NITROGEN FIXATION AND BULK ORGANIC REMOVAL IN A BLEACHED KRAFT PULP AND PAPER MILL TREATMENT SYSTEM

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NICOLA M FROST

Biology Department

University of Waikato

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ABSTRACT

A study was undertaken to assess some effects of nitrogen fixation and bulk organic substrate removal in a New Zealand bleached kraft pulp and paper mill wastewater treatment system. A novel wastewater biological treatment system based on nitrogen fixation has been identified as having significant potential for application in full scale treatment of pulp and paper and other nitrogen deficient wastewaters. This study contributes information about the significance and role of nitrogen fixing bacteria in such a system and will benefit development and optimisation of a wastewater treatment system based on nitrogen fixation.

The specific aims of the study were i) to identify the bulk organic substrates present in the mill's treatment systen, ii) to study some effects of changing the wastewater composition on the processes occurring in a nitrogen fixing biological treatment system and iii) to obtain and characterise some of the nitrogen fixing microorganisms present in this system.

Methanol, acetic acid and glucose accounted for approximately 43% of the soluble chemical oxygen demand entering the treatment system. The remaining 57% was unidentified in this study. Acetic acid was the first identified substrate removed from the treatment system, implicating this compound as the preferred substrate under these conditions.

A laboratory continuous culture experiment demonstrated that increases in acetate and methanol concentrations in the wastewater did not affect specific substrate removal and nitrogen fixation rates in the system but may have changed the microbial population. Low biomass nitrogen results were obtained in this experiment and attributed to microbial intracellular storage of poly-β-hydroxybutyrate.

Isolates were obtained from the mill's treatment system which were able to fix nitrogen under microaerophilic conditions. Four selected isolates were able to utilise methanol, acetic acid and glucose under nitrogen fixing conditions. All of these isolates removed

acetic acid from a mixed carbon substrate medium before methanol and glucose, consistent with individual substrate removal found in the Tasman treatment system.

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INTRODUCTION

1. INTRODUCTION

1.1 THE NEW ZEALAND PULP AND PAPER INDUSTRY

As the second largest manufacturing industry, forestry and forest product based industries are particularly important to the New Zealand economy. For the year ending June 1996, pulp and paper exports contributed about $900 million to the New Zealand economy, 35% of all forest products export earnings (Zwartz, 1997). There are presently eight pulp and/or paper mills in New Zealand. The location, type, capacity and effluent treatment process of each is given in Table 1.

Table 1: New Zealand pulp and paper mills, production and treatment data (Dell, Donald *et al.*, 1996)

Mill	Product	Processes	Effluent treatment process	Production (tonnes/ year)
Pan Pacific Forest Industries NZ Ltd	market pulp	TMP	screening ocean outfall	240 000
Carter Holt Harvey Pulp & Paper Kinleith	market pulp liner board	bleached kraft, NSSC	Segregated effluent 1 main wastewater - aerated lagoons 14 days 2 alkali bleaching - aerated lagoons 45 days	660 000
Carter Holt Harvey Pulp & Paper Penrose	corrugating medium	recycled paper	discharge to municipal sewer	64 000
Carter Holt Harvey Packaging Whakatane	carton board	SGW, NSSC	screening, clarifier underflow thickening screw press	85 000
Carter Holt Harvey Tissue Kawerau	market pulp tissue	bleached SCTMP	anaerobic screening, dissolved air flotation system	130 000
Carter Holt Harvey Pulp & Paper Mataura	market pulp recycle	market pulp recycle	screening and clarifiers	25 000
Tasman Pulp and Paper Co. Ltd	market pulp newsprint	SGW, RMP, bleached kraft	aerated lagoon 5-6 days retention	600 000
Winstone Pulp International	market pulp	CTMP, BCTMP	primary clarifier	130 000

TMP = Thermomechanical pulp;
CTMP = Chemithermomechanical pulp;
SCTMP = Sulphonated Chemithermomechanical pulp;
SGW = Stone Groundwood;
NSSC = Neutral Sulphite Semichemical;
RMP = Refiner Mechanical pulp;
BCTMP = Bleached Chemithermomechanical pulp;

1.2 ENVIRONMENTAL EFFECTS OF PULP AND PAPER MILL EFFLUENTS

The production of pulp and paper results in the production of large volumes of liquid waste which is usually treated and discharged into river, lake or marine environments. Typical discharges of constituents from a bleached kraft pulp and paper mill and their environmental concerns are outlined in Table 2. Some of these environmental concerns associated with the discharge are discussed below.

Table 2: Typical effluent discharges for bleached kraft mill (Stuthridge, Nicol *et al.*, 1997)

Parameter	Mill discharge tonne/day	Environmental concerns
Bulk organics	70	low dissolved oxygen
Solids	10	habitat blanketing
Colour	50	aesthetically unpleasant
Nitrogen	0.4	eutrophication
Phosphorus	0.1	eutrophication
Trace organics	0.7	toxicants

Bulk organics

Chemical and biological processes which occur naturally in the receiving environment oxidise and break down the bulk organic constituents. A high organic load to a receiving water environment exerts a high oxygen demand leading to the depletion of natural oxygen resources. Dissolved oxygen concentrations of 5-9 mg/L are required to maintain a healthy aquatic environment (Springer, 1993). Biochemical oxygen demand (BOD) and chemical oxygen demand (COD) are widely used to measure the organic content of a wastewater. BOD assays measure the amount of oxygen consumed by microorganisms in the biochemical oxidation of organic matter. BOD_5 is a measure of the amount of

oxygen consumed by the microorganisms in 5 days. In the COD assay, the amount of chemically oxidisable material is measured.

Solids

Suspended solids in bleached kraft mills consist of bark, wood fibre, and lime lost from the chemical recovery cycle (Springer, 1993). Suspended solids can lead to the development of sludge deposits which may smother the bottom of the recipient. The biological breakdown of sludges may exert an oxygen demand and result in odorous gas formation and solids flotation problems (Springer, 1993).

Colour

Dissolved lignin and carbohydrate fragments produced during pulping and bleaching carry the bulk of the chromophoric bodies that are responsible for the effluent colour. Coloured material adsorbs light reducing the photic depth of the receiving water which may decrease photosynthetic rates and effect aquatic productivity (Ministry for the Environment, 1994).

Nutrients

Kraft pulp and paper mill wastewaters typically contain low levels of nitrogen and phosphorus. To achieve effective biological treatment supplemental nutrients are often added to the wastewater. Although nutrients are essential to plant life, the discharge of surplus nutrients may promote excessive weed and phytoplankton growth, resulting in eutrophication (Springer, 1993).

Trace organics

Debarking, pulping and bleaching produce a wide variety of wood derived wastewater constituents. Resin acids, chlorinated resin acids and chlorophenolic compounds are responsible for most acute and chronic toxicity of bleached kraft pulp and paper mill wastewaters (Melcer, Schnell *et al.*, 1995). Elemental chlorine free (ECF) and totally chlorine free (TCF) bleaching technologies and secondary treatment have essentially eliminated chlorinated organic compounds and toxicity from mill discharges (Springer, 1993).

1.3 PULP AND PAPER EFFLUENT TREATMENT

Treatment of pulp and paper mill effluent is necessary in order to minimise the effect of the discharge on receiving waters. The treatment of kraft mill wastewaters in New Zealand is based around the use of biological systems called aerated lagoons. Aerated lagoons consist of an aerobic mixed liquor above an anaerobic sediment (Figure 1) (McFarlane, Clark *et al.*, 1993). The mixed liquor is made up largely of aerobic microorganisms that utilise the organic material in the wastewater as the carbon source for energy and the synthesis of new cell material. The primary organisms responsible for this conversion are bacteria, but treatment systems may also contain a variety of other organisms including algae, fungi, viruses, protozoa and rotifers (Springer, 1993).

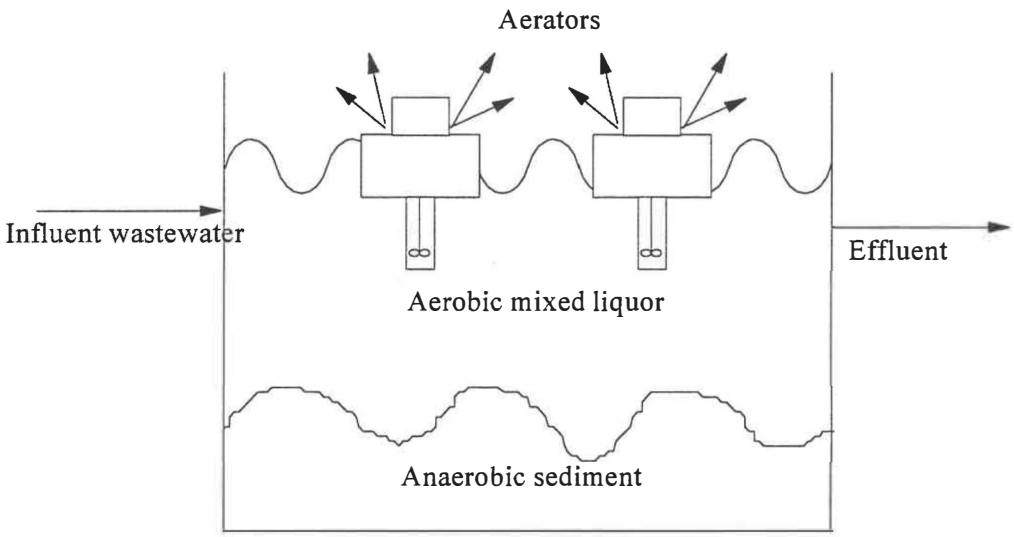

Figure 1: Aerated lagoon

Mechanical aerators are used to increase oxygen concentrations and to keep solids suspended in the mixed liquor. The microbial action within aerated lagoons removes non settleable colloidal solids and reduces the organic content of wastewaters. Typical BOD removals in an aerated lagoon system with retention times between 3-20 days are 80-90% (Springer, 1993). Colour is generally not removed and may sometimes increase in an aerated lagoon treatment system.

1.4 TASMAN PULP AND PAPER COMPANY LIMITED

The pulp and paper mills operated by the Tasman Pulp and Paper Company Limited (Tasman) have undergone many modernisations since commissioning in 1955. The three paper machines, two mechanical pulp mills and two kraft pulp mills process about 2 million tonnes of wood to produce approximately 200 000 tonnes of market kraft pulp and 400 000 tonnes of newsprint and directory paper each year. Tasman produces the newsprint for almost the entire New Zealand market and has a forty percent market share in Australia. Most of the pulp produced is exported to Australia, Asia and the United States.

The principal raw material used at Tasman is wood residue from plantation forests. The mill operates in an integrated manner to produce both newsprint paper and market kraft pulp. An overview of the process is shown in Figure 2.

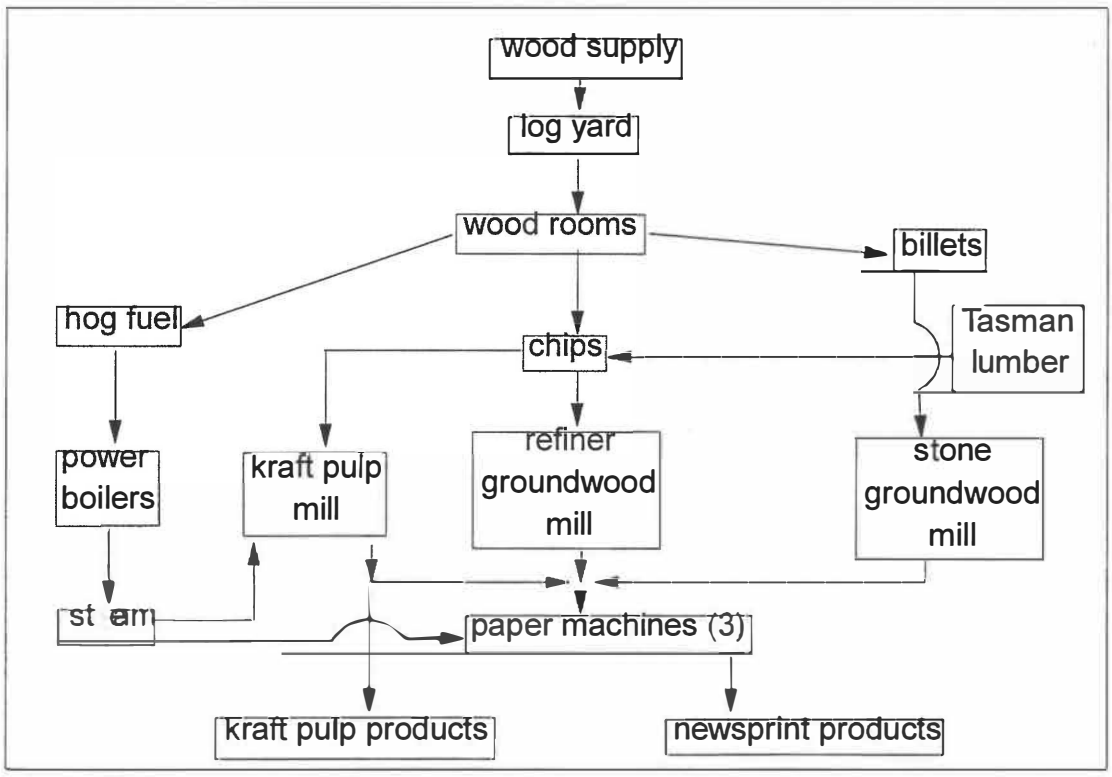

Figure 2: Tasman Pulp and Paper Company Ltd operation overview

Over $60 million in capital costs alone have been spent since 1984 on new processes or equipment upgrades and retrofits specifically designed to achieve significant

environmental improvements. More recent investment during 1996 - 1997, has seen a steam stripping plant installed to treat foul condensates and remove material which contributes about 15% of the biochemical oxygen demand discharged to the effluent treatment system.

1.5 TASMAN'S WASTEWATER TREATMENT SYSTEM

Approximately 150 000 m^3 of water is extracted from the Tarawera River daily and treated before use in Tasman's pulp and paper manufacturing process. Wastewater from the production of bleached and unbleached kraft pulp, stone and refiner groundwood pulp and newsprint is discharged to either the main process sewer or the acid sewer. The acid sewer takes about 10% of the total flow. The main process sewer undergoes both primary and secondary treatment. The acid sewer, because of its negligible suspended solid content, bypasses primary treatment.

The primary treatment plant includes bar screens and a primary clarifier. The primary clarifier is a standard circular sedimentation clarifier with a hydraulic capacity of about 20 000m^3, equivalent to a 3 hour retention time. The clarifier underflow is pumped into a dewatering system consisting of a pair of rotary screen thickeners followed by a screw press. The cake material that comes off the screw press is transferred to a landfill (Hunter, 1997).

Following clarification, the main process sewer is combined with the acid sewer and flows under gravity to the aerated lagoon treatment system. This system consists of 4 ponds in series with a total volume of 1.15 million m^3 (Figure 3).

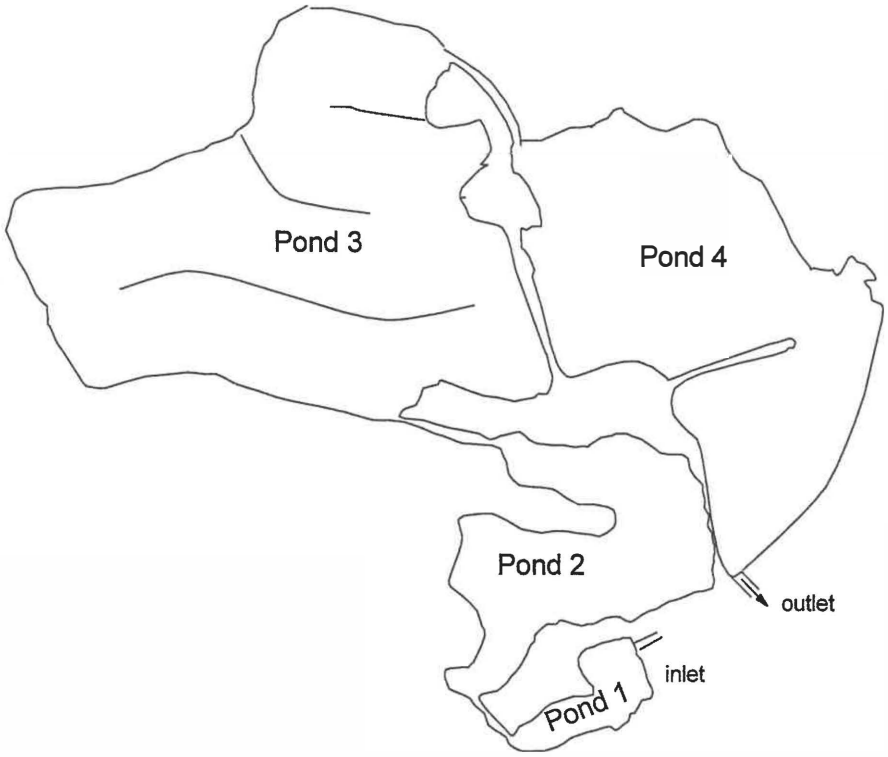

Figure 3: Tasman's aerated lagoon treatment system

At present wastewater flows of approximately 150 ML/d in this system provide a nominal hydraulic retention time of 7-8 days. Pond 1 is a small unaerated equalisation pond (Table 3). This pond provides approximately 3 hours of back-up settling and anaerobic respiration. From Pond 1, the flow passes through three aerated lagoons (Ponds 2, 3 and 4), prior to discharge to the Tarawera River. Pond 2 is the first aerated lagoon in the system and approximately 60% of the BOD_5 is removed in this pond. Pond 3 provides an environment where the BOD degradation is continued and also allows for some of the biosolids generated in the system to settle out. Pond 4 is an effluent polishing and settling pond.

Table 3: Tasman's aerated lagoon treatment system data

	Pond 1	Pond 2	Pond 3	Pond 4
Active volume (m^3)	34 000	220 000	490 000	410 000
Aerators	0	18	11	9
Power (kW)	0	1 026	730	500
kW/1000 m^3	0	4.67	1.50	1.2

Dredging is carried out periodically to remove the biomass generated in the ponds.

1.6 NITROGEN FIXATION AND THE TREATMENT OF PULP AND PAPER MILL WASTEWATERS

Pulp and paper wastewaters have low concentrations of the essential nutrient nitrogen. Many pulp and paper mills add nitrogen and phosphorus to their wastewaters to improve treatment performance. Hynninien and Viljakainen (1995) recommend that dissolved nitrogen concentrations in wastewater should typically be between 2-3 mg/L. The Tasman Pulp and Paper Ltd. aerated lagoon treatment system is operated without addition of supplemental nitrogen and still achieves excellent levels of biological removal of organic material (Slade, Leonard *et al.*, 1991). Studies of the aerated lagoon treatment system at Tasman Pulp and Paper Co. Ltd have shown that the total nitrogen concentration increases substantially across the treatment system. The total nitrogen concentration of the wastewater entering the system is approximately 1.5 mg/L and the total nitrogen concentration leaving this system is about 4 mg/L (Bruce and Clark, 1994).

By measuring acetylene reduction rates across Tasman treatment system, Bruce and Clark (1994) demonstrated that this increase in total nitrogen is due to bacterial fixation of atmospheric nitrogen. They concluded that the rates of nitrogen fixation estimated from the acetylene reduction data were sufficient to account for the fixation of at least 2-3 mg N/L.d in Pond 2.

Nitrogen fixation is an anaerobic or microaerophilic process for aerobic, facultatively anaerobic or anaerobic bacteria (Starr, Stolp *et al.*, 1981). Because of the sensitivity of the nitrogenase enzyme to oxygen (Dalton and Postgate, 1969b) and previous experience with high *Klebsiella* counts found in the wastewater of a thermomechanical pulp mill (Clark, Donnison *et al.*, 1992), previous studies on Tasman Pulp and Paper wastewater have concentrated on isolating anaerobic nitrogen fixing bacteria from the Tasman treatment system.

From Tasman Pulp and Paper treatment Pond 2, a moderately high level (3.0×10^4 CFU/mL) of *Klebsiella* was enumerated. However, only 3 of the 24 isolates demonstrated nitrogen fixing activity (Bruce and Clark, 1994). Nitrogen fixation in Pond 2 was occurring at a rate which accounted for the observed input of nitrogen into the system and it was concluded that other microorganisms, as well as *Klebsiella,* may be significantly involved in this nitrogen fixation process. Bruce and Clark (1994) used anaerobic enrichment techniques which would have isolated only anaerobic nitrogen fixing microorganisms and would have excluded microorganisms that required oxygen for nitrogen fixation.

Some aerobic nitrogen fixing microorganisms have special physiological mechanisms to protect the oxygen sensitive nitrogenase enzyme from air (Dalton and Postgate, 1969a). These mechanisms enable aerobic N-fixing organisms such as *Azotobacter spp.* to fix nitrogen at high dissolved oxygen concentrations compared to other free living N-fixing bacteria. *Azotobacter* is a free-living N-fixing obligate aerobe that is widely distributed in soil, water and rhizophere (Becking, 1981) and has previously been isolated from pulp mill effluents (Knowles, Neufeld *et al*., 1974).

Gapes and Clark (1995) demonstrated the advantages of operating an aerobic nitrogen fixation treatment system. The advantages of using such a system include:

- reduction in quantity of sludge produced. Elevated dissolved oxygen concentrations have been shown to reduce the biomass yield of a nitrogen fixing reactor system, possibly caused by an oxygen wasting mechanism used by the cells to protect the nitrogenase enzyme. Such a mechanism results in the consumption of organic substrate with less resultant cell mass production.
- substrate removal rates comparable to a system operating at low DO.
- elimination of costs associated with nitrogen addition.
- better control of nitrogen load being discharged to the receiving environment because bacteria only fix as much nitrogen as they require for growth, resulting in lower levels of ammoniacal nitrogen in the final effluent.

These findings with regard to nitrogen fixation in aerated lagoons and other nitrogen deficient wastewaters have significant potential for development of a biological treatment

system based on nitrogen fixation. Development and optimisation of such a wastewater treatment system requires an understanding of the fundamental microbiology in the system. However, little is known about the significance and role of nitrogen fixing bacteria in this treatment system. Further investigation into this area would be beneficial for future development.

1.7 OBJECTIVES OF THE WORK

The objectives of this work are:

1. To determine the composition of the organic substrate in Tasman whole mill effluent and the degradability of this substrate in Tasman's aerated lagoon treatment system.

2. To study some effects of changing the wastewater composition on the processes occurring in the nitrogen fixing biological treatment system by investigating associations between biomass composition, substrate removal efficiencies and N-fixation rates.

3. To obtain and characterise aerobic nitrogen fixing isolates from Tasman Pulp and Paper's aerated lagoon treatment system that are capable of organic substrate removal.

Section 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 NITROGEN-FIXING MICROORGANISMS

A diverse group of prokaryotes are able to utilise nitrogen gas as a source of nitrogen. These nitrogen-fixing (N-fixing) prokaryotes have been isolated from a wide variety of habitats including hot springs, acidic bogs and alkaline soils, water flooded and arid soils, marine and freshwaters, and even the gut of certain insects (Gordon, 1981). The N-fixing prokaryotes are genetically very diverse, including representatives of many unrelated bacterial families. N-fixing organisms may be aerobic, anaerobic or facultatively anaerobic. They may exist primarily as free-living organisms, or in associations with other micro or macro organisms.

The basic requirements for nitrogen fixation are the presence of the oxygen sensitive enzyme, nitrogenase, and the provision of ATP and reductant. The N-fixing organisms can be divided into various groups based on their metabolic diversity.

Free-living aerobic phototrophic bacteria

The cyanobacteria are able to generate unlimited ATP and reducing power provided sunlight is adequate. Some cyanobacteria have cells called heterocysts which contain nitrogenase and fix nitrogen. These cells are incapable of generating oxygen but are still able to produce ATP thus protecting the oxygen sensitive nitrogenase enzyme. Cyanobacteria are often the first colonisers of nutrient poor environments because they can assimilate their own carbon and nitrogen requirements (Fenchel and Blackburn, 1979).

Free-living anaerobic, phototrophic bacteria

Nitrogenase is present in members of the following groups; the Chromatiaceae, the Rhodospirillaceae, and the Chlorobiaceae. These bacteria have an unlimited ATP supply in a reduced environment where oxygen toxicity is not a problem (Fenchel and Blackburn, 1979).

Free-living aerobic and microaerophilic chemotrophic bacteria

This group contains a variety of bacteria which are dependent on oxygen as an electron acceptor for energy requirements. They have a number of special mechanisms to protect nitrogenase from oxygen. These aerobic chemotrophic bacteria are discussed in Section 2.1.1.

Free-living anaerobic chemotrophic bacteria

Only four genera of obligately anaerobic non-photosynthetic bacteria have been reported to contain N-fixing representatives: *Clostridium, Desulfovibrio, Desulfobacter* and *Desulfotomaculum* (Eady, 1992). Maintaining a reduced environment is not a problem for these organisms. Energy limitation is a problem because anaerobic bacteria have no respiratory chain phosphorylation system to generate ATP. All their ATP must be generated by substrate level phosphorylation. Some organisms belonging to the family Enterobacteriaceae and some *Bacillus* and *Vibrio* species are capable of heterotrophic growth under aerobic or anaerobic conditions, but nitrogen is fixed only under anaerobic or extremely microaerophilic conditions (Dalton, 1980).

Symbiotic aerobic phototrophic bacteria

Lichens are an association between cyanobacteria and fungi. The bacteria fix nitrogen when the lichen is located on a nitrogen deficient surface. Cyanobacteria also form associations with other bacteria, liverworts, mosses, ferns, gymnosperms and angiosperms (Fenchel and Blackburn, 1979).

Cyanobacteria and other phototrophs are not thought to make an important contribution to nitrogen fixation in pulp and paper aerated lagoon treatment systems because of the poor light penetration in these systems.

Symbiotic aerobic chemotrophic bacteria

These bacteria have solved the problem of energy supply by using the reduced carbon supply of plants. These associations can either be loose associations or very specific nodule forming associations. Bacteria belonging to the family Rhizobiaceae form associations with soybeans, peas, clover and other legumes (Fenchel and Blackburn, 1979).

2.1.1 The free-living aerobic and microaerophilic nitrogen-fixing bacteria

Free-living aerobic or microaerophilic N-fixing bacteria are a diverse group. At present, twenty two genera have been identified that contain all or some species which are capable of fixing nitrogen under suitable conditions (Eady, 1992).

The members of the Azotobacteraceae are amongst the most oxygen tolerant of the diazotrophs. In Bergey's Manual (Kreig, 1982) there are two genera in the Azotobacteraceae family; *Azotobacter* and *Azomonas*. These bacteria are widely distributed in soils and surface waters (Mulder and Brotonegoro, 1974). They have large ovoid cells which grow rapidly, form some extracellular slime and are catalase positive. Some *Azotobacter* species form small resting phase cysts which are less differentiated than spores and which contain novel lipids (Reusch and Sadoff, 1983).

Knowles (1974) isolated an *Azotobacter* species from aerobically incubated effluent from a neutral sulphite semi-chemical mill. The Azotobacter were very numerous and underwent significant proliferation during 24 hours of incubation. On the basis of the absence of cysts, its inability to utilise mannitol, rhamnose and starch and the apparent absence of an ultraviolet-fluorescent pigment, they tentatively identified the isolate as being close to *Azotobacter insignis* (now *Azomonas insignis*).

As well as being able to use a wide range of simple oxidisable carbohydrates, *Azotobacter* species are able to oxidise many aromatic compounds not susceptible to attack by many other bacteria (Hardisson, Sala-Trepat *et al.,* 1969). None of the *Azotobacter* isolates tested by Thompson and Skerman (1979) were able to utilise methanol as a carbon and energy source.

Beijerinckia and *Derxia* are genera comprised of bacteria capable of fixing nitrogen under atmospheric oxygen pressures. These genera have not been assigned to any particular family although they were previously members of the Azotobacteraceae (Kreig, 1982). These two genera were reclassified because DNA analyses and DNA hybridisation tests confirmed that they were genetically different from other members of the Azotobacteraceae (De Ley, 1968). Both *Beijerinckia* and *Derxia* are acid tolerant and

are able to fix nitrogen at pH 3.0 - 4.0 and have been commonly isolated from soils in tropical regions (Mulder and Brotonegoro, 1974).

The *Xanthobacter* are facultative hydrogen autotrophs that can fix nitrogen under either autotrophic or heterotrophic conditions. *Xanthobacter* are microaerophilic nitrogen fixers and are ubiquitous in environments with decaying organic matter or containing sufficient concentrations of H_2 and CO_2 and/or other products of anaerobic microbial activity such as organic acids and alcohols. When growing under heterotrophic conditions they can utilise methanol, ethanol, *n*-propanol, *n*-butanol and various organic acids as sole carbon sources. However, carbohydrate utilisation is limited and is frequently restricted to the utilisation of not more than three of the following; fructose, galactose, mannose and sucrose. For most strains glucose is not a preferred substrate (Kreig, 1982).

The Methylococcaceae are a diverse group of bacteria only able to utilise methane or methanol as sole energy and carbon source under aerobic or microaerophilic conditions. Some strains are nitrogen fixers and are extremely oxygen sensitive under nitrogen-fixing conditions (Kreig, 1982).

Azospirillum species are able to fix nitrogen under microaerophilic conditions and are capable of utilising a wide range of carbohydrates and organic acids. Five species of *Azospirillum* are recognised; *A. lipoferum, A. brasilense, A. amazonense, A. halopreferans,* and *A. iakense.* These bacteria are widely distributed in tropical and temperate soils. In semi-solid media they form a characteristic pellicle below the meniscus (Kreig, 1982).

Some members of the facultatively phototrophic bacteria are able to fix nitrogen in the presence of oxygen. Under anaerobic conditions these bacteria can utilise light energy for carbon assimilation and also for nitrogen fixation. Facultatively phototrophic members of the nonsulfur purple bacteria such as *Rhodobacter capsulatus* and *Rhodopseudomonas acidophilia* are able to fix nitrogen when they grow chemotrophically in the dark.

Several *Pseudomonas* strains, *P. stutzeri, P. saccharophilia, P. diazotrophica* and *P. pseudoflava,* are capable of diazotrophic growth under microaerophilic conditions. *Pseudomonas* species are the dominant organisms in many terrestrial and aquatic environments because of their catabolic diversity and tolerance to a wide range of temperatures (Eady, 1992).

The Beggiatoaceae are widespread in aquatic sulphide containing environments. All freshwater and marine isolates of the gliding sulphide-oxidising organism *Beggiatoa* and the type strain *B. alba* have been shown to be diazotrophic under microaerophilic conditions (Eady, 1992).

Seven out of eight strains of *Mycoplana dimorpha* are diazotrophic under microaerophilic conditions. These organisms, isolated from soil, are described as actinomycete-like, gram-negative, motile and branching. Little is known about their ecology and their taxonomy is uncertain.

Members of the Bacillaceae (of which only the genus *Bacillus* is facultative) the Vibrionaceae, the Enterobacteriaceae and the Rhodospirillaceae contain some members which are able to fix nitrogen. In all cases the organisms are capable of heterotrophic growth under aerobic or anaerobic conditions but nitrogen is only fixed under anaerobic or extremely microaerophilic conditions (Dalton, 1980). Neilson and Sparell (1976) were able to isolate from paper mill process waters representatives from the following genera; *Klebsiella, Enterobacter, Erwinia, Citrobacter* and *Escherichia.* In a later paper, Neilson and Allard (1985) concluded that endogenous N-fixing Enterobacteriaceae contained in some kinds of industrial wastewaters could successfully be used to diminish the addition of combined nitrogen to activated sludge treatment plants.

2.1.2 Special characteristics of some nitrogen-fixing bacteria

Facultative autotrophic growth on hydrogen

A number of N-fixing bacteria are able to obtain energy and reducing power from the oxidation of hydrogen for growth and CO_2 fixation. Included in this group are *Xanthobacter, Azospirillum, Derxia* and some strains of *Pseudomonas*, *Hydrogenophaga*

pseudoflava. It is possible that other N-fixing bacteria may grow under these conditions (Wiegel, 1992).

Storage of intracellular poly-β-hydroxybutyrate

One of the characteristics of many free-living aerobic N-fixing bacteria appears to be the production of intracellular granules consisting of poly-β-hydroxybutyrate (PHB). Species of *Azospirillum, Azotobacter, Methylococcaceae, Beijerinckia, Derxia* and *Xanthobacter* all produce these intracellular granules which may constitute up to 50% of their dry cellular weight when grown under N-fixing conditions (Kreig, 1982).

Polyhydroxybutyrates (PHB) are aliphatic polyester homopolymers which are stored as an energy reserve in cells of many bacteria. Microbial accumulation of PHB occurs often in environments where there is a transient abundance of carbon sources with respect to other nutrients such as nitrogen or phosphorus. Researchers have discovered that joining PHB with a copolymer produces a biodegradable plastic. This plastic stays flexible from sub-zero temperatures up to 130°C and completely breaks down into water and carbon dioxide in a few months. Commercial production is not currently viable because of high costs of the carbon substrate, low production volumes and maintenance of the large fermentation facility. However, the potential volume for the biodegradable plastic market is in the million ton range (Daniell and Guda, 1997).

Oxygen tolerance

Of the aerobic free-living N-fixing bacteria, members of the family Azotobacteraceae appear to be the most oxygen tolerant of the diazotrophs (Postgate, 1982). The other aerobic N-fixing bacteria described above prefer microaerophilic conditions for nitrogen fixation. Special physiological adaptations, which will be discussed below, allow the oxygen sensitive nitrogenase enzyme in *Azotobacter* species to function under aerobic conditions.

Growth at high temperatures

Three strains of thermophilic N-fixing bacteria have been recognised: one is a cyanobacterium; another is a methanogen; and the third is a *Clostridium.* These are all strictly anaerobic. For most N-fixing bacteria it seems that there is an upper limit to

nitrogen fixation at about 40°C. *Azotobacter* and *Beijerinckia* are particularly temperature sensitive and will not grow above 36°C (Postgate, 1987).

2.2 PHYSIOLOGY OF NITROGEN FIXATION

Nitrogen fixation results in the reduction of N_2 to ammonium which is then converted to an organic form:

$$N_2 + 2H^+ + 3H_2 \Leftrightarrow 2NH_4^+$$

At pH=7.0 this reaction can proceed spontaneously (ΔG^o = - 80 kJ/reaction), but due to the stability of dinitrogen, this is not observed (Haaker and Klugkist, 1987). Biochemically this reaction is catalysed by the enzyme nitrogenase where N_2 is coupled with a metal which lowers the activation energy for reduction.

