Oxygen Management for Optimisation of Nitrogen Removal in a Sequencing Batch Reactor

Katie Third

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Division of Science and Engineering

School of Biological Sciences and Biotechnology

Murdoch University, Western Australia

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I hereby declare that this thesis is my own account of my research and contains as its main content work that has not previously been submitted for a degree at any university

Katie Third

To my family

Contents

Project Summary

In today's progressively urbanised society, there is an increasing need for cost-effective, environmentally sound technologies for the removal of nutrients (carbon, phosphorous, nitrogen) from polluted water. Nitrogen removal from wastewater is the focus of this thesis. Conventional nitrogen removal requires the two processes of aerobic nitrification followed by anoxic denitrification, which is driven by remaining reducing power. While most wastewaters contain a significant fraction of reducing power in the form of organic substrate, it is difficult to preserve the reducing power required for denitrification, due to the necessary preceding aerobic oxidation step. Consequently, one of the major limitations to complete N-removal in traditional wastewater treatment systems is the shortage of organic carbon substrate for the reduction of oxidised nitrogen (NO_2^-, NO_3^-), produced from nitrification. This thesis followed two main research themes that aimed to address the problem of organic carbon limitation in nitrogen removal from wastewater, by management of the oxygen supply. The first theme was the study of N-removal by simultaneous nitrification and denitrification (SND) in the novel reactor type, the sequencing batch reactor (SBR). It was aimed to increase understanding of PHB metabolism and the limiting factors of SND and then to develop a suitable on-line control strategy to manage the oxygen supply and optimise nitrogen removal by SND. The second main research theme was the application of the CANON (Completely Autotrophic Nitrogen-removal Over Nitrite) process for nitrogen removal from wastewater; a novel process that requires minimal oxygen supply and has the potential to completely circumvent the requirement for organic substrate in nitrogen removal because it is catalysed by autotrophic microorganisms – Anammox (anaerobic ammonium oxidisers) and aerobic nitrifiers.

For study of the SND process, a completely automated 2 L sequencing batch reactor was developed with on-line monitoring of the dissolved oxygen concentration, pH and oxidation-reduction (ORP) potential. The SBR was operated continuously for up to 2 years and, due to its separation of different phases by time, enabled the study and optimisation of different microbial activities, including acetate uptake and conversion to PHB (feast phase), PHB hydrolysis and consumption (famine phase), nitrification and denitrification (and SND). All experimental work was performed using a mixed culture

and acetate as the organic substrate. Acetate consumption and PHB production was studied under different oxygen supply rates to establish conditions that allow maximum conversion of acetate to PHB during the feast phase. Lower DO supply rates (k_La 6 – 16 h^{-1}) resulted in preservation of a higher proportion of acetate as PHB than at higher DO supply rates (k_La 30 and 51 h^{-1}). Up to 77 % of the reducing equivalents available from acetate were converted to PHB under O_2-limitation, as opposed to only 54 % under O_2-excess conditions, where a higher fraction of acetate was used for biomass growth. A metabolic model based on biochemical stoichiometry was developed that could reproduce the trends of the effect of oxygen on PHB production. Experimental findings and simulated results highlighted the importance of oxygen control during the feast phase of an SBR in preserving reducing power as PHB.

To develop an oxygen management strategy for the aerobic famine phase, the effect of the dissolved oxygen (DO) concentration on SND, using PHB as the electron donor, was investigated. There was a clear compromise between the rate and the percentage of SND achieved at different DO concentrations. A DO setpoint of 1 mg L^{-1} was optimal for both the percentage of SND (61 %) and rate of SND (4.4 mmol N. Cmol X^{-1}. h^{-1}). Electron flux analysis showed that most SND activity occurred during the first hour of the aerobic famine period, when the oxygen uptake rate (due to NH_4^+ and PHB oxidation) was highest. Aerated denitrification ceased as soon as NH_4^+ was depleted. The presence of NH_4^+ provided an oxygen "shield", preventing excessive penetration of oxygen into the flocs and creating larger anoxic zones for SND. PHB degradation was first order with respect to the biomass PHB concentration ($df_{PHB}/dt = 0.19 \cdot f_{PHB}$). The slow nature of PHB degradation made it a suitable substrate for SND, as it was degraded at a similar rate to ammonium oxidation.

While DO control during the aerobic famine phase could increase nitrogen removal via SND, total N-removal in the SBR was still limited by the availability of reducing power (PHB) in the anoxic phase. The length of the aerobic phase needed to be minimised to prevent over-oxidation of PHB after NH_4^+ depletion. The specific oxygen uptake rate (SOUR) was found to be an effective on-line parameter that could reproducibly detect the end-point of nitrification. A simple method was developed for continuous, on-line measurement of the SOUR, which was used for automated adjustment of the aerobic phase length. Minimisation of the aerobic phase length by feedback control of the

SOUR improved nitrogen removal from 69 % (without phase length control) to 86 %, during one cycle. The SOUR-aeration control technique could successfully adapt the aerobic phase length to varying wastewater types and strengths and to varying aeration conditions. The medium- and long-term effects of oxygen management on nitrogen removal was investigated by operating the SBR continuously for up to one month using DO control throughout all stages of the SBR, i.e. oxygen-limitation during the feast phase, a DO setpoint of 1 mg L^{-1} during the famine phase and SOUR-controlled aerobic phase length. Complete oxygen management resulted in minimisation of the amount of PHB that was oxidised aerobically in each SBR cycle and caused an accumulation of cellular PHB over time. The increased availability of PHB during aeration resulted in a higher SOUR and increased N-removal by SND from 34 to 54 %. After one month of continuous SBR operation, the settling efficiency of the biomass improved from 110 mL . g^{-1}X to less than 70 mL . g^{-1}X and almost complete N-removal (97 %) was achieved via SND during aeration, however at a reduced rate (1.5 mmol Cmol X^{-1} h^{-1}). Therefore, long-term oxygen-management resulted in biomass with improved settling characteristics and a higher capacity for SND. Results of the first main research theme highlighted the importance of aeration control throughout all stages of the SBR for maximum N-removal via SND.

The CANON process was investigated as an alternative to the use of conventional activated sludge for treatment of wastewaters limited by organic carbon substrate. The initial study of the CANON process was performed at the Kluyver Laboratory in Delft, the Netherlands, using an already established Anammox enrichment culture. The effect of extended periods of NH_4^+-limitation on the CANON microbial populations was studied, to examine their ability to recover from major disturbances in feed composition. The CANON process was stable for long periods of time until the N-loading rate reached below 0.1 kg N m^{-3} day^{-1}, when a third population of bacteria developed in the system (aerobic nitrite oxidisers), resulting in a decrease in N-removal from 92 % to 57 %. Nitrite oxidisers developed due to increased levels of oxygen and nitrite. This highlighted the requirement for oxygen control during the CANON process to prevent increased DO levels and growth of undesired microbes. To initiate the CANON process from a local source, Anammox was enriched from local activated sludge (Perth, Western Australia). FISH analysis (fluorescence in situ hybridisation) of the enriched Anammox strain showed that it belonged to the Order *Planctomycetales*,

the same as all other identified Anammox strains, but represented a new species of Anammox. The enrichment culture was not inhibited by repeated exposure to oxygen, allowing initiation of an intermittently-aerated CANON process to achieve sustained, completely autotrophic ammonium removal (0.08 kg N m^{-3} day^{-1}) for an extended period of time, without any addition of organic carbon substrate. Dissolved oxygen control played a critical role in achieving alternating aerobic and anaerobic ammonium oxidation.

The main conclusion drawn from the study is the important role of oxygen management in achieving improved nitrogen removal. A careful oxygen management strategy can minimise wastage of reducing power to improve PHB-driven SND by activated sludge and can prevent major disturbances to the population balance in the CANON system. Oxygen management has the potential to reduce aeration costs while significantly improving nitrogen removal from wastewaters limited by organic carbon.

Nomenclature

Ac	= Acetate concentration (Cmol. L^{-1})
AcCoA	= Acetyl-CoA concentration (Cmol. L^{-1})
$\% Ac_{PHB}$	= Percentage of reducing equivalents from acetate preserved as PHB
c_L	= Steady state oxygen concentration in liquid phase (mg. L^{-1})
c_S	= Saturation oxygen concentration in the gas phase (mg. L^{-1})
COD	= Chemical Oxygen Demand (mg. L^{-1})
CoA	= Free Coenzyme A concentration (mol. L^{-1})
DO	= Dissolved oxygen (mg. L^{-1})
Feast phase	= Period where acetate is available as electron donor
Famine phase	= Period where PHB is the sole electron donor available
f_{AirOn}	= Air On Time Fraction
F:M ratio	= Food to microorganism ratio
f_{PHB}	= Fraction of PHB of active biomass (Cmol PHB/Cmol active X)
f_{PHB}^{max}	= Maximum PHB-saturation capacity (Cmol PHB/Cmol active X)
F_{PHB}	= Fraction of PHB of total biomass (Cmol PHB/Cmol total X)
k_La	= Oxygen transfer coefficient (h^{-1})
k_La_{full}	= Oxygen transfer coefficient (h^{-1}) for uninterrupted airflow
k_La_{int}	= Oxygen transfer coefficient (h^{-1}) during intermittent air supply
$k_{S,O2}$	= Half saturation constant for oxygen (mg. L^{-1})
$k_{S,O2(Het)}$	= Half saturation constant for O_2 of heterotrophic biomass (mg. L^{-1})
$k_{S,O2(Aut)}$	= Half saturation constant for O_2 of autotrophic biomass (mg. L^{-1})
$k_{S,NH4+}$	= Half saturation constant for ammonium (mol. L^{-1})
$k_{S,Ac}$	= Half saturation constant for acetate (Cmol. L^{-1})
MWtPHB	= Molecular weight of PHB (21.5 g $Cmol^{-1}$)
MWtX	= Molecular weight of biomass ($CH_{1.8}O_{0.5}N_{0.2}$) (24.6 g $Cmol^{-1}$)
NO_x^-	= Oxidised nitrogen, representing both nitrate (NO_3^-) and nitrite (NO_2^-)
PHA	= Polyhyroxyalkanoate
PHB	= Poly-β-hydroxybutyrate (Cmol. L^{-1})
OUR	= Oxygen uptake rate (mg. L^{-1}. h^{-1})
OTR	= Oxygen transfer rate (mg. L^{-1}. h^{-1})
R_{Ac}	= Rate of acetate consumption (Cmol. L^{-1}. h^{-1})

$R_{Ac(COD)}$ = Rate of acetate consumption (mg COD L^{-1} h^{-1})

$R_{PHB.Pr}$ = Rate of PHB production (Cmol. L^{-1}. h^{-1})

$R_{PHB.De}$ = Rate of PHB degradation (Cmol. L^{-1}. h^{-1})

R_{NH4^+} = Rate of ammonium oxidation (mol NH_4^+. L^{-1}. h^{-1})

R_{NO3^-} = Rate of nitrate production (mol NO_3^-. L^{-1}. h^{-1})

$R_{NO3^-(SND)}$ = Rate of simultaneous denitrification (mol NO_3^-. L^{-1}. h^{-1})

R_X = Rate of biomass production (Cmol X. L^{-1}. h^{-1})

R_{TCA} = Rate of NADH production by the TCA cycle (mol NADH. L^{-1}. h^{-1})

R_{O_2} = Rate of oxygen uptake (mol O_2.. L^{-1}. h^{-1})

$R_{e^- ->NO3-}$ = Rate of electron flow from PHB to nitrate (mol electrons. L^{-1}. h^{-1})

$R_{e^- ->O2}$ = Rate of electron flow from PHB to oxygen (mol electrons. L^{-1}. h^{-1})

r_{Ac} = Specific acetate consumption rate (Cmol Ac. Cmol X^{-1}. h^{-1})

r_{O2} = Specific oxygen consumption rate (mol. Cmol X^{-1}. h^{-1})

r_{PHB} = Specific PHB production rate (Cmol PHB. Cmol X^{-1}. h^{-1})

r_X = Specific growth rate (Cmol X. Cmol X^{-1}. h^{-1})

$r_{Ac}^{\max}$ = Maximum specific acetate consumption rate (Cmol. Cmol X^{-1}. h^{-1})

$r_{O_2}^{\max}$ = Maximum specific oxygen uptake rate (mol. Cmol X^{-1}. h^{-1})

$r_{PHB.De}^{\max}$ = Maximum specific PHB degradation rate (Cmol. Cmol X^{-1}. h^{-1})

$r_{PHB.\mathrm{Pr}}^{\max}$ = Maximum specific PHB production rate (Cmol. Cmol X^{-1}. h^{-1})

$r_{TCA}^{\max}$ = Maximum specific NADH production rate (mol. Cmol X^{-1}. h^{-1})

$r_X^{\max}$ = Maximum biomass production (growth) rate (Cmol. Cmol X^{-1}. h^{-1})

$r_{X.fe}^{\max}$ = Maximum specific growth rate in feast period (Cmol X. Cmol X^{-1}. h^{-1})

$r_{X.fam}^{\max}$ = Maximum specific growth rate in famine period (Cmol. Cmol X^{-1}. h^{-1})

S = Substrate (Cmol. L^{-1})

SBR = Sequencing Batch Reactor

SOUR = Specific oxygen uptake rate (mg $g^{-1}X$ h^{-1})

SRT = Sludge retention time (days)

SVI = Sludge Volume Index (mL $g^{-1}X$)

VFA = Volatile fatty acid

WWTP = Wastewater treatment plant

X	= Biomass concentration (g L^{-1})
$Y_{PHB/Ac}$	= Yield of PHB from acetate (Cmol/Cmol)
Y_{OBS}	= Observed yield (Cmol/Cmol)
$Y_{X/Ac}$	= Yield of biomass from acetate (Cmol/Cmol)

CHAPTER 1
Literature Review

1.1 Wastewater Treatment and The Activated Sludge Process

In our increasingly urbanised and industrialised society with a rapidly expanding world population, the need for cost-effective and environmentally sound technologies for wastewater treatment is obvious. Concern over management of water resources and increasingly stringent nutrient levels in discharged waters, set by government regulating bodies, make efficient wastewater treatment essential. The wastewater industry is constantly improving as our understanding of the microorganisms involved in nutrient removal evolves.

Activated sludge is at present the most widely used biological treatment process for both domestic and industrial wastewaters. The term "activated sludge" refers to a biological process that utilises a mixed community of miroorganisms that metabolise and transform organic and inorganic substances into environmentally acceptable forms. The typical microbiology of activated sludge consists of approximately 95 % bacteria and 5 % higher organisms such as protozoa, metazoa, fungi, rotifers etc (Irvine et al., 1997, Seviour and Blackall, 1999). The three primary nutrients that need to be removed to prevent deterioration of water bodies are carbon, nitrogen and phosphorous. Carbon is not considered difficult to remove biologically. On the contrary, one of the most significant problems with treatment of many wastewaters is a lack of organic carbon, as the removal of both nitrogen and phosphorous involve heterotrophic conversions, requiring an electron donor. Thus, treatment plants treating wastewaters containing low COD:N or COD:P ratios experience difficulties in removing residual nitrogen and phosphorous due to a shortage of organic substrate (Pitman, 1991, Zeghal et al., 1997b). As a consequence, research into control strategies to conserve organic substrate has gained attention in recent years and is the subject of research in this thesis (Wouterswasiak et al., 1994, Yu et al., 1997, Ra et al., 1998, Zhao et al., 1999, Yu et al., 2000).

1.1.1 Continuous Systems for Nitrogen Removal

Complete conventional nitrogen removal requires two biological processes: autotrophic nitrification, for oxidation of ammonium to nitrite or nitrate (Eqs 1, 2) and denitrification – a multi-step, heterotrophic process in which the oxidised nitrogen produced from nitrification (e.g. NO_2^-, NO_3^-) is reduced to dinitrogen gas (Eqs 3 – 7). The traditional view considered the two processes to be mutually exclusive, as nitrification requires oxygen and denitrification requires the absence of oxygen. The opposing oxygen requirements implied the need for strict separation (Munch et al., 1996, Hippen et al., 1997). Based on this assumption, conventional wastewater treatment plants are continuous flow systems (or plug-flow) that separate nitrification and denitrification by space.

Nitrification (Aerobic)

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + 2\ H_2O \quad \text{(Eq 1)}$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad \text{(Eq 2)}$$

Denitrification (Anaerobic) – using acetate as the electron donor

$$8\ NO_{3\,(aq)}^- + 2\ CH_3COOH \rightarrow 8\ NO_{2\,(aq)}^- + 4\ CO_2 + 4\ H_2O \quad \text{(Eq 3)}$$

$$8\ NO_{2\,(aq)}^- + CH_3COOH + 2\ H_2O \rightarrow 8\ NO_{(g)} + 2\ CO_2 + 8\ OH^- \quad \text{(Eq 4)}$$

$$8\ NO_{(g)} + CH_3COOH \rightarrow 4\ N_2O_{(g)} + 2\ CO_2 + 2\ H_2O \quad \text{(Eq 5)}$$

$$4\ N_2O_{(g)} + CH_3COOH \rightarrow 4\ N_{2(g)} + 2\ CO_2 + 2\ H_2O \quad \text{(Eq 6)}$$

$$8\ NO_{3\,(aq)}^- + 5\ CH_3COOH \rightarrow 4\ N_{2(g)} + 10\ CO_2 + 6\ H_2O + 8\ OH^- \quad \text{(Eq 7)}$$

The wastewater influent is generally mixed with returned activated sludge (RAS) as it enters the treatment reactor (Fig 1.1). There are several variations on the plug-flow theme, depending on the treatment objectives. For example, processes such as Biodenitro (Thornberg, 1993), Biodenipho, (Isaacs, 1997), the UCT (University of Cape Town) process (Ekama and Wentzel, 1997) and Bardenpho (Stevens et al., 1999) have been developed based on continuous design and involve different set-ups for achieving nitrification and/or aerobic-anoxic phosphorous removal. For continuous systems aimed primarily at removing nitrogen, the first section of the tank is often aerated, where the wastewater undergoes nitrification and carbon substrate oxidation as it moves along. High dissolved oxygen concentrations are typically applied to the aeration tank (> 2 mg L^{-1}) to achieve both ammonium oxidation and carbon oxidation (Spies and

Seyfried, 1988, Holmberg et al., 1989, Johansen et al., 1997, Ferrer et al., 1998). Nitrification followed by denitrification requires careful control because denitrification requires reducing equivalents that do occur in wastewater, but are difficult to preserve through the necessary preceding nitrification step.

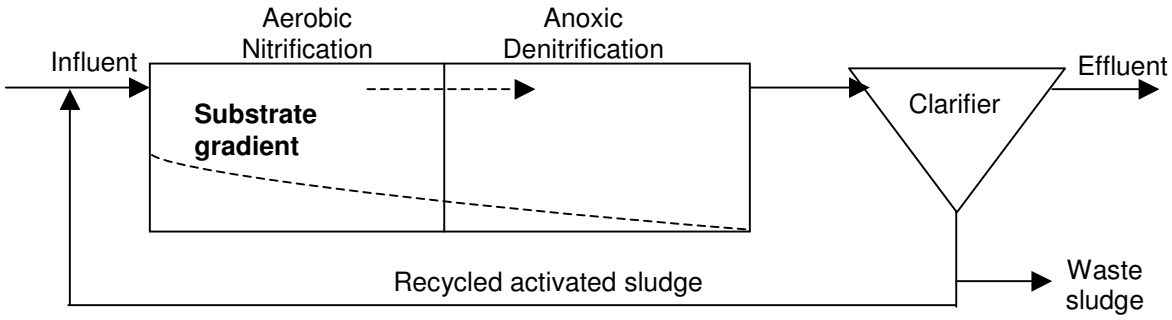

Figure 1.1: A simplified diagram of a conventional layout for a plug-flow wastewater treatment plant for the removal of nitrogen and carbon, showing the decreasing substrate concentration over the length of the reactor.

The wastewater is then pumped to an anoxic zone where denitrification is expected to occur. However, as mentioned above, the carbon in the wastewater is often already oxidised and a lack of reducing power prevents complete denitrification. This represents one shortcoming of conventional continuous systems. Strategies to overcome the lack of organic carbon in these systems have included recycling of nitrified waters back into contact with the incoming wastewater (anoxic feeding or predenitrification, Fig 1.2), or varying the feed point to bring organic-rich wastewater into contact with the nitrified water towards the end of the process (Jones et al., 1990a).

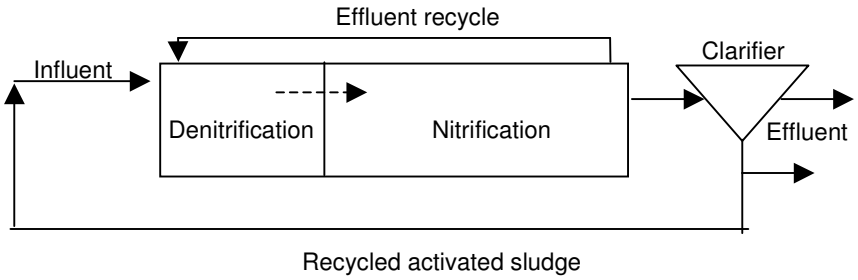

Figure 1.2: Simplified diagram of a predenitrification plug-flow treatment system aimed at efficiently using the reducing equivalents in the wastewater.

When organic carbon is lacking the controlled addition of methanol, acetate or propionate to the anoxic phase can result in significantly faster denitrification rates (Isaacs et al., 1994, Tam et al., 1994, Isaacs and Henze, 1995, Zeghal et al., 1997a, Zeghal et al., 1997b). However, these techniques are both costly and complicated. The use of predenitrification (Fig 1.2) is successful yet requires significant effluent recycling which is not only costly but also reduces the hydraulic capacity of the plant. At the end of the process a settling tank, or clarifier, separates the activated sludge from the treated water. Some activated sludge is wasted and the remaining concentrated sludge is returned to the start of the process.

In plug-flow systems the concentration of nutrients varies over the length of the reactor (Fig 1.1). Despite this, such systems are often designed with the potentially unrealistic expectation that they can be operated as steady state systems (Irvine et al., 1997). The desire for steady state operation was conventionally based on the idea that steady conditions are needed for effluent concentrations to be kept constant at permitted limits. Accordingly, facilities are often designed to dampen the impact of the system's unsteady character, such as flow equalisation tanks (Irvine and Ketchum, 1988). Due to their extended operation at a reduced substrate tension, conventional systems have a tendency to develop "bulking sludge", the term given to sludge with inefficient settling characteristics. Bulking sludge is caused by filamentous organisms that predominate at low substrate to microorganism ratios, or F/M ratios (Chudoba et al., 1973, Van den Eynde et al., 1984, Casey et al., 1994, Majone et al., 1996, Van Loosdrecht et al., 1997, Beccari et al., 1998, Spector, 1998, Seviour and Blackall, 1999). Bulking sludge is a serious problem for treatment plants as they rely on the settling characteristics of the sludge to achieve separation of the biomass. Another drawback of continuous plug-flow treatment systems is their requirement for a long sludge age (> 15 days) to ensure all year round nitrification (Ekama and Wentzel, 1997). Such disadvantages have led engineers and researchers to search for new alternatives in recent years, such as dynamic systems like the sequencing batch reactor (Orhon et al., 1986, Jones et al., 1990a, Ng et al., 1993, Rusten and Eliassen, 1993, Muniz et al., 1994).

1.1.2. The Sequencing Batch Reactor

The sequencing batch reactor (SBR) is increasingly being considered the preferred wastewater treatment reactor system for improved nutrient removal. Unlike the

conventional "steady state", or plug flow reactor types that separate aerobic and anaerobic microbial reactions by space, the SBR is a dynamic system that separates its phases by time. All reactions, including biomass separation, are performed within a single reactor (Okada and Sudo, 1986, Orhon et al., 1986, Irvine and Ketchum, 1988, Rusten and Eliassen, 1993). Operationally, the SBR is extremely flexible in its ability to meet different treatment objectives while physically it is very simple (Ketchum, 1997). The SBR has a reduced need for chemical alkalinity control and has the potential to better utilise the carbon already present in the wastewater by controlling microbial growth (Newland, 1998, Yoo et al., 1999). The SBR operates the separate phases in sequence to achieve an overall "continuous" operation (Irvine and Ketchum, 1988) (Fig 1.3).

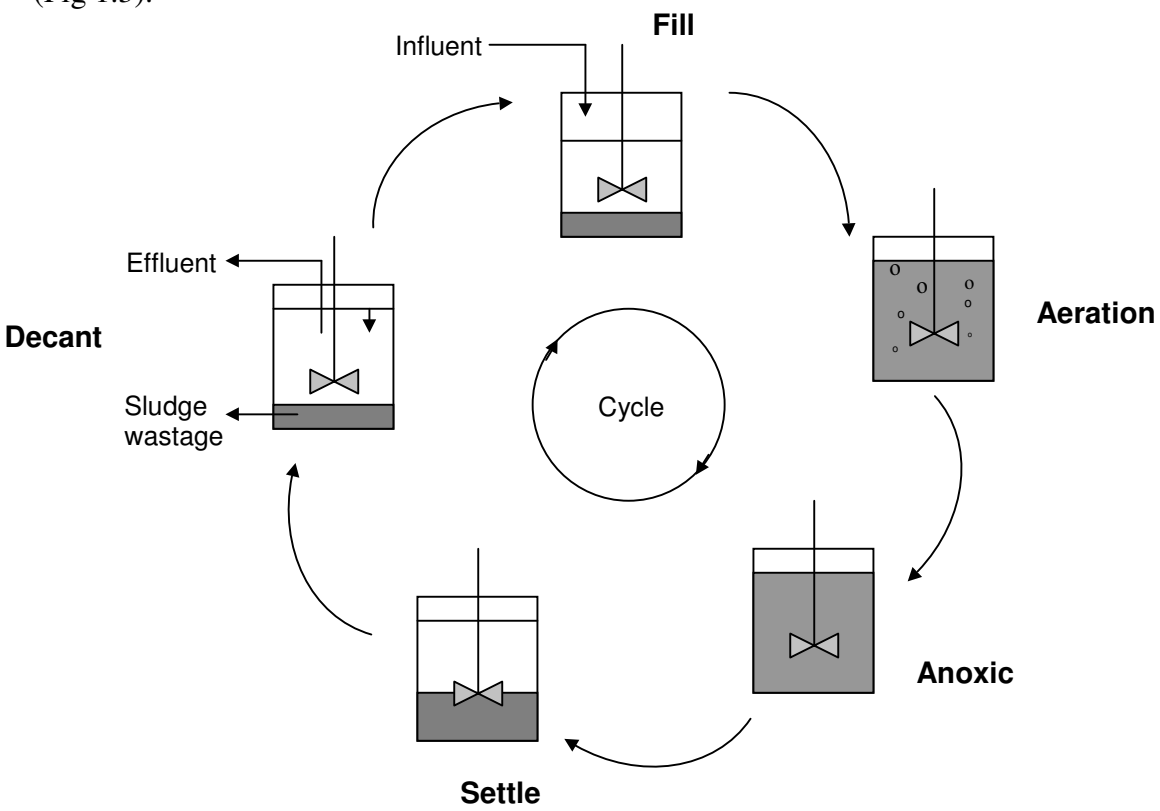

Figure 1.3: A typical SBR cycle for nitrogen removal demonstrating the sequence of the phases (adapted from Ketchum, 1997).

To fully achieve the potential advantages such a system as the SBR offers, a thorough understanding of the biological processes involved in a complex mixture of nitrifying and denitrifying bacteria is required. From a microbiological point of view, the key characteristic feature of SBR technology is the change between feast and famine during the reaction cycle, resulting in dynamic conditions (Van Loosdrecht et al., 1997).

Unlike continuous systems where microbes grow relatively continuously under substrate limitation, in the dynamic SBR the biomass undergoes periods of rapid growth and starvation, due to the fluctuating electron donor and acceptor concentrations. Microorganisms living in natural habitats have evolved effective strategies to cope with feast-famine conditions during evolution. One such survival strategy is the storage of soluble substrate as insoluble storage polymers such as polyphosphate and poly-β-hydroxybutyrate (*see* Section 1.3) (Fukase et al., 1985, Van Loosdrecht et al., 1997, Majone et al., 1999, Sudiana et al., 1999). Naturally fluctuating systems in which environmental parameters oscillate regularly work very well in terms of sustaining a rich, diverse and effective microbial population that can utilise even the lowest levels of nutrients and cope with changing conditions on various time levels (Chiesa et al., 1985, Stal, 1994, Irvine et al., 1997).

Figure 1.4: Two large-scale sequencing batch reactors treating domestic wastewater, operated in parallel (Busselton, Western Australia, ESI Ltd). The basin on the left was in the middle of the settle/decant phase, while the right basin was in the middle of the aeration phase. The decanting devices are visible at the far end of the basins.

1.2. Simultaneous Nitrification and Denitrification (SND)

Simultaneous (or concurrent) nitrification and denitrification implies that nitrification and denitrification occur concurrently in the same reaction vessel under identical operating conditions. SND can reduce or eliminate the need for separate tanks required in conventional treatment plants and consequently simplify the plant's design, saving

space and time (Keller et al., 1997, Sen and Dentel, 1998, Pochana et al., 1999, Tonkovic, 1999, Yoo et al., 1999, Zhao et al., 1999). Advantages of SND are that total nitrogen removal efficiencies achieved are similar to conventional nitrification-denitrification processes, without the need for an anoxic basin and a decrease in operational costs due to the low dissolved oxygen concentrations required (Collivignarelli and Bertanza, 1999). Many researchers have reported instances of SND under aerated conditions (Robertson et al., 1988, Albertson and Stensel, 1994, Gupta et al., 1994, Goronszy et al., 1996, Munch et al., 1996). However until recently, removal of nitrogen via SND has not typically been designed into full-scale wastewater treatment plants because the phenomenon has been perceived to be unpredictable (Tonkovic, 1999).

1.2.1. Explanation of SND

The explanations for SND can be classified into two categories – biological and physical (Munch et al., 1996). The biological explanation is that bacteria are more diversified than conventionally thought. Denitrifiers were originally assumed to be strictly anaerobic, but the existence of aerobic denitrifiers has been reported (Robertson et al., 1988, Van Loosdrecht and Jetten, 1998). For example, Kawakami et al. (1985) showed that in a pure culture of *Paracoccus denitrificans,* the enzyme nitrite reductase retained its activity through aerobiosis for up to 22 hours. Lloyd et al. (1987) monitored denitrification intermediates using on-line mass spectrometry and showed that a number of bacteria denitrify, even when the oxygen concentration approaches saturation. They concluded that aerobic denitrification, with the production of nitrogen gas or the intermediates NO and N_2O, may be as widespread and ecologically important as their anaerobic counterpart.

In addition to aerobic denitrification, it is becoming increasingly evident that a variety of heterotrophic bacteria can also contribute to nitrification (Sakai et al., 1997). Pure culture studies have shown that their activities are generally low, except under special conditions, where the process is thought to serve as an NADH-overflow mechanism (Robertson et al., 1988). Despite the low activities, Hall (1987) and Duggin et al. (1991) (cited in Sakai, 1997) proposed that the sum of their activities in the field might be comparable to those of the autotrophs because the amount of heterotrophic biomass is much larger than that of autotrophs. However until this stage this hypothesis remains

speculative, as there have been no conclusive studies to confirm a significant contribution to nitrogen removal by heterotrophic nitrifiers. Another novel group of microorganisms has been discovered in recent years that is capable of anaerobic ammonium oxidation (termed "Anammox"), which may contribute to ammonium removal at low DO concentrations (*see* section 1.4) (Mulder et al., 1995, Van De Graaf et al., 1995, Strous et al., 1997, Jetten et al., 1999, Strous et al., 1999).

In contrast to the biological explanation of SND, the physical explanation suggests that the opposing oxygen requirements of nitrification and denitrification are met by physical separation of zones within a floc. The hypothesis is that oxygen diffusion limitations within the floc create an anoxic micro-zone for heterotrophic denitrification to occur. The anoxic zone is not a discrete zone but rather a declining oxygen gradient from the surface to the centre of the floc (Munch et al., 1996). Until so far, there seems to be a general consensus in the literature that supports the dominance of the physical explanation of SND. It is likely that SND, as observed in existing plants, is a combination of both physical and biological mechanisms, yet the exact contribution of each mechanism is still unknown.

1.2.2. Factors Affecting SND

Since the recent observations of simultaneous nitrification and denitrification in large- and lab-scale reactors (Watanabe et al., 1992, Munch et al., 1996, Tonkovic, 1999), several studies have aimed at establishing criteria for optimising the process. There is a general agreement in the literature that the three most important factors affecting SND are the organic carbon source, the dissolved oxygen concentration and the floc size.

The Effect of Organic Carbon Substrate on SND

As heterotrophic denitrification requires reducing power in the form of organic substrate, it is not surprising that SND is enhanced by increased availability of organic carbon. According to Pochana and Keller (1999), complete denitrification can be achieved with a TCOD:TKN ratio of 7:1 and a ratio of 9:1 is required for both nitrogen and phosphorous removal. If the carbon to nitrogen ratio in the wastewater is lower than this, incomplete denitrification occurs. Pochana and Keller (1999) showed that the addition of an external carbon source to the aeration stage of their SBR resulted in over 90 % nitrate removal via SND, compared to a control reactor without external carbon

addition, which achieved only 47 % nitrate removal. Other studies have reported higher levels of SND with additional supply of organic carbon (Munch et al., 1996, Sen and Dentel, 1998, Chang and Tseng, 1999, Yoo et al., 1999, Zhao et al., 1999).

Without adding organic substrate, SND can be achieved by encouraging denitrifying bacteria to use intracellular stored substrates as the electron donor for denitrification. Operating strategies intended to promote substrate storage, such as the SBR, have the potential to reduce or eliminate the costly necessity for a supplemental carbon addition (Alleman and Irvine, 1980). While rates of denitrification using stored substrates are available, very few studies are available on the use of PHB during SND. Beun et al. (2001) showed that storage and degradation of PHB benefited the denitrification process and enabled SND during the aerobic phase. When no external carbon source was available the PHB was used as the electron donor for denitrification, provided that NO_3^- was the only electron acceptor available (i.e. if oxygen diffusion is limited by flocs). Using simulation results, they showed that SND was optimal at a dissolved oxygen concentration of 40 % saturation (3.2 mg L^{-1}), if sufficient stored substrate was available and granular biomass was used.

The Effect of Dissolved Oxygen on SND

Due to the contrasting DO requirements of the two nitrogen-removing reactions nitrification and denitrification, lowering the DO concentration in the bulk liquid phase clearly improves the denitrification process but slows down nitrification. Munch et al. (1996) proposed that for optimal nitrogen removal via SND, the rate of nitrification needs to equal the rate of denitrification and postulated this occurs at a DO concentration of 0.5 mg L^{-1}. Beun et al. (2001) showed that at a DO concentration of 4 mg L^{-1}, little nitrogen was removed via SND. When the DO was lowered to 1.6 mg L^{-1}, nitrification was slowed down slightly but 100 % of the nitrate formed by denitrification was denitrified. Pochana and Keller (1999) found that a DO concentration of 0.5 mg L^{-1} achieved SND (up to 95 %) at a moderate rate. Complete SND (i.e. 100 %) was achieved at a setpoint of 0.3 mg L^{-1}, but at a reduced rate. Painter (1977) studied denitrification by activated sludge at reduced DO concentrations and showed that at a DO concentration of 2 mg L^{-1}, the denitrification rate was only 10 % of the rate under strictly anaerobic conditions. At 0.2 mg L^{-1} DO the rate was 50 % of the anaerobic rate. He concluded that unless the immediate vicinity of a denitrifying cell is

devoid of oxygen, no denitrification will occur. It is difficult to compare studies that report different oxygen requirements for SND, because all studies are performed with differing biomass and floc sizes. While the amount of oxygen that an individual cell is exposed to is influenced by the dissolved oxygen concentration in the bulk liquid, it is more importantly influenced by the floc size (Goronszy et al., 1996, Oh and Silverstein, 1999, Pochana and Keller, 1999, Pochana et al., 1999).

The extent to which DO will penetrate into the floc depends on the thickness of the floc or biofilm (Watanabe et al., 1992), and also on other parameters such as the oxygen uptake rate of the bacteria. When the oxygen uptake rate is high, the depth of oxygen penetration will be less and simultaneous denitrification will be improved. Beun et al. (2001) showed that when ammonium was depleted, further denitrification did not occur. The authors concluded that denitrification probably stopped due to penetration of DO into the granules because nitrification was complete and the oxygen uptake rate decreased suddenly. Newland (1999) proposed that the amount of stored COD strongly influences the penetration of DO diffusion into the biological floc, since a high amount of COD will produce a high OUR and consequently an increased anoxic zone for simultaneous denitrification. It is clear from all the above studies that nitrification is decreased at low oxygen concentrations while denitrification is increased, but the extent of SND achieved depends on the separation between the aerobic and anoxic zones, which is in turn dependent on the floc size and the DO supply rate.

Operational experiences of SND at low DO concentrations

Several reports of SND occurring in large-scale treatment have appeared in recent years. The majority have been anecdotal observations when operating at reduced oxygen concentrations (Tonkovic, 1999). For example, during commissioning of the 10 ML day^{-1} capacity Lilydale treatment plant in Victoria, it was estimated that 45 % of the nitrate was denitrified in the aeration tanks at a DO concentration of 0.5 mg L^{-1} (Tonkovic and Nowak, 1999). During process optimisation at a treatment plant in Quakers Hill, NSW, it was found that when the DO was maintained between 0 – 2.5 mg L^{-1}, the total nitrogen removed doubled in comparison to trials where the DO was allowed to reach 6 mg L^{-1}. A review of large-scale treatment plant experiences of SND is available (Tonkovic, 1999). The common theme to all

of these reports is the increased potential for SND to occur in aeration basins when lower DO setpoints are applied.

Production of intermediates at low DO levels

While it has been established that low DO concentrations are important for achieving nitrogen removal via SND, some studies have reported increased levels of the toxic denitrification intermediates NO and N_2O when operating at low DO concentrations. These intermediates are greenhouse gases that are up to 300 times more potent than CO_2 (Hong et al., 1993). Von Schulthess et al. (1994) investigated the net production of denitrification intermediates NO and N_2O between DO concentrations of 0 to 4 mg O_2 L^{-1} and found that N_2O production is increased under conditions of high NO_2^- and oxygen. Fuerhacker et al. (2000) showed that NO emissions were especially high during denitrification conditions at low oxygen supply rates. Laboratory experiments by Hong et al. (1993) showed that N_2O production increased when the COD:N ratio decreased, suggesting lack of electron donor can also be the cause of incomplete denitrification. Thus, it is apparent that while an economic benefit may be achieved by encouraging SND in large-scale treatment plants, this may come at an environmental price. Several studies have investigated this subject further in order to understand ways in which the production of these gases can be reduced (Goreau et al., 1980, Jorgensen et al., 1984, Czepiel et al., 1995, Otte et al., 1996, Kester et al., 1997, Schonharting et al., 1998).

The Effect of Floc Size on SND

A study by Pochana and Keller (1999) aimed to systematically investigate the effect of decreasing floc size on SND rates by blending biomass and measuring the amount of SND achieved. Flocs that had an average diameter of 382 μm and 155 μm achieved 98.5 % and 26.3 % SND respectively, whereas the smaller flocs (77 μm) achieved only 4.3 % SND. The results of this study clearly showed that floc size dramatically affects the amount of SND achieved. Based on the knowledge that increased floc size or biofilm thickness enhances SND, several studies have looked at minimising oxygen diffusion by using biofilm systems.

Novel reactor types to improve SND by oxygen diffusion limitation

Sen and Dentel (1998) found up to 65 % nitrogen removal via SND in a fluidised bed reactor in which the biomass was immobilised on sand. The system provided high biomass retention and diffusion limitations due to the biofilm formation on the sand particles. Puznava et al. (2001) developed a biological aerated filter (BAF) for nitrogen removal based on SND. The process is completely aerated and relies on the depth of the biofilm to achieve anoxic zones for denitrification. When operating at a reduced oxygen tension of between 0.5 to 3 mg O_2 L^{-1} in a pilot scale BAF, they achieved the same final nitrogen level in the effluent (< 20 mg L^{-1} N) as their classical nitrification and denitrification BAF. The advantage of the SND process was that 50 % less air was required to achieve the same nitrogen removal.

Menoud et al. (1999) used porous, ring-type packing called Siporax^{TM} to demonstrate SND in a single plug-flow reactor. They found clear evidence that denitrification occurred inside the internal pore structure, where oxygen diffusion limitation was at its maximum. Chang and Tseng (1999) developed a double-membrane system for SND in a single tank. The tank contained several small tubes, on which thick biofilms formed. Methanol was fed into half of the tubes and the other tubes were fed with pure oxygen. The interference of organic carbon with nitrification and oxygen with denitrification were both prevented by the diffusion barriers in the biofilms and allowed two different niches for nitrifiers and denitrifiers to exist in a single tank. However, this particular set-up requires the continuous addition of methanol.

1.2.3. SND via Nitrite

An energy saving can be made by eliminating the nitrate production (i.e. nitrite oxidation) and consumption (i.e. nitrate reduction) steps from the SND process. Based on calculations by Turk and Mavinic (1986), SND via nitrite can potentially achieve 40 % reduction of the COD demand for denitrification (by eliminating Eq 3), 63 % higher rate of denitrification, 300 % lower biomass yield during anaerobic growth, and no apparent nitrite toxicity. Abeling and Seyfried (1992) suggested that 75 % less oxygen would be required for SND via nitrite, based on the stoichiometry of the complete nitrification reaction (Eqs 1, 2). Thus, if nitrification could be stopped at nitrite, this would allow conservation of organic substrate and consequently lower

nitrogen concentrations in the effluent could be achieved, particularly for wastewaters with low COD:N ratios.

To achieve simultaneous nitrification and denitrification via nitrite, the most common strategy employed is to deter the aerobic nitrite oxidisers. Nitrite oxidisers have been shown to have several distinct growth limitations that make it relatively easy to reduce their activity. Experimental results have shown that they experience lag periods in activity after exposure to anoxic or anaerobic conditions (Turk and Mavinic, 1986, Sakai, 1997). Studies incorporating an initial anoxic phase or an non-aerated fill phase have reported nitrite accumulation, for which they could not find an explanation (Munch et al., 1996, Pochana and Keller, 1999). It is possible that a lag in nitrite oxidising activity was the cause of nitrite accumulation in these studies. Due to their lower affinity for oxygen (i.e. higher half saturation constant for oxygen ($k_{S,O2}$) value), nitrite oxidisers do not compete well with ammonium oxidisers at the low DO concentrations that are usually applied to encourage SND (Hanaki et al., 1990, Hendrikus and Saskia, 1993, Laanbrock and Gerards, 1993, Dangcong et al., 2000).

Another growth disadvantage of nitrite oxidisers is their sensitivity to free ammonia concentrations above 10 mg L^{-1} (Neufeld et al., 1980, Abeling and Seyfried, 1992, Balmelle et al., 1992, Surmacz-Gorska et al., 1997). Due to this sensitivity, small changes in pH may cause significant inhibition of nitrite oxidising activity, as the ammonia-ammonium equilibrium is pH-dependent ($NH_3 + H^+ \Leftrightarrow NH_4^+$). Another growth restriction of nitrite oxidisers is their decrease in activity above 25 ^oC (Yoo et al., 1999). Based on all the above observations, Yoo et al. (1999) proposed the following criteria to achieve SND via nitrite: (1) Use SND and/or alternating nitrification/denitrification in the same reactor, to take advantage of the lag of nitrite oxidisers behind ammonium oxidisers, (2) Maintain low DO levels during aeration (< 2.5 mg L^{-1}) to take advantage of the higher $k_{S,O2}$ of nitrite oxidisers, (3) Raise the pH above 7.5 to increase the amount of free ammonia (NH_3) and (4) Maintain the temperature above 25^o C. The last two criteria are of course difficult to achieve in large-scale reactors, while the first two are relatively easy to implement.

