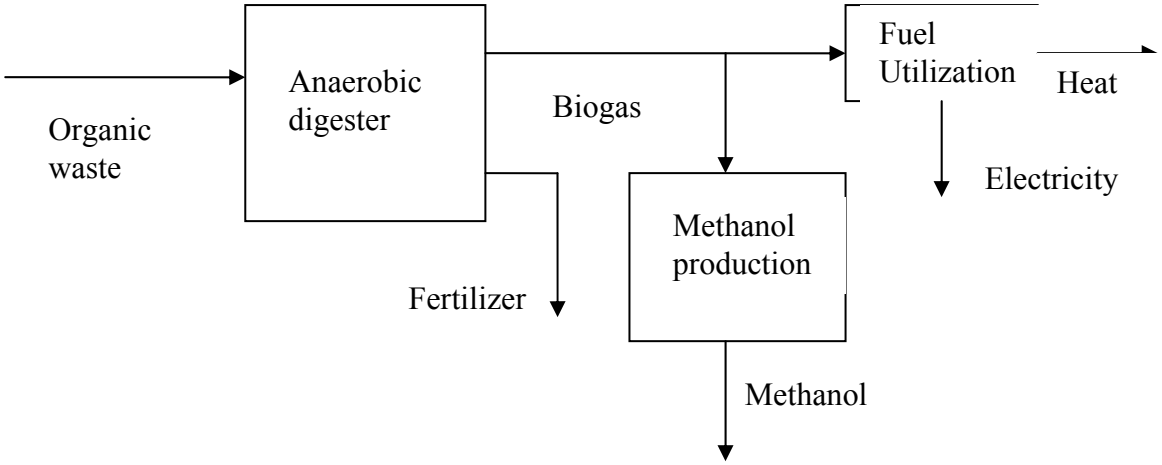

Figure 1.3: The general vision of an organic waste to liquid fuel process (Chapman, Gostomski, and Thiele 2004)

The research for this thesis focuses on the biological conversion of the methane to methanol using the nitrifying bacterium, *Nitrosomonas europaea*. Presently industrial methane to methanol conversion processes are thermochemical and are heavily dependent on economies of scale. The objective of this research is to develop an immobilized bacteria reactor for the biological conversion of biogas to methanol. *N. europaea* can co-metabolise methane to methanol. A biological conversion route would make the overall process economically viable for smaller applications. Once developed, the performance and methanol production capacity will be compared to reports of similar processes.

The following chapters introduce a variety of topics relevant to this research such as methanol production, nitrification and cell immobilization.

2 Methanol Production

2.1 Advantages of Methane to Methanol Conversion

The biogas produced by anaerobic digestion as mentioned (Section 1) consists predominantly of methane. It can therefore be used directly as a fuel for the production of heat or the co-generation of heat and electricity. Compared with methanol, methane has higher energy content on a per mole basis. However, because methanol is a liquid at ambient temperature and pressure, it has several advantages for storage, transport fuel and distribution. It can therefore be desirable to convert methane into methanol.

The enthalpies of combustions for methane and methanol are -802.62 kJ/mol and -638.49 kJ/mol respectively. This shows methane with a calorific advantage over methanol and is reflective of methanol being a product of partially oxidized methane.

$$CH_{4(g)} + 2O_{2(g)} \Rightarrow CO_{2(g)} + 2H_2O_{(g)} \qquad \Delta H°_{298} = -802.62 \text{ kJ} \qquad (1)$$

$$CH_3OH_{(l)} + \frac{3}{2}O_{2(g)} \Rightarrow CO_{2(g)} + 2H_2O_{(g)} \qquad \Delta H°_{298} = -638.49 \text{ kJ} \qquad (2)$$

However, since methanol is a liquid at ambient conditions and methane is a gas, methanol is easier to store than methane. The energy density of biomethanol is 15.8 MJ/l, and that of a similar fuel to biogas, natural gas, 38.2 x 10^{-3} MJ/l (Sims 2002). Therefore, larger volumes of methane would be needed to achieve a specific energy output. To avoid large volumes when storing methane, the gas is either liquefied or compressed. These processes are energy-intensive and lead to increased energy use and capital expenditure. Compressed gases are also very hazardous because of the high pressure; therefore storage and transport of methanol is simpler than for methane. Methane is also known to be a potent greenhouse gas (Ministry of Economic Development 2004). By converting it into methanol the risk of inadvertently adding to the greenhouse problem through leaking is lessened. However, leakage of methanol into the environment would not be without its own problems.

2.2 The Chemical Synthesis of Methanol from Methane

Methane can be chemically converted to methanol either directly through oxidation or indirectly by firstly converting the methane into syngas (CO + H_2) as an intermediate. The indirect route is described by Eqs. 3 - 6 (Zhang, He, and Zhu 2003):

$$CH_4 + H_2O \Rightarrow CO + 3H_2 \qquad \Delta H°_{298} = 206 \text{ kJ/mol} \qquad (3)$$

$$CH_4 + CO_2 \Rightarrow 2CO + 2H_2 \qquad \Delta H°_{298} = 247 \text{ kJ/mol} \qquad (4)$$

$$CH_4 + 1/2\,O_2 \Rightarrow CO + 2H_2 \qquad \Delta H°_{298} = -35 \text{ kJ/mol} \qquad (5)$$

$$CO + 2H_2 \Rightarrow CH_3OH \qquad \Delta H°_{298} = -90.7 \text{ kJ/mol} \qquad (6)$$

Eqs. 3 - 5 describe the production of the intermediate, syngas. Eq. 6 shows the conversion of the syngas to methanol. These reactions proceed in the presence of a catalyst (Skrzypek, Sloczynski, and Ledakowicz 1994; Zhang, He, and Zhu 2003). The production of the syngas from methane, also know as methane reforming, is an energy intensive process, requiring high temperatures (700 – 900 °C) and pressures (Laosiripojana and Assabumrungrat 2005). These extreme process conditions also mean a large capital cost. Methanol production via this process is therefore subject to economies of scale, requiring large processes for economic feasibility (Chapman, Gostomski, and Thiele 2004). Current conversion efficiencies for methanol production from syngas, according to Sims (2002) are 40 – 50 % conversion. This method of methanol production is the traditional commercial process. Presently it is common practice to use fossil based fuels, such as coal gasification or natural gas for methanol production.

Alternatively, the direct method for methanol production is the controlled partial oxidation of methane, such that there is an accumulation of methanol but only a limited accumulation of further oxidation products. The direct oxidation of methane to methanol is an exothermic process whereas the indirect method is endothermic. This indicates a lower energy input requirement for the direct oxidation (Eq. 7). Although the direct method has potential advantages, it is not commonly used commercially because of its low methanol yield. The low yield is due to the formation of other products that are more thermodynamically

favourable. Some of the possible products are; formaldehyde, carbon monoxide, carbon dioxide, ethane or ethene.

$$CH_4 + 1/2O_2 \Rightarrow CH_3OH \qquad \Delta H°_{298} = -128 \text{ kJ/mol} \qquad (7)$$

2.3 Biological Routes for Methanol Production

As opposed to chemical conversion, biological conversion of methane to methanol is possible. No known microbes naturally accumulate methanol as a metabolic endproduct. However there are two biological methods producing methanol as an intermediate product (Hyman and Wood 1983; Furuto, Takeguchi, and Okura 1999). These methods are: methane oxidation by methanotrophs or by nitrifying bacteria.

Methanotrophic bacteria can accumulate methanol in the presence of inhibitors (Furuto, Takeguchi, and Okura 1999; Lee *et al*. 2004). Methanotrophs oxidize methane to methanol through the enzyme, methane monooxygenase (MMO). This enzyme is not stable when purified therefore whole cells have been used for methane oxidation. The catabolism of methanotroph then uses the enzyme methanol dehydrogenase (MDH) to oxidize the methanol to formaldehyde, followed by a sequence of enzymatic oxidations to CO_2. In order to cause the accumulation of methanol, MDH is chemically inhibited. This method of methanol production has been researched by a variety of groups, including research on immobilized cell systems (Yu *et al.* 1998; Mehta, Mishra, and Ghose 1991)

Immobilization of cells offers advantages in biological processes (Section 5.0). Yu *et al.* (1998) and Mehta *et al.* (1991) investigated methanol production by methanotrophs in immobilized cell systems. Yu *et al.* (1998) immobilized cells on activated carbon investigating the effect the immobilization had on the cells' activity for the development of a biocatalyst. Mehta *et al.* (1991) immobilized cells with cross-linked cellulose for the development of a methanol biosynthesis process. Their results are shown in Table 2.1 which compares the activity to other results. The results show the activity is higher for the immobilized cells, indicating successful immobilization of the cells. Even though methanotrophs have shown good methanol production activity, both as free cells and immobilized cells, the process still has a drawback. Investigations have found that MMO is inhibited by methanol and have reported a decrease in its activity with increasing methanol concentration (5mM is inhibitory) (Takeguchi *et al*. 1997) with methanol accumulation

ceasing at 7.7 mM (Lee *et al*. 2004). Therefore the use of methanotrophs for a methanol biosythesis process is limited by the concentration of methanol .

Table 2.1: Methanol production by methanotrophic bacteria

Species	**Methanol Production Rate**	**Cell State**	**Reference**
Methylosinus trichosporium OB3b	1.52 μmol/h.mg. cells	Free cultures	(Takeguchi *et al*. 1997)
Methylosinus trichosporium OB3b	3.17 μmol/h.mg. cells	Suspended culture – semi continuous reactor	(Furuto, Takeguchi, and Okura 1999)
Methylosinus trichosporium	8.6 μmol/h.mg. cells	DEAE-cellulose linked cells *	(Mehta, Mishra, and Ghose 1991)
Methylomonas sp Z201	4.06 μmol/h.mg. cells	Immobilized by adsorption on activated carbon supports *	(Yu *et al.* 1998)

* Batch experiments

The other method, which is the focus of this research, uses the nitrifying bacterium *Nitrosomonas europaea* to co-oxidise methane to methanol using the ammonia mono-oxygenase system (Hyman and Wood 1983) (see Sec. 4.4). It was decided that this cometabolic pathway had potential for the following reasons (Chapman and Thiele 2003):

- The genome of *N. europaea* has recently been fully sequenced making the species amenable to potential directed genetic modification (Chain *et al.* 2003)
- *N. europaea* has been previously applied in biotechnological processes aimed at the bioremediation of waste water (Ely *et al.* 1997)
- *N. europaea* is a resilient environmental organism with a well understood physiology and is able to survive under starvation conditions (Prosser 1989)
- *N. europaea* is a chemolithoautotrophic bacterium, therefore it is unable to utilise methanol as energy source and uses CO_2 as the preferred carbon source (Prosser 1989).

Thus there is no need to add metabolic inhibitors to the culture in order to prevent methanol utilisation as an energy source.

3 Nitrification

3.1 Background

Nitrification is a biological, environmental process that is the stepwise oxidation of ammonia to nitrate via nitrite. It is a step in the nitrogen cycle in aquatic and terrestrial ecosystems. The nitrogen cycle describes the environmental recycling of nitrogen. Nitrate, ammonia and molecular nitrogen can be incorporated into cells as organic forms of nitrogen (proteins and amino acids). Upon degradation of these organic nitrogen compounds, ammonia is released in a process called deamination. The ammonia can subsequently be oxidized to nitrite and then to nitrate by nitrifying bacteria. This nitrate can be reduced to molecular nitrogen by denitrification. Fig. 3.1 is a simplified diagram of the nitrogen cycle (M^{c}Veigh 1998).

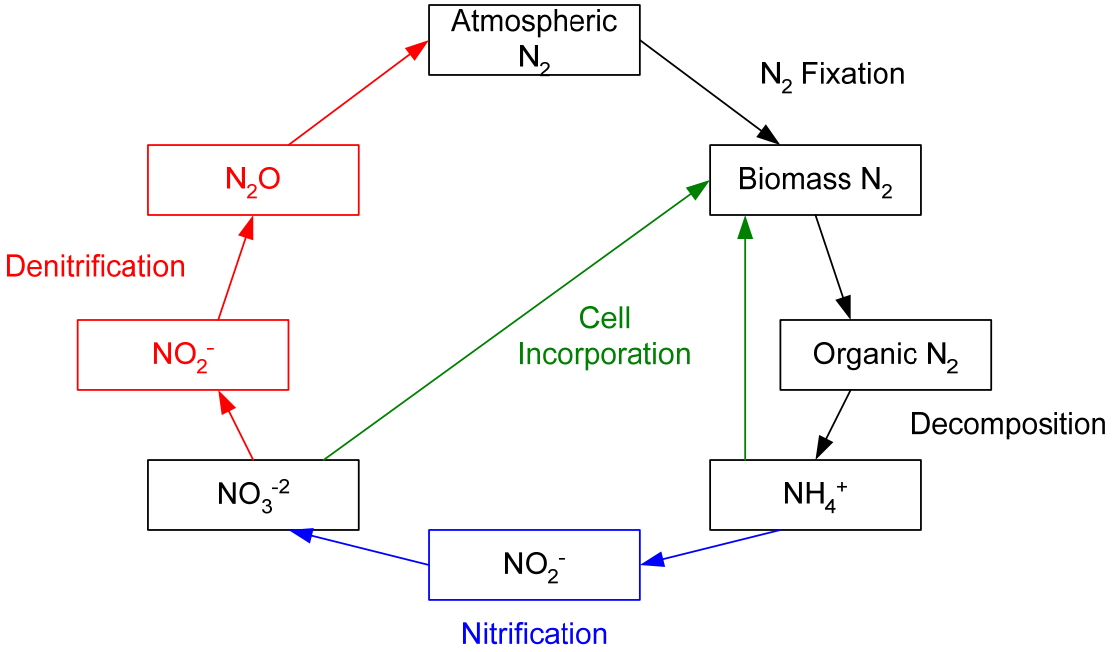

Figure 3.1: Simplified nitrogen cycle

A wide variety of bacteria can be involved in nitrification. They can be both heterotrophic bacteria or autotrophic. As this work investigates an autotrophic nitrifying bacterium, therefore all mention of nitrification assumes autotrophic nitrification.

This autotrophic nitrifying bacterium is a gram negative, aerobic bacterium. Nitrification is a two step oxidation process catalysed by two different families of bacteria. The first step is the oxidation of ammonia to nitrite by ammonia oxidizing bacteria, typified by the genus *Nitrosomonas*. Nitrite oxidising bacteria which oxidise nitrite to nitrate are typified by the

genus *Nitrobacter*. Ammonia oxidation is the slowest and therefore rate-limiting step in nitrification. Equations 8 - 10 describe the chemical reactions (Prosser 1989).

Ammonia oxidation

$$NH_4^+ + \frac{3}{2}O_2 \Rightarrow NO_2^- + H_2O + 2H^+ \qquad \Delta G° = -270 \text{ kJ/mol} \qquad (8)$$

Nitrite oxidation

$$NO_2^- + \frac{1}{2}O_2 \Rightarrow NO_3^- \qquad \Delta G° = -80 \text{ kJ/mol} \qquad (9)$$

Overall nitrification reaction

$$NH_4^+ + 2O_2 \Rightarrow NO_3^- + H_2O + 2H^+ \qquad \Delta G° = -350 \text{ kJ/mol} \qquad (10)$$

Inorganic nitrogen (in any form) is harmful if emitted into a receiving body of water. Nitrogen is an essential nutrient for biological growth and can damage waterways through excess unwanted growth (Gerardi 2002). Releasing ammonia into a receiving body of water has additional environmental impacts, such as: it is highly toxic to aquatic life (Richardson 1997) and has an unpleasant odour. It is therefore essential to remove ammonia from waste water. Nitrification is a widely-used biological method for the removal of ammonia from both domestic and industrial wastewater. There are various factors that influence the growth of the nitrifying ability of the bacteria (Table 3.1). It is advantageous to consider these factors when developing a nitrifying system to ensure an effective process.

Table 3.1: Factors influencing nitrification rates.

Influencing Factors	**Effect**
pH	Nitrification is pH dependent with an optimum in the range 7 – 9 (Henze 1995)
Dissolved oxygen concentration	Predominantly aerobic system requiring oxygen and is sensitive to changes in dissolved oxygen concentrations (Henze 1995).
Temperature	Optimum temperature range 30 – 35°C (Henze 1995)
BOD concentration	High BOD concentration leads to competition for oxygen with faster growing heterotrophic bacteria
Light	Activity of the nitrifying bacteria decreases in the presence of light (Shears and Wood 1985).

3.2 Ammonia Oxidation

This research uses an ammonia oxidising bacterium, *Nitrosomonas europaea*. This species is a common environmental bacterium found in New Zealand's aquatic and terrestrial ecosystems. This species has also been commonly researched in both pure and mixed cultures (Prosser 1989).

The biochemical mechanism for this bacterium is described by the following reactions (Eqs. 11 – 12). The ammonia is oxidised to hydroxylamine (Eq. 11), by the enzyme ammonia mono-oxygenase (AMO) that lies in the membrane of the cell. Hydroxylamine is then oxidised by the enzyme hydroxylamine oxidoreductase (HAO) to nitrous acid (nitrite) (Eq 12). The four protons that are produced in this reaction (Eq. 12) are used for the energy source for the AMO oxidation reactions (Prosser 1989). *N. europaea* is also capable of denitrification and anaerobic respiration but this is beyond the scope of this work.

$$NH_3 + O_2 + 2H^+ + 2e^- \Rightarrow NH_2OH + H_2O \quad (11)$$

$$NH_2OH + H_2O \Rightarrow HNO_2 + 4H^+ + 4e^- \quad (12)$$

3.3 Ammonia chemistry

Ammonia in solution has two forms: ionised and unionised ammonia (Eq 13). The pKa value of the system is 9.25. The equilibrium relationship between ionised and unionised ammonia is highly dependent on pH and temperature. An increase in pH favours the unionised form of ammonia. The speciation of the ammonia is important to this system, as the true substrate for *N. europaea* is unionised ammonia (Suzuki, Dular, and Kwok 1974):

$$NH_3 + H_2O \Leftrightarrow NH_4^+ + OH^- \quad (13)$$

3.4 Nitrification Kinetics

The growth of *N. europaea* is dependent on a variety of compounds. However the two major compounds involved in energy production are: ammonia and oxygen. The growth rate can be modelled based on Monod growth kinetics for a dual limited system, assuming oxygen and ammonia are limiting. If we assume the system is not oxygen limited the equation reduces to Eq. 14 (Prosser 1989):

$$\mu = \mu_{\max}\left[\frac{S_N}{K_{SN} + S_N}\right] \quad (14)$$

μ	growth rate, hr^{-1}
μ_{max}	maximum specific growth of biomass, hr^{-1}
K_{SN}	half saturation constant for ammonia, mM
S_N	substrate concentration for ammonia, mM

Table 3.2 shows some values for various growth parameters

Table 3.2: Various nitrification kinetic parameters

Species	**μ_{max} (1/h)**	**K_{sn} NH_4^+ (mM)**	**Yield (g biomass/mol NH_3)**	**Reference:**
Nitrosomonas sp	0.016-0.058		0.42-1.40	(Loveless and Painter 1968)
Nitrosomonas europaea	0.035	0.051	1.26-1.72	(Keen and Prosser 1987a)

4 *Nitrosomonas europaea* Co-metabolism

Nitrosomonas europaea can derive all its energy from ammonia oxidation and its carbon from carbon dioxide (Arp and Stein 2003; Chain et al. 2003). Chain *et al* (2003) sequenced the complete genome of *Nitrosomonas europaea* confirming ammonia as the major energy source of the bacterium and finding no other significant energy-gaining metabolism. Their findings also indicated a limited number of genes for catabolism of organic compounds and suggest that complete oxidation of simple organics could be possible. Hommes, Sayavedra-soto and Arp (2003) showed that fructose can be the sole source of carbon for growth but did not provide any energy benefit for the cell.