Nitrogenase is a major cellular constituent in N-fixing organisms comprising up to 15% of soluble cell protein. There are three genetically distinct, although related, nitrogenase systems: one based on molybdenum; another on vanadium; and a third that functions without either molybdenum or vanadium (Eady, 1992).

The nitrogenase system based on molybdenum consists of two components; component I containing iron and molybdenum (MoFe-protein or dinitrogenase) and component II containing iron but no molybdenum (Fe-Protein or dinitrogenase reductase) (Eady and Postgate, 1974).

Azotobacter vinelandii and *A. chroococcum* have both the Mo and V dependent nitrogenase systems. If the concentration of molybdenum in the environment is too low, these organisms are able to use the vanadium based enzyme for nitrogen fixation (Robson, 1979). The third nitrogenase system which functions in the absence of Mo and V has also been isolated from *A. vinelandii.*

Although large differences exist in the morphology and physiology of different types of N-fixing microorganisms, the overall reaction representing nitrogen fixation and the

nitrogenase enzyme are similar in all types of N-fixers, free living as well as symbiotic forms (Mulder, 1975):

N_2 + 8 e^-donor (reduced) + $8H^+$ + 16ATP $\rightarrow$ $2NH_3$ + H_2+ 8 e^-donor (oxidised) + 16ADP + 16P_i

The reduction of N_2 requires MgATP (mono-magnesium salt) in addition to reducing power in the form of 6 electrons. The electrons, derived from degradation of various types of carbon compounds, are transferred to an electron carrier. Ferredoxin is considered to be the immediate electron carrier to nitrogenase in all N-fixing organisms, with the exception of *Klebsiella pneumoniae* and possibly *Azotobacter* species which use flavodoxin. Under iron deficiency conditions several microorganisms form flavodoxin instead of ferredoxin. The redox potential of ferredoxin and flavodoxin is sufficiently low (-200 - -450 mV) to reduce the Fe protein. The electron transfer from Fe protein to MoFe protein (component one) is coupled to the hydrolysis of MgATP and ultimately dinitrogen, and protons are reduced at the MoFe protein (Figure 4) (Haaker and Klugkist, 1987).

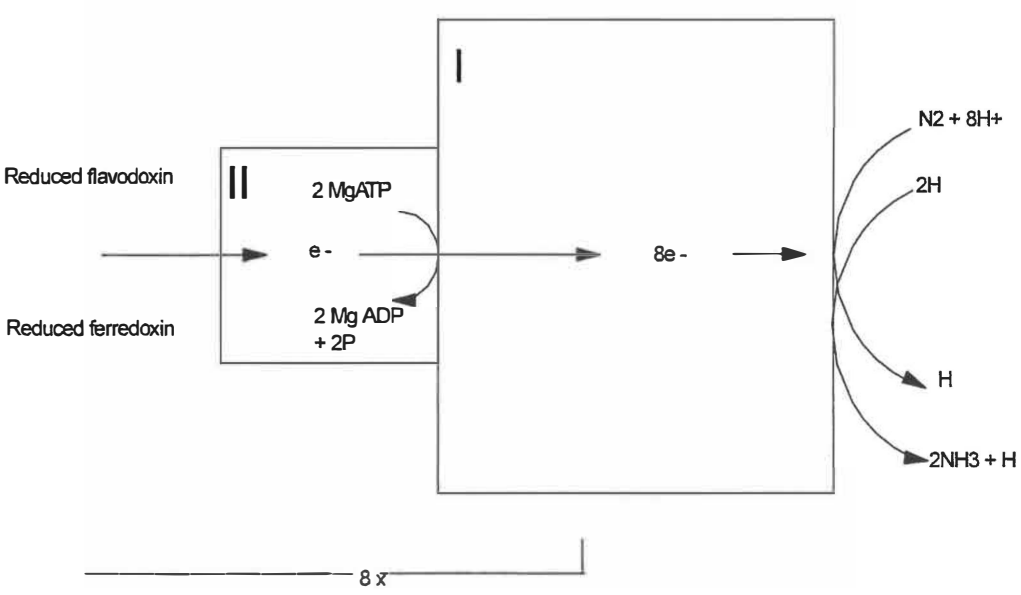

Figure 4: Reactions of the nitrogenase complex (from Haaker and Klugkist, 1987)

Haaker and Klugkist (1987) have proposed a model for the electron transport chain in aerobic N-fixing bacteria. In their model, a NADPH dehydrogenase in the cytoplasmic membrane is reduced by two electrons from NADPH. One electron (E_h = -160 mV) is donated to a respiratory chain component at the redox level of ubiquinone, oxygen being the ultimate acceptor. The other electron can be transferred to a redox centre with a low potential (e_h = -500 mV), and this centre can reduce flavodoxin (Figure 5).

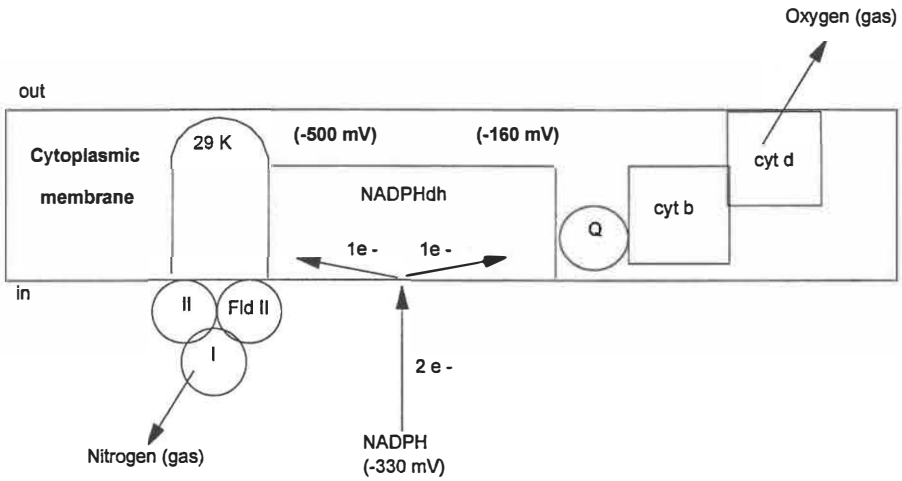

Figure 5: Model of the coupling of electron transport to nitrogenase with obligatory electron transport to dioxygen (from Haaker and Klugist, 1987)

According to this model, under high oxygen concentrations the components of the respiratory chain and all the redox centres in NADPH dehydrogenases are oxidised. All electrons are channelled away from the flavodoxin to oxygen. As well as offering an explanation for oxygen inhibition of nitrogenase, this model also gives an explanation for the tight coupling between electron transfer activity in the respiratory chain and nitrogenase activity, since for each electron used in nitrogen fixation at least one electron has to be oxidised by the respiratory chain (Haaker and Klugkist, 1987).

Nitrogenase tested from all organisms requires ATP for N-fixing activity (or for any other nitrogenase catalysed reductions). The ATP requirement for nitrogen fixation is very high. The stoichiometry has a minimum value of four ATP being split to ADP + P_i for each $2e^-$ transferred. However, the number of ATP molecules required by the nitrogenase depends strongly on such factors as temperature, pH, ratio of the nitrogenase

components and the electron flux through nitrogenase (Watt and Burns, 1977; Iman and Eady, 1980).

Although it has been calculated that only 6 electrons are required to reduce N_2, eight electrons are actually used, with two electrons being lost as hydrogen gas for each mole of N_2 reduced. It is possible that this hydrogen evolution is part of the reaction mechanism of nitrogenase (Brock, Madigan *et al.*, 1994). This evolved hydrogen can be oxidised by hydrogenase to recoup electrons and recover energy (Kennedy and Toukdarin, 1987).

Nitrogenase is not substrate specific and is capable of reducing acetylene, cyanide, azide, nitrous oxide and carbon monoxide. The reduction of acetylene to ethylene by nitrogenase is used to assay nitrogen fixation. The amount of ethylene produced can be measured by gas chromatography and is proportional to the amount of enzyme present.

2.3 THE GENETICS OF NITROGEN FIXATION

Much of the molecular research on the genetics of nitrogen fixation has focussed on *Klebsiella pneumoniae.* Seventeen genes have to be expressed for the synthesis of a functional nitrogenase in *Klebsiella pneumoniae.* These genes are organised in several operons forming the complex *nif* regulon. Genes H, M and S code for the Fe protein and genes D, K, E, N,V and B for the MoFe protein. In addition to nitrogenase structural genes, the genes for controlling a number of electron transport proteins and a number of regulatory genes are also present in the *nif* regulon. The products of the genes A and L are involved in regulation. Transcription of *nif* structural genes is activated by the NifA protein and is repressed by the NifL protein (Section 2.4.4).

The Fe and MoFe proteins are highly conserved (Eady, 1992). The structural genes encoding these proteins have been cloned and sequenced from many diazotrophs. The Fe protein is the most conserved within the nitrogenase system and the phylogenetic trees generated by *nifH* sequence data are largely in agreement with those generated by 16S RNA sequence data (Young, 1992). Nitrogenase activity in isolates can be confirmed by using the polymerase chain reaction to detect the presence of the Fe protein gene (*nifH).*

Studies have confirmed that molecular probes containing cloned *nifHDK* genes from one N-fixing bacterium will hybridise to DNA from virtually all nitrogen fixers, but not to DNA from non N-fixing bacteria (Brock, Madigan *et al*., 1994).

In *Klebsiella pneumoniae* there is no evidence of a nitrogenase other than the conventional Mo-containing enzyme. Differences in genetic systems of other diazotrophs are to be expected because of the range of oxygen tolerances, alternative nitrogenase systems, and also the distinct phylogenetic differences between the organisms.

2.4 EFFECT OF EXTERNAL FACTORS ON NITROGEN FIXATION

Nitrogen fixation by free-living bacteria may be affected by oxygen concentration, substrate supply, mineral nutrition and the presence of ammoniacal nitrogen.

2.4.1 Oxygen concentration

Protection of the nitrogenase complex from oxygen is not a problem for anaerobic organisms such as *Clostridium* and *Desulfovibrio*. By not fixing nitrogen under aerobic conditions, facultative anaerobes are also able to avoid nitrogenase inactivation by oxygen (Gordon, 1981).

Microaerophilic, motile diazotrophs (*Azospirillum brasilense*), grow in semisolid agar in a band below the surface at a depth which is dependent on the oxygen concentration of the gas phase. This avoidance behavour is a balance between oxygen inactivation of nitrogenase and the requirement for aerobic growth (Bergersen, 1984).

Some N-fixing bacteria such as *Beijerinckia* species, form large quantities of extracellular polysaccharides. It has been suggested that this slime layer impedes diffusion of oxygen to the bacteria within and represents a crude physical barrier against excessive oxygenation. However, gummy strains of *Klebsiella* have been found to be just as oxygen sensitive as non-gummy strains (Postgate, 1987).

Aerobic N-fixing bacteria require oxygen to generate ATP production and therefore require some physiological mechanisms to protect the oxygen sensitive component of nitrogenase from damage. Under a high degree of aeration, *Azotobacter* exhibit high respiratory rates (4000-5000 μL O_2/mg dry wt/hr) (Dalton and Postgate, 1969a). *Azotobacter* species also contain a multitude of cytochromes and redox proteins. It has been proposed that the electron transport system serves not only to produce energy but also to scavenge cytoplasmic oxygen, thus maintaining an essentially anoxic environment inside cells that nevertheless derives energy from aerobic metabolism (Yates, 1970). This mechanism is referred to as respiratory protection. Another observed response to increased oxygen levels is the formation of intracytoplasmic membranes which may be related to increased respiratory activity (Dingler and Oelze, 1985).

Oxygen stress is imposed when oxygen enters the cell more rapidly than it is removed by respiration. During oxygen stress the nitrogenase binds to a 2 Fe-2S protective protein to give an oxygen stable complex that is protected from oxygen damage but that is inert to nitrogenase substrates. This conformational protection is illustrated when nitrogenase is extracted from *Azotobacter vinelandii* or *A. chroococcum*. As a particulate extract, the enzyme is relatively insensitive to oxygen. However when the nitrogenase complex is split into its separate component fractions, component II (Fe protein) becomes very sensitive to oxygen and anaerobic handling is essential (Robson, 1979). Conformational protection is usually transitory because rates of respiration increase to meet the new demand, after which the complex dissociates to give active nitrogenase.

As well as this reversible inhibition, oxygen can also cause irreversible inhibition of nitrogenase activity. The regulation of *nif* expression by sources of fixed nitrogen has been more intensively studied. At present little is known about the regulation of nitrogenase synthesis by oxygen in aerobic N-fixing organisms. In *K. pneumoniae* the *nifL* gene is involved in oxygen regulation of nitrogenase synthesis. So far the only obligate aerobe identified with the *nifL* gene has been *Azotobacter vinelandii* (Blanco, 1993).

When the oxygen supply exceeds the demand, nitrogenase activity declines. A bell shaped curve is observed when nitrogenase activity is plotted against oxygen concentration. However, Drozd and Postgate (1970) first showed that adaptation of *A.*

chroococcum chemostat cultures to high pO_2 conditions was able to influence the shape of the resulting activity curve, as measured by acetylene reduction (Figure 6).

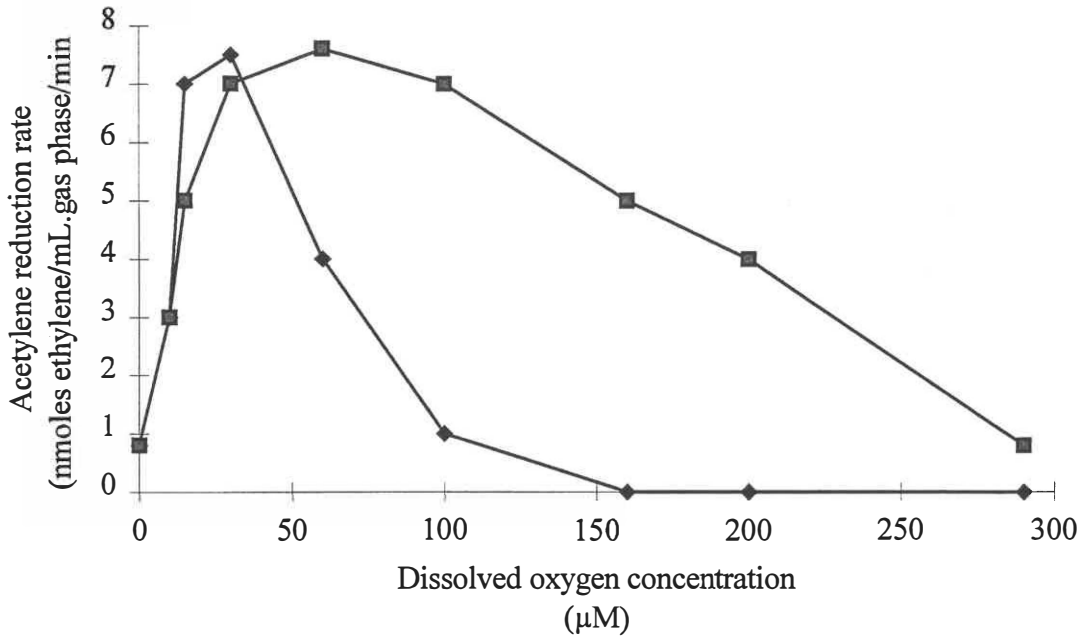

Figure 6: Rates of acetylene reduction at various DO concentrations by *Azotobacter vinelandii*. Populations grown at 0.09 atm O_2 (—) and at 0.55 atm O_2 (----) (from Drozd and Postgate, 1970

The optimum dissolved oxygen concentration (DO) varies for different N-fixing organisms and is influenced by the oxygen status during growth (Hill, 1988). The optimum DO of *Azotobacter chroococcum* is high compared to other free-living bacteria. If *A. chroococcum* is grown under high oxygen concentrations the optimum DO is significantly higher than when grown under lower oxygen concentrations (Table 4). The high optimum dissolved oxygen concentration in A*nabaena variabilis* (a cyanobacterium) is associated with the ability to form heterocysts (Jenson and Cox, 1983).

Table 4: Examples of the optimum dissolved oxygen concentration (DO) for nitrogenase activity in various diazotrophs (Hill, 1988)

N-fixing microorganism	Growth conditions	Optimum DO (μM)	Reference
Anabaena variabilis	high O_2 conditions	300	(Jenson and Holm, 1975)
Rhizobium ORS 571	free-living / high O_2	5.9	(Gebhardt, Turner *et al.*,
	free-living / low O_2	0.05	1984)
Klebsiella pneumoniae	microaerophilic	0.02-0.04	(Hill, Turner *et al,*. 1984)
Azotobacter chroococcum	high O_2	25.0	(Drozd and Postgate,
	low O_2	12.0	1970)

2.4.2 Substrate supply

Aerobic nitrogen fixation in free living organisms can be inefficient, especially when there is need to direct carbon sources into respiratory protection. Dalton and Postgate (1969a) showed that the efficiency of nitrogen fixation in chemostat cultures of *A. chroococcum* changed from 20 mg N fixed/g of C consumed to 40 mg N fixed/g of C consumed between high and low pO_2 values. Symbiotic microorganisms have been found to have values greater than 250 mg N fixed/g of C consumed. This is because they do not require large amounts of carbon and energy compounds to be used for the synthesis of cellular material, nor do they require respiratory protection (Postgate, 1982).

Most anaerobes are less efficient than aerobes at converting substrates into biomass because they have no respiratory chain phosphorylation system to generate ATP and all their ATP must be generated at the substrate level. It has been estimated that *Klebsiella* species fix about 5 mg N/g of C consumed (Bergersen, 1974).

2.4.3 Mineral nutrition

Lees and Postgate (1973) showed that phosphorus limited N-fixing cultures of *Azotobacter chroococcum* were unable to withstand increased oxygen concentrations due to the failure of their respiratory protection system (Lees and Postgate, 1973). However,

these observations may be restricted to particular conditions and it is unknown whether the harmful effect of limited phosphorus is a common phenomenon (Mulder and Brotonegoro, 1974). Bergerson (1974) used continuous cultures of *Klebsiella pneumoniae* grown with either N_2 or NH_4^+ as the nitrogen source and a range of limiting inorganic phosphate concentrations to show that N-fixing systems do not have a higher phosphate requirement than systems utilising combined N (Bergersen, 1974).

Potassium, calcium and magnesium are also essential elements for free-living, N-fixing bacteria (Mulder, 1975). Both molybdenum and iron are necessary for nitrogenase activity since they are components of the nitrogenase complex. The molybdenum requirement is variable depending on species and strain; for half maximal growth under N-fixing conditions, *Beijerinckia* species require between 0.004-0.034 ppm Mo. It is about 10-20 times higher in some *Azotobacter vinelandii* strains and about 2 times higher than in *Azomonas agilis,* whereas some strains of *Azotobacter chroococcum* have an equal to higher Mo requirement (Becking, 1962). Vanadium may substitute for molybdenum when molybdenum concentrations in the environment are low. The vanadium enzyme reduces substrates, particularly acetylene, less efficiently than the Mo nitrogenase (Kennedy and Toukdarin, 1987).

2.4.4 Presence of ammoniacal nitrogen

In most diazotrophs, nitrogenase can be affected both short term and long term by the presence of ammoniacal nitrogen. Little is known about the short term effect of ammonium on the process of nitrogen fixation. Inhibition is rapid and reversible, persisting as long as free NH_3 remains in the medium. Several studies have shown that NH_3 has to be assimilated with the formation of glutamine for inhibition to occur (Kennedy, Doetsch *et al,*. 1994). The mechanism of this short term control has been shown in some instances to involve covalent modification of the Fe protein. This modification involves reversible ADP-ribosylation of the Fe protein and has been demonstrated in *Azospirillum lipoferum,* and *A. brasilense* (Eady, 1992).

Azotobacter chroococcum and *A. vinelandii* also exhibit NH_3 "switch-off" but do not appear to have this modifying system. Some populations of *A. vinelandii* and *A. chroococcum* show only 30% - 50% NH_3 induced switch off (Drozd, Tubb *et al*., 1972)

but effects greater than 90% have also been reported and may be substrate dependent (Laane, Krone *et al.,* 1980). The degree to which NH_4^+ inhibits activity varies considerably with growth conditions such as O_2 input, respiratory rate, pH and growth stage (Klugist and Haaker, 1984).

In cells of *A.vinelandii* respiring at low rates, NH_4^+ can even stimulate nitrogenase activity as addition of NH_4^+ leads to increased respiration rates which overcome oxygen inhibition of nitrogenase activity (Klugist and Haaker, 1984). Of the other nitrogen sources such as nitrate, urea and amino acids, only nitrate apparently represses nitrogenase synthesis in *Azotobacter* (Drozd, Tubb *et al.* 1972).

Long term effects of ammonia involve the repression of nitrogenase synthesis (Laane, Krone *et al*., 1980). Organisation and control of the *nif* regulon is shown in Figure 7. In enteric bacteria, the expression of many genes in response to the global N status is modified by the *ntr* system. This system represses the synthesis of a number of enzymes yielding ammonia as long as ammonia is available to the cells. Under low cell ammonia concentrations the promoter of the *nifA* and *nifL* genes is activated by products of the nitrogen control genes, designated nA and nC. The protein of the *nifA* gene (A) subsequently activates the transcription of all other genes in the nif regulon. In the presence of ammonia product nB prevents activation at the nitrogen control level and product L at the *nif* regulon level (Gottschalk, 1986).

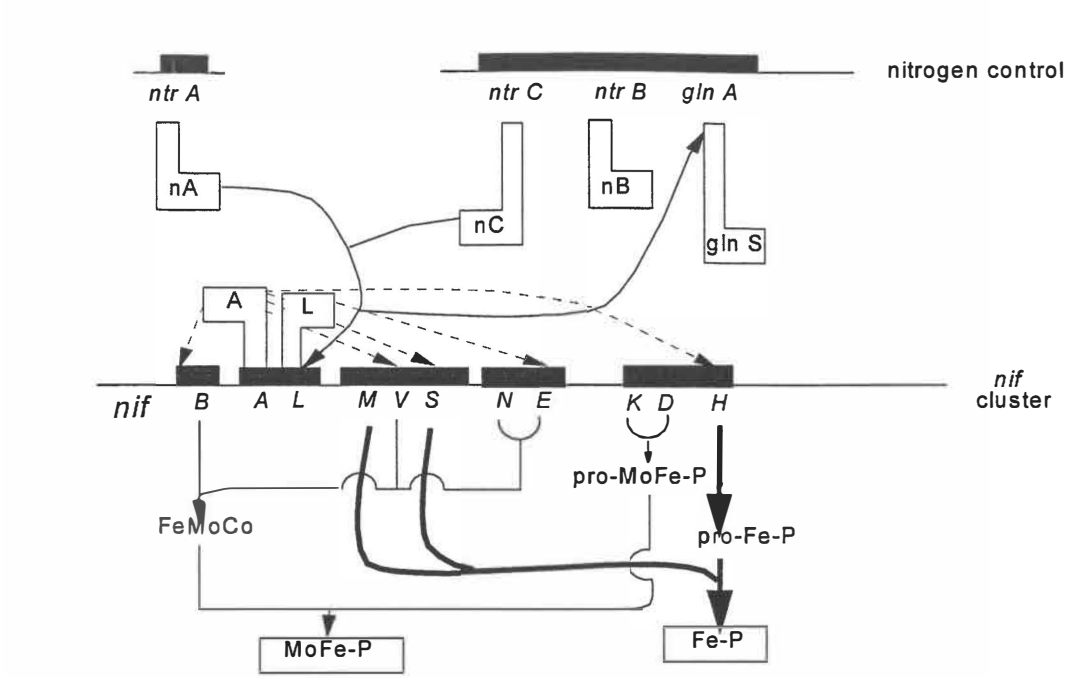

Figure 7: Organisation and control of the *nif* cluster in *Klebsiella pneumoniae* (from Gottschalk, 1986)

In *K. pneumoniae*, excessive concentrations of NH_4^+ (as well as oxygen) repress the synthesis of the nitrogenase complex via the *nifL* gene product, which under these conditions prevents the *nifA* product from activating expression of other *nif* genes.

It is to be expected that microorganisms with different physiologies will have developed modified regulatory strategies to control *nif* expression. The *nifA* gene has been identified by sequence or DNA hybridisation in 11 diazotrophs. In contrast, *nifL* has only been identified in *K. pneumoniae* and *A. vinelandii.*

2.5 CONSORTIAL NITROGEN FIXATION IN AQUATIC ENVIRONMENTS

Within an aquatic environment there exist well-defined highly dynamic spatial gradients and temporal fluctuations in factors controlling nitrogen fixation, including O_2 and NH_4^+ concentrations, pH, temperature and available organic carbon. Diverse autotrophic and heterotrophic N-fixing microorganisms populating these environments ensure a broad spectrum of physiological responses to these dynamic environmental constraints. A high

diversity of N-fixing bacteria allows N-fixing consortia to optimise nitrogen fixation when confronted with shifts in environmental conditions (Steppe, Olson *et al.,* 1996).

A large proportion of the research on consortial nitrogen fixation is concerned with cyanobacterial mats in the marine environment (Steppe, Olson *et al.,* 1996). Genetic techniques, in particular gene probing for microorganisms that contain the *nifH* gene, are now routinely used to determine the relationships that occur in the environment. The polymerase chain reaction (PCR) using degenerate primers enables diverse groups of nitrogen-fixing microorganisms to be detected, thus reflecting genetic potential for nitrogen fixation in a particular environment. By sequencing the amplified products from PCR, the dominant N-fixing taxonomic groups can be assessed. The results can be used to illustrate the importance of free-living versus particle associated nitrogen fixers, general quantification of *nif* genes under different spatial and temporal regimes, the relative importance of autotrophic versus heterotrophic nitrogen fixation, as well as many other unanswered questions.

An essential element of these N-fixing communities appears to be the presence and maintenance of anaerobic microzones which provide a locally reduced region where associative N-fixing bacteria can escape the potential inhibitory effects of oxygen on nitrogenase activity (Steppe, Olson *et al.*, 1996). Generally, lower overall oxygen concentrations are consistently accompanied by enhanced nitrogenase activity. Paerl and Carlton (1988) found that nitrogenase activity could be maximised by providing sufficient organic matter necessary to both create the oxygen depleted microzones and provide an energy source for supporting the N-fixers.

Also important for the development and proliferation of N-fixing communities are colonisable organic and inorganic surfaces as development of nitrogenase activity is often linked to the formation of detrital flocs. Within most natural environments, conditions are not the most favourable for bacteria as supplies of nutrients are low. Detrital flocs supply surfaces for microbial adhesion and biofilm growth. Within a biofilm, conditions may be more favourable because the bacteria are able to "catch" nutrients and the biofilm community may recycle nutrients between different species.

2.6 IMPLICATIONS FOR WASTEWATER TREATMENT OF PULP AND PAPER MILL WASTEWATER

In contrast with domestic sewage or food processing effluents, pulp and paper wastewaters have low concentrations of the key nutrient nitrogen, well below the levels conventionally recommended for aerobic treatment systems (Table 5).

Table 5: Comparison of total Kjeldahl nitrogen (TKN) wastewater concentrations from different sources

Wastewater	TKN concentration (mg/L)	Reference
Untreated domestic	20-85	(Tchobanoglous and Burton, 1991)
Meat processing	150	(Tipler and Borrie, 1997)
Poultry processing	120	(Couper and Maclennan, 1997)
Pulp and paper	0.70-3.0	Bruce, and Clark, 1994; Gapes and Clark, 1995; Nicol, 1997.

Hynninen and Viljakainen (1995) recommend typical guidelines of 2-3 mg/L of dissolved nitrogen in pulp and paper effluent for effective biological treatment. Work done by the Environmental Research Group, New Zealand Forest Research Institute has found that the dissolved nitrogen content of the wastewater entering Tasman's aerated treatment system is less than 1.5 mg/L (Bruce and Clark, 1994; Gapes and Clark, 1995; Gapes, 1996; Gapes, Frost *et al.,* 1997). Despite these low nitrogen concentrations, Tasman's and other pulp mill aerated lagoon treatment systems are operated with no addition of supplemental nitrogen, while achieving high levels of biological removal of organic material (Slade, Leonard *et al.*, 1991; Saunamaki, 1989).

Lower biomass yields were observed in a nitrogen fixing system compared to a nitrogen supplemented system in laboratory scale reactors operated under conditions similar to

Tasman's Pond 2 (Gapes, 1996). The lower biomass yields were attributed to the higher energy requirement for fixing atmospheric nitrogen under aerobic conditions. BOD removals were very similar with approximately 70% removals occurring in both systems. Soluble COD removals were slightly lower for the nitrogen fixing system (34-39%) than the nitrogen supplemented system (42-47%)(Gapes, 1996). The same system under N-fixing conditions removed >90% of resin acid compounds (Gapes, Robinson *et al.*, 1996). Recent studies have shown that a nitrogen fixing activated sludge system and a nitrogen fixing moving bed biofilm reactor system are very effective in the treatment of nitrogen deficient wastewaters from the pulp and paper industry (Gapes, Frost *et al.*, 1997).

The substrate inefficiency of aerobic bacteria fixing nitrogen under high oxygen concentrations has major implications for wastewater treatment. Operation of pulp and paper biological treatment systems with active nitrogen fixation at relatively high dissolved oxygen levels has the potential to significantly reduce biomass yields. This could lead to significant reductions in the amount of biological sludge requiring disposal.

At present a significant proportion of pulp and paper biological treatment systems rely on supplemental nitrogen addition (Valtilla, 1991). Nitrogen-fixing populations could be used to diminish the addition of combined nitrogen to activated sludge treatment plants and eliminate associated chemical costs (Neilson and Allard, 1985).

A nitrogen fixing wastewater treatment system has potential for application in producing biodegradable plastics. A number of free-living, aerobic, nitrogen fixing bacteria produce high levels of poly-β-hydroxybutyrate (PHB), an intracellular storage polymer. Commercially, PHB is of considerable interest because it is used to produce biodegradable plastics. The high cost of substrates as carbon sources for microbial PHB production has limited widespread commercialisation. Finding less expensive substrates would be important for the commercialisation of PHB. Using an industrial wastewater to provide the carbon substrate for PHB has been investigated (Son, Park *et al.*, 1996). Using wastewaters to provide carbon substrate for microbial PHB production has the potential to substantially reduce the cost of PHB production and at the same time creating an economic biosolids byproduct thus reducing costs for biosolid disposal.

The presence of combined nitrogen decreases total nitrogenase activity. Because N-fixing organisms only fix nitrogen when it is required and in quantities they require for growth, the soluble nitrogen in the effluent produced from nitrogen fixation based treatment systems should, in theory, be very low. This would be very advantageous for pulp and paper mills where nitrogen discharges are regulated, and for reducing the BOD of the treated wastewater, as nitrification can contribute significantly to the overall BOD load exerted in the receiving water (Davis and Cornwell, 1991).

Hynninen and Viljakainen (1995) state that if dissolved nitrogen in the effluent does not exceed 1.5-2 mg/L, the microorganisms will obtain nitrogen from the air, resulting in; accelerated degradation of biological sludge and increased effluent chemical oxygen demand, suspended solids and nutrient discharge. No further explanation was offered to substantiate these statements. Research completed by the Environmental Research Group does not support these statements but shows instead great potential for developing a full-scale treatment system based on the use of a nitrogen-fixing bacterial population.

N-fixing microaerophilic Enterobacteriaceae have been isolated from activated sludge plants treating wastes from paper and food industries (Neilson and Allard, 1985). However, it is likely that other microorganisms, as well as *Klebsiella* (a member of the Enterobacteriaceae), are involved in nitrogen fixation in Tasman Pulp and Paper Ltd's treatment system (Bruce and Clark, 1994). *Azotobacter* species have been isolated from a neutral sulphite chemical pulp mill (Knowles, Neufeld *et al.*, 1974). *Azotobacter* are able to fix nitrogen at relatively high oxygen concentrations and may represent a significant proportion of the N-fixing population in Tasman's treatment system. However, *Azotobacter* have not been shown to grow using methanol as a carbon substrate (Thompson and Skerman, 1979). Preliminary studies have shown that methanol, acetic acid and dissolved carbohydrates are important bulk organic constituents in Tasman's wastewater (Frost, McFarlane *et al.*, 1996).

The heterogeneity of aerated lagoon treatment systems provides many microenvironments capable of supporting a wide range of microorganisms with many different metabolic capabilities. In such a dynamic environment, a diverse nitrogen

fixing population would be most advantageous when confronting changes in environmental conditions.

2.7 SUMMARY

A diverse group of prokaryotes is able to fix nitrogen. While large differences exist in the morphology and physiology of these prokaryotes, the nitrogenase enzyme and the overall reaction representing nitrogen fixation are similar. Nitrogen fixation is an exothermic reaction but due to the stability of N_2 requires energy in the form of ATP. Because it is an energy expensive reaction, N-fixing organisms closely regulate nitrogen metabolism in the presence of combined nitrogen and under high oxygen concentrations.

Members of the Azotobacteraceae are among the least oxygen sensitive of the aerobic, free-living, heterotrophic, N-fixing bacteria. *Azotobacter* species are able to protect the oxygen sensitive nitrogenase enzyme by adjusting their respiration rate to the oxygen supply. Respiratory protection mechanisms enable *Azotobacter* to fix nitrogen at high dissolved oxygen concentrations compared to other free-living bacteria. Nitrogen fixation at high oxygen concentrations is inefficient and more substrate is required for cell production than under low oxygen conditions. A number of aerobic N-fixing bacteria fix nitrogen only under reduced oxygen concentrations (microaerophilic conditions) because they do not have the sophisticated physiological protection mechanisms that *Azotobacter* species have.

Over the past ten years genetic techniques, which are able to detect the presence of DNA homologous to the structural genes of the nitrogenase enzyme, have significantly increased the number of bacterial species that are recognised to fix nitrogen. These techniques can also be used to determine the relationships occurring in microbial communities existing in the environment. A diverse N-fixing population may be best suited to the dynamic conditions of an aquatic ecosystem.

N-fixing microorganisms have previously been isolated from paper and food industry wastewaters. Gapes and Clark (1995) demonstrated the advantages of operating an aerobic N-fixing treatment system for pulp and paper wastewaters. These advantages

include reduced sludge production, better control of nitrogen load to the discharge environment and no supplemental nitrogen addition.

It is necessary to study the fundamental microbiology of these nitrogen-fixing systems to understand how these systems operate. This study concentrates on how different organic substrates effect the performance of nitrogen fixing biological treatment systems. The information gained from this study will help improve and optimise treatment system design and performance.