1.3. Microbial Storage Compounds Relevant to the Activated Sludge Process

With the increasing use of dynamic treatment systems such as the SBR, researchers are slowly recognising the important role that microbial storage polymers play in such systems (Van Loosdrecht et al., 1997, Majone et al., 1999, Beun et al., 2000a). It is becoming increasingly evident that it is possible to take advantage of this metabolic ability to increase nutrient removal in dynamic systems. Wilkinson (1959) established three criteria for a compound to function as an energy-storage compound (Dawes and Senior, 1973): (1) The compound must be accumulated under conditions when the supply of energy from exogenous sources is in excess of that required by the cell for growth; (2) The compound must be utilised when the supply of exogenous sources is no longer sufficient for the optimal maintenance of the cell, and finally (3) The compound must be degraded to produce energy in a form utilisable by the cell and is utilised for some purpose which gives the cell a biological advantage in the struggle for existence over other cells. There are three main classes of microbial storage compounds that play a role in the activated sludge process, namely polysaccharides (e.g. glycogen), polyhydroxyalkanoates (PHAs, e.g PHB) and polyphosphates. The high molecular weight of these polymers means that they only slightly affect the internal osmostic pressure of the cell when they are synthesised (Dawes and Senior, 1973).

Polyphosphate can fulfil the role of ATP in some microorganisms. In general, cells do not have the capacity for directly storing large amounts of ATP or reducing power, even though there is always a demand for at least a small amount of ATP for cell maintenance (Cartledge, 1992). However, some cells have developed the capability to store polyphosphate, which produces ATP upon hydrolysis: $(P_i)_{n+1}$ + ADP ←→ $(P_i)n$ + ATP (Lehninger et al., 1993). Lipmann (1965) (cited in Dawes and Senior, 1973) suggested that the earliest microorganisms used polyphosphate as their prime energy carrier, and that the role of ATP as the universal energy carrier has arisen during the course of evolution. Glycogen and PHB are reduced organic polymers, consisting of glucose and hydroxy-acid monomers, respectively. Stanier et al. (1959) showed that the nature of the carbon substrate and the conditions of growth influence the flow of carbon into the different energy reserves PHB and glycogen. When substrates were metabolised via acetyl-CoA, without the intermediate formation of pyruvate, PHB accumulation predominated. Growth on substrates that were metabolised via pyruvate led to the formation of glycogen (Dawes and Senior, 1973). Therefore, glycogen is usually

formed when sugars are present in the liquid, and PHAs when volatile fatty acids (VFAs) are the substrate. The polyhydroxyalkanoate PHB is the subject of significant focus in this thesis.

1.3.1. Poly-Hydroxyalkanoates (PHA)

Polyhydroxyalkanoates are hydrophobic, reduced storage polymers that serve as carbon and energy reserves when no other external substrate is available and are accumulated by a wide variety of microbes under unbalanced growth conditions (Anderson and Dawes, 1990). They are a group of optically active microbial polyesters composed of hydroxyacyl monomer units (Dawes and Senior, 1973). Poly-β-hydroxybutyric acid (P(3HB) or PHB) is the most commonly found polyhydroxyalkanoate (Fig 1.5).

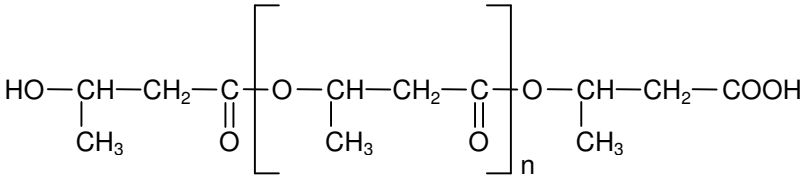

Figure 1.5: Chemical structure of poly-β-hydroxybutyric acid polyester (PHB).

The length of the carbon chain of the substrate determines the range of monomer units incorporated into PHA, since the β-hydroxyacid in the storage polymer contains the same chain length as the substrate (Anderson and Dawes, 1990). Since acetate contains two carbon atoms, the storage polymer produced is always poly-β-hydroxybutyric acid. The other commonly found storage polymer is poly-β-hydroxyvalerate, which has a carbon chain length of 5 and is formed when the substrate contains monomer chains of 3 carbons and 2 carbons. The synthesis of storage polymers is simpler than synthesis of the whole cell, and less adaptation is required than adapting the cellular composition during dynamic conditions and is therefore more rapid than a growth response (Daigger and Grady, 1982, Majone et al., 1999).

1.3.2. Poly-β-hydroxybutyric acid

A Short History

PHB was first isolated and described as an important bacterial product by Lemoigne in 1926 (Dawes and Senior, 1973). He later characterised PHB chemically and observed that it was involved in the sporulation of *Bacillus* spp. (Page, 1995). In 1958 Macrae and Wilkinson observed that the quantity of PHB accumulated increased as the carbon to nitrogen ratio of the growth medium was increased and concluded that PHB accumulation occurred in response to an imbalance of growth brought about by a nutrient limitation (Anderson and Dawes, 1990). An understanding of growth conditions on PHB metabolism enabled the first detailed studies on the metabolites and enzymes of the PHB pathway to be made (Merrick and Doudoroff, 1961, Sierra and Gibbons, 1962, Merrick and Doudoroff, 1964, Hippe, 1967, Griebel and Merrick, 1971). These studies established the cyclic nature of PHB synthesis and degradation pathway in the cell.

A sudden period of intense research was triggered in the early 1960's by the discovery that PHB is a biodegradable thermoplastic material with potential commercial applications (King, 1982). Environmental concern at the time over the persistence of non-biodegradable plastics in nature made PHB a promising alternative. In 1973 the first papers to shed light on regulation of the PHB pathway appeared from pure culture studies on *Azotobacter beijerincki* (Senior and Dawes, 1973) and *Hyrogenomonas eutropha* (Oeding and Schlegel, 1973), allowing attempts at optimisation of PHB production. Imperial Chemical Industries Ltd. (ICI) marketed their first bioplastic as *Biopol* in 1982. Recently the optimisation of PHA production has been accelerated by the use of fermentations using recombinant bacteria (Lee et al., 1997, Ryu et al., 1997, Wang and Lee, 1998, Shimizu, 2000, Du et al., 2001, Omar et al., 2001). Extensive review articles on microbial polyesters are available (Verhoogt et al., 1994, Steinbuchel et al., 1995, Iwata and Doi, 1999, Avella et al., 2000, Kim and Lenz, 2001).

PHB in Activated Sludge

The relevance of bacterial storage polymers in the activated sludge process remained largely unnoticed until the 1980's when the use of dynamic wastewater treatment systems such as the sequencing batch reactor became more prominent. Several studies

had reported the capacity of microbes in activated sludge to accumulate internal storage polymers (Chudoba et al., 1973, Zevenhuisen and Ebbinck, 1974, Van den Eynde et al., 1984). For example, Alleman and Irvine (1980) studied nitrogen removal in an SBR and reported increased endogenous respiration rates at the end of the aeration period, which they contributed to storage polymers. Yet these studies did not result in a general acceptance of a significant role of storage processes in activated sludge processes until recently (Van Loosdrecht et al., 1997, Gujer et al., 1999, Majone et al., 1999, Beun, 2001).

It was traditionally thought that PHB only accumulates when growth is restricted by an essential nutrient such as nitrogen, phosphorous or sulphur, while surplus carbon is available (Oeding and Schlegel, 1973, Senior and Dawes, 1973, Haywood et al., 1989b, Ratledge and Kristiansen, 2001). However it has been recognised that PHB can also accumulate under conditions without growth limitation. The driving force for PHB storage under these conditions is a dynamic feeding pattern, or a "feast-famine" regime (Van Loosdrecht et al., 1997, Majone et al., 1999, Beun et al., 2002). Activated sludge processes are highly dynamic with respect to the feed regime, especially when use is made of SBR processes. Even if the whole treatment process is considered to be in a steady state (e.g. continuous-flow systems), the biomass still grows under dynamic conditions, being continuously recycled among zones with different redox environments and substrate concentrations (Majone et al., 1999). Under dynamic conditions, bacteria that are capable of storing substrate can grow in a more balanced way, and are more independent of the external substrate concentration. They can use the stored substrate during times of starvation to gain a competitive advantage over bacteria without the capacity to produce storage polymers (Van Loosdrecht et al., 1997). Feast-famine environments logically convey a selection advantage to microbes capable of readily assimilating substrate materials and maintaining viability during extended starvation periods (Chiesa et al., 1985). The relevance of storage in dynamic conditions has been widely studied in the laboratory with cultures fed with synthetic substrates (*see* Sections 1.3.4 – 1.3.5).

Dynamic Conditions and Bulking Sludge

The influence of dynamic feeding patterns on sludge settling efficiency was recognised by Chudoba et al. (1973), who showed that the substrate to biomass ratio (i.e. S/X)

plays an important role in bacterial selection. Their study showed that a high initial S/X ratio selects for a robust population of floc-forming microbes over filamentous bacteria, the cause of bulking sludge. They explained their findings in the light of a kinetic selection theory, where filamentous bacteria have lower growth rates and higher substrate affinity (lower k_S for substrate) constants than floc-forming bacteria. According to the Monod equation (Eq 8), at high substrate concentrations the growth rate is controlled mainly by μ_{max}, while at low substrate concentrations the growth rate is controlled mainly by k_S. Therefore, the substrate concentration provides a selective pressure and floc-formers are favoured in systems that regularly introduce a high substrate gradient, as they can out-grow filamentous bacteria at high substrate concentrations (Chudoba et al., 1973, Chiesa et al., 1985, Kohno et al., 1991, Chudoba et al., 1992). Bioselectors have been designed, based on this theory, to contact the biomass with incoming organic-rich wastewater and allow for rapid removal and storage of readily biodegradable COD, while improving sludge settling characteristics (Chudoba et al., 1973, Newland, 1998).

$$\mu = \mu_{\max} . \frac{S}{k_S + S} \qquad \text{(Eq 8)}$$

where μ is the specific growth rate (h^{-1}), μ_{max} is the maximum specific growth rate (h^{-1}), k_S is the half saturation constant for the substrate (mg L^{-1}) and S is the substrate concentration (mg L^{-1}) (Monod, 1949).

The role of the substrate to biomass ratio in selection of PHB-storing bacteria can explain why storage compounds remained unnoticed to a large extent in conventional continuous-flow wastewater treatment systems. These reactors typically operate on the premise of reduced substrate tension and would therefore be unlikely to realise significant storage interaction unless stressed by a transient shock load (Alleman and Irvine, 1980). In contrast, dynamic systems such as the SBR impose conditions that enrich for substrate-storing microorganisms, by regular exposure to high substrate gradients. Storage is the main substrate removal mechanism in these reactors (Van Loosdrecht et al., 1997, Majone et al., 1999, Beun et al., 2000a, Beun et al., 2000b, Beun, 2001, Beun et al., 2002). Even though kinetic selection based on the storage response should allow predomination of floc-formers (Chudoba et al., 1992), Beccari et al. (1998) established a bulking sludge for long periods with a high capacity for storage.

Their study suggests that while the kinetic selection theory can describe general behaviour in dynamic systems, bacteria are diversified and exceptions to the rule exist.

1.3.3. PHB Synthesis and Degradation in the Cell

The cyclic pathway of PHB synthesis and degradation was established in 1973 for *Azotobacter beijerinckii* (Senior and Dawes, 1973) and *Hydrogemonas eutrophus* (Oeding and Schlegel, 1973). The synthetic pathway is similar in all microorganisms studied to date and consists of three steps; (1) a condensation reaction, where two moles of acetyl-CoA are condensed to form acetoacetyl-CoA, with the release of coenzyme A, (2) an NADPH-dependent reduction reaction to produce the stereospecific R-β-hydroxybutyryl-CoA, and (3) a polymerisation reaction where βHB units are added to the growing chain to produce PHB (Anderson and Dawes, 1990). The degradation pathway is a 4-step conversion resulting in the production of NADH and acetoacetate (Fig 1.6).

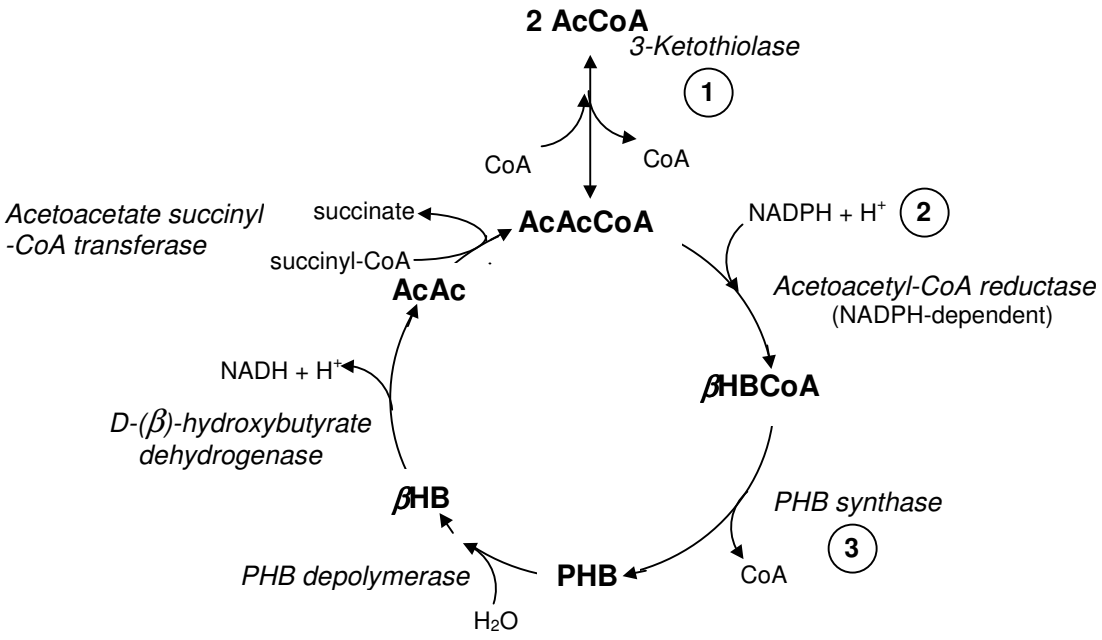

Figure 1.6: Cyclic process of PHB synthesis and degradation in the microbial cell where AcCoA = acetyl-CoA, AcAcCoA = acetoacetyl-CoA, βHBCoA = R-β-hydroxybutyryl-CoA, PHB = poly-β-hydroxybutyric acid, βHB = β-hydroxybutyrate, AcAc = Acetoacetate.

Regulation of PHB Metabolism

The fate of the central metabolite acetyl-CoA depends on environmental conditions. It can be diverted either to the TCA cycle and electron transport chain for the production of ATP and biomass, or to the PHB synthesis pathway. Conditions which stimulate PHB synthesis are high NAD(P)H, low CoA and high acetyl-CoA concentrations (Leaf and Srienc, 1998). PHB synthesis is especially sensitive to the *ratio* of acetyl-CoA/CoA (Van Wegen et al., 2001). This can be explained by the low equilibrium constant (6×10^{-5}) for the enzyme 3-ketothiolase of the first condensation reaction, which means that PHB synthesis is not favoured under equilibrium conditions (Reich and Sel'kov, 1981, Beun et al., 2001). When the ratio of the reaction substrate (acetyl-CoA) over the reaction product (acetoactate) increases, the equilibrium is driven into the direction of PHB synthesis, resulting in PHB production. Similarly, PHB synthesis is stimulated when the ratio of $NADH/NAD^+$ increases in the cell, for example during oxygen limitation (Van Wegen et al., 2001). In this respect, PHB formation serves as an electron sink, or NADH-overflow mechanism, since the production of one PHB monomer from acetyl-CoA requires two reducing equivalents (Fig 1.7) (Senior and Dawes, 1973, Van Niel et al., 1995, Van Aalst-Van Leeuwen et al., 1997).

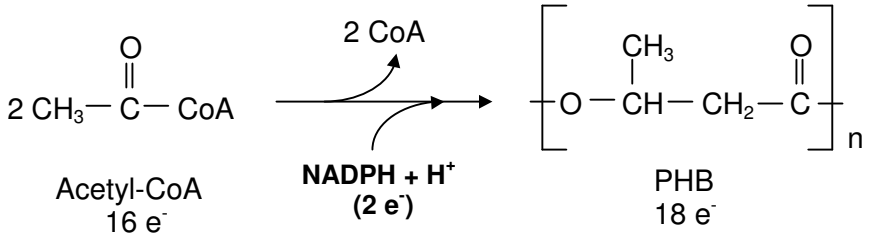

Figure 1.7: The production of PHB serves as an electron sink, storing reducing equivalents for later use during famine periods.

The first enzyme of the TCA cycle, citrate synthase, is inhibited by NADH, so acetyl-CoA is directed to PHB synthesis when NADH accumulates (Ratledge and Kristiansen, 2001). The first enzyme of the PHB synthesis pathway, 3-ketothiolase, is inhibited by free-CoA, which accumulates when the TCA cycle is highly active (Oeding and Schlegel, 1973, Senior and Dawes, 1973). This type of regulation has led most studies to conclude that the enzymes for PHB biosynthesis are constitutive, and regulation does not occur through control of enzyme concentrations, but rather that enzyme activity is

regulated through changes in metabolite concentrations (Haywood et al., 1988, Haywood et al., 1989a, Leaf and Srienc, 1998, Ratledge and Kristiansen, 2001, Van Wegen et al., 2001). This type of control allows the cell to immediately synthesise PHB when required. However, some studies suggest regulon control of PHB synthesis, where transcription of enzymes are alternatingly repressed or stimulated depending on environmental conditions (Yoo and Kim, 1994, Kessler and Witholt, 2001). It is likely that the type of control varies amongst microorganisms. Recent work with recombinant strains has showed that the biosynthesis *rate* of PHB is controlled by 3-ketothiolase and the PHB *content* is controlled by PHB synthase (Jung et al., 2000, Kessler and Witholt, 2001).

Much is known about extracellular PHB degradation, due to its commercial relevence to the bioplastic industry, however relatively little is known about intracellular PHB-degradation regulation. It is generally thought that the same conditions that stimulate PHB synthesis also inhibit its degradation, which prevents unrestricted cycling of metabolism (Dawes and Senior, 1973). Some studies have shown that many strains of PHB-storing bacteria repress PHB depolymerase gene expression in the presence of a soluble carbon source that permits high growth rates. After nutrient exhaustion the synthesis of PHB depolymerase is derepressed (Foster et al., 1995, Kessler and Witholt, 2001). However, in contrast to these data supporting regulon control of degradation, all bacteria studied until 1973 were shown to possess a constitutive NAD-specific D-(3)-hydroxybutyrate dehydrogenase (Dawes and Senior, 1973). A constitutive system controlled by metabolite concentrations is also supported by the recent findings that PHB depolymerase in *Pseudomonas oleovorans* is always present and active, and that PHB is synthesised and degraded simultaneously (Zinn, 1998, Kessler and Witholt, 2001). A similar result has been reported for *Alcaligenes eutropha* (Doi et al., 1992, Saito et al., 1995). The contrasting reports available regarding regulon control versus constitutive enzyme production for the PHB metabolic enzymes suggst that the type of control of PHB metabolism is likely to vary amongst microorganisms and needs to be studied on an individual basis. However, the vast majority of literature appears to be in favour of constitutive enzyme production and enzyme control by metabolite concentrations during PHB metabolism (Haywood et al., 1988, Haywood et al., 1989a, Leaf and Srienc, 1998, Ratledge and Kristiansen, 2001, Van Wegen et al., 2001).

1.3.4. Anaerobic PHB Storage

The role of PHB in the activated sludge process has been studied in the greatest detail with reference to anaerobic-aerobic processes for the phosphorous removal process known as Enhanced Biological Phosphorous Removal (EBPR). In this process, feeding is performed under anaerobic conditions, which favours growth of polyphosphate accumulating microorganisms (PAOs, poly-P or bio-P bacteria) (Mino et al., 1998, Dionisi et al., 2001). According to theory, under anaerobic conditions, substrates are fermented to fatty acids by a wide range of microbes. These volatile fatty acids (VFAs) form the dominant substrate for bio-P bacteria, which convert the VFAs to polyhydroxyalkanoates at the expense of energy released from the hydrolysis of polyphosphate, which is released from the cell into the liquid (Smolders et al., 1994, Carucci et al., 1995). When oxygen or nitrate becomes available the PHB is oxidised to generate energy for growth and restoration of the polyphosphate levels. During this phase, excess or "luxury" phosphate uptake occurs in which more phosphate is consumed than was initially released, resulting in P-removal. Storage polymers form a crucial part of metabolism of the bio-P bacteria (Mino et al., 1994, Smolders et al., 1995a, Smolders et al., 1995b, Mino et al., 1998).

Anaerobic uptake of VFAs is not limited to bio-P bacteria in activated sludge. The failure of EBPR systems is often reported to be due to the dominance of organisms that also take up organic carbon under anaerobic conditions, but whose metabolism appears to have no link to phosphate release (Satoh et al., 1994). These bacteria have been termed glycogen accumulating bacteria (GAOs) or "G bacteria" (Cech et al., 1994). They anaerobically consume VFAs and convert them to PHB by internally transforming glycogen into PHB to gain energy, instead of poly-P hydrolysis (Carucci et al., 1999a). They cause deterioration of P-removal systems by competing with bio-P organisms for their common substrate – VFAs – while not contributing to P-removal.

In addition to the accepted models of bio-P (Smolders et al., 1995c, Mino et al., 1998) and "G" bacteria (Cech et al., 1994), other pathways for anaerobic substrate consumption have been recognised. Some bacteria can take up glucose, instead of VFAs, under anaerobic conditions and transfer it directly to glycogen as the storage compound. The energy for the glucose conversion can be obtained from poly-P hydrolysis or lactic acid fermentation (Carucci et al., 1999b), which is different to the

metabolic pathways reported for PAOs and GAOs, as those groups require VFAs as the substrate for P-release. In the case of EBPR, this means that P-removal can be obtained with substrates other than VFAs (i.e. sugars), without their pre-conversion to VFAs or storage as PHB, but through direct storage as glycogen only. Liu et al. (1996) also established a population of bacteria capable of consuming glucose and acetate anaerobically, without poly-P hydrolysis. All sugars fed to the culture (glucose, fructose, maltose, sucrose, raffinose etc.) were stored as glycogen. The energy required for substrate uptake was generated from glycolysis of glycogen or from oxidation of a sugar, so did not involve glycogen conversion to PHB for energy, as in the case of bio-P and "G" bacteria. It is apparent that many different mechanisms of anaerobic substrate storage are available to microorganisms in nature and their relative contribution to P-removal requires more study.

1.3.5. Aerobic PHB Storage

Substrate storage as PHB under aerobic conditions is the major focus of organic substrate removal in this thesis. Aerobic PHB storage has traditionally been investigated in pure culture chemostats under oxygen-limitation because it was thought that PHB accumulation only occurred when a microorganism is in exponential growth but respiration is blocked, for example by oxygen limitation (Stockdale et al., 1968, Dawes and Senior, 1973, Anderson and Dawes, 1990). PHB storage in aerobic, dynamic cultures was only recently established as a major substrate removal mechanism in these systems (Van Aalst-Van Leeuwen et al., 1997, Van Loosdrecht et al., 1997, Gujer et al., 1999, Majone et al., 1999, Beun et al., 2000a, Beun et al., 2002). Aerobic storage phenomena have been mainly studied in pure and mixed cultures after cultivation in lab- or pilot-scale plants on defined synthetic media. High "observed" yields (Y_{OBS}) are the most common criterion used to suggest the occurrence of storage. An observed yield is derived from the ratio between oxidised substrate (as measured from the OUR) and removed substrate (Eq 9) (Majone et al., 1999). It is a measure of the substrate that is consumed without oxygen consumption and is therefore substrate that is either used for biomass growth or PHB synthesis. Y_{OBS} values greater than 0.5 are generally indicative of substrate storage. The storage response is a fast and high-yield response (Majone et al., 1996).

$$Y_{OBS} = 1 - \frac{O_2}{S} \qquad \text{(Eq 9)}$$

where Y_{OBS} is the observed yield, O_2 is the amount of oxygen consumed (COD) and S is the total substrate consumed (COD).

Studies of PHB Storage in Pure Cultures

Until recently all pure culture studies investigated PHB production during continuous cultivation with excess carbon source but limitation of a nutrient (N, S, P, Fe, Mg, K or O_2) (Oeding and Schlegel, 1973, Yoo and Kim, 1994, Mansfield et al., 1995, Page and Manchak, 1995, Saito et al., 1995, Steinbuchel et al., 1995, Leaf and Srienc, 1998, Kessler and Witholt, 2001, Van Wegen et al., 2001, York et al., 2001). Very few studies had looked at PHB storage under dynamic substrate supply until studies by Van den Eynde et al. (1983) and Van den Eynde et al. (1984), who investigated intermittently glucose-fed cultures of *Arthrobacter globiformis* and *Sphaerotilus natans* at low sludge retention times (SRT) of 0.8 – 1 day. Both cultures showed a much higher substrate uptake rate upon transition from chemostat to batch culture (up to 4.5 times higher), due to the sudden removal of substrate limitation. Increased observed yields were measured (Y_{OBS} 0.71 COD/COD for *A. globiformis* and 0.32 for *S. natans*), indicating the importance of storage in substrate removal after transition to batch culture.

Van Niel et al. (1995) studied PHB storage in a fast-growing culture of *Thiosphaera pantotropha,* a sulphur-reducing bacterium that can nitrify heterotrophically, denitrify aerobically, and store substrate as PHB. All three capabilities were thought to be NADH-overflow mechanisms. After transition from acetate-limitation (chemostat) to conditions of acetate in excess, the growth rate increased immediately (from 0.1 to 0.2 h^{-1}) and acetate was converted mainly to PHB (57 % w/w). Respiration measurements showed that only 29 % of the acetate taken up was oxidised and the remaining 14 % was used for growth. Another pioneering chemostat-study on aerobic acetate storage by Van Aalst-Van Leeuwen et al. (1997), using a pure culture of *Paracoccus pantotrophus,* showed that the amount of PHB accumulated depended strongly on the growth rate before addition of a pulse of acetate. At growth rates of 0.05 to 0.1 h^{-1}, 25 – 30 % of the acetate consumed was converted to PHB, while at a higher growth rate of 0.2 h^{-1} only 20 % was converted to PHB. The maximum specific acetate

uptake rates were independent of the initial growth rate, yet the amount of PHB stored decreased with increasing growth rates.

Studies of PHB Storage in Mixed Cultures

Majone et al. (1996) studied the effect of previous cultivation conditions on storage of PHB by aerobic, mixed cultures (DO > 6.5 mg L^{-1}). They selected for both a bulking sludge and a non-bulking sludge by continuous or intermittent feeding of a continuously stirred tank reactor (CSTR), respectively. Interestingly, when the cultures were exposed to batch conditions, both cultures showed that PHB storage was the prevailing mechanism of substrate removal. However, the intermittently fed, non-bulking culture showed a faster response to the acetate spike with a higher observed yield (Y_{OBS}) and PHB storage was practically the only substrate removal mechanism. The non-bulking sludge was more resistant to starvation and had a higher storage capacity than the filamentous (bulking) culture. This study highlighted the importance of dynamic feeding patterns in selecting for robust PHB-storing cultures.

Until the studies by Beun et al. (2000a, 2001, 2002) very little information was available on the stoichiometry and kinetics of PHB storage. In an SBR using activated sludge, Beun et al. (2000a) found that 66 – 100 % of the acetate consumed during the feast phase was converted to PHB. Acetate consumption and PHB production in the feast phase both proceeded with a zero-order rate. A literature search conducted by these authors showed that in all references, the ratio of the specific rate of acetate consumption over the specific rate of PHB production (referred to as q_{PHB}/q_{Ac}) had a constant value of 0.6 Cmol/Cmol. In all of these studies, the dissolved oxygen concentration was saturating (i.e. > 6.5 mg L^{-1}). They questioned whether this ratio has a constant value in general or whether it changes depending on the conditions applied in the system. In their later study, Beun et al. (2002) studied polymer storage in acetate-fed sludges with a wide range of fractions of PHB content values (f_{PHB}) (between 0 – 0.8 Cmol PHB/Cmol X), in order to verify whether the q_{PHB}/q_{Ac} ratio was in fact constant in general. During the feast period, the ratio was experimentally and theoretically constant for dynamically fed systems operating at sludge retention times (SRT) of > 2 days. This value was 0.6 Cmol/Cmol under aerobic conditions and 0.4 – 0.5 Cmol/Cmol under anoxic conditions, irrespective of the specific growth rate and the specific acetate uptake rate in the feast period. At SRT's shorter than 2 days, the ratio dropped to

0.4 Cmol/Cmol, suggesting that faster-growing cultures accumulate less PHB. It is interesting to note the effect of temperature on polymer storage (Krishna and Loosdrecht, 1999b). At 30^{o}C and higher, under aerobic conditions and continuous or pulse addition of acetate, the ratio q_{PHB}/q_{Ac} decreased from 0.6 to between 0.1 and 0.3. This suggests that wastewater treatment plants in tropical regions with warm temperatures would experience difficulties in encouraging microbial substrate storage.

Taking into account that wastewater is a mixed substrate, Carta et al. (2001) attempted to understand storage mechanisms with multiple substrates. They subjected a mixed bacterial population to dynamic feeding patterns with glucose and acetate. Both substrates were consumed simultaneously, and the relative contribution of growth and storage processes was similar as in systems fed with a single substrate only (where acetate was stored as PHB and glucose as glycogen). Acetate was consumed at a lower rate than glucose, although the specific rate was not influenced by the presence of glucose. The ratio of substrate uptake over substrate storage was again found to be 0.6 Cmol/Cmol for both substrates. The uptake of acetate was not influenced by the simultaneous uptake of glucose, and degradation kinetics of PHB and glycogen were also similar. They concluded that the global performance of the culture grown on mixed substrates could be described as the sum of the conversions observed in cultures fed with individual substrates.

Pulse Addition of Acetate to Unconditioned Activated Sludge

In an attempt to understand the contribution of storage polymers to metabolism by real activated sludge, some studies have aimed at quantifying storage in wastewater treatment plants. Dircks et al. (1999) studied respirograms (OUR data) of activated sludge after the addition of pulses of acetate. The respirograms could be divided into two phases of high and low OUR, and the shift between the two phases corresponded to the depletion of exogenous substrate. The observed yield of PHB on acetate was 0.71 COD/COD. In a later study, Dircks et al. (2001b) analysed the accumulation and degradation of PHB in activated sludge samples from pilot-scale and full-scale wastewater treatment plants, fed with acetate. Their results showed that PHB storage was the dominant process in the heterotrophic conversion of acetate in activated sludge under aerobic conditions. They found that a significant part of the total potential growth

(around 90 %) occurred during the famine period on stored PHB. A similar yield of PHB on acetate to their earlier study was found, of 0.67 COD/COD.

Studies of PHB Storage in Cultures Fed with Real Wastewater

Current knowledge about substrate removal mechanisms by real activated sludge in wastewater treatment plants and about the fate of substrates other than glucose and acetate is scarce. Yet this knowledge is essential as wastewater is a mixture of many different substrates (Henze et al., 1994, Beccari et al., 2002). Beccari et al. (2002) studied the addition of separate substrates to aerobic activated sludge cultures such as acetate, ethanol and glutamic acid and compared this data to measurements obtained with raw and filtered wastewater. PHB was stored when the substrate was ethanol or acetate, but no appreciable PHB was measured with glutamate. However, only those substrates that feed into metabolism at acetyl-CoA have been found to result in PHB production (Jenkins, 1992). Only five of the twenty amino acids feed in at acetyl-CoA, and glutamate is not one of them. Thus, it may not be surprising that the cultures did not produce PHB from glutamate. A low amount of storage was observed in the cultures fed with raw and filtered wastewaters, although the soluble COD content of their wastewater was quite low (70 mg L^{-1} filtered and 150 mg L^{-1} unfiltered) and acetate contributed to less than 20 mg L^{-1} of the available COD. From the results of Beccari et al. (2002) with synthetic substrates, the growth, storage and oxidation in the COD balance accounted for only 75 % of the substrate removal, so they concluded that the rest was due to low-energy processes such as biosorption (since no OUR corresponded to the substrate uptake).

Carucci et al. (2001) aimed to give direct evidence of storage phenomena when “real” activated sludge was mixed with influent wastewater in aerobic batch tests. PHB storage was the main contribution to acetate removal when fed with a synthetic medium, and in the case of wastewater, PHB was also formed from substrates other than acetate. However, it only accounted for 18 – 22 % of overall soluble COD removal, suggesting other unidentified storage compounds or other non-storage phenomena play a role in substrate removal. The OUR in the feast phase was higher for the wastewater tests than in batch tests with acetate, suggesting a significant contribution of external soluble COD other than acetate in the wastewater tests. The respective contributions for PHB versus external COD utilisation could not be easily

separated because both substrates were present simultaneously in both raw and filtered wastewaters. They concluded that many more storage compounds need to be identified and quantified to confirm the conceptual structure of the recent IAWQ model that includes storage, ASM3. In the ASM3 it is assumed that all soluble COD is converted to storage products (*see* Section 1.3.10).

Hanada et al. (2002) showed that the ratio of heterotrophic organisms capable of storing substrate in activated sludge varied among different municipal and laboratory sludges. In ASM3 it is hypothesised that all heterotrophic microorganisms can store substrate. In this study, they proposed a "dual biomass" model, which assumes there are two types of heterotrophs – those that cannot store substrate (as in ASM1) and those that can store substrate (as in ASM3). They estimated numbers of PHB-storing heterotrophs by Nile Blue and DAPI staining under a fluorescent microscope of activated sludge from 5 full scale plants. Their study showed that the acetate uptake rates and PHB storage rates were up to 30 times faster for cultures in the laboratory that were acclimatised to acetate and that lab studies may over-estimate the actual amount of storage achievable in wastewater treatment plants.

1.3.6. Anoxic PHB storage

Until the studies by Beun et al. (2000b), Dionisi et al. (2001) and Majone et al. (1998), very little attention had focused on substrate storage under anoxic conditions. Dionisi et al. (2001) showed that anoxic acetate removal rates did not vary significantly from the aerobic rates. This was in contrast to the findings of Beun et al. (2000b) who reported three times faster acetate uptake rate during aerobiosis compared to anoxia, for biomass cultivated under anoxic conditions. However, it was evident from the results of Dionisi et al. (2001) that the previous cultivation conditions affected the transient response of the biomass to acetate pulses. For biomass grown under anoxic conditions, the adaptation to aerobic conditions was immediate. But the biomass already acclimated to aerobic conditions showed a much slower response to anoxic conditions. The slower response to nitrate respiration by aerobically selected mixed cultures could not be attributed to non-denitrifying bacteria, since they calculated that this could not be more than 10 % (Majone et al., 1998). Dionisi et al. (2001) concluded that biomass adaptation from aerobic to anoxic conditions is much slower than the opposite one. A time lag in respiration when shifting from aerobic to anoxic conditions was also observed by Liu et

al. (1998). Other studies have shown that PHB-storing cultures selected under aerobic conditions have a low capacity for anoxic substrate uptake. Falvo et al. (2001) studied the capacity of *Amaricoccus kaplicensus* to store PHB in an aerobic SBR. The microorganisms showed a high capacity for aerobic storage of acetate as PHB, but showed no anaerobic storage and very slow anoxic storage. Similarly, Beccari et al. (1998) enriched a bulking sludge that had a high aerobic storage capacity, but which was inactive under anoxic conditions.

1.3.7. PHB-Saturation Capacities of Microbial Cultures

The maximum PHB capacity of cultures varies significantly among different studies. In all experimental studies, PHB storage has been shown to follow saturating kinetics. But reported storage capacities are often high (up to 20 times higher than reported in wastewater treatment plants), so this dependence could be of minor importance under usual sludge loading (Majone et al., 1999). Beccari et al. (1998) established a bulking sludge that could store up to 47 % on a w/w basis of stored polymer per total solids. They kept feeding their culture and showed that the acetate removal rate and the OUR increased exponentially, while the PHB production rate decreased, indicating growth was becoming more important with continued feeding. Their results showed that even though the storage capacity had been saturated, the new biomass was still able to store PHB at its maximum rate. PHB storage was shown to be an integral part of metabolism, even during exponential growth. This in contrast to the original belief that PHB storage occurs only during growth limitation (Oeding and Schlegel, 1973, Senior and Dawes, 1973, Anderson and Dawes, 1990). A pure culture of *Thiosphaera pantotropha* stored up to 42 % of its dry weight (Van Niel et al., 1995). In the study by Beun et al. (2002), the maximum theoretical and experimental PHB content was 80 % (w/w). All of these studies concluded that such high capacities will not easily be reached in the wastewater treatment plant situation, and a decline in the PHB production rate due to filling up of the cells would not be expected. Thus, the PHB production rate can be considered constant and not limited by the PHB content in plant situations of relatively low substrate loading. Van Aalst-Van Leeuwen et al. (1997) showed that the maximum fraction of PHB obtainable depended strongly on the growth rate before the pulse addition. It was clear the maximum amount of substrate stored decreased as the growth rate increased.

1.3.8. Growth of Bacteria on Stored Polymers

The growth of bacteria on storage polymers has been studied little in the literature. There are few reports on pure cultures growing on storage polymers and documented kinetic relationships for these processes are not available either. The synthesis of PHB has been well researched in pure culture studies because it has had the commercial drive from the application of PHB as a bioplastic. However, until the use of sequencing batch reactors in the activated sludge process in recent times, there has been little commercial interest to study the growth process on stored polymers, or the PHB degradation process.

Van Aalst-Van Leeuwen et al. (1997) showed for a pure culture of *Paracoccus pantotrophus* that the growth yield using PHB as the electron donor was only 6 % less than the yield of direct growth on acetate and concluded that the ability of these bacteria to afford energetically efficient growth on the stored polymer gives them a competitive advantage to non-storing bacteria. Beun et al. (2000a) predicted biomass yields on acetate and PHB at different ATP/NADH ratios between 1 and 3 (i.e. the theoretical maximum and minimum efficiency of oxidative phosphorylation). According to their model, a decrease in the ATP/NADH ratio from 3 to 1 led to a reduction in the net biomass yield (on PHB) of only 10 to 4 %, compared to direct growth on acetate. Therefore, even when oxidative phosphorylation is operating at minimum efficiency (e.g. during slow growth), the biomass yield is only 10 % lower than direct growth on acetate. These authors also concluded that the storage mechanism of PHB is energetically efficient. The implication of this in large-scale water treatment is that stimulating the pathway for PHB production would only marginally decrease heterotrophic sludge production.

Dircks et al. (2001a) demonstrated that storage of glycogen and subsequent growth occurred without significant loss of energy at all (< 2 %) and was comparable to direct growth on glucose. Formation and consumption of glycogen appeared to be much faster than for PHB. Kohno et al. (1991) reported similar findings where cellular carbohydrate was found to accumulate and degrade much faster than PHB. Storage of substrate as PHB thus appears to slow down oxidation of reducing power in comparison to storage of substrate as glycogen, and allows slightly less growth (biomass yield) than on glycogen.

1.3.9. PHB Degradation Kinetics

Beun et al. (2000a) found that PHB degradation occurred slower when their mixed culture biomass contained a low fraction of PHB (i.e. low f_{PHB} values), than when the culture contained a high fraction of PHB (high f_{PHB}). They suggested that at low PHB levels, PHB is not evenly distributed over all cells and there will be cells containing no PHB. Dircks et al. (2001b) also showed PHB degradation patterns by activated sludge were dependent on the amount of PHB present in the cells at the end of the feast period. Beun et al. (2002) later showed that it was possible to describe PHB degradation with one equation, depending only on the PHB content of the biomass, according to $df_{PHB}/dt = -0.15(f_{PHB})$. All data that had previously been obtained at different f_{PHB} values and different biomass growth rates (SRT's) could be explained by their first order kinetics. Degradation appeared to be independent of the type of electron acceptor present in the system and independent of the SRT of the system. Dircks et al. (2001b) found that multiple order kinetics gave a good description of the rate of PHB degradation, rather than a single first order rate expression as found by Beun et al. (2002). Their experiments showed that it started with zero order kinetics and then changed to 2/3 order kinetics, then 1^{st} order kinetics and finally 2^{nd} order kinetics. Therefore, the order increased as the cellular PHB content (f_{PHB}) decreased. They also gave the explanation that PHB is not uniformly distributed in all cells, leading to different times at which the cells become PHB-limited.

Beun et al. (2002) suggested that the internal PHB hydrolysis reaction limits the PHB degradation rate, as the type and concentration of electron acceptor available did not affect the degradation rate. Thus, it could be assumed that the PHB degradation rate is the growth rate-determining process, rather than the consumption of PHB is driven by the growth rate of the organisms. Their theory is in accordance with the findings of Van Aalst-van Leeuwen et al. (1997), who showed that the growth rate of their pure culture in the famine period (0.05 h^{-1}) was lower than the steady state growth rate at which the reactor was normally operated (0.2 h^{-1}). This meant that the growth rate decreased suddenly when the bacteria were forced to use stored substrate, due to the slower rate of electron donor availability. Although Beun et al. (2002) proposed that the PHB degradation process is independent of the growth rate of the organisms, Van Aalst-Van Leeuwen et al. (1997) showed that the rate of PHB consumption increased with the growth rate under which the cells were cultivated. Similarly, Dircks et al. (2001b)

showed that a sludge with a sludge retention time of 4 days degraded PHB faster than sludges with longer SRTs at high fractions of PHB. Beun et al. (2002) explained these findings by proposing there is a direct relationship between the PHB degradation rate, the actual growth rate and the type of electron acceptor. They proposed that the average specific growth rate depends on the fraction of PHB in the cells (f_{PHB}). This means that when the cells are cultivated at a certain SRT, the average fraction of PHB in the cells will adjust such that the average growth rate of the cells equals the sludge wastage rate. The first order degradation rate that fitted all their data means that at temporary high loading rates, the accumulated PHB is degraded relatively quickly, whereas at low loading slow PHB conversion occurs. This proposal appeared to fit the results found by Van Aalst-Van Leeuwen et al. (1997) and Dircks et al. (2001b).

1.3.10. Modelling of PHB Metabolism

Unstructured PHB Models

When using an unstructured (or black box) model to describe PHB consumption and degradation, changes in the internal composition of the biomass are totally ignored. The activities of the biomass are specified only by the amount of biomass present (Majone et al., 1999). Unstructured models (e.g. the Monod model) are only realistic if there are no significant changes in the environment, or these changes are very slow compared to changes in biomass composition. In unstructured models, the separate yields of biomass and PHB from substrate are not considered and only a total yield is used, even though the phenomena are completely different. The consumption of stored products is assumed to contribute to endogenous metabolism, working at a higher rate than usual (Majone et al., 1999). Unstructured models allow a good description of the process when storage is not too important. They are restricted due to fundamental shortcomings and cannot predict high transient phases such as fed-batch or dynamic behaviour (Yoo and Kim, 1994).