A variety of other hydrocarbons can be oxidized by this bacterium. These molecules range from: simple straight chain hydrocarbons (Hyman, Murton, and Arp 1988; Hyman and Wood 1983; Hyman and Wood 1984; Voysey and Wood 1987, Hyman, 1984 #77); halogenated hydrocarbons (Keener and Arp 1994; Duddleston *et al*. 2000; Rasche, Hyman, and Arp 1991; Rasche, Hyman, and Arp 1990; Hyman, Page, and Arp 1994; Ely *et al*. 1997) to larger, more complex molecules, including estrogens (Shi *et al*. 2004) and polycyclic aromatic hydrocarbons (PAHs) (Chang, Hyman, and Williamson 2002). Table 4.1 shows some of the variety of investigated hydrocarbons and their corresponding products.

Table 4.1: A selection of various hydrocarbons that have been shown to be co-metabolized by *Nitrosomonas europaea*

Hydrocarbon Substrate	Product	Reference
Methane	Methanol	(Hyman and Wood 1983) (Voysey and Wood 1987)
Alkanes up to C8	Corresponding primary or secondary alcohol	(Hyman, Murton, and Arp 1988)
Alkenes up to C8	The corresponding epoxide	(Hyman, Murton, and Arp 1988)
Methanol	Formaldehyde	(Voysey and Wood 1987)
Formaldehyde	Formate	(Voysey and Wood 1987)
Benzene	Phenol	(Hyman *et al.* 1985) (Keener and Arp 1994)
Phenol	Hydroquinone	(Hyman *et al.* 1985)
Trichloroethylene	N/A	(Ely *et al*. 1997)
Methyl Bromide	N/A	(Rasche, Hyman, and Arp 1990)
Estrone	N/A	(Shi *et al.* 2004)
Napthalene	2-napthol	(Chang, Hyman, and Williamson 2002)

N/A – not available, or not published

The reaction mechanism of each of these reactions is a single oxygen molecule insertion into a C-H bond (Arp and Stein 2003). This reaction is synonymous with the AMO catalysed ammonia to hydroxylamine reaction and this enzyme is thought to be responsible for the oxidation (Hyman and Wood 1983). The rates of oxidation of the various hydrocarbons are reported to be much lower than ammonia oxidation rates (Hyman *et al.* 1985; Chang, Hyman, and Williamson 2002; Voysey and Wood 1987).

4.1 Methane Oxidation by Nitrosomonas europaea

Methane oxidation by *Nitrosomonas europaea* has been studied by several authors. Methane was investigated as an alternative substrate because of AMO's similarity with the methanotrophic bacteria's enzyme MMO (Suzuki, Kwok, and Dular 1976; Hyman and Wood 1983). Several have found that methane is oxidized to methanol (Hyman and Wood 1983; Hyman, Murton, and Arp 1988). Eq. 15 is the reaction for the conversion of methane to methanol occurring by a similar mechanism to that producing hydroxylamine (Section 3.2). The important difference is that no substrate for the HAO enzyme is produced in methane oxidation and therefore no protons can be produced through this enzyme for the reductant pool. Methanol can itself be oxidised (by the AMO enzyme) according to Voysey and Wood (1987). The product of this reaction (Eq. 16) is formaldehyde. The formaldehyde produced can react with the hydroxylamine to produce formaldoxime (Eq. 17) Formaldoxime was shown to inhibit HAO (Voysey and Wood 1987), thus affecting the reducing power of the bacterium. Jones and Morita (1983) showed that methane can be completely oxidised to carbon dioxide (Eq. 18) and also showed cell incorporation of the methane.

$$CH_4 + O_2 + 2H^+ + 2e^- \Rightarrow CH_3OH + H_2O \tag{15}$$

$$CH_3OH + O_2 + 2H^+ + 2e^- \Rightarrow CH_2O + 2H_2O \tag{16}$$

$$CH_2O + NH_2OH \Rightarrow H_2CNOH + H_2O \tag{17}$$

$$CH_2O + O_2 \Rightarrow CO_2 + H_2O \tag{18}$$

Suzuki *et al.* (1976) have investigated the effect of methane, carbon monoxide and methanol on oxygen uptake of cell-free extracts of *N. europaea*. They have found that none of the species were oxidized by the extract. Ammonia oxidation, however, was shown to be inhibited by all three substances and that the degree of inhibition was dependent on ammonia concentration. They conclude that the behaviour was competitive inhibition of ammonia oxidation.

Hyman *et al.* (1983) used whole cells of *N. europaea* and showed that methanol was produced at a rate of 1.06 mmol/g dry wt.h. Their investigation looked at the subsequent effect on

ammonia oxidation and oxygen uptake. The methane driven oxygen uptake and methane inhibition using an AMO inhibitor, led them to conclude that methane is an alternative substrate for AMO.

Research by Jones *et al.* (1983) on a variety of strains of nitrifiers, in the presence of methane including *N. europaea,* showed a significant amount of methane oxidation to carbon dioxide. Their results also showed incorporation of the methane into cellular material.

Hyman *et al* (1988) investigated hydrocarbon oxidation by *N. europaea* for a variety of alkanes and alkenes. They report that all straight-chained hydrocarbons up to C8 could be oxidized by the bacterium. A methanol production rate of 0.5 mmol/g dry wt.h was their highest reported rate and was seen in the presence of 10 mM NH_4^+. Their results showed a decrease in the methanol production rate with decreasing ammonia concentrations due to the competition for reductant. The authors suggest that the oxidation of hydrocarbons is through the AMO enzyme.

Chapman *et al*. (2004) investigated methane oxidation by *N. europaea* in both batch experiments and in a chemostat with cell recycle. They found methanol production rates ranging between 0.17 – 2.23 mmol/g dry weight.hr. Their results did not show the simple competitive inhibition proposed by the other authors. They suggested that it was not AMO but another activity which was responsible for the methane oxidation. They also suggest that the methane oxidation rate varies with both the methane concentration and bacteria growth rate. Their discussion of reactor development suggested a higher biomass was needed, recommending a biomass concentration of 5 – 7 g/l. Their investigation also recommended a concentration of 100 mM needed to be achieved before a commercial process may be feasible.

5 Immobilization

5.1 Background

Immobilization is the restricting or stopping of the movement of micro-organisms or enzymes in order to keep them in a system. There are five different principal methods of immobilisation: adsorption of the cells to a surface; covalent bonding of the cells to a surface; entrapment of the cells in a lattice; encapsulation the cells in a semi-permeable membrane; or cross-linking of the cell either chemically (covalent crosslinks) or physically (flocculation) (Bickerstaff 1997) (Figure 5.1).

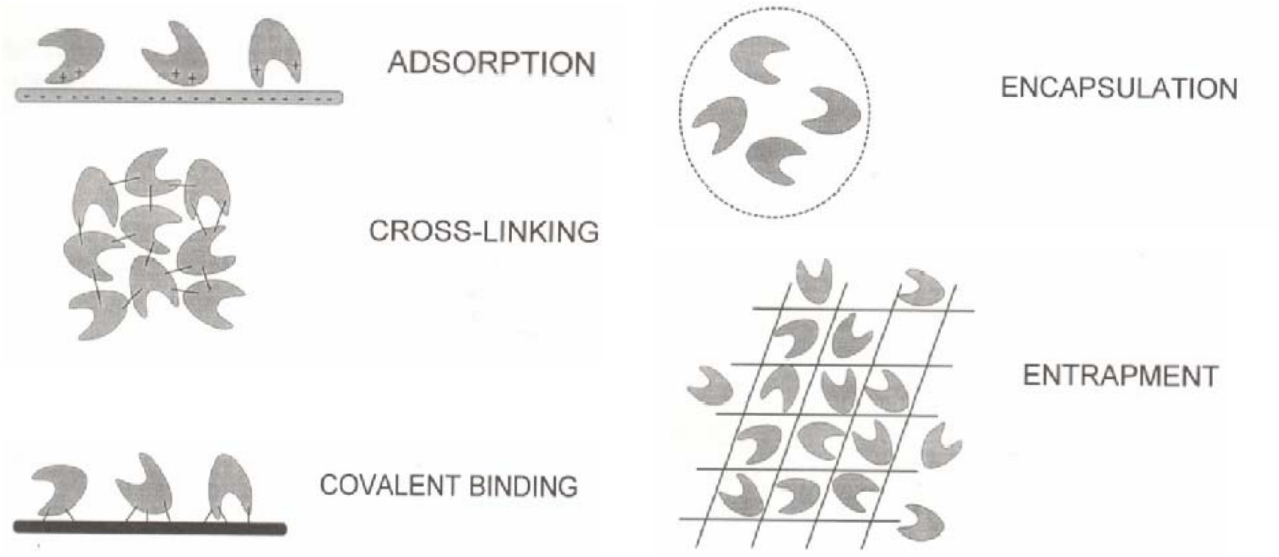

Figure 5.1: Schematic diagrams of principal methods of immobilization modified from (Bickerstaff 1997).

Immobilization has several advantages over planktonic cells. They include: higher biomass concentrations (Strotmann and Windecker 1997); higher hydraulic and substrate loading (Rostron, Stuckey, and Young 2001); and different responses to environmental conditions (Batchelor *et al.* 1997; De Boer *et al.* 1991; Allison and Prosser 1993; Powell and Prosser 1992; Rostron, Stuckey, and Young 2001).

Rostron, *et al* (2001) compared a free culture nitrification system to various different immobilized supports in a chemostat (biological CSTR) bioreactor. They investigated the effect of hydraulic loading on nitrification rate. They concluded that the hydraulic retention

time for the immobilized system could be 12 hours still having partial nitrification while complete washout of the free culture had occurred at 24 hours. The rates of nitrification presented for the various immobilized systems were higher than those in a free cell system.

Nitrification is sensitive to a variety of environmental conditions (Section 3.1). However when the cells are immobilized the activity of the cells is less drastically altered by some of these conditions. De Boer *et al.* (1991) and Allison *et al.* (1993) have both shown that nitrifying cells immobilized in aggregates (De Boer *et al.* 1991) and in a biofilm (Allison and Prosser 1993) continue to nitrify at lower pH than free cells. Rostron *et al.* (2001) investigated nitrification at temperatures from 25°C to 16°C with immobilized nitrifiers. They suggested that the decrease in the nitrification rate, in relation to decreasing temperature, was more likely to be caused by mass transfer limitations than a decrease in the specific activity of the bacteria. Powell *et al.* (1992) found that immobilised *N. europaea* cells were less affected by the potent nitrification inhibitor nitrypirin. They suggested either low growth rate or protection of the cells by extracellular material was responsible for the protection of the cells. Batchelor *et al.* (1997) showed that the recovery time of *N. europaea* in a biofilm (described in Section 5.2) following a period of starvation was significantly less than for free cells.

5.2 Biofilms

Some bacteria spend much of their time in the natural environment not as free cells, but as colonies immobilized in biofilms. A biofilm is described by Bryer (2000), summarizing a previous definition, as "a surface accumulation, which is not necessarily uniform in time or space, that comprises cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin" (Figure 5.2). Nitrifying bacteria, including *N. europaea*, are capable of forming biofilms (Prosser 1989).

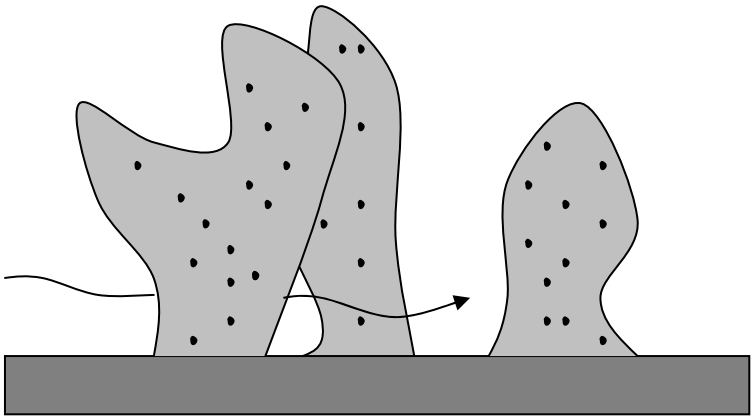

Figure 5.2: Schematic diagram of a biofilm. The black specks are colonies of bacteria; the light grey is exopolysaccharide; the dark grey is the support surface; and the arrow represents the flow of water through channels in the biofilm.

The following summarizes the steps of the growth and persistence of biofilms (Bryers 2000):

1. Biasing or preconditioning of the substratum either by macromolecules which are present in the bulk liquid or which are intentionally coated on to the substratum (support surface)
2. Transport of planktonic cells from the bulk liquid to the substratum
3. Adsorption of cells at the substratum for a finite time
4. Desorption of the reversibly adsorbed cells
5. Irreversible adsorption of bacterial cells at a surface
6. Transport of substrates to and within the biofilm
7. Substrate metabolism by the biofilm-bound cells and transport of the products out of the biofilm. These processes are accompanied by cellular growth, replication and extra-cellular polymer production
8. Biofilm removal (detachment or sloughing).

In order for the biofilm to grow, it is essential that the bacteria attach to the surface. This attachment can be reversible or irreversible. Reversible attachment occurs through Van der Waals forces and irreversible attachment is caused by covalent binding to the surface. Various investigations have revealed methods for encouraging biofilm growth.

5.2.1 Encouraging Biofilm Growth

Genetic approaches to investigating biofilms have revealed bacterial properties that encourage biofilm growth. These properties include: bacterial motility; cell surface proteins; extracellular polysaccharides (EPS); external environmental cues; and bacterial signalling (Kjelleberg and Molin 2002; Pratt and Kolter 1999; Hall-Stoodley and Stoodley 2002). An investigation on *N. europaea* by Schmidt *et al.* (2004) found a difference in some expressed proteins when comparing free cells with those in a biofilm. This differing expression of proteins was linked to nitric oxide using proteomic and physiological evidence and they concluded that nitric oxide was an environmental initiator for biofilm growth.

Moreover, various authors have shown that the supports' surfaces are also responsible for the attachment of bacteria to a surface and the subsequent growth of biofilm. Properties that have been shown to be important include: surface charge (Kida *et al.* 1992; Sousa *et al.* 1997; Li and Logan 2004; Teixeira and Oliveira 1998), hydrophobicity (Li and Logan 2004; Sousa *et al.* 1997; Teixeira and Oliveira 1998) and porosity (Bickerstaff 1997; Messing and Oppermann 1979, Teixeira, 1998 #26).

Teixeira *et al.* (1998) investigated surface properties of support material leading to stable biofilm growth. Their research indicated the importance of the supports' surface electrical charges in bacterial attachment. They found that the adhesion of a nitrifying consortium (*Nitrosomonas and Nitrobacter*) was strongly affected by electrical charge of the surface, measured as the electrophoretic mobility. Both bacteria were found to present a negative surface charge (Teixeira and Oliveira 1998) therefore they showed a preference to positively charged particle surfaces. They also found the hydrophobicity of the surface had an effect but it was less dominant. Sousa *et al's.* (1997) work with a nitrifying consortium investigating attachment to polymeric supports also showed that electrostatic force between the bacteria and the support surfaces were important, but concluded that the high hydrophobicity of the surface was the governing attraction.

Porous particles can be suitable immobilization supports (Bickerstaff 1997; Teixeira and Oliveira 1998). The porosity of the particle relates to the available surface area and the biomass space available for cell occupation. Messing *et al.* (1979) investigated the relationship between the dimensions of the microbes (that reproduce by fission) and their accumulation in porous inorganic structures. Their findings showed in order to achieve high

accumulation of microbes at least 70% of the pores should have a diameter larger than the smallest major dimension of the microbe but less than five times its largest dimension.

5.3 Immobilized Nitrifying Bacteria

A variety of different supports have been assessed in literature for their ability to immobilize nitrifying bacteria. The most common methods are the entrapment in a polymer matrix (Leenen et al. 1996; Matsumura et al. 1997; Sumino et al. 1992; Rostron, Stuckey, and Young 2001) or biofilm formation on a surface (Teixeira and Oliveira 1998; Rostron, Stuckey, and Young 2001; M^{c}Veigh 1998; Strotmann and Windecker 1997; Park, Sool Lee, and Il Yoon 2002).

Leenen *et al.* (1996) compared synthetic gels to natural gels as material for entrapment and found that synthetic gel has a superior mechanical stability but that natural gels had better diffusivity. Consequently they decided that synthetic gels were promising immobilization material. Rostron *et al.* (2001) investigated the use of PVA-entrapped nitrifiers finding that this material also shows potential. Sumino *et al.* (1992) investigated the entrapment of the bacteria into urethane gel beginning with six different pre-polymers. They reported significant retention of the initial cell activity, concluding that it was a dramatic improvement over conventional acrylamide immobilization.

A variety of materials have been investigated for their ability to support biofilm growth. Teixeira *et al.* (1998) compared a variety of minerals including sand, limestone, basalt, pumice and poraver. They found that limestone and basalt showed high ammonia removal efficiency owing to surface charge and that poraver was efficient because of its high porosity. Strotmann *et al.* (1997) successfully immobilized nitrifiers on porous glass (SIRAN^{TM}), concluding that the increase in nitrification was due to the increase in biomass. Natural zeolites, especially clinoptilolite, have also been shown to be a successful biofilm support. Zeolites are well known for their use in ammonia treatment of wastewater because of their ion exchange properties. Nitrifying biofilm grow readily on this material because of the high ammonia concentration on the surface of the material. This combination of technologies has shown increased performance when compared to standard clinoptilolite (M^{c}Veigh 1998). Park *et al.* (2002) found accelerated nitrification rates when nitrifying bacteria were immobilized to clinoptilolite as compared with an activated sludge nitrifying system.

When entrapment-immobilized nitrifiers were directly compared to biofilms by Rostron *et al.* (2001) it was found that the encapsulation in PVA had superior nitrification rates to two adsorption materials, Kaldnes (polyethylene) and Linpor (sponge material). The rates were Linpor: 0.57 g Nm^{-3}-reactor d^{-1}; Kaldnes: 0.53 g Nm^{-3}-reactor d^{-1}; PVA: 0.70 g Nm^{-3}-reactor d^{-1}.

6 Objectives

The objective of this research is to develop an immobilized cell bioreactor, using the nitrifying bacteria, *Nitrosomonas europaea*, for the purpose of biologically producing methanol from methane.

To accomplish this objective the following aims were addressed:

- Develop a pure culture of *Nitrosomonas europaea* immobilized in a bioreactor system with a higher biomass concentration than free cell systems
- Investigate the methanol production capacity of the system
- Compare the methanol production by the immobilized cells to other biological methanol production investigations
- Investigate the feasibility of a methanol biosynthesis process using the developed reactor system.

7 Experimental Methods

7.1 The Bacterium

Nitrosomonas europaea Winogradsky 1892 was received from the International Collection of Microorganisms from Plants (ICMP) at Landcare Research, Auckland, New Zealand (ATCC no. 25978; ICMP no. 13139). The bacterium was originally used in Foundation of Research Science and Technology contract WASX 0201: "Production and Application of Renewable Fuels" (Chapman, Gostomski, and Thiele 2004). A pure culture of this bacterium was used throughout this research. If contamination of the experiment by other bacteria occurred, the experiment was either discontinued or the level of contamination was measured according to Section 7.5.