Section 3

COMPOSTION OF BULK ORGANIC SUBSTRATES IN TASMAN PULP AND PAPER'S WASTEWATER

3. COMPOSITION OF BULK ORGANIC SUBSTRATES IN TASMAN PULP AND PAPER'S WASTEWATER

3.1 INTRODUCTION

In New Zealand aerated lagoon systems are used for secondary treatment of kraft mill effluents. These aerated lagoons are responsible for the removal of oxygen demanding substances from the wastewater. Bulk organic constituents are largely responsible for the oxygen demand of the effluent. Therefore the efficient removal of the bulk organic constituents of pulp and paper mill effluents is a critical issue for these waste treatment systems.

The organic load of pulp and paper effluents consists of a wide variety of compounds. Not all of these compounds are readily degradable in the biological systems used to treat these effluents. A preliminary study at Tasman Pulp and Paper Ltd's aerated lagoon treatment system indicated that methanol, dissolved carbohydrates and acetic acid were readily degradable and completely removed by the treatment system and accounted for a significant proportion of the biodegradable oxygen demand (Frost, McFarlane *et al.*, 1996).

The availability and nature of the readily degradable organic substrate effects the composition of the microbiological consortium within a treatment system. *Azotobacter*, among the most oxygen tolerant of nitrogen fixing bacteria, have previously been found in pulp and paper mill effluents (Knowles, 1974) and are known to utilise a wide range of carbohydrates, alcohols and organic acids. *Azotobacter,* however, are unable to utilise methanol as a carbon and energy source (Thompson and Skerman, 1979). In contrast *Xanthobacter* are microaerophilic nitrogen fixing bacteria that are able to utilise methanol, other alcohols and organic acids but have only limited ability to utilise carbohydrates (Kreig, 1982).

The identification of the significant readily degradable substrates in Tasman's wastewater will provide information for the optimisation of pond treatment performance. Bulk organic composition and concentration data gained from this work will be used to study

the effects of different substrate compositions on the processes occurring within a biological treatment system and for isolating bacteria responsible for the degradation of these individual substrates in a laboratory biological reactor system.

3.2 OBJECTIVE OF THIS STUDY

To determine the composition of the organic components in Tasman's wastewater and the degradability of these components within Tasman's aerated lagoon treatment system.

3.3 METHODS

3.3.1 Sample collection

Wastewater discharges from the production of bleached and unbleached kraft pulp, stone and refiner groundwood pulp and newsprint contribute to the combined mill effluent at Tasman Pulp and Paper Ltd. The combined mill effluent enters Pond 1 after undergoing primary clarification. Pond 1 is an unaerated settling pond. From Pond 1 the flow passes through three aerated lagoons (Ponds 2, 3 and 4) prior to discharge to the Tarawera River.

Four sites at Tasman Pulp and Paper Ltd's treatment system were sampled over a ten day period during March 1997. During the sampling period no spills were recorded and the system appeared to operating normally. Figure 8 illustrates these sample sites.

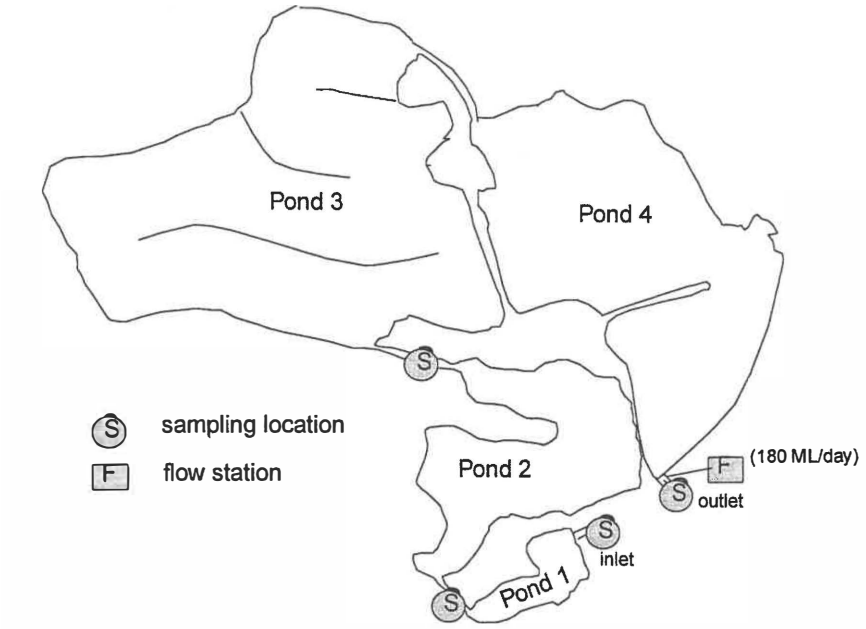

Figure 8: Sample sites and flow measurement at Tasman Pulp and Paper Ltd. Treatment system

Table 6 describes the abbreviations used in this report for each sampling site. Flow data were recorded at the outlet to Pond 4.

Table 6: Nomenclature of sites sampled at Tasman treatment system

Sampling site	Abbreviation used in this report
Pond 1 inlet	Pond 1 in
Pond 1-2 cutting	Pond 2 in
Pond 2-3 cutting	Pond 2 out
Pond 4 outlet	Pond 4 out

Twenty four hour composite wastewater samples were collected and stored on ice from these sites. Samples were collected from the main flow, clear of floating scum and bottom sludge.

The samples were transported on ice and analysed for COD and methanol at the laboratory. Samples for dissolved carbohydrate and volatile fatty acid analysis were filtered through a GF/C filter paper and frozen on arrival back at the laboratory.

3.3.2 Analytical methods

Total and soluble COD

The total chemical oxygen demand (CODt) was determined by using a microscale adaptation of APHA Method 5220D (APHA, 1992). Samples taken for soluble chemical oxygen demand (CODs) determination were filtered through GF/C filter paper prior to analysis. A full method description is given in Appendix A.

Volatile fatty acid analysis

Volatile fatty acids (VFA); acetic, propionic, iso-butyric and n-butyric acids were determined by GC-FID using an in-house method of the Environmental Research Group at the New Zealand Forest Research Institute. A sample was injected onto a capillary column gas chromatograph (Nukol™ 30m column, operating at 130°C) and the presence of volatile fatty acids were detected using a flame ionisation detector. Concentrations were determined by reference to an iso-valeric acid internal standard via calibration to a standard mixture. The detection limit for acetic acid was 1 mg/L. A full method description is given in Appendix A.

Methanol and ethanol analysis

Methanol, ethanol and acetone were analysed by gas chromatography on a 0.53 mm i.d x 30 m column coated with a thick film of BP-wax and detection via a flame ionisation detector. The aqueous samples were injected directly onto the column using a cool on column injector operating at 140° C. Peak identities were established by comparison of retention times with those of authentic samples. The concentration of methanol and ethanol was quantified with reference to an external standard. The detection limit for methanol was less than 0.5 mg/L. A full method description is found in Appendix A.

Dissolved carbohydrate analysis

Dissolved carbohydrates were analysed by the Analytical Chemistry Laboratory at the New Zealand Forest Research Institute. Total dissolved carbohydrates were analysed using a method based on that of Petterson and Schwandt (1991). Pre-filtered samples were hydrolysed in sulphuric acid, separated by anion exchange chromatography and quantitatively measured with a pulsed amperometric detector. Detection limits were 0.1

mg/L arabinose, 0.7 mg/L galactose, 0.3 mg/L glucose, 0.3 mg/L xylose and 0.5 mg/L mannose.

3.3.3 Data interpretation

Individual organic components have a theoretical COD that can be calculated to determine the contribution of identified compounds to the overall COD of a mixture. This COD balancing approach is used in the following section. Example calculations are given in Table 7 and Table 8 for converting the concentration of specific compounds to their theoretical COD and the amount of soluble COD recovered from substrate analyses.

Table 7: Theoretical COD calculations for substrates recovered

Substrate	Equation	COD conversion factor
Acetone	$CH_3COCH_3 + 4O_2 \rightarrow 3CO_2 + 3H_2O$	2.2
Methanol	$2CH_3OH + 3O_2 \rightarrow 2CO_2 + 4H_2O$	1.5
Ethanol	$C_2H_5OH + 3O_2 \rightarrow 2CO_2 + 3H_2O$	2.08
Dissolved carbohydrates	$C_6H_{12}O_6 + 6O_2 \rightarrow 6H_2O + 6CO_2$	1.07
Acetic acid	$CH_3COOH + 2O_2 \rightarrow 2CO_2 + 2H_2O$	1.07
Propanoic acid	$C_2H_5COOH + 3.5O_2 \rightarrow 3CO_2 + 3H_2O$	1.51

The COD was measured on solutions containing methanol, acetic acid and glucose. The actual COD of these solutions compared favourably with the calculated theoretical COD.

Table 8: COD balance example from Pond 2 in - 17 March - 26 March 1997

	Mean concentration mg/L	COD conversion factor	Theoretical COD mg/L
measured $COD_{(s)}$	535		
acetone	1.2	2.2	2.6
methanol	39.9	1.5	59.9
ethanol	2.4	2.08	5.0
dissolved carbohydrates	49.7	1.07	53.2
acetic acid	15.4	1.07	16.5
propanoic acid	3.4	1.51	5.1
TOTAL COD detected			142.3

Pond 4 out $COD_{(s)}$ = 204 mg/L

Assuming Pond 4 out $COD_{(s)}$ is an estimation of non biodegradable $COD_{(s)}$

Biodegradable $COD_{(s)}$ at Pond 2 in = Pond 2 in $COD_{(s)}$ - Pond 4 out $COD_{(s)}$

= 535 - 204

= 331 mg/L

Theoretical COD recovered from analysis = 142.3 mg/L

% of biodegradable $COD_{(s)}$ recovered = 43%

% of $COD_{(s)}$ recovered = 27 %

3.4 RESULTS

Raw data is presented in Appendix B.

3.4.1 Substrate removal across Tasman Pulp and Paper treatment system

Figure 9 shows a chronological plot of soluble COD concentrations at Tasman Pond 1 in, Pond 2 in, Pond 2 out, and Pond 4 out during the sampling period. Soluble COD concentrations were similar at sites Pond 1 in and Pond 2 in. The mean soluble COD concentration at Pond 2 in was 540 mg/L. Pond 2 out soluble COD was less variable than Pond 1 in and Pond 2 in with an average concentration of approximately 320 mg/L. At Pond 4 out the soluble COD concentration was consistently 200 mg/L.

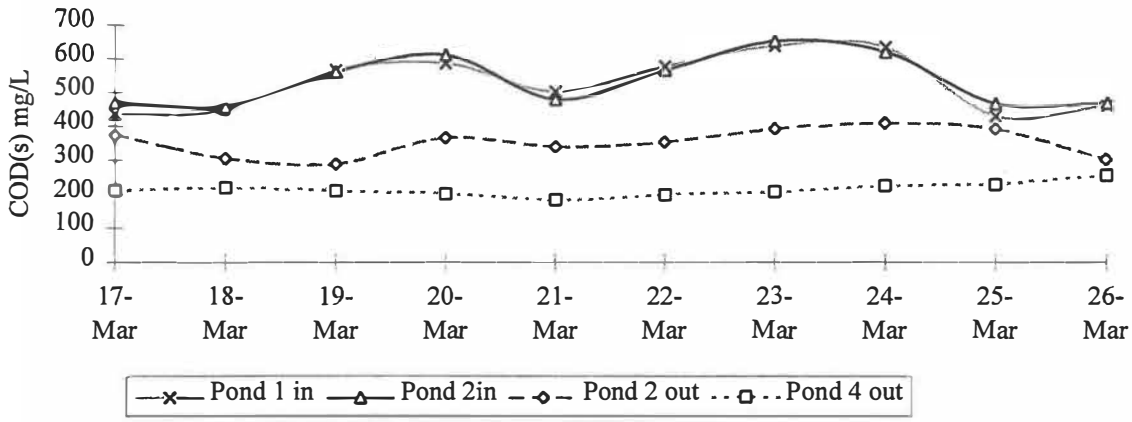

Figure 9: Chronological plot of soluble COD concentrations in Tasman's treatment system

Figure 10 illustrates mean substrate concentrations across Tasman's treatment system. This figure shows that the largest soluble COD removals occurred in Pond 2. Approximately 200 mg/L of soluble COD was removed from Pond 2 and a further 100 mg/L between Pond 2 out and Pond 4 out. A small amount of acetic acid appeared to have been produced in Pond 1 but this was completely removed during treatment in Pond 2. Methanol and dissolved carbohydrates were less degradable than acetic acid as concentrations were still detectable at Pond 2 out, however, these substrates were all degraded by Pond 4 out.

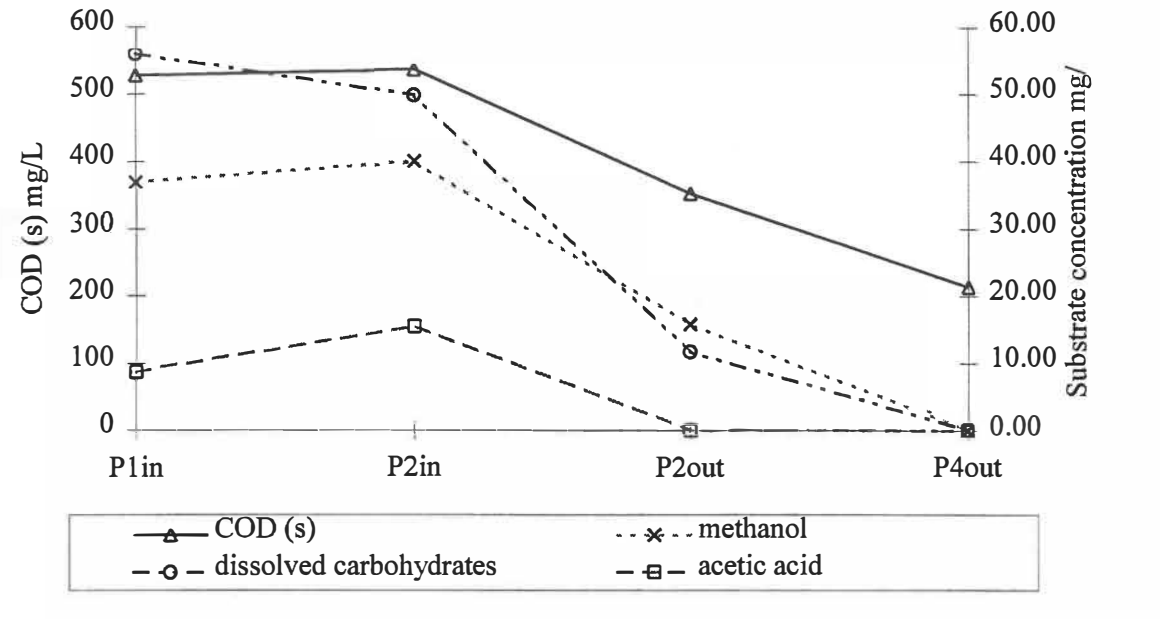

Figure 10: Mean substrate concentrations across Tasman treatment system over 10 day period

In this report the soluble COD at Pond 4 out has been defined as the non-biodegradable soluble COD. In this context, non-biodegradable soluble COD refers to the soluble COD that is not readily degradable in this treatment system. At Pond 4 out, the total BOD was approximately 10% of the soluble COD (Appendix B) and therefore it is assumed that the soluble COD present at Pond 4 out is not readily biodegradable in this treatment system. The term biodegradable as used in this work, includes substrate removal by adsorption processes as well as assimilation by microorganisms.

Figure 11 illustrates the composition of soluble COD throughout Tasman's wastewater treatment system. Substrate concentrations were converted to theoretical COD concentrations as detailed previously (Section 3.3.3). This figure shows that approximately 35% of the soluble COD at Pond 1 and Pond 2 consists of unidentified degradable substrates, 40% of unidentified non-degradable substrates and the remaining 25% consisting of methanol, dissolved carbohydrates and acetic acid. By Pond 4 out all identified and unidentified degradable substrates had been consumed.

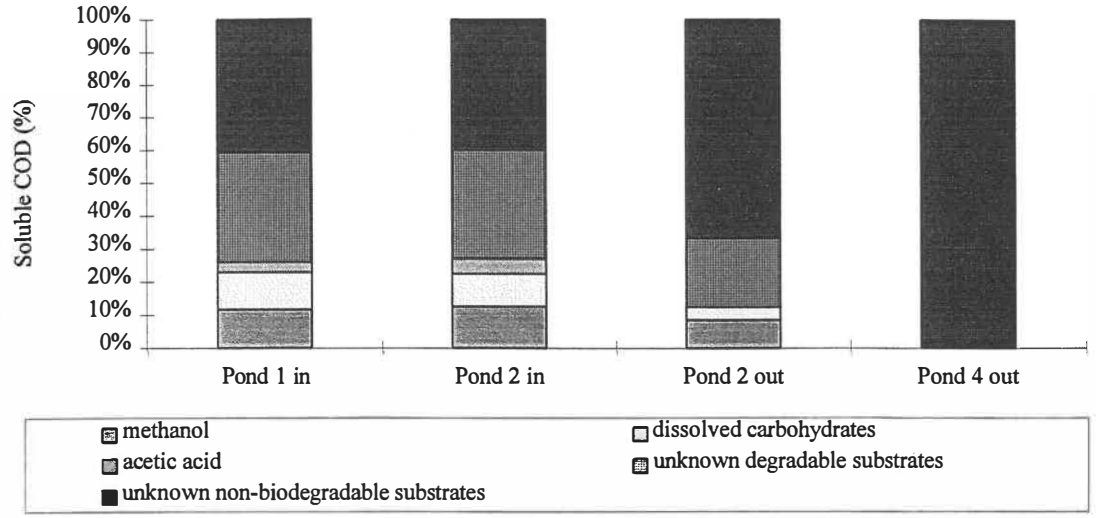

Figure 11: Composition of soluble COD throughout Tasman treatment system

3.4.2 Mean substrate concentration - Tasman Pulp and Paper Pond 2

The composition of the soluble COD at Pond 2 in during the sampling period is illustrated in Figure 12. All individual substrate concentrations were converted to theoretical COD as described previously.

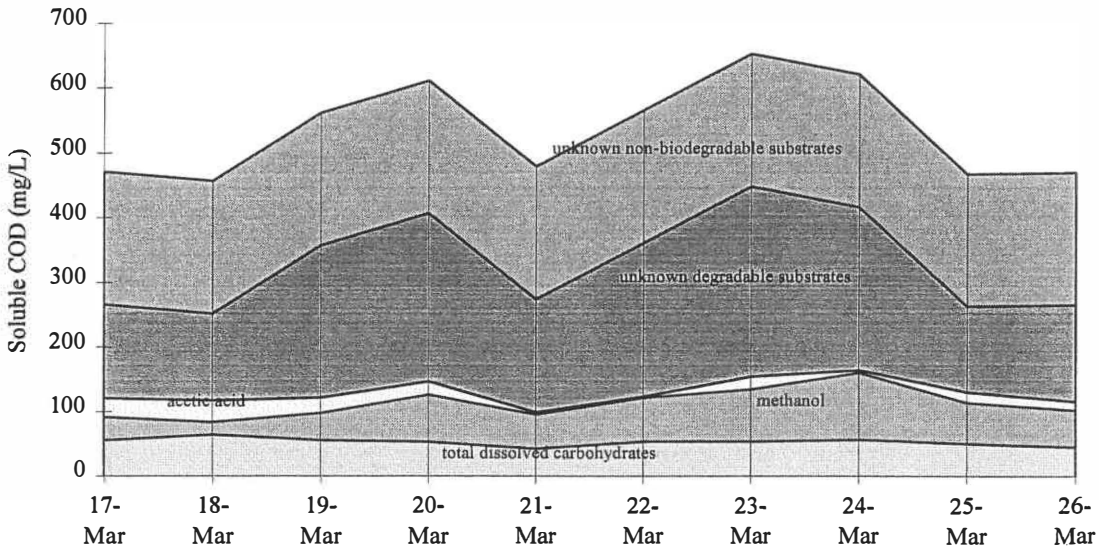

Figure 12: Chronological plot of soluble COD composition at Pond 2 in

Total carbohydrates and methanol made up the bulk of the known readily degradable organic compounds entering Pond 2. Whilst total carbohydrate concentrations appeared to be consistent at Pond 2 in, acetic acid and methanol concentrations were extremely

variable. Unknown degradable substrates contribute a significant proportion (150 - 300 mg/L) of the soluble COD. Unknown non biodegradable substrate contributed the largest proportion of soluble COD.

Figure 13 illustrates the composition of soluble COD at Pond 2 out during the sampling period. This figure shows that a significant proportion of the degradable substrates was removed in Pond 2.

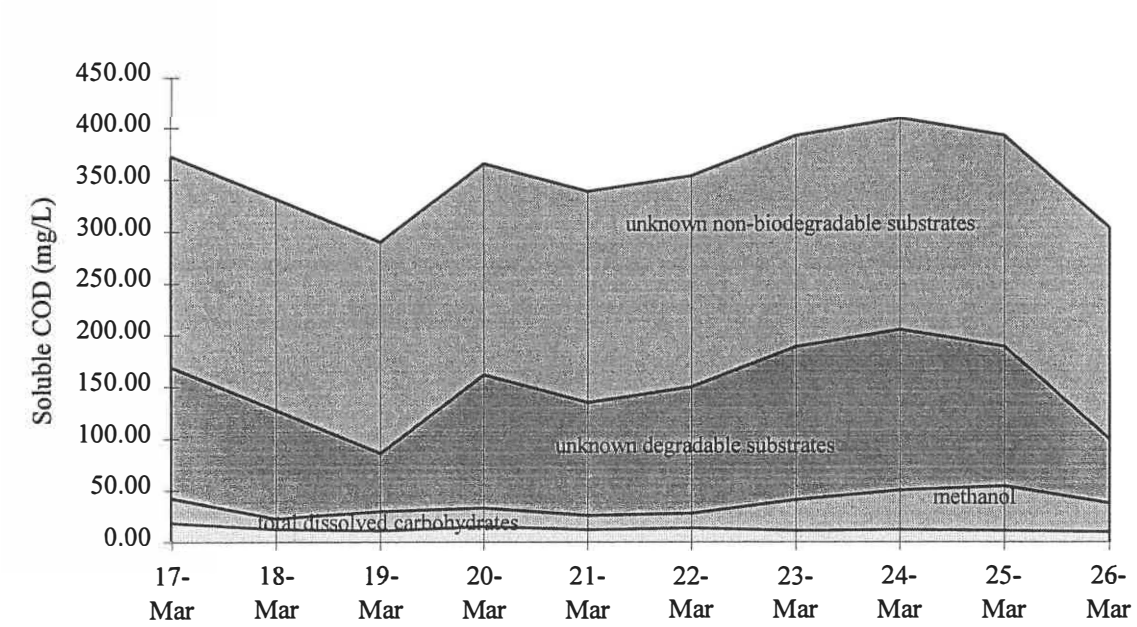

Figure 13: Chronological plot of soluble COD composition at Pond 2 in

3.4.3 Comparison with 1996 Tasman treatment system survey

Table 9 compares the mean bulk organic data collected during the 1997 sampling period with data collected during a similar study in 1996 (Frost, McFarlane et al. 1996).

Table 9: Comparison of March 1996 (Frost *et al,* 1996) and March 1997 data collected from Pond 2, Tasman wastewater treatment system

Units are	Pond 2 in		Pond 2 out	
(mg/L)	1996	1997	1996	1997
$COD_{(t)}$	650.4	859.9	502.6	602.4
$COD_{(s)}$	416.9	535.1	261.9	321.7
Methanol	37.7	39.9	23.4	15.8
Total dissolved carbohydrates	38.7	49.7	6.9	11.7
Acetic acid	30.9	15.4	nd	nd

nd = not detected nt = not tested

Higher COD concentrations were recorded in 1997 possibly due to dredging in Pond 1 during the sampling period. Methanol concentrations were similar to those recorded in 1996. Lower levels of acetic acid were detected in the 1997 samples. Dredging was occurring in Pond 1 during the sampling period and may have affected the performance of Pond 1 preventing the anaerobic conversion of substrate to acetic acid. Total dissolved carbohydrate concentrations increased in 1997 because the analysis was broadened to include cyclotol compounds.

3.5 DISCUSSION

Soluble COD concentrations at Pond 1 in and Pond 2 in were similar suggesting that little removal occurred in Pond 1, which functioned only as a holding pond. In a previous study Pond 1 was thought to be operating as an anaerobic pond, converting carbohydrate polymers to more readily degradable acetic acid (Frost, McFarlane *et al.,* 1996). During the 1996 study higher acetic acid concentrations were observed entering Pond 2 than in the present study. Dredging of Pond 1 during the current study may have affected the concentration of acetic acid entering Pond 2.

In the present study, the majority of organic substrate degradation occurred in Pond 2, the first aerated lagoon in the treatment system. Sixty percent (200 mg/L) of the soluble COD was removed in this pond.

Approximately 25% of the soluble COD at Pond 2 in consisted of identified degradable substrates. Of these identified substrates, dissolved carbohydrates, methanol and acetic acid contributed about 43% of the biodegradable soluble COD in this pond. Biodegradable soluble COD was defined as the soluble COD that was removed in this treatment system.

The remainder of the soluble COD consisted of unidentified compounds. Approximately 40% of the soluble COD was non-degradable in this treatment system and 35% was degradable but unidentified. Approximately 60% of this degradable fraction was removed within Pond 2. Assuming that most of this unknown fraction was degraded biologically, the unknown fraction would affect the composition of the microbiological consortium present in the treatment system. Further study is required to identify this unknown fraction.

Mean concentrations of identified substrates at Pond 2 in during the sample periods are shown in Table 10.

Table 10: Summary table of Pond 2 mean substrate concentrations

Parameter (mg/L)	Pond 2 in	Pond 2 out
$COD_{(t)}$	859.9	602.4
$COD_{(s)}$	535.1	321.7
DOC	190.1	118.8
Methanol	39.9	15.8
Total dissolved carbohydrates	49.7	11.7
Acetic acid	15.4	nd

nd = not detected

During this period, methanol and acetic acid concentrations were quite variable producing the variation of COD concentrations observed at Pond 1 in and Pond 2 in. Commissioning problems with a foul condensate stripper resulted in variable methanol loadings to the treatment system during the sampling period. Variable acetic acid concentrations may have been due to the dredging occurring in Pond 1 during the study.

Because of the "U" shape and subsequent flow characteristics of this pond (Slade, Leonard *et al.*, 1991) the microorganisms in Pond 2 are likely to be exposed to an intermediate substrate concentration, somewhere between the concentrations observed at Pond 2 in and Pond 2 out.

Acetic acid was completely removed from the system at some point before Pond 2 out. This is consistent with previous work (Frost, McFarlane *et al.*, 1996) which found that acetic acid was degraded faster in the treatment system than methanol and total dissolved carbohydrates. Methanol and total dissolved carbohydrates were completely removed from the treatment system by Pond 4 out.

3.6 CONCLUSIONS

The conclusions that can be drawn from this field study of the Tasman treatment system are:

- The bulk of the degradable soluble COD was removed in Pond 2, the first aerated lagoon of Tasman's treatment system. At Pond 2 in, the soluble COD consisted of approximately 25% identified degradable substrates (methanol, acetic acid and total dissolved carbohydrates), 40% unidentified degradable substrates and 35% unidentified non-biodegradable substrates. Forty percent of the degradable substrates was removed in Pond 2.

- Acetic acid was the most readily degradable of the identified substrates consistent with results obtained during the 1996 study (Frost, McFarlane *et al.,* 1996). Methanol and total dissolved carbohydrates were degradable and completely removed from the system by Pond 4 out.

- The large proportion (40%) of unidentified degradable substrates are likely to influence the microbial consortium in the treatment system. More work is needed to identify this unknown fraction.

- The acetic acid concentrations observed during this sampling period were lower than those obtained from the 1996 study and more variable. Dredging in Pond 1 during the sampling period may have inhibited the anaerobic conversion of substrate polymers to acetic acid. Methanol concentrations were variable, most likely due to the intermittent operation of the newly commissioned foul condensate stripper.

Section 4

ASSESSMENT OF SOME EFFECTS OF WASTEWATER COMPOSITION ON NITROGEN FIXATION IN A BIOLOGICAL TREATMENT SYSTEM

4. ASSESSMENT OF SOME EFFECTS OF WASTEWATER COMPOSITION ON NITROGEN FIXATION IN A BIOLOGICAL TREATMENT SYSTEM

4.1 INTRODUCTION

Studies of the aerated lagoon system at Tasman Pulp and Paper Co. Ltd have shown that bacterial growth on nutrient deficient wastewaters is dependent upon bacterial fixation of atmospheric nitrogen (Clark and Bruce, 1994). Gapes and Clark (1995) have suggested that the nitrogen fixing bacteria are responsible for the majority of removal of organic material in the wastewater.

The bulk organic substrate study (Section 3) identified that dissolved carbohydrates, methanol and acetic acid contributed to approximately 40% of the degradable soluble COD in Pond 2 of Tasman's treatment system. Methanol and acetic acid concentrations entering Pond 2 were highly variable. A substantial change in the concentration of one of these substrates may alter the microbiological consortium present, affecting nitrogen fixation rates and COD removals.

To study the effect of changing wastewater composition on the nitrogen fixation processes occurring in Tasman's aerated treatment system, a laboratory scale biological reactor was used. By simulating aerated lagoon treatment in a bioreactor it is feasible to control the wastewater composition and feed rate. This control would not be possible using the full scale Tasman treatment system.

A continuous stirred tank reactor (CSTR) was used as the biological reactor for this work. CSTRs are easy to run, allow accurate determination of biological growth parameters and are also considered suitable for simulating Pond 2 of Tasman Pulp and Paper Ltd's aerated lagoon treatment system (Gapes and Clark, 1995). Previous work has shown that this pond functions as a number of CSTR's acting in series (Slade, 1989).

4.2 OBJECTIVE OF THIS STUDY

To study some effects of changing the wastewater composition on the processes occurring in the nitrogen fixing biological treatment system of bleached kraft mill effluent by investigating associations between biomass composition, substrate removal efficiencies and N-fixation rates.

4.3 METHODS AND MATERIALS

4.3.1 Sample collection

Six hundred litres of wastewater from Tasman Pulp and Paper Ltd were collected from Pond 2 in on the 23rd April 1996. The wastewater was mixed in a 1000 L container and solids were left to settle out overnight. The wastewater was stored in 20 L containers at -30°C. The sample containers were thawed and kept at 4°C as required.

4.3.2 Culture techniques

The studies were carried out using a 7L Chemap (Switzerland) fermenter. The use of this system allowed measurement and control of dissolved oxygen (DO), temperature, agitation speed and reactor volume. A schematic diagram of a typical CSTR apparatus is presented in Figure 14.

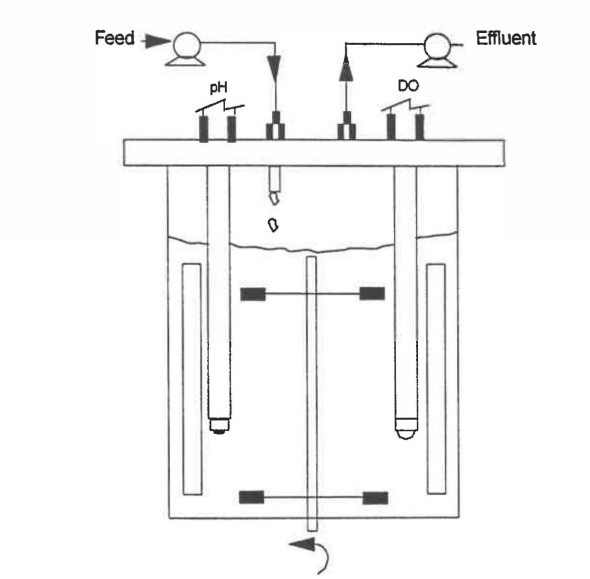

Figure 14: Schematic diagram of a laboratory CSTR and ancillary equipment

Dissolved oxygen control

Aeration was provided by surface reaeration caused by mixing of vessel contents. Precise control of the dissolved oxygen concentration was effected by automatic control of the stirrer speed. A feedback control mechanism adjusted the speed of the stirrer in proportion to the magnitude of the difference between the DO setpoint and the actual DO of the system.

The reactor was operated at a dissolved oxygen concentration of 2.2 mg/L (30% saturation). Previously, this DO concentration had been used by Gapes and Clark (1995) to operate an effective aerobic N-fixing treatment system with wastewater from Tasman's Pond 2. The DO probe was regularly calibrated; at the 0% saturation level by suspending the head of the probe into a 250 mL Erlenmeyer flask and sparging this with argon gas for 5 - 10 minutes to strip out any oxygen; and at the 100% saturation level by suspending the probe in distilled water that had been stirred overnight in air to ensure its oxygen saturation. The reactor was operated at a temperature of 30.8°C. At this temperature the saturation dissolved oxygen concentration in water is 7.46 mg/L (APHA, 1992).

Reactor volume control

The volume of the liquid in the reactor was controlled at 4.7 L, with a 3.16 L headspace volume above the surface of the liquid. The working volume was maintained by way of a level control probe that activated the effluent pump.

The wastewater sample was fed to the reactor using a Masterflex peristaltic pump (Cole Palmer Instrument Co. Catalogue No. 7554-30, 1-100 rpm) that was attached to a Masterflex variable speed controller. The wastewater was fed at a rate of 10 L/day (HRT≈0.5 day).

The effluent draw-off was effected by a Chemap AG (Switzerland) peristaltic pump, attached to the reactor's sample removal port. This consisted of a pipe immersed vertically in the reactor in a zone of complete mixing. In order to remove a representative sample of the reactor contents, the draw off pump was set to operate at maximum speed when activated by the reactor's level controller.

Feed preparation

The feed for the reactor was prepared by filtering through Whatman No.3 filter paper, and then through Whatman GF/B filter paper, ensuring that the feed entering the reactor only consisted of soluble material.

As a precaution against phosphorus limitation, phosphorus, in the form of sodium dihydrogen orthophosphate was added to the reactor feed to give a BOD_5:P ratio of approximately 100:1.

Reactor operation

The experimental work involved controlling the DO concentration in the reactor at 2.24 mg/L at a temperature of 30.8°C. The HRT in the reactor was maintained at 0.5 day.

Initially, a two litre sample of Tasman Pond 2 effluent was added to the reactor as the seed source containing the microorganisms. The reactor was considered to have reached steady state conditions after four HRTs had elapsed. During this start up period, soluble chemical oxygen demand ($COD_{(s)}$) of both the feed and the effluent were measured.