The IAWQ Task Group on Mathematical Modelling introduced the Activated Sludge Models (ASM) in 1987 for Design and Operation of Wastewater Treatment Processes for simulation of activated sludge processes (Henze et al., 1999). All are examples of unstructured models. In ASM1 the biomass is considered as one compound with a yield of total biomass (including PHB if present) on substrate. Stored substrate is accounted for as active biomass and consumption of stored substrate is accounted for in the

biomass decay process, characterised as slowly biodegradable substrate. The ASM models 2 and 2d (Gujer et al., 1995, Henze et al., 1999) are an extension of ASM1, introducing bioP removal. Recently the IAWQ task group introduced ASM3 (Gujer et al., 1999) as a possible replacement for ASM1. The main difference between the new model and the earlier model is the recognition of the importance of storage polymers in heterotrophic metabolism. In the ASM3, all readily biodegradable COD is first stored as internal cell products (X_{sto}) and is later used for growth. Substrate uptake and storage requires energy in the form of ATP that is obtained from respiration. As an improvement to the earlier models, the biomass yields are separated into a biomass yield and a PHB yield.

In the ASM3 model, PHB kinetics are presumed to be described by Monod kinetics (Gujer et al., 1999). Monod kinetics were developed for growth on soluble substrates and probably do not give a correct description of the conversion of internal stored particulate compounds like PHB. Also, ASM3 does not allow growth on external substrate. Krishna and Loosdrecht (1999a) evaluated the ASM3 model for its capacity to describe aerobic COD conversions. They showed that growth rates in the feast and famine period could not be described correctly using ASM3. This is because ASM3 allows growth on stored polymers only. They proposed a modification, where growth was allowed on external substrate. Growth on PHB was modelled as being inhibited by soluble acetate, so that when soluble substrate is around growth occurs predominantly on the soluble substrate, and not on both PHB and acetate. The improved model could describe the kinetics more accurately than ASM3.

Structured Models of PHB Metabolism

Structured models of PHB metabolism incorporate changes in the organism's composition (i.e. including storage products) and their activity is related to their composition (Majone et al., 1999). The advantage of a metabolic model is that yield and maintenance coefficients depend on the same metabolic parameters. Van Aalst-Van Leeuwen et al. (1997) developed a structured metabolic model for *Paracoccus pantotrophus* in which acetate is aerobically stored as PHB under dynamic conditions. All other papers until this point had looked at modelling of PHB storage due to a nutrient limitation. This study was one of the first to look at modelling under dynamic substrate supply. To facilitate modelling, the results of all pulse experiments were

divided into 2 phases, (1) PHB production phase, where acetate is the sole energy and carbon source for growth and PHB production, and (2) PHB consumption phase, where PHB is the sole carbon and energy source. The model was based on seven internal reactions and resulted in two linear equations describing the conversion processes (one for the presence of external substrate and one for its absence). The linear equations in the model contain 2 unknown parameters, the ratio between ATP produced and electrons transferred from NADH to an electron acceptor (ATP/NADH ratio) and the ATP consumption due to maintenance processes (m_{ATP}). These values were assumed to be independent of the SRT. The only unknown that had to be measured was the "split factor", or the ratio of substrate going to active biomass over substrate going to PHB. Kinetic expressions were introduced that were based on experimentally rates of growth, acetate consumption, PHB production and consumption. The structure of this metabolic model was subsequently used in other studies (Beun et al., 2000a, Beun, 2001).

Leaf and Srienc (1998) recognised that Monod kinetics (or Michaelis-Menten for enzyme kinetics) were not sufficient to explain PHB synthesis and degradation pathways, as they can only describe irreversible reactions. Since the first two steps of PHB synthesis are reversible reactions (i.e. the thiolase reaction and the reductase reaction), these authors used complex rate expressions for enzyme-catalysed reactions that reflect both the reversibility of the reactions and the reaction mechanisms. Use of these rate expressions dramatically changed the behaviour of the system compared to a simple model containing only Michaelis-Menten kinetics, and could predict the different responses to changes in enzyme activities as well as inhibition of flux by the reaction products CoASH and $NADP^+$. Van Wegen et al. (2001) applied these reversible kinetics to a recombinant *Escherichia coli* strain to study PHB synthesis and measured intracellular concentrations of acetyl-CoA and 3-hydroxybutyryl-CoA for the first time. They established that the PHB flux was highly sensitive to the acetyl-CoA/CoA ratio, the total acetyl-CoA + CoA concentration and the pH, as measured by "response coefficients". It was less sensitive to the NAD(P)H/NAD(P) ratio. Reversible kinetics could accurately describe the behaviour of PHB synthesis in this strain.

1.3.11. The Role of PHB in Nutrient Removal

PHB and Denitrification

Very few reports are available on the use of PHB in nitrogen removal by activated sludge. Alleman and Irvine (1980) recognised that operating strategies intended to promote cellular storage, such as that offered by the SBR, may consequently reduce or eliminate the costly necessity for a supplemental carbon addition. These authors measured denitrification rates in an SBR after allowing the biomass to store different amounts of organic carbon (up to 750 mg L^{-1} BOD). When only 50 mg L^{-1} carbon was stored, the rate of denitrification was 4 mg N $g^{-1}X$ h^{-1}, which was similar to rates observed when soluble carbon (as tryptose soya broth, BOD 400 mg L^{-1}) was available (5 mg N $g^{-1}X$ h^{-1}). The denitrification rate reached saturation at 250 mg L^{-1} BOD of stored substrate, and the rates were higher than observed with soluble substrate (8.4 mg gX^{-1} h^{-1}). Jones et al. (1990b) observed similar denitrification rates using sequestered organics, ranging from 3.8 – 4.2 mg N g X^{-1} h^{-1}. The lowest rate was correlated with the test that was exposed to the longest aeration time (45 minutes), which was presumed to lower the amount of stored substrate available for denitrification. Another study by Beun et al. (2001) found that storage and subsequent PHB degradation benefited denitrification. However, to date these are the only studies available linking together nitrogen removal and PHB degradation.

1.4. The Anammox Process for Nitrogen Removal

As highlighted in earlier sections, complete nitrogen removal from wastewater can be limited by the availability of sufficient electron donor in the form of organic substrate. Provided the process is controlled appropriately, complete nitrogen removal can be achieved with a carbon to nitrogen (C:N) ratio of 7:1 (Pochana and Keller, 1999). However, there are many types of wastewater that have a high ammonium and very low organic carbon content, resulting in low C:N ratios (< 1:1). In these cases, conventional nitrification-denitrification is uneconomical, due to the requirement for large quantities of carbon supplements. This has caused researchers to search for novel nitrogen removal processes in recent years. A major breakthrough came in 1986, with the discovery of Anammox, a completely autotrophic nitrogen removal process.

1.4.1. A Short History of Anammox

ANaerobic AMMonium OXidation (Anammox) is the microbiological conversion of ammonium and nitrite to dinitrogen gas in the absence of organic carbon (Eq 10). It is a very recent addition to our understanding of the biological nitrogen cycle and is so far the most unexplored part of nitrogen metabolism. The Anammox reaction was first predicted to be capable of supporting autotrophic growth by Engelbert Broda in 1977, based on the exergonic Gibbs free energy of the reaction (-357 kJ/mol) in comparison to aerobic ammonium oxidation (-118 kJ/mol). It was not until 10 years later (1986) that the Anammox process was discovered by Arnold Mulder in a denitrifying pilot plant for the treatment of wastewater from the Gist Brocades yeast factory in Delft, the Netherlands (Mulder et al., 1995). For almost a century, the nitrogen cycle had been considered completed: ammonium could not be oxidised under anoxic conditions. Research of the last five years has proved this is not the case.

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad \text{(Eq 10)}$$

Since the introduction of nitrogen removal from wastewater in the 1960's, ammonium removal has been engineered based on traditional microbiological knowledge of the nitrogen cycle. Initially, the cumbersome process of nitrification-denitrification that microbiology seemed to require puzzled the wastewater engineers. Focht and Chang (1975) wrote "… this seemingly peculiar aspect of the nitrogen cycle necessitates that ammonium must first be oxidised to nitrite or nitrate and then be subsequently reduced

to produce molecular nitrogen …". In contrast to the lengthy conventional nitrogen removal process, the anammox process converts ammonium directly into dinitrogen gas, without the requirement for an organic electron donor (Fig 1.8).

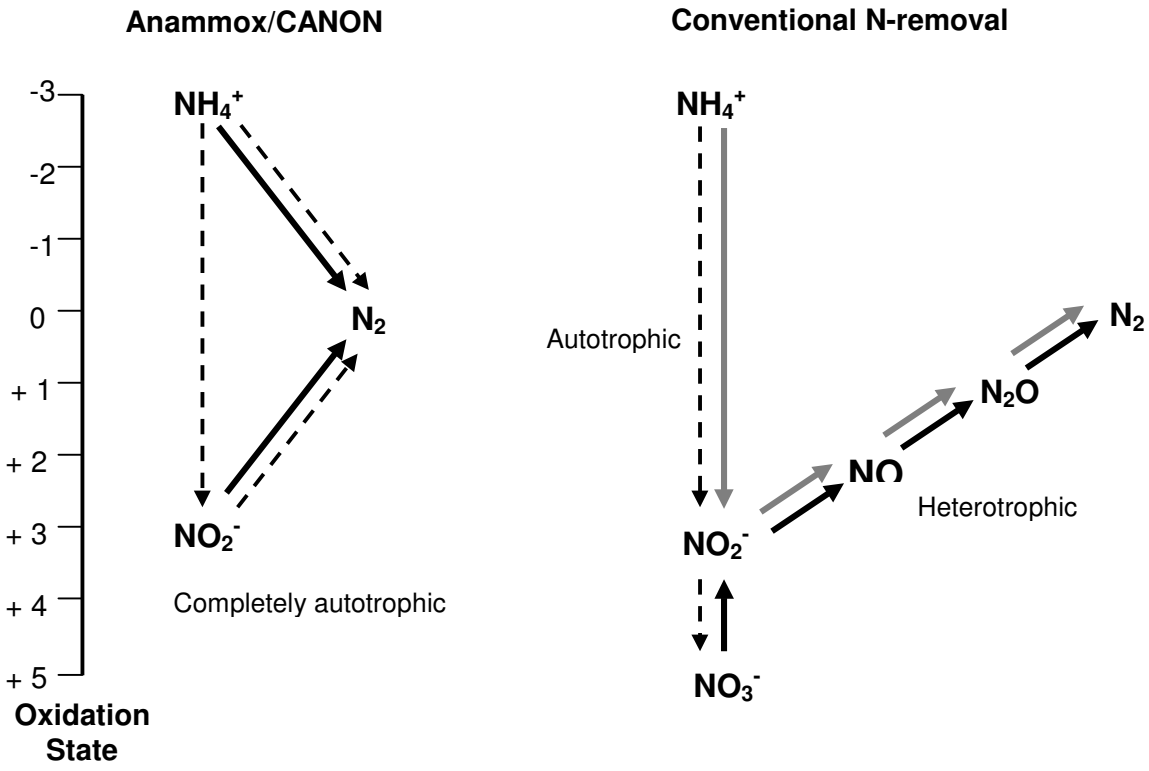

Figure 1.8: Nitrogen removal by; Left: The Anammox (solid line) and CANON processes (dotted line, i.e. nitrification and Anammox) as opposed to; Right: Conventional nitrogen removal via nitrification (dotted line) and denitrification (solid line). Grey arrows represent N-removal via nitrite (used in SND via nitrite).

After the initial discovery of Anammox, nitrate was believed to be the electron acceptor for ammonium oxidation. Only after the role of nitrite was recognised, a closer investigation of the microorganism was possible (Van de Graaf et al., 1996, 1997). A bottleneck in the progress of microbiological research into the Anammox process was the lack of a pure culture of the responsible organism. All initial attempts to purify the microorganism failed, since up to 80 % of the Anammox activity was lost after taking the biomass from the enrichment reactors (Van de Graaf et al., 1996). The available microbiological cultivation techniques were not designed to deal with very slowly growing microorganisms. Enrichment and study of the Anammox organisms demanded a new experimental approach. Due to the very low maximum growth rate of Anammox

cells (0.003 h^{-1}), an enrichment culture with full biomass retention was required. Strous (2000) applied the sequencing batch reactor (SBR) to successfully enrich an exponentially growing Anammox culture and was able to produce large quantities of Anammox biomass of relatively defined microbial composition. This enabled physiological study of the bacteria, leading to the discovery of the stimulatory role of hydrazine (N_2H_4) and the reversibly inhibitory role of oxygen. The enrichment culture also enabled the discovery that nitrite serves not only as the electron acceptor, but also as the electron donor for the formation of biomass from carbon dioxide. Hence, the formation of nitrate in the reaction (Eq 10) is stoichiometrically coupled to growth (Strous, 2000).

It was attempted to serially dilute the cells from SBR aggregates and incubate them aseptically under anoxic conditions to isolate a single Anammox cell, yet this failed to lead to isolation of any Anammox microbe. Finally, a physical purification method was applied, in which mild sonication and density gradient centrifugation was combined to purify the dominant morphotypical cells from the heterogeneous aggregates obtained from the SBR. After optimisation, the maximum purification of Anammox achieved was 99.5 – 99.8 %. The activity of these purified cells was dependent on cell density and on the addition of trace amounts (50 μM) of either hydrazine or hydroxylamine. Anammox was found to be incapable of growing in 100 % pure culture (Strous, 2000). The dominant 16S rDNA sequence of the purified organism was analysed phylogenetically and it was shown that the new bacterium was a new, deep-branching planctomycete. The microorganism was named Candidatus *Brocadia anammoxidans*. Since then, a different genus of Anammox organisms growing in biofilms (Munich, Germany) has been genetically identified and named Candidatus *Kuenenia stuttgartiensis* (Schmid et al., 2000). Of the twenty-five 16S rDNA fragments amplified from the biofilm, nine were affiliated to the Planctomycetales. Comparative analysis showed that those sequences were more than 98.9 % similar to each other, but only distantly related to *Brocadia anammoxidans*, even though they belong to the same phylogenetic branch. Since the discovery of Anammox, several reports have appeared in which Anammox activity has been detected, such as in marine sediments in the Baltic North-Sea (Dalsgaard and Thamdrup, 2002). Anammox has recently been enriched from municipal wastewater sludges in Sydney, Australia (Toh et al., 2002a) and applied

to treatment of high-ammonium containing wastewater from coke ovens (Toh and Ashbolt, 2002b).

1.4.2. Application of Anammox and the Development of the CANON Process

Anammox is particularly suited to the treatment of high-strength industrial wastewaters in which the ammonium content is high and the organic carbon content very low. By means of a mass balance, Strous (2000) showed that in biomass treating wastewaters containing organic substrates that allow heterotrophic growth, Anammox bacteria would comprise a very minor portion of the biomass. Up to now, most applied Anammox research has been directed towards ammonium removal from liquid effluents of sludge digesters (Jetten et al., 1997a). These secondary wastewater streams are generally returned to primary treatment and can make up 20 % of the total N-load. Since these effluents are warm, concentrated (0.5 – 2 g N L^{-1}) and contain no organic electron donor, they may be treatable by the Anammox process (Jetten et al., 1999). Until recently, the physical method of steam stripping of NH_3 has been the method of choice for ammonium removal from industrial wastewater. Conventional biological treatment of such wastewaters was not competitive because there was often insufficient carbon source available in the water to support complete denitrification. Supplementation of the water with methanol has been applied, but is relatively expensive (Strous, 2000). With the development of the Anammox process, a new opportunity has been created for biological nitrogen removal from industrial wastewater (Mulder et al., 1995, Van Loosdrecht and Jetten, 1998). However, the Anammox reaction by itself requires not only ammonium, but also even higher amounts of nitrite (Eq 10). The need to treat waste streams containing concentrated ammonium only has led to the development of processes such as SHARON-Anammox (Hellinga et al., 1998, Van Dongen et al., 2001), CANON (Strous, 1997, Sliekers et al., 2002a) and OLAND (Kuai and Verstraete, 1998). The result in all cases is the completely autotrophic oxidation of ammonium to dinitrogen gas and small amounts of nitrate (13 %).

In the SHARON-Anammox process (Single reactor High activity Ammonia Removal Over Nitrite), the Anammox process (requiring NH_4^+ and NO_2^-) is preceded by a partial nitrification step (producing NH_4^+ and NO_2^-). In the reactor designed for partial nitrification, use of high temperatures and short residence times (< 1 day) ensures the aerobic nitrite oxidisers are washed out of the system (Hellinga et al., 1998). The

CANON process (Completely Autotrophic Nitrogen-removal Over Nitrite) performs both Anammox and partial nitrification in the same reactor simultaneously, under oxygen-limitation. The process relies on the simultaneous interaction of the aerobic and anaerobic ammonium oxidisers. The nitrite produced by the aerobic ammonium oxidisers is used by Anammox, resulting in oxidation of ammonium to dinitrogen gas under O_2-limitation (Eq 11):

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \qquad \text{(Eq 11)}$$

The process can be initiated simply by introducing small amounts of oxygen into an active Anammox population (Sliekers et al., 2002a). Nitrogen removal rates of up to 1.5 kg N m^{-3} day^{-1} can be achieved with the CANON process (Sliekers et al., 2002b). However, very little is currently known about its optimisation and consequently its large-scale application has not yet been realised.

The OLAND process (Oxygen Limited Autotrophic Nitrification Denitrification) also achieves almost complete ammonium removal to dinitrogen gas, but uses normal nitrifying sludge as the biocatalyst. The process makes use of the ability of *Nitrosomonas* cells to denitrify using ammonium as the electron donor and nitrite as the electron acceptor, together with their ability to perform anaerobic ammonium oxidation in the presence of gaseous NO_2 (Bock, 1995, Schmidt and Bock, 1997). Under oxygen-limitation, ammonium is oxidised to nitrite and, due to the shortage of electron acceptor, the cells consume their own nitrite in order to oxidise another mole of ammonium. This process of oxidative-reductive N-removal is referred to as nitrite dismutation (Verstraete and Phillips, 1998). However, the maximum rates of the OLAND process reported to date are relatively low: 0.05 kg N m^{-3} day^{-1} in an SBR (Kuai and Verstraete, 1998) and 0.0015 kg N m^{-2} day^{-1} in a rotating biological contactor (Pynaert et al., 2002). The overall savings achieved by the above-mentioned autotrophic N-removal processes amount to 58 % reduction in aeration costs and 100 % saving in electron donor requirement (Eqs 1 - 7 versus Eq 11).

Derivation of Thesis Objectives

While there has been considerable research conducted into the separate areas of SND and PHB storage, there is clearly a gap in our understanding of the link between the two subjects. Rates of SND achievable on defined stored substrates are not available and the optimisation of SND, using PHB or other storage products as the electron donor, is an unexplored area of research. As autotrophic NH_4^+-oxidation is a relatively slow process in comparison to heterotrophic oxidation processes, the presence of a slowly degradable carbon source during aeration, such as PHB, should increase the availability of reducing power for simultaneous denitrification at low DO concentrations. Previous research has proven the economical advantages of SND for nitrogen removal, however there is a requirement for the study of PHB-driven SND if the concept is to be applied on large-scale.

The objectives of this thesis run along two main research themes. The first is the study of SND for nitrogen removal in an SBR. The aim is to increase understanding of the kinetics of heterotrophic PHB metabolism and the limiting factors of SND using PHB as the electron donor and to then use this improved understanding to develop a suitable on-line control strategy for optimisation of N-removal in a laboratory-SBR, via management of the oxygen supply. Oxygen represents a major fraction of wastewater treatment plant costs and is thus a critical parameter to manage. The second research theme is the study of the CANON process as an alternative to the use of conventional activated sludge. CANON allows nitrogen removal with even less oxygen requirement than conventional activated sludge processes, without the addition of organic carbon and is therefore potentially a viable technology for treatment of low-COD wastewaters.

The specific objectives of this thesis are:

- Develop a suitable automated sequencing batch reactor, with appropriate process monitoring, that can be continuously operated for extended periods of time. As the SBR system is differentiated into different phases by time, this should allow the investigation of different microbial activities, such as acetate uptake and conversion to PHB (feast phase), PHB hydrolysis and consumption (famine phase), nitrification and denitrification (and SND).

- Determine the oxygen requirement during the feast phase for maximum conversion of acetate to PHB, to maximise SND in the following famine phase.
- Generate sufficient understanding of the kinetics of acetate consumption and PHB production in order to develop a structured metabolic model for prediction of reactor behaviour.
- Determine the oxygen requirement for SND during the famine phase, in order to maximise the amount of PHB used for NO_x^- reduction (instead of aerobic oxidation), while still enabling complete nitrification.
- Establish the optimal length of the SBR aerobic phase such that nitrification is completed, yet total aeration time is minimised.
- Develop an on-line process control technology that uses the understanding generated to optimise N-removal performance.
- Establish the extent to which the previous "history" of the biomass affects the nitrogen removal capacity.
- Examine the autotrophic CANON system as the extreme low-oxygen, high ammonium treatment process, as an alternative to activated sludge and to achieve a direct comparison between the efficiency of conventional nitrogen removal vs the CANON process.

CHAPTER 2

The Effect of Dissolved Oxygen on PHB Accumulation in Activated Sludge Cultures[1]

Abstract

Nitrogen removal from wastewater is often limited by the availability of reducing power to perform denitrification, especially when treating wastewaters with a low carbon to nitrogen ratio. In the increasingly popular sequencing batch reactor (SBR), bacteria have the opportunity to preserve reducing power from incoming chemical oxygen demand (COD) as poly-β-hydroxybutyrate (PHB). The current study uses laboratory experiments and mathematical modelling in an attempt to generate a better understanding of the effect of oxygen on microbial conversion of COD into PHB. Results from a laboratory SBR with acetate as the organic carbon source showed that the aerobic acetate uptake process was oxygen-dependent, producing higher uptake rates at higher dissolved oxygen (DO) supply rates. However at the lower DO supply rates (k_La 6 – 16 h^{-1}, 0 mg L^{-1} DO) a higher proportion of the substrate was preserved as PHB than at higher DO supply rates (k_La 30, 51 h^{-1}, DO > 0.9 mg L^{-1}). Up to 77 % of the reducing equivalents available from acetate were converted to PHB under oxygen limitation ($Y_{PHB/Ac}$ 0.68 Cmol/Cmol), as opposed to only 54 % under oxygen-excess conditions ($Y_{PHB/Ac}$ 0.48 Cmol/Cmol), where a higher fraction of acetate was used for biomass growth. It was calculated that by oxygen management during the feast phase, the amount of PHB preserved (1.4 Cmmol L^{-1} PHB) accounted for an additional denitrification potential of up to 18 mg L^{-1} nitrate-nitrogen. The trends of the effect of oxygen (and hence ATP availability) on PHB accumulation could be reproduced by the simulation model, which was based on biochemical stoichiometry and maximum rates obtained from experiments. Simulated data showed that at low DO concentrations, the limited availability of ATP prevented significant biomass growth and most ATP was used for acetate transport into the cell. In contrast, high DO supply rates provided surplus ATP and hence higher growth rates, resulting in decreased PHB yields. The results suggest that oxygen management is crucial to conserving reducing power during the feast phase of SBR operation, as excessive aeration rates decrease the PHB yield and allow higher biomass growth.

[1] This chapter is published in Biotechnology & Bioengineering 82 (2) 238-250.

2.1. Introduction

The sequencing batch reactor (SBR) is increasingly being considered the preferred wastewater treatment reactor system for improved nutrient removal. If conditions are controlled appropriately, removal of all nutrients including nitrogen, phosphorous and organic carbon can be achieved in a single reactor. The most distinct difference between the SBR and a conventional plug-flow activated sludge system is the dynamic and periodic nature of the sequencing batch reactor. The biomass is exposed to fluctuating concentrations of electron donors and acceptors over time, which is often referred to as "feast and famine" conditions (Krishna and Loosdrecht, 1999a, Van Loosdrecht et al., 1997, Beun et al., 2000a). It has been recognised in the last decade that bacterial populations adopt a survival strategy when exposed to feast and famine conditions, whereby they store a large fraction of the soluble substrate, when available, as storage polymers such as poly-β-hydroxybutryic acid (PHB). The production of reduced storage polymers is thought to serve as an NADH-overflow mechanism to control the redox state of heterotrophic cells during unbalanced growth conditions (Senior and Dawes, 1973, Van Niel et al., 1995) (Fig 2.1). In the SBR, bacteria capable of storing substrate have a competitive advantage because they are able to balance their growth under continuously changing conditions (Van Loosdrecht et al., 1997).

In the absence of oxygen, or under microaerophilic conditions, storage polymers can serve as the electron donor for denitrification when all external substrate has been depleted (Jones et al., 1990a, Jones et al., 1990b, Beun et al., 2001). Alleman and Irvine (1980) recognised that operating strategies intended to promote cellular storage, such as that offered by the SBR, may reduce or eliminate the costly necessity for a supplemental carbon addition that is often required when treating wastewaters with a low carbon to nitrogen ratio. In conventional wastewater treatment systems where storage does not play a significant role, readily biodegradable organic substrate is rapidly oxidised during the aeration phase and plays a minor role in nitrogen removal. Bacterial storage of organic substrate in dynamic systems represents a convenient means of conserving reducing power. It is generally accepted in the literature that the aerobic respiration rate of stored substrate is much lower than the respiration rate of soluble substrate (Beccari et al., 1998, Carta et al., 2001, Beun et al., 2000a, Majone et al., 1999, Dircks et al., 1999). For example, the Activated Sludge Model 3 (ASM3)

models the aerobic respiration rate of PHB as equal to the endogenous respiration rate (0.2 day^{-1}) (Gujer et al. 1999, Krishna and Loosdrecht, 1999a). This effectively means that the rapid oxidation of organic substrate to carbon dioxide is slowed down by its storage as PHB. The idea of slowing down organic carbon oxidation is that when the aeration phase is complete, reducing power can still be available for denitrification. Alternatively, it can be used as the electron donor during simultaneous nitrification and denitrification (SND) at low dissolved oxygen concentrations during the aeration phase (Beun et al., 2001). Up to 100 % nitrogen can be removed via SND when sufficient reducing power is available during the aerobic phase of an SBR (Pochana Keller, 1999). Reducing power could be preserved through storage as a polymer, however this potential has not been quantitatively investigated in the literature.

Heterotrophic PHB-storing bacteria in activated sludge can use organic carbon in the influent wastewater either to form new biomass or to store the reducing power as PHB, while oxidising a small fraction for energy generation. In terms of nitrogen removal, the most efficient metabolic route would be to convert reducing power to PHB for use as the electron donor during SND. The use of substrate for biomass growth is undesirable, as microbial growth plays an insignificant role in nitrogen removal. In addition, it is desirable to limit heterotrophic growth due to the large expense associated with excess sludge treatment. It is therefore important to operate the fill phase such that the maximum amount of reducing equivalents from the substrate are stored for nitrogen removal, rather than either being oxidised by oxygen or assimilated into biomass. PHB metabolism in mixed cultures has been studied intensively in recent years (Majone et al., 1996, Van Loosdrecht et al., 1997, Beun et al., 2000a, 2002, Beun, 2001), yet little attention has been paid to the effect of the oxygen supply rate on acetate conversion to PHB. This study investigates the production of PHB by a mixed culture in a laboratory SBR using acetate as substrate. The objective was to establish the effect of oxygen availability on acetate uptake and PHB production, to allow improved oxygen management for SBR wastewater treatment plants. Fully aerobic conditions are expected to oxidise too much acetate and insufficient oxygen may inhibit COD uptake. In order to verify whether established biochemical metabolic pathways of bacterial cells support the observed results, a simple fundamental model was developed and contrasted against experimental findings.

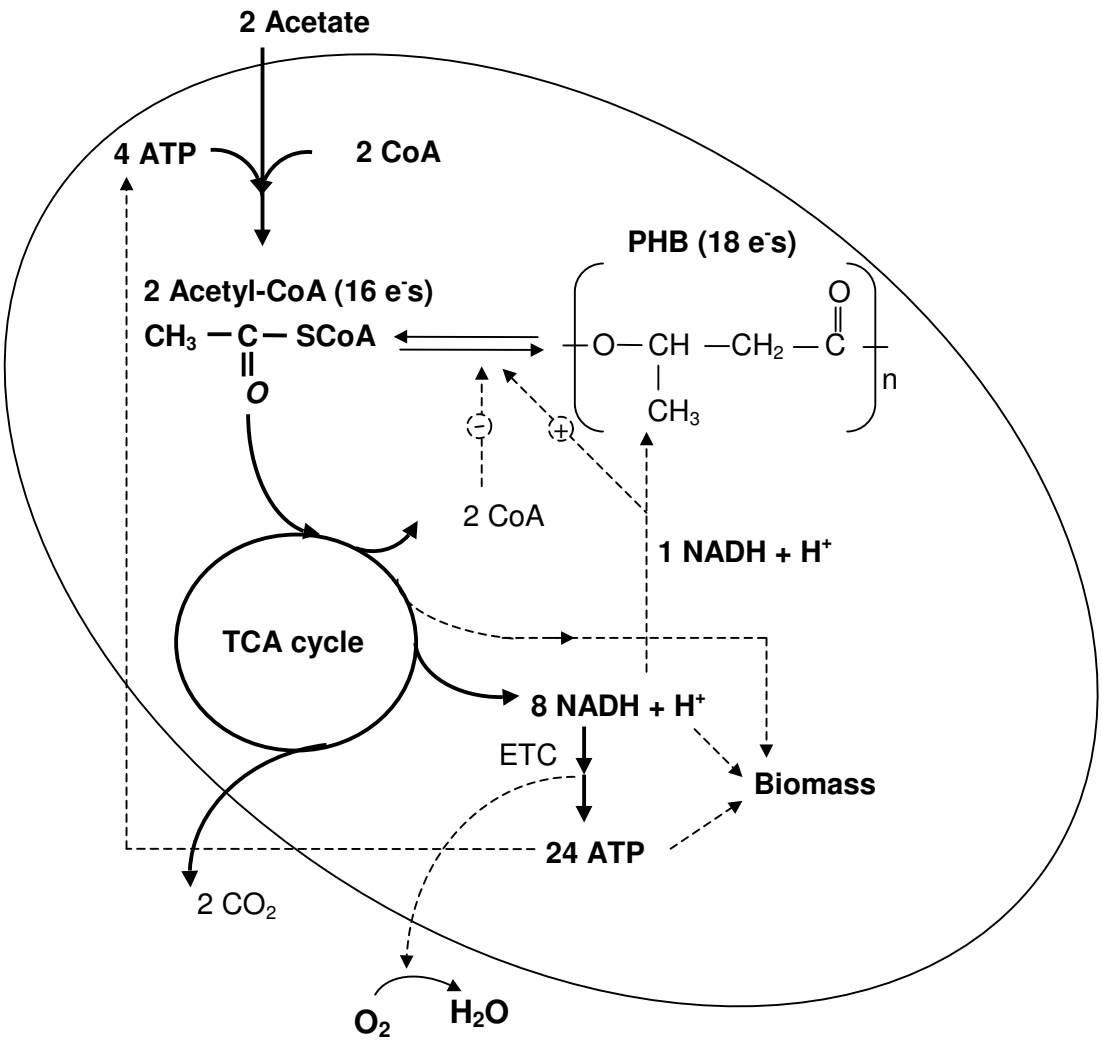

Figure 2.1: A diagramatic representation of the mechanism (as understood) for PHB production from acetate in heterotrophic cells. Bold parts of the diagram represent general metabolism of acetyl-CoA via the TCA cycle and electron transport chain (ETC) to generate energy (ATP) and biomass precursors used for growth. If the rate of the TCA cycle and respiration is slower than the rate at which acetate is being brought into the cell, NADH and acetyl-CoA accumulate. PHB acts as an electron- and acetate-sink, producing a reduced storage polymer that can be used in times of starvation. One mole of ATP is required to transport one carbon molecule of acetate into the cell and activate it to acetyl-CoA (Stouthamer, 1973, Beun et al., 2002). If oxidative phosphorylation operates at maximum theoretical efficiency (i.e. 3 moles ATP per mole of reducing equivalents) (Lehninger et al., 1993), 6 moles of ATP are produced from 1 Cmol of acetate. The energy required for transport and activation of acetate corresponds to 16.7 % of the ATP budget (i.e. 1/6 ATP per Cmol Ac). Therefore, at least 16.7 % of acetate must be oxidised to enable substrate import and storage as PHB.

2.2. Materials and Methods

2.2.1. Experimental Setup

A sequencing batch reactor (SBR) with a working volume of 2 L was used for all experiments (Fig 2.2). The glass reactor (height 0.25 m, diameter 0.15 m) was fitted with a fermentor lid containing a turbine propeller stirrer, a feed inflow tube, an air line with attached bubble diffuser, a dissolved oxygen probe, pH probe, oxidation-reduction potential (ORP) probe, acid and base inflow tubes for pH control, a temperature sensor and heating element, and an effluent withdrawal line. Temperature was maintained constant at 25^{o}C. Dissolved oxygen was measured using an InPro 6000 polarographic oxygen sensor (Mettler Toledo, Australia). The airflow was maintained constant at the required airflow rate by a needle valve mass-flow controller attached to the main airline. The stirring rate was maintained constant in all experiments at 80±5 rpm, except where the k_La was required to be much higher, when it was increased up to 200 rpm. The lower stirring speed (80 rpm) was found to be low enough to maintain efficient floc formation (floc diameter 0.5 - 2 mm, SVI < 100 mL g X^{-1}). Where the biomass was blended to allow maximum oxygen transfer to the cells, this is specified in the results section. The k_La for all experiments ranged from 6 to 51 h^{-1}, and was measured before the start of each experiment (see k_La determination).

The SBR was operated continuously in cycles of around 400 minutes. One standard cycle consisted of a rapid fill phase (3 mins), an aeration phase (240 mins), an anoxic phase (120 mins), a settle phase (17 mins), and a 20 min effluent withdrawal phase, referred to as "standard conditions". The SBR was completely automated, with all pumps, stirrers, airflow valves and phase lengths controlled by National Instruments instrumentation control software LabView^{TM} (Version 6). Dissolved oxygen (DO), pH and redox potential were monitored continuously on-line and the data was logged into a spreadsheet. LabView^{TM} was used to create the metabolic model used to simulate experimental results. The dry weight and the Sludge Volume Index (SVI) of the sludge were measured according to Standard Methods (Greenberg et al., 1992). The sludge retention time (SRT) was calculated from the amount of excess sludge in the effluent. Sludge from a conventional activated sludge wastewater treatment plant (Subiaco, Western Australia) was used as inoculum. A sludge blanket of 800 mL was inoculated into the reactor, and the remaining 1.2 L was filled with feed during subsequent cycling.

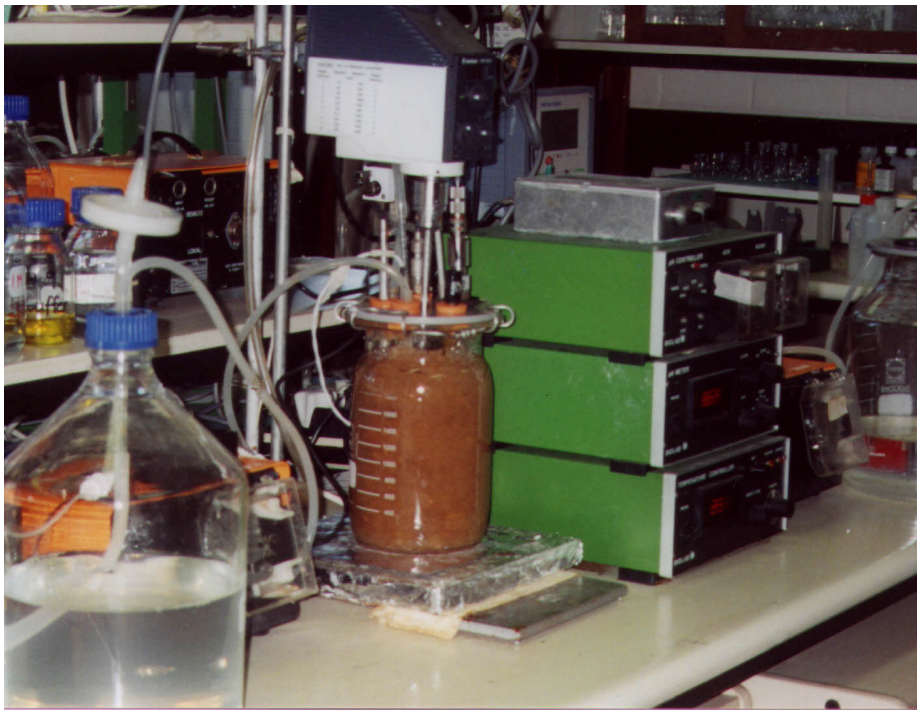

Figure 2.2: Experimental setup used for all experimental work in Chapters 2 – 5, showing the fully automated 2 L SBR with on-line monitoring of DO, pH and oxidation-reduction potential (ORP), temperature control (25 ^{o}C) and DO control.

2.2.2. Synthetic Wastewater

The standard composition of the nutrient medium was (mg L^{-1}): CH_3COONa 500, NH_4Cl 125, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4.7H_2O$ 51, $CaCl_2.2H_2O$ 300, $FeSO_4.7H_2O$ 6.25, yeast extract 50 and 1.25 mL L^{-1} of trace element solution. Where higher concentrations of nutrients were used, the concentrations are specified in the results section. The medium was autoclaved to prevent bacterial activity in the feed vessel. The trace element solution was added after autoclaving and contained (g L^{-1}) (adapted from Strous, 2000): EDTA 15, $ZnSO_4.7H_2O$ 0.43, $CoCl_2.6H_2O$ 0.24, $MnCl_2.4H_2O$ 0.99, $CuSO_4.5H_2O$ 0.25, $NaMoO_4.2H_2O$ 0.22, $NiCl_2.6H_2O$ 0.19, $NaSeO_4.10H_2O$ 0.21, H_3BO_4 0.014 and $NaWO_4.2H_2O$ 0.050.

2.2.3. Sampling and Analytical Procedures

Nitrogen Compounds: All nitrogen compounds were measured spectrophotometrically. Nitrate was measured at 420 nm after reaction (10 min) of 40 µL sample (containing 0.1 to 5 mM nitrate) with 10 µL saturated ammonium amidosulphonate (to remove nitrite), 0.2 mL 5% (w/v) sodium salicylate in 98% sulphuric acid and 2 mL cold (4 ^{o}C) 4 M

NaOH (Cataldo et al., 1975). Nitrite was measured at 540 nm after reaction (30 min) of 1 mL sample (containing 0.002-0.05 mM NO_2^-) with 1 mL 1 % (w/v) sulphanilic acid in 1 M HCl and 1 mL 0.1 % N-1-naphtylethyldiamine dihydrochloride in H_2O (Strous, 2000). Ammonium was determined by the Nesslerization method (Greenberg et al., 1992). The sample was diluted to between 0.01 – 0.2 mM NH_4^+, and then pre-treated with mineral stabiliser (10 µL) and a polyvinyl alcohol-dispersing agent (10 µl) (supplied by Hach™) to inhibit precipitation of calcium, magnesium, iron and sulphide when treated with the Nessler reagent. A 2 mL sample was reacted (1 min) with 80 µL of Nessler reagent (10 % (w/v) HgI_2 and 7 % (w/v) KI in 4 M NaOH) and the absorbance read at 425 nm.

Phosphorous: Soluble orthophsophate was determined by the ascorbic acid method (Greenberg et al., 1992). A 10 mL sample was reacted (2 mins) with a reagent powder pillow (Hach™) containing ammonium molybdate, potassium antimonyl tartrate, and ascorbic acid. A phosphomolybdic acid complex forms in this method that is then reduced to molybdenum blue by ascorbic acid. The absorbance at 890 nm was directly related to the phosphorous concentration.

Acetate and Poly-(β)-Hydroxybutyric Acid: The acetate concentration in the supernatant and the PHB content of lyophilised biomass were measured by gas chromatography (GC) using a Varian Star 3400 Gas Chromatograph with autosampler. The PHB content of biomass was determined by the method developed in Smolders et al. (1994). The PHB was extracted, hydrolysed and esterified in a one-step digestion mixture containing HCl, 1-propanol and dichloroethane at 150 ^oC for 2 hours. The resulting organic phase was extracted with deionised water to remove free acids, dried over water-free Na_2SO_4 and analysed by GC using benzoic acid as an internal standard. Hydroxybutryic acid standards (1-5 mM) for calibration were prepared from the sodium salt of L-(+)-*β*-hydroxybutyric acid (Sigma H3145).

2.2.4. Calculations

For the purpose of all calculations involving biomass, the exact composition of the biomass was not measured, but one carbon mole was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (Ratledge and Kristiansen, 2001).

Determination of the Volumetric Oxygen Transfer Coefficient (k_La) and Oxygen Uptake Rate (OUR)

The k_La was measured experimentally before each test at the specific operating stirrer speed, airflow rate and mixed liquor viscosity. The culture was aerated until a steady state of oxygen was reached. Under steady state conditions the oxygen transfer rate (OTR) into solution is equal to the oxygen uptake rate (OUR) of the biomass. To determine the oxygen uptake rate of the culture, the air was then switched off and the rate of decrease in dissolved oxygen measured. The k_La could then be calculated according to Eq 1.

$$k_L a(h^{-1}) = \frac{OTR}{(c_S - c_L)} \qquad \text{(Eq 1)}$$

where the OTR is the oxygen transfer rate (mg O_2 L^{-1} h^{-1}), which is equal to the OUR at steady state, c_S the saturating concentration of oxygen in the gas phase (mg L^{-1}) and c_L the concentration of oxygen in the liquid phase (mg L^{-1}). The value obtained by this method was verified by the de-gassing method for k_La determination, in which the reactor was filled with water and the DO concentration reduced to zero by the addition of a small amount of sodium dithionite. The liquid was then aerated and the DO concentration measured over time. By plotting the OTR versus the saturation deficit ($c_S - c_L$) , the k_La could be determined from the slope of the curve (Eq 1). Once the k_La was known, the oxygen uptake rate could be calculated from steady state dissolved oxygen measurements according to Eq 2;

$$\text{OUR (mg } L^{-1} h^{-1}) = k_L a.\ (c_S - c_L) \qquad \text{(Eq 2)}$$

Calculation of Biomass PHB Content

GC measurements of PHB content were calculated as a PHB concentration per mass of cell matter analysed;

$$F_{PHB}(Cmol/Cmol) = \frac{PHB}{X}\left(\frac{Cmol.L^{-1}}{Cmol.L^{-1}}\right) \qquad \text{(Eq 3)}$$

The mass of dried cell matter analysed was measured as g L^{-1} on a four-point balance and converted to Cmol L^{-1} (21.5 g/Cmol X). The PHB fraction of active biomass (f_{PHB}) (i.e. biomass not including PHB) was calculated as;

$$f_{PHB}(Cmol/Cmol) = \frac{PHB}{X - PHB} \qquad \text{(Eq 4)}$$

The total concentration of PHB inside the reactor was calculated by multiplying the biomass PHB fraction (F_{PHB}) by the biomass concentration;

$$PHB(Cmol.L^{-1}) = F_{PHB}\left(\frac{CmolPHB}{CmolX}\right).X\left(\frac{CmolX}{L}\right) \qquad \text{(Eq 5)}$$

Calculation of the Observed Yield (Y_{OBS})

The fraction of substrate consumed for non-oxidative purposes (i.e. the total observed yield including PHB and biomass production) was calculated from the oxygen uptake rate (mg O_2 L^{-1} h^{-1}) and the acetate consumption rate expressed in terms of COD ($R_{Ac(COD)}$) (mg COD L^{-1} h^{-1});

$$Y_{OBS} = 1 - \left(\frac{OUR}{R_{Ac(COD)}}\right) \qquad \text{(Eq 6)}$$

Calculation of the Yield of PHB from Acetate ($Y_{PHB/Ac}$)

Where the concentration of PHB was measured, the fraction of substrate converted to PHB could be calculated. The total amount of PHB produced was calculated as the final concentration of PHB at the end of the acetate consumption period, minus the starting concentration of PHB inside the cells at time zero. The yield was calculated according to;

$$Y_{PHB}(Cmol/Cmol) = \frac{PHB_{produced}}{Acetate_{consumed}} \qquad \text{(Eq 7)}$$

where PHB and acetate concentrations are both expressed in Cmol L^{-1}. One carbon mole of acetate represents 4 electrons (i.e. analogous to the concept of a degree of reduction of 4), and one carbon monomer of hydroxy-butyric acid (HB) represents 4.5

electrons. Thus, the percentage of reducing equivalents transferred from acetate to PHB at the end of the feast phase was calculated as;

$$\%Ac_{PHB} = Y_{PHB} \cdot \left(\frac{4.5}{4}\right) x100 \qquad \text{(Eq 8)}$$

Calculation of the Biomass Growth Rate

Due to the large background of biomass and the resulting insensitivity of dry weight measurements over the time span of the experiments (15 – 180 minutes), growth rates were predicted indirectly from the electron balance, assuming biomass is more reduced than the substrate, acetyl-CoA. Acetate consumed by the bacteria is used for PHB production, oxidation or assimilation into biomass. The oxidation state of carbon in the assumed biomass molecule ($CH_{1.8}O_{0.5}N_{0.2}$) is –0.2 and therefore requires 4.2 electrons/Cmol for its production (degree of reduction 4.2). The rates of electron distribution were calculated from measurements of acetate, PHB and the specific oxygen uptake rate (SOUR) as follows;

Reaction	e- mol/Cmol X/h	
e- available from acetate	+4 (r_{Ac})	Measured
e- conserved as PHB	- 4.5 (r_{PHB})	Measured
e- for oxygen reduction (ox. phosph)	- 4 (SOUR)	Measured
e- used for X production	- 4.2 (r_X)	-

$$\text{Where } + (4.r_{Ac}) - (4.5.r_{PHB}) - (4.SOUR) - (4.2.r_X) = 0$$

$$r_X \text{ (Cmol X/Cmol X/h)} = [+ (4.r_{Ac}) - (4.5.r_{PHB}) - (4.SOUR)]/4.2 \qquad \text{(Eq 9)}$$

2.3. Results

The SBR was operated under standard conditions and monitored until a steady state was reached, as determined by a constant biomass concentration for more than seven days and reproducible behaviour of on-line data. The average sludge retention time (SRT) was 11 days, and the culture had been adapted to the dynamic SBR conditions for more than 8 weeks when the experiments were performed.