7.2 Growth Medium

Table 7.1 shows the constituents and their concentration in the growth medium used throughout (Hyman and Wood 1983). $(NH_4)_2SO_4$ concentration was adjusted to achieve the desired ammonia concentration. This medium has been successfully used for *N. europaea* growth and methane oxidation (Hyman and Wood 1983; Chapman, Gostomski, and Thiele 2004).

Table 7.1: Constituents and their concentrations in the growth medium (Hyman and Wood 1983).

Chemical	**Concentration (mM)**	**Chemical**	**Concentration (μM)**
$(NH_4)_2SO_4$	10 – 30	$FeSO_4$	0.24
$MgSO_4$	0.272	EDTA	0.174
$CaCl_2$	0.6	Phenol Red	1.4
K_2HPO_4	3.0		

7.3 Sterilization

Sterilization was achieved using steam autoclaving at 121°C and 15 psig for a period of at least 15 minutes and up to 90 minutes for larger volumes. Some liquids and gases were sterilized using filter sterilization through sterile 0.45 μm filters (Millipore Millex^{TM}). All

sterile work was in the presence of a flame or in a class II biological safety cabinet (Clyde Apac, Clean Air).

7.4 Maintaining Cultures

Active cultures of *N. europaea* were maintained in 60 ml serum bottles containing 30 ml M2 medium and sealed using butyl rubber stoppers. The pH was controlled using 0.5 – 1.5 ml of sterile 1 M ammonium bicarbonate on a daily basis and given 50 ml fresh air through a sterile filter (Chapman et al. 2005).

7.5 Contamination

Samples were taken from the system aseptically and 0.1 ml of the sample plated on organic rich plate count agar (Table 7.2). *N. europaea* could not grow on these plates, therefore after a plate was inoculated, if no growth occurred on the plate after 4 days, the system was considered uncontaminated.

Table 7.2: The concentrations of ingredients in nutrient agar plates

Chemical	**Concentration g/l**
Peptone	5
Yeast extract	5
Citric acid	1.5
Na_2HPO_4	2.7
Sucrose (Chelsea white)	50
Agar	20

Contamination levels were determined using serial dilutions, plating on agar plate (Table 7.2) and counting of the colony forming units.

7.6 Batch Biofilm Growth Experiments

Initial screening of biofilm support material was undertaken to assess their suitability. Types assessed included: limestone, basalt, ceramic, polyethylene (kaldness particles), glass particles (Advanced Filtration Media), and activated carbon. These experiments were undertaken by inoculating 60 ml serum bottles containing 30 ml of 50 mM NH_4^+ medium and

10 ml of dry particles. The serum bottles were maintained as in Section 7.2. Then after 1-2 weeks of growth, the particles were removed under aseptic conditions and rinsed thoroughly with sterile medium and added to fresh 50 mM NH_4^+ medium that was buffered with 50 mM, pH 7.7 phosphate buffer. The nitrite production was measured over a short period of time (1 – 4 hours) (Section 7.7.2) and the protein on the support surface was measured according to Section 7.7.4.

7.7 Experimental Method

7.7.1 Immobilized Cell Bioreactor

A trickle bed reactor (Figure 7.1) was chosen as the type of biological reactor for this work because:

- The substrate for the desired reaction, methane, is a gas and in a trickle bed gas is the continuous phase (Doran 1995)
- Good mass transfer can be achieved (Andrews and Noah 1995)
- High biomass concentration can be achieved (Tyagi and Vembu 1990)
- Higher conversion can be achieved due to plug flow through a packed bed (Andrews and Noah 1995).

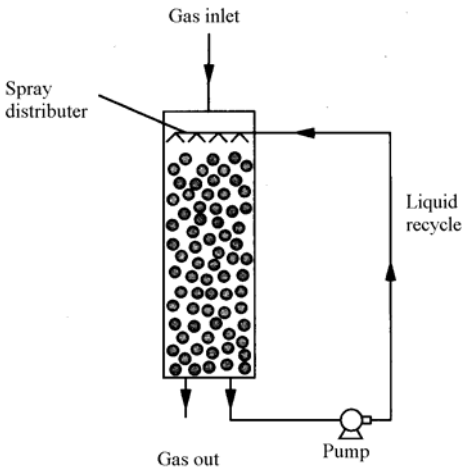

Figure 7.1: Simplified diagram of a co-current trickle bed. Modified from Doran (1995)

7.7.2 Experimental apparatus

A laboratory scale co-current trickling filter bioreactor was developed. A co-current configuration was chosen to enhance liquid removal, since only one outlet is used for both gas and liquid. If a counter-current configuration was used it could have caused excessive liquid

holdup. The trickle bed consisted of a 100 mm diameter tube 370 mm in length which contained a packed bed of a depth with a depth of 300mm. The packed bed consisted of 6 mm ceramic raschig rings (see Section 8.2). Appendix A contains the mechanical drawings of the trickle bed. The experimental system consisted of two major parts: a trickle bed (plug flow reactor) and a stirred vessel (CSTR). The stirred vessel was a New Brunswick Multigen fermenter that had a working volume of 1690 ml. The purpose of the stirred vessel was to enable the system to be operated in recycle and pH and temperature to be controlled. Aeration of the trickle bed was with a Precision SR-9500 aquarium pump. A process flow diagram of the reactor (Figure 7.2) shows the layout of the experiment.

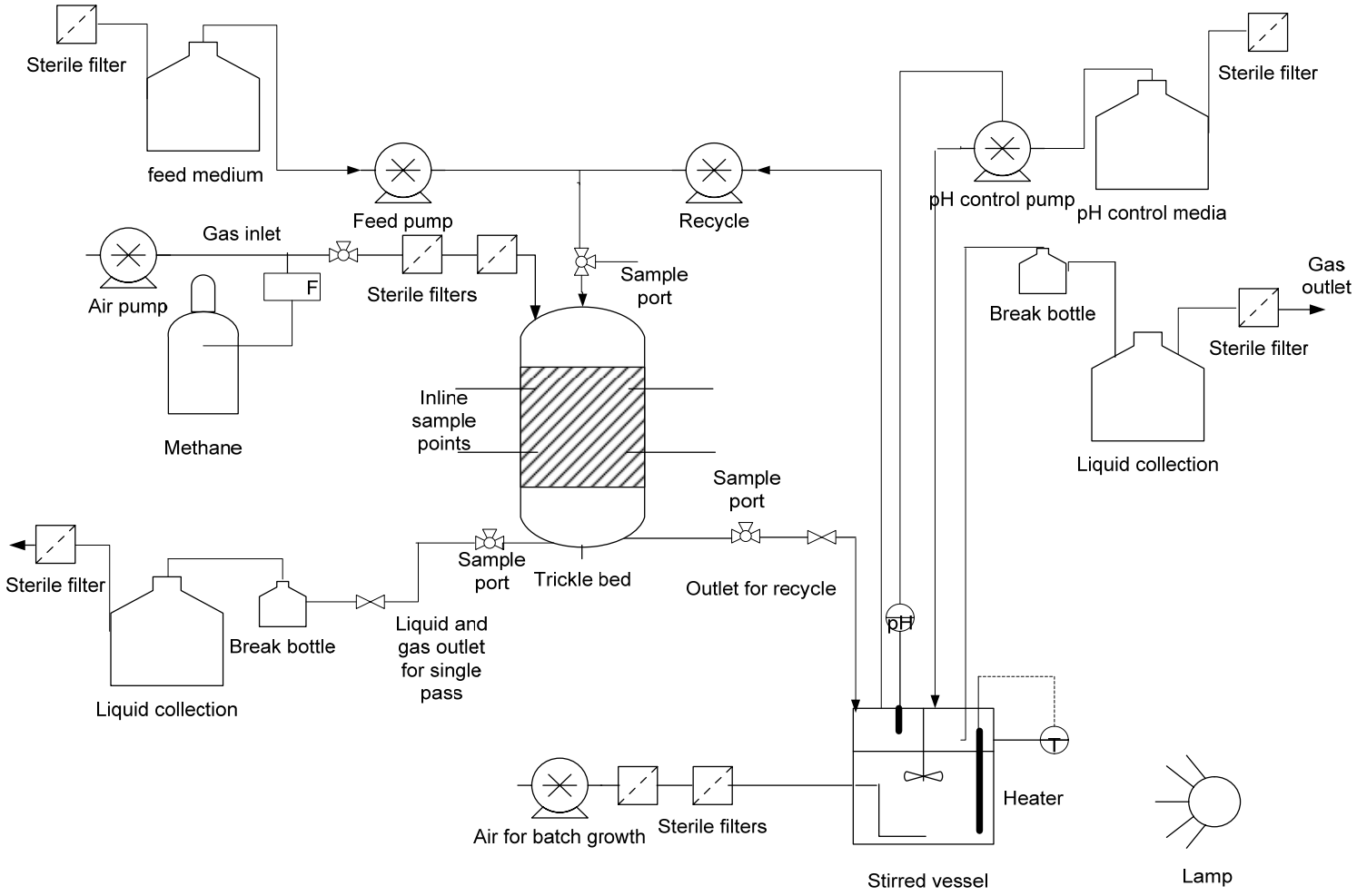

Figure 7.2: A Process flow diagram of the experimental setup

7.7.2.1 pH Control

The pH was controlled in the stirred vessel at a pH 7.8 ± 0.1 using a Hanna pH controller connected to a Phoenix Fermprobe autoclavable pH electrode, controlling a Masterflex peristaltic pump. The pH control liquid was either 1 M $NaHCO_3$ or 0.5 M Na_2CO_3.

7.7.2.2 Temperature Control

Temperature of the recycled medium was controlled at 30°C by an electrical element that was part of the New Brunswick Multigen fermenter. The feed medium, which was combined with the recycled medium at the top of the trickle bed, was at ambient temperature. Therefore the temperature of the medium at the point of distribution onto the packed bed was likely to be slightly lower that 30°C.

7.7.2.3 Reactor inoculation

The reactor system was inoculated with *N. europaea* into the stirred vessel using 60 ml of inoculum grown according to Section 7.4. The part of the stirred vessel was then operated in batch mode and air was sparged into it. The stirred vessel at this stage was covered to prevent light inhibition. Once the free cell bacterial culture had reached stationary phase (optical density of 0.32) the trickle bed was inoculated by recycling the planktonic cell around the trickle bed for 1 week. If the system became ammonia limited, 25 ml of sterile 1.14 M $(NH_4)_2SO_4$ was added.

7.7.2.4 Sampling

Sterile syringes were used to take 1.1 ml samples from the outlet of the trickle bed (Figure 7.2) through a sterile plastic three-way valve. Sampling was done in the presence of a flame to minimise the risk of contamination.

7.7.2.5 Sample Storage

Samples were stored in 1.5 ml micro centrifuge tubes and frozen at -18 °C.

7.7.2.6 Minimising Planktonic Cells

After inoculation of the trickle bed, the cover on the stirred vessel was removed and a lamp was shone at the vessel. Light has been shown to inhibit *N. europaea* (Shears and Wood 1985). This was to minimise the activity and growth of the free cells.

7.7.2.7 Methane Experiments

Four experiments in which the system where completed. The flow conditions are described in Table 7.3. Runs i and ii were where the system was run without recycle.

Table 7.3: Flow conditions for methane experiments

Run	Feed Flow (ml/min)	Gas Flow (ml/min) Natural gas:air (1:1)	Recycle Flow (ml/min)
i	98	1.6	0
ii	72	1.5	0
1	8.2	1.1	88
2	2.1	1.1	88
3	2.0	0.75	88
4	2.0	0.86	88

The variation in gas flows is due to changes in the pressure drop of the system. This was not expected to matter since the flows were sufficiently high to not be limiting. The composition of the gas was as described in Section 7.8.6.

7.8 Analysis Techniques

7.8.1 Spectrophotometry

Spectrometric measurements were made on a Shimadzu Multispec-1500 spectrophotometer at the required light wavelength (see specific assay). The curvettes used were 1 ml plastic curette with a 1 cm optical path.

7.8.2 Nitrite Assay

Nitrite concentration was measured using a colorimetric method number 4500-NO2 (Eaton et al. 1995). The assay is a colorimetric assay using a colour reagent; containing 8.5% phosphoric acid, 10 g/l sulfanilamide and 1 g/l N-(1-naphthyl)-ethylenediamine. The sample was compared to a potassium nitrite standard in the range of 0 μg/l to 1500 μg/l to establish a concentration.

7.8.2.1 Procedure

- A 5 – 20 μl sample was diluted into 5 ml pure water for the nitrite concentration to be in the range of 0 μg/l to 1500 μg/l.

- Then 0.2 ml of colour reagent was added, mixed and then left for 10 minutes but not longer than 2 hours
- The absorbance was then measured on a spectrophotometer (Section 7.7.1) at 543 nm.

7.8.3 Ammonia Assay

Ammonia was measured as the ammonium ion using a colorimetric method, known as the phenate method (Eaton et al. 1995). The colour of the solution was compared to an ammonium standard solution 0 – 1 mg N/l.

Table 7.4: Reagents for ammonia assay

Reagent	**Constituents**
Phenol reagent	10 g phenol in 95 ml ethanol and 5 ml n-propanol.
Sodium nitroprusside solution	5 g/l sodium nitroprusside
Alkaline solution	300 g/l sodium citrate; 10 g/l NaOH
Oxidising solution	4:1 mixture of alkaline solution and sodium hypochorite

7.8.3.1 Procedure

A 5 – 50 ml sample was diluted to be the range 0 – 1 mg N/l to a volume of 5 ml. To the 5 ml of diluted sample:

- 0.2 ml phenol was added and mixed well
- Then 0.2 ml sodium nitroprusside solution was added and mixed well
- Then 0.5 ml of oxidising solution was added and mixed well
- The mixture was then incubated at room temperature for 1 hour
- The absorbance was then measured on a spectrophotometer (Section 7.7.1) at 640 nm

7.8.4 Protein Assay

The method used to determine protein was a colorimetric assay, the Lowry protein assay (Chart 1994). The colour of the solution is determined by the following procedure and is compared to a standard prepared using bovine serum albumin (BSA) in the range 0 – 100 μg/ml.

Table 7.5: Reagents for Lowry protein assay

Reagent	**Constituents**
Protein standard	0 – 100 μg/ml BSA
A	0.01% $CuSO_4$ and 0.02% Sodium tartrate in 2% $NaCO_3$
B	1N Folin & Ciocalteau Phenol Reagent

7.8.4.1 Procedure

- Mix 100 μl of sample with 100 μl 2N NaOH and heated at 90°C for 10 minutes to lyse the cells.
- Add 1 ml Solution A, mix and then incubated at room temperature for 10 minutes
- Add 0.1 ml Solution B, mix immediately and then incubated for 10 minutess
- Measure in spectraphotometer 750 nm

7.8.5 Methanol

Methanol was measured using a Varian CP-3800 Gas Chromatograph with a Chrompack Capillary Column CP Sil 5CB. The detector was a flame ionisation detector (FID). Table 7.6 shows the operating conditions of the gas chromatograph. The area count was measured and compared to an external standard methanol plot to establish a concentration. Injections were repeated at least four times.

Table 7.6: GC conditions for detecting methanol

GC	Varian Star 3800
Column	Varian Chrompack capillary column
Length x ID x film thickness	15m x 0.32mm x 1.00 μm
Column temperature, °C	45
Carrier gas pressure, PSI	4.4 in head space
Carrier gas flow rate, ml/min	5.0
Sample injected, μl	1
Carrier gas	Helium
Injector temperature, °C	220
Detector temperature, °C	100

7.8.6 Methane

The methane concentration in the gas was estimated based on the flows of air and natural gas. The natural gas used was compressed natural gas from BOC Gas and had a methane content of approximately 80%. The gas flow of the air was measured before the addition of methane and the flow of natural gas was set to maintain a 1:1 ratio of air to methane giving a methane concentration of 40%. Although the system was being developed for biogas, natural gas was used to develop the system.

7.8.7 Attached Biomass Estimation

Biomass immobilized on the support material was estimated by volatile suspended solid using the method number 2540 E (Eaton *et al*. 1995). The entire content of the trickle bed was rinsed, firstly with fresh sterile medium to remove planktonic cells then with deionised water. The particles were then removed from the reactor. The remaining biomass was scraped for the reactor walls and was then added to the particles. The entire contents of the reactor were dried at 103°C overnight in a vacuum oven to remove the water. The mass of the particles was then measured. The particles were then placed in a muffle furnace at 550 °C to ash for at least one hour. The particles were then removed from the furnace and were allowed to cool in the vacuum oven under vacuum 500 mmHg at ambient temperature. The method suggests a desiccator, but one of sufficient size was not available. The mass of the particles was then measured and the loss of mass that occurred due to the ashing process was the volatile solids. This method measured the total amount of biomass in the reactor but did not give an indication of biofilm structure or distribution in the reactor.

7.8.8 Free Cell Measurement

The free cells in the system were measured by optical density at 600 nm using the spectrophotometer described in Section 7.7.1., Deionised water was used as a blank.

8 Results and Discussion

Six methane experiments were completed under the conditions described in Table 7.3. The two experiments in which the system was run without the recycle (run i and ii), showed no methanol production and the results are not shown. The results of the other four experiments, where the system was run with recycle, are presented below. The results show the effects the methane had on the system, including the effect on the ammonia oxidation and the production of methanol.

8.1 The Effect of Methane on Ammonia Oxidation

The following results demonstrate the effect which the addition of methane had on the reactor system (with recycle) under differing residual ammonia conditions. The methane was added in the feed gas at a ratio of 1:1 (air : natural gas). Two vertical lines on the figures indicate the period of methane addition; the first indicates the beginning of methane addition, the second indicates the end of methane addition. Shown in the figures are the nitrite concentration, the residual ammonium concentration (measured at the trickle bed outlet), feed ammonium concentration (before dilution with recycle stream) and the optical density of the solution in the trickle bed outlet.

Figure 8.1 shows an experiment in which the inlet flow rate was increased from 2.04 ml/min to 8.20 ml/min at 174 minutes in order to increase the amount of excess ammonia in the system (Run 1). Few conclusions can be drawn about the effect of the methane on the ammonia oxidation because of the large change the dilution rate had on the system. For subsequent experiments, the residual ammonia concentrations were adjusted by changing the concentration of the feed ammonia and not adjusting the dilution rate.