Stable $COD_{(s)}$ removal rates indicated that the reactor was operating under steady state conditions.

The experimental study was carried out in 3 different stages:

Stage 1:

The first thirteen days of reactor operation were used to determine control conditions. During this period the reactor was fed only with Tasman Pond 2 wastewater.

Stage 2:

Acetic acid was added to the Pond 2 wastewater on day 14 to day 26.

Stage 3:

On day 41, the reactor was reseeded with a Pond 2 out wastewater sample. Methanol was added to the feed during day 41- day 57.

After additional substrate supplementation, four HRTs elapsed before sample measurement commenced.

Reactor maintenance

All surfaces within the reactor were scrubbed daily to prevent adhering microbial growth from becoming a significant factor in the experiment. Similarly, the effluent removal pipe, the tubing from the feed reservoir to the reactor, and the tubing from the reactor to the effluent drain were cleaned daily to prevent significant microbial buildup.

Sample measurement

For each of the three substrate operating conditions, samples for COD, nutrient, and total suspended solids analysis were collected from the reactor effluent on 3 separate sampling days. Oxygen uptake rate (OUR) measurements and acetylene reduction assay measurements were carried out on the reactor the day preceding the sampling day. COD, and total solid analyses were completed the same day as sampled. Nutrient analysis were frozen for a maximum of 2 weeks before analysis. All analyses were completed at least in duplicate.

4.3.3 Sample analysis

Biomass determination

The total suspended solids (TSS) concentration was used to estimate the biomass concentration of the sample. The reactor feed was filtered through a GF/B glass fibre filter paper (Whatman) and considered to be totally soluble. Any suspended solid material in the reactor was therefore assumed to be biological material from the growth of microorganisms in the system.

Total and volatile suspended solids were measured on the mixed liquor in the reactor and the effluent using methods based on APHA Standard Method 5220 C-E (APHA, 1992). Full method descriptions are found in Appendix A. The effluent samples were collected on ice to limit any changes that might occur on standing.

Samples were centrifuged and the supernatant was removed and filtered through pre-washed and weighed 0.45 μM cellulose acetate filter paper (Sartorius), and the filtrate evaporated to a constant weight in a pre-weighed beaker for the determination of dissolved solids. Filtered solids were dried to a constant weight at 105°C for the determination of suspended solids. The centrifuged solid pellet was removed and dried overnight at 105°C in a pre-weighed moisture dish to determine suspended solids concentration. The suspended solid concentration of the supernatant and the pellet were added to determine total suspended solids concentration.

Volatile suspended solids (VSS) measurements were also carried out by placing the crucibles from the TSS analysis into a muffle furnace at 550°C for one hour. The samples were removed, cooled in a desiccator to ambient temperature and reweighed.

Oxygen uptake rate

The oxygen uptake rate (OUR) of the microorganisms in the reactor was measured by sealing the headspace during continuous operation of the reactor and taking syringe samples of the headspace gas through a rubber septum at a number of time intervals.

These samples were analysed for oxygen concentration on a BASIC™ gas chromatograph (Carle Instruments INC. Model GC 8700) using two columns, a Poropak pre-column for separating carbon dioxide from the nitrogen and oxygen, and a molecular sieve column for effecting the separation of nitrogen and oxygen. The columns were operated at 65°C with helium as the carrier gas, at an inlet pressure of 200 kPa. A thermal conductivity detector was used to record the oxygen and nitrogen as they passed off the column. The nitrogen peak on the chromatogram was used as an internal standard in determining the oxygen concentration of the sample.

It was assumed the effect of the dissolved oxygen concentration differences between the feed and final effluent was insignificant, and that the measurement of oxygen transfer from the headspace to the reactor mixed liquor was therefore equal to the oxygen uptake by the microorganisms. Gapes and Clark (1995) showed that, for the worst case scenarios under this assumption, the maximum uncertainty in the OUR measurement was less than 12%. A full method description is given in Appendix A.

Chemical oxygen demand

The chemical oxygen demand (COD) of a sample was determined by using a microscale adaptation of APHA Method 5220D (APHA, 1992). A full method description is given in Appendix A. Samples taken for soluble COD determination were filtered through a GF/C glass fibre filter paper (Whatman) prior to analysis. Total and soluble COD was taken from the reactor mixed liquor, while the feed COD was measured on unfiltered samples as the filtering process used to prepare the feed was considered adequate to give a totally soluble sample. Mixed liquor samples taken for total COD were disintegrated in a Waring blender for 30 seconds prior to analysis, in order to obtain a homogenised sample.

Nitrogen and phosphorus

Total Kjeldahl nitrogen (TKN), total phosphorus (TP) and dissolved reactive phosphorus (DRP) analyses were carried out on samples by the Rotorua District Council Environmental Laboratory using modified NWASCO methods (NWASCO, 1982). Total oxidised nitrogen (TOXN), measuring the nitrate and nitrite concentration of a sample, was determined by the Rotorua District Council Environmental Laboratory using a

modification of the method by Downs (Downs, 1978). Nitrogen fixation was determined by mass balance of TKN across the reactors.

Acetylene reduction assay

Nitrogen fixation activity in the reactors was measured using batch acetylene reduction assays, based upon the method described by Bruce and Clark (1994). Assays were conducted under an argon atmosphere and were initiated by addition of acetylene gas. The samples were incubated at 30°C with shaking at 120 rpm for 30 mins. At the completion of the incubation period, a sample of the head space gas was withdrawn and the formation of ethylene was quantified by gas chromatography on a 1.5 m x 6.4 mm packed column of Poropak N: carrier gas (N_2) 20 mL/min; H_2 20 mL/min; air, 300 mL/min; oven temperature 78°C; 1 mL sample loop; FID detector temperature, 150°C. The concentration of ethylene was quantified with reference to a standard curve. Full method description is given in Appendix A.

Cell counts

Samples were withdrawn from the mixed liquor in the reactor with a sterile pipette and diluted using sterile phosphate buffered water (APHA, 1992, Section 9050C). A dilution series was set up from 10^{-1} - 10^{-7} so that plates contained between 30 and 300 colonies. The spread plate agar technique was used as determined by the method specified in APHA (Section 9215C). The appropriate dilutions were pipetted onto the surface of a nitrogen free mineral salts (NFMS) agar in 0.1 mL aliquots. Using a sterile glass rod the liquid was distributed evenly over the pre-dried agar plates. The plates were prepared in duplicate and incubated aerobically in air tight containers at 30°C for 5 - 7 days.

The NFMS medium was based on Brown, Burlingham *et al.* (1962) (Table 11). Full details of preparation are given in Appendix A.

Table 11: NFMS medium (Brown, Burlingham *et al*, 1962)

Ingredient (per litre)	NFMS
Glucose	10 g
K_2HPO_4	0.8 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2$	0.1 g
$FeSO_4.7H_2O$	2.5 mg
H_3BO_3	2.9 mg
$CuSO_4.5H_2O$	0.1 mg
$MnCl_2.4H_2O$	0.09 mg
$CoSO_4.7H_2O$	1.2 mg
$Na_2MoO_4.2H_2O$	2.5 mg
$ZnSO_4.7H_2O$	1.2 mg
Nitrilotriacetic acid	100 mg
Agar	15 g

NFMS agar was prepared containing a range of different carbon substrates including; 1% glucose, 1% acetic acid, and 1% methanol. NFMS agar plates containing 1% glucose recorded the highest colony numbers and these plates were therefore counted and used to record the number of CFU/mL.

NFMS agar plates containing between 30 and 300 colonies were counted immediately after incubation. The bacterial count/mL was computed by multiplying the average number of colonies per plate by the reciprocal of the dilution used. Counts were reported in units of CFU/mL.

Enrichment for pure isolates

After incubation, 28 colonies were randomly selected from the NFMS agar plates containing the different carbon sources. These colonies were successively transferred to NFMS agar and incubated until pure isolates were obtained. Isolates were checked for purity by microscopic examination under phase contrast illumination and by doing a gram stain and examining under bright field illumination.

Culture maintenance

Isolates were preserved by freeze-drying. Cultures incubated for 5 days were harvested from the NFMS agar plate using a beef extract suspending fluid. To obtain sufficient inoculum cultures had to be harvested from at least three NFMS agar plates. The freeze drying technique used, was an in-house method of the Microbiology Department, University of Waikato. A full method description is presented in Appendix A. The freeze-dried isolates were stored at 4°C. To retrieve the isolates, nutrient broth was used to resuspend the culture.

Isolates were also preserved using the Microbank System (Pro-Lab Diagnostics, Canada). A dense suspension of the isolate was inoculated into cryogenic vials containing small plastic beads. After mixing, the fluid was aseptically removed and the vial frozen at -30°C. To retrieve the cultures a bead was removed from the vial and inoculated onto nutrient agar.

Microscopic examination

Phase contrast

A wet mount of the mixed liquor from the reactor was routinely observed under phase contrast illumination at 40x and 100x (oil immersion) magnification using a light microscope (Leica DM-RB). Pure isolates, grown on NFMS and nutrient agar, were examined for motility and cell morphology in the same manner.

Gram stain

Pure isolates were gram stained using the Hucker staining method (Gerhardt, Murray *et al.*, 1994) with prepared stains (DIFCO Laboratories) and examined under bright field illumination using a light microscope (Leica DM-RB).

Poly-ß-hydroxybutyrate (PHB) stain

To stain for PHB a heat fixed film was immersed in a filtered (% wt/vol) Sudan Black B made up in ethylene and stained for 10 minutes. The slide was removed and blotted with a piece of filter paper to remove most of the dye and then placed in xylol to complete decolourisation. The slide was then dried carefully with filter paper and counterstained for 5 to 10 seconds with 0.5% (wt/vol) aqueous Safarin (DIFCO Laboratories). Under oil

immersion using bright field illumination PHB granules stain darkly against a pink background (Gerhardt, Murray *et al*., 1994).

Biochemical reactions

Biochemical reactions of isolates were determined using a commercially available test kit, Microbact 12A (Disposable Products Pty. Ltd, South Australia). The isolates were inoculated and examined according to the manufacturer's instructions.

4.4 RESULTS

The experimental results are given in Appendix C. The three different reactor stages are described in Table 12.

Table 12: Reactor operating conditions during the 3 stages of the experiment

Stage	Time (d)	Feed composition	$COD_{(s)}$ (mg/L)
1 (control)	0-13	Pond 2 in wastewater	450
2	14-25	Pond 2 in wastewater +350 mg/L acetic acid	840
3	41-57	Pond 2 in wastewater + 200 mg/L methanol	630

4.4.1 COD

Figure 15 displays the chemical oxygen demand (COD) data collected from the experiments. The addition of 350 mg/L of acetate in Stage 2 of the experiment increased the feed COD from 450 mg/L to 840 mg/L. In Stage 3 of the experiment approximately 200 mg/L of methanol was added resulting in a feed COD of 630 mg/L. The soluble COD remaining in the effluent in all 3 stages was a constant 230 mg/L.

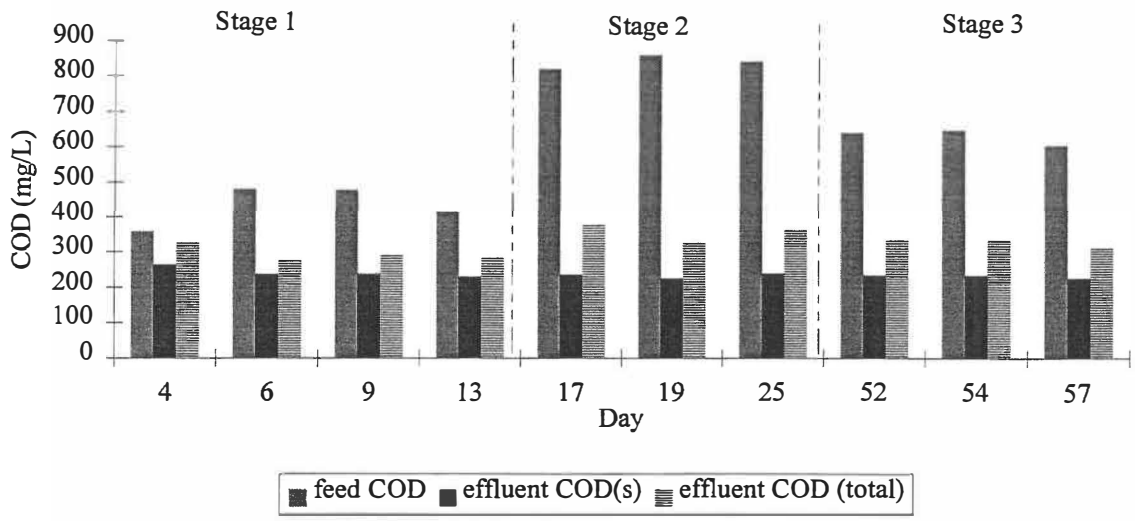

Figure 15: COD concentration (mg/L) data

Figure 16 shows the soluble COD removals achieved during the 3 stages of the experiment. During increased acetate and methanol stages, the percentage of soluble COD removed increased from approximately 50% to 73% and 65% respectively indicating that the additional substrate was oxidised within the reactor.

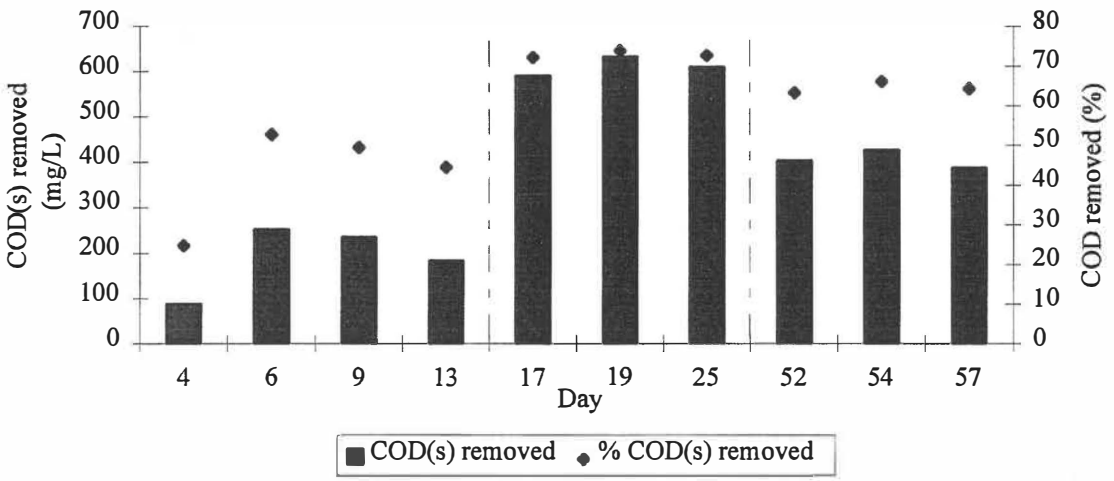

Figure 16: Soluble COD removals

Figure 17 demonstrates that the specific substrate removal rate was not affected by additions of acetate and methanol. At a 95% level of confidence (ANOVA analysis, Appendix B) there was no significant difference between the mean substrate removal rates for each of the three stages.

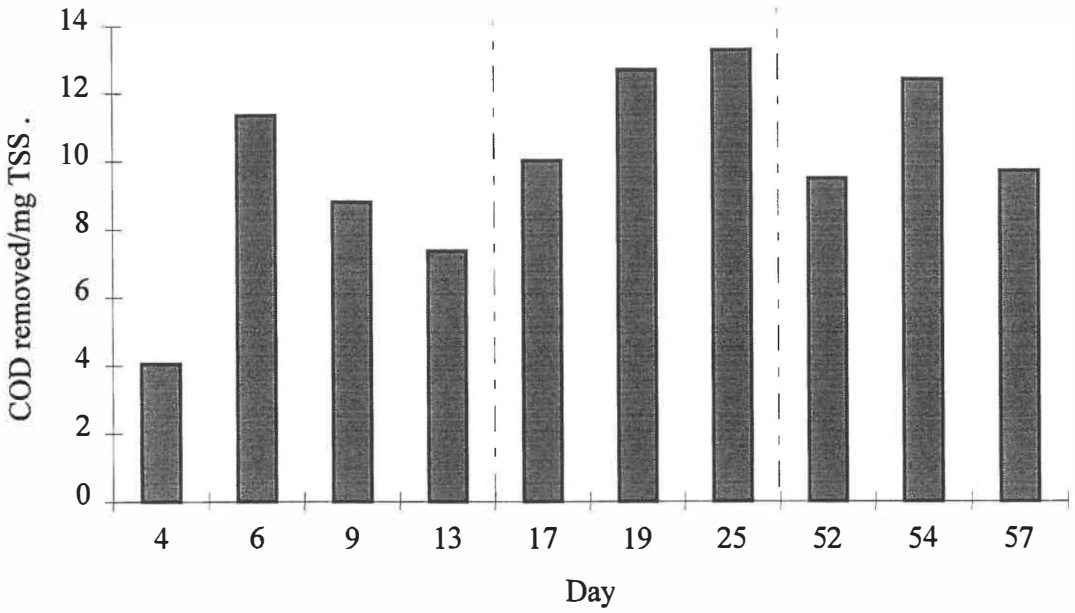

Figure 17: Specific COD removal rates

4.4.2 Nitrogen

The nitrogen concentrations in the feed and effluent are shown in Figure 18.

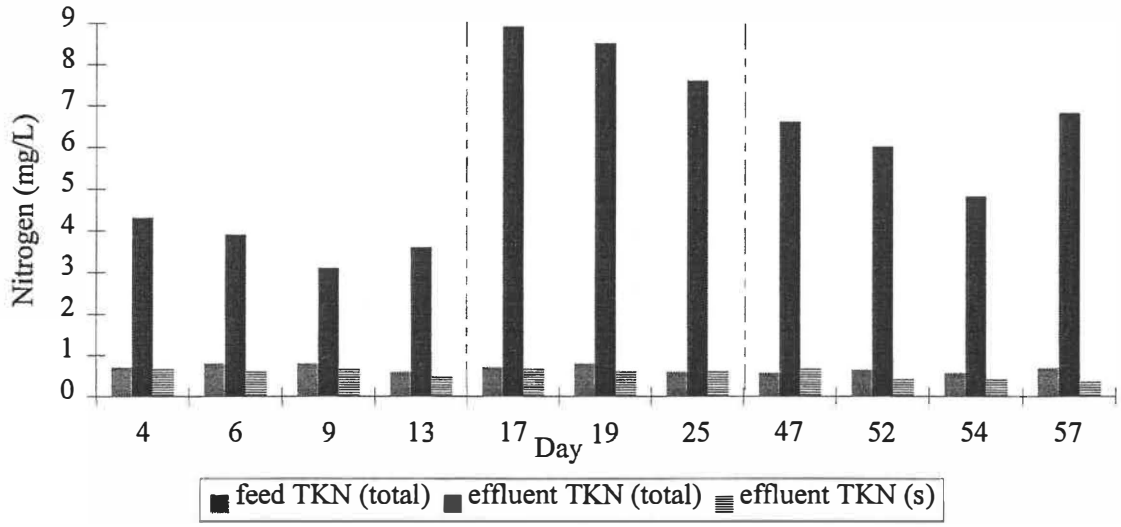

Figure 18: Total Kjeldahl nitrogen concentration mg/L

Under the 3 different operating conditions; control, increased acetate concentration and increased methanol concentration, the total Kjeldahl nitrogen (TKN) concentration in the

feed was approximately 0.7 mg/L. Average effluent total TKN concentrations ranged from 3.5 mg/L for the reactor operating under control conditions to 8.3 mg/L under increased acetate conditions. Effluent soluble TKN (TKNs) concentrations were low, at approximately 0.5 mg/L.

4.4.3 Nitrogen fixation

Acetylene reduction

No or low acetylene reduction activity was observed using the method of Bruce and Clark (1994). The nitrogenase enzyme is inhibited by oxygen, yet aerobic and microaerophilic nitrogen-fixing bacteria still require oxygen to obtain energy to fix atmospheric nitrogen. This method involved conducting the assay under anaerobic conditions and therefore the aerobic bacteria were possibly limited by oxygen.

Addition of a small quantity of oxygen to the headspace was trialed and gave positive results confirming the presence of nitrogen fixation within the reactor system. However, Drozd and Postgate (1970) obtained distinct maxima when plotting acetylene rate against oxygen partial pressure. These maxima varied depending on the bacterial species and the partial pressure under which the bacterial culture was grown. To obtain quantitative results from this assay, the acetylene reduction rate needed to be assessed over a range of air concentrations. This was not able to be accomplished within the time allocated to this experiment.

Organic nitrogen fixation

A mass balance of TKN nitrogen across the reactor enabled nitrogen fixation rates to be estimated. Nitrogen fixation can be observed when the effluent nitrogen levels are significantly higher than the feed. Nitrogen fixation rates were calculated from the difference in total TKN concentrations in the feed and the effluent.

Figure 19 shows that the nitrogen fixation rates under increased acetate and methanol concentrations were higher than under control conditions.

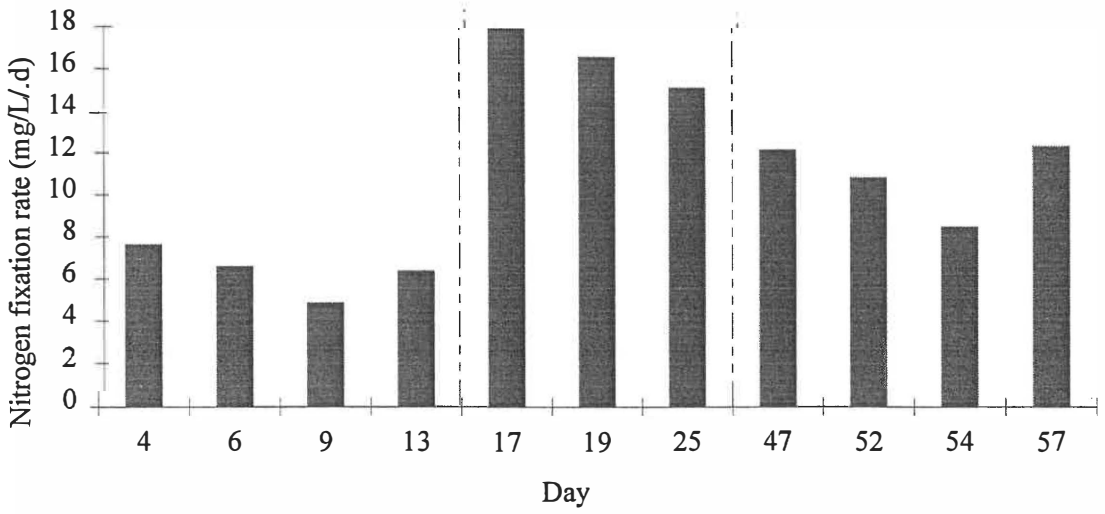

Figure 19: Nitrogen fixation rate of reactor biomass

The majority of this increased rate is accounted for by the increase in biomass concentrations under these operation conditions. The nitrogen fixation rate per unit of biomass is shown in Figure 20.

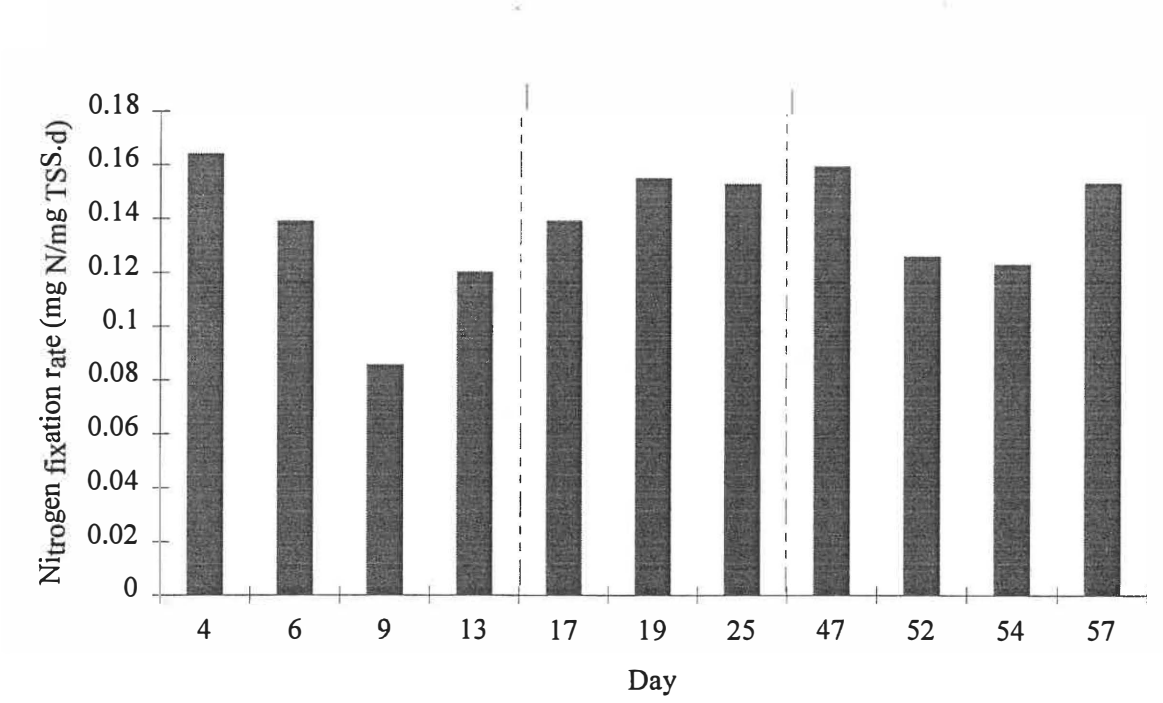

Figure 20: Specific nitrogen fixation rate of biomass

The specific rate of nitrogen fixation, as shown in Figure 20, was relatively constant under all reactor operating conditions indicating that the nitrogen fixation rate of the

biomass was not affected by increased additions of acetate and methanol and that the critical factor affecting nitrogen fixation rate was the biomass concentration. At a 95% level of confidence (ANOVA analysis, Appendix B) there was no significant difference between the mean specific nitrogen fixation rates of the 3 stages.

The nitrogen content of the microorganisms in the system has been assumed to be equal to the difference between the total and soluble nitrogen concentrations in the effluent. The difference between the total nitrogen concentration in the effluent and the feed is equal to the amount of nitrogen biologically fixed. Therefore, the ratio of nitrogen fixed to the total nitrogen content of the effluent gives an indication of the significance of nitrogen fixation and the role of nitrogen fixers in the overall microbial population.

Table 13 shows the ratio of nitrogen fixed to the nitrogen content of the system.

Table 13: Ratio of nitrogen fixation to total nitrogen content

Stage	N fixed/total N content
1	0.955
2	0.99
3	0.972

From the ratios calculated in Table 13 it can be seen that nitrogen fixation accounted for more than 95% of the nitrogen content of the biomass. These ratios indicate that most microorganisms present in this system were nitrogen fixers.

Figure 21 presents data on the nitrogen content of the biomass which was calculated by dividing the difference between effluent total and soluble total Kjeldahl nitrogen (TKN) by total suspended solids (TSS) concentration. Under all three operating conditions the nitrogen content of the biomass was approximately 0.07 mg N/mg TSS. Pirt (1975) states that based on an average composition of cell tissue of $C_5H_7NO_2$, cell nitrogen constitutes approximately 12% of the dry weight of bacteria. The low value in this system could be due to intracellular or extracellular storage of polymers resulting in an increased C:N ratio. The production of intracellular granules consisting of poly-β-

hydroxybutyrate (PHB) is a characteristic of a number of nitrogen fixing bacteria. Microbial accumulation of PHB commonly occurs under high carbon and limited nitrogen or phosphorus concentrations (Doi 1990).

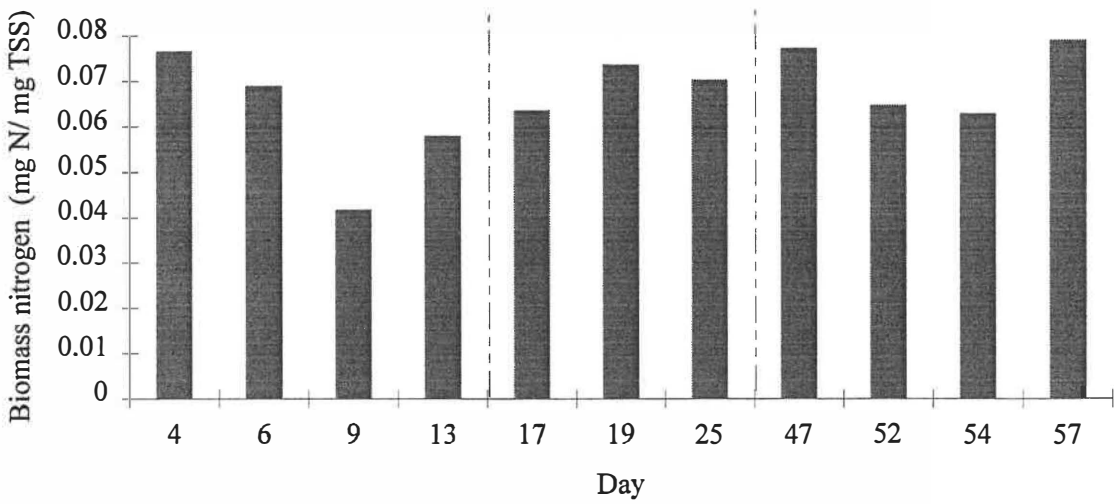

Figure 21: Nitrogen content of biomass

4.4.4 Phosphorus

To ensure the system was not phosphorus limited, phosphorus was added to feed in a ratio of BOD_5:P = 100:1. On day 9, too much phosphorus was inadvertently added to the feed, resulting in the high phosphorus level on this day.

Figure 22 shows the phosphorus concentrations in the feed and reactor effluent. The effluent phosphorus concentrations were similar to the feed phosphorus concentrations. As the reactor feed and effluent flow rates were identical, these results indicate that there was a good phosphorus balance across the system.

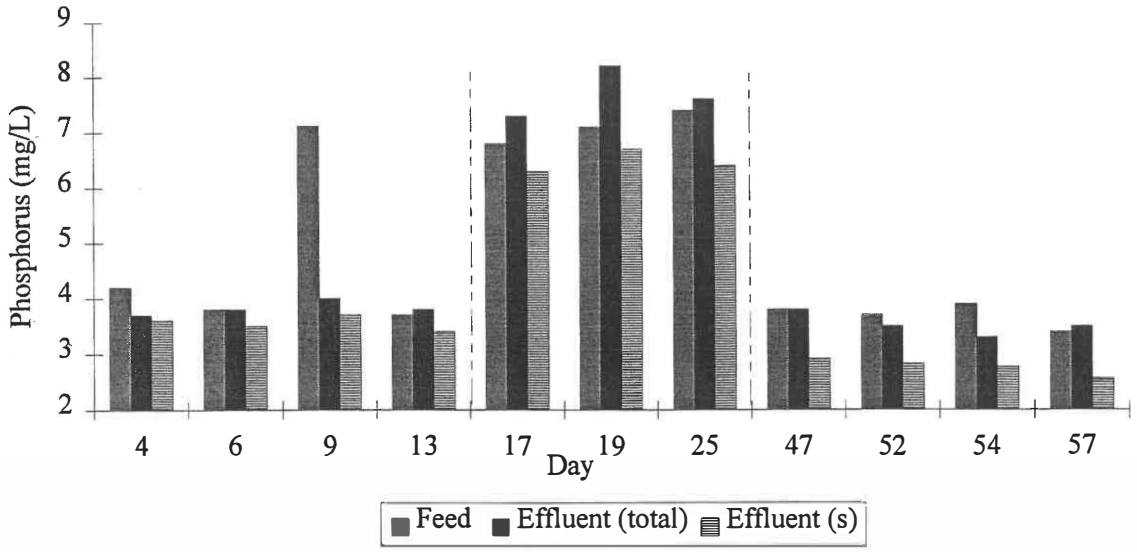

Figure 22: Feed and effluent total phosphorus concentrations

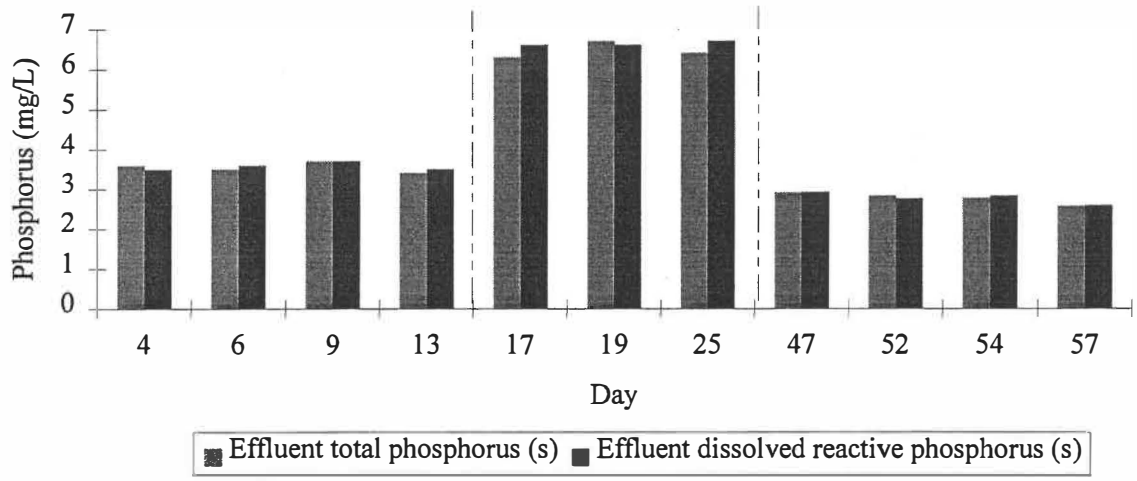

Figure 23: Effluent total and dissolved phosphorus concentrations

Figure 23 compares total and dissolved phosphorus concentrations in the reactor effluent. Phosphorus present in the form of dissolved reactive phosphorus (DRP) is readily available to microorganisms. Figure 23 shows that all the soluble phosphorus present in the system existed as DRP.

The difference between effluent total and soluble total phosphorus (TP) per unit weight of biological solids (TSS) was used to estimate the biomass phosphorus content (Figure

24). Pirt (1975) stated that the phosphorus content of bacterial cells is about 1.5% of the dry biomass, but the content increases with growth rate. The difference in means for the phosphorus content of the biomass was significant for the 3 different operating stages at a 95% level of confidence (ANOVA analysis, Appendix B). The phosphorus content appeared to increase under the increased acetate concentrations of Stage 2, with values ranging from 0.8%-1.4%. Gapes (1996) found that the biomass phosphorus of nitrogen-fixing organisms, operating under similar conditions to Stage 1, was approximately 0.006 mgP/mg TSS, similar to the values found in this work.