Typical Behaviour of Aerobic PHB Production under Standard Reactor Operating Conditions

Figure 2.3 shows acetate consumption and PHB production behaviour observed reproducibly during a standard SBR cycle. Of the reducing equivalents available from acetate, 75 % of the electrons were preserved as PHB at the end of the feast phase, resulting in a yield of PHB from acetate ($Y_{PHB/Ac}$) of 0.67 Cmol/Cmol. The rates of acetate consumption and PHB production were constant during the feast phase but PHB was degraded at a decreasing rate over the course of the famine period (Fig 2.3).

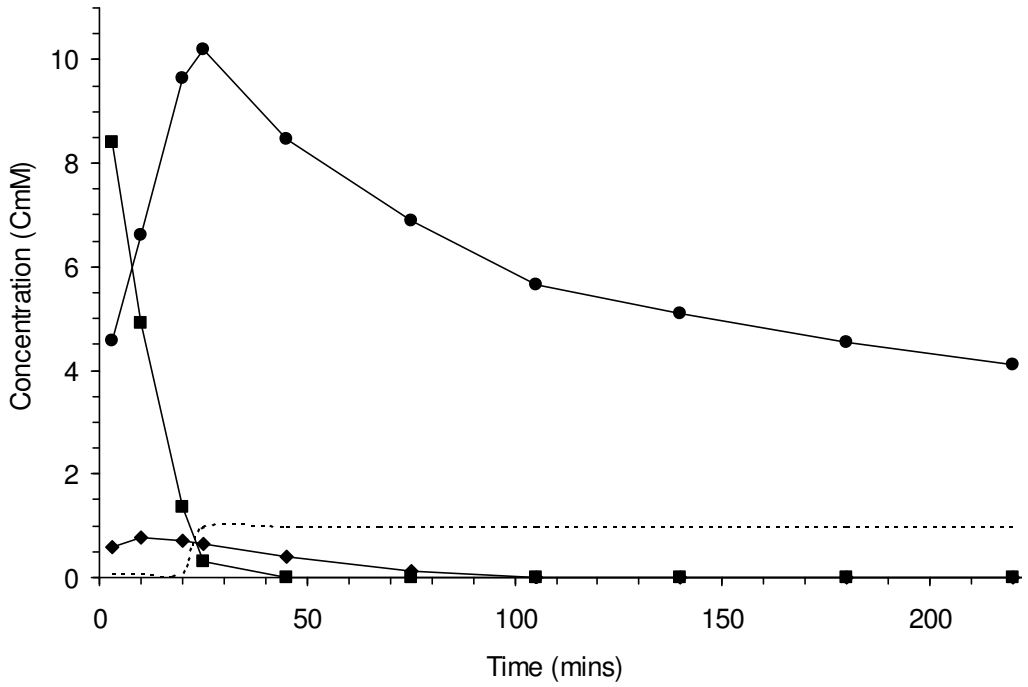

Figure 2.3: Acetate consumption and PHB production during an SBR cycle under standard operating conditions (k_La 16 h^{-1}): Acetate (■), PHB (●), phosphate (◆) and dissolved oxygen (dotted line). The SVI of the sludge in this cycle was 86 mL $g^{-1}X$.

Effect of the Oxygen Supply Rate on Acetate Conversion to PHB

To establish which conditions maximise the aerobic conservation of reducing power as PHB, the acetate consumption and PHB production kinetics were investigated under different aeration conditions (Fig 2.4, Table 2.1). All rates increased with increased oxygen supply to the biomass, showing that not only the acetate uptake rate, but also the PHB synthesis rate was limited by the oxygen supply. However, for effective preservation of reducing power as PHB it is the fraction of substrate converted to PHB, rather than the rate of the process that is important. The fate of the non-oxidised acetate changed markedly at the higher oxygen supply rates (k_La of 30 and 51 h^{-1}), where a higher proportion of the carbon from acetate was assimilated into biomass (Fig 2.5A). Only 54 – 55 % of the total reducing equivalents were conserved as PHB ($Y_{PHB/Ac}$ 0.48 – 0.49 Cmol/Cmol) as opposed to 71 – 78 % at the lower oxygen supply rates ($Y_{PHB/Ac}$ 0.63 – 0.68). It was found that the proportion of acetate oxidised, as derived from the electron flow to oxygen, was reasonably constant at around 20 % (Fig 2.5A). This was unexpected, as the production of biomass requires energy, thus one expects that if biomass production increases, oxygen consumption should also increase. The increased fraction of PHB preserved at lower DO concentrations represents 16 – 24 % of the influent COD (1.4 CmM PHB), which may be significant for improved nitrogen removal via SND, as the efficiency of SND increases with increased reducing power (Pochana and Keller, 1999). For the most efficient conversion of reducing equivalents to PHB a moderate dissolved oxygen supply appears to be appropriate, where the DO concentration may not be detectable in the bulk liquid.

The acetate consumption rate was far in excess of the acetate oxidation rate and the growth rate at all oxygen supply rates (Table 2.1). Under oxygen-excess conditions, the acetate uptake rate was 6 times higher than the oxidation rate (r_{O2}/r_{Ac} 0.17) and 4 times higher than the growth rate (r_X/r_{Ac} 0.27). This implies that even when growth is not limited, the cells accumulate large amounts of acetyl-CoA internally that encourage PHB production. However, the relative proportion of acetyl-CoA channelled into PHB production instead of biomass growth changes with increasing oxygen supply.

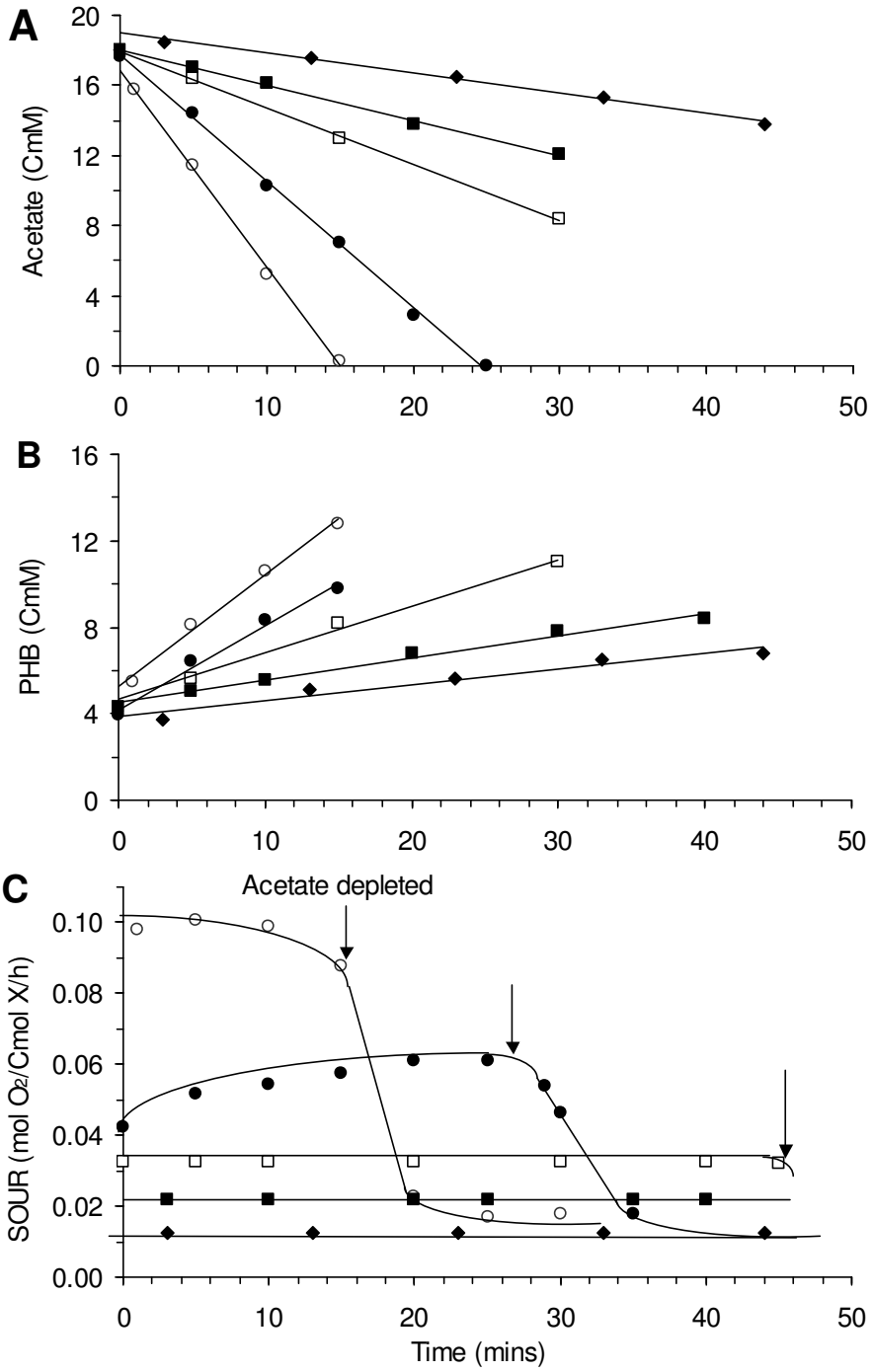

Figure 2.4: The effect of the oxygen supply rate during the filling phase of the SBR on; A: Acetate consumption, B: PHB production and C: Oxygen consumption (k_La: 6 h^{-1} (♦), 12 h^{-1} (■) 16 h^{-1} (□), 30 h^{-1} (●) and 51 h^{-1} (❍)). In the trial using k_La 51 h^{-1}, the biomass was blended to allow maximum oxygen transfer to the cells, decreasing the SVI from 91 mL g^{-1}X before blending to 290 mL g^{-1}X. At the lower k_La values (6, 12 and 16 h^{-1}), oxygen was not detectable in the bulk liquid (i.e. 0 mg L^{-1}), while at the highest oxygen supply rates (30 and 51 h^{-1}) the DO concentration was > 0.9 mg L^{-1}.

Table 2.1: Summary of aerobic acetate consumption and PHB production characteristics at different DO supply rates.

	Oxygen-limited (0 mg L^{-1})			DO > 0.9 mg L^{-1}	
	k_La 6 h^{-1}	k_La 12 h^{-1}	k_La 16 h^{-1}	k_La 30 h^{-1}	k_La 51 h^{-1}
r_{Ac} (Cmol. Cmol X^{-1}. h^{-1})	0.05	0.13	0.15	0.37	0.59
r_{PHB} (Cmol. Cmol X^{-1}. h^{-1})	0.03	0.09	0.09	0.20	0.28
r_{O2} (mol O_2. Cmol X^{-1}. h^{-1})	0.012	0.024	0.031	0.064	0.10
r_X (h^{-1})	0.01	0.02	0.03	0.09	0.16
r_{PHB}/r_{Ac}	0.60	0.65	0.63	0.55	0.47
r_{O2}/r_{Ac}	0.24	0.18	0.21	0.17	0.17
r_X/r_{Ac}	0.20	0.18	0.20	0.24	0.27
Y_{OBS} (Cmol/Cmol)	0.77	0.82	0.81	0.83	0.83
$Y_{PHB/Ac}$ (Cmol PHB/Cmol Ac)	0.63	0.65	0.68	0.49	0.48
$Y_{X/Ac}$ (Cmol X/Cmol Ac)	0.14	0.17	0.13	0.34	0.35

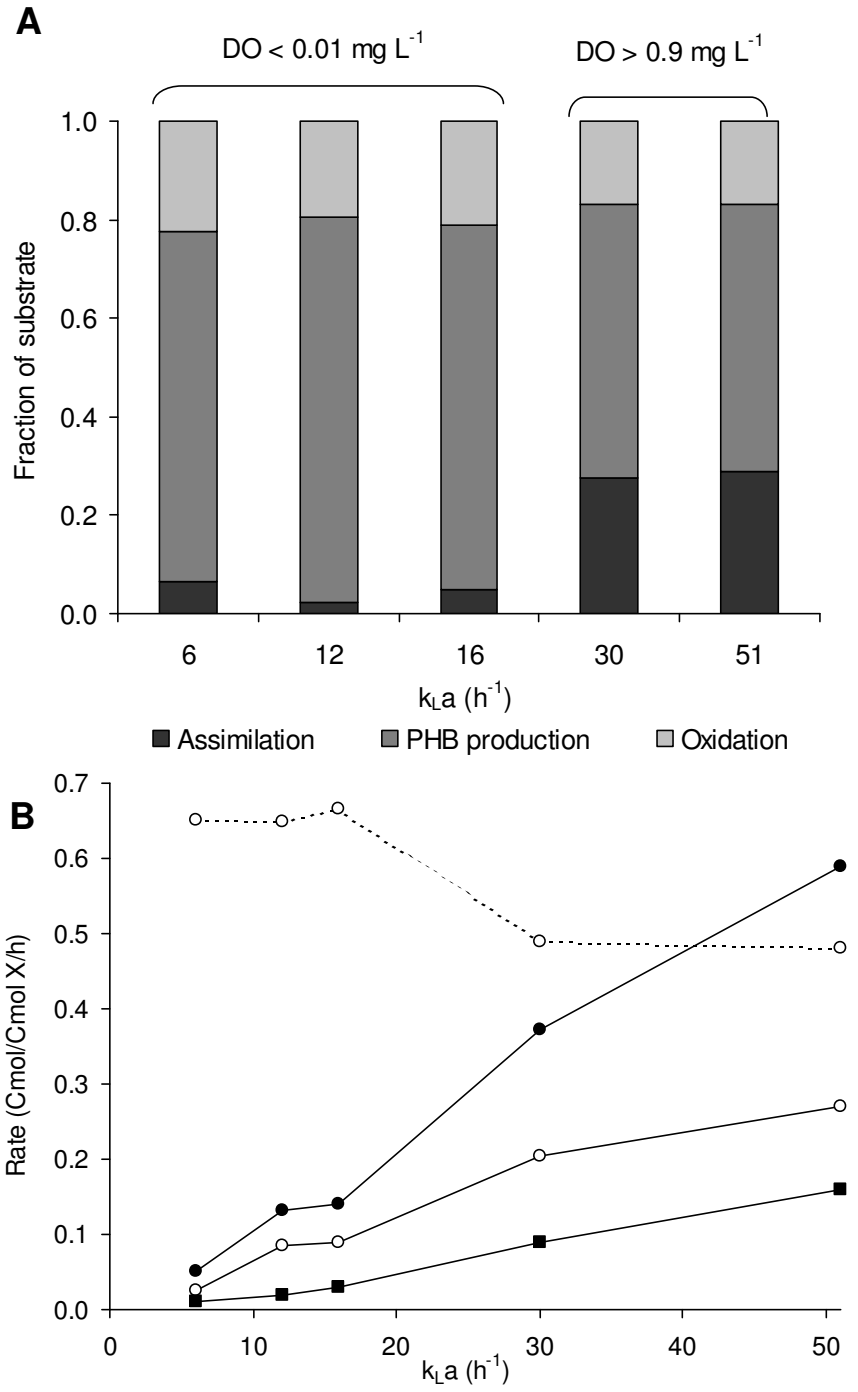

Figure 2.5: The effect of the oxygen supply rate during the filling phase of the SBR on; A: The distribution of acetate between PHB synthesis, growth and oxidation (given as a fraction of the total substrate consumed, based on reducing equivalents); B: Rates of acetate consumption (●), PHB production (❍), biomass growth (■) and PHB yield from acetate ($Y_{PHB/Ac}$) (dotted line).

Rationale for the Development of a Metabolic Model Based on Intracellular Metabolite Ratios

The objective was to use the simplest approach possible to produce a metabolic model that could explain the behaviour observed. In accordance with established literature, our model assumes that the enzymes for PHB biosynthesis are constitutive, and regulation does not occur through control of enzyme concentrations, but rather that enzyme activity is regulated through changes in metabolite concentrations (Haywood et al., 1988a, 1988b, 1989, Oeding and Schlegel, 1973, Anderson and Dawes, 1990, Leaf and Srienc, 1998, Van Wegen et al., 2001). It was assumed for simplicity that the cell maintains a constant pool size of the metabolic couples NADH + NAD^+, acetyl-CoA + CoA and ATP + ADP, and that it is the ratio of these couples (e.g. NADH/(NADH+NAD^+)) that influences the usage of acetyl-CoA by the different metabolic pathways. Research has shown that PHB metabolism is highly sensitive to these ratios in the cell (Van Wegen et al., 2001, Leaf and Srienc, 1998).

PHB production requires acetyl-CoA and reducing power (NADH), while biomass synthesis requires acetyl-CoA and ATP (and some NADPH). Thus, the distribution of acetyl-CoA between these two pathways should depend on the availability of NADH and ATP, respectively. In this manner, our model produces growth as the consequence of intracellular concentrations of growth metabolites. Hence the growth rate itself is not the cause of changes in cell behaviour, but rather it is the consequence of electron and carbon fluxes directed by kinetic and stoichiometric constants. This is in contrast to approaches based on Monod kinetics where the growth rate is determined from extra-cellular substrate concentrations. The model incorporated standard Michaelis-Menten kinetics for uptake of external substrates (acetate and oxygen), while internal intermediary steps were controlled by ratios of reaction couples that, due to the fixed pool size, have a value of between 0 and 1 (Table 2.2) and exhibit a 2^{nd} or 3^{rd} order effect (depending on the number of reactants) on the maximum rate.

Model Assumptions

- The transport of acetate and its activation to acetyl-CoA requires 1 ATP per carbon mole of acetate (Stouthamer, 1973, Van Aalst-Van Leeuwen et al., 1997, Beun et al., 2000a).
- The efficiency of oxidative phosphorylation operates at its theoretical maximum efficiency of 3 moles ATP produced per mole of reducing equivalents (NADH + H^+) passed through the electron transport chain (Lehninger et al., 1993).
- Nutrients for biomass growth (i.e. nitrogen, phosphorous etc) are not limiting.
- The total amount of ATP needed for the production of biomass from acetyl-CoA is 2.16 mol ATP/Cmol biomass (Stouthamer, 1973, Verduyn et al., 1991, Beun, 2001).
- Total PHB degradation is dependent only on the PHB concentration. Beun et al. (2002) showed that the specific rate of PHB degradation is first order and depends only on the fraction of PHB per unit of biomass (Cmol PHB/Cmol X). Thus, the total rate of PHB degradation (i.e. multiplied by the biomass concentration) is independent of biomass concentration.
- Intracellular metabolite concentrations are expressed in terms of mmol per litre of reactor volume, rather than specifying a certain cell volume. Thus the specific metabolite concentrations are expressed in terms of mol metabolite/Cmol biomass (adapted from Van Wegen et al., 2001).
- The cell maintains a constant pool size of NAD(P)H/($NAD(P)^+$+NAD(P)H), acetyl-CoA/(CoA+acteyl-CoA) and ATP/(ADP+ATP);

Pool	Pool size (μM) and literature source
$NAD(P)^+ + NAD(P)H + H^+$	300 (Van Wegen et al., 2001)*
Acetyl-CoA + CoA	1100 (Leaf and Srienc, 1998)
ATP + ADP	100 (Lehninger et al., 1993)

* Values determined during rapid PHB production in recombinant *E.coli*.

- There are no diffusion barriers in the form of flocs; all bacterial cells are freely suspended and exposed to the same oxygen concentration.
- PHB degradation has no ATP requirement, therefore the yield of biomass from PHB is the same as for direct growth on acetate.

Table 2.2: Stoichiometry of metabolic reactions involved in cellular growth and PHB metabolism and their rate equations incorporated into the metabolic model.

Process	Stoichiometries and Rate Equations
Acetate Uptake	0.5 Ac + 0.5 CoA + ATP → 0.5 AcCoA + ADP + P_i $-R_{Ac} = -r_{Ac}^{\max}.X.\left(\frac{Ac}{k_{s,Ac}+Ac}\right).\left(\frac{ATP}{ATP+ADP}\right).\left(\frac{CoA}{AcCoA+CoA}\right)$
Respiration	1 NADH + H^+ + 0.5 O_2 + 3 ADP + 3 P_i → 3 ATP + NAD^+ + H_2O $-R_{O_2} = -r_{O_2}^{\max}.X.\left(\frac{O_2}{k_{s,O_2}+O_2}\right).\left(\frac{NADH}{NAD^+ + NADH}\right).\left(\frac{ADP}{ATP+ADP}\right)$
Growth	0.5 AcCoA + 2.16 ATP → 1 Cmol X + 2.16 ADP + 2.16 P_i $R_X = r_X^{\max}.X.\left(\frac{AcCoA}{AcCoA+CoA}\right).\left(\frac{ATP}{ATP+ADP}\right)$
TCA Cycle	AcCoA + 4 NAD^+ + H_2O → 4 NADH + H^+ + 2 CO_2 $R_{TCA} = r_{TCA}^{\max}.X.\left(\frac{AcCoA}{AcCoA+CoA}\right).\left(\frac{NAD^+}{NADH+NAD}\right)$
PHB Production	0.5 AcCoA + 0.25 NADH + H^+ → 0.25 PHB + 0.5 CoA + 0.25 NAD^+ $R_{PHB.\Pr} = r_{PHB.\Pr}^{\max}.X.\left(\frac{AcCoA}{AcCoA+CoA}\right).\left(\frac{NADH}{NADH+NAD^+}\right).\left(1-\frac{f_{PHB}}{f_{PHB}^{\max}}\right)$
PHB Degradation	0.25 PHB + 0.5 CoA + 0.25 NAD^+ → 0.5 AcCoA + 0.25 NADH + H^+ $-R_{PHB.De} = -r_{PHB.De}^{\max}.(PHB).\left(\frac{CoA}{AcCoA+CoA}\right).\left(\frac{NAD}{NADH+NAD^+}\right)$

General Model Behaviour

Using the rate constants derived from experimental findings (Table 2.3), the simple biochemical model showed simultaneous acetate consumption, PHB production and biomass growth, at rates similar to those measured experimentally (Figs 2.6, 2.3). The effect of oxygen supply in the model reflected the trends in the experimental findings: between DO concentrations of 0.1 mg L^{-1} and 1.0 mg L^{-1} the yield of PHB decreased from 0.68 Cmol/Cmol, to 0.52 Cmol/Cmol (Figs 2.6, 2.7). It was interesting to note that the acetate consumption and PHB production rates reached saturation at a DO setpoint of around 0.75 mg L^{-1}, well before the biomass growth rate reached its maximum rate (> 2 mg L^{-1}) (Fig 2.7). According to the model, DO setpoints higher than 0.75 mg L^{-1} shift the metabolism in favour of biomass growth, at the expense of PHB production, while not significantly improving the acetate consumption rate. The model suggests that DO concentrations lower than the half saturation constant for oxygen ($k_{S,O2}$) (0.6 mg L^{-1} in this simulation) minimise growth and preserve reducing power.

Table 2.3: Default rate constants and half saturation constants used in the metabolic model.

Maximum Specific Rate Constants	Values used	Units
Acetate consumption[1] ($r_{Ac}^{\max}$)	0.60	Cmol. Cmol X^{-1}. h^{-1}
PHB production[1] ($r_{PHB.\Pr}^{\max}$)	0.30	Cmol. Cmol X^{-1}. h^{-1}
PHB degradation[1] ($r_{PHB.De}^{\max}$)	12	h^{-1}
Growth rate[1] ($r_{X}^{\max}$)	0.20	Cmol. Cmol X^{-1}. h^{-1}
Rate of TCA cycle[2] ($r_{TCA}^{\max}$)	0.10	Cmol. Cmol X^{-1}. h^{-1}
Oxygen uptake rate[1] ($r_{O_2}^{\max}$)	0.10	Cmol. Cmol X^{-1}. h^{-1}
Heterotrophic Half Saturation Constants		
k_{s,O_2} [3]	0.60	mg L^{-1}
$k_{S,Ac}$ [4]	0.02	Cmmol L^{-1}
PHB saturation capacity of biomass		
$f_{PHB}^{\max}$ *	0.6	Cmol PHB/Cmol X

[1] These values were derived from the experimental section of this paper.
[2] Rate constant of TCA cycle was assumed to be same as for oxygen uptake, so it did not become limiting.
[3] A relatively high $k_{S,O2}$ was chosen for the simulation to imitate a situation with flocs.
[4] Suggested half saturation constant of the ASM3 model (Gujer et al., 1999).

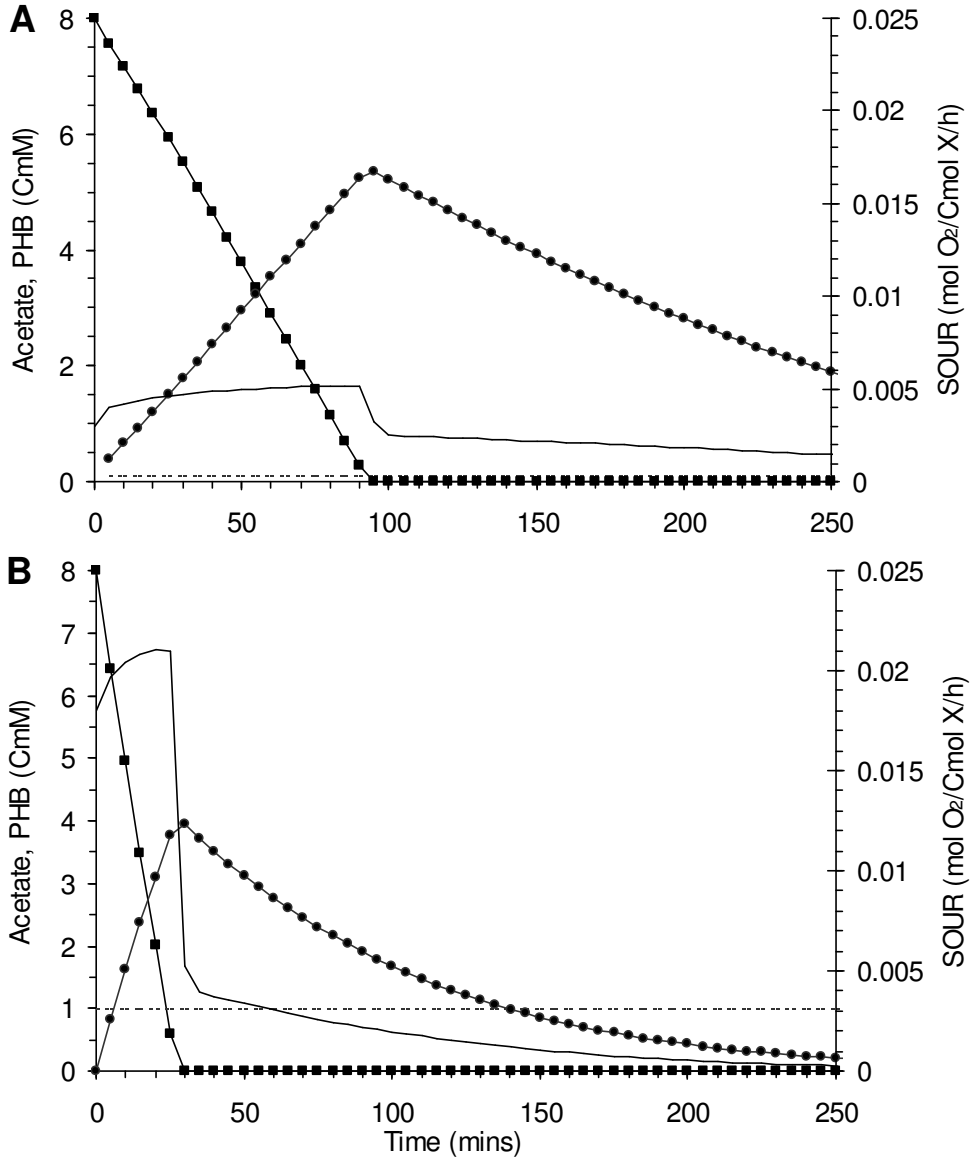

Figure 2.6: General behaviour of the model with default parameter settings (Table 2.3) during a standard SBR cycle with; A: DO setpoint 0.01 mg L^{-1}, and B: DO setpoint 1.0 mg L^{-1} (Acetate (■), PHB (●), SOUR (solid line) and dissolved oxygen concentration (dotted line).

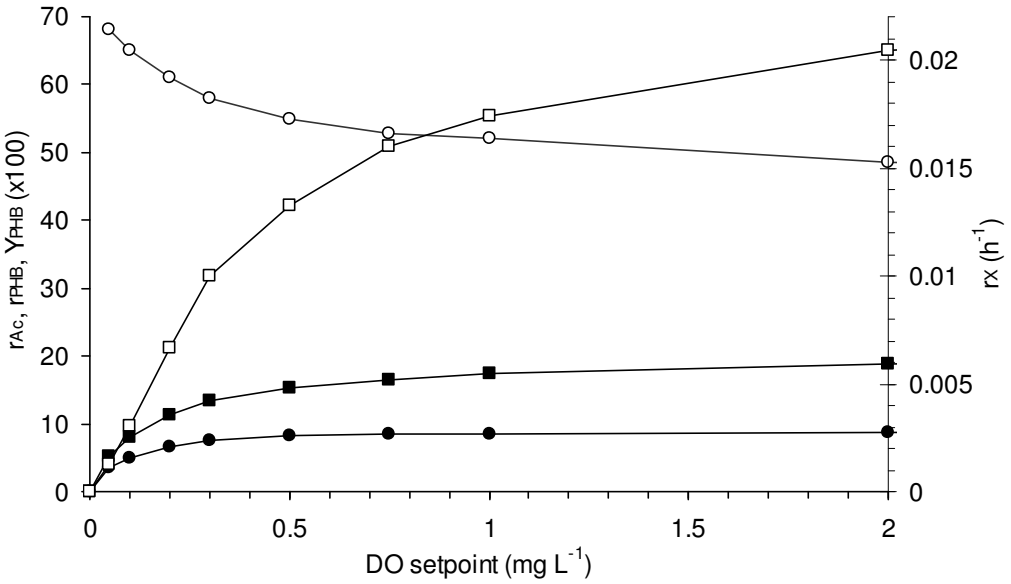

Figure 2.7: Simulated data showing the effect of the dissolved oxygen concentration during the fill phase of a standard SBR cycle on; Acetate consumption rate (■), PHB production rate (●), yield of PHB x 100 (Y_{PHB}) (❍) and the biomass growth rate (□).

Plots of the intracellular metabolite ratios over time at different DO setpoints (Fig 2.8) showed that during the feast phase (presence of acetate), increased dissolved oxygen concentrations favoured the prevalence of ATP and NAD^+ while low dissolved oxygen concentrations favoured the inverse (high ADP and NADH). Given that PHB synthesis requires NADH and biomass synthesis requires ATP, the modelled metabolite levels provide a sensible explanation why oxygen limitation shifts the flux of acetyl-CoA towards more PHB synthesis (Fig 2.7). As soon as acetate was depleted, there was an abrupt change in all metabolite concentrations (Fig 2.8). As expected, acetate depletion caused a decrease in intracellular acetyl-CoA levels. As acetate transport into the cell represents an ATP-sink, the removal of this sink caused all internal ATP levels to rise.

In accordance with literature findings (Van Aalst-Van Leeuwen et al., 1997, Van Loosdrecht et al., 1997), the model predicted that the rate of growth can change markedly during the transition from feast to famine phase (Fig 2.9). At the higher DO concentrations, the transition caused a decrease in growth rate, while at lower DO concentrations it caused an increase. The change in intracellular levels of ATP and acetyl-CoA during the transition provides the explanation, as the product of the ATP and acetyl-CoA ratios determines the growth rate. At low DO concentrations the ATP availability was low so that ATP dependent reactions such as growth were severely

disadvantaged. After depletion of acetate the ATP level increased about 10-fold, compared to only 2-fold at the highest DO concentrations (Fig 2.8). This rapid ATP availability accelerated growth rates at lower DO concentrations but not at higher DO concentrations, which were also affected by a 4-fold drop in acetyl-CoA.

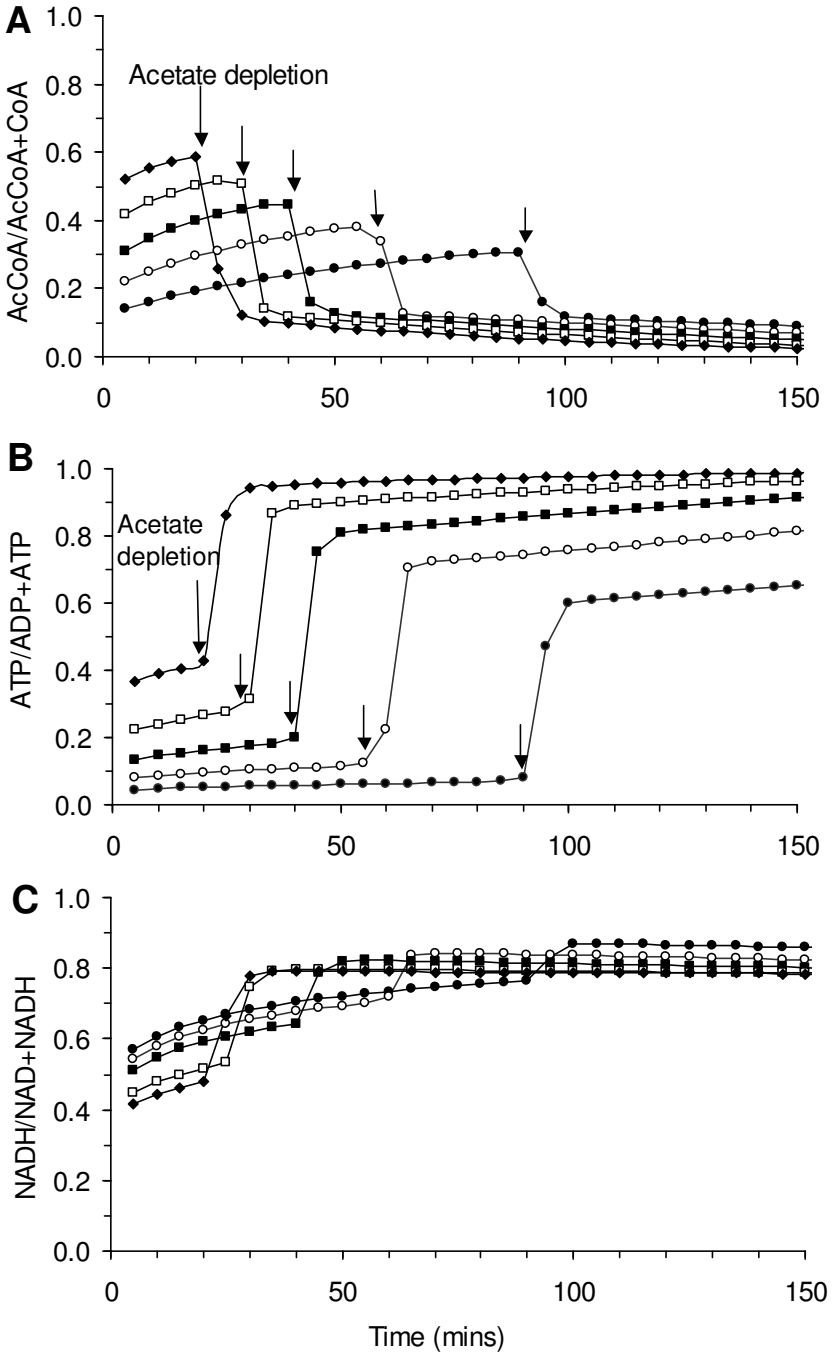

Figure 2.8: Simulated data showing the effect of the dissolved oxygen concentration during the fill phase of the SBR on intracellular metabolite ratios; DO setpoint 0.05 mg L^{-1} (●), 0.1 mg L^{-1} (❍), 0.2 mg L^{-1} (■), 1 mg L^{-1} (□) and 4 mg L^{-1} (◆).

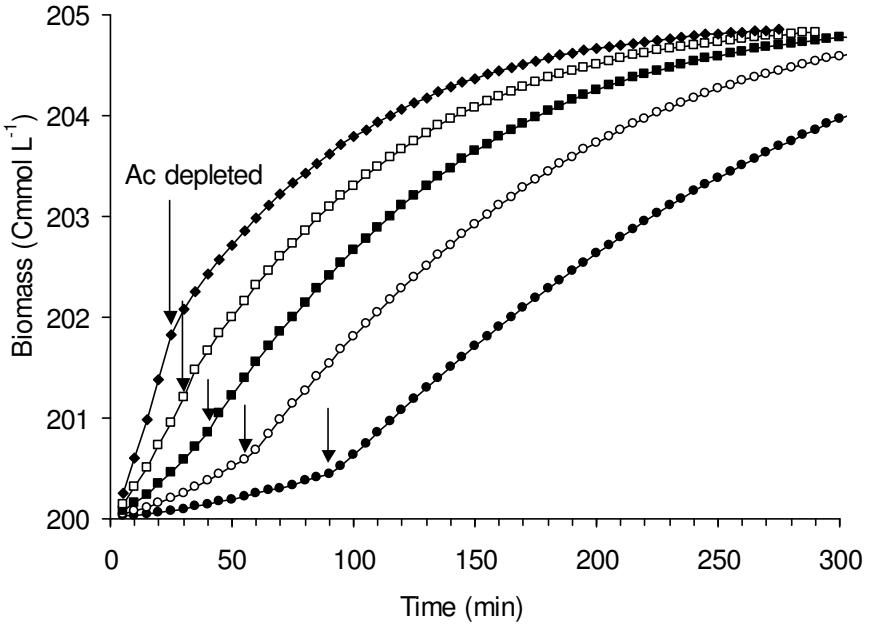

Figure 2.9: Change in biomass concentration during the SBR fill phase over time at different DO concentrations: 0.05 mg L^{-1} (●), 0.1 mg L^{-1} (❍), 0.2 mg L^{-1} (■), 1 mg L^{-1} (□) and 4 mg L^{-1} (◆) (all using default model parameters, Table 2.3).

Significance of the Half Saturation Constant for Oxygen $\left(k_{S_{O2}}\right)$ of Heterotrophic Biomass

As PHB metabolism was shown experimentally and theoretically to be dependent on the dissolved oxygen concentration, the model was used to assess the effect of the k_S for oxygen on PHB metabolism (Fig 2.10). At low DO concentrations, a higher k_S value slowed down the rates of acetate consumption and growth more strongly than the rate of PHB production, resulting in an increase in PHB yield. In terms of maximising PHB yield from substrate, a high k_S for oxygen, or the presence of oxygen diffusion barriers such as flocs, are beneficial. The modelled results also confirmed the trend that DO concentrations higher than the $k_{S,O2}$ significantly decrease the PHB yield without a large increase in the rate.

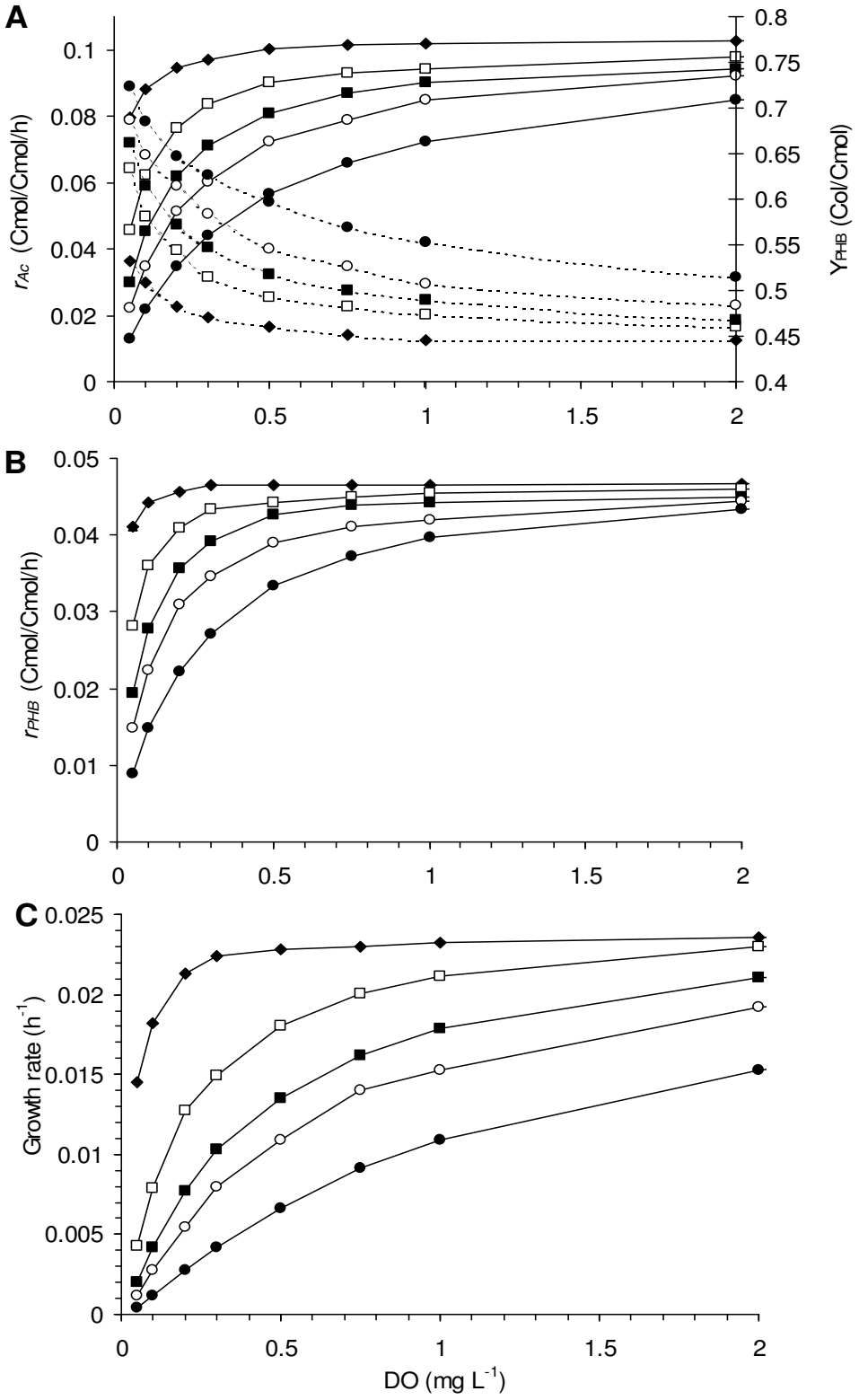

Figure 2.10: The effect of the substrate affinity constant for oxygen ($k_{S,O2}$) on; A: Acetate consumption rate (solid lines) and Y_{PHB} (dotted lines); B: PHB production rate and; C: Biomass growth rate; k_S 0.05 mg L^{-1} (◆), 0.25 mg L^{-1} (□), 0.5 mg L^{-1} (■), 0.8 mg L^{-1} (○), 1.5 mg L^{-1} (●).