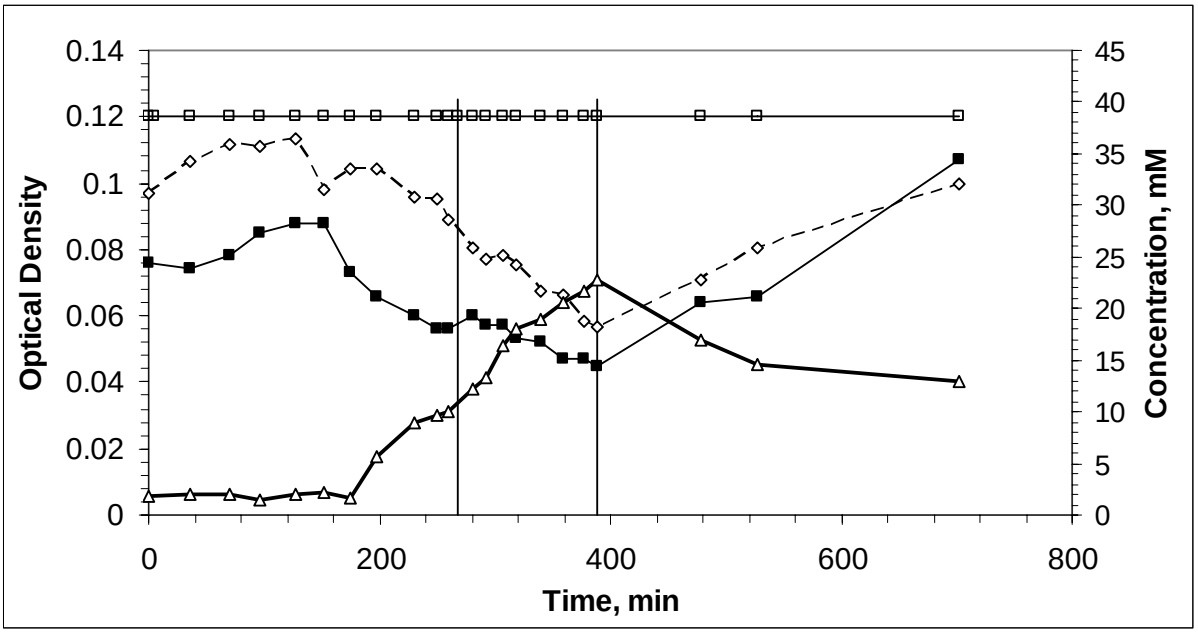

Figure 8.1: Methane addition at increased dilution rate (run 1) to cause excess ammonia conditions. The parameters shown are optical density (■), nitrite concentration (◊), the residual ammonia (Δ) and the inlet ammonia concentration (□).

Figure 8.2 shows an experiment where methane was added to the system that had a residual ammonia concentration of 2.2 mM (Run 2). It can be seen that the system was not at a steady state on the addition of methane. The residual ammonia concentration was decreasing at the time of methane addition. When methane was added to the system, the decrease stopped and the residual ammonia concentration increased from 1.32 mM to 2.66 mM. The residual ammonia continued to decrease when the methane was stopped. The nitrite concentration dropped when the methane was added. The nitrite concentration was above the inlet ammonia concentration. This is assumed to be a consequence of the higher concentration of ammonia in the feed medium prior to the experiment. The optical density of the solution decreased from 0.044 to 0.027. At steady state the growth rate is equal to the detachment rate. A decrease in the optical density could perhaps suggest an effect on the detachment or the growth rate of the bacteria.

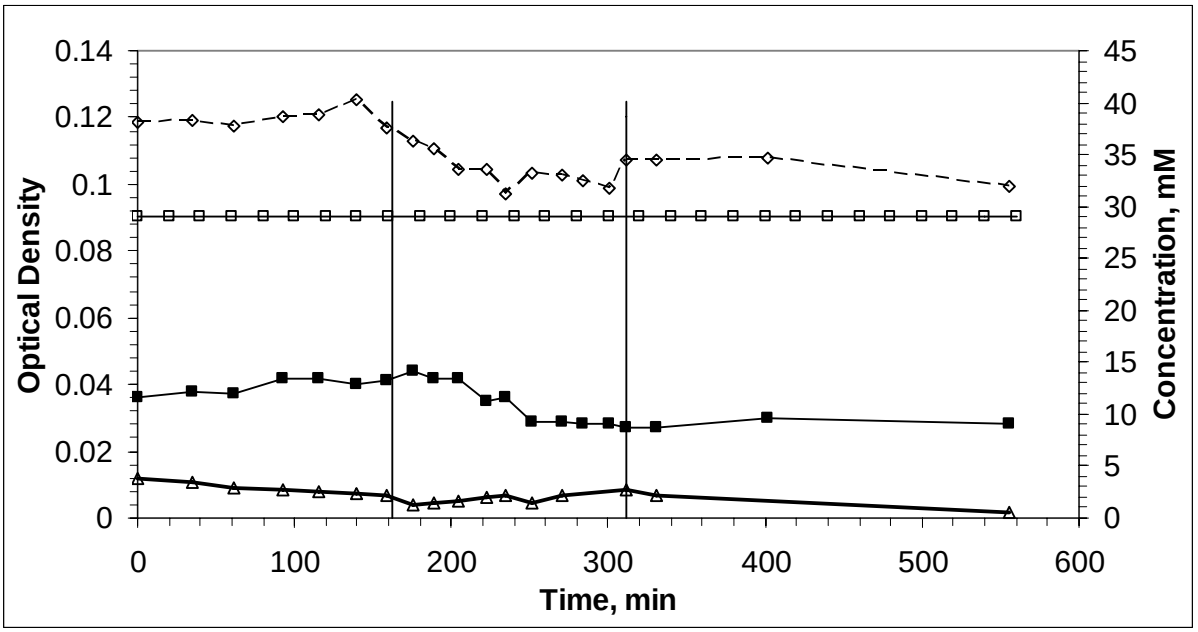

Figure 8.2: Methane addition in the presence of excess ammonia, run 2. The parameters shown are optical density (■), nitrite concentration (◊), the residual ammonia (Δ) and the inlet ammonia concentration (□).

Figure 8.3 shows the effect methane had on the system when it was added to the inlet air. The system had a low residual ammonia concentration (0.3 mM). The graph shows an increase in the residual ammonia over the period when methane was added. Once the methane was stopped, the residual ammonia began to drop again. The concentration of nitrite was much higher than the amount that could be formed from the inlet ammonia. This is due to the higher ammonia concentration in the feed medium in the day prior to the methane experimentation. The optical density of the medium in the system remained constant except for a rapid unexplained jump at minute 265.

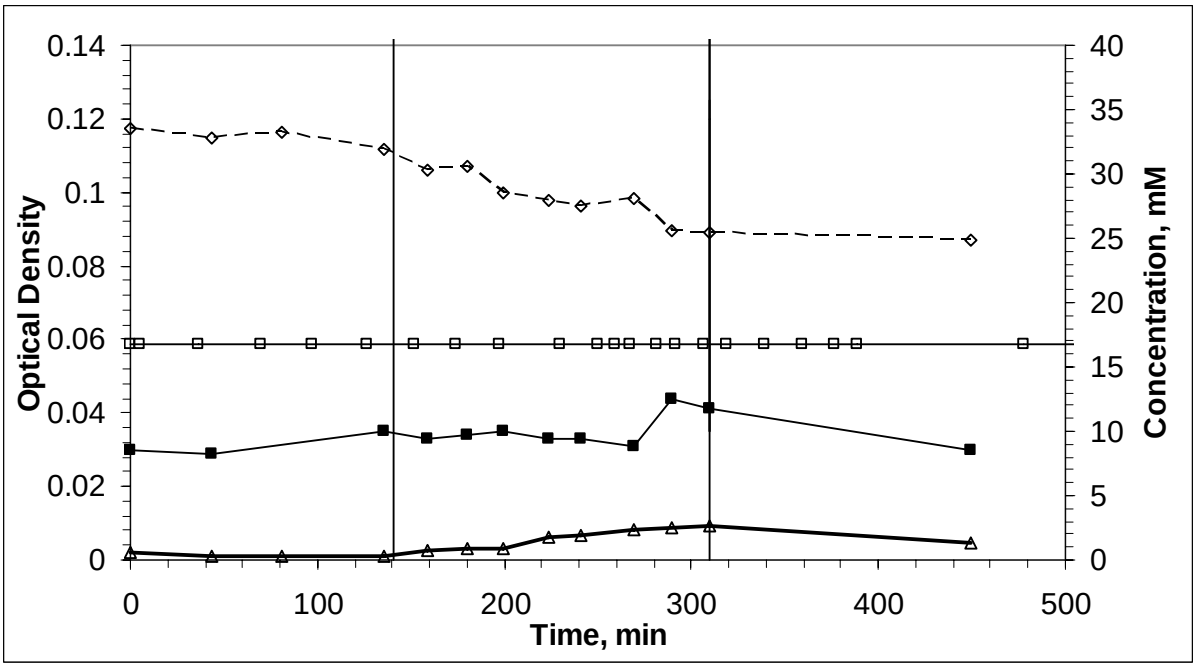

Figure 8.3: The effect of methane on a system with low residual ammonia, run 3. The parameters shown are optical density (■), nitrite concentration (◊), the residual ammonia (Δ) and the inlet ammonia concentration (□).

Figure 8.4 shows a repeat of the experiment shown in Figure 8.3. The length of the time the system had methane pumped into it was extended from 170 minutes to 385 minutes for this run. This experiment was undertaken when the system had low levels of contamination (81 x 10^3 CFUs/ml) in the free solution. This value was three orders of magnitude below the concentration of free cells in the system (4 x 10^6 cells/ml) and was not expected to have a large impact. The figure shows an increase in the residual ammonia concentration up until minute 220, when the residual ammonia became constant. The nitrite concentration decreases over the entire period of methane addition. The optical density decreased from 0.041 to 0.030 after an initial slight increase.

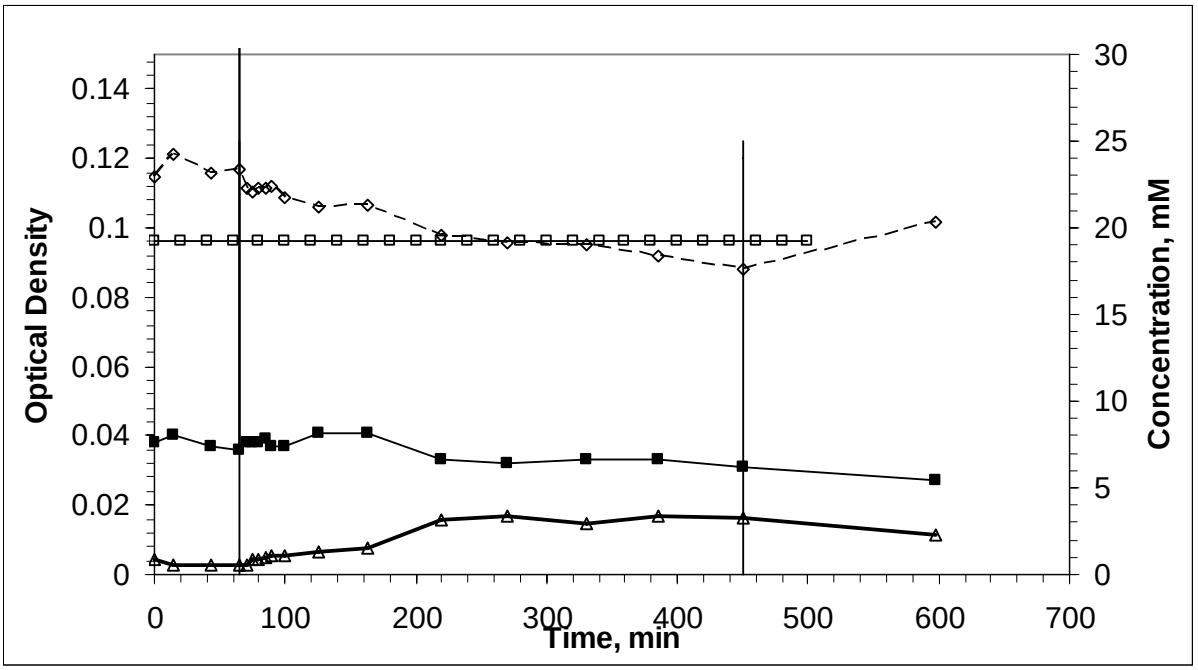

Figure 8.4: Repeat of experiment at low residual ammonia over an increased time frame, run 4. The parameters shown are optical density (■), nitrite concentration (◊), the residual ammonia (Δ) and the inlet ammonia concentration (□).

Figure 8.2, 8.3 and 8.4 consistently show an effect on the ammonia oxidation following the addition of methane to the gas stream. The residual ammonia increased after the addition of methane. Investigation into the ammonia oxidation rates showed that the initial ammonia oxidation activity was inhibited by up to almost 70%, calculated 60 minutes after the addition of methane (Table 8.1)

Table 8.1: Inhibition of ammonia oxidation upon methane addition.

Run	Initial ammonia oxidation activity (mmol/ g VSS hr)[a]	Ammonia oxidation activity 60 minutes after methane addition (mmol/ g VSS hr) *	Percent inhibition at 60 minutes after methane addition
2	1.73 ± 0.005[b]	0.73± 0.194	58 ± 9.4 %
3	0.77 ± 0.001[c]	0.24 ± 0.031	69 ± 1.5 %
4	0.88 ± 0.002[c]	0.28± 0.068	68 ± 3.3 %

[a] ammonia oxidation activity before methane addition

[b] estimated by unsteady state mass balance, using the average change in ammonia before the methane addition.

[C] based on steady state mass balance.

* calculated solving unsteady state substrate balances, using the average change in ammonia concentration over the first 60 min of methane addition.

Table 8.2 shows the nitrite production rates before and after the addition of methane. The values were estimated by solving unsteady state mass balances. The values are an average of results of two possible trend lines and the errors are the standard deviation of the calculated results using those trend lines. The values do not agree with the ammonia oxidation rates within experimental error. Both runs 3 and 4 have a higher initial nitrite production rate than ammonia oxidation rates. This is unexpected and suggests that there was extra error in the nitrite or ammonium measurement or an unaccounted for source of nitrite. The production rate after the addition of methane is negative; this indicates the removal of nitrite was higher than the loss of nitrite due to dilution. This would suggest that denitrification occurred in the system, upon addition of methane. Denitrification by *N. europaea* is the reduction of nitrite to nitrogen gases and is known to occur under oxygen limited conditions (Schmidt *et al.* 2004; Arp and Stein 2003). The denitrification could have been caused by methane or, more likely, by oxygen limitation caused by dilution of its concentration by methane (see below).

Table 8.2: Nitrite production activities during methane addition

Run	Initial nitrite production activity (mmol/ g VSS hr)[a]	Nitrite production activity 60 minutes after methane addition (mmol/ g VSS hr) *
2	1.07 ± 0.17	-0.83 ± 0.43
3	0.99 ± 0.09	-0.97 ± 0.77
4	1.10 ± 0.15	-0.29 ± 0.64

[a] nitrite production activity calculated at the time of methane addition using an average change in nitrite over time before methane addition.

* calculated solving unsteady state substrate balances, using the average change in nitrite concentration over the first 60 min of methane addition.

The experimental procedure combined feed air with natural gas at equal flow rates, giving approximately 40 % methane in the feed stream. This addition of natural gas also resulted in a dilution of the gas phase oxygen concentration to 10.5% and subsequently the reduction of the dissolved oxygen concentration. The inhibition of the ammonia oxidation could therefore be a result of: the presence of methane; the presence of methane's oxidised derivatives; the decreased dissolved oxygen concentration; or a combination of them all.

The concentration of oxygen in air is 21 mole %. Assuming equilibrium with the liquid, the dissolved oxygen concentration can be estimated using Henry's law (at 30°C with pure water) giving a value of 8.05 mg/l. When methane was added to the gas stream (1:1, air:natural gas) the dissolved oxygen concentration was reduced to 4.02 mg/l. Stenstrom and Poduska (1980) reviewed research on the effect dissolved oxygen had on nitrification, stating that reported values for the half saturation constant (K_s) varied widely in the range of 0.3 mg/l to up to 4.0 mg/l. Loveless and Painter (1968) reported a value for a pure culture of *N. europaea* as 0.3 mg/l, much lower than the concentration expected in the trickle bed reactor. With these conditions, the dissolved oxygen concentration probably did not influence the ammonia oxidation. However, these parameters were for free cell systems. For cells immobilized in a biofilm, the dissolved oxygen concentration drives the penetration of oxygen into the biofilm, subsequently affecting the biological oxidation activity of the cells (Hibiya *et al.* 2004). It is therefore possible that the decreased dissolved oxygen concentration lowered the ammonia oxidation rate. The penetration depth is dependent on biofilm density and the biofilm thickness (Hibiya *et al*. 2004). The structure and distribution of the biofilm was not investigated, making it difficult to quantify the possible loss in ammonia oxidation activity this may have caused. However, an experiment without methane and reduced oxygen concentration would clarify the cause.

Assuming the reduced oxygen concentration decreased the ammonia oxidation, there are several solutions: using a more pure oxygen supply rather than air or decreasing the ratio of natural gas to air. Both solutions would decrease the feasibility of the process. Using a higher purity source of oxygen would increase the cost of such a process either through the purchasing of oxygen or process equipment to obtain a concentrated oxygen supply. The other option of decreasing the flow of methane would decrease the concentration of methane in the gas stream. The methanol production activity can decrease in lower methane concentrations (Chapman, Gostomski, and Thiele 2004). This would also affect the feasibility of a biosynthesis process.

Figures 8.2 and 8.4 showed a decrease in the optical density of 0.044 ± 0.005 to 0.027 ± 0.005 and 0.041 ± 0.005 to 0.030 ± 0.005 for runs 2 and 4. The error is estimated from the standard deviation of the measurements. Run 3 however had a sudden increase during the addition (Figure 8.3), most likely due to sloughing at the time of sampling. It is difficult to determine

what the cause of the changes may be. Possible causes include: a decrease in the cellular growth in the system (either biofilm or free cell); or a change in the attachment/detachment processes of the biofilm. Table 8.1 shows that there is a decrease in the ammonia oxidation activity after the addition of methane. With such a substantial decrease in ammonia oxidation it could be assumed that there is a subsequent effect on the growth rate of the bacteria. Chapman *et al.* (2004) used a chemosat with cell recycle, controlling cell growth rate, while investigating methane co-metabolism. They did not report an effect on the growth rate during short term exposure to methane, but saw a decrease in the growth rate in a run over an extended period of time (>30 hours). It could be speculated that the addition of methane somehow affected the attachment or detachment of the biofilm. Denitrification products have been shown to be responsible for *N. europaea* attachment and detachment processes (Schmidt *et al.* 2004). It can be seen from the nitrite production results (Table 8.2) that denitrification apparently occurred in this system after the addition of methane. This would suggest that methane may encourage a combined nitrification/denitrification process as well as influence the cell state. However, in the absence of further evidence the most likely cause for the decrease in optical density was a change in the growth rate. Further research on the effect and the links between various gases, on both biofilm formation and nitrogen removal, could be beneficial for development of nitrification processes.

Methane competes with ammonia for the AMO active site (Hyman and Wood 1983; Hyman, Murton, and Arp 1988; Keener and Arp 1994; Suzuki, Kwok, and Dular 1976), thus inhibiting ammonia oxidation. Suzuki *et al.* (1976) investigated the effect methane had on the oxygen uptake but did not measure the direct effect of the methane on ammonia oxidation. Hyman *et al.* (1983) investigated the effect of methane on oxygen uptake and on ammonia oxidation in the presence of 600 μM CH_4. They reported a 17% inhibition of ammonia utilization, at an ammonium concentration of 530 μM and 21% at 340 μM. These initial ammonia conditions are similar to the conditions for runs 3 and 4 (Figure 8.3 and Figure 8.4) and the dissolved methane concentration is 554 μM. Jones *et al.* (1983) showed no inhibitory effects of methane on ammonia oxidation up to a dissolved methane concentration of 1.0 mM. The drop in the activity of the cells (Table 8.1) is higher than reported by Hyman *et al.* (1983). This implies that, if methane inhibition was the sole cause for the adverse effect on the cells, they were more susceptible to inhibitors when in a biofilm. This is contrary to previous investigations that have indicated cells in a biofilm are protected against harmful chemicals

(Bryers 2000; Powell and Prosser 1992). Powell *et al.* (1992) investigated this phenomenon looking at biofilms of *N. europaea* and the inhibitory affects of nitrapyrin on nitrite production. They concluded that the cells were less sensitive to the inhibitor due to low growth rate of biofilm cells and the presence of extracellular polymeric material. It is therefore possible that methane could be inhibiting ammonia oxidation but is unlikely to be the sole cause of the inhibition. It should be noted that in run 4, ammonia oxidation does not completely shut down over the methane addition period of 450 minutes. Chapman *et al.* (2004) investigated the effect methane addition on free cells over a longer period of time (up 4500 min). Their results showed almost complete loss of ammonia oxidation at 3000 minutes. Longer methane addition experiments would be needed to investigate whether biofilm cells were more stable over longer periods of time than free cells.