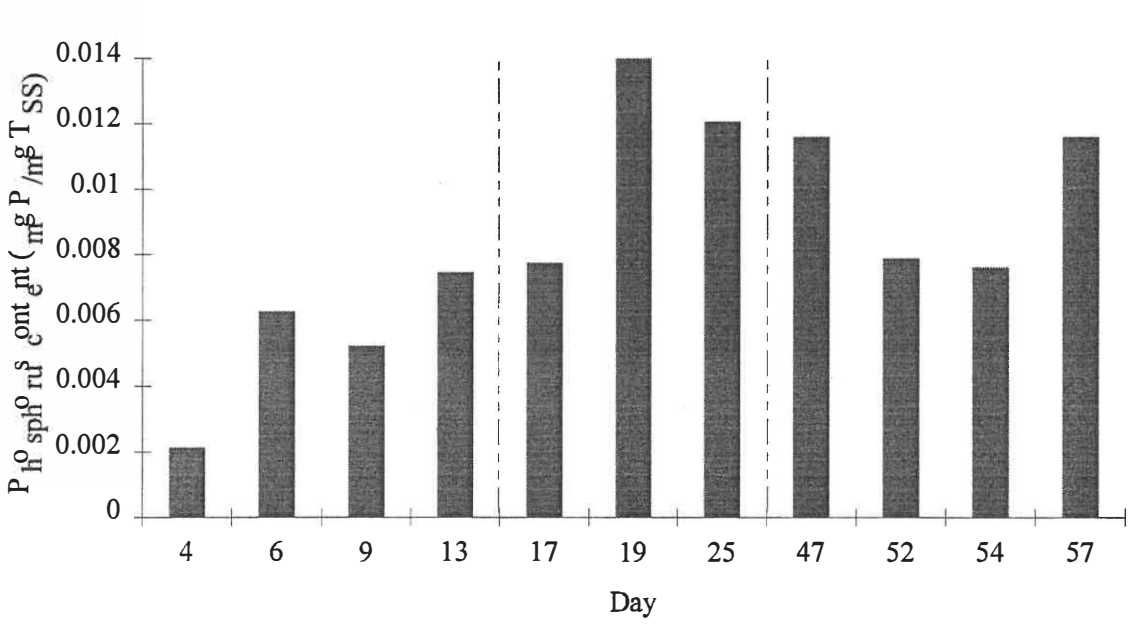

Figure 24: Phosphorus content of the biomass

4.4.5 Solids concentration

Figure 25 presents the total suspended solids (TSS) and volatile suspended solids (VSS) data for the 3 stages of the experiment. VSS concentrations were very similar to their corresponding TSS concentrations indicating that most of the suspended material was volatile (combustible at 550° C) and therefore the use of TSS as a measure of cell biomass was justifiable.

Under increased acetate concentrations, the biomass concentration in the reactor was approximately double that of the control conditions. Under increased methanol concentrations the biomass concentrations also increased.

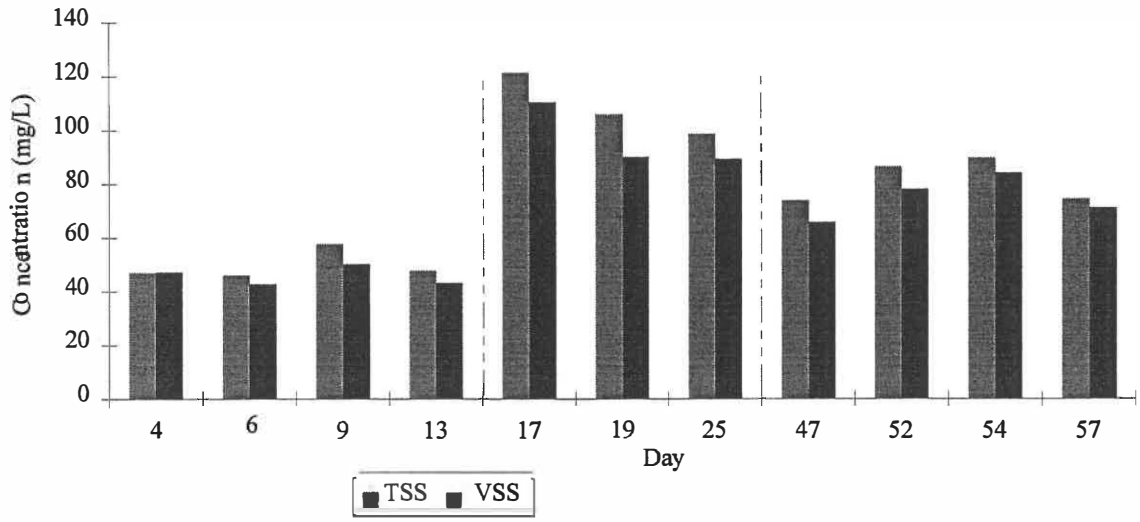

Figure 25: Biomass concentration

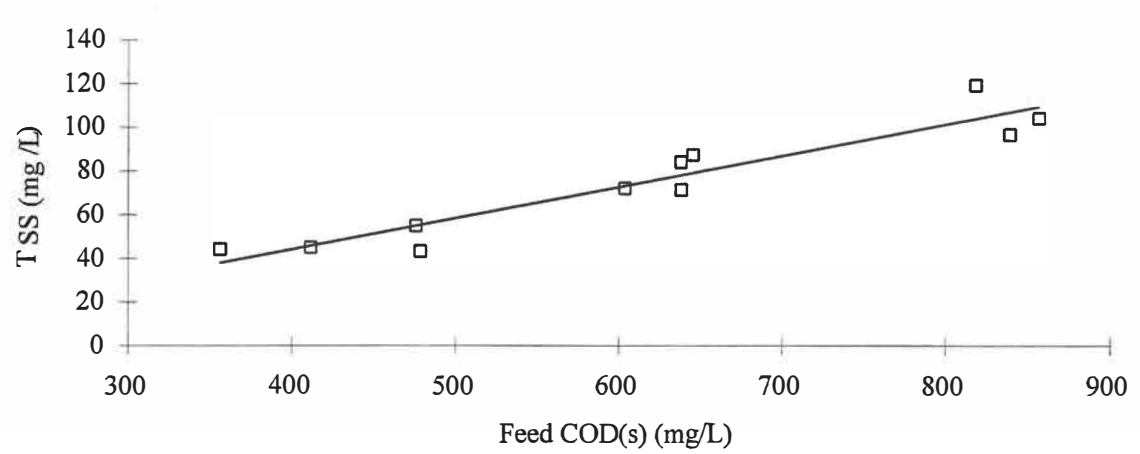

Figure 26: Relationship between biomass and feed COD

Figure 26 shows the relationship between the biomass (measured by total suspended solids) and the $COD_{(s)}$ of the feed. The increase in reactor biomass was proportional to the feed COD.

4.4.6 Observed yield

The observed biomass yield is used to describe the amount of cell mass produced by the consumption of substrate within the reactor. Figure 27 gives the observed growth yield coefficient Y_o during the 3 stages of the experiment.

Observed yield is defined by (Pirt,1975):

$$Y_o = \frac{X_e - X_o}{S_o - S_e}$$

Y_o = observed growth yield (mg biomass/mg COD)

X_e = biomass concentration in the effluent

X_o = biomass concentration in feed (mg/L). In this study $X_o = 0$

S_o = soluble COD concentration in feed (mg/L)

S_e = soluble COD concentration in effluent (mg/L)

Under control conditions, the mean observed cell yield was approximately 0.22 mg TSS/mg COD. Gapes and Clark (1995) found that the cell yield of nitrogen fixing organisms operating under similar conditions was approximately 0.23 mg TSS/mg COD. The mean observed yields for each stage are given in Table 17. At a 95% level of confidence (ANOVA analysis, Appendix B) there was no significant difference between the mean observed yield for the 3 operating stages.

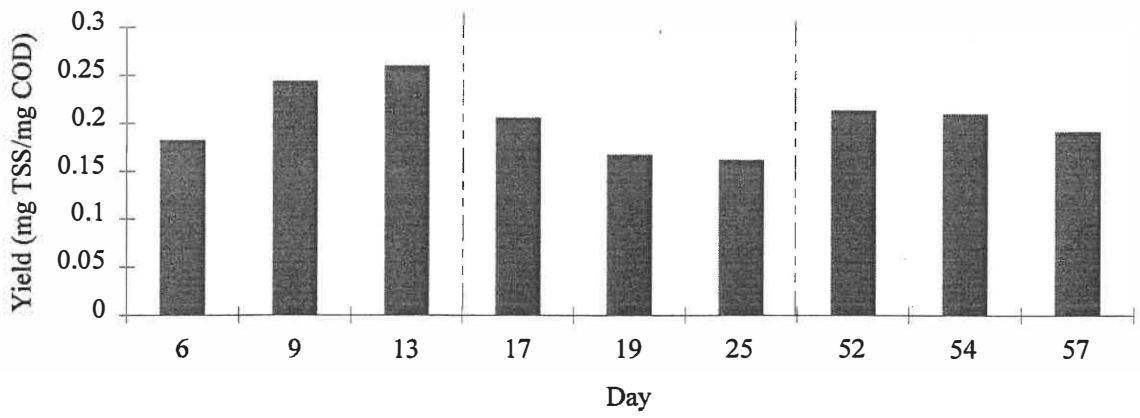

Figure 27: Observed biomass yields

4.4.7 Oxygen uptake rate

The oxygen uptake rate (OUR) gives a measure of the respiratory activity of the microorganisms within the reactor. The OUR's measured in this work are given in Figure 28. The OUR of the microorganisms under increased acetate concentrations was significantly greater than control organisms. Under these conditions the active biomass was greater, increasing the numbers of respiring cells.

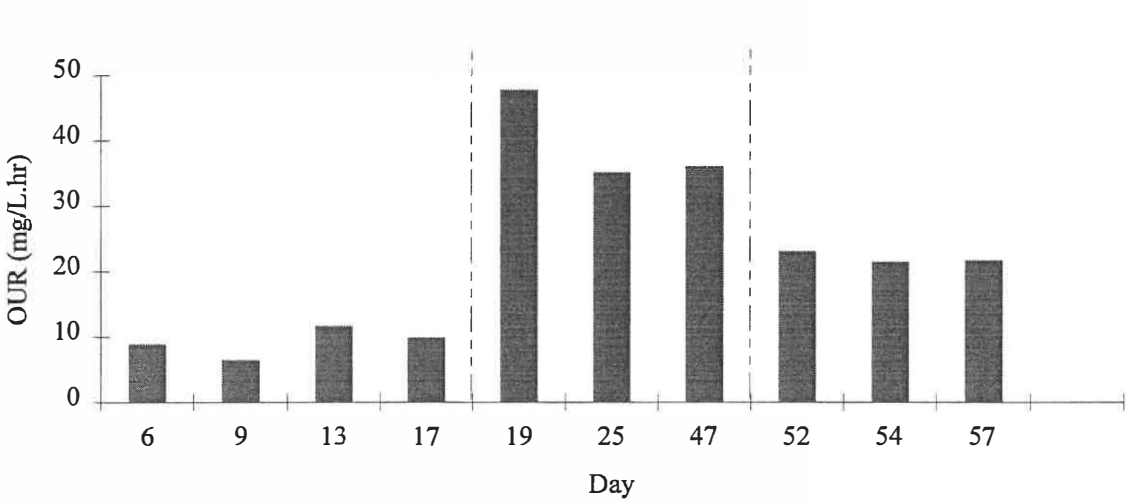

Figure 28: OUR data

The specific oxygen uptake rate (SOUR) is defined as the oxygen uptake rate per unit of biomass within the reactor. This parameter can be used to compare the relative respiration rates of biomass from systems with different biomass concentrations, with high SOUR corresponding to high respiratory activity. Figure 29 shows the SOUR of the biomass under the three different operating conditions. Gapes and Clark (1995) obtained a SOUR of 0.21 when operating a reactor under similar conditions to Stage 1 of this work.

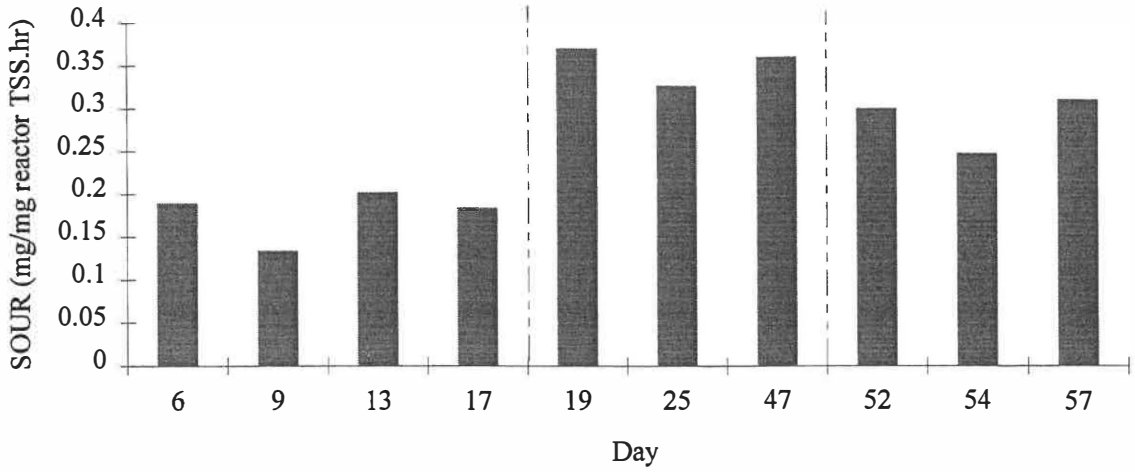

Figure 29: Specific oxygen uptake rate (SOUR)

Figure 29 indicates that under increased acetate and methanol operating conditions the mean SOUR was higher than under the control conditions. This difference was found to be significant at the 95% level of confidence (ANOVA analysis, Appendix B).

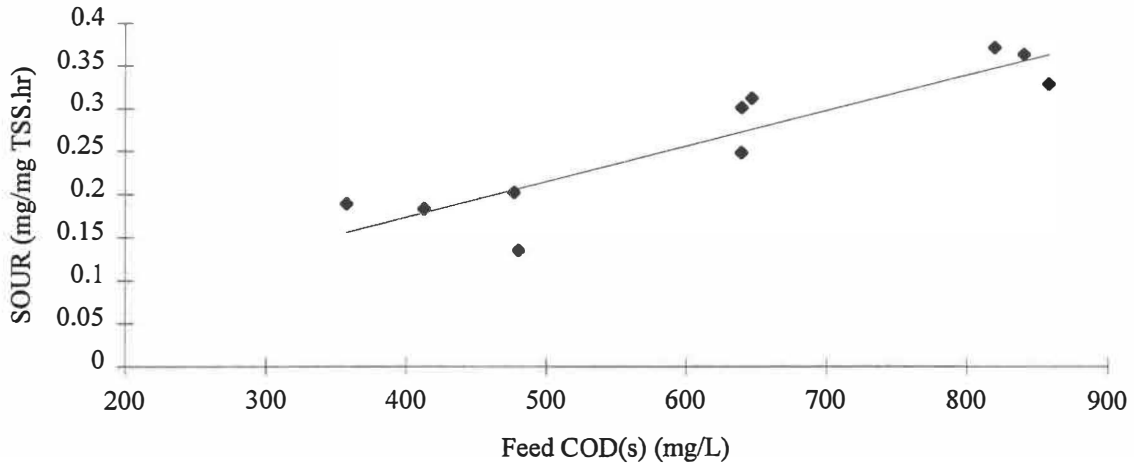

Figure 30: Comparison of specific oxygen uptake rate (SOUR) at different feed COD concentrations

Figure 30 illustrates that the SOUR was proportional to the COD concentration of the feed. An increase in the SOUR would be expected if the system was operating under carbon limited conditions during Stage 1, and then during Stages 2 and 3 more degradable substrate was made available resulting in increased metabolic reactions and cell growth rates. However, this explanation is not satisfactory because specific nitrogen

fixation rates and observed yields did not increase but remained stable throughout the duration of the experiment.

The increases in respiratory requirement may be due to a change in microorganism composition in Stage 2 and 3 where the organisms may be more oxygen sensitive, requiring a higher level of respiratory protection and therefore demonstrating a higher specific oxygen uptake rate.

4.4.8 Cell counts

Two types of colony morphology could be distinguished on NFMS agar; pinprick size transparent colonies and 3-6 mm cream colonies. Cell counts of these colony morphologies are shown in Table 14 and Figure 31.

Table 14: Cell counts (CFU/mL)

	Stage 1			Stage 2			Stage 3		
day	4	6	9	17	19	25	47	52	54
cream	$1x10^5$	$1x10^6$	$3x10^5$	$9x10^6$	$9x10^6$	$2x10^7$	$2x10^5$	$2x10^5$	$3x10^5$
transparent	$3x10^6$	$5x10^6$	$2x10^6$	$3x10^{10}$	$3x10^{10}$	$3x10^{10}$	$8x10^6$	$7x10^6$	$8x10^6$

Under control conditions the transparent colonies were present in numbers at least 5 times greater than the cream colonies. Both cream and transparent colonies increased at least 100 fold during Stage 2. Cell counts for transparent colonies under these conditions could only be reported as $>3x10^{10}$ CFU/mL because there were greater than 300 colonies present on the lowest dilution. Under methanol supplemented conditions (Stage 3) cream cell counts were very low. With approximately 180 mg/L of extra soluble COD, transparent cell counts during this third stage were expected to be considerably greater than those observed during Stage 1. These low counts may be the result of some other microorganisms proliferating in the reactor which could not be cultivated under the enrichment conditions used.

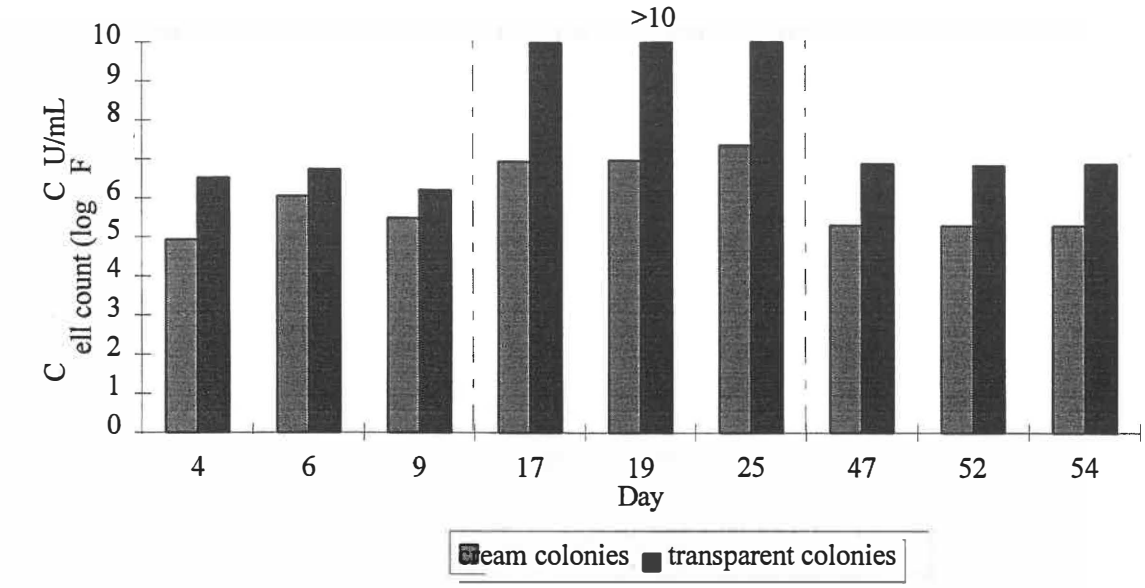

Figure 31: Cell counts

4.4.9 Microscopic examination

Direct microscopic examination under phase contrast illumination revealed many *Azotobacter* type cells under Stages 1 and 2. No differences in the microbial composition could be distinguished between these two stages. In Stage 2 more free-swimming bacteria were apparent in the mixed liquor.

Mixed liquor samples taken from the reactor during the third stage (increased methanol) revealed a completely different consortium with many filamentous cells (Figure 32).

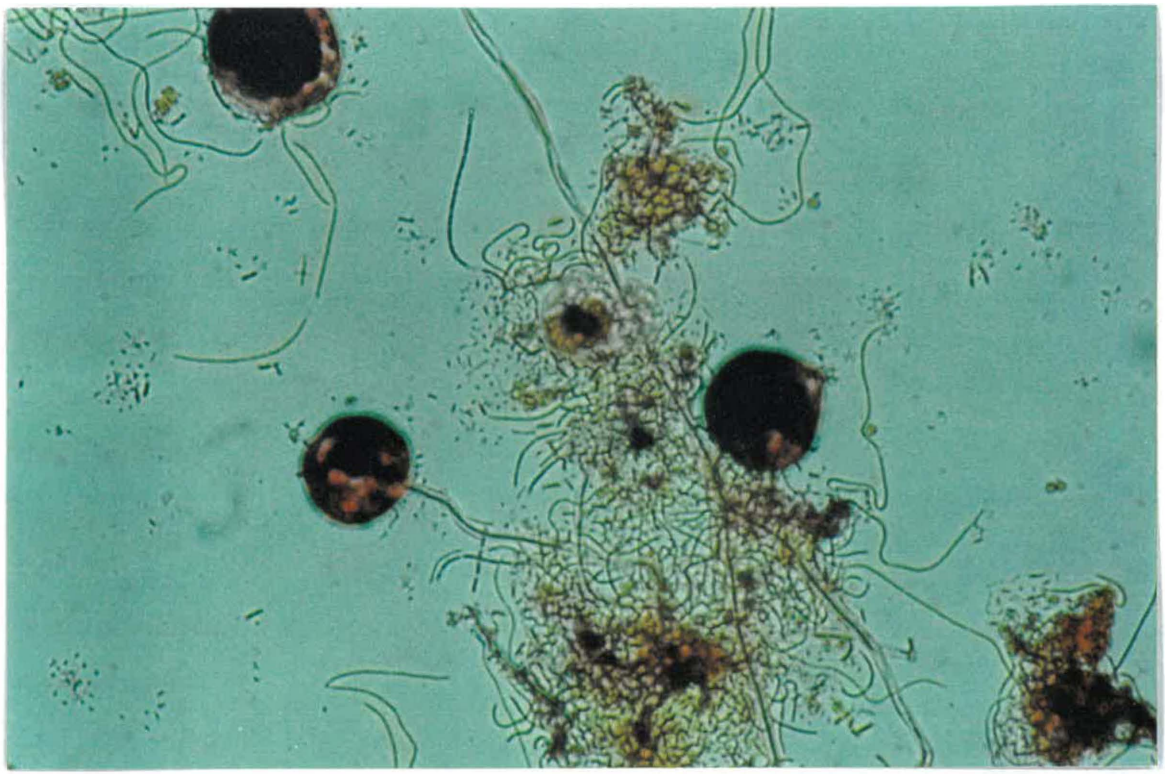

Figure 32: Photomicrograph showing Stage 3 mixed liquor (Gram stain, bright field illumination, 400 x magnification)

4.4.10 Isolates

From successive streaking on NFMS agar six pure isolates were distinguished by microscopic examination under phase contrast illumination and by doing a gram stain and examining under bright field illumination. Table 15 gives a brief description of these isolates.

Table 15: Isolates obtained from NFMS agar

	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6
Isolated from reactor under stage:	1 and 2	1 and 2	1	3	3	3
Agar isolated on:	1% glu[a]	1% glu[a]	1% meth[b]	1% glu[a]	1% meth[b]	1% glu[a]
Colony morphology NFMS agar	cream	transp[c] white	transp[c] mucous	transp[c] opaque	transp[c]	transp[c]
Colony diameter(mm)	3-6	1-4	1	1	1	2-4
Colony pigmentation on nutrient agar	cream	cream	bright yellow	cream	dry yellow	pale pink
Cell morphology	large yeast like	small ovoid	small rod	small rod	branched cell	elongated "S"
Gram stain	-	-	-	-	+	-
PHB stain	+	-	+	+	+	-
Oxidase	+	-	-	+	+	+

[a]NFMS with 1% glucose as sole carbon source

[b]NFMS with 1% methanol as sole carbon source

[c] transparent

In pure culture only Isolate 1 was able to fix nitrogen in filter sterilised (0.2μm) Tasman Pulp and Paper whole mill effluent at a DO concentration of 2.2 mg/L. Isolate 1 has been

tentatively identified as a *Azomonas spp* because of its "yeast-like" size and its inability to utilise as a sole carbon source mannitol, rhamnose and lactose (Table 16) (Knowles, Neufeld *et al.*, 1974).

Table 16: Isolate 1 - carbon utilisation test results

Ability to utilise as sole carbon source	isolate 1
glucose, maltose, acetate, sucrose	+ve
methanol, mannitol, rhamnose, starch, lactose	-ve

The cell morphology of this isolate is shown in Figure 33.

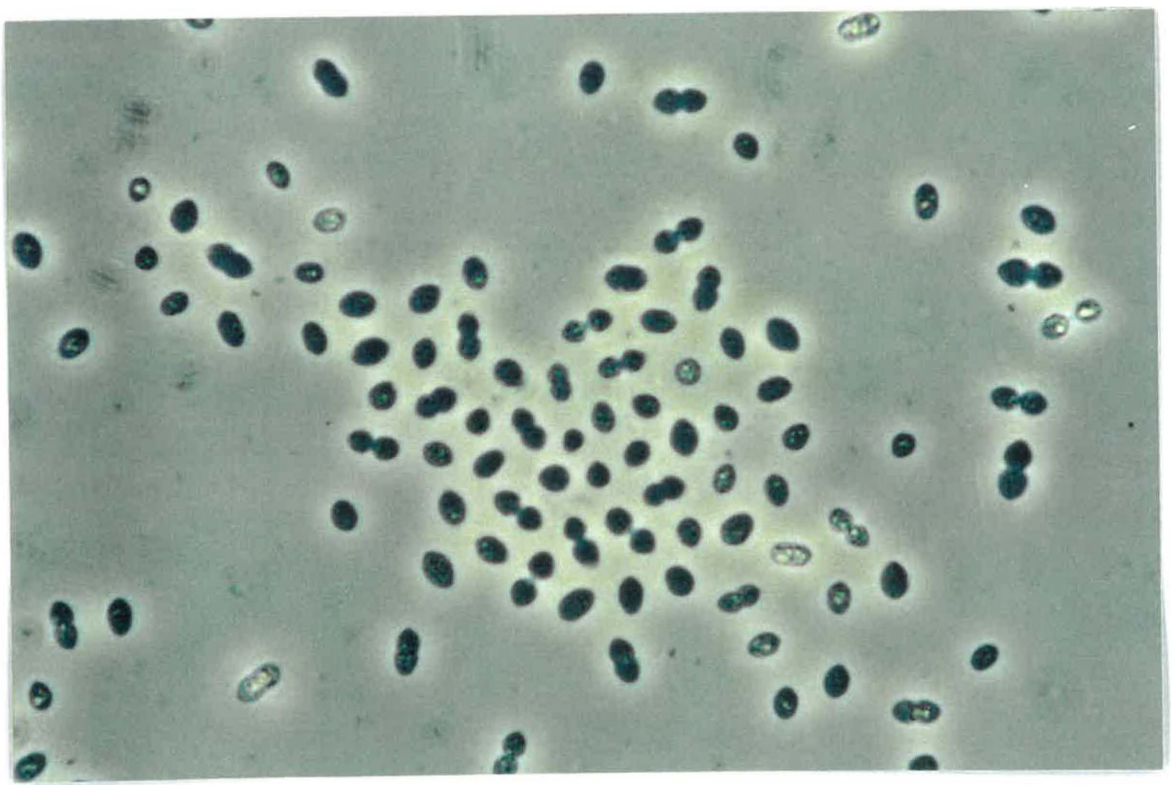

Figure 33: Photomicrograph of Isolate 1 (phase contrast, 1000x magnification)

Isolate 2 gave a good positive ID as *Enterobacter agglomerans* using the Microbact 12A identification system. Using the Microbact system none of the other isolates registered positive identifications. Isolate 5 may belong to the genus *Xanthobacter* due to its positive gram stain, cell morphology and its ability to grow using methanol as sole carbon and energy source (Kreig, 1982). The poor growth of these transparent isolates on NFMS agar made characterisation extremely difficult. Attempts to preserve these isolates were largely unsuccessful. The isolates could not be revived after freeze-drying, possibly due to the poor growth of the culture used for freeze-drying.

4.5 DISCUSSION

Table 17 gives a summary of the main parameters obtained in this work for the three different operating stages. Included in this summary table is the data obtained by Gapes and Clark (1995) operating a bioreactor under conditions similar to Stage 1.

Table 17: Summary of parameters

Parameter		Stage 1	Stage 2	Stage 3	Gapes and Clark (1995)
Observed yield	mgTSS/mgCOD	0.18-0.26	0.14-0.19	0.14-0.19	0.23
COD(s) removed	mg/L	180-252	590-630	388-426	240
	%	44-53	72-74	63-66	46
Reactor biomass	mg/L	47-57	107-129	70-86	56
N-fixation rate	mgN/mgTSS.d	0.09-0.16	0.13-0.15	0.12-0.15	0.07
Biomass N	mgN/mgTSS	0.04-0.07	0.06-0.07	0.06-0.07	0.05
Biomass P	mgP/mgTSS	.002-.007	.007-.013	0.07-.011	.008
Specific OUR	mgO_2/mgTSS.d	0.13-0.20	0.32-0.37	0.25-0.31	0.21

The results obtained from Stage 1 were very similar to those obtained by Gapes and Clark (1995).

COD removals in the bioreactor system were consistent with those detected in Pond 2 of Tasman treatment system. Approximately 200 mg/L of soluble COD was removed from Pond 2, similar to the values obtained under Stage 1. The amount of COD removed increased with substrate supplementation. Undegradable soluble COD concentrations remained constant under increased acetate and methanol conditions (230 mg/L). This indicates that all added acetic acid and methanol were degraded within the reactor and that methanol and acetic acid are readily utilised by the nitrogen fixing bacteria in the system. Increased methanol and acetate concentrations in the reactor did not adversely affect COD removals.

The reactor biomass increased during Stages 2 and 3. At the 95% level of confidence no difference was detected between the mean observed yield for the three operating stages. A significant difference was observed at a 95% level of confidence between the means of the three operating stages for the specific oxygen uptake rate. An increase in specific OUR was observed during Stages 2 and 3 and this increase was proportional to the COD loading rate. These results are surprising because there were no significant differences detected in the specific nitrogen fixation rates and observed yields throughout the study as would be expected if the amount of available substrate was increased so the system was no longer carbon limited.

The ratio of the amount of nitrogen obtained from nitrogen fixation to the total nitrogen content of the reactor biomass indicated that nitrogen fixation provided the majority (>95%) of the nitrogen requirement. Increased acetate and methanol conditions did not affect this ratio and no significant differences were detected in the mean specific nitrogen fixation rate of reactor biomass under increased acetate and methanol concentrations.

The nitrogen content of the biomass was low (0.07 mg N/mg TSS) compared to 12%, calculated from the average composition of cell tissue (Pirt, 1975) and remained unaffected throughout the entire study. These observed low values could be due to a high cellular carbon content. The formation of intracellular storage products such as polyhydroxybutyrate (PHB) is common in many nitrogen fixing bacteria. Four of the six isolates obtained during this study exhibited positive results when stained for PHB.

Microorganisms that can remove substrate rapidly from the mixed liquor and accumulate this as a substrate storage supply within their cells will have a competitive advantage over microbes that cannot, particularly under transient substrate conditions. It has been suggested that a function of PHB in *Azotobacter* species is to provide an oxidisable substrate for respiratory protection of nitrogenase under low environmental substrate conditions (Senior and Dawes, 1971). For nitrogen fixing bacteria present in the Tasman treatment system the endogenous catabolism of intracellular storage substrate would be an advantage under lower substrate conditions found further down the treatment system.

Previous studies (Clark and Gapes, 1995; Gapes, 1996), under conditions similar to Stage 1, have revealed that the phosphorus content of the biomass in nitrogen fixing systems to

be lower than 1.5%, the value calculated from the average composition of cell tissue (Pirt, 1975). The phosphorus content of the biomass for Stage 1 is in agreement with these results. Unlike the nitrogen content of the biomass, this value increased as substrate concentration increased. Nitrogen fixing bacteria such as *Xanthobacter* have been reported to accumulate intracellular polyphosphates (Cloete and Muyima, 1997). Storage of an intracellular polyphosphate would increase the phosphorus content of the biomass.

Some significant differences in microbial composition were observed during the study. Total cell counts increased with increased substrate concentrations. Cell counts under methanol conditions were lower than expected. The counts were only slightly higher than those obtained under Stage 1, with 180 mg/L of additional COD. The low counts recorded may be due to the presence of some other organism not selected by enrichment techniques. The cream colonies were present in low numbers under Stage 1 and 3 but proliferated under increased acetate concentrations.

Isolate 1 was purified from a cream colony. This isolate was tentatively identified as belonging to the *Azomonas* genus because of its distinctive cell morphology and carbon substrate utilisation patterns. *Azomonas* species have previously been isolated from aerobically incubated effluent from a neutral sulphite semi-chemical mill (Knowles, 1974).

On the NFMS medium, the transparent colonies grew poorly and confirmation of nitrogen fixing ability as well as further characterisation was difficult. Some of these isolates grew very well on a complex medium containing nitrogen (eg Nutrient agar). Isolate 2 was identified as *Enterobacter agglomerans.* Nitrogen fixing strains of *E.agglomerans* have been previously isolated from decaying fir trees (Dalton, 1980).

4.6 CONCLUSIONS

The conclusions that can be drawn from this bioreactor study are:

- The laboratory reactor was found to give an adequate simulation of the COD removal of Pond 2 at the Tasman Pulp and Paper treatment system.

- Increased concentrations of methanol and acetate did not significantly affect the specific nitrogen fixation rate or the specific COD removals rates of the reactor biomass. However, an increased specific oxygen uptake rate during Stages 2 and 3, cell counts and microscopic observations indicate that the microbiological consortia did change, particularly during Stage 3.