Effect of Model Assumptions on Simulation Outcomes (Parameter Sensitivity Analysis)

As the model developed in this study was based on several assumptions, the effect of changes in these assumptions on simulated trends was assessed for three assumed values; the ATP/NADH ratio, the ATP requirement for acetate transport and the maximum rate of the TCA cycle (Table 2.4). While the number of ATP molecules that is produced per pair of electrons passed through the electron transport is a mechanistically fixed number of three ATP per pair of electrons, the actual number of the resulting ATP molecules that can be used for growth and acetate transport depends on the maintenance requirements of the cell. A number of studies have quantified the ATP to NADH ratio (or $P/2e^-$) of activated sludge cultures and showed that the number of ATP molecules available to the cell after maintenance, per NADH molecule, ranges between 1.85 – 2.4 (Beun et al., 2000a, 2000b, Smolders et al., 1994, Kuba et al., 1996). The modelled results presented in this study are based on the assumption that all three ATP molecules are available (i.e. maximum theoretical $P/2e^-$ ratio of 3) and thus simulated data may over-estimate maximum achievable PHB yields. Parameter sensitivity analysis showed that a change in the ATP/NADH ratio from 3 to 2 scaled the yield of PHB from acetate ($Y_{PHB/Ac}$) down by 13 – 15 % at both DO setpoints of 0.1 and 1 mg L^{-1}. Thus, the absolute PHB yield achieved depends on the available ATP/NADH ratio and hence on the cellular maintenance requirement. Fast-growing cells are expected to have a lower relative maintenance requirement (Stouthamer, 1973) and would thus be likely to produce higher values of $Y_{PHB/Ac}$. Changes in the two other model assumptions had a similar scaling up or down effect. However, the underlying trends in the simulated data were not affected by the assumed values. In all cases, the yield of PHB from acetate was increased at a lower DO setpoint in comparison to a higher DO setpoint, independent of the assumed model values (Table 2.4).

Table 2.4: Parameter sensitivity analysis of three model assumptions to quantify the effect of a change in the assumptions on modelled outcomes. Bold values represent default parameters used in the model.

	DO setpoint 1 mg L^{-1}			**DO setpoint 0.1 mg L^{-1}**		
	$Y_{PHB/Ac}$	$Y_{X/Ac}$	r_{PHB}	$Y_{PHB/Ac}$	$Y_{X/Ac}$	r_{PHB}
ATP/NADH ratio						
3.0	**0.49**	**0.20**	**0.04**	**0.67**	**0.09**	**0.03**
2.7	0.47	0.17	0.04	0.60	0.08	0.02
2.0	0.43	0.15	0.03	0.55	0.07	0.02
1.5	0.37	0.15	0.02	0.49	0.06	0.01
Maximum rate of TCA cycle r_{TCA}^{max}						
0.15	0.53	0.26	0.05	0.64	0.10	0.03
0.10	**0.49**	**0.20**	**0.04**	**0.63**	**0.09**	**0.02**
0.09	0.48	0.19	0.04	0.62	0.09	0.02
0.05	0.42	0.17	0.03	0.59	0.08	0.02
ATP required for Ac transport (ATP/Cmol)						
1.5	0.43	0.15	0.03	0.57	0.08	0.02
1.0	**0.49**	**0.20**	**0.04**	**0.63**	**0.09**	**0.03**
0.75	0.51	0.21	0.05	0.64	0.10	0.03
0.50	0.52	0.22	0.06	0.67	0.11	0.04

2.4. Discussion

Oxygen control is recognised as being essential to enhance nitrogen removal via simultaneous nitrification and denitrification during the famine phase of sequencing batch reactors (Pochana and Keller, 1999, Munch et al., 1996, Beun et al., 2001), but it has been largely ignored for the feast phase. Our study shows that oxygen management is equally important in the acetate uptake phase for effective preservation of reducing power, required for improved nitrogen removal via SND in the famine phase.

Energy Requirement for COD Uptake

Using acetate as the model substrate for COD, the microbial uptake of acetate and its conversion to PHB is strictly oxygen dependent. In the absence of oxygen, ATP cannot be synthesized and the transport of acetate into the cell is not enabled. Results have also shown that an oversupply of oxygen can compromise PHB production. It should be noted that in real wastewater treatment plants some anoxic substrate uptake can occur when either fermentable organic substrate (e.g. sugars, glycogen) or nitrate is available as an ATP source (Beun et al., 2000b, Dionisi et al., 2001). Furthermore the adaptation to enhanced biological phosphate removal (EBPR) has been shown to provide an anaerobic ATP source and hence some COD uptake due to polyphosphate hydrolysis (Smolders et al., 1995, Carucci et al., 1999, Liu et al., 2000).

Oxygen Management During COD Uptake

When sufficient oxygen was supplied for maximum COD uptake kinetics, the yield of PHB from acetate decreased significantly to 0.5 Cmol/Cmol. In a typical wastewater treatment plant (e.g. feed COD 400 mg L^{-1}), this could waste sufficient reducing power for removal of 30 mg L^{-1} nitrate-nitrogen via SND during the aeration phase. Our experimental and modelled data suggest that a compromise between fast COD uptake and satisfactory levels of PHB generation can be accomplished by controlled oxygen supply. To prevent unnecessary oxidation of acetate, the oxygen concentration should be kept lower than the oxygen half saturation constant of the heterotrophic biomass (Fig 2.10). Depending on floc size and wastewater composition the oxygen concentration may need to be kept below detection limits.

Effect of PHB Production on Biomass Synthesis

The heterotrophic biomass yield at the end of the feast phase was up to 3 times lower under oxygen limitation than with high oxygen supply rates (Fig 2.5A). According to the model, the reason for this was that at low DO concentrations virtually all available ATP was used for acetate uptake, severely compromising the assimilation reaction. At high DO supply rates, a surplus of ATP allowed simultaneous growth. However the oxidation of PHB during the famine phase will produce ATP and allow biomass synthesis. As the model showed, if there is no energy requirement for PHB degradation, the yield of biomass from PHB is the same as for acetate, resulting in the same final biomass yield (Fig 2.9). In reality, aerobic biomass growth on PHB results in a 4 – 10 % lower yield than direct growth on acetate (Beun et al., 2000a), which does not greatly reduce biomass production. However, if PHB can be encouraged to reduce nitrate (during SND) during the famine phase by operating at low DO concentrations, this would further decrease the biomass yield by 20 %, since the anoxic biomass yield on PHB has been reported to be 0.53 Cmol/Cmol, compared to 0.67 Cmol/Cmol aerobic yield (Beun et al., 2000b). For the wastewater treatment plant operator this is of benefit as a decrease in heterotrophic biomass results in an increase of the fraction of nitrifiers in the biosolids. An increase in the percentage of nitrifiers would allow a shortening of the aeration phase as it is limited by the rate of ammonium oxidation. To achieve a total lower biomass yield after each cycle, oxygen management is important during both the feast and the famine phase and the requirement for low-DO supply could significantly reduce aeration costs.

The Relationship Between PHB Synthesis and the Specific Growth Rate

In general, literature studies have concluded that limiting the microbial growth rate can enhance PHB synthesis. Nitrogen limitation is well known to favour PHB synthesis in microbial cells (Senior and Dawes, 1973, Oeding and Schlegel, 1973, Page et al., 1995, Anderson and Dawes, 1990). Recent studies on PHB synthesis in SBRs concluded that higher PHB yields are produced at longer solid retention times when the cells are growing slowly (< 0.01 h^{-1}) (Van Aalst-Van Leeuwen et al., 1997, Beun et al., 2000a, Pagni et al., 1992, Van Niel et al., 1995). Beun et al. (2000a) showed that at SRTs of 9.5 and 19.8 days the ratio of PHB produced over acetate consumed (r_{PHB}/r_{Ac}) was 0.60 Cmol PHB/Cmol, similar to the values observed in this study at low oxygen supply rates (Table 2.1). However when an SRT of 3.8 days was applied and the average

growth rate of the biomass increased, this ratio decreased to 0.41, resulting in a lower PHB yield. Our results and model predictions of limited oxygen supply offer a further possibility of slowing down growth and enhancing PHB synthesis, by oxygen limitation. The biochemical explanation is that by slowing down those reactions that compete with PHB synthesis for acetyl CoA (i.e. assimilation, dissimilation), a larger proportion of acetyl-CoA will flow to PHB. Operation at long sludge retention times slows down assimilation, while oxygen limitation slows both assimilation and dissimilation. Thus, a combination of O_2-limitation and a long SRT should ensure maximum PHB storage with minimum growth.

The Effect of Sludge Retention Time (SRT) on Predicted PHB Yield

Beun et al. (2002) suggested that SBR operation at a sludge retention time of longer than 2 days will favour PHB production over acetate consumption (r_{PHB}/r_{Ac} 0.6 Cmol/Cmol), compared to a shorter SRT (r_{PHB}/r_{Ac} 0.4 Cmol/Cmol). We used our biochemical model to simulate shorter SRTs by using lower start-up biomass concentrations, since at low SRTs more biomass is wasted during a cycle, producing a higher initial substrate to microorganism ratio. The model showed that a higher initial biomass concentration (simulating a long SRT) resulted in a shorter feast phase and a higher PHB yield than at a low starting biomass concentration (Fig 2.11). At low initial biomass concentrations (or short SRT), a single cell experiences feast conditions for a longer period of time and the higher substrate to microbe ratio allows more growth, reducing the PHB yield. The model is therefore in full accordance with the results of Beun et al (2000a, 2002).

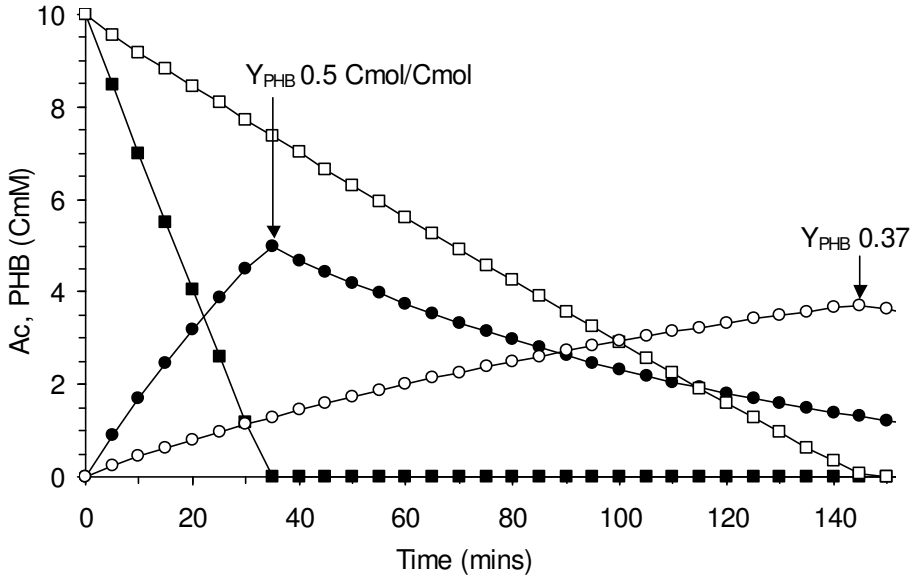

Figure 2.11: Simulation of the effect of sludge retention time on the PHB yield by using different starting biomass concentrations of 50 Cmmol L^{-1} (□ acetate and ❍ PHB) and 200 Cmmol L^{-1} (■ acetate and ● PHB).

2.5. Conclusions

To enable efficient denitrification in a SBR, PHB is valuable resource to manage. Theoretical as well as experimental results show that this can be improved by oxygen control. Oxygen limitation was necessary for optimum PHB yields with minimal biomass growth. The exact oxygen concentration advisable for treatment plants may depend on the general operation conditions. As a preliminary estimate, dissolved oxygen concentrations approximate to the heterotrophic $k_{S,O2}$ appear reasonable for acetate-type wastewaters. Wastewaters characterised by carbohydrates, phosphates or other fatty acids may require a different oxygen management that could be derived by future studies. For treatment plant operators, operation of an SBR at low DO concentrations, not only during the famine phase but also throughout the feast phase, has two advantages; aeration costs are decreased and the availability of reducing power in the famine phase is increased. High oxygen supply rates are potentially wasteful and allow higher biomass growth at the expense of lower PHB production and consequently lower nitrogen removal.

CHAPTER 3

Simultaneous Nitrification and Denitrification Using Stored Substrate (PHB) as the Electron Donor in an SBR[1]

Abstract

The potential for PHB to serve as the electron donor for effective simultaneous nitrification and denitrification (SND) was investigated in a 2 L sequencing batch reactor (SBR) using a mixed culture and acetate as the organic substrate. During the feast period (i.e. acetate present), heterotrophic respiration activity was high and nitrification was competitively inhibited by oxygen. Once acetate was depleted the oxidation rate of PHB was up to 6 times slower than that of soluble acetate and nitrification could proceed due to the decreased competition for oxygen. The slow nature of PHB degradation meant that it was an effective substrate for SND, as it was oxidised at a similar rate to ammonium and was therefore available for SND throughout the entire aerobic period. The percentage of nitrogen removed via SND increased at lower DO concentrations during the famine period, with up to 78 % SND achieved at a DO concentration of 0.5 mg L^{-1}. However, the increased percentage of SND at a low DO concentration was compromised by a 2-times slower rate of nitrogen removal. A moderate DO concentration of 1 mg L^{-1} was optimal for both SND efficiency (61 %) and rate (4.4 mmol N. Cmol X^{-1}. h^{-1}). Electron flux analysis showed that the period of highest SND activity occurred during the first hour of the aerobic famine period, when the specific oxygen uptake rate (SOUR) was highest. It is postulated that a high SOUR due to NH_4^+ and PHB oxidation decreases oxygen penetration into the floc, creating larger zones for anoxic denitrification. The accumulation of nitrate towards the end of the SND period showed that SND was finally limited by the rate of denitrification. As PHB degradation was found to follow first order kinetics ($df_{PHB}/dt = - 0.19 \cdot f_{PHB}$), higher PHB concentrations would be expected to drive SND faster by increasing the availability rate of reducing power and reducing penetration of oxygen into the floc, due to the corresponding increased SOUR. Process control techniques to accumulate higher internal PHB concentrations to improve PHB-driven SND are discussed.

[1] This chapter has been accepted for publication in Biotechnology & Bioengineering

3.1. Introduction

Simultaneous nitrification and denitrification (SND) has gained significant attention in recent years due to its potential to eliminate the need for separate tanks required in conventional treatment plants and consequently to simplify the plant's design, saving space and time (Keller et al., 1997, Sen and Dentel, 1998, Yoo et al., 1999, Zhao et al., 1999). Advantages of SND are that nitrogen removal efficiencies achieved are similar to conventional nitrification-denitrification processes, without the need for an anoxic basin, together with a decrease in operational costs due to the low dissolved oxygen concentrations and shorter treatment times required (Collivignarelli and Bertanza, 1999). However, until recently, removal of nitrogen via SND has not typically been designed into full-scale wastewater treatment plants because the phenomenon has been perceived to be unpredictable (Tonkovic, 1999). An increased understanding of the controlling factors of SND is necessary before optimisation of large-scale plants can be successful.

SND relies on concurrent aerobic ammonium oxidation and anaerobic denitrification under identical operating conditions. As ammonium oxidation is a relatively slow process, SND requires a slowly degradable carbon substrate such that reducing power is available for denitrification throughout the ammonium oxidation process, instead of being rapidly oxidised to CO_2 within the early stages of aeration. The storage of soluble substrate as storage polymers (e.g. PHB) by heterotrophic bacteria in dynamic systems is well established in the literature (Beun et al. 2002, Majone et al. 1999, Van Loosdrecht et al. 1997). This internal carbon source is degraded much slower than soluble substrate and can be used as the electron donor for denitrification when no external substrate is available (Beun et al. 2001, Jones et al., 1990a, 1990b, Majone et al., 1998). The ability of heterotrophs to rapidly remove soluble substrate and store it as a slowly degradable polymer represents a convenient opportunity to preserve reducing power for simultaneous nitrification and denitrification.

A significant amount of research has focused on the aerobic storage of soluble substrate as PHB and its subsequent degradation in activated sludge cultures (Beun et al., 2000a, 2000b, 2002, Dircks et al., 1999, 2001, Krishna and Loosdrecht, 1999a, 1999b, Majone et al., 1996, 1999). However, very little literature is available on the role of PHB

metabolism in nitrogen removal. The objectives of this study were to investigate the effect of the dissolved oxygen concentration on the rate and quantity of SND achieved during aeration and to determine the nature of PHB degradation kinetics and its relation to N-removal. With this information it was aimed to establish the limiting factors of SND in an SBR.

3.2. Materials and Methods

3.2.1. Experimental Setup

A sequencing batch reactor (SBR) with a working volume of 2 L and on-line measurement of dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) was used for all experiments, as described in Chapter 2. The SBR was completely automated, with all pumps, stirrers, airflow valves and phase lengths controlled by National Instruments instrumentation control software LabView^{TM} (Australia). Temperature was maintained at 25 ^{o}C and the airflow rate and stirring speed were 100 L h^{-1} and 80 ± 2 rpm, respectively. The k_La for all experiments was 25 h^{-1}, and was checked before the start of each experiment (see Measurement of k_La). Sludge from a conventional activated sludge wastewater treatment plant (Subiaco, Western Australia) was used as inoculum. One standard SBR cycle, referred to as "standard conditions", consisted of a rapid fill phase (3 mins), an aeration phase (240 mins), an anoxic phase (120 mins), a settle phase (17 mins) and a 20 min effluent withdrawal phase. The sludge volume was 0.8 L and the reactor was filled with 1.2 L of influent during each fill period. The effluent withdrawal line was located directly above the sludge blanket at the 0.8 L mark (Fig 3.1). In this manner, sludge that had not settled during the settle phase was immediately withdrawn during the decant period. This method of sludge removal resulted in biomass of consistent and efficient settlability (sludge volume index < 100 mL g^{-1} X). The sludge retention time (SRT) that resulted from the SBR operation was calculated from the amount of excess sludge that was removed in each cycle. During steady state SBR operation, the SRT and biomass concentration (specified in Result section) were stable for long periods of time (> 4 months). Based on a total SBR cycle length of 400 mins and feed volume of 1.2 L (in a working volume of 2 L), the SBR operation resulted in a hydraulic retention time (HRT) of 11.2 hours and an organic loading rate of 1.2 Cmol acetate L^{-1} h^{-1}.

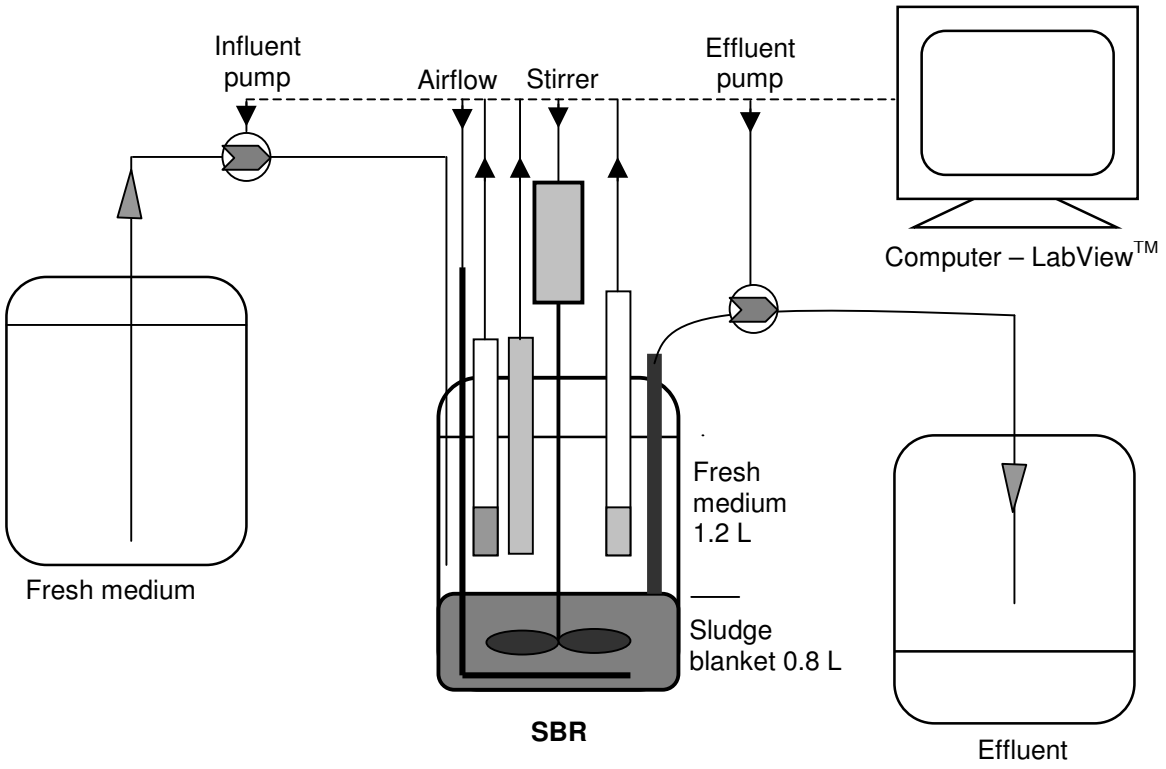

Figure 3.1: Diagrammatic representation of the experimental setup used for all experimental work.

3.2.2. Synthetic Wastewater

The standard composition of the nutrient medium was (mg L^{-1}): CH_3COONa 500, NH_4Cl 125, KH_2PO_4 44, $NaHCO_3$ 125, $MgSO_4.7H_2O$ 51, $CaCl_2.2H_2O$ 300, $FeSO_4.7H_2O$ 6.25, yeast extract 50 and 1.25 mL L^{-1} of trace element solution. The composition of the trace element solution is Chapter 2. Where higher concentrations of nutrients were used, the concentrations are specified in the results section. The medium was autoclaved to prevent bacterial activity in the feed vessel.

3.2.3. Sampling and Analytical Procedures

All nitrogen compounds were measured spectrophotometrically as Chapter 2, while acetate and the PHB content of lyophilised biomass were measured by gas chromatography, according to Smolders et al. (1994). The dry weight (measured as total suspended solids) and the Sludge Volume Index (SVI) of the sludge were measured according to Standard Methods (Greenberg et al., 1992).

3.2.4. Measurement of the Volumetric Oxygen Transfer Coefficient (k_La)

The k_La was measured before each experiment for the specific operating stirring speed, airflow rate mixed liquor viscosity. The culture was aerated until a steady state of oxygen was reached. Under steady state conditions the oxygen transfer rate (OTR) into solution is equal to the oxygen uptake rate (OUR) of the biomass. To determine the OUR of the culture, the air was then switched off and the rate of decrease in dissolved oxygen measured. The k_La could then be calculated from Eq 1.

$$OTR = k_L a.(c_S - c_L) \qquad \text{(Eq 1)}$$

where the OTR is the oxygen transfer rate (mg O_2 L^{-1} h^{-1}), which is equal to the OUR at steady state, c_S the saturating concentration of oxygen in the gas phase (mg L^{-1}) and c_L the concentration of oxygen in the liquid phase (mg L^{-1}). The value obtained by this method was verified by the de-gassing method for k_La determination, in which the reactor was filled with water and the DO concentration reduced to zero by the addition of a small amount of sodium dithionite. The liquid was then aerated and the DO concentration measured over time. By plotting the OTR versus the saturation deficit (i.e. $c_S - c_L$), the k_La could be determined from the slope of the curve (Eq 1).

3.2.5. Calculations

On-Line Calculation of the Specific Oxygen Uptake Rate (SOUR)

Where dissolved oxygen control was used, the DO concentration was controlled about the setpoint using PID (Proportional, Integral, Differential) control, developed by the authors in LabView™ (USA). The airflow rate and stirrer speed were maintained constant at all times, resulting in a constant k_La when air was supplied continuously (k_La_{full}) (measured as above). To maintain the DO concentration at the setpoint, the oxygen transfer rate (OTR) needed to be continuously adjusted, according to the PID control algorithm. The controller achieved varying oxygen transfer rates by digital on-off switching of the air supply, via a solenoid valve, resulting in intermittent air supply of varying frequency. The controller varied the frequency of air supply (f_{AirOn}) between 0 and 1; a fraction of 1 implied that air was supplied 100 % of the time, a fraction of 0.5 indicated air was supplied for 1 second out of 2 etc. The DO concentration was checked every 2 seconds and the frequency of air supply (f_{AirOn}) adjusted accordingly by the controller. The PID control maintained the oxygen concentration to within 0.02 mg L^{-1}

of the setpoint, except during the feast phase, when the culture was oxygen-limited. As the dissolved oxygen concentration was constant during the aerobic famine period, the OUR could be calculated on-line directly from the steady state DO concentration, once the k_La was known. The actual k_La during intermittent air supply (k_La_{int}) was measured for different air supply fractions (f_{AirOn}), which resulted in a linear relationship between f_{AirOn} and k_La_{int} (Eq 2).

$$k_La_{int}\,(h^{-1}) = 24.6 \cdot f_{AirOn} + 0.4\,(h^{-1}) \qquad \text{(Eq 2)}$$

where the y-intercept of the linear equation (0.4 h^{-1}) was the k_La produced due to stirring only, without airflow (f_{AirOn} = 0) and the k_La for full aeration (f_{AirOn} = 1) was 25 h^{-1}. The oxygen transfer rate into solution was then calculated on-line (Eq 1) by substituting in k_La_{int} for k_La. The OUR values calculated on-line were verified regularly by interrupting the DO control, supplying full air and measuring the actual OUR mid-cycle (as under K_La Measurement). The OUR was converted to the specific oxygen uptake rate (SOUR) according to;

$$SOUR\,(mg.\,g\,X^{-1}.\,h^{-1}) = \frac{OTR}{X}\left(\frac{mg.L^{-1}.h^{-1}}{g.L^{-1}}\right) \qquad \text{(Eq 3)}$$

On-line SOUR calculation by this method relied on the assumption that the biomass concentration did not change significantly over one cycle (measurements showed it increased by 20 % maximum). In experiments without dissolved oxygen control, where air was supplied continuously, the OUR was calculated off-line from dissolved oxygen data (Eq 4). The change in the dissolved oxygen concentration at any time (dc_L/dt) is equal to the oxygen transfer rate minus the oxygen uptake rate;

$$\frac{dc_L}{dt}(mg.L^{-1}.h^{-1}) = OTR - OUR \qquad \text{(Eq 4)}$$

where the OTR was calculated according to Eq 1 and dc_L/dt was calculated over a small time interval ($t_0 - t_1$) (Eq 5).

$$\frac{dc_L}{dt}(mg.L^{-1}.h^{-1}) = \frac{c_{L,t1} - c_{L,t0}}{t_1 - t_0} \qquad \text{(Eq 5)}$$

where $c_{L,t1}$ and $c_{L,t0}$ were the dissolved oxygen concentrations at the beginning and end of the time interval, respectively. The SOUR was then calculated from the OUR according to Eq 3.

PHB Content of Biomass

For the purpose of all calculations involving biomass, the exact composition of the biomass was not measured, but one carbon mole was assumed to be $CH_{1.8}O_{0.5}N_{0.2}$ (Ratledge and Kristiansen, 2001). GC measurements of PHB content were calculated as a PHB concentration per mass of cell matter analysed;

$$F_{PHB}(Cmol/Cmol) = \frac{PHB}{X}\left(\frac{Cmol.L^{-1}}{Cmol.L^{-1}}\right) \qquad \text{(Eq 6)}$$

Calculation by this method includes a small imprecision as PHB has a slightly different composition and molecular weight ($CH_{1.5}O_{0.5}$, 21.5 g $Cmol^{-1}$) to active biomass ($CH_{1.8}O_{0.5}N_{0.2}$, 24.6 g $Cmol^{-1}$). However, as the following results aim to determine rates and trends of PHB metabolism (independent of absolute values), the error was considered negligible. The PHB fraction of active biomass (f_{PHB}) (i.e. biomass not including PHB) was calculated as;

$$f_{PHB}(Cmol/Cmol) = \frac{PHB}{X - PHB} \qquad \text{(Eq 7)}$$

The percentage of PHB of the total biomass (on a mass basis) was calculated according to;

$$\%PHB(g/g) = \frac{PHB}{X}.\frac{MWtPHB}{MWtX}x100 \qquad \text{(Eq 8)}$$

The total concentration of PHB inside the reactor was calculated by multiplying the biomass PHB fraction (F_{PHB}) by the biomass concentration;

$$PHB(Cmol.L^{-1}) = F_{PHB}\left(\frac{CmolPHB}{CmolX}\right).X\left(\frac{CmolX}{L}\right) \qquad \text{(Eq 9)}$$

The yield of PHB from acetate ($Y_{PHB/Ac}$) was calculated by dividing the total PHB produced (CmM) by the total acetate consumed (CmM) during the feast phase;

$$Y_{PHB/Ac}(Cmol/Cmol) = \frac{PHB_{produced}}{Ac_{consumed}} \qquad \text{(Eq 10)}$$

Calculation of SND and Total Nitrogen Removal

The amount of nitrogen removed via simultaneous nitrification and denitrification (SND) was calculated as the amount of ammonium that did not appear as either nitrate or nitrite at the end of the ammonium oxidation period. SND was defined as only occurring during the period when ammonium was present (Eq 11);

$$\%SND = \left(1 - \frac{NO_x^{-}{}_{produced}}{NH_4^{+}{}_{oxidised}}\right) x100 \qquad \text{(Eq 11)}$$

where $NO_x^-{}_{produced}$ was the sum of the nitrite and nitrate present when ammonium was depleted (mM) and $NH_4^+{}_{oxidised}$ was the amount of ammonium oxidised during the aerobic famine period, calculated as the total ammonium consumption minus NH_4^+ assimilated into biomass (mM). The amount of NH_4^+ assimilated ($NH_4^+{}_{assim}$) was calculated from PHB measurements, assuming a heterotrophic biomass yield of 0.5 $Cmol_X/Cmol_{PHB}$ and a related NH_4^+ consumption of 0.2 mM per Cmol of PHB assimilated (i.e. assumed biomass molecule $CH_{1.8}O_{0.5}N_{0.2}$) (Eq 12). The autotrophic biomass yield was assumed to be negligible during one SBR cycle.

$$NH_4^+{}_{assim}\,(mM) = 0.2 \cdot (PHB_{t1} - PHB_{t0}) \qquad \text{(Eq 12)}$$

where PHB_{t0} and PHB_{t1} were the PHB concentrations at the beginning and end of the measurement interval (CmM), respectively. The rate of SND was calculated as the amount of NO_x^- simultaneously denitrified by the end of the NH_4^+ oxidation period, divided by the length of time required to remove all ammonium (hours);

$$R_{SND}\,(mmol.CmolX^{-1}.h^{-1}) = \frac{NH_4^{+}{}_{oxidised} - NO_x^{-}{}_{produced}\,(mM)}{time(h).X\,(CmM)} \qquad \text{(Eq 13)}$$

The percentage of total nitrogen removed was calculated from the total nitrogen remaining at the end of the SBR cycle (N_{final}) and the total nitrogen present at the start of the cycle ($N_{initial}$);

$$\%N_{removal} = \left(1 - \frac{N_{final}}{N_{initial}}\right) x100 \qquad \text{(Eq 14)}$$

where $N_{initial}$ and N_{final} were calculated as the sum of ammonium, nitrite and nitrate present (mM) at the beginning and end of the SBR cycle, respectively.

Calculation of the Electron Flow to NO_x^- and Oxygen

The following calculations apply to Fig 3.8 only. Rates of electron flow to NO_x^- and O_2 were calculated from NO_x^- and O_2 consumption rates. As NO_x^- was simultaneously produced and consumed during periods when NH_4^+ was present, the NO_x^- consumption rate (R_{NOx^-Cons}) was defined as the difference between the ammonium oxidation rate ($R_{NH_4^+Ox}$) and the NO_x^- production rate (R_{NOx^-Prod}) (Eqs 15 – 16). Therefore, when ammonium was absent the NO_x^- consumption rate was due to nitrite or nitrate consumption only;

$$R_{NO2^-Cons}\ (\text{mmol. L}^{-1}\ \text{h}^{-1}) = R_{NH4^+Ox} - R_{NO2^-Prod} \qquad \text{(Eq 15)}$$

$$R_{NO3^-Cons}\ (\text{mmol. L}^{-1}\ \text{h}^{-1}) = R_{NH4^+Ox} - R_{NO3^-Prod} \qquad \text{(Eq 16)}$$

$$\text{where}\quad R_{NH_4^+Ox} = \frac{NH_4^{+}{}_{t0} - NH_4^{+}{}_{t1}}{t_1 - t_0} \qquad \text{(Eq 17)}$$

where $NH_4^+{}_{t0}$ and $NH_4^+{}_{t1}$ were the ammonium concentrations (mM) at the beginning and end of the measurement interval, respectively, after subtracting the ammonium assimilated into heterotrophic biomass in the time interval (Eq 12). Nitrite/nitrate production rates were calculated as;

$$R_{NO_x^-Prod} = \frac{NO_x^{-}{}_{t1} - NO_x^{-}{}_{t0}}{t_1 - t_0} \qquad \text{(Eq 18)}$$

It was assumed that nitrate was first reduced to nitrite, requiring 2 electrons from an electron donor, per mole of nitrate. For the reduction of one molecule of NO_2^- to N_2, 3 electrons from an electron donor are required. Thus, the total rate of electron transfer from PHB to NO_x^- ($R_{e^-\rightarrow NOx^-}$) was calculated as;

$$R_{e^-\rightarrow NOx^-} = 2.(R_{NO_3^-\rightarrow NO_2^-}) + 3.(R_{NO_2^-\rightarrow N_2}) \qquad \text{(Eq 19)}$$

As it was assumed that nitrite was reduced all the way through to N_2 (accepting 3 electrons), the electron flow to NO_2^- may be over-estimated if some NO_2^- was only reduced to the intermediates NO and N_2O. In the worst-case scenario (no NO_2^- reduced beyond NO), the electron flow to NO_2^- would be overestimated by 67 % (electron transfer of only 1 instead of the assumed 3). This would represent an over-estimation of 40 % in the total electron transfer to NO_x^- (electron transfer of 3 instead of the assumed total of 5).

The electron flow to oxygen was calculated from OUR data. The oxygen uptake rate due to PHB oxidation (OUR_{PHB}) was calculated as the difference between the total OUR (OUR_{total}) and the OUR due to ammonium oxidation (OUR_{NH4}^{+}) (Eq 20);

$$OUR_{PHB}\ (\text{mmol } O_2.\ L^{-1}.\ h^{-1}) = OUR_{total} - OUR_{NH4}^{+} \qquad \text{(Eq 20)}$$

where OUR_{total} was measured on-line (Eqs 1, 2). The OUR due to ammonium oxidation (OUR_{NH4+}) was calculated from ammonium measurements, after subtracting NH_4^+ assimilated into biomass (Eq 12). It was assumed that ammonium was oxidised completely to NO_3^-, requiring 2 moles of oxygen per mole of NH_4^+. It is possible that some ammonium was nitrified to nitrite only, resulting in SND via nitrite, requiring only 1.5 moles O_2 per mole NH_4^+. Thus, rates of electron flow to oxygen may be over-estimated by a maximum of 25 %.

$$OUR_{NH_4^+}(mmolO_2.L^{-1}.h^{-1}) = 2.R_{NH_4^+} \qquad \text{(Eq 21)}$$

As the reduction of one mole of oxygen requires 4 electrons, the rate of electron flow to oxygen from PHB ($R_{e^-_{\rightarrow}O2}$) was calculated as;

$$R_{e^-\rightarrow O_2}(e^-mmol.L^{-1}.h^{-1}) = 4.OUR_{PHB} \qquad \text{(Eq 22)}$$

3.3. Results

The SBR was operated under standard conditions and monitored until a steady state was reached, as determined by a constant biomass concentration for more than seven days and reproducible behaviour of on-line data. It generally took less than one week to reach steady state after inoculation with sludge from a local treatment plant. A constant k_La was used during full and intermittent aeration (25 h^{-1}), which resulted in O_2-limitation during the feast phase. The previous chapter showed that acetate conversion to PHB is maximised under O_2-limitation (Chapter 2). The average sludge retention time (SRT) was 11 days during the experimental period and the culture had been adapted to the dynamic SBR conditions for more than 8 weeks when the experiments were performed. The following results represent typical data sets that were obtained reproducibly over the duration of this study.

Typical Behaviour of the SBR

Using the defined acetate based medium, the laboratory SBR produced a repetitive pattern of acetate, PHB, and nitrogen metabolism (Fig 3.2). Three distinct stages could be detected during the aerobic phase of the SBR that were due to the predominance of specific microbial activities (Fig 3.2B): 1. Bacterial uptake of acetate and PHB synthesis, 2. Nitrification and PHB degradation, 3. Residual PHB degradation and maintenance respiration. These aerobic stages could be clearly recognised from online data showing a high specific oxygen uptake rate (SOUR) in the first stage, a steadily decreasing SOUR in the second phase, terminating with a significant drop at the end and a low residual SOUR in the final stage after ammonium was depleted. Due to the lack of dissolved oxygen control during the aerobic phase under standard conditions, the dissolved oxygen concentration was high (> 3 mg L^{-1}), resulting in only a small fraction of nitrogen removal via SND (31 %) by the end of ammonium oxidation phase (Eq 11, Fig 3.2A). Most of the PHB produced during the feast phase was oxidised by the end of the aerobic period, resulting in a lack of electron donor for denitrification during the anoxic phase and poor overall nitrogen removal (41 %) (Eq 14).

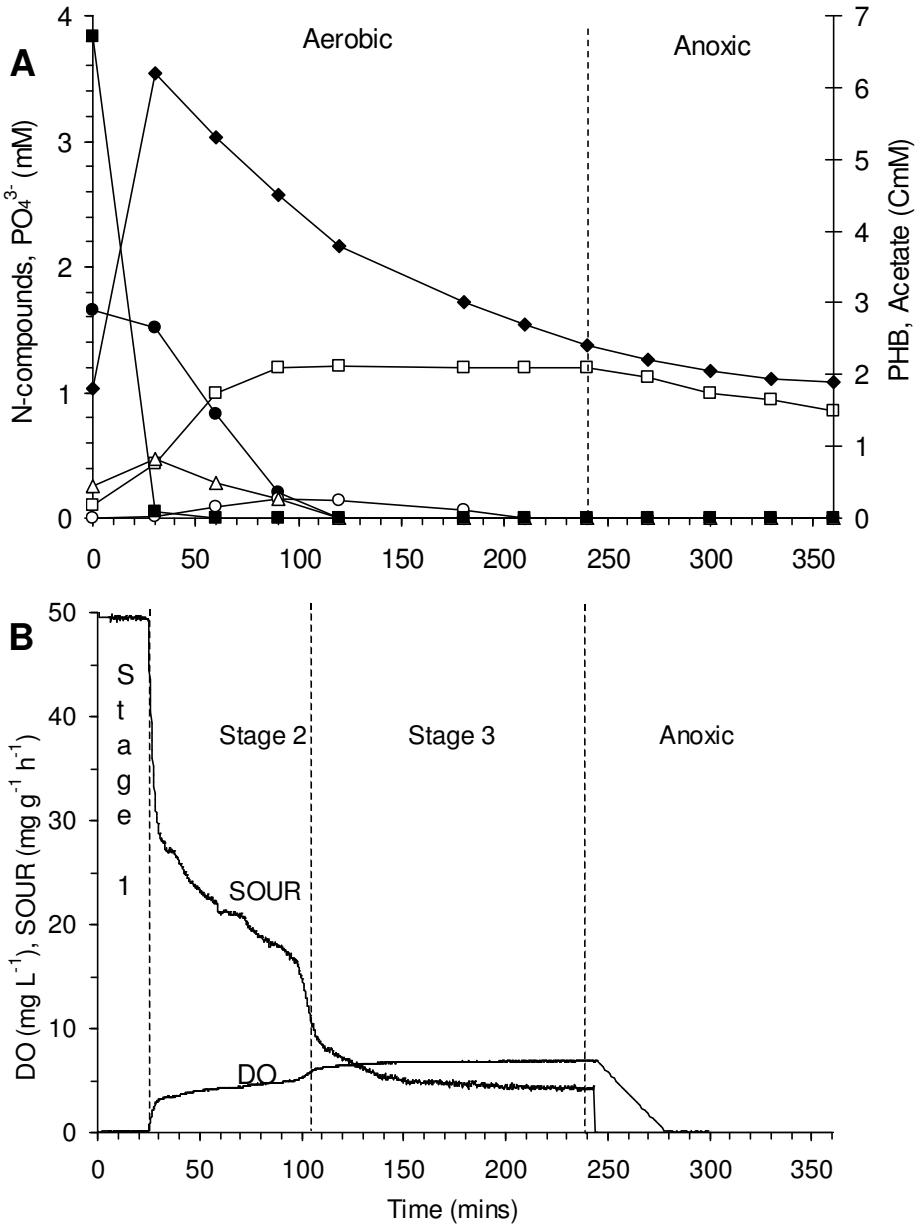

Figure 3.2: Nutrient removal and on-line measurements during a standard SBR cycle with unrestricted airflow (k_La 25 h^{-1}, biomass concentration 0.16 Cmol L^{-1}), A: Acetate (■), PHB (◆), NH_4^+ (●), NO_3^- (□), NO_2^- (❍) and PO_4^{3-} (Δ), and B: Dissolved oxygen and specific oxygen uptake rate (SOUR), calculated according to Eqs 1, 3, 4 & 5.