It has been suggested that *N. europaea* is capable of completely oxidizing methane to carbon dioxide (Jones and Morita 1983). It could therefore be possible that a further oxidative product could be inhibiting AMO. Methanol has been shown to inhibit ammonia oxidation in *N. europaea* but at 5 mM (Chapman, Gostomski, and Thiele 2004). This is much higher than the concentrations seen in this reactor (Section 8.3) and is therefore unlikely to have been the cause of the loss of activity. The oxidized product of methanol, formaldehyde, is also inhibitory to nitrite production, having an effect at concentrations as low as 0.1 mM (Voysey and Wood 1987). This would affect the ammonia oxidation either through inhibition of AMO or indirectly through the HAO and the cells' reducing power available for ammonia oxidation. Formaldyhyde has also been shown to react with the ammonia oxidation reaction intermediate, hydroxylamine to form formaldoxine (Section 4.1) (Voysey and Wood 1987), which is a potent inhibitor of ammonia oxidation. However, the rapid change in ammonia oxidation activity suggests that either methane inhibition or oxygen limitation was the probable cause of the change in ammonia oxidation because the other products have had no time to accumulate. This does not completely exclude the effects of these oxidized carbon products.

Whether the cause of the decrease in ammonia oxidation is the lower oxygen concentration or inhibition by methane or its oxidized products, a decrease in the only known catabolic process would be detrimental to the system. In a trickle bed system like the one used, the growth rate is low (Section 8.2) and much of the energy from ammonia oxidation is used for cellular

maintenance. Insufficient energy to support maintenance could ultimately result in death of the cells.

The energy deficit in the cells could be overcome by supplementing the system with hydrazine. Hydrazine is an alternative substrate for the HAO enzyme (section 3), the energy producing step in ammonia oxidation, and is capable of supplying the cells with energy. Chapman *et al*. (2004) tested the concept, however, their results showed the addition of hydrazine caused the accumulation of hydroxylamine, inhibiting methanol and nitrite production. These results imply that their system was not energy limited. However, since such a dramatic decrease in ammonia oxidation was witnessed in the trickle bed system, low cellular energy may still be a problem. The addition of hydrazine to supplement the catabolism, causes the build up of inhibitory products (Chapman, Gostomski, and Thiele 2004) and is therefore not a straightforward solution. Another possible solution is using a cyclic approach to methanol production (Chapman, Gostomski, and Thiele 2004). Periodically exposing the cells to methane followed by a period without methane may give the cells time to recover their energy. Biofilm cells have been shown to recover quickly from starvation (Batchelor *et al*. 1997). These results would also suggest the system was capable of a rapid recovery after the methane had been stopped (Figure 8.2, 8.3 and 8.4). This quick recovery from adverse conditions would allow for short down times for cell recovery. It should be noted this would only be beneficial if methanol production rates and the maximum achievable concentration were sufficient for a viable process (Section 8.3).

8.2 Immobilization and Biomass

Various support materials have been used for immobilizing nitrifying bacteria (Section 5.3). Although good results have been achieved with entrapment methods of immobilization, for simplicity growing a biofilm was chosen. A variety of support materials were considered for this research based on results and recommendations in the literature. They included: limestone, basalt, ceramic, polyethylene (Kaldnes particles), clinoptilolite, glass partilcles (Advance Filtration Media) and activated carbon. Initial batch experiment results, reporting specific nitrite production activity, (Appendix B) showed active nitrite-producing biofilm growth was possible on a variety of these materials including: limestone, basalt, ceramic, polyethylene and clinoptilolite. The glass particles and activated carbon showed poor nitrite production activity. A fair comparison of the ability for the material to grow biofilm could

not be made since the cells may have been in different growth phases. Based on the preliminary results, ceramic raschig rings (6mm) were chosen as the support material for the trickle bed reactor. Ceramic was chosen as a support material because it is inert. This material should not adsorb or react to either ammonia or methane. Other materials may have superior ability to grow biofilm but in order to investigate the behaviour of the *N. europaea* biofilm, an inert surface was advantageous. Zeolite is a commonly used material for nitrification applications but because of the ion exchange characteristics could hide any effect the methane may have on ammonia oxidation capacity. *N. europaea* consistently formed an active biofilm on ceramic raschig rings in batch cultures (Appendix B). Raschig rings have also been well used in packed bed applications and have well defined properties (Perry, Green, and Maloney 1997). Limestone and basalt could not be obtained in sufficient quantity to be used in the reactor. The polyethylene particles (Kaldnes particles) were not used because it was not known how they would behave in a packed bed arrangement.

A laboratory scale experimental apparatus was developed that successfully immobilized a pure culture of *N. europaea.* The initial attempts at maintaining a pure culture in the system were unsuccessful and the slow growth of *N. europaea* made start-up slow. After several attempts, the system was maintained without contamination. The system ran uncontaminated for several months despite various difficulties. Contamination was checked every few days as described in Section 7.5. The difficulties, during setup of the bioreactor due to contamination, could pose a significant problem in the development of a commercial process.

The growth rate of *N. europaea* is dependent on both oxygen and ammonia concentrations. The concentrations of these chemicals within the biofilm vary with the depth of biofilm because of simultaneous diffusion and reaction. The growth rate and activity of the cells will also depend on diffusion of ammonia and oxygen (as they become limiting). The packed bed is a plug flow reactor and the concentration of ammonia in the liquid phase decreases along the length of the reactor. Assuming the concentration becomes limiting, the growth rate and activities of the cells within the system may vary along the length of the packed bed. Therefore, there will be a distribution of these parameters within the system. The estimated quantity of biofilm in the system was based on the measurement of the entire biomass within the trickle bed. Using this method of biomass measurement it is impossible to distinguish between the possible different growth rates (and activities) in the reactor. Consequently,

results based on this measurement, such as growth rate, methanol production activity and ammonia oxidation activity of the cells are averages over the entire contents of the trickle bed.

An advantage of an immobilized system is they have higher biomass concentrations than free cell chemostat systems. When this reactor system was dismantled, biological growth was seen on the surface of the particles. The top of the trickle bed showed biofilm growth only where the distributed liquid had contacted. Further down the packed bed the packing was more completely covered with growth. The biofilm was pink and the growth was not uniform across the surface (Figure 1.1 A). The cells in the biofilm were easily disturbed, suggesting the cells were reversibly attached to the particles. There also was significant growth on the walls of the reactor. The cells on the reactor walls were scraped and added to the particles for the volatile suspended solids analysis.

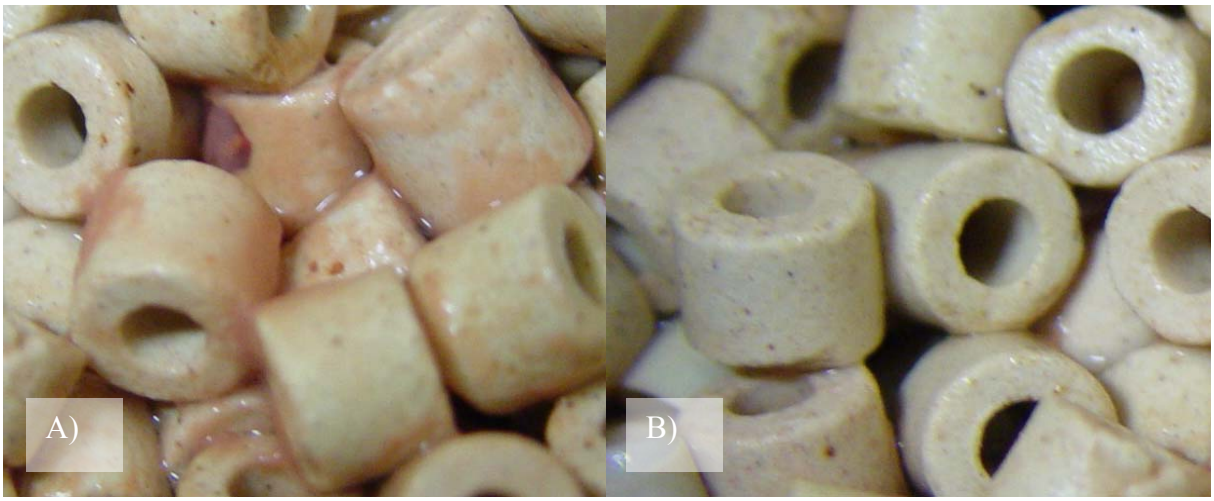

Figure 8.5: Images of the support material at the top of the trickle bed. A) shows some biofilm growth compared with B) that does not have visible biofilm growth.

The amount of volatile suspended solids was measured to be 2.6 ± 0.1 g VSS (Sec. 7.8.7). The amount of free cells in the system was in the range 13 - 26 mg dry weight, much lower than the amount of immobilized cells in the system, assuming dry weight and volatile suspended solids are directly comparable. The growth rate of the biofilm was estimated to be 0.00035 1/hr. The estimation of the growth rate was by solving a steady state mass balance over the biofilm and free cell concentrations (Appendix C). It was assumed that the attachment of free cells was negligible compared with the growth and detachment of the biofilm and that the free cell growth and activity was negligible. The activity of the free cells in the system was not assessed. Retrospectively, an investigation into the activity of these free cells would have been beneficial to confirm the assumption of low activity.

The method of biomass estimation required the destruction of the biofilm. The experiments were done over a two month period and the biomass measured at the end. The system was continuously run with a medium containing approximately 50 mM NH_4^+ until the medium was changed to one with a lower concentration for the methane experiments. This situation was not ideal for accurately knowing biomass concentration at the specific time of the experiments, but was necessary to avoid the risk of contamination of the system by sampling the biomass during operation.

The static liquid hold-up in the trickle bed was measured to be 48.96 ± 9.24 ml and the operating hold-up estimated using dimensionless correlation (Perry, Green, and Maloney 1997) giving a value of 283 ± 9 ml giving a total liquid hold-up in the trickle bed as 332 ± 13 ml. Therefore the average biomass concentration as calculated using these values was 7.82 ± 0.43 g VSS/l. The volume of the packed bed was 2.35 l. This gives a concentration per unit bed volume of 1.11 ± 0.04 g/l. The total volume of liquid in the reactor (including: liquid hold-up in the trickle bed; the volume of the stirred vessel; and the tubing) was 2050 ± 80 ml.

This biomass concentration was much higher that previous work investigating methanol production with *N. europaea*. The biomass concentrations worked with by other author were: 0.005 g/l (Hyman and Wood 1983), 0.07 g/l (Hyman, Murton, and Arp 1988), 0.33 – 0.45 g/l (Chapman, Gostomski, and Thiele 2004). A higher biomass concentration would inevitably mean a higher methanol accumulation rate for a specific volume of reactor, assuming methanol production rates similar to previous reports. Chapman *et al.* (2004) estimated that to achieve an economically feasible process would require a biomass concentration of 5-7 g/l with an activity as shown in batch cultures of 2-3 mmole/g hr, assuming no product inhibition. A higher biomass concentration was successfully achieved with this reactor configuration and immobilization technique. However, the methanol production activity was dramatically less than other reports (Section 8.3).

8.3 Methanol Production by Nitrosomonas europaea.

During the period of methane addition of the reactor, the accumulation of methanol in the system was monitored. The following results show the accumulation of methanol within the reactor system for various runs. The conditions of the system for the various runs is described in Section 7.6.2.3. The starting point of each figure is where the methane addition began.

The error bars on each graph indicate the standard deviation of the gas chromatograph results of at least four repeat injections. The large uncertainties found were a result of measuring concentrations close to the analysis limit of the instrument.

Figure 8.6 shows the accumulation of methanol in the system during run 1. The inlet medium flow of 8.20 ml/min ensured the system had excess ammonia present. The inlet ammonium concentration was 38 mM. The initial rate (over the first 20 mins) of methanol accumulation was 0.025 ± 0.004 mM/hr and the system reached a steady state concentration of 0.037 ± 0.012 mM.

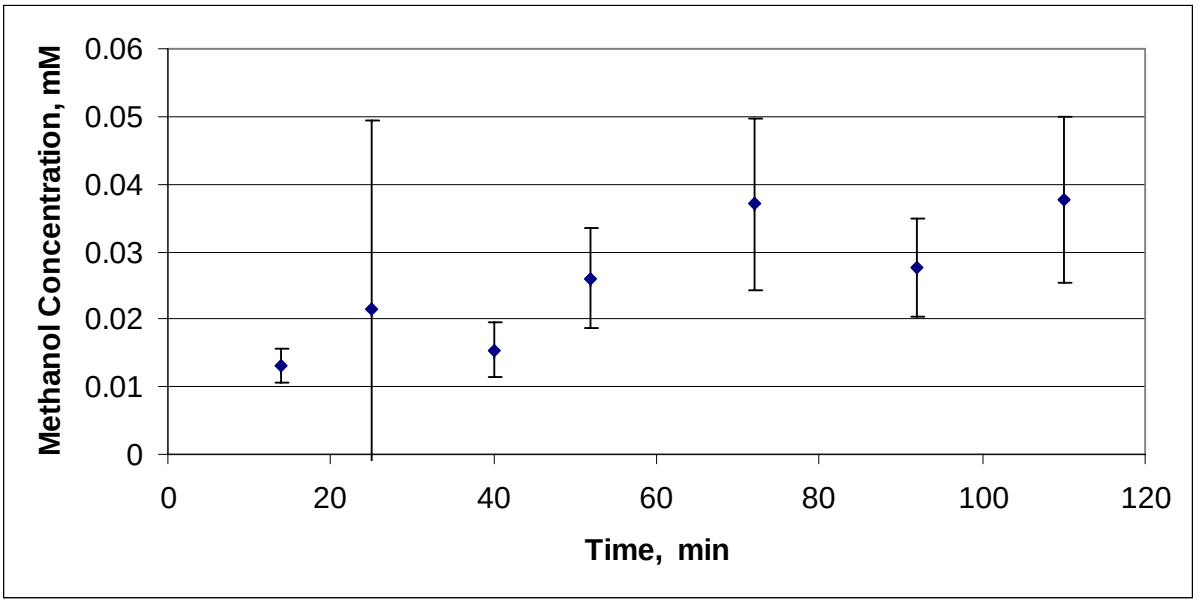

Figure 8.6: Methanol accumulation during run 1. The medium feed flow rate was 8.2 ml/min with an initial excess ammonium concentration of 10 mM.

Figure 8.7 shows the accumulation of methanol in the system during run 2. The inlet medium flow of 2.08 ml/min the excess ammonia in the system upon addition of methane was 2.2 mM. The inlet ammonium concentration was 29 mM. The initial rate of methanol accumulation was 0.082 ± 0.051mM/hr and the system reached a steady state value of 0.040 ± 0.034 mM.

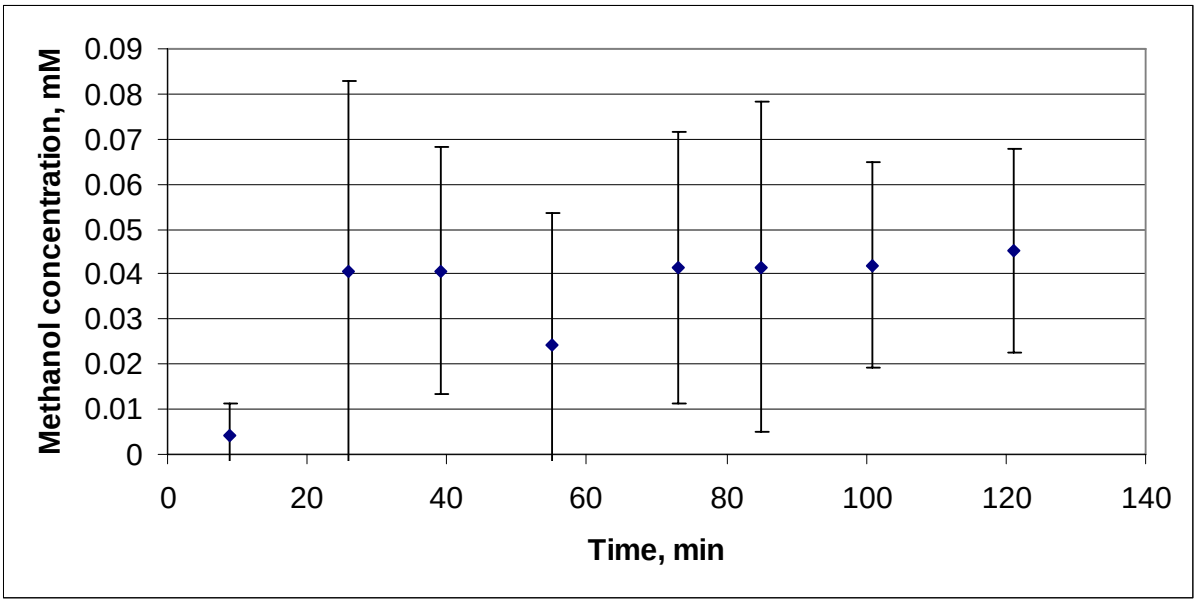

Figure 8.7: Methanol accumulation during run 2**.** The medium feed flow rate was 2.08 ml/min with an initial excess ammonium concentration of 2.2 mM.

Figure 8.8 shows the accumulation of methanol in the system during run 3. The inlet medium flow of 2.04 ml/min the excess ammonia in the system upon addition of methane was 0.3 mM. The inlet ammonium concentration was 16.7 mM. The initial rate of methanol accumulation was 0.143 ± 0.094 mM/hr and the system reached a steady state value of 0.080 ± 0.034 mM

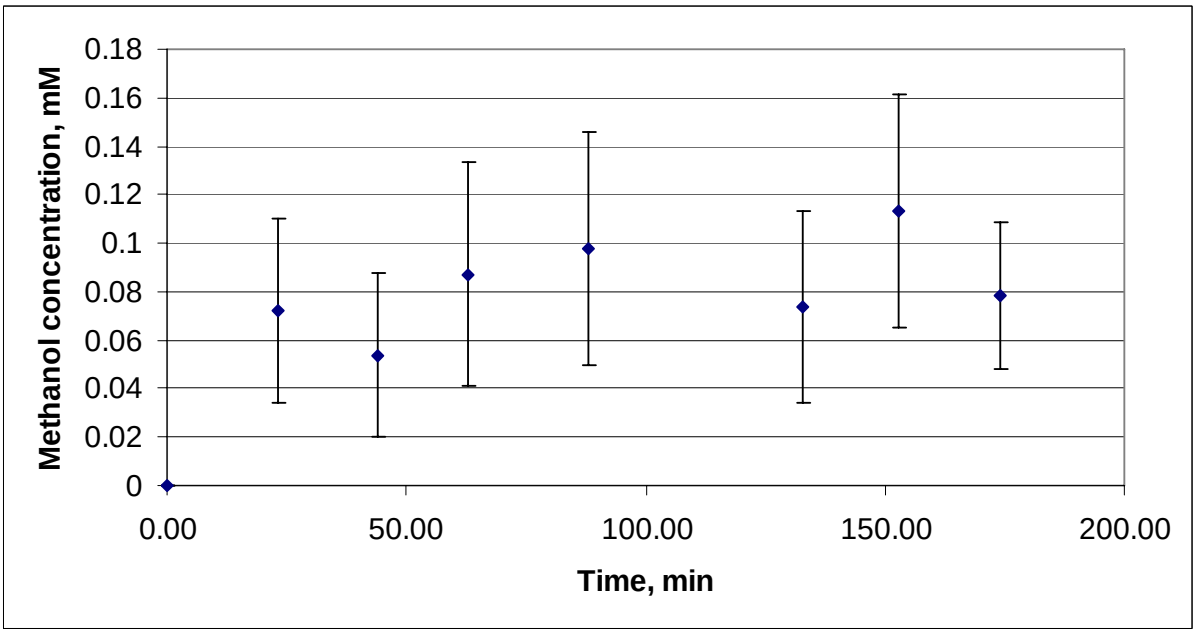

Figure 8.8: Methanol accumulation during run 3**.** The medium feed flow rate was 2.04 ml/min with an initial excess ammonium concentration of 0.3 mM.