- Low biomass nitrogen content was observed in the reactor systems. This could have been due to the intracellular storage of polyhydroxybutyrate (PHB). Many nitrogen fixing bacteria are able to form intracellular PHB granules which may act as a selective advantage under transient substrate conditions such as those in pulp and paper mill effluent treatment systems.

- Microbiological analyses suggest that a diverse range of aerobic micro-organisms with nitrogen-fixing capabilities are able to grow in a bioreactor simulating Tasman's treatment system.

Section 5

PURE CULTURE STUDIES

5. PURE CULTURE STUDIES

5.1 INTRODUCTION

Nitrogen fixation has been clearly established as a significant activity in Pond 2 of Tasman's aerated lagoon treatment system (Bruce and Clark, 1994).

The continuous culture experiment (Section 4), operating at dissolved oxygen concentrations of 2.2 mg/L, illustrated that changes in acetate and methanol concentrations may have changed the bacterial population but did not affect specific substrate removal or specific nitrogen fixation rates in the system. Even at this elevated dissolved oxygen concentration many different nitrogen fixing isolates were able to be isolated from the system.

Extreme difficulties were experienced when growing and preserving the cultures isolated in this work (Section 4.4.10). Poor growth was obtained on NFMS agar and broth after 10 day incubation periods with all transparent isolates. Slightly better growth was obtained using Nutrient Broth (Difco), and preservation by freeze-drying was attempted. However, these isolates could not be resuscitated after freeze-drying.

To continue the pure culture study it was necessary to develop a better enrichment and culture medium to obtain some new isolates. Previous studies on Tasman's treatment system have concentrated on isolating anaerobic nitrogen-fixing bacteria such as *Klebsiella* species (Bruce and Clark, 1994). From these studies it was concluded that *Klebsiella* did not make a major contribution to the amount of nitrogen fixed and was not significantly involved in the nitrogen fixation occurring in this treatment system.

Many free-living aerobic or microaerophilic nitrogen-fixing bacteria have been identified (Section 2.1.1). These bacteria are widely distributed in the environment and are able to protect their nitrogenase enzyme from oxygen in a variety of ways. Aerobic techniques were used in this study to isolate and study nitrogen-fixing bacteria from Pond 2 of Tasman's treatment system, since Pond 2 is essentially aerobic and the substrates of interest (acetate, methanol and carbohydrate) are more likely to be degraded by aerobic than anaerobic organisms.

5.2 OBJECTIVES OF THIS STUDY

To isolate and characterise aerobic nitrogen fixing bacteria from the microbial consortium present in Pond 2 of Tasman treatment system capable of substrate removal.

5.3 METHOD AND MATERIALS

5.3.1 Enrichment and isolation of pure cultures from Tasman Pond 2 effluent

The Spread Plate Agar technique (APHA, 1992, Section 9215C) was used to enrich for nitrogen fixing isolates from Pond 2 effluent at Tasman treatment system. Samples were diluted using sterile phosphate buffered water (APHA section 9050C). The appropriate dilutions were pipetted onto the surface of nitrogen free agar in 0.1 mL aliquot's. The spread plates were incubated aerobically at 30°C for 5-7 days.

To improve cultivation of aerobic nitrogen fixing organisms, three different nitrogen free media were used (Table 18). The carbon source for the amended NFMS (NFMSA) medium contained a combination of carbon substrates, each of which contributes significantly to the organic load in Tasman's wastewater. These substrates were approximately one hundred times more concentrated than Tasman Pond 2 wastewater so that the total carbon substrate added to the NFMSA medium was approximately 10 g/L. Substrate carbon concentrations of this value appear to be standard in enrichment media for the isolation of nitrogen fixing microorganisms (Becking, 1981).

Table 18: Media used for the isolation and cultivation of nitrogen fixing bacteria from Tasman's Pond 2

Ingredient (g per litre unless specified)	NFMS (Brown, Burlingham *et al.* 1962)	Azospirillum (Becking, 1981)	Amended NFMSA
L-Malic acid		5	
Sodium acetate			3
Methanol			4
Galactose			2
Glucose	10		1
K_2HPO_4	0.8	0.8	0.6
$MgSO_4.7H_2O$	0.2	0.2	0.2
$CaCl_2$	0.1	0.02	0.1
NaCl	0.1		0.1
Biotin	0.01 mg		0.01 mg
Trace element solution[1]	5 mL	5 mL	5 mL
Bromythmol blue soln (0.5%)		5 mL	
Agar	15	15	15

[1]See Appendix A

Following incubation, under nitrogen fixing conditions, colonies were repeatedly streaked on amended NFMS (NFMSA) agar containing 20 mg/L NH_4Cl until pure cultures were obtained. The ammonium chloride was included to initiate growth and to permit the organism to grow at higher oxygen tensions during the earlier stages of growth (Dalton, 1980). Isolates were checked for purity by microscopic examination. For all further experiments the NFMSA agar was used.

5.3.2 Ability of isolates to reduce acetylene

Pure isolates were transferred to NFMSA agar slopes containing 20 mg/L NH_4Cl and incubated for 5 days at 30°C. After incubation, 1 mL of NFMSA broth was added to wash the bacterial growth off the slope, creating a suspension. The 1 mL suspension was then transferred to sterile Hungate tubes containing 4 mL of NFMSA broth.

Nitrogen fixation activity of isolates was determined immediately using batch acetylene reduction assays. Assays were performed in 16.6 mL Hungate tubes containing 5 mL of culture. Assays were conducted in a mixture of 95% argon, 5% air in the head space and were initiated by 0.6 mL of acetylene gas (0.6 mL of headspace was withdrawn from the tube prior to acetylene addition). Tubes were incubated at 30°C with shaking at 120 rpm for 30 minutes. Assays were conducted in triplicate and blanks were assayed as above using 5 mL of distilled water. Full details are given in Appendix A.

5.3.3 Ability of isolates to grow under chemolithoautotrophic conditions

An inoculum was prepared from a NFMSA (with 20 mg/L NH_4Cl) slope culture incubated for 5 days at 30°C. One mL of NFMSA broth was added to the slope culture to wash off the bacterial growth and the suspension was transferred to 25 mL of NFMSA broth and incubated overnight. This culture was then transferred to 700 mL of Basic Mineral Medium (Wiegel, 1992), consisting of ingredients as described in Table 19.

Table 19: Basic mineral medium used for the cultivation under chemolithotrophic conditions (Wiegel, 1992)

Ingredient (per litre)	
K_2HPO_4	1 g
KH_2PO_4	0.5 g
$NaHCO_3$	2 g
$MgSO_4.7H_2O$	0.1 g
$CaCl_2.2H_2O$	0.04 g
Trace element solution[1]	5 mL
$NiCl_2.6H_2O$	0.02 mg
Biotin	0.01 mg

[1]see Appendix A

The medium was sparged with a 10% CO_2, 10% H_2, 5% air and 75% N_2 atmosphere and incubated with shaking at 150 rpm at 30°C.

5.3.4 Ability of isolates to grow on different carbon substrates

The isolates were tested to determine whether they could use glucose, methanol, acetic acid and peptone as the sole carbon and energy source. Isolates were streaked on NFMSA agar containing only the selected carbon substrate and incubated for 5 days at 30°C.

5.3.5 Biochemical reactions

Biochemical reactions of some of the isolates were determined by the Rotorua Hospital Laboratory. Commercially available test kits; API 20 NE (API Laboratory Products, Basingstoke) and Microbact 24E (Disposable Products Pty. Ltd, South Australia) were used. The isolates were inoculated and examined according to the manufacturer's instructions.

5.3.6 Substrate utilisation

Preparation of inoculum

A well-isolated colony was selected from a NFMSA agar plate and inoculated into 100 mL of NFMSA broth (containing 100 mg/L NH_4Cl to initiate growth) and incubated under aerobic conditions at 30°C for 5 days. Fifty mL of this inoculum was transferred to a 1 litre flask containing 700 mL of NFMSA broth (+ 100mg/L NH_4 Cl). The carbon substrate consisted of approximately 3g/L of methanol, 3g/L of acetic acid and 0.5g/L of glucose. Such a large volume of broth was required because approximately 20 mL of broth was removed each time for glucose, methanol, acetic acid, pH and optical density measurements.

The cultures were incubated without shaking for 2 days at 30°C and then transferred to a 30°C shaking incubator and mixed at approximately 150 rpm for the duration of the experiment. The pH of the cultures was monitored and if necessary adjusted to pH 7 ± 0.2. Samples were withdrawn aseptically at regular intervals to measure optical density of the culture at 660 nm. Samples were also aseptically withdrawn, filtered through a 0.22 μM Polyethersulfone Membrane (Whatman) and frozen for further analysis.

Microscopic analysis and NFMSA spread plate technique (Section 5.3.1) were used to check culture purity.

COD analysis

COD was determined by using a microscale adaptation of APHA Method 5220D. Full method descriptions are given in Appendix A.

Methanol analysis

Methanol was analysed by GC-FID, using an in-house method of the Environmental Research Group at the New Zealand Forest Research Institute. Details of the method are given in Appendix A.

Acetic acid analysis

Acetic acid was determined by GC-FID, using an in-house method of the Environmental Research Group at the New Zealand Forest Research Institute. A full method description is given in Appendix A.

Glucose analysis

Glucose was analysed using a colorimetric technique (Boehringer Mannheim test kit 124028 GLU MPR2) based on converting glucose via enzymatic reaction to a coloured complex and measuring absorbance at 610nm. Glucose concentrations were quantified by using an external standard. A full method description is give in Appendix A.

5.4 RESULTS

5.4.1 Enrichment of isolates

Figure 34 illustrates the bacterial growth obtained after 7 days incubation on a NFMSA agar plate. NFMSA supported better growth than the NFMS medium used in the previous experiment (Section 4.3.3).

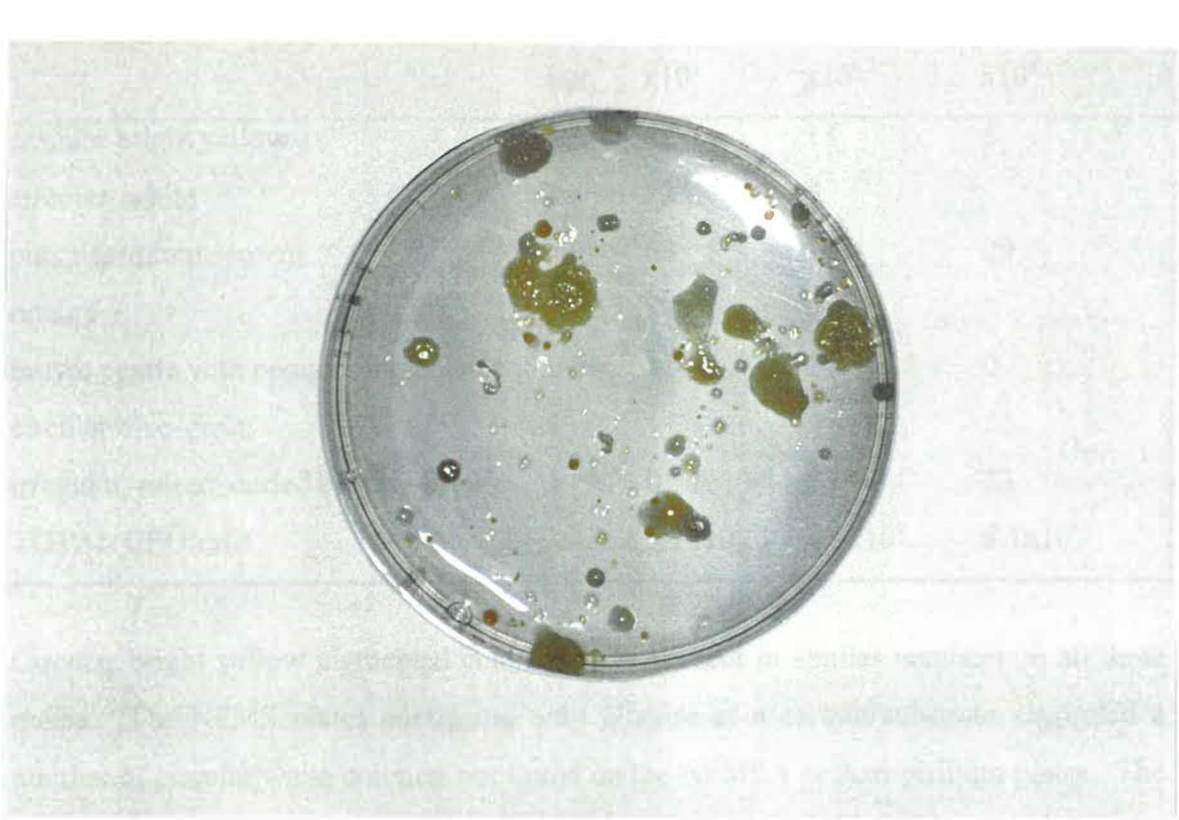

Figure 34: NFMSA agar plate from Tasman Pond 2

NFMSA medium contained 0.1 g/L NaCl, 0.01 mg/L of biotin and a carbon substrate consisting of 4 g/L methanol, 3 g/L acetate, 2 g/L galactose and 1 g/L of glucose.

Descriptions and number of colonies from the three agars used to enrich for nitrogen fixing isolates from Tasman Pond 2 effluent are presented in Table 20. These counts were obtained from spreading 0.1 mL of a 1 x 10^4 dilution of Pond 2 effluent onto the agar and incubating for 6 days.

Table 20: Colony description and mean counts from enrichment media

Colony description	Size mm	NFMS $x10^6$	NFMSA $x10^6$	Azospirillum $x10^6$
circular bright yellow	1	12	13	5
circular, white	1	22	0	
punctiform transparent	<1	62	147	29
orange	1-3	3	0	0
brown centre with opaque ring outside	2-3	11	14	0
circular blue-green	1			25
irregular, raised, curled creamy-brown	3	0	15	22
TOTAL CFU/mL		$1.1x10^7$	$1.9x10^7$	$8.1x10^6$

Circular bright yellow pigmented colonies were present in similar numbers on all three media. The NFMS plates containing only glucose as a carbon substrate supported a number of circular white colonies not found on the NFMSA or Azospirillum plates. The Azospirillum plates produced many small blue green colonies, pigmented by the Bromythmol Blue in this medium, which may be similar to the white colonies found on the NFMS plates.

Very small transparent colonies predominated on the NFMSA plates but were also present on the other media. Approximately 10% of the plate count on NFMSA and Azospirillum plates consisted of raised, tenacious, creamy-brown colonies. These were absent on the NFMS plates. The large cream *Azomonas* type colony morphology seen in Section 4.4.10 was absent and further enrichments from Tasman Pond 2 out wastewater failed to culture this isolate.

Total plate counts were consistently 1.8 - 2.0 x 10^7 CFU/mL on NFMSA plates and slightly lower on NFMS and Azospirillum plates.

The colonies were not well isolated and under microscopic examination various cell morphologies could be detected within each colony. Various colony types from each of

the three media were selected and these were streaked on all media and incubated aerobically at 30° C. The results after 10 days incubation are shown in Table 21.

It took many (approximately 5-8) transfers using NFMSA media until pure cultures were obtained. Many strains grew poorly or not at all after successive transfers. Five different isolate types, that grew within 5 days on aerobically incubated (30°C) NFMSA agar, could be distinguished after purification (Table 22). It is extremely difficult to relate these pure isolates with the colony morphologies seen in Table 20 because of the number of transfers needed to obtain pure cultures.

Table 21: Growth of colonies selected from enrichment plates on NFMS, NFMSA and Azospirillum agar (incubated at 30°C for 10 days)

Colony source (agar)	Colony description	Growth on NFMS	Growth on NFMSA	Growth on Azospirillum
Azospirillum	circular blue-green	poor/opaque	poor/opaque	none
Azospirillum	circular bright yellow	good/opaque	good/yellow	good/yellow
Azospirillum	irregular, raised, cream	poor/transp.	poor/cream	poor/cream
Azospirillum	transp. opaque	poor/opaque	good/opaque	poor/opaque
NFMSA	circular bright yellow	good/yellow	good/yellow	poor/opaque
NFMSA	irregular raised cream	good/transp.	good/cream	poor/opaque
NFMSA	raised creamy brown	poor/opaque	good/pink	poor/opaque
NFMSA	transp. raised	none	poor/transp.	none
NFMSA	cream raised	very poor /transp.	none	none
NFMS	circular white	none	poor/transp.	none
NFMS	transp.	good/transp.	good/cream and transp.	poor/transp.
NFMS	pink/orange	good/transp.	none	poor

transp. = transparent

Table 22: Description of 5 isolates enriched from Tasman Pond 2 effluent using NFMSA medium

	Isolate 7	Isolate 8	Isolate 9	Isolate 10	Isolate 11
colony morphology on NFMSA agar	large, brown, irregular, raised, tough	yellow, round smooth, glistening	opaque, irregular	bright yellow, dry, small, round	creamy -brown, round, smooth
cell morphology (5 day old cultures on NFMS agar)	Figure 35 curved rods	Figure 36 dumbell rods	plump curved rods	Figure 37 small dumbell rods	Figure 38 long dumbell rods
gram stain	-	variable	-	-	variable
PHB stain	+	+	-	+	+
oxidase	+	+	-	+	+
catalase	-	+	+	+	weak +
growth on methanol	+	+	+	+	+
growth on glucose	+	+	+	+	+
growth on acetic	+	+	+	+	+
growth on peptone	+	+	+	+	+
ability to grow chemolithotrophi-cally	not tested	weak?	not tested	weak?	not tested
ability to reduce acetylene	+	+	1-	+	+

[1]Isolate 9 was unable to reduce acetylene under the experimental conditions. A range of different oxygen concentrations was tried (from 0.2%-1.8%) but this isolate still failed to reduce acetylene and therefore was excluded from subsequent experimental work.

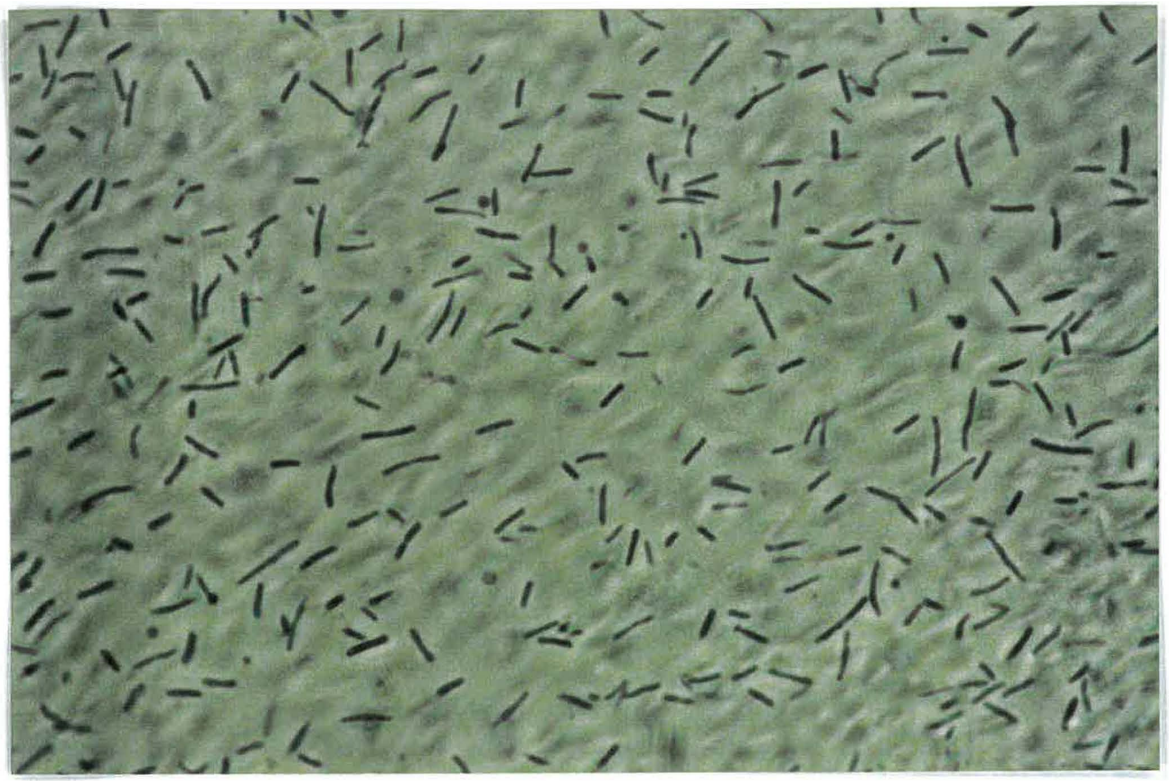

Figure 35: Cell morphology of Isolate 7 (phase contrast x 1000)

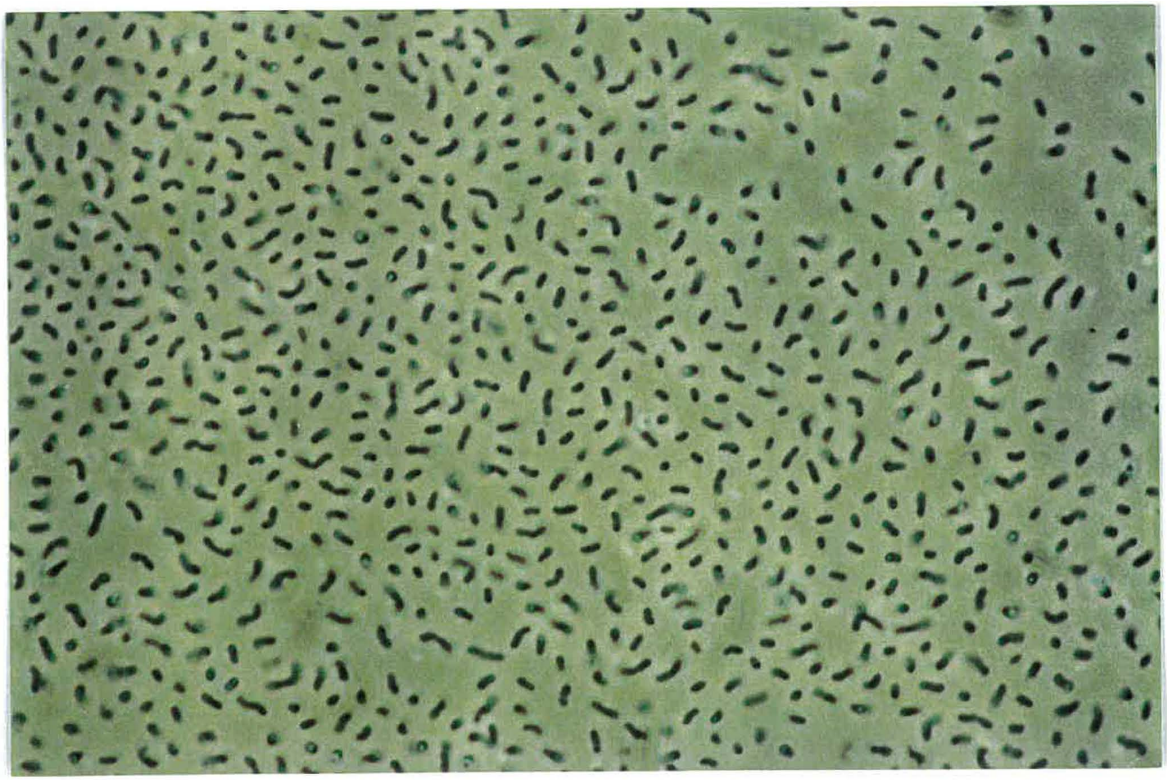

Figure 36: Cell morphology of Isolate 8 (phase contrast x 1000)

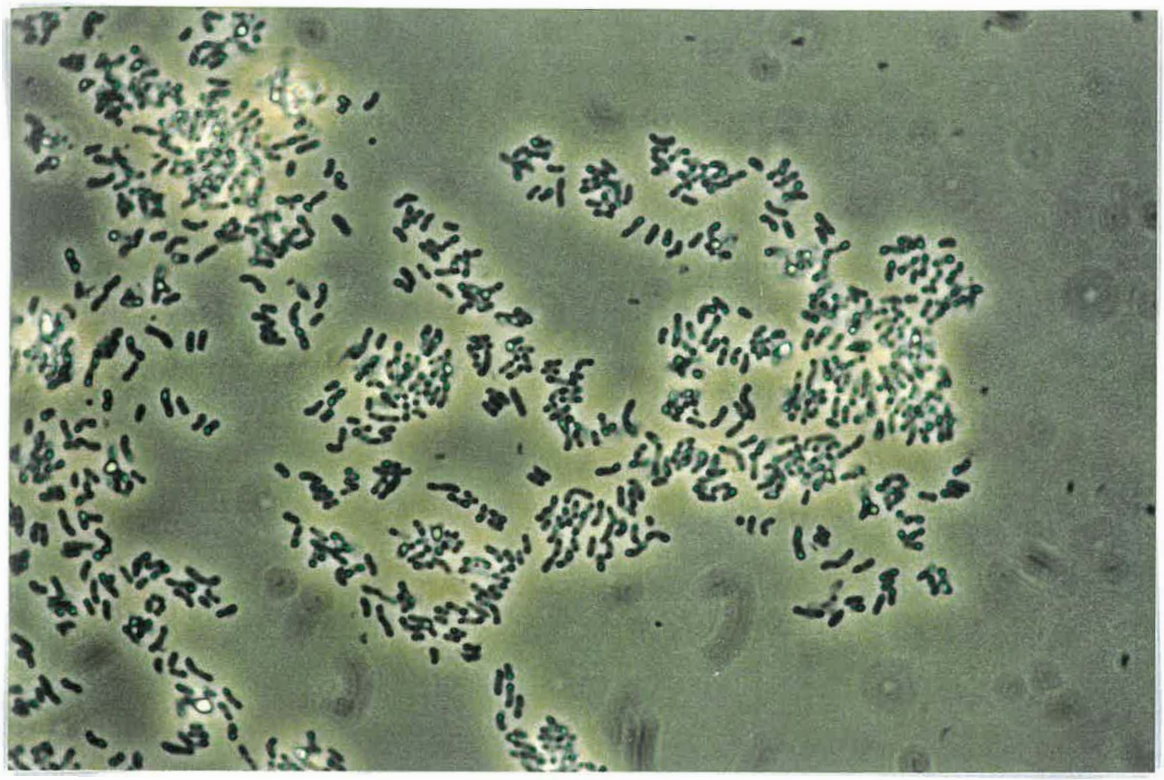

Figure 37: Cell morphology of Isolate 10 (phase contrast x 1000)

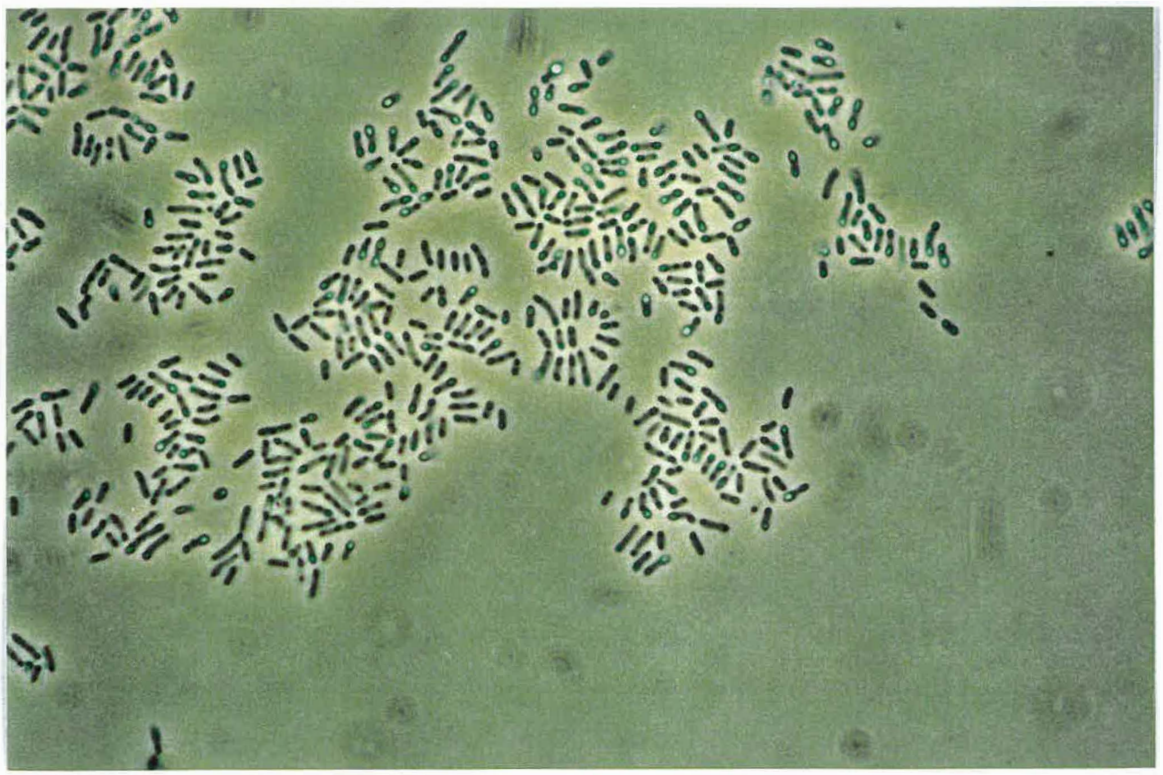

Figure 38: Cell morphology of Isolate 11 (phase contrast x 1000)

5.4.2 Biochemical test kit results

The results of the biochemical tests in Microbact 24E kit for Isolates 7, 8, and 10 are presented in Table 23. Isolate 11 did not grow in the time frame required by this kit therefore biochemical results are unavailable for this isolate.

Table 23: Microbact 24E test kit results for Isolates 7,8 and 10

	Isolate 7	Isolate 8	Isolate 10
Lysine decarboxylase	+	+	-
Ornithine decarboxylase	-	-	-
H_2S production	-	-	-
Glucose fermentation	+	-	-
Mannitol fermentation	-	-	-
Xylose fermentation	-	-	-
ß-galactosidase	-	-	-
Indole	-	-	-
Urease	+	-	+
Voges-proskauer	-	-	-
Citrate utilisation	-	-	-
Trytophan deaminase	-	-	-
Gelatin hydrolysis	-	-	-
Malonate	+	+	+
Inositol	-	-	-
Sorbitol	-	-	-
Rhamnose	-	-	-
Sucrose	-	-	-
Lactose	-	-	-
Arabinose	+	-	-
Adonitol	-	-	-
Raffinose	-	-	-
Salicin	-	-	-
Aginine	-	-	-

The results of the biochemical tests in API 20 NE kit for Isolates 7, 8, and 10 are presented in Table 24. Isolate 11 did not grow in the time frame required by this kit therefore biochemical results are unavailable for this isolate.

Table 24: API 20 NE test kit results for Isolates 7,8 and 10

	Isolate 7	Isolate 8	Isolate 10
Nitrate reduction	+	-	-
Indole	-	-	-
Glucose acidification	+	-	-
Arginine hydrolase	-	-	-
Urease	+	-	-
Esculin hydrolysis	-	-	-
Gelatin hydrolysis	-	-	-
ß-galactosidase	-	-	-
Glucose	+	-	-
Arabinose	-	-	-
Mannose	-	-	-
Mannitol	-	-	-
N-acetyl-glucosamine	-	-	-
Maltose	-	-	-
Gluconate	+	+	-
Caprate	-	-	-
Adipate	-	-	-
Malate	+	+	-
Citrate	-	+	-
Phenyl-acetate	-	-	-

Incubation periods for both the biochemical test kits are short, 18-24 hours. Under these conditions slow growing cultures may not have sufficient time to produce a positive reaction. Isolates 8 and 10 could not assimilate glucose using the biochemical test kits. Both these isolates, however, were able to assimilate glucose after 5 days growth on NFMS medium containing glucose as a sole carbon substrate (Table 22). Therefore, all

negative results from the biochemical tests are questionable. Only positive results from these tests were used for isolate characterisation.

The large, irregular, raised and tough colony morphology of Isolate 7 resembles the description of *Beijerinckia* (Kreig, 1984). This isolate decarboxylated lysine, fermented glucose, hydrolysed urea, reduced nitrate to nitrite and assimilated glucose, gluconate, arabinose, malate and malonate. Apart from malonate, species of *Beijerinckia* have been reported to assimilate these carbon substrates (Becking, 1984). This isolate was able to grow on NFMSA agar with only peptone as a carbon substrate, did not utilise sucrose and was catalase negative. *Beijerinckia* species cannot grow on peptone, can utilise sucrose and are catalase positive (Table 22). Isolate 7 does not match the description of other well-known aerobic free-living nitrogen fixing bacteria such as *Azotobacter, Azomonas, Azospirillum, Derxia,* and *Xanthobacter.* The characteristics of the isolates obtained from the reactor study (Section 4, Table 15) do not resemble those of Isolate 7.

Isolate 8 decarboxylated lysine and assimilated malonate, gluconate, malate and citrate under test kit conditions. However on NFMS agar with a five day incubation period this organism could assimilate methanol, glucose and acetic acid (Table 22). This isolate may belong to the genus *Xanthobacter* due to its bright yellow pigmentation, its rod shaped cells and its limited ability to utilise carbohydrates. The colony and cell morphology of this rod resembles Isolate 3 from the reactor experiment (Section 4, Table 15).

Isolate 10 hydrolysed urea under Microbact 24E test kit conditions but not under API 20 NE test kit conditions. Only malonate was able to be assimilated by this organism under the test kit conditions. On NFMS agar isolate 10 could assimilate glucose, methanol and acetic acid (Table 22). This isolate was also pigmented bright yellow but differed from Isolate 8 because the yellow pigmentation was dry and because it was non motile. This isolate may also belong to the genus *Xanthobacter*. Isolate 10 has similar cell and colony morphology characteristics to Isolate 5 from the reactor experiment (Section 4, Table 15)

To confirm that Isolates 8 and 10 belong to the *Xanthobacter* genus, their ability to grow under nitrogen fixing chemolithoautotrothic conditions was tested. Results from these tests were inconclusive due to organic substrate contamination of the media (Table 22).

5.4.3 Substrate utilisation study

The results from the substrate utilisation study for Isolates 7,8,10,11 are presented in Figures 39 - 42. Nitrogen concentration at day 0 and day 60 was determined by total Kjeldahl nitrogen (TKN) and the results from this analysis are shown in Table 25.