Aerobic Stage 1: Acetate Uptake and PHB Synthesis (Feast Phase)

As long as acetate was present the dissolved oxygen concentration stayed below detection limit (less than 0.02 mg L^{-1}) and ammonium oxidation was insignificant (Fig 3.2A). Of the acetate consumed, 65 % was preserved as PHB ($Y_{PHB/Ac}$ 0.65 Cmol/Cmol). Ammonium oxidation only started once acetate was depleted, which was indicated on-line by the first significant drop in SOUR (Fig 3.2B). To test whether the inhibition of ammonium oxidation was due to the presence of organic material or to heterotrophic bacteria out-competing autotrophs for oxygen, a control experiment was run which showed that provided excessive amounts of oxygen were supplied, nitrification could run concurrently with acetate uptake in the feast period (Fig 3.3), confirming competitive inhibition of nitrification by oxygen. However, it was necessary to keep the oxygen concentration higher than 2 mg L^{-1} during the feast phase, as trials with less aeration (DO around 1 mg L^{-1}) showed that ammonium oxidation was still inhibited while acetate was present (Fig 3.4). The SOUR during acetate consumption was 6 times higher than during PHB oxidation, causing increased competition for oxygen during the feast phase. The nitrifying bacteria were unable to utilise the oxygen in the bulk liquid at 1 mg L^{-1} DO, although this was well above their half saturation constant (about 0.5 mg L^{-1}, data not shown), indicating a diffusion limitation of oxygen to autotrophs within the biomass flocs during periods of high heterotrophic respiration activity. During oxygen-limited conditions in the feast period some phosphate release was observed (Fig 3.2A). However, the ratio of phosphate released per acetate consumed (0.03 Pmol/Cmol Ac) was up to 25 times lower than reported for enriched (> 95 %) poly-P cultures (0.21 – 0.75 Pmol/Cmol) (Smolders et al., 1994). During the subsequent famine phase, the ratio of PHB to phosphate consumption (0.2 mol P/Cmol PHB) was 10 times lower than enriched poly-P cultures (2 mol P/Cmol PHB) (Smolders et al., 1994). Combined, these ratios indicate that poly-P organisms constitute less than 10 % of the total heterotrophic population.

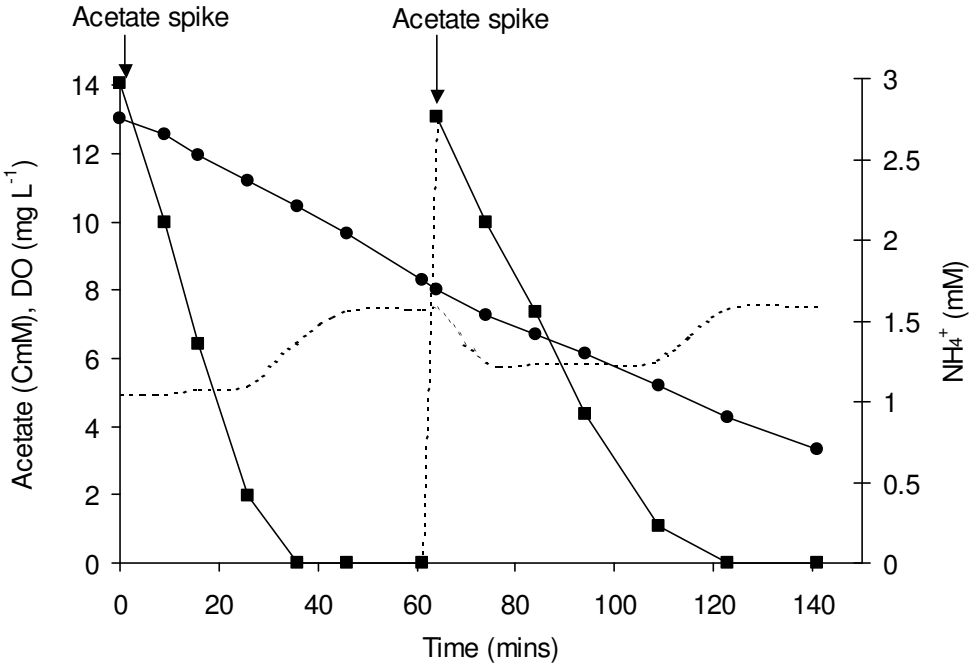

Figure 3.3: Effect of acetate addition to a nitrifying culture in the presence of a high DO concentration: Acetate (■), NH_4^+ (●) and dissolved oxygen (dotted line).

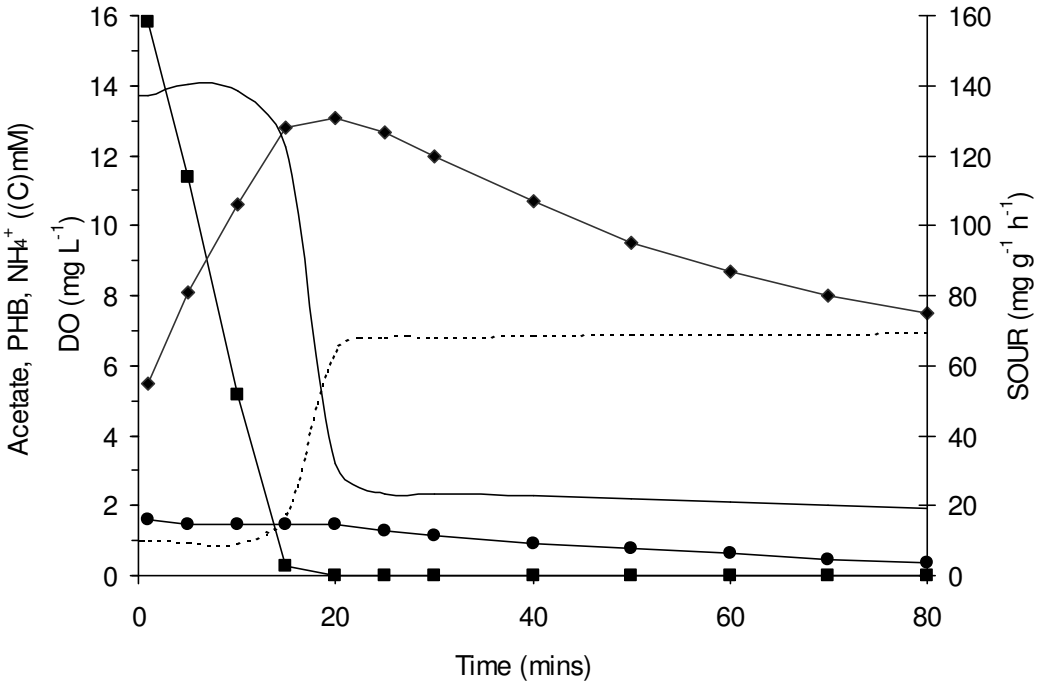

Figure 3.4: Acetate consumption and nitrification at low DO (1 mg L^{-1}): acetate (■), PHB (◆), NH_4^+ (●), DO (dotted line) and SOUR (solid line). The maximum f_{PHB} reached was 0.07 Cmol/Cmol.

Aerobic Stage 2: Nitrification and PHB Degradation

As soon as acetate was depleted the SOUR dropped to a lower level and gradually decreased as PHB and ammonium were oxidised (Fig 3.2B). The depletion of ammonium caused a marked drop in the SOUR that could be used as a reliable indicator of nitrification termination. Trial runs without ammonium in the feed did not display any such behaviour (Fig 3.5A). The SOUR due to PHB oxidation alone showed that PHB was degraded at a decreasing rate over the course of the aerobic period. When the SBR was fed with an acetate-free medium, the SOUR was constant (Fig 3.5B), showing that the ammonium concentration was saturating over the whole nitrification period (i.e. $[NH_4^+] >> k_{S,NH4}^+$). The sharp drop in the SOUR after ammonium depletion suggests a low $k_{S,NH4}^+$ of the nitrifiers. The overall SOUR behaviour under standard conditions reflects the combined respiration activity of ammonium and PHB oxidation, as the sum of the two curves produces the typical SOUR behaviour under standard SBR conditions (Fig 3.5C).

Aerobic Stage 3: Residual PHB Degradation and Maintenance Respiration

PHB was still available after ammonium was depleted, which was slowly oxidised over the remainder of the aerobic period (Fig 3.2A). It is interesting to note that at the end of the aerobic phase (240 mins), significant PHB was measurable in the cells (1.9 CmM, Fig 3.2A). However, the presence of PHB during the anoxic phase did not allow for significant denitrification. To test whether the PHB measured inside the cells at the end of the cycle represented available reducing power or PHB that was integral cell matter, a biomass sample was starved for 3 days in a shake flask with sufficient growth nutrients. The residual PHB remaining at the end of the starvation period was 0.008 Cmol/Cmol, which corresponded to a total of 1.4 CmM PHB for the biomass concentration in Fig 3.2A (0.17 CmM biomass). Therefore, the cells therefore appear to contain a fraction of non-degradable PHB that is not available for nutrient removal.

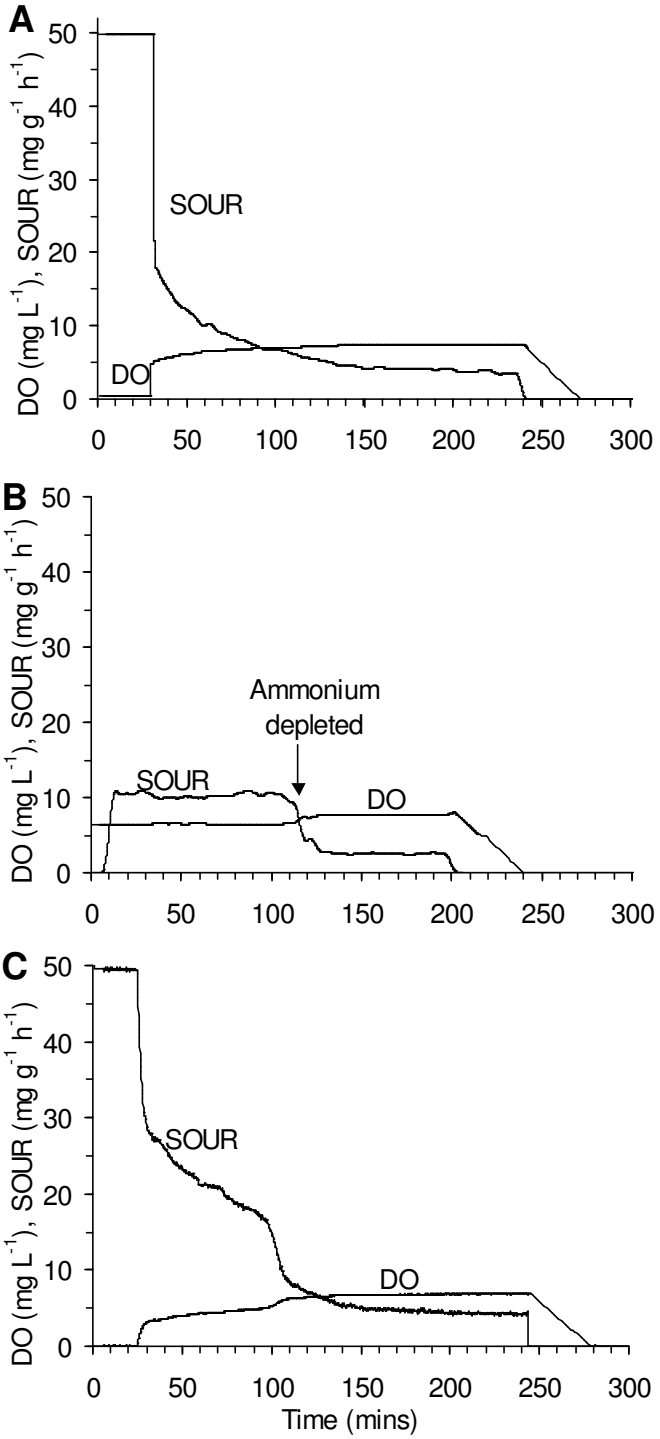

Figure 3.5: On-line measurement of the SOUR (Eqs 1, 3, 4, 5) and DO concentration during an SBR cycle fed with A: Ammonium-free medium, B: Acetate-free medium, and C: Feed containing both acetate and ammonium.

Effect of the Oxygen Supply on SND

The nitrogen removal performance of the SBR at full aeration (DO concentration > 3 mg L^{-1} during the second aerobic stage) resulted in complete nitrification but only a small percentage of SND (31 %) (Fig 3.2). This indicated that the oxygen supply was too high to allow for significant denitrification to occur simultaneously. To test to what

extent a decreased oxygen supply could improve SND, nitrogen removal was investigated at three dissolved oxygen concentrations of 1.5, 1.0 and 0.5 mg L^{-1}. The ammonium oxidation rate decreased at the lower dissolved oxygen concentrations and the percentage of nitrogen removed via SND increased (Fig 3.6, Table 3.1). The extended time required for nitrification at the lower DO setpoints was visible from the longer time required for the second SOUR-drop in on-line data (Fig 3.7). To visualise the periods of highest SND activity, the electron flow from PHB to both oxygen and nitrate was plotted for each dissolved oxygen setpoint (Fig 3.8). In all cases, electron flow to nitrate was highest during the first 100 minutes of the aerobic famine phase when the SOUR was also high (Fig 3.7). Electron flow to nitrate decreased suddenly when all ammonium was depleted, possibly due to greater penetration of oxygen into the flocs due to the sudden drop in the SOUR. The rate of electron flow to nitrate at the lowest DO setpoint (0.5 mg L^{-1}) was initially limited by the availability of nitrate from nitrification (Fig 3.8C). Therefore, while the total quantity of SND was increased at the lowest DO setpoint, the rate of SND was compromised (Table 3.1). A plot of the percentage of SND and the rate of SND versus the DO concentration showed that both the SND percentage and rate decreased at the highest DO setpoints (Fig 3.9). To assess the area of optimum rate and quantity of SND, the rate of SND was multiplied by the fraction of SND and plotted also against the DO concentration (Fig 3.9). This showed that there was a window of optimum SND rate and quantity between 0.8 – 1.2 mg L^{-1} for the biomass used in this experimental work.

Table 3.1: Nitrogen removal characteristics at different average DO concentrations.

Average DO concentration (mg L^{-1})	**5**	**1.5**	**1.0**	**0.5**
NH_4^+ oxidation rate (mmol NH_4^+. Cmol X^{-1} h^{-1})	8.0	7.5	6.1	3.3
Duration of NH_4^+ oxidation (mins)	100	105	125	250
% SND (Eq 11)	31	55	61	78
Rate of SND (Eq 13) (mmol N. Cmol X^{-1} h^{-1})	3.5	5.2	4.4	2.6
Total N removal (%) (Eq 14)	44	63	69	93
Biomass concentration[1] (Cmol L^{-1})	0.17	0.15	0.17	0.15

[1] Where one carbon mole of biomass was assumed to be 24.6 g $Cmol^{-1}$ ($CH_{1.8}O_{0.5}N_{0.2}$)

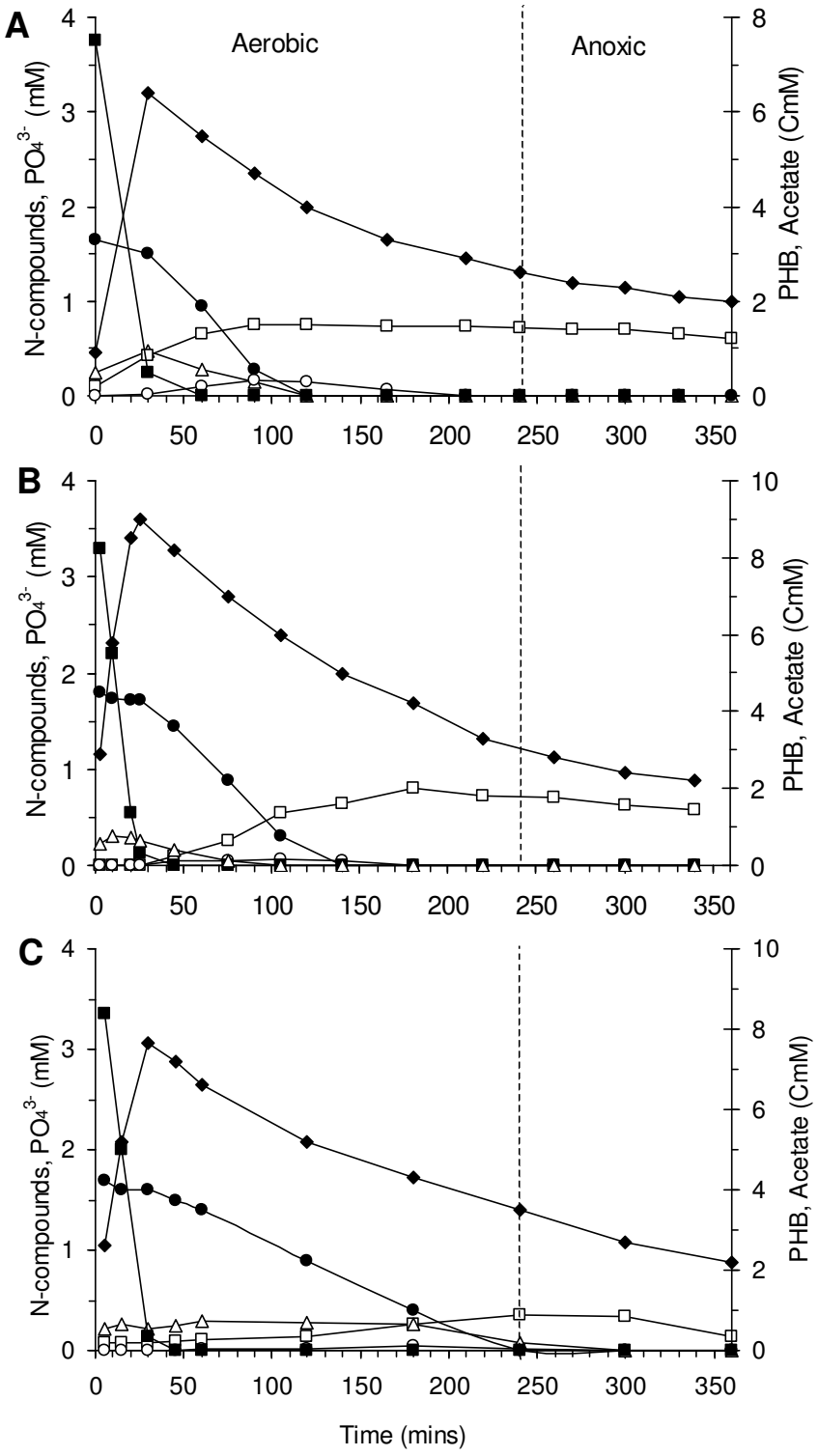

Figure 3.6: Nutrient removal in the SBR at different DO concentrations during the famine phase; A. 1.5 mg L^{-1}, B. 1 mg L^{-1} and C. 0.5 mg $L^{-1:}$ Acetate (■), PHB (◆), NH_4^+ (●), NO_3^- (□), NO_2^- (❍) and phosphate (Δ). All feast phases were operated under oxygen limitation (0 mg L^{-1}, k_La 25 h^{-1}).

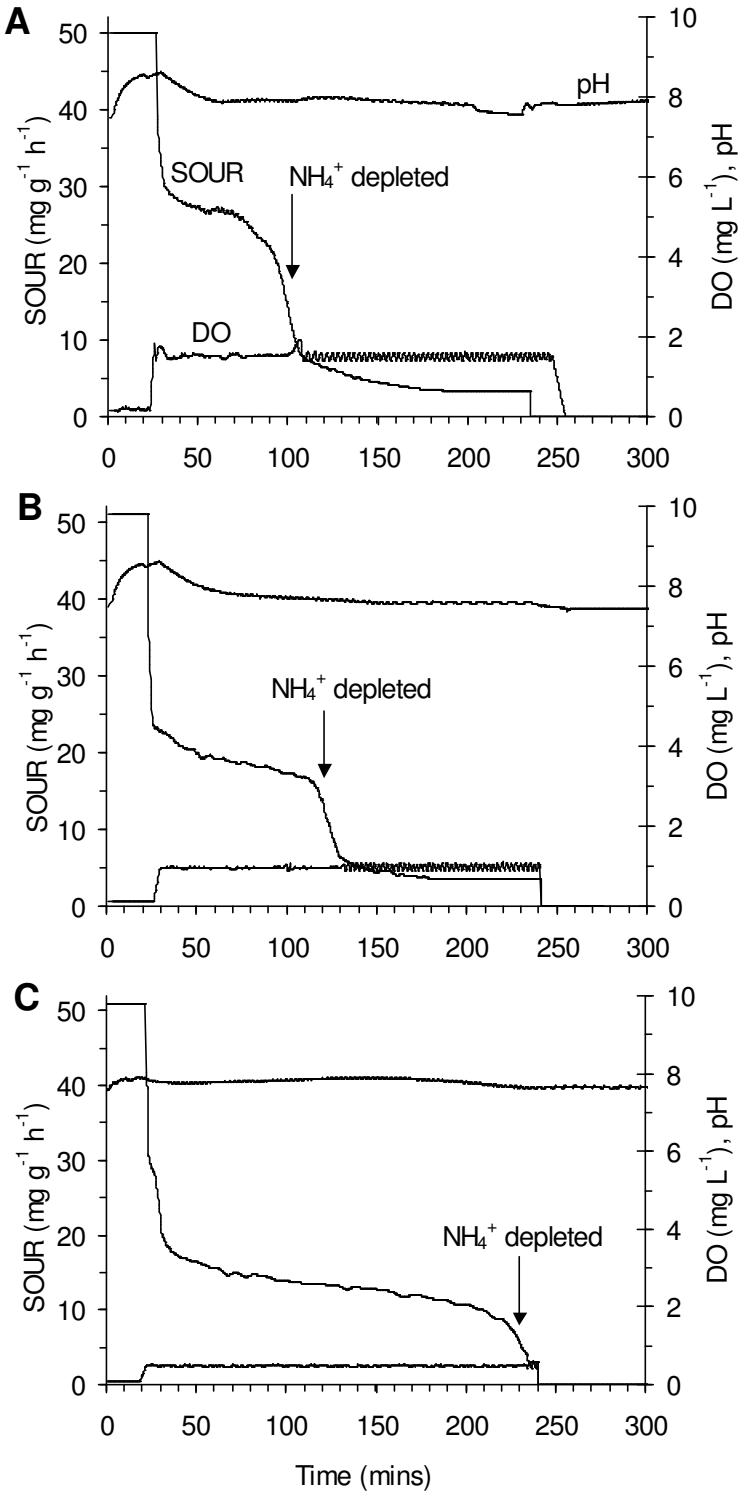

Figure 3.7: On-line measurement of the SOUR, DO concentration and pH during SBR cycling with a DO setpoint of A. 1.5 mg L^{-1}, B. 1 mg L^{-1} and C. 0.5 mg L^{-1}.

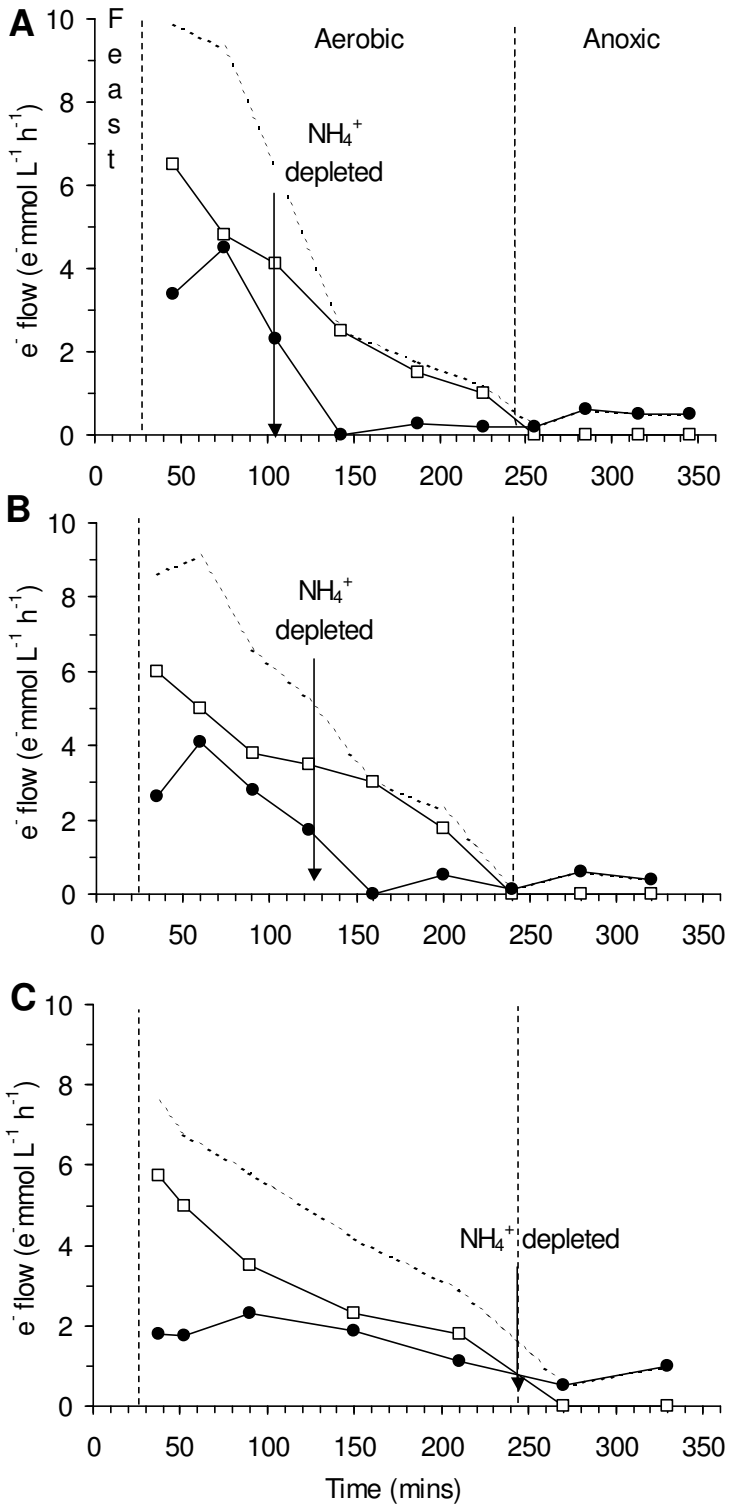

Figure 3.8: Plots of the total electron flow from PHB to an electron acceptor (dotted line), electron flow from PHB to O_2 (□) and electron flow to NO_x^- (●) during SBR cycles at different DO concentrations; A. 1.5 mg L^{-1}, B. 1 mg L^{-1} and C. 0.5 mg L^{-1}.

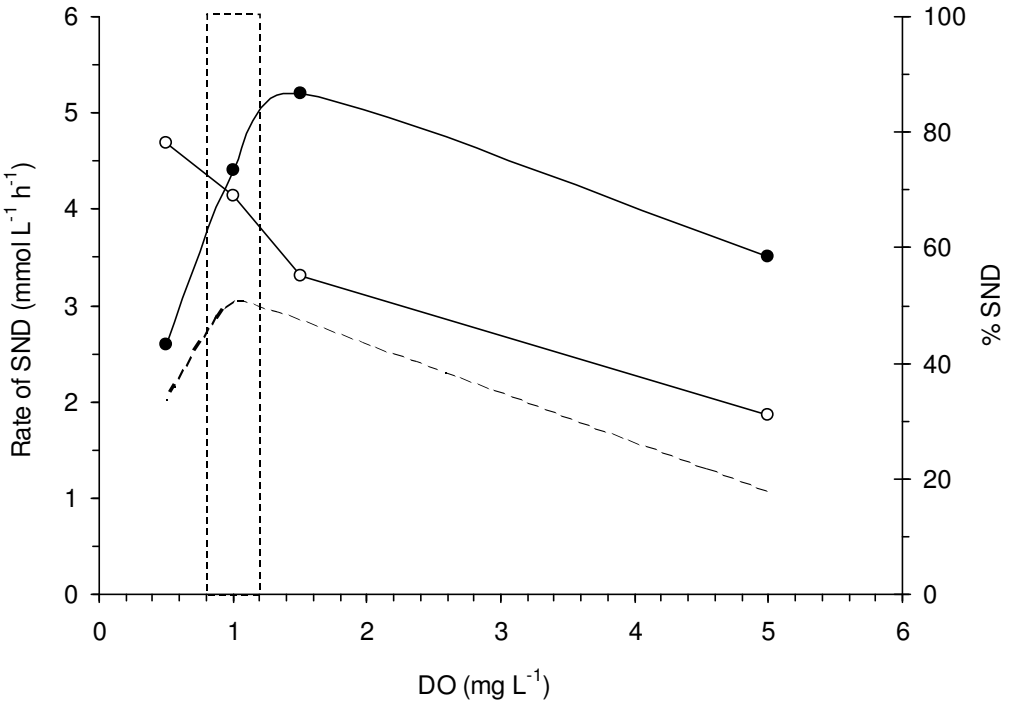

Figure 3.9: Plot of the percentage of SND (❍) and the rate of SND (●) and the SND rate multiplied by the percentage of SND (÷ 100) (dotted line) versus the dissolved oxygen concentration, for analysis of the DO concentration of optimum SND rate and quantity.

Effect of the DO Concentration on PHB Degradation

To analyse the effect of the dissolved oxygen concentration on PHB degradation kinetics, the specific rate of PHB degradation ($-d f_{PHB}/dt$, multiplied by –1) was plotted against the fraction of PHB in the active biomass (f_{PHB}) for the PHB data obtained at different DO setpoints (Fig 3.10). The curves suggested that the degradation rates fit first order kinetics with respect to the PHB cell fraction. The rate constants were similar at all DO concentrations, showing that PHB degradation proceeded at the same rate, independent of the bulk liquid DO concentration over the DO concentrations tested (0.5 – 5 mg L^{-1}). This indicated that in contrast to the nitrifying bacteria, oxygen limitation did not apply to heterotrophic PHB oxidation. All curves intercepted the x-axis at approximately 0.008 Cmol/Cmol, corresponding to the non-degradable PHB fraction (Fig 3.10).

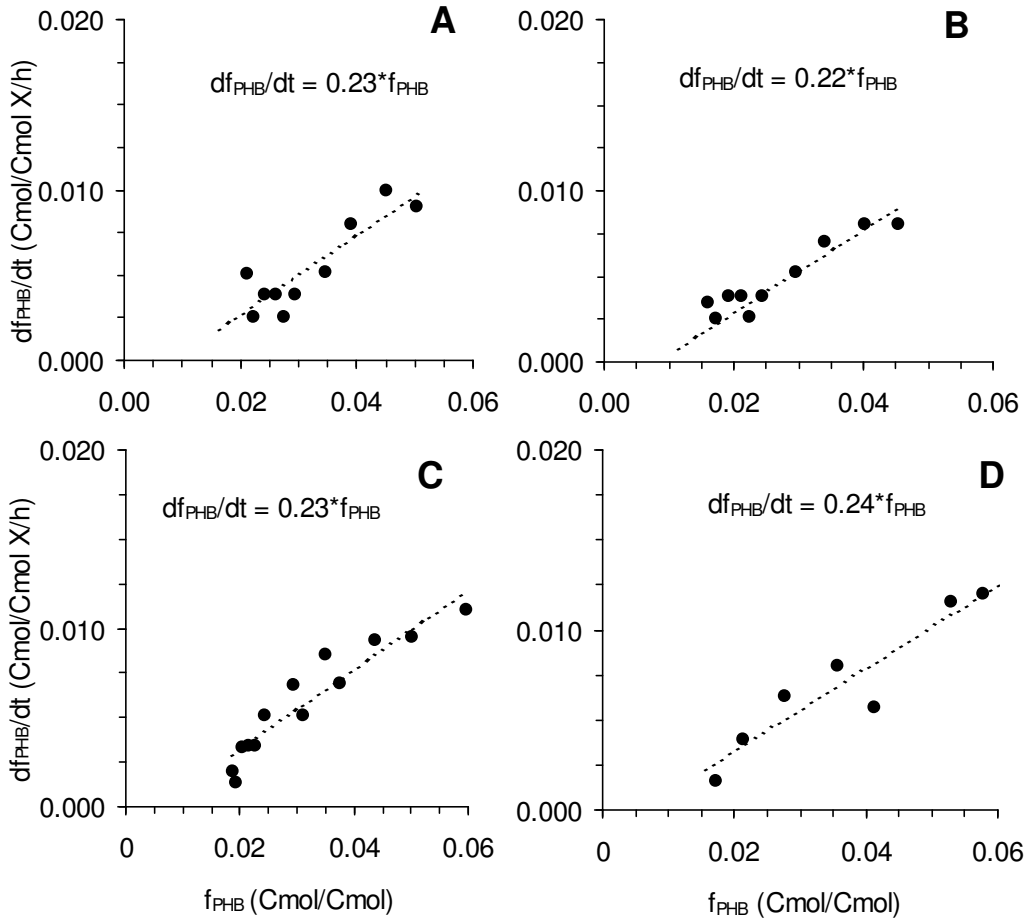

Figure 3.10: Analysis of PHB degradation kinetics under different DO concentrations by plotting the specific PHB degradation rate ($-df_{PHB}/dt$) (multiplied by –1) versus the fraction of PHB in the cells (f_{PHB}) for each of the DO concentrations, A: > 3 mg L^{-1}, B: 1.5 mg L^{-1}, C: 1 mg L^{-1} and D: 0.5 mg L^{-1}. The biomass concentrations in each test are specified in Table 3.1.

PHB Degradation at High Internal PHB Concentrations

The PHB degradation rate clearly increased with a higher fraction of PHB available (Fig 3.10). To determine the maximum possible PHB degradation rate, the biomass was fed with a very high loading of acetate (200 CmM) (Fig 3.11). The aim was to determine the saturating PHB-capacity of the heterotrophic biomass and the nature of PHB degradation kinetics at high levels of f_{PHB}. Ammonium was added in small amounts for heterotrophic growth, but was not sufficient for either nitrification or denitrification. Acetate uptake and PHB production were constant until around 200 minutes when the PHB concentration stabilised, reaching a final cell fraction (f_{PHB}) of 0.61 Cmol/Cmol (54 % total dry wt). The slowing down of PHB production may have

been due to growth limitation of the heterotrophic biomass by ammonium, as NH_4^+ was no longer measurable in the liquid when the PHB storage rate started to decline (Fig 3.11). The final f_{PHB} value reached (0.61 Cmol/Cmol) was around 15 times higher than measured under standard operating conditions (0.04 Cmol/Cmol). Microscopic analysis of the biomass after lipophilic staining was useful in visualising the increase in size of individual cells during the PHB production phase (Fig 3.12). A plot of the PHB degradation rate against the PHB cell fraction confirmed first order kinetics even at the highest f_{PHB} values (Fig 3.13). The rate constant measured over the whole range of f_{PHB} values ($df_{PHB}/dt = 0.19 \cdot f_{PHB}$) was slightly lower than observed at low COD-loading (average rate constant 0.23, Fig 3.10). The rate constants determined under standard reactor operating conditions only covered f_{PHB} values less than 0.06 Cmol/Cmol, thus it would be expected that the rate constant determined over the whole range of values (0 – 0.6 Cmol/Cmol) would represent reality more accurately. Alternatively, PHB degradation may not be exactly first order at the lower PHB fractions and warrants further investigation.

The maximum biomass growth rate and the specific oxygen uptake rate during growth on PHB were estimated from dry weight measurements (Fig 3.11) and compared with maximum rates reported in Chapter 2 for the feast phase of PHB metabolism, where acetate was the sole electron donor (Table 3.2). Although the values estimated from Fig 3.11 are estimates only, comparison of these values with maximum rates of the feast phase showed that the maximum respiration rate of PHB is 2.2 times lower than the maximum respiration rate of acetate (Table 3.2). Thus, even when cells are degrading PHB at maximum rate, the oxidation rate of insoluble reducing power (PHB) is still significantly slower than oxidation of soluble substrate (acetate). Thus, PHB provides a slowly degradable carbon source during the absence of an external soluble carbon source.

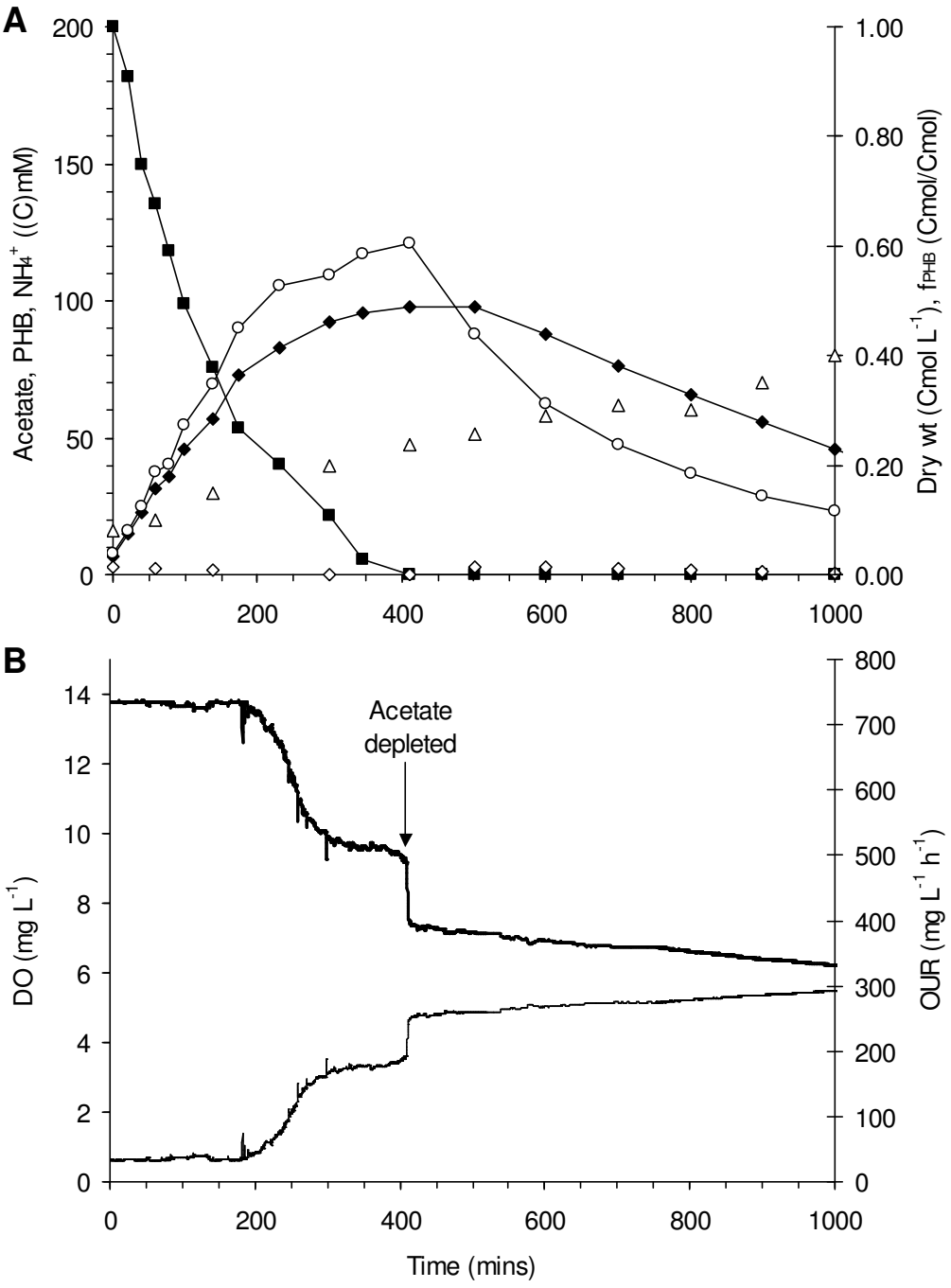

Figure 3.11: PHB production and degradation at a high acetate loading rate, A: Acetate (■), PHB (◆), biomass concentration (Δ), NH_4^+ (✧) and PHB cell fraction (f_{PHB}) (❍), and B: DO concentration (thin line) and OUR (thick line).

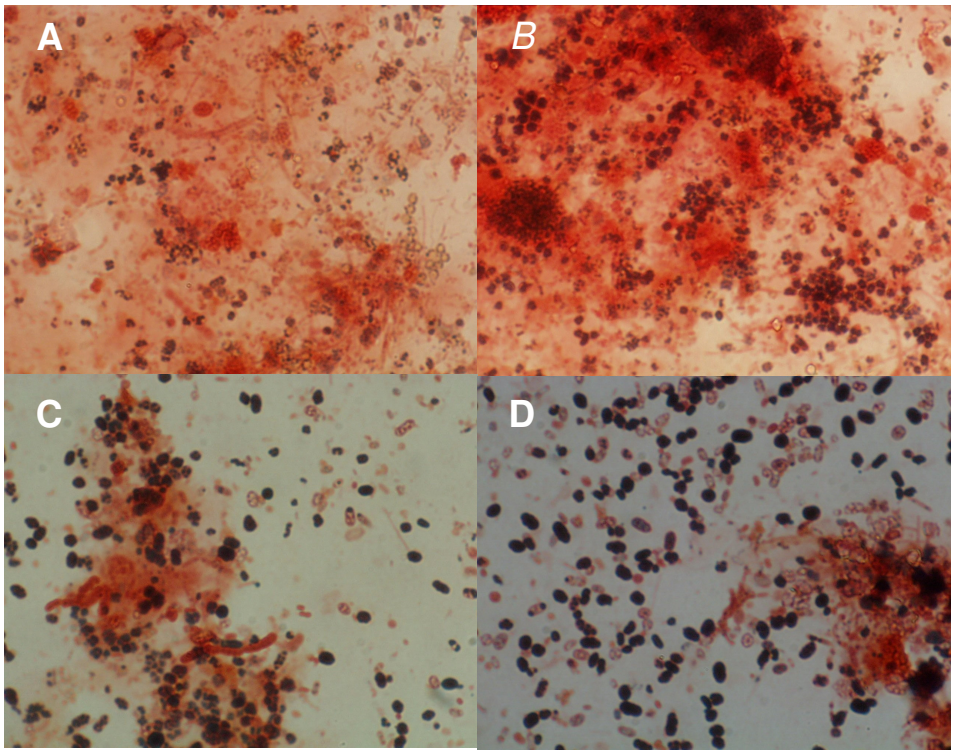

Figure 3.12: Staining of biomass with Sudan Black to observe the increase in PHB content of the heterotrophic cells during the PHB-filling experiment; A: 0 minutes, B: 100 minutes, C: 300 minutes and D: 400 minutes (all at a magnification of 1000x). Black staining indicates lipophilic cellular material (i.e. PHB), and pink areas show non-lipophilic cellular material.