Figure 8.9 shows the accumulation of methanol in the system during run 4. Methane was pumped into the system for a period of 6 hour 25 minutes during this run, 4 hours more than the previous runs. This inlet medium flow of 2.04 ml/min the excess ammonia in the system upon addition of methane was 0.5 mM. The inlet ammonium concentration was 19.22 mM. The initial rate of methanol accumulation was 0.143 ± 0.094 mM/hr the system did not reach a steady state, but the rate of accumulation decreased to 0.02 ± 0.01 mM/hr

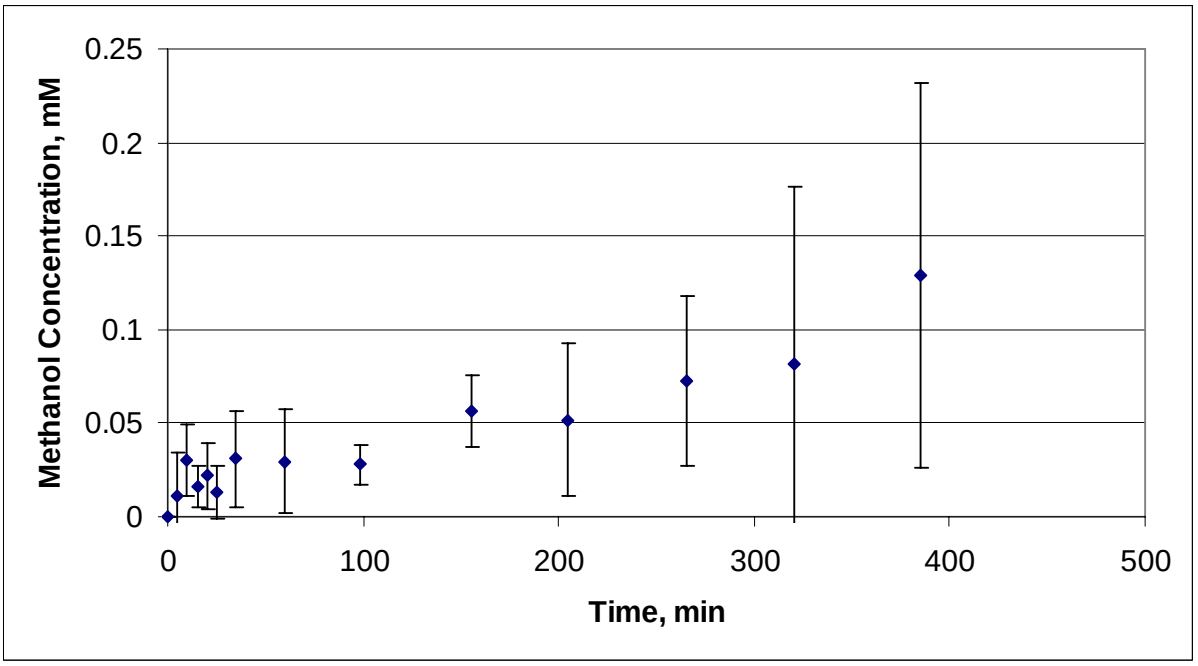

Figure 8.9: Methanol accumulation during run 4**.** The medium feed flow rate was 2.04 ml/min with an initial excess ammonium concentration of 0.5 mM.

The initial methanol accumulation rate was obtained from the figures 8.6-8.9 and the initial methanol production activity was determined over the first 20 minutes of each run. The steady state methanol production activity was also calculated based on steady state methanol concentrations. The results do not show a clear correlation between the residual ammonia and the specific methanol activity.

In a continuous flow system containing cells with a constant activity, the system should reach a steady state methanol concentration. However, estimation of the steady state concentrations based on the initial methanol production activity showed that there was a change in the rate of methanol accumulation. This could be caused by either a change in the rate of production of methanol or an increase in the consumption of the produced methanol. For each of the runs, the rate of accumulation decreased as time progressed and the system approached a steady

state. Table 8.3 compares the initial methanol production activity and the steady state production rate activity. These values are described as the apparent methanol production activities. The results clearly show a decrease in the observed activity.

Table 8.3: Table of methanol production rates

Experiment Description	**Residual ammonium at start of methane addition (mM)**	**Initial Methanol accumulation Rate (mM/hr)**	**Apparent Steady state methanol production Activity (mmol/ g VSS.hr)**	**Apparent Initial Methanol Production Activity (mmol/ g VSS.hr) ***
Run 1	10	0.025 ± 0.004	0.01 ± 0.002	0.02 ± 0.004
Run 2	2.2	0.082 ± 0.051	0.002 ± 0.002	0.07 ± 0.041
Run 3	0.3	0.143 ± 0.094	0.004 ± 0.002	0.12 ± 0.076
Run 4	0.5	0.053 ± 0.044	0.006 ± 0.005$_a$	0.04 ± 0.035

* estimated solving unsteady state mass balance over the first 20 min of each run

$_a$ estimated using final measured value in run 4 for a steady state value

A change in the production rate of methanol would change the rate of accumulation of methanol within the system. Such a change may be caused by a build up of inhibitory products within the system. Products that could be responsible for inhibition of the AMO enzyme are methanol, formaldehyde and/or formaldoxine (Voysey and Wood 1987).

Methanol, formaldehyde and formaldoxine have been shown to inhibit AMO (as discussed above). However, the concentration for which methanol was inhibitory is higher than that achieved in this reactor (5 mM) (Chapman, Gostomski, and Thiele 2004). Formaldehyde and formaldoxine may also be present in the system, although were not specifically measured. Figures 8.2, 8.3 and 8.4 clearly showed that the addition of methane to the system affected ammonia oxidation. However, if the production of any of these further oxidized products caused inhibition of AMO, it would be expected to affect the ammonia oxidation at similar times to the change in methane oxidation, during the experiment. There is no obvious change in the ammonia oxidation activity following that caused by the methane, indicating this not the cause of the change in methanol production rate.

A loss of endogenous reductant could change the activity of the cells (Hyman, Murton, and Arp 1988). Both the oxidation of ammonia and methane by AMO require energy. Therefore as well as competing for AMO's active site they also compete for energy (Hyman, Murton, and Arp 1988). The energy gaining step in the oxidation of ammonia to nitrite is the conversion of hydroxylamine to nitrite. The decrease in the ammonia oxidation as shown in Section 8.1 would lead to a decrease in the amount of hydroxylamine available for energy production, consequently leading to an insufficient amount of reducing power to drive both methane oxidation and ammonia oxidation. *N. europaea* has a higher affinity for ammonia than for methane (Hyman and Wood 1983) implying a decrease in the methanol production rate would be seen before a further effect on ammonia oxidation. Voysey and Wood (1987) compared values of relative specificity of an enzyme for different substrates (k_{cat}/k_m), stating values of 0.008 for methanol, 0.004 for methane compared to 1 for ammonia. They concluded methane was a slightly poorer substrate than methanol, and both are much poorer than ammonia. Chapman *et al.* (2004) investigated this as a possible cause of the rate change and concluded this was not the cause. However, their results did not show a large effect on ammonia oxidation upon addition of methane and it cannot be ruled out as a possibility in this case.

The other cause for the change in the accumulation rate could be the consumption of methanol. The variety of chemicals that can be oxidized by AMO suggests the enzyme is not very specific (Section 4.0). As well as the oxidation of methane, methanol can also be oxidized forming formaldehyde (Voysey and Wood 1987). Methanol consumption has been shown by various authors (Voysey and Wood 1987; Jones and Morita 1983; Chapman, Gostomski, and Thiele 2004). Voysey *et al.* (1987) suggest that *N. europaea* prefers to oxidize methanol rather than methane (see above). The increase in the consumption of the methanol may be due to the increase in the methanol concentration. A decrease in the production of methanol was also reported by Chapman *et al*. (2004) in both batch cultures and a cell recycle chemostat. The methanol concentration that they achieved was much higher than these experiments (0.75 mM in batch, 0.6 mM in chemostat). Their results supported methane-induced methanol degradation, concluding that the decrease in the methanol accumulation was caused by methanol degradation by *N. europaea.*

From the results obtained it is impossible to establish the cause of the change in the methanol accumulation rate. The most likely possibilities are the consumption of methanol and/or the

loss of endogenous reductant. Either possibility would indicate this system is not suitable for a methanol production process.

Table 8.4 shows the maximum achieved methanol concentrations in the system. These values are much lower than reported maximum concentration. Chapman *et al.* (2004) were able to achieve 0.75 mM in a continuous system (not at steady state) and approximately 2 mM in batch experiments. It can be concluded that whatever is responsible for the decrease in methanol production, it was not improved (in fact worsened) in biofilm immobilized cells compared to free cells.

Table 8.4: Maximum methanol concentration

Run	Maximum Measured Methanol Concentration mM
1	0.037 ± 0.012
2	0.040 ± 0.034
3	0.113 ± 0.048
4	0.129 ± 0.102

Table 8.5 shows a comparison of the initial specific methanol production activity measured compared with the activities as reported by other authors. The specific rates of methanol production measured during these experiments were considerably lower than those in other reports (approximately 10 - 20 fold less). There is a range of possible explanations that could either be solely responsible or jointly responsible for the low rates. These are: the low growth rate of cells in the biofilm; protection of the cells by extracellular material; low selectivity for methane in the presence of ammonia; or oxygen limitation within the biofilm.

Table 8.5: Comparison of *Nitrosomonas europaea* methanol production rates by various authors.

Reference	**Specific Methanol Production Activity, (mmol/g dry weight. hr)**	**Growth Rate (1/hr)**
Hyman *et al.* (1983)	1.06	N/A
Hyman *et al.* (1988)	0.28 - 0.5	N/A
Chapman *et al.* (2004)a	1.00 – 2.23	N/A
Chapman *et al.* (2004)b	0.17 – 0.7	0.0008 - 0.0016
Chapman *et al.* (2004)$_b$	0.85 – 1.1	0.0042 - 0.0091
This work $_d$	0.02 – 0.12	0.00035 $_c$

a batch culture

b recycle chemostat system

c average value estimated using a steady state mass balance assuming negligible free cell growth

N/A not published or unavailable

d measured as volatile suspended solids g VSS

Low growth rates and extracellular material have been proposed as explanations why cells in biofilms are less affected by inhibitory substances (Powell and Prosser 1992). The methanol production rate could be expected to be lower than for free cells due to other authors' previous observations of increased resistance to inhibitory or toxic material (Powell and Prosser 1992; Bryers 2000; Powell and Prosser 1991). However, the extent to which the biofilm affected the methanol production was unexpectedly large.

The low methanol production activity may be caused by the low growth rate of the cells. Low growth rates affect enzyme kinetics either through low activity of the enzyme or low expression of the enzyme. Methane oxidation in *N. europaea* is said to occur competitively with ammonia oxidation through the AMO enzyme. Ammonia oxidation is the main energy yielding reaction in *N. europaea* and therefore AMO activity is closely coupled to the growth rate (Chain et al. 2003). The average growth rate of the cells in the trickle bed was 0.00035 1/hr (Appendix C for calculation). This was lower than the growth rates in other methanol production experiments (Chapman, Gostomski, and Thiele 2004). Although the growth rate of the cells in batch systems used by Hyman *et al*. (1983), Hyman *et al.* (1988) and Chapman *et al.'s* (2004) was not quantified, the cells used were at some point in the exponential growth

phase. It can be assumed, therefore, that the growth rate was significantly higher than those in the biofilm. The work of Chapman *et al.* (2004) with a chemostat system showed lower methanol production at lower growth rates down to a growth rate of 0.0008 1/hr. It is therefore likely that low methanol production is at least partly due to low growth rate, supporting the conclusions of Chapman *et al.* (2004) that methanol production is dependent on growth rate.

Since methane behaves competitively with ammonia, it may be possible that the presence of residual ammonia caused the low methanol production rate. Chapman *et al.* (2004) showed conflicting results regarding the role of ammonia in methanol oxidation. Their batch study indicated that residual ammonia had no effect on the methanol production rate. However, results from a cell recycle fermenter indicated a need for the residual ammonia to be less that 0.5 mM for methanol production. Hyman *et al.'s* (1988) work showed that methanol can be produced with ammonia present. No significant difference that could account for such a low methanol production could be seen from the results at varying ammonium concentration (Table 8.3). All the specific productions rates over the range tested were significantly lower than other reported results.

The oxidation of methane is an oxygen insertion reaction synonymous with the oxidation of ammonia by *N. europaea* (Section 3.0). Therefore methane oxidation would also have been affected by the oxygen limitation exerted on the system through the addition of methane, proposed above (Section 8.1) for the loss of ammonia oxidation. The loss of ammonia oxidation that resulted from the addition of methane was a maximum of 70%. If it is assumed that this ammonia oxidation inhibition was entirely the fault of oxygen limitation then the maximum possible loss of methanol production activity (compared to free cells) is 70%. The measured methanol production rates at 5 – 10 % of the reported values. Therefore oxygen limitation could not solely account for the low methanol production activity but it may contribute.

The growth rate, residual ammonia concentration and oxygen limitation of the system may all have an impact of the methanol production activity of the cell. However, the low growth rate and the subsequent low AMO activity of the cells was likely the dominant influencing factor.

In order to improve the specific methanol production rate, the cells need to be growing at a higher growth rate. An attempt to increase the biomass concentration with a free cell culture has been attempted using a cell recycle reactor (Chapman, Gostomski, and Thiele 2004). This reactor configuration allows the growth rate to be controlled. However the maximum biomass concentration was limited by an unknown limiting nutrient. In order to encourage a high growth rate in a biofilm reactor, a thin, active layer of biofilm needs to be maintained. Using a trickle bed, a thinner layer can be encouraged by having a large surface available for biofilm growth. The hydraulics of the system can also be used to influence of the biofilm thickness. By increasing the hydraulic loading the shear forces on the biofilm are increased, increasing the sloughing of the biofilm, creating a thinner, actively growing biofilm. The hydraulic loading could be increased in this system by increasing the recycle medium flow rate. Alternatively, reactor systems with higher shear forces, such as a fluidized bed, could be used. However changing the reactor configuration would lose the advantage of having a continuous gas phase. It would also require a reconsideration of support material.

8.4 Biomethanol Production Processes

Two biological routes for methanol production from methane have been identified. They are the use of methanotrophic bacteria with inhibition of methanol dehydrogenase (Section 2.3) or use of nitrifier co-metabolism, as investigated in this research. Investigations attempting to produce methanol via either of these routes have focused on the need to increase the methanol accumulation rate by increasing the methanol production activity or through increasing the biomass concentration in the system. Table 8.6 is a summary of reported results compared with this work, indicating important parameters such as biomass concentration and methanol production activity.

Table 8.6: Summary of results from various biological methanol production investigation

Reference	**Species**	**Cell State**	**Biomass Concentration (g/l)**	**Maximum Methanol Production Activity (mmol/g dry weight·hr)**
Chapman *et al.* (2004)	N. europaea	Free cells in a cell recycle chemostat	0.33 – 0.45	1.2
This work	N. europaea	Immobilized in a biofilm	7.82 ± 0.43*	0.12 ± 0.08*
Furuto *et al.* (1999)	*Methylosinus trichosporium* OB3b	Free cells in a semi-contiuous chemostat	0.04	3.17
Mehta *et al.* (1991)	*Methylosinus trichosporium*	Immobilized in cellulose in a chemostat	2.00	25.00 [a]

* mass in grams volatile suspended solids

[a] optimised reactor system

The trickle bed bioreactor developed for this research had a higher biomass concentration than other reactor systems (Table 8.6). The developed reactor was successful in creating an environment with a high concentration of biomass. However, the advantage of high biomass concentrations was offset by the low activity of the cells (Section 8.3). Methanotrophic bacteria show good methanol production activity with reported values ranging from 3.17 mmol/g dry weight.hr (Furuto, Takeguchi, and Okura 1999) to 25 mmol/g dry weight. hr (Mehta, Mishra, and Ghose 1991). These values are higher than those for methanol production using *N. europaea*, where reported values range between 0.5 and 2.23 mmol/ g dry weight.hr. Methane oxidation by methanotrophs is the first step in of the main energy-gaining metabolism. The oxidation of methane by *N. europaea*, however, does not yield energy (Chain *et al*. 2003). Therefore methane oxidation is likely to be a favourable metabolic process compared with nitrification co-metabolism.

Authors have immobilized methanothrophs successfully using both adsorption (Yu *et al.* 1998) and cross linking (Mehta, Mishra, and Ghose 1991), retaining significantly higher activities than those seen in *N. europaea*. Both groups have demonstrated a good retention of

previous free cell activity following immobilization. Yu *et al.* (1998) found that by adsorbing the cells onto activated carbon, over 50% of the free cell activity could be retained. They also found that using a combination of adsorption and entrapment in agar resulted in a further retention of activity. Mehta *et al*'s (1991) DEAE-cellulose linked cells retained 80% of the activity of the free cells' activity. The results for *N. europaea* showed a significant loss of methanol production activity after immobilization in a biofilm. Compared to the results of free cell measurements which have an activity of 1.0 mmol/g dry weight.hr (Chapman, Gostomski, and Thiele 2004) the cells have in the range of 1% - 6% of the free cells' activity. These values were also optimistic as they are based on the higher initial rates seen in the reactor and were not retained for extended times.

In order to utilize either of these bacteria for a biological synthesis process, the rates of methanol production need to be improved. Two essential methods for improved rates are: higher methanol production activity and/or increased biomass concentrations. Due to the higher activity and the more successful immobilization techniques (Mehta, Mishra, and Ghose 1991; Yu *et al*. 1998) methanotrophic bacteria offer an improved rate for methanol production, when compared with *N. europaea*.

However, overcoming the barrier of sufficient rates is not the only problem in methanol process development. Both *N. europaea* and methanotrophic bacteria are sensitive to the methanol concentration in the system. Methane oxidation of methane by methanotrophs becomes inhibited at 6 mM (Furuto, Takeguchi, and Okura 1999). *N. europaea* also shows inhibition at 5 mM (Chapman, Gostomski, and Thiele 2004). This concentration is significantly lower than the 100 mM that is suggested to be a minimum requirement for an economically feasible process (Chapman, Gostomski, and Thiele 2004). It is therefore unlikely that either methanotrophic bacteria or *N. europaea* could be used for a biosynthesis process.