Table 25: Total Kjeldahl nitrogen concentration data from substrate utilisation studies (mg/L)

mg/L	Isolate 7	Isolate 8	Isolate 10	Isolate 11
TKN at day 0	27	27	27	27
TKN at day 60	67	107	152	79
Dissolved TKN at day 60	42	15.4	19.9	13.5

The substantial increases in TKN concentration from day 0 to day 60 indicated that nitrogen fixation occurred in all cultures. Isolate 7 had a high dissolved TKN concentration at day 60, probably an effect of the sampling difficulties associated with the pellicle growth in this culture.

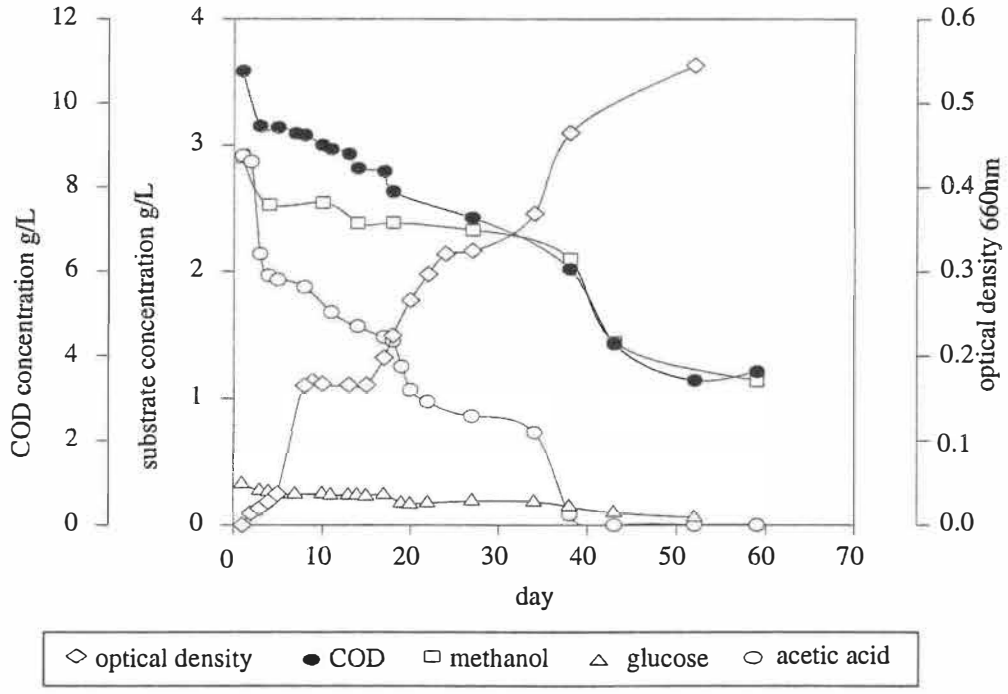

Figure 39: Time course for batch growth of Isolate 7 in NFMSA medium with a mixed carbon source

Isolate 7 grew as a thick elastic pellicle suspended in the NFMS medium. Because of this pellicle growth, representative sampling was difficult and optical density measurements were likely to be underestimated. A lag phase was not evident for Isolate 7 and cell growth appeared to occur steadily. Approximately 70% of the COD was removed from the medium by day 60. Acetic acid removal rate was the greatest of the substrates and by day 40 all acetic acid had been consumed. Methanol concentrations decreased steadily until day 40. Once acetic acid had been completely removed the rate of methanol removal increased. By day 60 approximately 1000 mg/L of methanol remained, accounting for a significant proportion of the residual COD concentration. A gradual decline in glucose concentration was observed over the 60 day period.

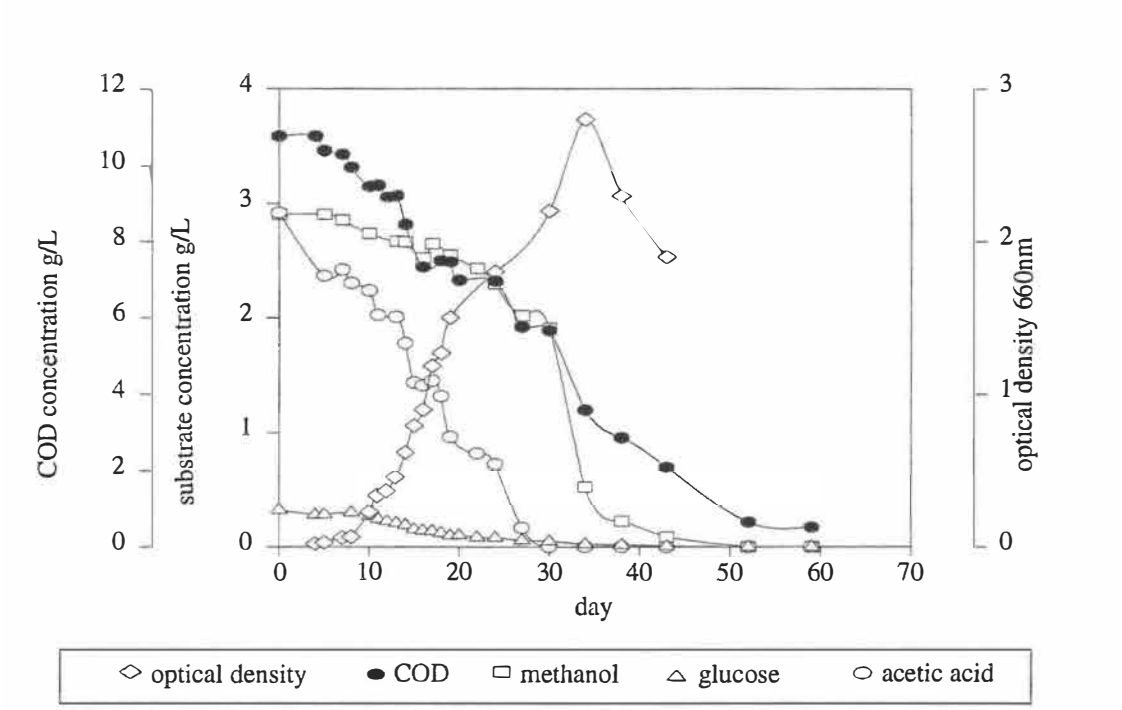

Figure 40: Time course for batch growth of Isolate 8 in NFMSA medium with a mixed carbon source

Isolate 8 experienced a 10 day lag phase after which a rapid increase in optical density occurred. This isolate recorded the highest optical density readings of all the isolates. The culture was dark yellow. Optical density peaked at day 35 and then decreased rapidly, probably due to cell lysis. The rate of COD removal from the NFMS medium was the most rapid and complete of all isolates with 95% removal occurring by day 60, resulting in negligible residual COD. Acetic acid removal was more rapid than for the

other substrates, with complete removal by day 30. This isolate was able to utilise acetic acid and methanol simultaneously. As for Isolate 7, an increase in methanol removal rates occurred at day 30 after all the acetic acid had been removed. By day 52 all methanol had been consumed. Of all the isolates, glucose removal rate was greatest for this isolate with glucose being completely consumed by day 30. TKN concentration at day 60 was lower than Isolate 10 because of endogenous decay and lower biomass levels affecting nitrogen fixation.

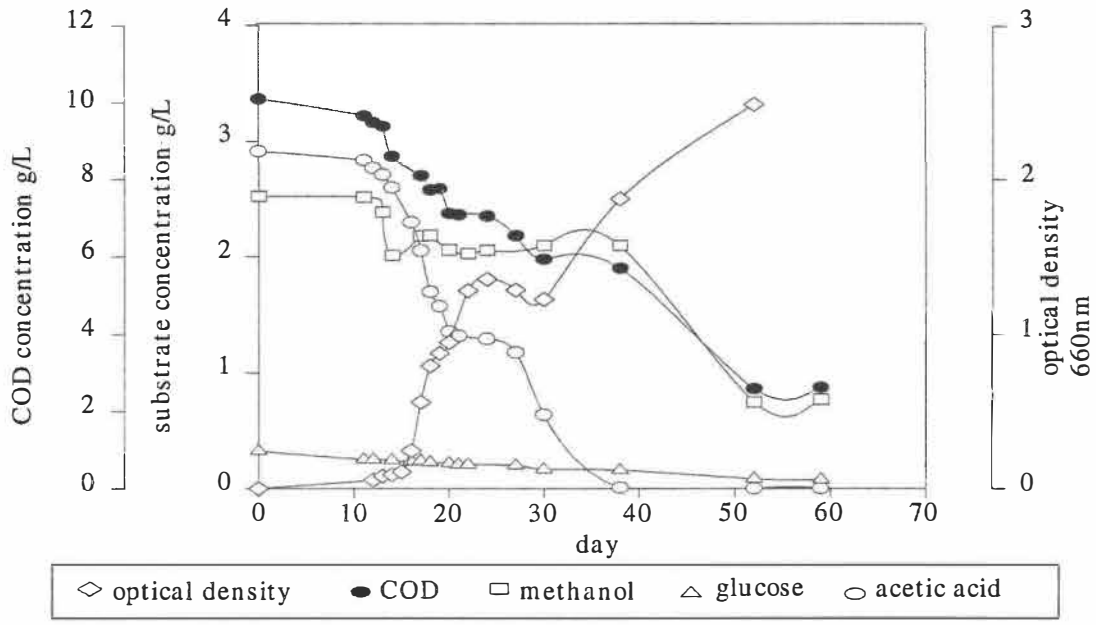

Figure 41: Time course for batch growth of Isolate 10 in NFMSA medium with a mixed carbon source

Isolate 10 had a 15 day lag phase before a period of rapid growth. This growth levelled off at day 25, but from day 35 another period of rapid growth was observed. At day 60, cell growth had still not reached a maximum. High TKN concentrations were recorded at day 60 because the cells in this culture were still growing rapidly. By day 60, 80% of the COD had been removed from the medium. Of all substrates, acetic acid removal rates were greatest and all of this had been consumed by day 40. Methanol removal rates appeared to be reasonably steady until day 40 followed by a rapid decrease over a 10 day period. Glucose concentrations decreased gradually throughout the experiment. The optical density and substrate removal rates of this culture indicate a diauxic growth

pattern. A significant proportion of the residual COD present at day 60 could be accounted for by the undegraded methanol present in the medium.

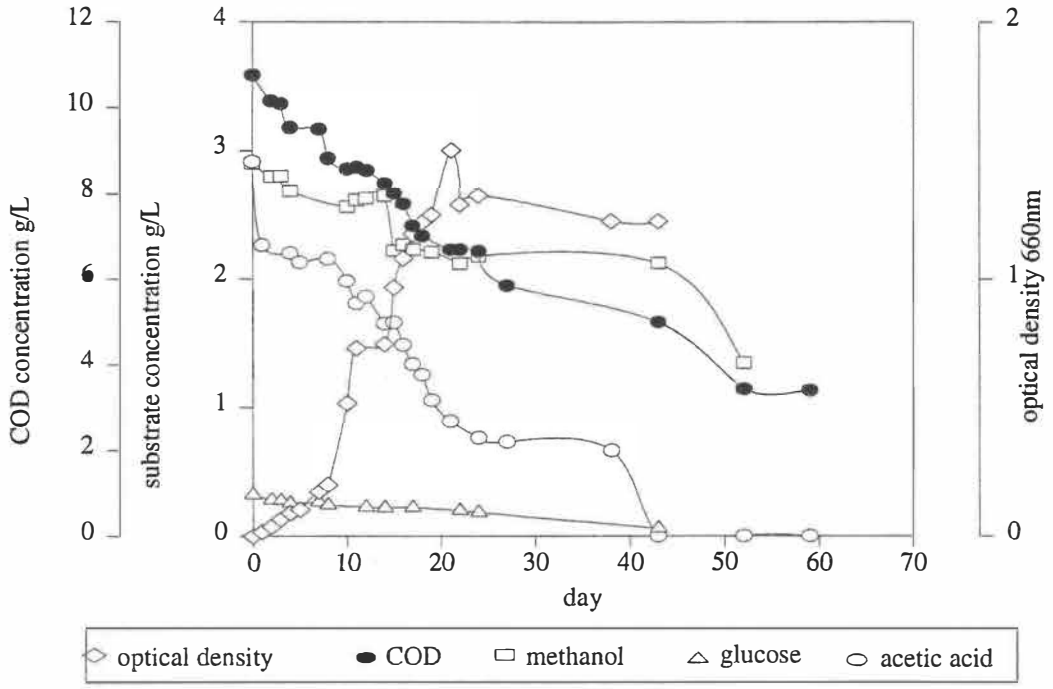

Figure 42: Time course for batch growth of Isolate 11 in NFMSA medium with a mixed carbon substrate

Isolate 11 experienced rapid growth initially with no lag period. Lower optical density values were observed in this culture compared to Isolates 8 and 10. Optical density measurements indicated that stationary phase had been reached by day 20. COD removal rate was steady and by day 60, 70% COD removal had occurred. Acetic acid removal rates were rapid and this substrate was consumed by day 40. Methanol removal rates were less than acetic acid but steady. The rate of methanol consumption increased at day 40 when all acetic acid had been consumed. Glucose concentrations decreased gradually throughout the experiment. The lower TKN concentrations measured at day 60 reflect the lower biomass concentration as indicated by the optical density of this culture.

All four isolates grew slowly and in most cases after 60 days substrate removal was still not complete. The residual substrate could be accounted for by undegraded methanol.

Only Isolate 8 was able to completely remove methanol from the medium within the 60 day time frame.

To confirm that these isolates were not being inhibited in some way by the NFMS culture medium or that they were lacking some vital growth factor, Isolates 7,8,10 and 11 were inoculated into 0.2 μM filter sterilised Pond 2 in wastewater. The carbon substrate used in the substrate utilisation study was added to the wastewater. Optical density was monitored over a 15 day period. After 15 days, under the same incubation conditions as previously, optical densities of 0.055 to 0.125 were recorded. The optical density of the wastewater cultures was not significantly different from the optical densities measured in the substrate utilisation study. Therefore, it is unlikely that the slow growth of these isolates was caused by the culture medium.

5.5 DISCUSSION

In Section 4 a standard nitrogen free mineral salts (NFMS) agar was used to enrich for nitrogen fixing bacteria. NFMS agar and broth has previously been used successfully by a number of researchers to isolate aerobic nitrogen fixing bacteria (Brown, Burlingham *et al.*, 1962; Knowles, Neufeld *et al.,* 1974; Dalton, 1980). For the work in Section 4 this medium satisfactorily isolated *Azomonas* type cells but produced poor growth of transparent colonies which were present in dominant numbers. Better growth was observed using a similar medium but with the addition of 0.1 g/L of NaCl and 0.01 mg/L of biotin with enrichments directly from Tasman Pond 2. An amended NFMS (NFMSA) medium containing methanol and acetic acid as well as glucose was also used for enrichment and slightly more growth was evident. Many tiny transparent colonies still formed on this medium.

Aerobic enrichment techniques using NFMSA agar enumerated a high level (1.8×10^7 CFU/mL) of nitrogen fixing bacteria from Pond 2 of Tasman's treatment system. A number of different colony morphologies was apparent. Many colonies did not grow well on the NFMSA agar after consecutive transfers to obtain pure cultures. The isolates which were characterised were those that grew best on NFMSA agar. The substrate utilisation studies confirmed that these isolates are very slow growing organisms. Inoculating the isolates into a Pond 2 wastewater enriched with a mixed carbon substrate did not improve the growth of these organisms, confirming that the composition of NFMSA was not inhibiting growth.

Gapes (1996) found that a nitrogen fixing system had a lower growth rate than a nitrogen supplemented system under aerobic conditions. Aerobic nitrogen fixation in free-living organisms can be inefficient, especially when there is a need to direct carbon sources into respiratory protection (Dalton and Postgate, 1969a). The slow growth of the isolates observed in this experiment may be due to low substrate efficiency under aerobic nitrogen fixating conditions. Slow growth rates may also be a result of culture conditions such as dissolved oxygen concentration, inoculum size, the absence of beneficial

microbial associations in a pure culture or perhaps due to the lack of colonisable surfaces present in the soluble NFMSA medium and 0.2 μM filter sterilised Pond 2 effluent.

During the isolation procedure it was noticed that a number of different cell morphologies existed within a colony. Much work had to be done to obtain pure cultures and many failed to grow after consecutive transfers. These observations may indicate that microbial associations can play an important role within this community. A possible explanation for the inability to isolate in pure culture is that some of these organisms may have in fact been scavenging nitrogen from the system rather than actively fixing nitrogen.

Conventional microbiological techniques were applied to isolate, characterise and identify the organisms used in this pure culture study. The emphasis in this study was placed on characterising the substrate utilisation patterns of these organisms rather than a complete identification of the nitrogen fixing isolates.

Four isolates that were studied in detail were able to utilise methanol, acetic acid and glucose as sole carbon substrates under nitrogen fixing conditions. Incubation in NFMSA broth with a combined methanol, acetic acid and glucose substrate showed several properties of these isolates. Isolate 10 demonstrated a diauxic growth pattern with sequential removal of acetic acid and then methanol. The other isolates all demonstrated preferential removal of acetic acid over methanol. The residual COD at the end of the 60 incubation day period could be accounted for by the undegraded methanol. Isolate 8, which was able to utilise methanol nearly as effectively as acetic acid, was the only isolate to remove all substrates by day 60.

Diauxic growth has been observed in *Azotobacter vinelandii* grown in a medium containing both acetate and glucose. Glucose was not metabolised until acetate was removed from the medium (George, Costenbader *et al.*, 1985). These authors suggest that acetate or its metabolites act to repress the glucose utilisation system in *A. vinelandii.* During their study they found that the rate of nitrogen fixation under diauxic conditions for cells growing on acetate was 50% of that during growth on glucose. They were unable to draw any conclusions between a direct correlation of nitrogen fixation with ATP pool levels because a number of other factors which can affect nitrogenase activity

needed to be considered first. Haaker and Veeger (1976) determined that cells grown on sucrose and pyruvate have different nitrogen fixing capabilities. These findings suggest that the specific nitrogen fixation rate of the biomass should decrease when grown on acetate. During Stage 2 of the reactor experiment we observed no such change (Section 4, Figure 20). More work needs to be done in this area. If different substrate compositions affect nitrogen fixation rates, this will have implications for optimising wastewater treatment systems based on nitrogen fixation.

The study of bulk organic substrate composition (Section 3) revealed that all acetic acid was consumed in Pond 2 but methanol and carbohydrates are not completely degraded until further down the system. This observation supports the findings above that for all isolates acetic acid was consumed at a higher rate than methanol and glucose.

Commercial biochemical test kits were used to identify the isolates but because these target fast-growing medical bacterial strains the results were inconclusive. Isolates 8 and 10 may be grouped as presumptive *Xanthobacter* species.

Xanthobacter are microaerophilic nitrogen fixers that are commonly found in environments with decaying organic matter or containing sufficient concentrations of H_2 and CO_2 and/or other products of anaerobic microbial activity, such as organic acids and alcohols. These organisms grow on methanol and other organic acids but carbohydrate utilisation is limited. For most strains glucose is not a preferred substrate (Kreig 1982). *Xanthobacter* are metabolically versatile and under carbon substrate limiting conditions these are able to grow as hydrogen oxidisers. It would seem reasonable that these bacteria could be present in the Tasman treatment system.

Isolate 7 does not fit well into any of the well-known classification schemes for nitrogen fixing bacteria. Ideally genotyping methods need to be employed to sequence and identify these organisms.

The Tasman treatment system provides many micro-environments capable of supporting a wide range of microorganisms with different metabolic capabilities. Previous studies have used anaerobic enrichment techniques which have enumerated *Klebsiella* species from Tasman Pond 2 (Bruce and Clark, 1994). The reactor study (Section 4) detected the

presence of the *Azomonas* type cells (Isolate 1) but these were not isolated from the treatment system in this study.

Conventional microbiological enrichment techniques are very selective and may only recover a small percentage of the microorganisms present (Wagner and Amann, 1997). Dominant nitrogen fixing bacteria present in marine and terrestrial cyanobacterial mats have been assessed using oligonucleotide probes to screen for microorganisms containing the *nifH* gene (Steppe, 1996). Because culturing of the microorganisms is not a requirement these genetic techniques offer more comprehensive insights to the interrelationships that exist between the microorganisms and their environment. Further work in this area is needed to understand the community structure and function so that nitrogen fixing treatment systems can be managed to optimise treatment performance.

5.6 CONCLUSIONS

The conclusions drawn from the pure culture work were:

- A number of different nitrogen-fixing isolates were obtained under aerobic conditions from Tasman's Pond 2 wastewater using an amended nitrogen free mineral salts medium with a carbon substrate consisting of methanol, acetic acid, galactose and glucose.

- Substrate utilisation studies demonstrated that each of the 4 selected isolates were able to utilise acetic acid, methanol and glucose as a carbon substrate. All isolates removed acetic acid from the medium before methanol which is consistent with the bulk organic removal study results (Section 3).

- The isolates were all characterised by slow growth rates in both the nitrogen free mineral salts medium and Pond 2 wastewater. Under aerobic conditions slower growth rates were observed in a nitrogen fixing system compared to a nitrogen supplemented system (Gapes, 1996). As well, growth of these isolates may have been affected by culture conditions.

- Inconclusive results were obtained from identification tests using conventional microbiological techniques. Two isolates may belong to *Xanthobacter* species due to their distinctive colony and cell morphology, ability to utilise methanol and limited ability to use carbohydrates.

- The results of this work and previous work (Bruce, 1994) suggest that a diverse range of microorganisms with nitrogen fixing capabilities is represented within the Tasman treatment system.

Section 6

SUMMARY AND RECOMMENDATIONS FOR FURTHER WORK

6. SUMMARY AND RECOMMENDATIONS FOR FURTHER WORK

6.1 SUMMARY

Studies of the aerated lagoon treatment system at Tasman Pulp and Paper Co. Ltd have shown that an increase in total nitrogen concentration across Pond 2 is due to bacterial fixation of atmospheric nitrogen (Bruce, 1994). Because of the typically low concentration of dissolved nitrogen in pulp and paper wastewaters as well as the fact that this treatment system receives no nitrogen supplementation, the microorganisms must fix atmospheric nitrogen to satisfy their nitrogen requirements for cell synthesis.

Gapes and Clark (1995) demonstrated the advantages of operating a nitrogen fixing system treating bleached kraft mill effluent at a dissolved oxygen concentration of 2.2 mg/L. Elevated oxygen concentrations have been shown to reduce the biomass yield of a nitrogen fixing laboratory reactor system. These findings have significant implications for reducing biosolid disposal requirements of waste treatment systems. As well as reduced sludge production, the advantages include better control of the nitrogen load to the discharge environment and no requirement for nitrogen supplementation.

Pulp and paper production is not a stable operation and as a result wastewater,from the mill is subjected to varying loads due to spills and changing mill operations. The availability and nature of the degradable organic substrate in the wastewater affects the nitrogen fixing microorganisms that proliferate in a treatment system. For example, *Azotobacter* are not able to utilise methanol while this study (Section 3) showed that the soluble degradable COD entering Tasman's treatment system consisted of approximately 20% methanol.

An important objective of this study was to see if an increase in concentration of a wastewater constituent affected the composition of the microbial consortia, nitrogen fixation rates and substrate removals. The composition of the soluble organic material entering Tasman treatment Pond 2 was determined (Section 3). These results were used

to decide which organic substrate concentrations could be varied to study the effects of the modified wastewater composition on the nitrogen fixing system.

Methanol and acetic acid were selected because they were present in the wastewater at significant concentrations and their loadings to the treatment system were variable. However, these compounds and total dissolved carbohydrates only accounted for approximately 43% of the degradable soluble COD. The remaining 57% could not be identified but is likely to have significant influence on the microbial consortia. A key finding of this study was that acetic acid was the first identified substrate removed from the treatment system, implicating this compound as the preferred substrate under these conditions.

The laboratory bioreactor experiment demonstrated that increases in acetate and methanol concentrations did not affect specific substrate removal and nitrogen fixation rates in the system but may have changed the microbial population (Section 4). The dissolved oxygen concentration in the laboratory system was controlled at 2.2 mg/L. Although this system was found to give an adequate simulation of COD removal in Pond 2, the system was unable to provide the variety of oxygen micro-environments available in an aerated lagoon system. Even at this high oxygen concentration many different isolates were obtained suggesting that a diverse range of aerobic microorganisms with nitrogen fixing capabilities was present. One of these isolates was tentatively identified as an *Azomonas* species. This organism has previously been isolated from pulp and paper wastewater (Knowles, Neufeld *et al.*, 1974).

An interesting result obtained throughout the bioreactor study was the lower than average nitrogen content of the biomass. Low biomass nitrogen was attributed to microbial intracellular storage of poly-β-hydroxybutyrate (PHB). Many nitrogen fixing bacteria form intracellular PHB granules which may act as a selective advantage under the transient substrate conditions found in pulp and paper mill effluent treatment systems.

Isolates were obtained from Tasman treatment Pond 2 which were able to fix nitrogen under microaerophilic conditions. Four selected isolates were able to utilise methanol, acetic acid and glucose as a carbon substrate. All of these isolates removed acetic acid from the mixed carbon substrate medium before methanol and glucose which is

consistent with individual substrate removal found in Tasman treatment system. Two isolates may belong to *Xanthobacter* species because of their distinctive colony and cell morphology and substrate utilisation patterns. *Xanthobacter* are microaerophilic nitrogen fixers that are commonly found in environments with decaying organic matter. These bacteria are metabolically versatile and it would be reasonable to expect to find them in the Tasman treatment system. Members of the Azotobacteraceae family were not isolated directly from Tasman's treatment system.

In conclusion, the Tasman treatment system provides a heterogeneous environment capable of supporting a diverse range of nitrogen fixing microorganisms. The laboratory bioreactor study suggests that the nitrogen fixing system is resilient, adapting to large concentration increases of both methanol and acetic acid with no adverse effects on substrate removal or specific nitrogen fixation rates. This resilience is also observed in the Tasman treatment system, where despite process changes and mill spills, good organic removals are consistently achieved. A requisite for good treatment performance in this dynamic environment would appear to be a nitrogen fixing microbial population with diverse metabolic and physiological capabilities.

6.2 RECOMMENDATIONS FOR FURTHER WORK

6.2.1 Complete identification of soluble degradable substrate entering Tasman Pond 2

Methanol, acetic acid and total dissolved carbohydrates accounted for just under half of the soluble biodegradable COD entering Tasman's Pond 2. Approximately 60% of this unidentified substrate was removed within Pond 2. Assuming that most of this was removed by biodegradation, this unknown substrate will be influencing the composition of the microbial consortium present in Pond 2. To gain a comprehensive view of how different substrate compositions affect treatment performance in this system requires that these substrates be identified.

6.2.2 Microbiological survey of Tasman Pond 2 using molecular biological tools

A wide range of bacteria can be isolated using conventional techniques such as spread plate inoculation. These techniques bias the types of microorganisms that are obtained in pure culture and many microorganisms are unable to be isolated using them. Molecular biological techniques enable identification and phylogenetic characterisation of microorganisms without cultivation. Using rRNA oligonucleotide probes targeting important phylogenetic groups in a top to bottom approach allows a direct and rapid characterisation of the microbial community structure (Wagner and Amann, 1997). Probes targeting microorganisms that contain the *nifH* gene can be used to detect diverse groups of nitrogen-fixing microorganisms reflecting genetic potential for nitrogen fixation in a particular environment (Kirshetein, Paerl *et a,*. 1991).

A survey throughout Tasman Pond 2 using these molecular tools to monitor the nitrogen fixing community over time would provide valuable information on the functioning of this treatment system. Later more specific studies could be carried out manipulating the microbial community with the aim of optimising treatment performance.

16S RNA sequencing can be used to confirm the identification of the nitrogen fixing isolates obtained in Section 5.

6.2.3 Biodegradable plastics from a nitrogen fixing system

Disposal of biosolids is a key issue confronting operation of waste treatment systems. Low biomass nitrogen in the bioreactor study was attributed to microbial storage of polyhydroxybutyrate (PHB). Commercially PHB is of considerable interest because it is used to produce biodegradable plastics. Using the biosolids from a nitrogen fixing waste treatment system to produce biodegradable plastics has potential economical and environmental benefits and is worthy of further investigation.

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Appendix A

COMPILATION OF ANALYTICAL METHODS

(A1) Chemical oxygen demand

(A2) Volatile fatty acids

(A3) Methanol

(A4) Glucose

(A5) Acetylene reduction assay

(A6) Biomass determination

(A7) Oxygen uptake rate

(A8) Preparation of nitrogen free mineral agar

(A9) Maintenance of bacteria by freeze-drying

COMPILATION OF ANALYTICAL METHODS

This appendix contains full method descriptions for non-contracted parameters used in this study.

A.1 CHEMICAL OXYGEN DEMAND

Total chemical oxygen demand (COD) and soluble chemical oxygen demand (COD_s) were measured using APHA-AWWA-WPCF Standard method 5220 D (APHA, 1992).

A dichromate solution was prepared in the following manner; 24.518 g of $K_2Cr_2O_7$ (Analar grade, dried at 105°C), 100 mL of concentrated H_2SO_4, and 26.6 g $HgSO_4$ were dissolved in deionised water and made up to 1 L. A sulphuric acid solution was prepared by dissolving 22 g Ag_2SO_4 in 2.5 L of H_2SO_4. A stock standard solution, with a COD of 5000 mg/L, was prepared by dissolving 4.25 g potassium hydrogen phthalate (Analar grade, dried at 105°C) was dissolved in 500 mL deionised water with 2 mL concentrated H_2SO_4 and made up to 1 L. Working standards of 100 mg/L, 250 mg/L, 500 mg/L and 1000 mg/L were made up from stock standard weekly.

To measure COD, 2 mL of homogeneous sample was pipetted into a 10 mL borosilicate digestion tube. O.75 mL dichromate solution and 2.25 mL of sulphuric acid solution were added by automatic dispenser. The vial was capped, mixed and digested at 160°C for 3 hours. Standards and two distilled water blanks were run with each set of samples. A Pye Unicam SP-550 spectrophotometer was used to measure the absorbance of samples and standards at 620 nm.

A standard curve was constructed, and the slope and intercept calculated. A linear function was used to calculate sample concentrations. Samples were diluted quantitatively if necessary, to give absorbances within the range of standards used.

Soluble COD was performed in a similar manner on effluent filtered through GF/C glass-fibre filter disks.

A.2 VOLATILE FATTY ACIDS

Volatile fatty acids were determined by GC-FID, using an in-house method of the Environmental Research Group at the New Zealand Forest Research Institute. Concentrations of the short chain volatile fatty acids, acetic, propionic, iso-butyric, and n-butyric were measured using gas chromatography. A sample (2 mL) was mixed with iso-valeric internal standard (0.4 mL of 1000 ppm solution) and analysed on a 9 ft x 0.25 in. packed column of 10 % FFAP on Chromosorb G (100 - 200 mesh) support. Carrier gas N_2 40mL/min was passed over 50% formic acid solution, to saturate it with formic acid. The separation was performed at 105° C using a 1μL injection volume. The concentration of VFAs was quantified by reference to the internal standard via calibration using a standard mixture containing 1000 ppm iso-valeric, 250 ppm acetic, 250 ppm propanoic, 250 ppm iso-butyric, and 250 ppm n-butyric acids.

A.3 METHANOL

Methanol was determined by GC-FID, using an in-house method of the Environmental Research Group at the New Zealand Forest Research Institute. Aqueous samples were injected directly onto a 0.53 mm i.d x 30m column coated with a thick film of BP-wax, using a cool on column injector operating at 140°C.

Peak identities were established by comparison of retention times with those of authentic samples. The concentration of methanol, ethanol and acetone was quantified with reference to external standards. A standard curve was constructed, and slope and intercept calculated. A linear function was used to calculate sample concentrations. The detection limit for methanol was less than 0.5 mg/L.

A.4 GLUCOSE

Glucose was analysed using a colourmetric technique. The analysis was carried out using a test kit supplied by Boehringer Mannheim (124028 GLU MPR2). Glucose was converted via an enzymatic reaction to a coloured complex:

$Glucose + O_2 + H_2O \rightarrow gluconate + H_2O_2$

$H_2O_2 + ABTS^{®} \rightarrow coloured\ complex + H_2O_2$

The absorbance of the coloured complex was measured at 610 nm. Glucose concentrations were quantified using a 9.1 mg/L glucose standard supplied by the manufacturer. Samples were diluted so glucose concentrations were between 6 and 10 mg/L.

Solution 1 was the 9.1 mg/L glucose standard. Solution 2 (containing ABTS) was prepared by dissolving contents of premix 1 in 300mL of distilled water. This solution was stored in amber glass bottle. Stored at 2°C to 8°C it is stable for six weeks. Samples were prepared as below.

	Distilled H_2O	Sample	Solution 1	Solution 2
Blank	0.2 mL			5 mL
Standard			0.2 mL	5 mL
Sample		0.2mL		5 mL

The solutions were mixed and incubated at 25°C for 25 - 50 minutes in shaking water bath. The absorbance of samples and standard was read at an absorbance of 610 nm Glucose concentrations were calculated using the following equation.

$C\ (mg/L) = diln \times 10 \times ABS_{sample}/ABS_{std}$

A.5 ACETYLENE REDUCTION ASSAY

Nitrogen fixation activity in Section 4 was determined using batch acetylene reduction assays, based upon the method described by Bruce and Clark (1994).

Acetylene was manufactured daily from the reaction of calcium carbide (CaC_2) with water. The gas was collected and stored in a rubber soccer ball bladder attached to a

plastic T-connector plugged with rubber bungs. The bladder was flushed with argon to exclude all air before collecting the acetylene gas.

Assays were performed in 160 mL serum bottles containing 45 mL of mixed liquor sample (straight from the reactor). The bottles were sparged for 3 minutes with argon gas to remove dissolved oxygen and then sealed. Before the assay 10 ml of the headspace was extracted. To initiate the assay 10 ml of acetylene gas was injected into the bottle (partial pressure of C_2H_2 is approximately 0.09 atm). The bottles were incubated with shaking for 30 minutes. Samples were analysed in triplicate. Blanks containing distilled water were prepared and incubated in a similar manner.

At the completion of the incubation period, approximately 4-5 mL of head space was extracted and stored in disposable plastic syringes with the needles plunged into rubber bungs.

Formation of ethylene gas was quantified by gas chromatography on a 5ft x 0.25in packed column of Poropak N: carrier gas (N_2) 20 mL/min; H_2 20 mL/min; air 300 mL/min; oven temperature 78°C; 1mL sample loop; FID detector temperature 150°C.

The concentration of ethylene was quantified by reference to a standard curve. A cylinder of 1000 ppm ethylene in nitrogen provided gas to prepare a standard curve. Various standards were made by injecting known amounts into a sealed separating flask which was previously thoroughly flushed with air. Glass beads were added to the flask to obtain satisfactory mixing. Reproducibility of the assay under anaerobic conditions is good with 0.3% - 10% standard deviation expressed as a percentage of the mean.