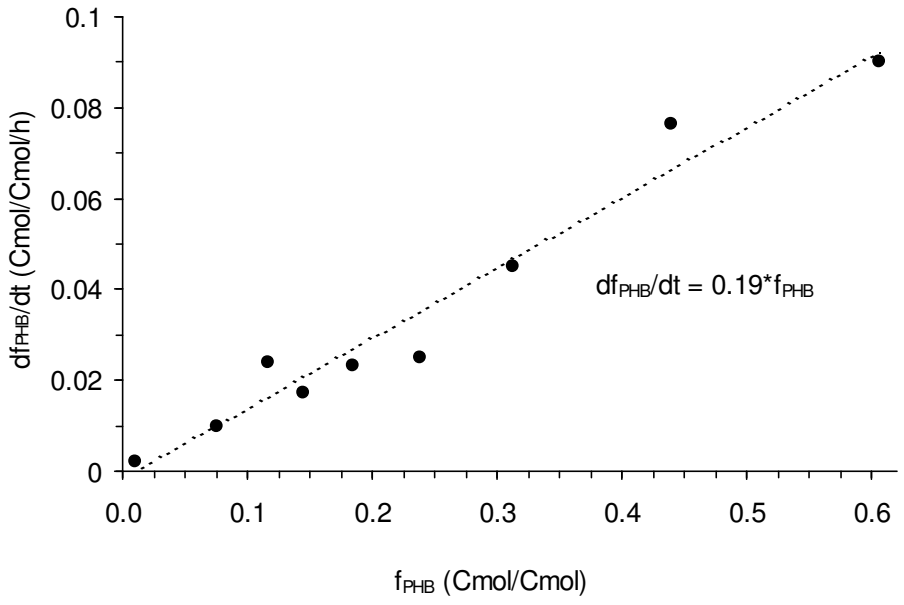

Figure 3.13: Plot of the specific PHB degradation rate ($-df_{PHB}/dt$, multiplied by -1) versus the fraction of PHB (f_{PHB}) in the cells over the whole range of f_{PHB} values.

Table 3.2: Comparison of maximum rate constants during growth on acetate and PHB. Rates for PHB degradation were estimated from experimental data (Fig 3.11), while rates from the feast period are reported in Chapter 2.

	Famine Period	Feast Period
Electron donor	PHB	Acetate
Max. rate of e^- donor consumption (Cmol. Cmol X^{-1}. h^{-1})	0.09	0.60
Max. rate of PHB production (Cmol. Cmol X^{-1}. h^{-1})	-	0.30
Max. respiration rate (mol O_2. Cmol X^{-1}. h^{-1})	0.055	0.12
Max. biomass growth rate (h^{-1})	0.045	0.13

3.4. Discussion

Substrate Storage as PHB Preserves Reducing Power

To achieve effective simultaneous nitrification and denitrification, the separate processes of nitrification and denitrification should proceed at similar rates. As autotrophic nitrification is generally slow in comparison to heterotrophic metabolism, SND requires a slowly degradable carbon substrate to provide reducing power for denitrification during the nitrification process. When treating complex wastewaters there is an increased likelihood that slowly degradable or particulate organic material will be available to serve as the electron donor for denitrification. Up to 100 % SND can be achieved when treating such wastewaters at low DO (Pochana and Keller, 1999). However when particulate substrate is not available, storage of soluble substrate as PHB represents an opportunity to conserve organic carbon as a slowly degradable polymer for denitrification (Jones et al., 1990a, 1990b, Majone et al., 1998, Beun et al., 2001). At a low PHB cell content (0.07 Cmol/Cmol), the respiration rate with PHB was up to 6 times slower than with acetate (Fig 3.4), and even at saturating levels of PHB (f_{PHB} 0.61 Cmol/Cmol), the PHB oxidation rate was still 2.2 times slower than the acetate oxidation rate (Fig 3.11, Table 3.2). This shows that PHB storage significantly slows down the rate of carbon oxidation and prevents its rapid oxidation to CO_2. PHB was thus available as the electron donor for SND throughout the entire famine period

(Fig 3.6). Interestingly, reported rates of denitrification using PHB as the electron donor are very similar to reported rates of ammonium oxidation (Table 3.3), which suggests that these two processes are well matched to achieve SND.

The comparison of maximum rates of PHB production and consumption for the feast and famine phase (Table 3.2) confirmed that PHB degradation is the slowest reaction in the whole PHB metabolic process. This can explain why the PHB degradation rate was apparently independent of the dissolved oxygen concentration (Fig 3.10), as it is likely that PHB oxidation was limited by the internal PHB hydrolysis rate and not by the DO concentration over the range tested (DO > 0.5 mg L^{-1}). The maximum estimated biomass growth rate on PHB was 3 times lower than that on soluble acetate (Table 3.2), suggesting that both growth and respiration rates of the biomass when growing on PHB were determined by the PHB hydrolysis rate. These results agree with the work by Beun et al. (2002) who also showed that it was possible to describe PHB degradation with first order kinetics ($df_{PHB}/dt = -0.15 \cdot f_{PHB}$), depending only on the PHB content of the biomass. The rate constant was similar to that reported in this study ($df_{PHB}/dt = -0.19 \cdot f_{PHB}$, Fig 10). Beun et al. (2002) found that PHB degradation rates were independent of the growth rate and the electron acceptor type or concentration, leading the authors to conclude that PHB hydrolysis is the rate-limiting step in PHB degradation. This hypothesis is supported by the results of this study and others (Dircks et al., 2001, Van Aalst-Van Leeuwen et al., 1997). The slow nature of PHB degradation would be advantageous for heterotrophic cells in nature, as rapid oxidation of reducing power during starvation periods would be prevented. The availability of a slowly degradable substrate during starvation periods provides cells with a competitive advantage over other cells that do not have the availability of an electron donor during famine periods (Van Loosdrecht et al., 1999). Coincidentally, the slow nature of PHB degradation is advantageous for the SND process in activated sludge by providing a slowly degradable carbon source for denitrification during the relatively slow nitrification process.

Table 3.3: Comparison of maximum rates reported in the literature for aerobic ammonium oxidation and denitrification using stored polymers. All units cited were converted by the authors to mol.Cmol $X^{-1}.h^{-1}$ by assuming 1 carbon mole biomass = 24.6 g and 1 g biomass contains 0.5 g protein.

Nitrification	NH_4^+ oxidation rate (mmol Cmol X^{-1}. h^{-1})	**Denitrification**	NO_3^- reduction rate (mmol Cmol X^{-1}. h^{-1})
Strous, 2000	4.5[1]	Beun et al., 2000b	9[2]
Jetten et al., 1997a	3.1[1]	Alleman and Irvine 1980	4
Jetten et al., 1997b	15	Jones et al., 1990a	5.5[1]
This study (Table 3.1)	8	Meyer 1985 (cited in Jones et al. 1990a)	4.6[1]
		This study (Table 3.1)	3.9*

[1] Values were averaged from a range of values given.
[2] The rate was calculated by the author from the maximum anoxic PHB degradation reported (0.022 Cmol. $Cmol^{-1}$. h^{-1}), assuming half the PHB is oxidised by nitrate.

DO Concentration and SND – A Compromise Between Rate and Quantity

SND relies on two reactions that have opposing requirements; Ammonium oxidation requires oxygen and is limited at low DO concentrations, while denitrification is limited by the availability of an electron donor and requires the absence of oxygen. In accordance with the literature (Beun et al., 2001, Munch et al., 1996, Pochana and Keller, 1999, Pochana et al., 1999) our study showed that the percentage of SND could be improved by lowering the oxygen supply during the famine period (Table 3.1, Fig 3.6). However for satisfactory nutrient removal from wastewater it is not only the percentage of SND but also the rate that is important. As expected, lowering the oxygen supply slowed down the nitrogen removal process (Table 3.1), an observation that has been reported recently: When lowering the DO concentration from 0.5 to 0.3 mg L^{-1}, Pochana and Keller (1999) increased the percentage of SND from 95 to 100 %, but at a much reduced rate. From our results, the maximum rate of SND was achieved at a DO concentration of 1.5 mg L^{-1}, yet the percentage of SND at this DO concentration was not optimal (Fig 3.9). DO concentrations higher than 1.5 mg L^{-1} decreased both the rate and quantity of SND achieved, while low setpoints (< 1 mg L^{-1}) increased the quantity but decreased the rate of SND. Thus, there is a compromise between the rate at which SND occurs and the total nitrogen removal possible. Rates of SND are scarcely reported

in the literature, making it difficult to compare optimal rates of SND in this study with others. SND is typically reported as a percentage and the optimal DO concentrations for maximum percentages of SND vary largely among studies, depending on floc size and aeration conditions. For example, Beun et al. (2001) achieved 100 % SND at a DO concentration of 1.6 mg L^{-1} when using granular biomass (floc size up to 5 mm diameter), compared to only 55 % SND at 1.5 mg L^{-1} in this study with non-granular biomass (floc size < 100 µm, measured by haemocytometer, data not shown). In contrast, Pochana and Keller (1999) required a DO concentration of 0.3 mg L^{-1} to achieve 100 % SND when using non-granular activated sludge. Pochana et al. (1999) showed that a reduction in floc size from 100 µm to 40 – 50 µm diameter reduced the percentage of SND achieved from 95 % to 21 %. Therefore, the floc size and thus the extent of DO penetration into the floc has a large impact on the amount of SND achievable. SND would need to be optimised on a plant-to-plant basis, depending on the sludge and aeration characteristics. Multiplication of the rate of SND by the percentage of SND was useful for finding the DO concentration of optimal SND rate and quantity (Fig 3.9) and could potentially be used as a suitable SND performance indicator for optimisation of SND in large-scale plants.

Electron Flow Analysis shows Aerobic vs Anoxic PHB Usage during Aeration

Plots of electron flow rates to both nitrate and oxygen were useful in visualising the areas of maximum SND activity throughout the SBR cycle (Fig 3.8). In all cases, total electron flow from PHB decreased over the course of the aeration period, in accordance with PHB measurements (Fig 3.6). The rate of electron flow to nitrate was highest during periods where the SOUR was also high (Figs 3.7, 3.8), which may indicate that higher rates of nitrate reduction were achieved due to reduced penetration of oxygen into the centre of the flocs. Once ammonium was depleted, the rate of electron flow to nitrate was minimal during the remainder of the aeration period (Fig 3.8). Beun et al. (2001) reported a similar finding, whereby denitrification during the aerobic period stopped completely once ammonium was depleted. These authors also contributed the lack of SND after ammonium depletion to deeper penetration of oxygen into the granule. Thus, the presence of ammonium appears to act as an oxygen "shield", protecting heterotrophic cells from oxygen penetration and allowing simultaneous denitrification. Higher oxygen uptake rates due to both PHB and NH_4^+ oxidation would be expected to create larger anoxic zones in the floc and allow greater denitrification

rates. In all cases, electron flow from PHB to nitrate during the anoxic phase was low, probably due to a lack of reducing power in the form of PHB. The use of shorter aeration periods would improve this problem.

Possible Improvements to SND and Nitrogen Removal in the SBR

At all dissolved oxygen setpoints tested, nitrate accumulated towards the end of the ammonium oxidation (i.e. SND) period (Fig 3.6). This indicated that SND became limited by the rate of denitrification. The decreasing denitrification rate was also indicated by the decreasing electron flow to nitrate after the initial 75 minutes of the aerobic famine phase (Fig 3.8). This may have been due to two factors; (1) Increased penetration of oxygen into the floc due to the decreasing SOUR over the course of the aeration period, causing increased competition for PHB by oxygen (Fig 3.7), and/or (2) A gradual decrease in the rate of PHB availability, resulting in limitation of reducing power for denitrification. With regard to the first limitation, the oxygen uptake rate of the culture decreased over the course of the SND period (Fig 3.7). Thus, a decrease in the oxygen supply rate would be expected to reduce the penetration of oxygen into the floc and favour improved denitrification towards the end of ammonium oxidation. As a consequence, a downward sliding DO setpoint would be expected to improve SND. In addition, both limitations to SND could theoretically be improved by operating at increased cellular PHB levels. As shown in this study and very recent work (Beun et al., 2002), higher cell fractions of PHB are equivalent to higher organic substrate availability and are hence expected to improve the rate at which nitrate is denitrified during SND. A higher availability of PHB would also increase the SOUR due to higher aerobic respiration activity and possibly create larger anoxic zones. In our study, the fraction of PHB in the biomass was very low (0.01 – 0.055 Cmol/Cmol), when compared to the maximum capacity of the biomass measured (0.61 Cmol/Cmol). One way in which the PHB content inside the cells could be increased over time would be to reduce the length of the aeration period, such that the PHB remaining in the cells accumulates over time. When operating at DO concentrations of 1 and 1.5 mg L^{-1} – the DO concentrations where the optimum rate of SND was achieved – the aerobic phase length of 240 minutes was too long as all ammonium was oxidised by 105 and 125 minutes, respectively. Immediate termination of the aeration phase after ammonium depletion would prevent unnecessary aerobic oxidation of PHB and potentially allow the build-up of PHB over the long term, driving faster PHB hydrolysis and thus faster

SND. On-line monitoring of the SOUR could reproducibly detect the end-point of ammonium oxidation (Figs 3.2B, 3.7). The ability to use this on-line signal for the management of cycle lengths is currently being investigated in our laboratory.

3.5. Conclusions

This study helps to improve the overall understanding of PHB metabolism in mixed cultures and its relation to SND and nitrogen removal. PHB metabolism was shown to be the slowest reaction of the whole cellular PHB metabolic process. The relative maximum rates of electron flow during PHB metabolism are depicted in Fig 3.14. The slow nature of PHB degradation makes the storage polymer a suitable substrate for SND, as ammonium oxidation can proceed at a similar rate to PHB oxidation (and thus denitrification) at low DO concentrations (< 1.5 mg L^{-1}). Electron flow analysis was useful in visualising the contribution of nitrate reduction to overall PHB oxidation. Under the conditions tested, the period of highest SND activity was during the first hour of the aerobic famine period, when the SOUR was also high. It is likely that a high SOUR decreases oxygen penetration into the floc and creates larger anoxic zones for simultaneous denitrification. The build up of higher levels of PHB to increase the SOUR and denitrification rates, together with improved oxygen management, should improve PHB-driven SND.

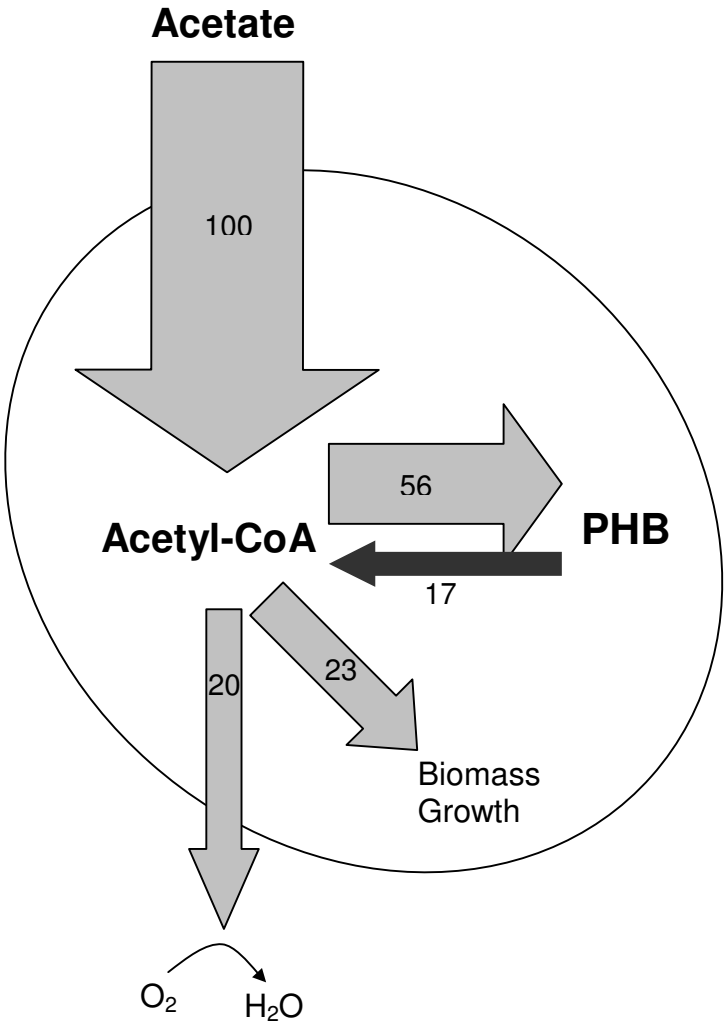

Figure 3.14: A conceptual diagram to compare the overall maximum rates of electron flow during PHB metabolism (Table 3.2). The thickness of the arrows provides a visual comparison of the difference in rates, assuming the cell has a total acetate inflow representing 100 electron mol.Cmol $X^{-1}.h^{-1}$.

CHAPTER 4

The Specific Oxygen Uptake Rate (SOUR) as an On-Line Control Parameter for Improved N-Removal in an SBR[1]

Abstract

In processes where denitrification is designed to occur after aerobic nitrification, complete nitrogen removal can be limited by the availability of reducing power in the form of organic substrate. To maximise the amount of reducing power available for anoxic denitrification in a sequencing batch reactor (SBR), the length of the initial aerobic phase should be no longer than required for ammonium oxidation, such that organic substrate is not oxidised unnecessarily. In this study, a simple method was developed for measuring the specific oxygen uptake rate (SOUR) on-line, which could clearly detect the end-point of nitrification and be used for automated adjustment of the aerobic phase length. The laboratory SBR was operated such that all organic substrate (acetate) was rapidly converted to PHB, which then served as the electron donor for nitrogen removal via simultaneous nitrification and denitrification (SND) during the aerobic phase (up to 70 % SND). During SBR cycling with a fixed aeration length (240 minutes), PHB was unnecessarily oxidised after ammonium depletion, resulting in little denitrification and poor total nitrogen removal (69 %). However, when the aerobic phase length was controlled via the SOUR, up to 1.8 CmM PHB (58 mg L^{-1} COD) could be preserved, enabling improved total nitrogen removal (86 %). The drop in the SOUR after ammonium depletion was a reproducible event that could be detected even when using raw wastewater and fresh activated sludge. The SOUR-control technique was able to successfully detect the endpoint of nitrification and adapt the aerobic phase length to varying wastewater types and strengths and to different dissolved oxygen setpoints.

[1] This chapter has been submitted to Applied Microbiology & Biotechnology.

4.1. Introduction

Nitrogen removal from wastewater is often limited by the availability of organic carbon, especially when treating wastewaters with a low carbon to nitrogen (C:N) ratio. Conventional biological nitrogen removal involves two sequential processes: aerobic nitrification followed by anoxic denitrification (Jetten et al., 1997b). Due to the fast nature of heterotrophic metabolism in comparison to autotrophic nitrification, readily biodegradable organic substrate is generally oxidised during the early stages of the aeration phase and has little opportunity to participate in subsequent denitrification. Strategies to overcome the lack of organic carbon in conventional plug-flow systems have included the controlled addition of carbon (methanol, acetate or propionate) to anoxic reactors (Isaacs et al., 1994, Isaacs and Henze, 1995, Tam et al., 1994, Zeghal et al., 1997, Puznava et al., 1998), or the re-cycling of nitrified waters back into contact with the organic-rich wastewater (anoxic feeding) (Jones et al., 1990b). However, these techniques can be complicated and often costly.

Dynamic reactor types such as the sequencing batch reactor (SBR) offer an opportunity to utilise organic carbon in the influent more efficiently. By promoting microbial substrate storage, dynamic systems have the potential to reduce or eliminate the costly necessity for a supplemental carbon supply (Alleman and Irvine, 1980). The high substrate gradient imposed at the beginning of each SBR cycle (feast period) encourages the growth of bacteria that are able to rapidly import substrate and store it as a reduced polymer, such as poly-β-hydroxybutyric acid (PHB). Substrate-storing bacteria have a competitive advantage in the SBR due to their ability to grow in the absence of an external carbon source (famine period), while other bacteria undergo starvation (Van Loosdrecht et al., 1997). The storage of organic substrate as PHB has another advantage: In mixed bacterial cultures using acetate as the organic carbon source, the oxidation rate of PHB is 2 – 6 times lower than oxidation of soluble substrate (Chapter 3). Thus, the conversion of a readily degradable organic substrate to PHB helps to preserve reducing power throughout the aeration phase of an SBR. As PHB can also serve as electron donor for denitrification (Jones et al., 1990a, 1990b), this will improve nitrogen removal. Furthermore, under microaerophilic conditions, the availability of PHB enables nitrogen removal via simultaneous nitrification and denitrification (SND) (Beun et al., 2001, Chapter 3).

To ensure that sufficient PHB is available for anoxic denitrification after completion of the aerobic phase of an SBR, the length of the aerobic phase needs to be managed. Since the primary objective of the aerobic period is the oxidation of ammonium to nitrite or nitrate, the oxygen supply should cease as soon as ammonium has been depleted. Methods for determining the endpoint of nitrification include pH and oxidation-reduction potential (ORP) breakpoints in on-line measurements (Hao and Huang, 1996, Sasaki et al., 1993, Paul et al., 1998, Yu et al., 1997, Yu et al., 2000). However, these measurements have been perceived to be unreliable, due to the large variety of influences that can obscure identifiable points of interest in full-scale plants (Zipper et al., 1998). Measurement of respiration activity is a reliable means for detecting changes in microbial activity and can be used to detect the endpoint of ammonium oxidation (Johansen et al., 1997, Spanjers et al., 1999). Several respirometry devices have been developed for the purpose of measuring respiration activity in activated sludge. The majority involve complicated systems for periodic or continuous removal of a mixed liquor sample for testing in a separate respirometer (Temmink et al., 1993, Watts and Garber 1994, Klapwijk et al., 1998). This study presents a method for continuous on-line measurement of the specific oxygen uptake rate (SOUR) of a mixed culture in a laboratory SBR, by means of a dissolved oxygen probe and a simple calculation procedure based on the specific oxygen mass transfer coefficient (k_La). The study aims to evaluate the usefulness of SOUR monitoring for on-line detection of ammonium depletion and control of the aerobic phase length, in an attempt to avoid "over-oxidation" of organic substrate after ammonium depletion. The effect of this control strategy on nitrogen removal is described and the feasibility of using SOUR-controlled aerobic phase length with real wastewater and fresh activated sludge is investigated.

4.2. Materials And Methods

4.2.1. Experimental Setup

A 2 L sequencing batch reactor (SBR) with on-line measurement of dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) was used for all experiments, as described in Chapters 2, 3. The airflow rate and stirring speed were maintained constant at 100 L h^{-1} and 80 ± 2 rpm, respectively. The k_La in all experiments was 21 h^{-1}, and was checked before the start of each experiment. One standard cycle SBR consisted of a rapid aerobic fill phase (3 mins), an aeration phase (up to 240 mins), an anoxic phase (120 mins), a settle phase (5 – 20 mins), and a 20 min effluent withdrawal phase. A sludge blanket of 800 mL was inoculated into the reactor, and the remaining 1.2 L was filled with feed during subsequent cycling.

4.2.2. Dissolved Oxygen Control

These experiments were performed before PID control of the DO concentration had been developed. The simplest form of oxygen control was used, whereby the DO concentration was controlled about the setpoint by simple digital on-off switching of the air supply, i.e. "on" if the DO is below the setpoint and "off" if above the setpoint. This resulted in oscillations of the DO about the setpoint (± 0.4 mg L^{-1}). Due to the oscillating DO conditions, the average dissolved oxygen concentration was calculated for each aerobic phase.

4.2.3. On-Line Calculation of the Specific Oxygen Uptake Rate (SOUR)

On-line calculation of the oxygen uptake rate (OUR) required knowledge of the changing k_La over time. In principle, once the k_La is known the OUR can be calculated from the actual steady state oxygen concentration. However, because of the undulating behaviour of the oxygen concentration, true steady state conditions were not reached. For accurate on-line monitoring of the OUR, a more complex procedure was necessary. As the airflow was supplied intermittently, the actual k_La for intermittent air supply (k_La_{int}) could be estimated by multiplying the k_La value for uninterrupted airflow (k_La_{full}) (measured as in Chapter 2) by the fraction of time that the airflow was on over a defined period (Eq 1). The Air On Time fraction (f_{AirOn}) could be measured precisely by LabView (Fig 4.1A). ;

$$k_L a_{int}\,(h^{-1}) = f_{AirOn} \cdot k_L a_{full}\,(h^{-1}) \qquad \text{(Eq 1)}$$

where the k_La for uninterrupted airflow (k_La_{full}) had been determined before the experiment. According to this calculation, for an air supply that was "on" for half the time (during a DO undulation about the setpoint), the k_La was half the value it would be if air were supplied continuously. The oxygen transfer rate (OTR) into solution during one DO undulation about the setpoint was then calculated according to;

$$OTR\,(mg\,L^{-1}\,h^{-1}) = k_L a_{int} \cdot (c_S - c_L) \qquad \text{(Eq 2)}$$

where c_L (mg L^{-1}) was calculated on-line as a running average of the DO concentration (for 30 on-line DO data points) and c_S was the saturation concentration of oxygen in the gas phase (mg L^{-1}). On-line calculation of the OUR by this method was based on two assumptions; 1. The DO concentration was at steady state, and 2. Each time the air was "on" the k_La was immediately at its maximum value and whenever the air was "off", the k_La was zero. It was apparent from dissolved oxygen data that the simplified view of the k_La (assumption 2) was not accurate, since when the oxygen was turned off, the DO continued to rise for some time, showing that oxygen transfer did not stop immediately (Fig 4.1A). To quantify the error caused by the k_La assumptions, the real OUR was periodically measured by aerating the culture mid-cycle until a steady state was reached and measuring the oxygen consumption rate after turning off the air supply, while flushing the reactor headspace with nitrogen gas. It was found that the real OUR was consistently 14 – 15 % higher than the on-line calculated OUR. The source of this error was the simplified k_La assumption. To determine the actual k_La during undulating oxygen conditions, the DO data were used to calculate the changing OTR, given that the rate of change of the DO concentration (dc_L/dt) at any point is equal to the oxygen transfer rate minus the oxygen uptake rate;

$$\frac{dc_L}{dt} = OTR - OUR \qquad \text{(Eq 3)}$$

As the real OUR could be measured and dc_L/dt was obtained on-line from DO data, the OTR over time was known (Eq 3) and the real k_La could be determined (Eq 2) (Appendix 1). The real k_La varied distinctly from the assumed k_La and responded much slower to changes in the air supply than expected (Fig 4.1B). Although the short-term dynamics of oxygen transfer were very different between the assumed k_La and the real scenario, the total amount of oxygen transferred over each oscillation under the two

different scenarios differed by only 14 %, as calculated by integrating the oxygen transfer rate curves (Appendix 1). To compensate for the under-estimation of OUR values calculated by the on-line method, the program was modified to include a 14 % scale-up of the on-line values, which subsequently resulted in an accurate approximation of the true oxygen uptake rate that could be confirmed reproducibly by measurements of the actual OUR during a cycle.

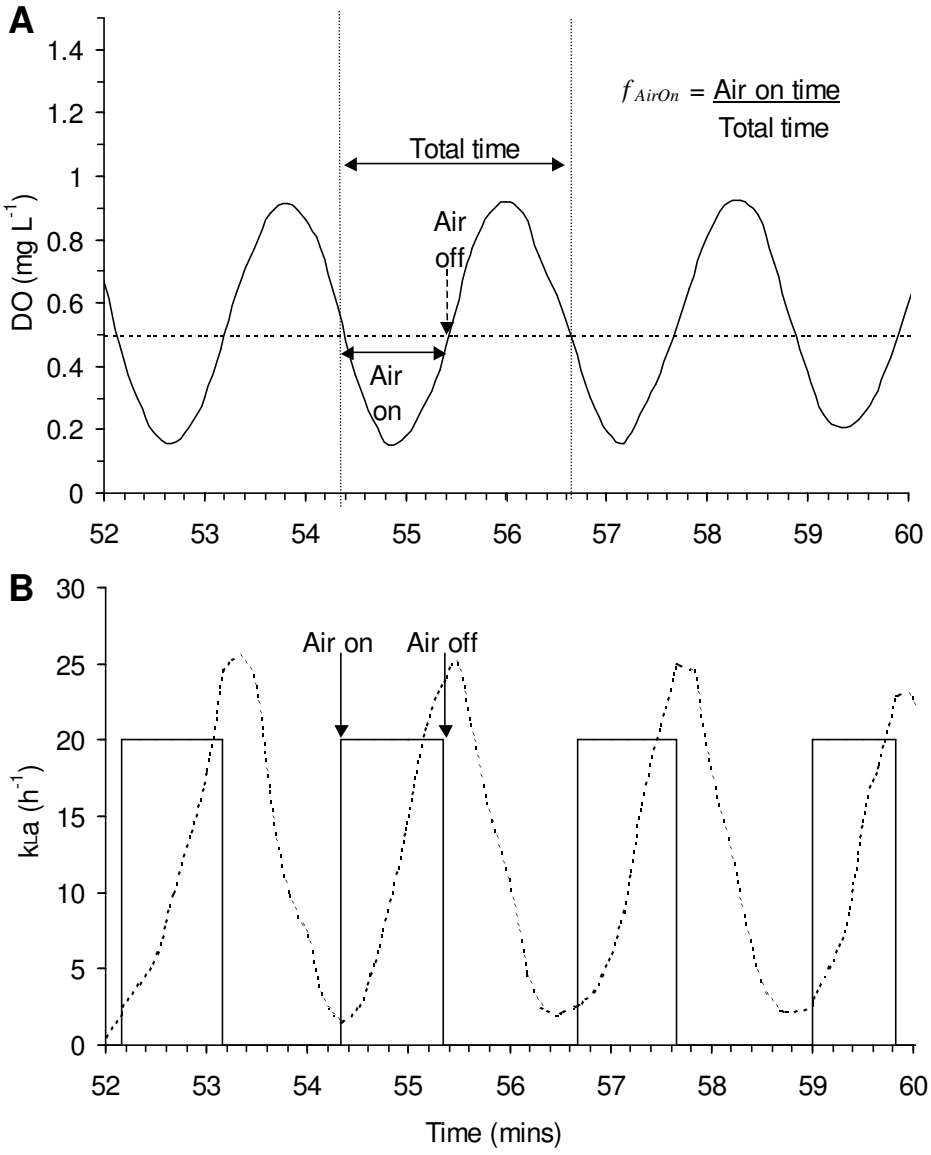

Figure 4.1: Method for on-line calculation of the SOUR, A: Calculation of the fraction of "air on" time of the total time (f_{AirOn}) for a DO undulation about the setpoint, and B: Comparison of the real k_La (dotted line) with the assumed k_La (solid line) used for calculating the SOUR.

To convert the oxygen uptake rate into a specific oxygen uptake rate (SOUR), the biomass concentration was measured before the start of each experiment and the SOUR was then calculated as;

$$SOUR\ (mg.\ g^{-1}\ X.\ h^{-1}) = \frac{OTR}{X}\left(\frac{mg.L^{-1}.h^{-1}}{gX.L^{-1}}\right) \qquad \text{(Eq 4)}$$

On-line SOUR calculation by this method relied on the assumption that the biomass concentration did not change significantly over one cycle (measurements showed it increased by 20 % maximum). While the results presented in the following study rely on a trend in the SOUR, rather than absolute values, we aimed to monitor the SOUR as accurately as possible such that comparisons of SOUR values could be made with other studies.

4.2.4. Synthetic Wastewater

The composition of the nutrient medium used is described in Chapter 2, resulting in a final carbon to nitrogen ratio (C:N) of 10:1. Where higher concentrations of nutrients were used, the concentrations are specified in the results section. The medium was autoclaved to prevent bacterial activity in the feed vessel.

4.2.5. Raw Wastewater and Activated Sludge

The wastewater and fresh activated sludge used in this study were obtained from a large-scale Sequencing Batch Reactor treating 160 ML/day of domestic wastewater (Woodman Point, Western Australia, Environmental Solutions International (ESI) Ltd). The wastewater was taken before the primary treatment stage and was therefore filtered before use to remove solids (50 µm pore size). The composition of the wastewater (after filtering) was (mg L^{-1}): Total COD 540, BOD 200, particulate COD 380, Acetate COD 13 (i.e. 7 % of total BOD), ammonium 40 and total Kjeldahl nitrogen (TKN) 60. This resulted in a final total COD: N ratio of 9:1 and a soluble BOD: N ratio of 3:1.

4.2.6. Sampling and Analytical Procedures

All compounds, dry weight and sludge volume index were measured as described in Chapter 2.

4.2.7. Calculations

The PHB content of biomass and yields were calculated as in Chapter 2. The yield of PHB from acetate ($Y_{PHB/Ac}$) (COD/COD) could be calculated from PHB and acetate measurements (Cmol L^{-1}) by considering that one carbon mole of PHB is equivalent to 36 mg COD and one carbon mole of acetate 32 mg COD;

$$Y_{PHB/Ac}(COD/COD) = \frac{PHB_{produced}}{Ac_{consumed}}\left(\frac{Cmol}{Cmol}\right).\frac{36}{32}\left(\frac{gCOD/CmolPHB}{gCOD/CmolAc}\right) \qquad \text{(Eq 5)}$$

4.3. Results

Previous experiments showed that acetate conversion to PHB during the feast phase of an SBR is maximised under O_2-limitation (Chapter 2). Thus, the feast phase in all SBR cycles (i.e. soluble substrate present) was operated under oxygen limitation (k_La 21 h^{-1}, [DO] 0 mg L^{-1}). The DO concentration was controlled to around 1 mg L^{-1} during the famine phase of all experiments. The sludge age of the laboratory-biomass was 11 days and the biomass had been adapted to the dynamic lab-SBR conditions for more than 4 weeks when the experiments were performed. The biomass contained a fraction of PHB that was not degradable (0.008 Cmol PHB/Cmol X) (Chapter 3), which was subtracted from all PHB measurements to show only the available PHB. Where fresh activated sludge was used, the sludge age at the time of sampling from the WWTP was 20 days. The following results represent typical data sets observed repeatedly over the 2-year duration of this study.

Typical SBR Behaviour during Standard Operation

During dynamic SBR operation with the defined acetate-based medium, the SOUR showed a reproducible pattern of behaviour, consisting of three distinct stages of biomass activity (Fig 4.2). The first stage of highest activity was due to acetate consumption and PHB production (feast phase). During this time, up to 71 % of the reducing equivalents available from acetate were preserved as PHB ($Y_{PHB/Ac}$ 0.71 COD/COD) (Fig 4.3A). The second, lower stage of SOUR was due to simultaneous ammonium and PHB oxidation. The SOUR decreased gradually until ammonium was depleted, at which point there was a clear, sharp drop in the SOUR (Fig 4.2). The sudden drop in the SOUR is consistent with the low half saturation constant for ammonium ($k_{S,NH4+}$) of nitrifying bacteria. This SOUR-drop could be used as an on-line

indicator of nitrification termination. The third phase of very low SOUR was due to residual PHB oxidation and maintenance respiration. Due to the low DO concentration during the famine period (average 1 mg L^{-1}), significant nitrogen was removed via simultaneous nitrification and denitrification (SND) using PHB as the electron donor (59 %) (Fig 4.3A). When ammonium was depleted, as indicated by the second drop in the SOUR, some PHB was still available (2.5 CmM). This would have been sufficient reducing power to denitrify 1.1 mM nitrate if anoxic conditions had been applied immediately (since 1 Cmol PHB can reduce 0.45 mol NO_3^-, assuming a biomass yield of 0.5 Cmol/Cmol). However, due to the continued supply of oxygen, this available PHB was subsequently "wasted" by aerobic oxidation. Consequently, little denitrification occurred and the overall nitrogen removal was low (69 %).

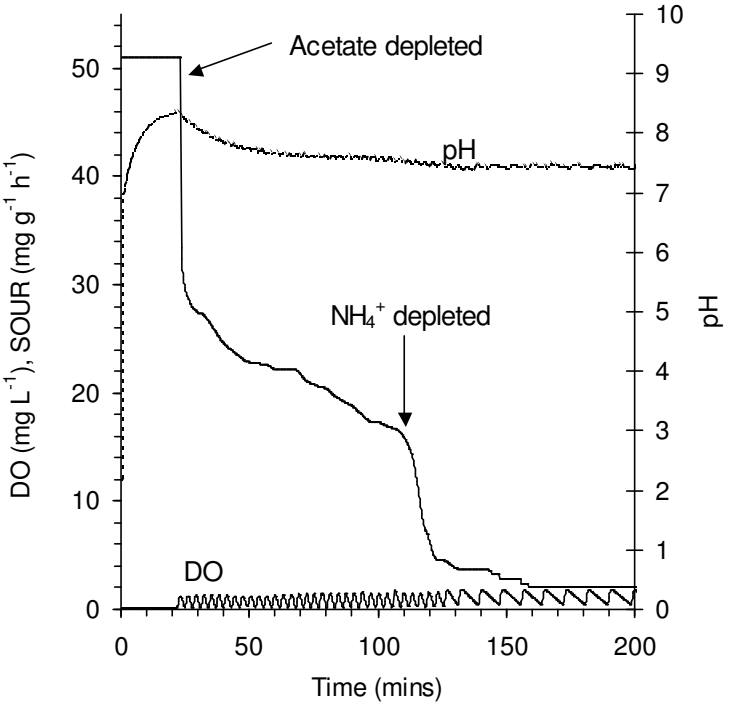

Figure 4.2: Typical behaviour of the SOUR, DO and pH during standard SBR operation with a DO setpoint of 1 mg L^{-1}. The biomass concentration in the reactor was 3.3 g L^{-1}, with an SVI of 92 mL g^{-1}X.

SOUR-Control of the Aerobic Phase Length

To test whether termination of the aerobic phase as soon as ammonium was depleted (as indicated by the second drop in the SOUR) could improve nitrogen removal during one cycle, the control program was modified to automatically terminate the aeration phase as soon as the SOUR dropped to below 10 mg g^{-1} X h^{-1}. This enforced immediate anoxic conditions as soon as ammonium was depleted and avoided further aerobic PHB oxidation (Fig 4.3B), resulting in more PHB being available at the start of the anoxic phase (2.7 CmM). This enabled subsequent denitrification and improved the total nitrogen removal to 86 %, in comparison to only 69 % with fixed aeration length (Fig 4.3A). Furthermore, the total cycle length was reduced by 100 minutes, allowing a faster throughput of wastewater.

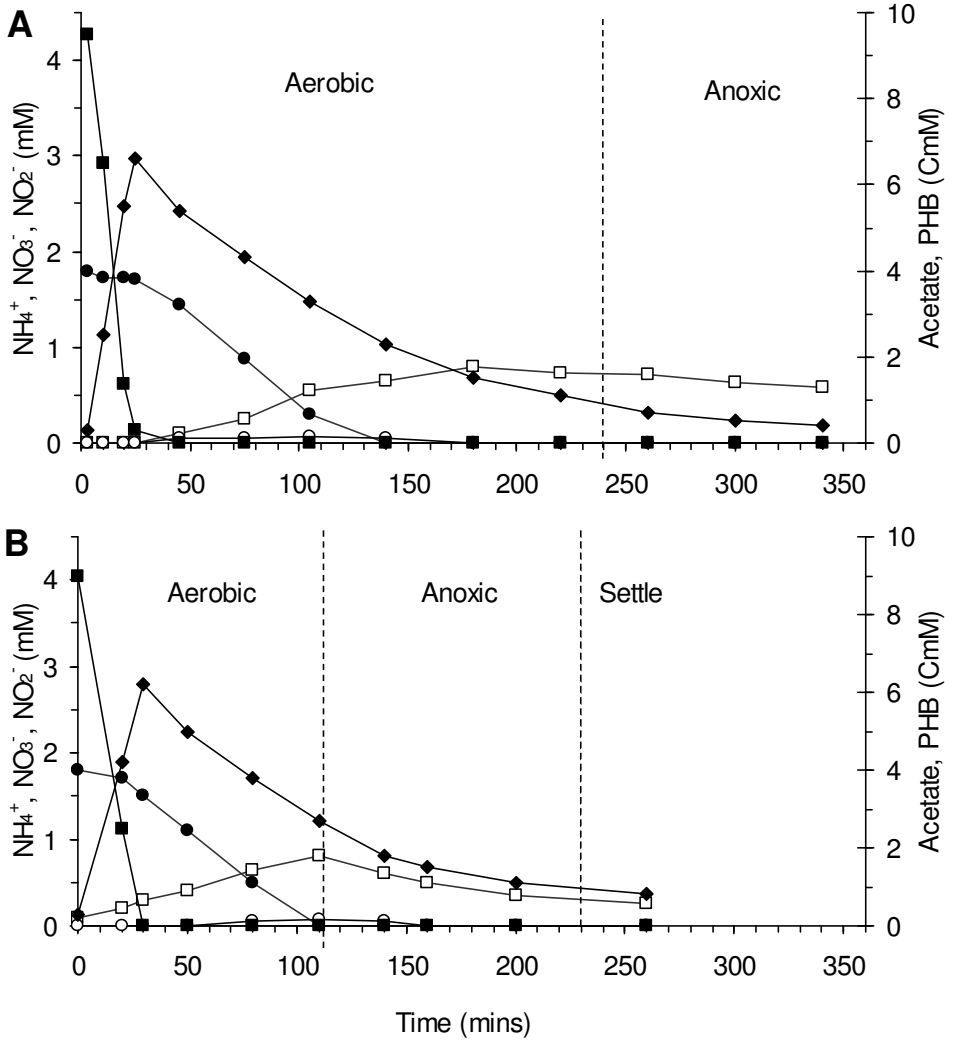

Figure 4.3: Nutrient removal in the SBR during A: Standard SBR operation with a fixed aerobic phase length (240 minutes) and, B: SBR operation with SOUR-controlled aerobic phase length (Acetate (■), PHB (◆), NH_4^+ (●), NO_3^- (□), NO_2^- (❍)). The average DO concentration was 1 mg L^{-1} during the famine period in both cycles.

Adaptability of the SOUR-Control Method to Varying Feed Strength

Over many cycles the endpoint of nitrification could be easily detected, under standard SBR operating conditions. To determine whether it could also be detected and utilised with varying feed concentration, the SBR was fed with a 2 x concentrated medium. The same aeration capacity was applied as in previous cycles (k_La 21 h^{-1}). The doubling of both the ammonium and acetate concentration extended the time required for complete oxidation by a factor of about two (Fig 4.4). The control program correctly adjusted the required length of the aerobic period to 200 minutes (compared to only 110 minutes with standard feed composition). The removal of 89 % nitrogen by the end of the anoxic phase indicated that reducing power had been preserved by automated termination of the aerobic phase length. The control method could successfully adapt the aeration length to the strength of the feed.

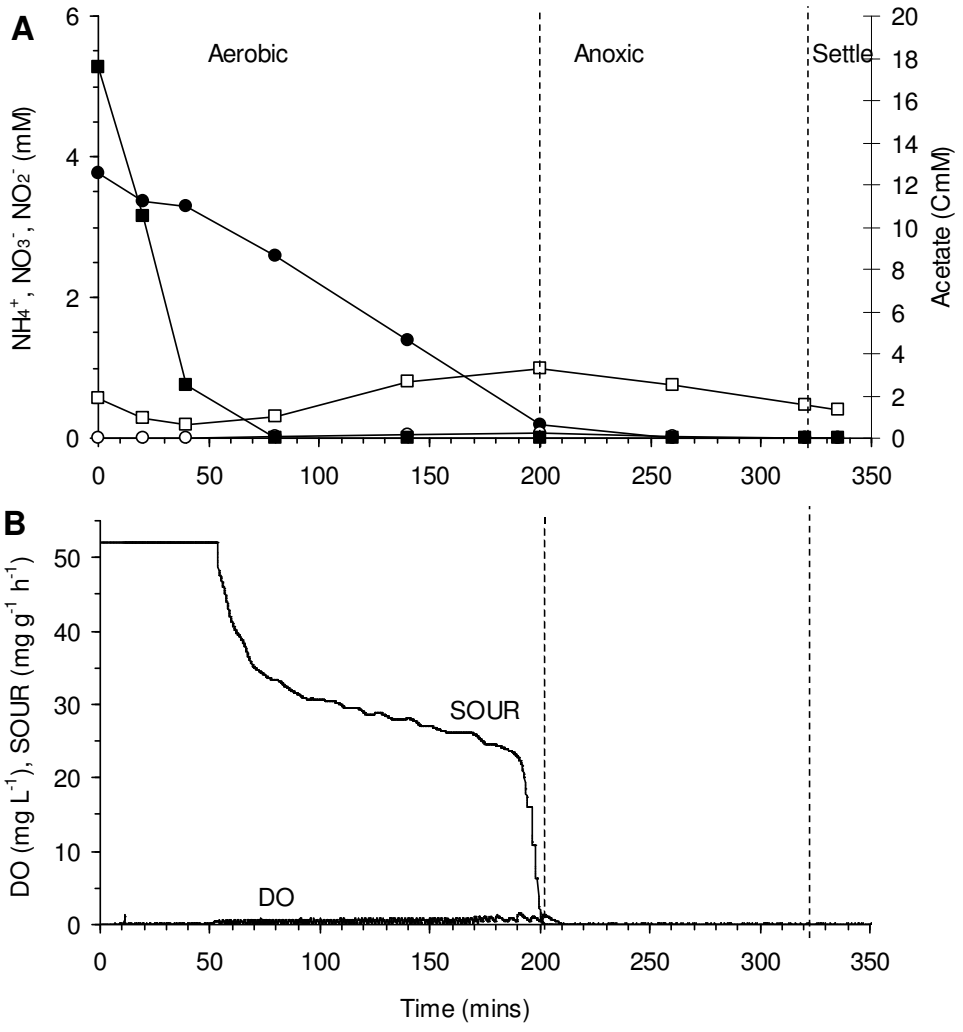

Figure 4.4: Nutrient removal and on-line data in the SBR during SOUR-controlled aerobic phase length with double the concentration of acetate and ammonium in the feed, A: Acetate (■), PHB (◆), NH_4^+ (●), NO_3^- (□), NO_2^- (❍), and B: Specific oxygen uptake rate and DO concentration.