9 Conclusions

The overall aim of this research was the development of a process in which methanol could be effectively produced from methane. Specifically the project was to use cell immobilization of *Nitrosomonas europaea* to overcome some of the metabolic restrictions of this bacterium, such as low biomass concentrations and inhibition by methane and its oxidized derivatives. It was possible that a process using immobilized bacteria could produce methanol to a higher concentration and at an improved rate.

A trickle bed bioreactor with a recycle loop containing pH and temperature control was developed. The reactor successfully contained cells immobilized in a biofilm. Through this immobilization a high biomass concentration of 7.82 ± 0.43 g VSS/l was achieved, meeting a target suggested by Chapman *et al.* (2004) as a suitable amount of biomass required to meet a minimum 100 mM concentration of methanol required for commercial development. Other investigations have used much lower biomass concentration such as 0.33 – 0.45 g dry weight/ l. The biomass is a critical parameter in achieving a higher methanol production but needs to be coupled with sufficiently high methanol production activity.

The methanol production activity of the immobilized cells in this work was significantly lower than previous reported activities, being approximately 10 - 20 % of their activity. This low activity was likely due to a combination of low average growth rate in the reactor and oxygen limitation. The initial rate of methanol accumulation within the system was shown not to reach the steady state value. The results showed a drop in the methanol accumulation rate, owing to the increasing consumption of methanol and/or the result of reduced endogenous reducing power.

The addition of methane to the system had a detrimental effect on the ammonia oxidation within the system. On addition of methane the ammonia oxidation activity dropped to as low as 30% of the pre-methane activity. This inhibition was possibly due to decreased oxygen concentration caused by the dilution of the oxygen in the gas stream and possibly the inhibition of AMO by methane.

Overcoming the challenges of obtaining a high biomass concentration by immobilization needs to be coupled with increased high growth rates for an immobilized system to be able to achieve high production rates. However, reaching a higher production rate is not a complete solution. *Nitrosomonas europaea* is inhibited by methanol at 5 mM. Immobilization of the bacterium did not allow methanol to accumulate up to this concentration and offers no possibility of exceeding it. A methanol biosynthesis route using *N. europaea* in their natural forms is therefore unlikely to be capable of being used in such a process.

10 Recommendations

The immobilized *Nitrosomonas europaea* trickle bed bioreactor did not show any results that suggest that this reactor configuration using this bacterium could be developed into a commercial methanol production process. However, the reactor system that was developed will have use for further research. A feature of this trickle bed reactor contrary to other trickle bed setups is the complete isolation of the system allowing investigation into areas that a typical trickle bed would not be capable of (or inferior in). The reactor system that was developed may prove to be a valuable research tool for the investigation of biofilm based processes.

The way the system has been developed allows the species in the system to be controlled. The system could be used for a pure culture or for mixed cultures. This would allow investigation into how bacteria behave in a reactor system alone or with other species and could be a valuable tool for the investigation of spatial organisation of bacteria under a variety of conditions.

Typical trickle bed reactors are supplied oxygen though natural flows. This system is completely isolated from the external environment and sterile oxygen is pumped in. This allows the gas flows and composition of gases to be controlled. By controlling the gas composition the effect various gases have on a system could be investigated.

The ability of the system to control a range of parameters, including liquid flows and compositions, gas flows and composition, pH and temperature, and bacterial communities also makes the system ideal for model verification.

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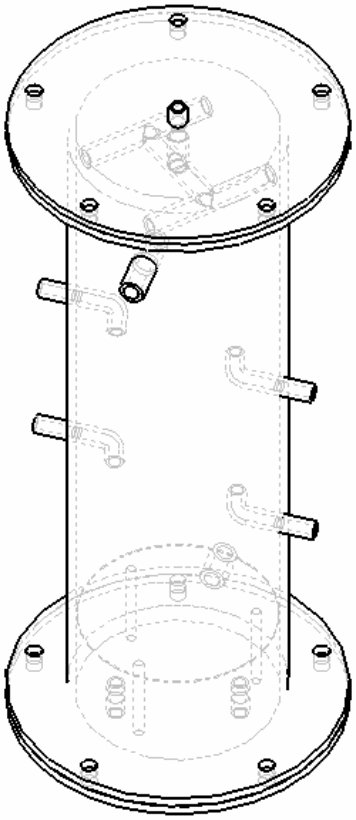
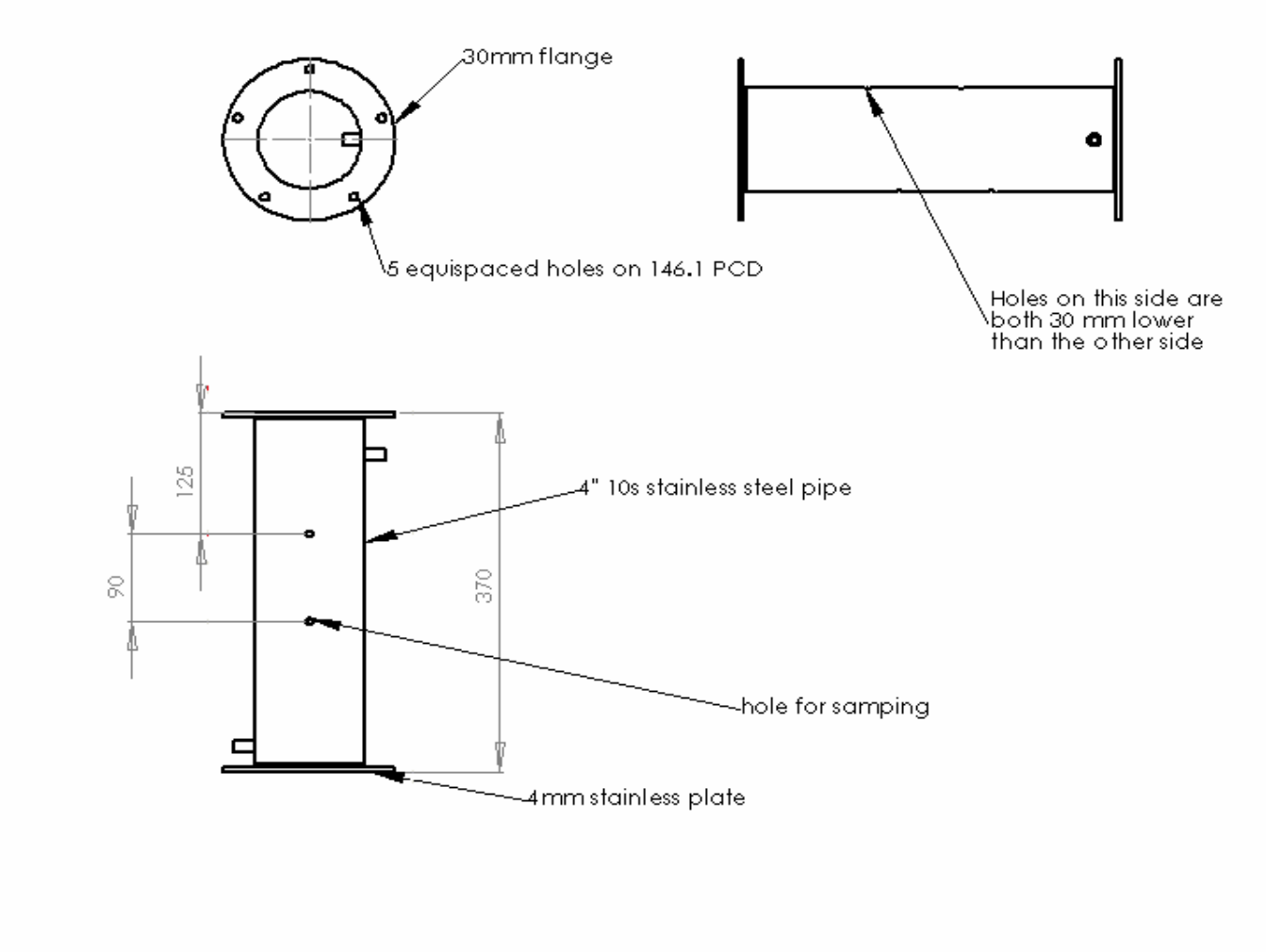
Appendix A Engineering Drawings

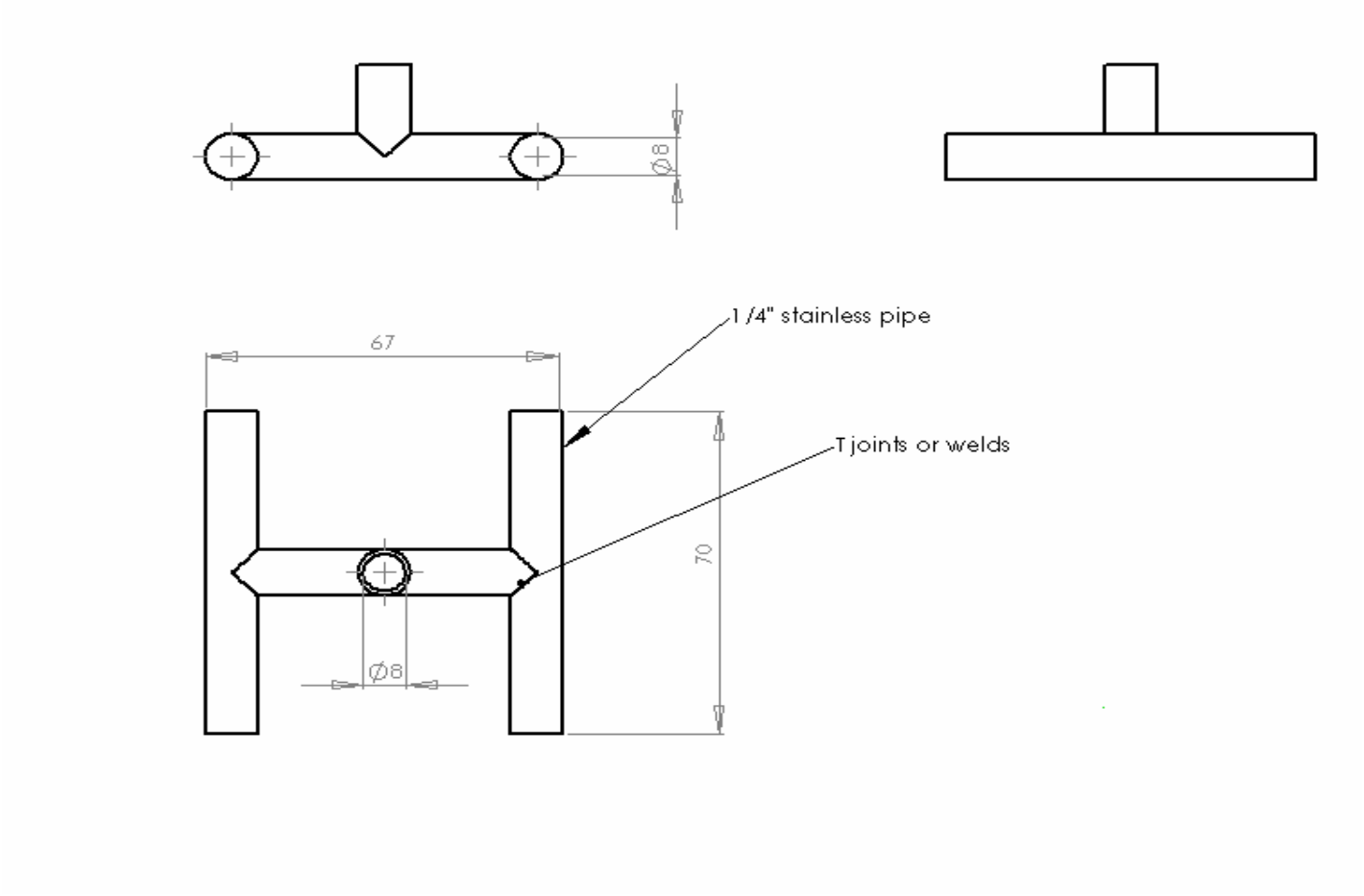
The following are the engineering drawings of the trickle bed and its parts



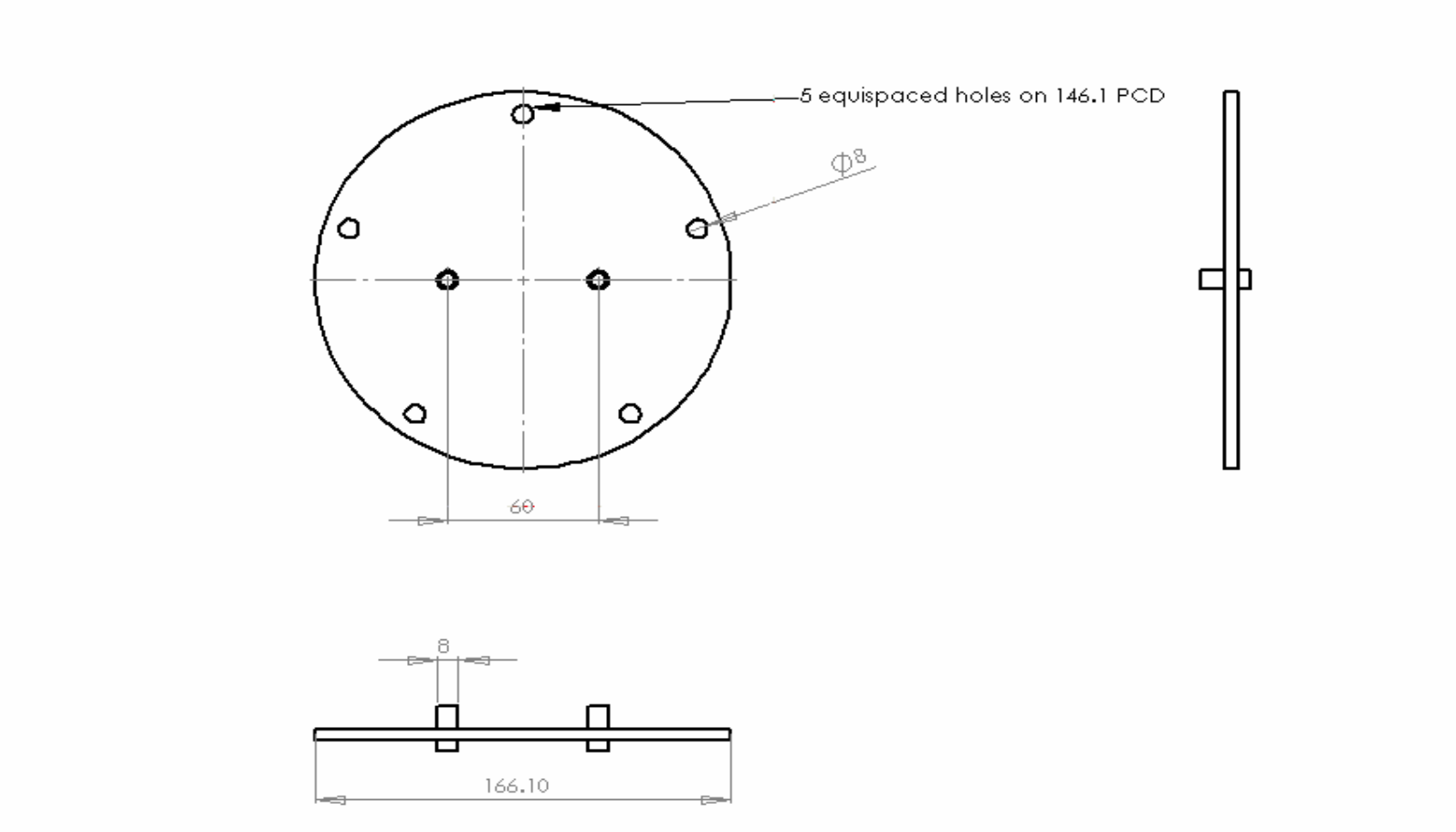
Engineering drawings of the main body of the trickle bed.



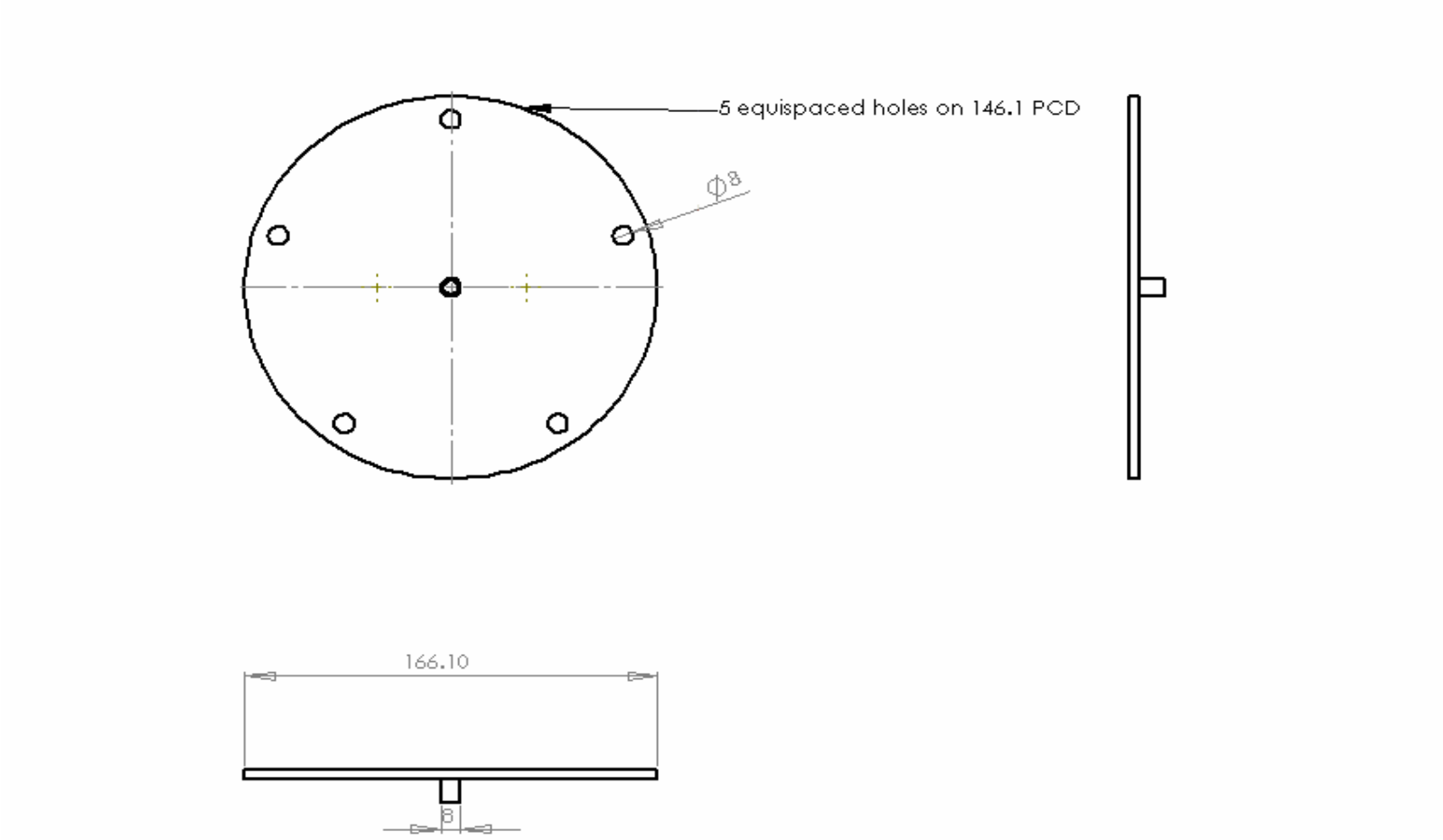
Drawing of the liquid distributor positioned about the packed bed.