Sample preparation for the acetylene reduction assay in Section 5 differed from the above method and is outlined below. Pure isolates were transferred to NFMS agar slopes containing 20 mg/L NH_4Cl and incubated for 5 days at 30°C. After incubation period, 1 mL of NFMS broth was added to wash the bacterial growth off the slope, creating a suspension. The 1 mL suspension was then transferred to sterile Hungate tubes containing 4 mL of NFMS broth.

Assays were performed in 16.6 mL Hungate tubes containing 5 mL of sample. Tubes were sparged with argon gas for 3 minutes and then sealed. Assays were conducted in a mixture of 95% argon, 5% air in the head space and were initiated by 0.6 mL of acetylene gas (0.6 mL of headspace was withdrawn from the tube prior to acetylene addition). Tubes were incubated at 30°C with shaking at 120 rpm for 30 minutes. Assays were conducted in triplicate and blanks were assayed as above using 5 mL of distilled water. Headspace samples were withdrawn and analysed for ethylene production, as described above.

Conversion of results to rate of nitrogen fixed

read ppm C_2H_4 off standard curve

ppm C_2H_4 = μL

headspace volume in bottle = 160 - 45 = 115 mL or (16.6 - 5 = 11.6 mL)

at 20°C, 1 μmole gas = p/nRT

101.3/1x8.314x293

24.0 μL

at 20°C, 1 nmole gas 0.0240 μL

$$\text{nmoles } C_2H_2/\text{hour} = \frac{(\text{ppm } C_2H_2) \times \text{headspace volume}}{0.240\ \mu\text{L} \times 0.5\text{hr}}$$

can divide by mL of culture used to give nmoles C_2H_2/mL.hr

A.6 BIOMASS DETERMINATION

Solids concentrations were measured on the reactor mixed liquor and effluent as follows:

Three successive portions of 20 mL of distilled water were washed through a 0.45μm cellulose acetate filter paper (Sartorius). The filter paper was dried at 104° C for one hour and then weighed. A blank was prepared in the same manner.

A 400 -500 mL aliquot of sample was centrifuged in a Sorvall high speed centrifuge at 10000 rpm for 10 minutes.

The supernatent was removed and 100 mL of this was filtered through the pre-weighed 0.45μ filter paper. A 40 mL portion of this filtrate was dried overnight at 105°C in a pre-weighed moisture dish. The filter paper was dried for 60 minutes and weighed.

$$\text{Supernatent dissolved solids concentration } \underline{\text{DS}} \text{ (mg/L)} = \frac{\text{solids in dish after drying}}{\text{initial volume in dish}}$$

$$\text{Supernatent suspended solids concentration } \underline{\text{FS}} \text{ (mg/L)} = \frac{\text{solids on filter paper after drying}}{\text{volume filtered}}$$

The centrifuged solid pellet and the residual supernatent remaining with the pellet (and the washings required to get it from the tube) were dried overnight at 105° C in a pre-weighed moisture dish.

$$\text{Residual supernatent (in with pellet) (L)} = \text{Initial volume added to centrifuge tube} - \text{supernatent volume removed}$$

$$\text{Dissolved solids in dish with pellet (mg)} = \text{DS} \times \text{residual supernatent (in with pellet)}$$

$$\text{Total suspended solids in dish } \underline{\text{MS}} \text{(mg/L)} = \frac{\text{solids in dish after drying} - \text{dissolved solids in dish}}{\text{volume centrifuged}}$$

$$\text{Total suspended solids } \underline{\text{TSS}} \text{ (mg/L)} = \text{FS} + \text{MS}$$

The reactor feed was filtered through a 0.45 μM glass fibre filter paper (Whatman) and therefore considered to be totally soluble. Any suspended solid material in the reactor was assumed to be biological material from the growth of microorganisms in the system. The total suspended solids (TSS) concentration was therefore an estimate of the biomass concentration of the sample.

Volatile suspended solids (VSS) measurements were also carried out by placing the crucibles from the TSS analysis into a muffle furnace at 550°C for one hour. The samples were removed, cooled in a dessicator to ambient temperature and reweighed.

$$\text{Dissolved ash concentration } \underline{\text{DA}} \text{ (mg/L)} = \frac{\text{solids in dissolved solids dish after drying at 550°C}}{\text{Initial volume in dish}}$$

$$\text{Dissolved ash in dish with pellet (mg)} = \underline{\text{DA}} \times \text{residual supernatent (in with pellet)}$$

$$\text{Total suspended ash in dish with pellet } \underline{\text{SA}} \text{ (mg/L)} = \frac{\text{solids in dish after drying 550°C} - \text{diss. ash in dish with pellet}}{\text{volume centrifuged}}$$

$$\text{VSS as \% of } \underline{\text{TSS}} \text{ in dish with pellet } \underline{(\%\text{VSS})} = \frac{(\underline{\text{MS}} - \underline{\text{SA}}) \times 100}{\underline{\text{MS}}}$$

$$\underline{\text{VSS}} \text{ of sample} = \underline{\text{VSS}} \times \underline{\text{TSS}} \text{ of sample}$$

The TSS and VSS measurements were carried out in duplicate for each analysis of solids concentration. The analysis was carried out on both reactor mixed liquor and effluent samples. The effluent sample was collected on ice to limit changes occurring to the biological solids concentration on standing.

A.7 OXYGEN UPTAKE RATE

The oxygen uptake rate (OUR) of the microrganisms in the reactor was measured by sealing the headspace during continuous operation of the reactor and taking syringe samples of the headspace gas through a rubber septum at a number of time intervals.

These samples were analysed for oxygen concentration on a BASIC™ gas chromatograph (Carle Instruments INC. Model GC 8700) using two columns, a Poropak pre-column for separating carbon dioxide from the nitrogen and oxygen, and a molecular sieve column for effecting the separation of nitrogen and oxygen. The columns were operated at 65°C with helium as the carrier gas, at an inlet pressure of 200 kPa. The

sample was injected onto the pre-column. After 55 seconds the nitrogen and oxygen had passed onto the molecular sieve, while the CO_2 was still passing through the initial column. The system was then switched over for the carrier gas to bypass the molecular sieve, allowing the carbon dioxide to pass out of the system, with the N_2/O_2 remaining stationary within the molecular seive. After 165 seconds, the carrier gas was switched back to pass through the molecular seive, effecting the separation of oxygen from nitrogen. A thermal conductivity detector was used to record the oxygen and nitrogen as they passed off the column. Assuming that the nitrogen concentration in the headspace was constant over the period of the analysis, and that nitrogen and oxygen were the only significant gases present in the sample, the nitrogen peak on the chromatogram was used as an internal standard in determining the oxygen concentration of the sample.

The oxygen concenetration was calculated as follows:

$$\text{Oxygen concentration (mol/L) of sample at time t } (O_t) = \frac{\text{Initial } N_2 \text{ conc (mol/L)}}{\%\ N_2} \times \%\ O_2$$

$\%\ N_2$ = percent nitrogen in the sample, from the chromatogram

$\%\ O_2$ = percent oxygen in the sample, from the chromatogram

Initial N_2 concentration was calculated from the percentage of N_2 in the sample taken at t=0

$$\text{Initial } N_2 \text{ concentration (mol/L)} = \text{Initial } \%\ N_2/100 \times 101.3/(8.314 \times T)$$

T = temperature of sample (K). This was assumed to be 303K.

$$\text{Weight (g) oxygen in headspace at time t } (W_o) = O_t \times \text{headspace volume (L)} \times 32$$

The slope of the line formed by plotting R vs time gave the oxygen uptake rate for the sample.

It was assumed the effect of the dissolved oxygen concentration differences between the feed and final effluent was insignificant, and that the measurement of oxygen transfer from the headspace to the reactor mixed liquor was therefore equal to the oxygen uptake by the microorganisms. Gapes and Clark (1995) showed that, for the worst case

scenarios under this assumption, the maximum uncertainty in the OUR measurement was less than 12%.

A.8 PREPARATION OF NITROGEN FREE MINERAL SALTS AGAR

2% Magnesium Salts Solution

$MgSO_4.7H_2O$	10 g
$d.H_2O$	500 mL

1% Calcium Salts Solution

$CaCl_2.2H_2O$	4.65 g
$d.H_2O$	500 mL

100 % Trace Element Solution

$FeSO_4.7H_2O$	0.25 g/L
H_3BO_3	0.29 g/L
$CoSO_4.7H_2O$	0.12 g/L
$CuSO_4.5H_2O$	0.01 g/L
$MnCl_2.4H_2O$	9.0 mg/L
$Na_2MoO_4.2H_2O$	0.25 g/L
$ZnSO_4.7H_2O$	0.12 g/L
Nitrilotriacetic acid	10 g/L

Dissolve nitrilotriacetic acid in distilled water and adjust pH to 6.5 with 3N KOH. Add trace elements and make up to 1 L

20% Carbon Source Soln.

	20% Concentrated Solution (250 mL)
Methanol	55 mL
Acetic acid	61.95 g Na acetate
Glucose	45 g

N.B 45 mg/L of acetic acid is equivalent to 61.95 mg/L of sodium acetate

Specific gravity of 95% ethanol and methanol at 20°C is 0.81 g/cm^3

GMS BROTH (2000 mL bottle)

double .distilled H_2O	1840 mL	
100% Mg Salts Soln.	20 mL	(0.02%)
100% Ca Salts Soln	20 mL	(0.01%)
Autoclave when cool add the following sterile solutions		
100% Trace elements	20 mL	(1%)
40% K_2HPO_4	4 mL	(0.08%)
20% Carbon source soln.	100mL	(1.0%)

MS AGAR (500 mL Bottle)

Purified agar	7.5 g	
d.H_2O	460 mL	
100% Mg Salts soln.	5 mL	(0.02%)
100% Ca Salts soln.	5 mL	(0.01%)
Boil to dissolve and autoclave. When cool add the following sterile solutions:		
100% Trace elements	5 mL	(1%)
40% K_2HPO_4	1 mL	(0.08%)
20% Carbon source soln.	25.0 mL	(1.0%)

A.8 MAINTENANCE OF BACTERIA BY FREEZE DRYING

This method is the in-house method used by Microbiology Department, University of Waikato.

Six ampoules were used for each isolate. Labels were prepared by typing the identification and date on blotting paper. The labels were then folded, added to the ampoule and the ampoules plugged with non absorbant cotton wool. The ampoules were sterilised by autoclaving at 121° C for 30 minutes.

Fresh cell material was harvested from an agar plate using the folowing suspending fluid:

Beef extract	10g/ 100 mL RO water
Meso-inositol	5g
Peptone (trypticase)	10g
NaCl	1g

Dissolve and adjust pH to 7.5. Dispense in 10 mL amounts into universals.
Autoclave for 20 mins

Ampoules were filled with the suspension using a sterile pasteur pipette; approximately 0.1-0.2 mL was delivered to the bottom of each ampoule. The cotton wool was replaced after flaming the top of the ampoule.

The ampoules were placed in liquid nitrogen to freeze the suspension. The cotton wool was then pushed down the ampoule. The ampoules were placed on the freeze-dryer and the valves opened slowly to full release. They were left to dry overnight

Constriction of ampoules must be carried out quickly to avoid prolonged exposure of ampoule contents to air. The ampoules were removed from the drier and rotated in a flame to produce a short capillary section. The ampoules were cooled and placed back on the drier for 30 minutes under full vacuum.

The ampoules were sealed in situ on the drier, while still under vacuum. A flame was waved gently across the constriction from all sides. The glass was sealed and pulled gently away from the freeze drier.

Appendix B

SUPPLEMENTAL DATA

(B1) Section 3 data

(B2) Section 4 data

(B3) Statistical analysis (ANOVA) of Section 4 data

B.1 SECTION 3: TASMAN FIELD STUDY DATA

Oxygen demand, acetic acid methanol and ethanol data

		COD(t)	COD(s)	DOC(s)	BOD	acetic	methanol	ethanol
17-Mar	pond 1 in	1092.7	435	137.8	209	30.0	22.3	1.2
	pond 2 in	717.2	470.3	161	188	27.1	24.0	nd
	pond 2 out	629.8	372.8	119.8	95	nd	15.9	nd
	pond 4 out	375.4	210.3	71.3	27	nd	nd	nd
18-Mar	pond 1 in	727.3	447	148	218	11.0	10.9	1.2
	pond 2 in	826.8	456.4	153.6	243	31.1	12.9	2.2
	pond 2 out	450.8	nm	95.91	89	nd	6.8	nd
	pond 4 out	344.9	218.8	70.44	27	nd	nd	nd
19-Mar	pond 1 in	806.8	565.5	189.3	229	10	29.4	2.7
	pond 2 in	754	561.3	188.6	275	22	28.6	3.1
	pond 2 out	599.1	289.7	95.25	93	nd	12.3	nd
	pond 4 out	318.4	210.5	74.28	26	nd	1.8	nd
20-Mar	pond 1 in	896.2	585.3	194.5	197	6	42.2	3.1
	pond 2 in	957.4	611	201.8	236	19	49.1	3.3
	pond 2 out	598.6	365.6	116.4	123	nd	12.1	nd
	pond 4 out	349.6	201.3	70.09	12	nd	nd	nd
21-Mar	pond 1 in	869.4	502.1	177.1	182	7	33.8	nd
	pond 2 in	784.7	478.6	164.6	nm	3	35.3	nd
	pond 2 out	521.6	338.8	114.7	nm	nd	8.9	nd
	pond 4 out	356.6	183.1	66.83	14	nd	nd	nd
22-Mar	pond 1 in	678.9	576.9	207	210	9.4	42.0	1.8
	pond 2 in	851	565.1	202.2	nm	2	45.4	1.4
	pond 2 out	583.7	353.5	121.9	nm	nd	9.6	nd
	pond 4 out	355.6	198.3	71.27	17	nd	nd	nd
23-Mar	pond 1 in	834	637.3	224.5	230	9	52.0	2.0
	pond 2 in	929.9	651.8	229.2	273	18	54.4	2.5
	pond 2 out	602.1	392.2	136.2	115	nd	19.7	nd
	pond 4 out	403.6	206.4	73.17	20	nd	nd	nd
24-Mar	pond 1 in	709.3	634.6	227.6	208	nd	63.4	0.4
	pond 2 in	986.5	620.2	219.7	nm	4	68.9	3.3
	pond 2 out	673.3	408.9	150.6	nm	nd	25.4	nd
	pond 4 out	342.1	224.9	84.98	25	nd	nd	nd

		COD(t)	COD(s)	DOC(s)	BOD	acetic	methanol	ethanol
25-Mar	pond 1 in	970.2	429.5	nm	193	2	36.3	2.2
	pond 2 in	1126.7	466.8	nm	173	15	42.4	2.3
	pond 2 out	717.2	392.2	nm	105	nd	28.8	nd
	pond 4 out	448.6	229.7	nm	27	nd	nd	nd
26-Mar	pond 1 in	571.3	461	nm	159	3	nm	nm
	pond 2 in	665	469.3	nm	225	13	37.9	0.9
	pond 2 out	647.6	303.1	nm	107	nd	18.4	0.8
	pond 4 out	399.6	255.8	nm	30	nd	nd	nd

nm = not measured nd = not detected

Total dissolved carbohydrate data

		arabinose	galactose	glucose	xylose	mannose	cyclotols	total CHO
17-Mar	pond 1 in	5.5	22.4	7.3	3.6	4.1	8.8	51.7
	pond 2 in	4.8	22.6	6.4	3.5	4.9	9.7	51.9
	pond 2 out	1	6.7	2.2	1.6	0.6	5.	17.4
	pond 4 out		nd	nd	nd	nd	nd	nd
18-Mar	pond 1 in	5.1	27.9	7.2	3.5	5.5	12.2	61.4
	pond 2 in	4.8	26.6	6.4	3.5	5.6	13.4	60.3
	pond 2 out	0.8	4.4	1.9	1.4	nd	3.0	11.5
	pond 4 out		nd	nd	nd	nd	nd	nd
19-Mar	pond 1 in	4.4	22.2	8.1	5.4	6.6	9.9	56.6
	pond 2 in	4.3	20.9	6.7	4.5	5.1	10.4	51.9
	pond 2 out	0.7	3.3	2.1	1.4	nd	2.6	10.1
	pond 4 out		nd	nd	nd	nd	nd	nd
20-Mar	pond 1 in	4.6	19.6	7.7	4.7	4.6	8.5	49.7
	pond 2 in	3.9	18.1	7.4	5.6	5.3	9.2	49.5
	pond 2 out	0.8	3.1	3.1	2.1	0.6	4.3	14.0
	pond 4 out		nd	nd	nd	nd	nd	nd
21-Mar	pond 1 in	4	20.3	7.7	4	5.3	10.5	51.8
	pond 2 in	2.5	16.3	4.9	4	2.8	9.5	40
	pond 2 out	0.6	2.2	2	1.4	0.7	4.4	11.3
	pond 4 out		nd	nd	nd	nd	nd	nd
22-Mar	pond 1 in	3.8	20.4	8.2	4.1	5.7	9.6	51.8
	pond 2 in	3.4	19.1	8.1	4.8	4.9	9.9	50.2
	pond 2 out	0.7	2.8	2.5	1.7	0.8	3.9	12.4
	pond 4 out		nd	nd	nd	nd	nd	nd

		arabinose	galactose	glucose	xylose	mannose	cyclotols	total CHO
23-Mar	pond 1 in	4.4	20.9	9	4.2	4.6	8.7	51.8
	pond 2 in	3.8	18.8	7.5	4.5	4.4	11.1	50.1
	pond 2 out	0.7	3	1.8	1.5	0.7	2.66	10.4
	pond 4 out		nd	nd	nd	nd	nd	nd
24-Mar	pond 1 in	7.2	28.4	17.1	5.7	10.9	12.5	81.8
	pond 2 in	3.9	21.6	6.2	4.8	6	10.5	53.1
	pond 2 out	0.7	3.3	2.2	1.8	0.7	2.3	11.0
	pond 4 out	nd	nd	nd	nd	nd	nd	nd
25-Mar	pond 1 in	3.7	22.8	5.4	3.2	7.4	9.7	52.2
	pond 2 in	2.9	20.3	4.2	3.2	6.1	10.3	47.1
	pond 2 out	0.5	2.4	1.5	1	0.5	4.3	10.2
	pond 4 out		nd	nd	nd	nd	nd	nd
26-Mar	pond 1 in	5.4	21.9	6.6	3.5	4.9	8.2	50.5
	pond 2 in	3	19.8	4.7	3.1	3.9	8.02	42.5
	pond 2 out	0.4	2.1	1.6	1.1	0.6	3.3	9.1
	pond 4 out		nd	nd	nd	nd	nd	nd

nd = not detected

B.2 SECTION 4: BIOREACTOR STUDY DATA

Stage 1

		30-Apr	2-May	5-May	9-May	Average
Day		4	6	9	13	
Reactor volume	(L)	4.7	4.7	4.7	4.7	4.7
Flow rate	(L/d)	10.07	10.1	10.08	10.1	10.09
HRT	(d)	0.47	0.46	0.47	0.46	0.47
u	(1/d)	2.14	2.15	2.14	2.15	2.15
Feed COD	(mg/L)	357.8	480.2	477.4	413.1	432.1
Mixed liquor CODs	(mg/L)	264	236.2	236.7	229.6	241.6
Effluent CODs	(mg/L)	269.2	227.6	241.8	229.6	242.0
Mixed liquor CODt	(mg/L)	330.4	279	293.1	284.3	296.7
Effluent CODt	(mg/L)	323.9	279	289.2	288.5	295.15
CODs removed	(mg/L)	88.6	252.55	235.6	183.5	190.1
% CODs removed		24.8	52.6	49.4	44.4	42.8
Mixed liquor TSS	(mg/L)	46.98	46.08	57.54	47.73	49.58
Effluent TSS	(mg/L)	46.98	47.88	57.54	53.58	51.49
Mixed liquor VSS	(mg/L)	47.30	42.73	50.11	43.26	45.85
Effluent VSS	(mg/L)	47.30	45.06	50.11	48.48	47.74
Observed yield	(mg eff VSS/mg COD)	0.53?	0.18	0.21	0.26	0..30
	(mg eff TSS/mg COD)	0.53?	0.18	0.24	0.26	0.30
Carbon yield	(mg TSS/mgTOC)	1.92	0.55	nm	0.88	1.12
OUR	(mg/L.hr)	8.91	6.43	11.65	9.84	9.2075
SOUR	(mg/mg reactor TSS.hr)	0.19	0.13	0.20	0.18	0.18
Feed TOC	(mg/L)	124.9	176.1	155.5	154.4	152.7
Mixed liquor TOCs	(mg/L)	99.8	91.1	nm	93.8	94.9
Effluent TOCs	(mg/L)	100.4	89.0	nm	93.7	94.3
TOCs removed	(mg/L)	24.5	87.1	nm	60.7	57.4
% TOCs removed		19.6	49.5	nm	39.3	36.1
Feed VFAs	(mg/L)		49.0	48.5	46.3	47.9
Effluent VFAs	(mg/L)	nd	nd	nd	nd	nd

Stage 1 (continued)

		30-Apr	2-May	5-May	9-May	Average
Day		4	6	9	13	
Feed TKN	(mg/L)	0.7	0.8	0.8	0.6	0.7
Effluent TKNt	(mg/L)	4.3	3.9	3.1	3.6	3.7
Effluent TKNs	(mg/L)	0.7	0.6	0.7	0.5	0.6
N requirement	(mg/L)	3.6	3.3	2.4	3.1	3.1
N content of biomass	(mg N/mg TSS)	0.077	0.069	0.042	0.058	0.061
Ratio Nfix: total N uptake		1	0.94	0.96	0.97	0.97
N_2 fixation rate	(mg/d)	36.25	31.31	23.184	30.3	30.26
N_2 fixation rate	(mg/L/d)	7.71	6.66	4.93	6.45	6.44
N_2 fixation rate	(mg/mg/d)	0.164	0.139	0.086	0.120	0.127
N_2 fixation rate	(nmol/day)	1.29	1.12	0.828	1.08	1.08
N_2 fixation rate	(nmol/mL/hr)	11.48	9.91	7.34	9.59	9.58
N_2 fixation rate	(nmol/mg/hr)	244.31	207.05	127.57	179.05	189.50
Feed TOXN	(mg/L)	0.035	0.039	0.034	0.037	0.0362
Effluent TOXNs	(mg/L)	0.027	0.039	0.028	0.026	0.03
Feed TP	(mg/L)	4.2	3.8	7.1	3.7	4.7
Effluent TP	(mg/L)	3.7	3.8	4	3.8	3.82
Effluent TPs	(mg/L)	3.6	3.5	3.7	3.4	3.6
P content of biomass	(mg P/mg TSS)	0.002	0.006	0.005	0.007	0.005
Feed DRP	(mg/L)	4	3.4	6.4	3.7	4.38
Effluent DRPs	(mg/L)	3.5	3.6	3.7	3.5	3.58
Cell count - cream	(CFU/ mL)	$9.5x10^4$	$1.1x10^6$	$3.0x10^5$	$7.7x10^5$	$5.6x10^5$
-transparent	(CFU/ mL)	$3.3x10^6$	$5.3x10^6$	$1.6x10^6$	$6.1x10^6$	$4.1x10^6$

nm = not measured nd = not detected

Stage 2

		13-May	15-May	21-May	Average
Day		17	19	25	
Reactor volume	(L)	4.65	4.65	4.65	4.65
Flow rate	(L/d)	10.18	10.04	10.1	10.12
HRT	(d)	0.46	0.46	0.46	0.46
u	(1/d)	2.19	2.16	2.17	2.17
Feed COD	(mg/L)	820.2	858.3	840.7	839.7
Mixed liquor CODs	(mg/L)	235	224.7	238.5	232.7
Effluent CODs	(mg/L)	229.8	226.5	231.6	229.3
Mixed liquor CODt	(mg/L)	380.6	329.3	362.5	357.5
Effluent CODt	(mg/L)	362.3	376.3	414.8	384.5
CODs removed	(mg/L)	590.4	631.8	609.1	610.4
% CODs removed		72.0	73.6	72.4	72.7
Mixed liquor TSS	(mg/L)	121.37	105.89	98.67	108.64
Effluent TSS	(mg/L)	129	107.24	99.5	111.91
Mixed liquor VSS	(mg/L)	110.33	90.00	89.17	96.50
Effluent VSS	(mg/L)	115.26	88.72	89.10	97.69
Observed yield	(mg eff VSS/mg COD)	0..20	0.14	0.15	0.16
	(mg eff TSS/mg COD)	0.21	0.17	0.16	0.18
Carbon yield	(mg TSS/mgTOC)	0.65	0.53	0.50	0.56
OUR	(mg/L/hr)	47.75	35.14	36.05	39.65
SOUR	(mg/mg reactor TSS/hr)	0.370	0.328	0.362	0.353
Feed TOC	(mg/L)	315.7	313.7	313.3	314.2
Mixed liquor TOCs	(mg/L)	118.4	116.1	114.5	116.3
Effluent TOCs	(mg/L)	117.7	112.8	112.5	114.3
TOCs removed	(mg/L)	198	200.9	200.8	199.9
% TOCs removed		62.7	64.0	64.1	63.6
Feed VFAs	(mg/L)	378.02	441.58	395.7	405.1

Stage 2 (continued)

		13-May	15-May	21-May	Average
Day		17	19	25	
Feed TKN	(mg/L)	0.7	0.8	0.6	0.7
Effluent TKNt	(mg/L)	8.9	8.5	7.6	8.3
Effluent TKNs	(mg/L)	0.7	0.6	0.6	0.63
N requirement	(mg/L)	8.2	7.9	7	7.7
N content of biomass	(mg N/mg TSS)	0.064	0.074	0.070	0.069
Ratio Nfix: total N uptake		1	0.975	1	0.99
N_2 fixation rate	(mg/d)	83.48	77.31	70.7	77.16
N_2 fixation rate	(mg/L/d)	17.95	16.62	15.20	16.59
N_2 fixation rate	(mg/mg/d)	0.139	0.155	0.153	0.149
N_2 fixation rate	(nmol/day)	2.98	2.76	2.52	2.76
N_2 fixation rate	(nmol/mL/hr)	26.43	24.48	22.38	24.43
N_2 fixation rate	(nmol/mg/hr)	204.88	228.25	224.97	219.37
Feed TOXN	(mg/L)	0.039	0.042	0.041	0.041
Effluent TOXNs	(mg/L)	0.028	0.025	0.024	0.026
Feed TP	(mg/L)	6.8	7.1	7.4	7.1
Effluent TP	(mg/L)	7.3	8.2	7.6	7.7
Effluent TPs	(mg/L)	6.3	6.7	6.4	6.5
P content of biomass	(mg P/mg TSS)	0.008	0.014	0.012	0.011
Feed DRP	(mg/L)	6.9	7	7.4	7.1
Effluent DRPs	(mg/L)	6.6	6.6	6.7	6.6
Cell count - cream	(CFU/mL)	$8.7x10^6$	$9.4x10^6$	$2.3x10^7$	$1.4x10^7$
-transparent	(CFU/mL)	$>3x10^{10}$	$>3x10^{10}$	$>3x10^{10}$	$>3x10^7$

nd = not detected nm = not measured

Stage 3

		12-Jun	17-Jun	19-Jun	22-Jun	Average
Day		47	52	54	57	
Reactor volume	(L)	4.7	4.7	4.7	4.7	4.7
Flow rate	(L/d)	9.54	9.54	9.53	9.54	9.54
HRT	(d)	0.49	0.49	0.49	0.49	0.49
u	(1/d)	2.03	2.03	2.03	2.03	2.03
Feed COD	(mg/L)	nm	639.9	647.1	605.3	630.8
Mixed liquor CODs	(mg/L)	250.5	233.5	233.3	224.5	235.45
Effluent CODs	(mg/L)	247.2	236	220.4	216.7	230.1
Mixed liquor CODt	(mg/L)	338.5	332.5	333.1	313.2	329.3
Effluent CODt	(mg/L)	346.15	325.7	333.1	324	332.2
CODs removed	(mg/L)	nm	403.9	426.7	388.6	406.4
% CODs removed		nm	63.12	65.94	64.20	64.42
Mixed liquor TSS	(mg/L)	73.85	86.34	89.63	74.5	81.08
Effluent TSS	(mg/L)	76.7	86.34	69.6	81.02	78.42
Mixed liquor VSS	(mg/L)	65.71	78.03	83.93	71.12	74.70
Effluent VSS	(mg/L)	70.89	78.45	61.29	77.01	71.91
Observed yield	(mg eff VSS/mg COD)	nm	0.19	0.14	0.20	0.18
	(mg eff TSS/mg COD)	nm	0.21	0.21	0.19	0.21
C0arbon yield	(mg TSS/mgTOC)	0.66	0.67	0.53	nm	0.62
OUR	(mg/L/hr)	23.04	21.42	21.66	nm	22.04
SOUR	(mg/mg reactor TSS/hr)	0.30	0.25	0.31	nm	0.29
Feed TOC	(mg/L)	196	219.7	218.7	nm	211.47
Mixed liquor TOCs	(mg/L)	84.13	91.9	90.42	nm	88.82
Effluent TOCs	(mg/L)	80.3	90.6	87.02	nm	85.97
TOCs removed	(mg/L)	115.7	129.1	131.68	nm	125.49
% TOCs removed		59.03	58.76	60.21	nm	59.33
Feed methanol	(mg/L)	178.2	194.4	222.8	nm	198.47
Effluent methanol	(mg/L)	0	0		m,	0.00

Stage 3 (continued)

		12-Jun	17-Jun	19-Jun	22-Jun	Average
Day		47	52	54	57	
Feed TKN	(mg/L)	0.58	0.64	0.58	0.69	0.62
Effluent TKNt	(mg/L)	6.6	6	4.8	6.8	6.05
Effluent TKNs	(mg/L)	0.67	0.41	0.42	0.38	0.47
N requirement	(mg/L)	5.93	5.59	4.38	6.42	5.58
N content of biomass	(mg N/mg TSS)	0.077	0.065	0.063	0.079	0.071
Ratio Nfix: total N uptake		1.02	0.96	0.96	0.95	0.97
N_2 fixation rate	(mg/d)	57.43	51.13	40.22	58.29	51.77
N_2 fixation rate	(mg/L/d)	12.22	10.88	8.56	12.40	11.01
N_2 fixation rate	(mg/mg/d)	0.16	0.13	0.12	0.15	0.14
N_2 fixation rate	(nmol/day)	2.05	1.83	1.44	2.08	1.85
N_2 fixation rate	(nmol/mL/hr)	18.18	16.19	12.73	18.46	16.39
N_2 fixation rate	(nmol/mg/hr)	237.07	187.51	182.95	227.79	208.83
Feed TOXN	(mg/L)	<0.2	<0.2	<0.2	<0.2	<0.2
Effluent TOXNs	(mg/L)	<0.2	<0.2	<0.2	<0.2	<0.2
Feed TP	(mg/L)	3.8	3.7	3.9	3.4	3.70
Effluent TP	(mg/L)	3.8	3.5	3.3	3.5	3.53
Effluent TPs	(mg/L)	2.91	2.82	2.77	2.56	2.77
P content of biomass	(mg P/mg TSS)	0.012	0.008	0.008	0.012	0.010
Feed DRP	(mg/L)	3.4	3.5	3.7	3.2	3.45
Effluent DRPs	(mg/L)	2.92	2.76	2.83	2.58	2.77
Cell count - cream	(CFU/mL)	$2.0x10^5$	$2x10^5$	$3x10^5$	nm	$2.3x10^3$
-transparent	(CFU/mL)	$8x10^6$	$7x10^6$	$8x10^6$	nm	$7.7x10^7$

nm = not measured nd = not detected

B.3 STATISTICAL ANALYSIS (ANOVA) SECTION 4 DATA

Parameters are considered significantly different at the 95% level of confidence if the calculated F value is greater than F critical.

N-fixation rate (mg/mg/d)

Groups	*Count*	*Sum*	*Average*	*Variance*
Stage 1	4	0.509	0.127	0.001
Stage 3	4	0.561	0.140	0.000
Stage 2	3	0.454	0.151	0.000

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.001	2	0.000	0.876	0.453	4.459
Within Groups	0.004	8	0.001			
	5					
Total	0.005	10				
	5					

F < F crit. ∴ No significant difference between the means of the 3 different operating conditions for specific nitrogen fixation rates. ($\alpha = 0.05$)

COD removal (mg/L) / TSS (mg/L).d

Groups	*Count*	*Sum*	*Average*	*Variance*
Stage 1	4	31.517	7.88	9.254
Stage 2	3	36.037	12.0125	3.061
Stage 3	3	31.665	10.555	2.690

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	31.00	2	15.50	2.76	0.130	4.737
Within Groups	39.26	7	5.61			
Total	70.26	9				

$F < F$ crit. $\therefore$ No significant difference between the means of the 3 different operating conditions for specific COD removal rates. ($\alpha = 0.05$).

P content (mg P/ mg TSS)

Groups	*Count*	*Sum*	*Average*	*Variance*
Stage 1	4	0.021	0.005	0.000
Stage 3	4	0.039	0.010	0.000
Stage 2	3	0.034	0.011	0.000

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	7E-05	2	0.000	5.512	0.031	4.459

Within Groups	5E-05	8	0.000
Total	0.0001	10	

F > F crit. ∴ At 95% confidence interval there is a significant difference between the means of the 3 different operating conditions for P content of the biomass.

Observed yields (mg eff TSS/ mg COD)

Groups	*Count*	*Sum*	*Average*	*Variance*
Stage 1	3	0.687	0.229	0.002
Stage 2	3	0.535	0.178	0.001
Stage 3	3	0.616	0.205	0.000

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.004	2	0.002	2.409	0.171	5.143
Within Groups	0.005	6	0.001			
Total	0.009	8				

F < F crit. ∴ No significant difference between the means of the 3 different operating conditions for the observed

yield. ($\alpha = 0.05$).

Specific OUR (mg/mg TSS/hr)

Groups	*Count*	*Sum*	*Average*	*Variance*
Stage 1	3	0.520	0.173	0.001
Stage 2	3	1.060	0.353	0.001
Stage 3	3	0.860	0.287	0.001

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.050	2	0.025	25.757	0.001	5.143
Within Groups	0.006	6	0.001			
Total	0.055	8				

1. F > F crit. ∴ At 95% confidence interval there is a significant difference between the means of the 3 different operating conditions for oxygen uptake rates.