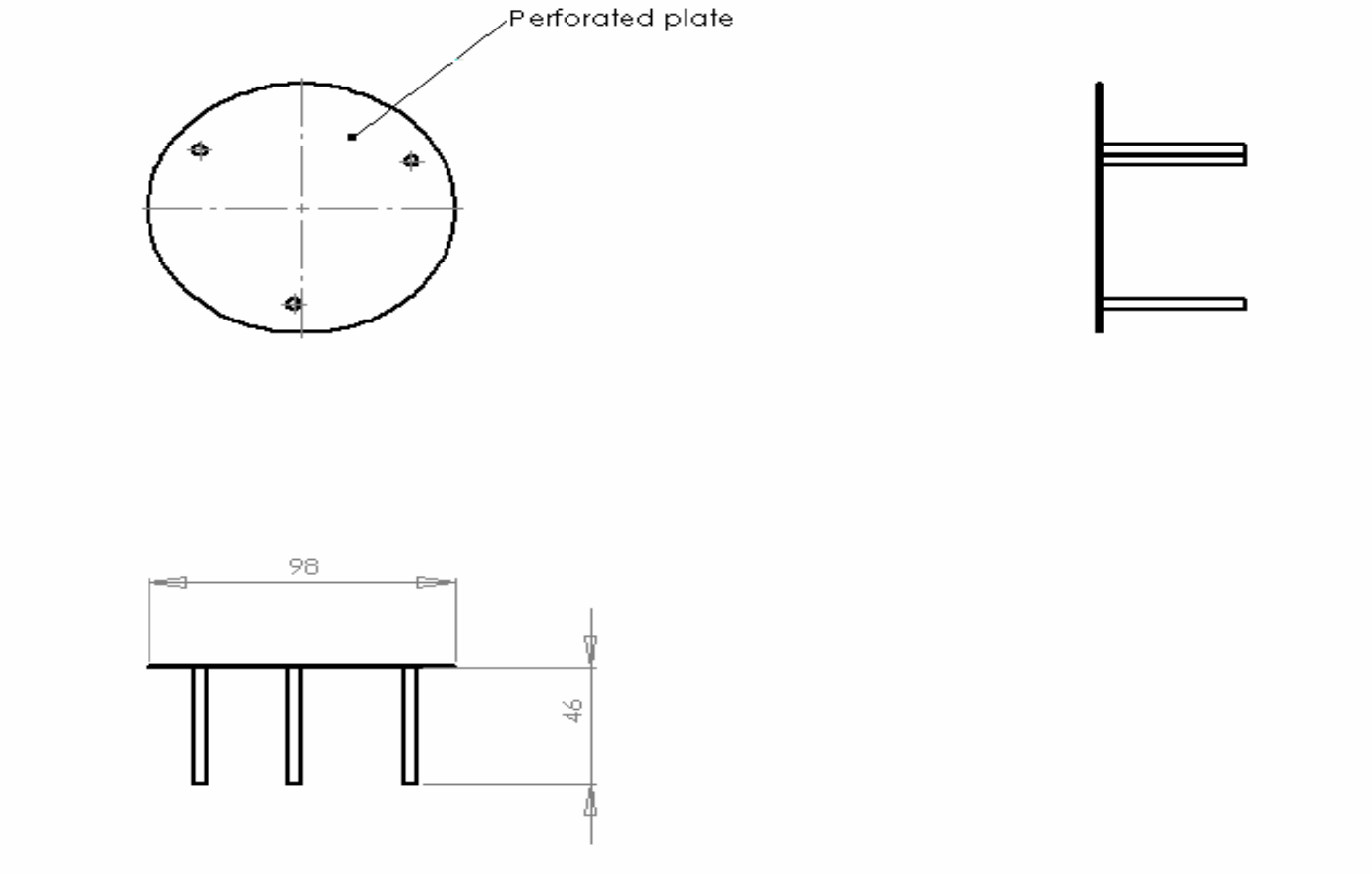
Drawing of the base plate of the trickle bed.



Drawings of the top plate of the trickle bed.



Drawing of the perforated tray that supported the packed bed.



Appendix B Batch activity test results

Table 1.1: Table of biofilm nitrite production activities (μmol/μgprotein/hr) on various supports

	Run 1		**Run 2**		**Run 3**		**Run 4**	
Free Cell or support type	**Flask 1**	**Flask 2**	**Flask 1**	**Flask 2**	**Flask 1**	**Flask 2**	**Flask 1**	**Flask 2**
Free suspension	4.80E-02	3.78E-02	1.49E-02	7/16E-03	5.76E-03	6.88E-03	7.49E-03	3.32E-03
Activated carbon	No growth pH changed	No growth pH changed	-	-	-	-	-	-
Glass particles	1.23E-04	N/A	Contaminated	Contaminated	-	-	-	-
Kaldnes polyethylene	-	-	7.52E-05	Contaminated	No growth	7.34E-05	6.61E-04	5.59E-04
Clinoptilolite	-	-	-	-	6.66E-04	3.87E-3	2.09E-04	No growth
Ceramic raschig rings	-	-	-	-	8.53E-3	7.65E-3	8.30E-05	8.79E-04
Limestone	-	-	-	-	-	-	2.71E-03	7.37E-03
Basalt	-	-	-	-	-	-	3.38E-03	5.16E-03

Appendix C Sample calculation

Growth Rate Calculation

The mass balances of the bacteria in a continuous reactor system are shown in Eqs. 18 - 20. Equation 18 is a mass balance of the biofilm, with a term for the growth of the biofilm, one for the attachment of biofilm and one for biofilm detachment.

$$\frac{dB}{dt} = \left(r_{dep} + r_g - r_{\det}\right) \qquad (18)$$

Where:

$\frac{dB}{dt}$ change in biofilm mass (B) with respect to time (t), mg dry weight. hr^{-1}

r_{dep} rate of bacterial deposit on surface, mg dry weight. hr^{-1}

r_g rate of biofilm growth on surface, mg dry weight. hr^{-1}

r_{det} rate of biofilm detachment from the surface, mg dry weight. hr^{-}

Assuming the rate of attachment is negligible compared to the detachment and growth then at steady state the growth rate is equal to the rate of detachment (equation 3).

$$r_g = r_{\det} \qquad (19)$$

Eq. 20 is a mass balance for the bacteria in free suspension including: biomass flowing into the system; biomass out of the system; free cell biomass growth; and detachment of biomass from the biofilm.

$$\frac{dX}{dt} = \frac{F_{in}}{V} X_{in} - \frac{F_{out}}{V} X_{out} + \mu X + \frac{r_{\det}}{V} \qquad (20)$$

Where:

$\frac{dX}{dt}$ change in free cell concentration with respect to time (t), mg dry weight L^{-1} hr^{-}

1

F_{in}	flow into the system, L hr^{-1}
F_{out}	flow out of the system, L hr^{-1}
V	working volume, L
X	free cell concentration, mg dry weight L^{-1}
μ	cell growth rate, hr^{-1}

Assuming the biomass concentration in the inlet was zero; and in steady state the equation becomes:

$$\frac{r_{\det}}{V} = \frac{F_{out}}{V} X_{out} - \mu X \qquad (21)$$

Assuming free cell growth is negligible

$$r_g = F_{out} X_{out} \qquad (22)$$

$$F_{out} = F_{in} = 2.04 g/ml = 122 ml/hr$$

$$X_{outOD} = 0.04(OD_{600})$$

Converting optical density to mg/ml using conversion from Chapman *et al*. (2003).

$$X_{out} = (0.1866)(0.04)$$

$$X_{out} = 0.0075 mg/ml$$

$$r_g = (0.0075)(122)$$

$$r_g = 0.915 mg/hr$$

The total mass of the biofilm (B) was 2600 mg VS, therefore the growth rate is

$$\mu_{biofilm} = \frac{0.915}{2600} = 0.00035 \frac{1}{hr}$$

Mass balance over the entire reactor system for ammonia

$$V\frac{dC_{NH4}}{dT} = F_iC_{NH4i} - F_oC_{NH4o} + R_{generation} - R_{Consumption}$$

$$D = \frac{F_i}{V}; R_{generation} = 0;$$

$$\frac{dC_{NH4}}{dT} = DC_{NH4i} - DC_{NH4o} - \frac{R_{Consumption}}{V}$$

$$R_{Consumption} = \left(DC_{NH4i} - DC_{NH4o} - \frac{dC_{NH4}}{dT} \right)V$$

Where:

$\frac{dC_{NH4}}{dT}$ Change in ammonium concentration (mM) with time (min) estimated as an average over the first 60 after methane addition

$R_{consumption}$ Rate of consumption of ammonium (mMol/min)

V Total volume of the reactor (ml) = 2.05 l

At steady state $dC_{NH4}/dT = 0$ and the equation becomes

$$R_{Consumption} = (DC_{NH4i} - DC_{NH4o})V$$

Calculation for run 3

Steady state (before methane addition)

$R_{consumption}$ = $(DC_{NH4i} - DC_{NH4o})V$

= $((9.92 \times 10^{-4})(16.74) - (9.92 \times 10^{-4})(0.52))2.05$

= 0.033 mmol/min

$ActivityNH_4$ = $60(R_{consumption})/B$

= (60)(0.033)/(2.6)

= 0.77 mMol/ gVSS hr

Unsteady state (after methane addition)

dC_{NH4}/dT for run 3 from figure 8.3 over first 60 mins = 0.011 mM/min

$R_{consumption}$ $= (DC_{NH4i} - DC_{NH4o} - dC_{NH4}/dT)V$

$= ((9.92 \times 10^{-4})(16.74) - (9.92 \times 10^{-4})(0.52) - 0.011)2.05$

$= 0.010$ mmol/min

$ActivityNH_4$ $= 60(R_{consumption})/B$

$= (60)(0.010)/(2.6)$

$= 0.23$ mMol/ gVSS hr

Mass balance over the entire reactor system for nitrite

$$V\frac{dC_{NO_2^-}}{dT} = F_i C_{NO_2^-} - F_o C_{NO_2^-} + R_{generation\ NO_2^-} - R_{Consumption}$$

$$D = \frac{F_i}{V}; R_{consumption\ NO_2^-} = 0; C_{NO_2^-} = 0$$

$$\frac{dC_{NO_2^-}}{dT} = \frac{R_{generation\ NO_2^-}}{V} - DC_{NO_2^-}$$

$$R_{gereration_{NO_2^-}} = \left(DC_{NO_2^-} + \frac{dC_{NO_2^-}}{dT} \right) V$$

Where:

$\frac{dC_{NO_2^-}}{dT}$ Change in nitrite concentration (mM) with time (min) estimated as an average over the first 60 after methane addition

$R_{gernerationNO2}$ Rate of generation of nitrite (mMol/min)

V Total volume of the reactor (ml)

At steady state $dC_{NO2-}/dT = 0$ and the equation becomes

$$R_{gereration_{NO_2^-}} = \left(DC_{NO_2^-} \right) V$$

Calculation for run 2

Steady state

$R_{gernerationNO2}$ $= (DC_{N02-})V$

$= (1.03 \times 10^{-3})(22.26)2.05$

$= 0.046$ mmol/min

$ActivityNH_4$ $= 60(R_{gernerationNO2})/B$

$= (60)(0.046)/(2.6)$

$= 1.07$ mMol/ gVSS hr

Unsteady state

dC_{NO2}/dT for run 2 from figure 8.3 over first 60 mins = -0.039 mM/min

$R_{gernerationNO2}$ $= (DC_{NO2} + dC_{N02}/dT)V$

$=((1.01 \times 10^{-3})(21.27))-0.034)2.05$

$= -0.036$ mmol/min

$Activity_{NO2-}$ $= 60(R_{gernerationNO2})/B$

$= (60)(-0.036)/(2.6)$

$= -0.83$ mMol/ gVSS hr

Mass balance over the entire reactor system for methanol

$$V\frac{dC_{MeOH}}{dT} = F_i C_{MeOHin} - F_o C_{MeOHo} + R_{generation} - R_{Consumption}$$

$$D = \frac{F_i}{V}; R_{consumtion} = 0; C_{MeOHin} = 0$$

$$\frac{dC_{MeOH}}{dT} = \frac{R_{Generation}}{V} - DC_{MeOHo}$$

$$R_{Generation} = \left(\frac{dC_{MeOH}}{dT} + DC_{MeOHo}\right)V$$

Where:

$\frac{dC_{MeOH}}{dT}$ Change in methanol concentration (mM) with time (min) estimated as an average over the first 20 min of the experiment.

$R_{generation}$ Rate of consumption of ammonium (mmol/min)

V Total volume of the reactor = 2.05 l

At steady state

$$\frac{dC_{MeOH}}{dT} = 0$$

$$R_{Generation} = (DC_{MeOH})V$$

Calculation for run 2

Steady state

C_{MeOH} at steady state = 0.04 mM

$R_{gernerationMeOH}$ = $(DC_{MeOH})V$

= $(1.03 \times 10^{-3})(0.04)2.05$

= 8.22×10^{-05} mmol/min

$Activity_{MeOH}$ = $60(R_{gernerationMeOH})/B$

= $(60)(8.22 \times 10^{-05})/(2.6)$

= 0.002 mmol/ gVSS hr

Unsteady state

dC_{MeOH}/dT for run 2 from figure 8.3 over first 20 mins = 1.37×10^{-03} mM/min

C_{MeOH} at 20 mins = 0.027 mM

$R_{gernerationMeOH} = (DC_{MeOH} + dC_{MeOH}/dT)V$

$= ((1.01 \times 10^{-3})(0.027)) + 1.37 \times 10^{-03})2.05$

$= 0.003$ mmol/min

$Activity_{MeOH} = 60(R_{gernerationNO2})/B$

$= (60)(0.003)/(2.6)$

$= 0.07$ mmol/ gVSS hr

Appendix D Data Tables

Run 1

Feed flow rate	F_i	2.04	g/min
Feed flow rate	F_i	2.04	ml/min
Feed NH4+ concentration	C_{NH4i}	38.70	mM
Fi/V	D	8.15E-01	min^{-1}
Fi/V		4.89E+01	hr^{-1}
Gas flow rate	G	1.09	l/min
gas composition		40%	

Time	T, minutes	O.D.	NO2-, mM	NH4+, mM	Methanol, mM
08:38	0.00	0.076	31.11	1.74	-
08:45	5.00	-	-	-	-
09:14	36.00	0.074	34.16	1.95	-
09:48	70.00	0.078	35.90	2.04	-
10:15	97.00	0.085	35.73	1.54	-
10:45	127.00	0.088	36.45	1.97	-
11:10	152.00	0.088	31.60	2.21	-
11:32	174	0.073	33.46	1.65	-
11:55	197	0.066	33.46	5.60	-
12:28	230	0.06	30.86	8.87	-
12:48	250	0.056	30.60	9.57	-
12:57	259	0.056	28.54	9.98	0.000
13:05	267	-	-	-	-
13:19	281	0.06	25.87	12.19	0.013
13:30	292	0.057	24.83	13.23	0.021
13:45	307	0.057	25.18	16.38	0.015
13:57	319	0.053	24.21	17.97	0.026
14:17	339	0.052	21.63	19.03	0.037
14:37	359	0.047	21.37	20.57	0.028
14:55	377	0.047	18.78	21.64	0.038
15:07	389	0.045	18.17	22.84	-
16:36	478	0.064	22.74	16.97	-
17:25	527	0.066	25.87	14.57	-
20:20	702	0.107	32.14	12.98	-

Run 2

Feed flow rate	F_i	2.08	g/min
Feed flow rate	F_i	2.08	ml/min
Feed NH4+ concentration	C_{NH4i}	29.12	mM
Fi/V	D	1.01E-03	min^{-1}
Fi/V		6.08E-02	hr^{-1}
Gas flow rate	G	1.07	l/min
Gas composition		40.00	%

Time	T, minutes	O.D.	NO2-, mM	NH4+, mM	Methanol, mM
10:04	0.00	0.036	38.04	3.73	-
10:39	35.00	0.038	38.24	3.37	-
11:05	61.00	0.037	37.72	2.97	-
11:36	92.00	0.042	38.56	2.76	-
12:00	116.00	0.042	38.77	2.56	-
12:24	140.00	0.04	40.25	2.35	-
12:43	159.00	0.041	37.54	2.21	0.004
13:00	176	0.044	36.20	1.33	0.040
13:13	189	0.042	35.57	1.49	0.041
13:29	205	0.042	33.57	1.72	0.024
13:47	223	0.035	33.53	2.02	0.041
13:59	235	0.036	31.18	2.11	0.042
14:15	251	0.029	33.14	1.48	0.042
14:35	271	0.029	33.04	2.12	0.045
14:48	284	0.028	32.51	-	-
15:05	301	0.028	31.70	-	-
15:15	311	0.027	34.41	2.67	-
15:35	331	0.027	34.55	2.15	-
16:46	402	0.03	34.62	-	-
19:20	556	0.028	31.98	0.51	-

Run 3

Feed flow rate	F_i	2.04	g/min
Feed flow rate	F_i	2.04	ml/min
Feed NH4+ concentration	C_{NH4i}	16.74	mM
Fi/V	D	9.92E-04	min^{-1}
Fi/V		5.95E-02	hr^{-1}
Gas flow rate	G	0.75	l/min
Gas composition		40.00	%

Time	T, minutes	O.D.	NO2-, mM	NH4+, mM	Methanol, mM
09:01	0.00	0.03	33.53	0.52	-
09:45	44.00	0.029	32.81	0.28	-
10:22	81.00	-	33.25	0.27	-
11:17	136.00	0.035	31.98	0.32	0.013
11:40	159	0.033	30.25	0.67	0.012
12:01	180	0.034	30.53	0.84	0.012
12:20	199	0.035	28.50	0.95	0.011
12:45	224	0.033	27.91	1.70	0.011
13:02	241	0.033	27.43	1.98	0.011
13:30	269	0.031	28.15	2.31	0.011
13:50	289	0.044	25.57	2.44	0.010
14:11	310	0.041	25.43	2.69	-
16:30	449	0.03	24.91	1.28	-
20:35	694	-	-	0.95	-

Run 4

Feed flow rate	F_i	2.04	g/min
Feed flow rate	F_i	2.04	ml/min
Feed NH4+ concentration	C_{NH4i}	19.22	mM
Fi/V	D	9.92E-04	min^{-1}
Fi/V		5.95E-02	hr^{-1}
Gas flow rate	G	0.86	l/min
Gas composition		40.00	%

Time	T, minutes	O.D.	NO2-, mM	NH4+, mM	Methanol, mM
09:30	0.00	0.038	22.91	0.82	-
09:45	15.00	0.04	24.28	0.50	-
10:14	44.00	0.037	23.15	0.50	-
10:35	65.00	0.036	23.42	0.55	0.000
10:40	70	0.038	22.31	0.54	0.011
10:45	75	0.038	22.03	0.84	0.030
10:50	80	0.038	22.26	0.84	0.016
10:55	85	0.039	22.25	0.92	0.022
11:00	90	0.037	22.44	1.09	0.013
11:10	100	0.037	21.78	1.05	0.031
11:35	125	0.041	21.20	1.30	0.030
12:13	163	0.041	21.27	1.56	0.028
13:10	220	0.033	19.55	3.14	0.056
14:00	270	0.032	19.12	3.34	0.052
15:00	330	0.033	19.05	2.94	0.072
15:55	385	0.033	18.40	3.34	0.082
17:00	450	0.031	17.64	3.25	0.129
19:28	598	0.027	20.31	2.23	-