The total SOUR was higher when fed with the concentrated medium (average SOUR 36 mg $g^{-1}X\ h^{-1}$, Fig 4.4B) than with standard feed (23 mg $g^{-1}X\ h^{-1}$, Fig 2). The SOUR due to ammonium oxidation was constant during the famine phase, as calculated from the ammonium oxidation rate (Fig 4.4A). Thus, the higher SOUR must have been caused by a higher oxidation rate of PHB. SND was improved to 70 % during the aerobic phase when fed with the stronger medium (compared to 59 % with standard feed concentration), suggesting that a higher availability of PHB can improve SND during the aerobic phase.

Adaptability of the SOUR-Control Method to Different Aeration Conditions

In order for wastewater treatment plants to use such a control technique it must be flexible enough to adapt to different DO settings used during the aerobic period. The ability of the SOUR-control method to detect the endpoint of nitrification under varying oxygen supply rates was tested by operating the SBR at different DO setpoints of 0.3, 0.7 and 3 mg L^{-1} (Fig 4.5). Due to oxygen limitation at the lower setpoints, the time required for ammonium oxidation was longer and the control program adapted the aeration length accordingly. The SOUR cut-off setpoint that was used previously (10 mg g X^{-1} h^{-1}) needed to be lowered for the lower DO setpoints (Fig 4.5). The ammonium oxidation rate was slowed down more than 2-fold at the lowest DO-setpoint, but was only slowed down 0.8-fold at 0.7 mg L^{-1}. As the half-saturation constant for oxygen ($k_{S,O2}$) is the DO concentration where the rate is slowed down to half its maximum rate, the on-line SOUR data imply that the apparent $k_{S,O2}$ of the ammonium oxidisers is between 0.3 and 0.7 mg L^{-1}. However, due to the oscillating oxygen concentration during the aerobic periods, these values are estimates only.

Adaptability of the SOUR-Control Method to Real Wastewater

The SOUR-control technique was effective at detecting the endpoint of nitrification for the defined, synthetic wastewater prepared in the laboratory. As the composition of real wastewater is expected to be more complex, it was decided to investigate how the control technique behaved when the SBR was fed with domestic wastewater from a large-scale SBR. The characteristics of the wastewater are shown in Materials and Methods. The biomass used was the same as in the previous experiments (i.e. adapted to SBR conditions). The SOUR showed a similar pattern as in cycles with the artificial medium, even from the very first cycle after feeding with wastewater (Fig 4.6). There

was a defined period of high respiration activity during the first 20 –30 minutes of each SBR cycle due to the oxidation and/or storage of the soluble COD.

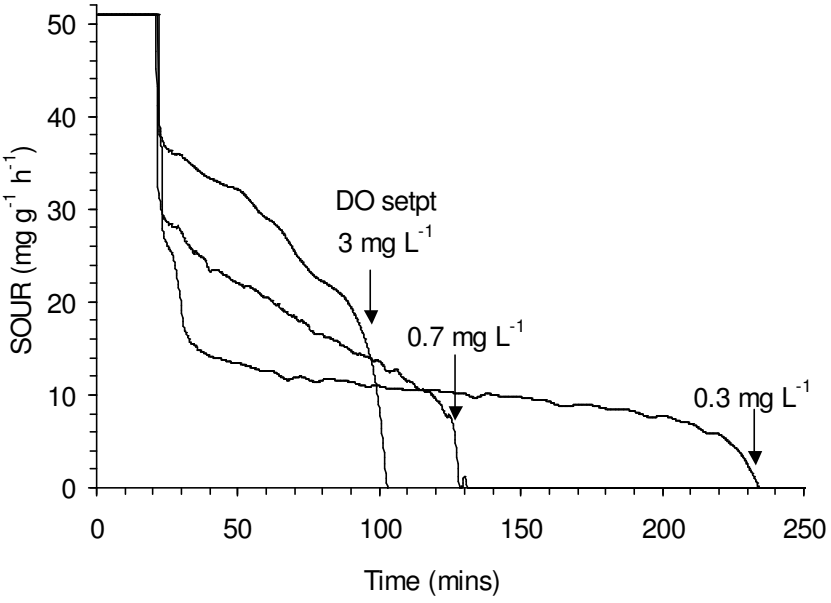

Figure 4.5: Adaptation of the aerobic phase length via SOUR-controlled aeration to varying ammonium oxidation rates at different DO setpoints of 3, 0.7 and 0.3 mg L^{-1}. Arrows indicate points of aerobic phase termination.

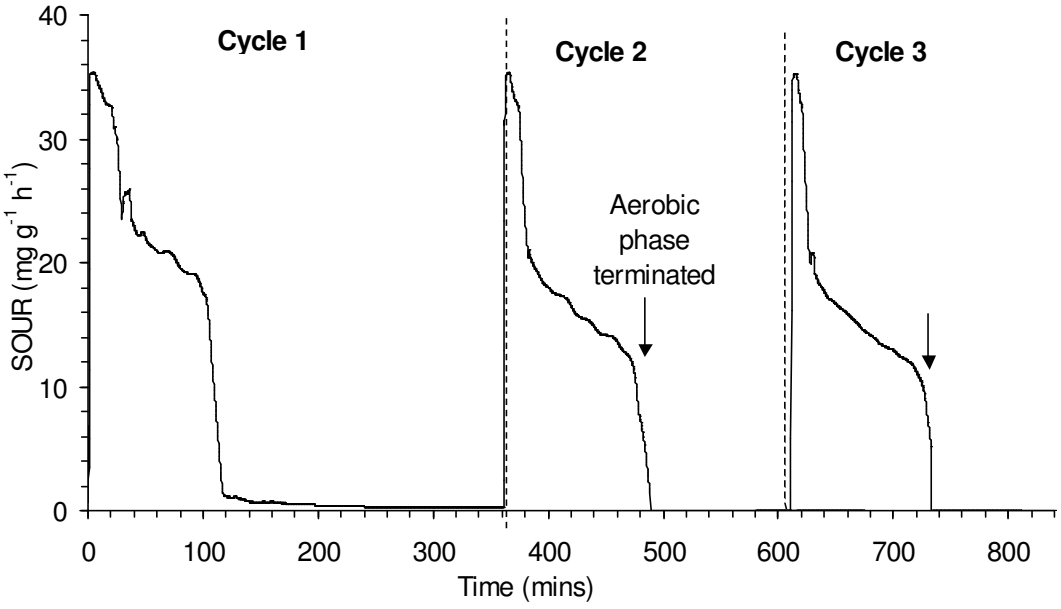

Figure 4.6: On-line measurement of the SOUR during SBR-feeding with real wastewater. The first cycle was operated with a fixed aerobic phase (240 minutes) to test whether the nitrification endpoint could be detected from the SOUR. The following cycles were operated with SOUR-controlled aerobic phase length.

After 120 minutes of each SBR cycle, the characteristic drop in the SOUR occurred when ammonium was depleted, which was confirmed by ammonium measurements (data not shown). As the first cycle showed that the nitrification end-point was clearly detectable with real wastewater, subsequent cycles were operated with SOUR-controlled aerobic phase length, which caused immediate transition to the anoxic phase after ammonium depletion (Fig 4.6, cycles 2, 3). As the wastewater contained a significant fraction of particulate COD (380 mg L^{-1}) it was expected that a significant amount of SOUR would be visible after ammonium depletion. However, the respiration activity of the biomass after ammonium was oxidised was close to zero, suggesting the absence of significant biodegradable COD or PHB. This could imply that the particulate COD measured in the wastewater could have been due to bacterial cells themselves.

Usefulness of SOUR Monitoring with Raw Wastewater and Fresh Activated Sludge

The typical SOUR pattern observed with real wastewater may have been caused by the "history" of the biomass, as the biomass used had previously been adapted to the dynamic laboratory SBR conditions. To test whether a similar SOUR pattern occurred when using unconditioned biomass, the SBR biomass was replaced with fresh activated sludge from the large-scale SBR. The SOUR was monitored on-line after feeding with raw wastewater. Again, a similar pattern of SOUR behaviour was observed (Fig 4.7), showing that the typical SOUR pattern obtained with laboratory-conditioned biomass and artificial wastewater was not just a laboratory artefact and could be reproduced using real wastewater and activated sludge. After consumption of all soluble COD (as indicated by the first SOUR-drop), the pH decreased during ammonium oxidation and needed to be controlled. During the second cycle, the pH was not controlled and consequently it decreased to a pH of 5.9 by the end of the aerobic period (data not shown). This decrease in pH affected the shape of the SOUR-drop after ammonium depletion to a longer, less sudden decrease, compared to the sudden, sharp drop when the pH was controlled to above 7 (cycle 1, Fig 4.7). Despite the less distinct drop, the SOUR-control technique was still able to correctly adjust the length of the aerobic phase after ammonium depletion. The amount of SND achieved by the end of the aerobic period was less than 30 % (data not shown), which may have been due to the lower concentration of soluble organics in the raw wastewater (200 mg L^{-1}), compared to the laboratory medium (300 mg L^{-1}).

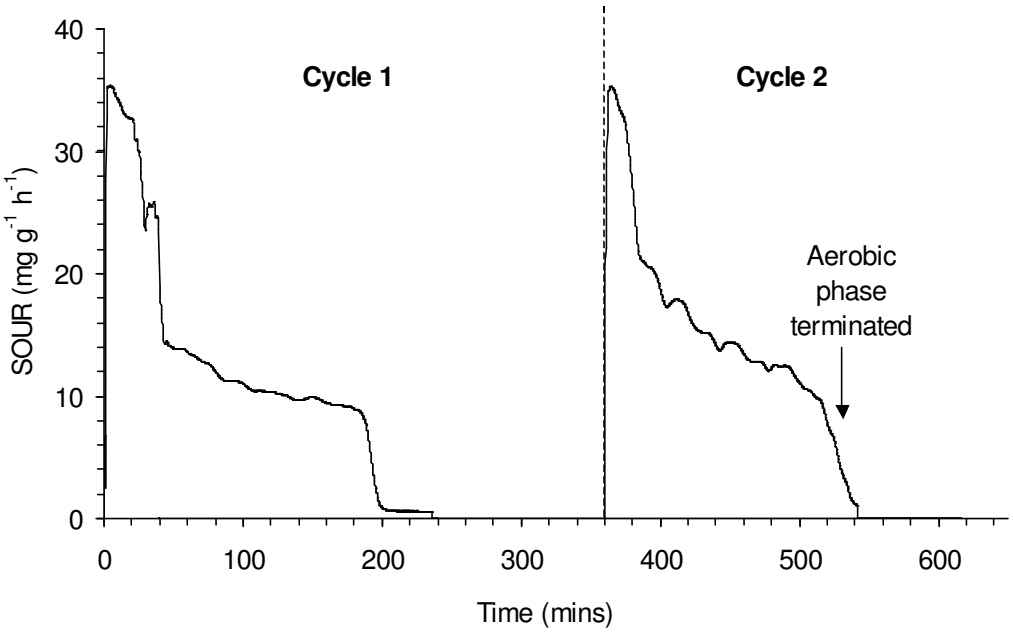

Figure 4.7: On-line measurement of the SOUR after inoculation of the SBR with fresh activated sludge and feeding with raw wastewater. The first cycle was operated with a fixed aerobic phase length (240 minutes) and subsequent cycles were operated with SOUR-controlled aerobic phase length.

4.4. Discussion

Oxygen Management Allows More Efficient Use of Organic Substrate

For optimum nitrogen removal in an SBR designed for aerobic nitrification (or SND) followed by anoxic denitrification, a very tight oxygen management strategy is necessary on three fronts; 1. The benefit of supplying low oxygen transfer rates during the feast period has been described (Chapter 2), 2. The need for limiting oxygen concentrations during the famine phase is generally established to be essential for SND (Beun et al., 2001, Pochana and Keller, 1999, Pochana et al., 1999, Munch et al., 1996, Zhao et al., 1999, Chapter 3) and is supported by this study (Figs 4.3, 4.4), and 3. This study showed that by terminating the aerobic phase and beginning the anoxic phase as early as possible, a further increase in nitrogen removal is obtained by enhancing denitrification in the following anoxic phase. This is not a new concept, as many wastewater treatment plants incorporate systems for preventing over-aeration during the aerobic phase (Yu et al., 1997, Zhao et al., 1994, Thornberg et al., 1993). The amount of organic carbon conserved by automated aerobic phase termination was quantified in this study. By incorporating on-line management of the aerobic phase length, a surplus of around 1.8 CmM PHB (58 mg L^{-1} COD) was preserved for the anoxic phase, compared to a cycle without aeration control (Fig 4.3). This was sufficient to denitrify 70 % of the residual nitrate and improve total nitrogen removal to 86 %. The carbon to nitrogen ratio of the synthetic wastewater used in this study was 10:1, representing a typical medium-strength wastewater. Thus, the nitrogen removal achieved here should also be achievable with real wastewaters, provided the oxygen supply is managed. Automated control of the aerobic phase length minimised the amount of organic substrate "wasted" by aerobic oxidation, allowing improved nitrogen removal (Fig 4.3). For optimum use of the limited amount of organic carbon in wastewater, oxygen management is necessary throughout all aerobic stages of the SBR process.

Operation at low DO concentrations during aeration has traditionally been found to encourage filamentous bacteria in continuous feeding systems (Casey et al., 1994). However, in dynamic systems such as the SBR, the substrate gradient favours floc-formers (Majone et al., 1996, Van Loosdrecht et al., 1997). In this study, biomass with efficient settling characteristics (ie. floc size > 200 µm, SVI < 100 mL g^{-1}X) was established in the SBR operating continuously at low DO (1 mg L^{-1}) for extended

periods of time (up to 2 years). Other studies have reported efficient floc formation during continuous low-DO operation in sequencing batch reactors (Dangcong et al. 2001, Chapter 3).

Usefulness of the SOUR-Control Method

Measurement of microbial respiration activity during the aerobic phase of activated sludge processes can clearly detect the end-point of ammonium oxidation, but can require expensive equipment to do so (Johansen et al., 1997, Spanjers et al., 1999). The method developed in this study was simple, requiring dissolved oxygen measurement and knowledge of the relative changes in oxygen mass transfer. This method could theoretically be used to predict the SOUR in plants where DO setpoint control is used. The decreasing power demand on air blowers required to keep the dissolved oxygen at the setpoint gives an indication of the changing OUR and could serve a function similar to the air time fraction (f_{AirOn}) described here. Knowledge of the precise k_La value is not absolutely necessary, as it serves only as a scaling factor. An OUR trend would be expected to be more important for detecting ammonium depletion, rather than an absolute value. The SOUR-control method described is also possible with more precise DO control, such as PID control of the DO concentration. The same principle can be used, whereby the rate of air supply required to maintain the dissolved oxygen at the setpoint is measured and related to the SOUR (Eqs 2, 4). This technique is currently being applied successfully in our laboratory with PID dissolved oxygen control.

The adaptability of a control method to large-scale plants is an important consideration. Very few treatment plants have an influent of constant flow-rate and composition. Typically, influent characteristics, flowrates and nutrient loadings vary by factors of 2 – 10 (Henze et al., 1994). The developed SOUR-control method relied on a sudden SOUR drop, which was clearly discernible after ammonium depletion (Fig 4.2). It was therefore independent of changing feed composition, concentration and air supply (Figs 4.4, 4.5, 4.6, 4.7). However, one shortcoming of the SOUR-control technique used in this study was that it relied on the use of an arbitrary SOUR cut-off value. The magnitude of the SOUR is strictly dependent on the rate of oxygen supply, as evidenced in experiments where the DO setpoint was varied (Fig 4.5). Oxygen-limitation at the lower DO setpoints resulted in a lower SOUR and consequently, the SOUR cut-off value that was effective in previous experiments (10 mg $g^{-1}X\ h^{-1}$) was too high at lower

DO setpoints and would have resulted in premature termination of the aerobic phase. Rather than using a fixed SOUR cut-off value as in this study, the use of the second derivative of the dissolved oxygen concentration (i.e. the change in the SOUR) would be a reliable means for detecting ammonium depletion (Fig 4.8). The second derivative amplifies changes in the SOUR and the nitrification endpoint is clearly detectable, also when raw wastewater and fresh activated sludge are used (Fig 4.8B). The advantage of this method over use of a fixed value is that it would be independent of the aeration conditions and would therefore be more effective during fluctuating plant conditions. The SOUR-aeration control technique developed in this study has been shown to be effective in batch reactors where the feed is supplied within a short time period and consequently nutrients are depleted all at once. However, these points of depletion would not be as clearly detectable in intermittently-fed or continuously-fed systems.

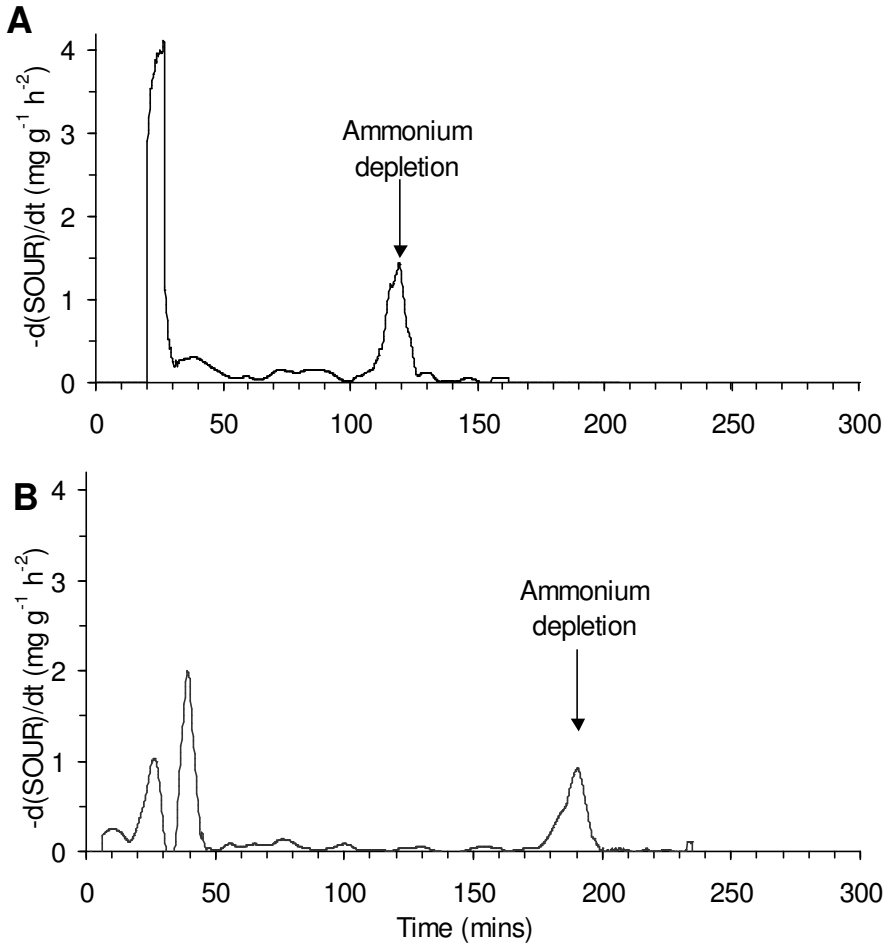

Figure 4.8: Use of the second derivative of the DO concentration (multiplied by –1) for detection of the nitrification endpoint during A: SBR cycling with the acetate-based laboratory medium, and B: SBR cycling with raw wastewater and raw activated sludge.

Application of the SOUR-Control Technique to Raw Wastewater

The results of this study showed that a similar pattern of SOUR behaviour was obtained with both the simplified acetate-based medium and with raw wastewater in the laboratory-SBR (Figs 4.2, 4.6, 4.7). The SOUR pattern in all cases was characterised by a period of high respiration activity during soluble COD oxidation and a second phase of lower SOUR during ammonium oxidation. The relative drop in the SOUR due to ammonium depletion is an event that is preserved, as it is due to a low half saturation constant of ammonium ($k_{S,NH4+}$) of the nitrifying population (< 0.25 mM NH_4^+ , Wiesmann, 1994, Henze et al., 1987, Gujer et al., 1999). These results show that the SOUR behaviour observed during laboratory-SBR cycling is not a product of "adapted" biomass or a simplified feed, but is representative of any activated sludge sample that is fed with wastewater containing a soluble COD fraction and ammonium. As most wastewaters contain both of these components, it is expected that similar behaviour would also be observed in large-scale sequencing batch reactors, provided rapid filling can be achieved.

Very little nitrogen was removed via SND when using raw wastewater (< 30 %, data not shown). This may have been due to the low fraction of BOD in the raw wastewater (200 mg L^{-1}) compared to the laboratory medium (300 mg L^{-1}), resulting in storage of less substrate and consequently a lower availability of reducing power during aeration. The lack of SND during aeration meant that it was necessary to control the pH when using raw wastewater, as it dropped below 6 during the ammonium oxidation phase (data not shown). This lowering of pH is in turn known to slow down the aerobic nitrification process (Anthonisen et al. 1976, Neufeld et al., 1980). This highlights one significant advantage of nitrogen removal via simultaneous nitrification and denitrification, as opposed to sequential nitrification and denitrification. During SBR operation with the acetate-based medium, the pH did not decrease below 7.5 during the famine phase (Fig 4.2) and consequently pH control was not required. Denitrification is an alkaline-producing reaction (Eq 7) and hence buffers the proton production from ammonium oxidation (Eq 6). It also appears that oxidation of organic substrate contributes to alkalinity through CO_2 stripping (Yoo et al., 1999), as evidenced by a reproducible increase in the pH during the initial feast phase (Fig 4.2). Therefore, PHB oxidation and the resulting CO_2 stripping may contribute to a buffering of the pH. The simultaneous occurrence of denitrification, PHB oxidation and nitrification results in a

relatively balanced pH during aeration (Figs 4.2, 4.3). Thus, SND has the potential to save costs in chemicals required for pH control on the large-scale. Operating strategies that encourage soluble substrate storage and nitrogen removal via SND should reduce the need for pH control.

Nitrification: $NH_4^+ + 2\ O_2 \rightarrow NO_3^- + 2\ H^+ + H_2O$ (Eq 6)

Denitrification: $5\ CH_3COOH + 8\ NO_3^- \rightarrow 4\ N_2 + 10\ CO_2 + 6\ H_2O + 8\ OH^-$ (Eq 7)

In this study, up to 71 % of the total COD could be preserved as PHB (Fig 4.3), which meant that sufficient PHB remained after ammonium was depleted and excess PHB was available for denitrification. This amount of COD storage may not be possible on the large scale, particularly if the wastewater has a low soluble COD fraction, resulting in a low food to microorganism ratio (F/M). Some studies have tried to quantify the role of storage in real activated sludge. Carucci et al. (2001) showed that PHB was produced in real activated sludge, not only from acetate but also from other unknown substrates, but accounted for only 18 – 22 % of the total soluble COD. The food to microorganism ratio used in their study (30 mg COD $g^{-1}X\ h^{-1}$) was similar to this study (23 – 30 mg COD $g^{-1}X\ h^{-1}$) and hence similar results may be expected. Hanada et al. (2002) showed the fraction of heterotrophic organisms capable of PHA storage in activated sludge varied among different municipal sludges between 15 – 35 % of the total biomass. Thus, laboratory studies may over-estimate the amount of storage that can be achieved on large scale and represent an 'ideal' situation. Despite this, the principle of SOUR-controlled aerobic phase length is still applicable to large-scale reactors. Even if significant PHB cannot be preserved by automated termination of the aerobic phase, the supply of oxygen beyond ammonium depletion can be prevented, resulting in decreased aeration costs and faster wastewater throughput rates (Fig 4.3). A faster throughput of wastewater may imply that SBRs of smaller volume could be used, resulting in decreased operating costs.

4.5. Conclusions

The simple method developed for measuring the SOUR enabled on-line detection of the end-point of nitrification. This allowed automated control of the aerobic phase length, preventing unnecessary oxidation of organic substrate during the aerobic phase and increased the amount of nitrogen removal achieved over one SBR cycle. The improved nitrogen removal was accompanied by a decrease in the oxygen requirement and a faster throughput of feed. Oxygen management in large-scale sequencing batch reactors would be expected to achieve savings in operation costs while improving effluent quality and avoiding the need for an additional carbon supplement to achieve complete nitrogen removal. Tests with raw wastewater and fresh activated sludge showed that the control technique is adaptable to large-scale reactors, as ammonium depletion is a conserved signal and is independent of the aeration conditions and type of wastewater.

CHAPTER 5

Long-Term Oxygen Management for Improved N-Removal via SND in an SBR[1]

Abstract

Oxygen management can improve nitrogen removal during SBR operation by minimising the amount of organic substrate that is oxidised aerobically. Over-aeration results in a shortage of organic substrate, required for anoxic denitrification and nitrogen removal via simultaneous nitrification and denitrification (SND). This study investigates the long-term effect of aeration control on SND by a mixed culture in a 2 L acetate-fed SBR, using PHB as the electron donor for nitrogen removal. The reactor was operated continuously with automated termination of the aerobic phase after ammonium depletion, using the specific oxygen uptake rate (SOUR) as the control parameter. Over the first 12 cycles of operation in this mode, the PHB content of the biomass gradually increased 3-fold from 0.007 Cmol/Cmol to 0.022 Cmol/Cmol. This increasing biomass PHB content resulted in a progressively increasing SOUR, which allowed an increased amount of nitrogen removal via SND from 34 % to 52 %. It is proposed that the higher SOUR improved SND by reducing the penetration of dissolved oxygen into the flocs and creating larger anoxic zones. After one month of continuous SBR operation with SOUR-controlled aerobic phase length, it was found that the settling efficiency of the biomass had improved, as shown by a decrease in the sludge volume index (SVI) from 110 $mL \cdot g^{-1}X$ to less than 70 $mL \cdot g^{-1}X$. Measurements showed that although the ammonium oxidation rate had slowed down 2-fold after one month (from 0.3 to 0.13 mmol NH_4^+ $g^{-1}X$ h^{-1}), almost complete nitrogen removal (97 %) was achieved via SND during the aerobic phase, within 3 hours. Thus, in the laboratory, long-term oxygen management resulted in biomass with improved settling characteristics and significantly higher capacity for N-removal via SND. The SOUR-control technique could also be applied to fresh activated sludge and real domestic wastewater in the laboratory to increase the SOUR of the biomass over time. Our results may help to explain long-term history effects of N-removal capabilities of wastewater treatment plants and indicate that short-term over-oxidation can have longer-term effects on nitrogen removal.

[1] This chapter has been submitted to Water Research

5.1. Introduction

Due to the increasingly stringent nutrient level standards of discharged waters set by government regulating bodies, the need for cost-effective wastewater treatment technology is becoming more important. To achieve a higher effluent quality without an increase in operational costs, an increased understanding of the controlling factors of nitrogen removal is necessary. In wastewater treatment processes designed for nitrogen removal, the cost of aeration can amount to 50 % of the total electrical power consumption of the plant (Ferrer et al., 1998). Thus, any decrease in the amount of oxygen required to achieve nitrogen removal represents economical savings. Traditionally it has been assumed that the two processes of nitrogen removal – aerobic nitrification and anaerobic denitrification – need to be strictly separated due to their opposing oxygen requirements. However, in the last decade it has become clear that, if the dissolved oxygen concentration is carefully controlled, both processes can occur under identical operating conditions by a process referred to as Simultaneous Nitrification and Denitrification (SND) (Collivignarelli and Bertanza et al., 1999, Munch et al., 1996, Beun et al., 2001, Chapter 3). SND is an attractive alternative technology for nitrogen removal that has gained significant attention from engineers and scientists in recent years, due to its potential to eliminate the need for separate tanks as required in conventional treatment plants, resulting in a simplified and smaller plant design and thus savings in capital costs and aeration requirements (Keller et al., 1997, Sen and Dentel, 1998, Pochana and Keller, 1999, Tonkovic, 1999, Yoo et al., 1999, Zhao et al., 1999).

The efficiency of SND has been suggested to be dependent on three factors; the dissolved oxygen concentration, the floc size and the availability of sufficient organic carbon substrate (Pochana and Keller, 1999). Low dissolved oxygen concentrations and large flocs are generally thought to be essential for optimal SND (Pochana and Keller, 1999, Munch et al., 1996). For complete nitrogen removal via SND, the rate of ammonium oxidation should approximately equal the rate of denitrification. As autotrophic nitrification is generally slow in comparison to heterotrophic metabolism, SND requires a slowly degradable organic substrate to provide reducing power for denitrification during the nitrification process. The conversion of readily biodegradable COD into a bacterial storage polymer such as PHB (poly-β-hydroxybutyric acid)

preserves soluble COD as a slowly degradable substrate, since the oxidation rate of PHB is slow in comparison to that of soluble substrate (Carta et al., 2001, Beun et al., 2000a, Majone et al., 1999, Dircks et al., 1999, Chapter 3). Microbial storage of soluble substrate as PHB is prominent in dynamic reactor types such as the sequencing batch reactor (SBR). PHB can serve as an effective electron donor for nitrogen removal via SND in sequencing batch reactors (Beun et al., 2001, Chapter 3).

An increased concentration of available COD leads to a direct increase in SND (Sen and Dentel, 1998). Studies have shown that activated sludge containing a high PHB content (f_{PHB}) is equivalent to a wastewater with a high COD content, as the rate of PHB hydrolysis (and thus PHB availability) increases with an increasing cellular fraction of PHB (Beun et al., 2002, Chapter 3). Thus, operation of reactors with biomass containing a high PHB content should show an increased efficiency of SND. Newland (1998) proposed that a high concentration of stored substrate should increase the oxygen uptake rate (OUR) of the biomass, reduce the penetration of dissolved oxygen into the floc and hence increase the amount of SND achieved. But how is it possible to increase the PHB content of the biomass when operating with wastewater of a relatively low COD composition? Effective control of the aeration supply may provide the answer.

Management of the air supply plays an important role in maximising the use of PHB for nitrogen removal during SBR operation. Research has shown that PHB can be used most efficiently by three forms of oxygen management; 1. The use of oxygen limitation during the feast phase of an SBR increases the amount of reducing power conserved as PHB (Chapter 2), 2. The use of low dissolved oxygen setpoints (< 2 mg L^{-1} DO) during the famine period. This enables an increased fraction of PHB oxidation using nitrate as the electron acceptor, instead of oxygen, thus improving SND during aeration (Chapter 3), and 3. Termination of the aerobic phase as soon as ammonium is completely oxidised (e.g. via measurement of the SOUR) (Chapter 4). This prevents unnecessary oxidation of organic substrate by oxygen beyond ammonium depletion and increases the amount of PHB available for denitrification, thus improving total nitrogen removal over one cycle. The aim of this study is to investigate whether long-term SOUR-controlled aerobic phase length in a laboratory-SBR can gradually increase the biomass PHB content over a number of cycles and produce a biomass with a higher capacity for nitrogen removal via SND. Systematic studies quantifying the so-

called "history" effect of biomass are rare and have not yet focused on SND in a sequencing batch reactor.

5.2. Materials and Methods

5.2.1. Experimental Setup

A completely automated 2 L sequencing batch reactor (SBR) with on-line measurement of dissolved oxygen (DO), pH and oxidation-reduction potential (ORP) was used for all experiments, as described in Chapters 2, 3. Temperature was maintained at 25^{o}C and the airflow rate and stirring rate were 100 L h^{-1} and 80 ± 5 rpm, respectively. The k_La during full aeration in all experiments was 21 h^{-1}, and was checked before the start of each experiment. One standard SBR cycle consisted of a rapid fill phase (3 mins), an aeration phase (controlled length up to 180 mins), an anoxic phase (120 mins), a settle phase (17 mins), and a 20 min effluent withdrawal phase.

5.2.2. Dissolved Oxygen Control and On-Line Calculation of the Specific Oxygen Uptake Rate (SOUR)

The dissolved oxygen concentration was controlled about the setpoint using PID (Proportional, Integral, Differential) control, developed by the authors in LabView^{TM} (USA). The SOUR was calculated on-line as described in Chapter 3.

5.2.3. Synthetic Wastewater

The composition of the nutrient medium used is described in Chapter 2, resulting in a final carbon to nitrogen ratio (C:N) of 10:1. The medium was autoclaved to prevent bacterial activity in the feed vessel.

5.2.4. Raw Wastewater and Activated Sludge

The source of the wastewater and fresh activated sludge used in this study was the same as described in Chapter 4, with a COD:N ratio of 9:1 and a soluble BOD:N ratio of 3:1.

5.2.5. Sampling and Analytical Procedures

All compounds, dry weight and sludge volume index were measured as in Chapter 2.

5.2.6. Calculations

The percentage of SND, rate of SND and electron flow to both oxygen and nitrate during SND were calculated as described in Chapter 3 (Eqs 11 – 22).

5.3. Results

The feast phase in all SBR cycles (i.e. acetate present) was operated under oxygen limitation, as previous experiments showed that aerobic acetate conversion to PHB during the feast phase of an SBR is maximised under O_2-limitation (Chapter 2). As soon as acetate was depleted, the DO concentration was controlled to 1 mg L^{-1} in all SBR cycles. The biomass contained a fraction of PHB that is not degradable (0.008 Cmol PHB/Cmol X) (Chapter 3), which was subtracted from all PHB measurements to show only the available PHB. The sludge age of the biomass during the experimental period was 11 days.

Typical SOUR Behaviour During a Standard SBR Cycle

The SOUR was calculated by the method originally developed in Chapter 4, with the exception that this study incorporated improved dissolved oxygen control (via PID control, as in Chapter 3). This resulted in a constant DO concentration during the aerobic period (Fig 5.1A), as opposed to oscillating DO conditions used in Chapter 4. To confirm that the SOUR pattern obtained in the previous chapter was not an artefact caused by the oscillating DO concentration, the SOUR during a standard SBR cycle was monitored with PID DO-control (Fig 5.1A). Biomass adapted to the dynamic laboratory SBR operation and defined acetate-based medium showed the characteristic SOUR behaviour observed reproducibly in previous chapters (Chapters 3, 4). Three distinct stages were visible in the SOUR that were due to the predominance of different microbial activities: 1. Acetate consumption and PHB production (0 – 25 minutes) (Fig 5.1B), 2. Nitrification and PHB oxidation (25 – 140 minutes), and 3. Residual PHB oxidation and maintenance respiration (140 – 240 minutes). The SOUR terminated with a significant drop when ammonium was completely oxidised. This sudden decrease was clearly detectable in every SBR cycle and could be used as an on-line indicator for the termination of nitrification. Automated termination of the aeration phase as soon as ammonium is depleted, as indicated by the second SOUR-drop, has been shown to improve total nitrogen removal over one cycle from 69 to 86 % (Chapter 4). The objective of the following experiments was to test whether continuous operation with SOUR-controlled aerobic phase length could improve nitrogen removal beyond one cycle.

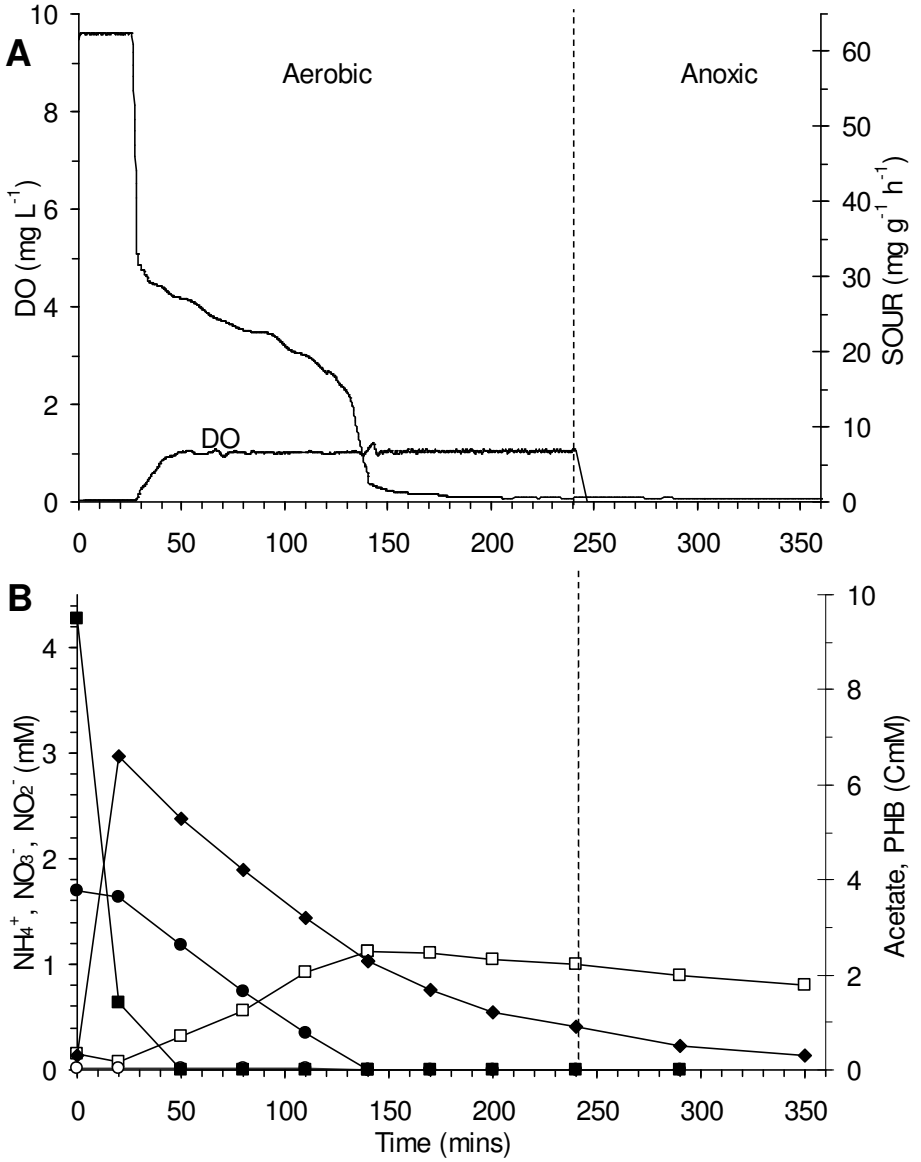

Figure 5.1: Typical behaviour of the SBR during a standard cycle with a DO setpoint of 1 mg L^{-1} using PID control, A: On-line measurement of the SOUR and DO concentration, and B: NH_4^+ (●), NO_3^- (□), NO_2^- (❍), PHB (▯) and acetate (♡). The biomass concentration and the SVI were 2.7 g L^{-1} and 110 mL. g^{-1}X, respectively.

Continuous SBR Operation with SOUR-Controlled Aerobic Phase Length

The SBR was operated continuously with SOUR-controlled aeration length for several days to test whether PHB could accumulate gradually, providing a higher start-up PHB concentration in the subsequent cycles. Gradual PHB accumulation should allow for improved nitrogen removal. Before starting the experiment, the culture was aerated for one day in order to begin the experiment with starved biomass. The first indication that conditions were changing during continuous SOUR-controlled operation over the first 12 cycles (2 days) was given by an increasing SOUR (Fig 5.2). Beyond 12 cycles, the

SOUR did not increase further. The ammonium concentration in the feed was constant and similarly the ammonium oxidation rate was constant, as indicated by the time required for the second SOUR-drop in each SBR cycle (Fig 5.2). This meant that the increasing SOUR must have been due to a higher oxidation rate of PHB. Measurements of the biomass PHB content at the end of the aerobic phase showed that there was indeed an increase in cellular PHB content over 12 cycles (Fig 5.3). The PHB fraction of the biomass (f_{PHB}) appeared to increase in a linear fashion, increasing by around 0.0014 Cmol/Cmol per cycle, which is equivalent to an increase in the total reactor concentration of 0.2 CmM PHB per cycle. To determine the effect of increased PHB availability on nitrogen removal, the nitrogen metabolism was investigated during cycles 1, 5 and 12 (Fig 5.4). Over the tested 12 cycles, the amount of nitrogen removed via SND increased from 34 to 52 % and total nitrogen removal from 65 to 85 % (Table 5.1). Thus, the small amount of PHB preserved at the end of each cycle was apparently sufficient to significantly improve nitrogen removal over progressive cycles.

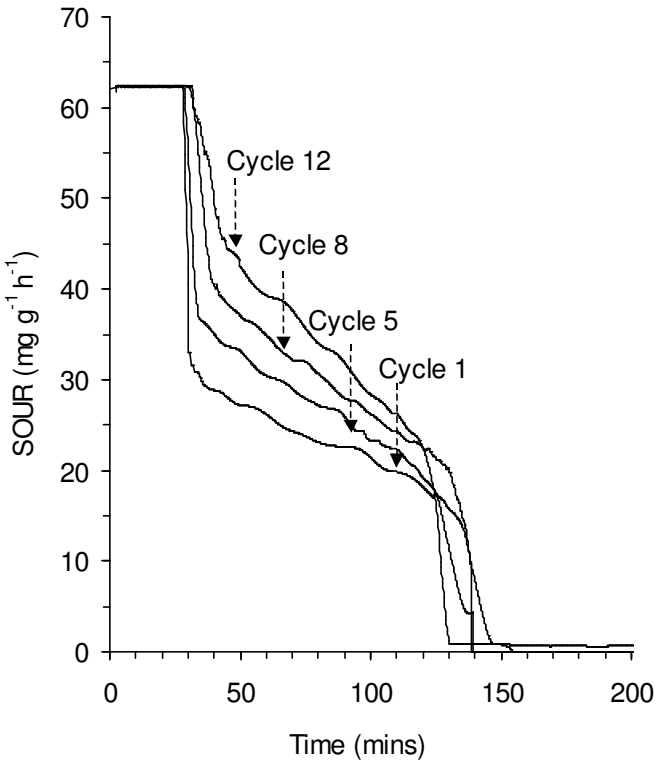

Figure 5.2: Change in the SOUR over time (from cycle 1 until cycle 12) during continuous SBR cycling with SOUR-controlled aerobic phase length.

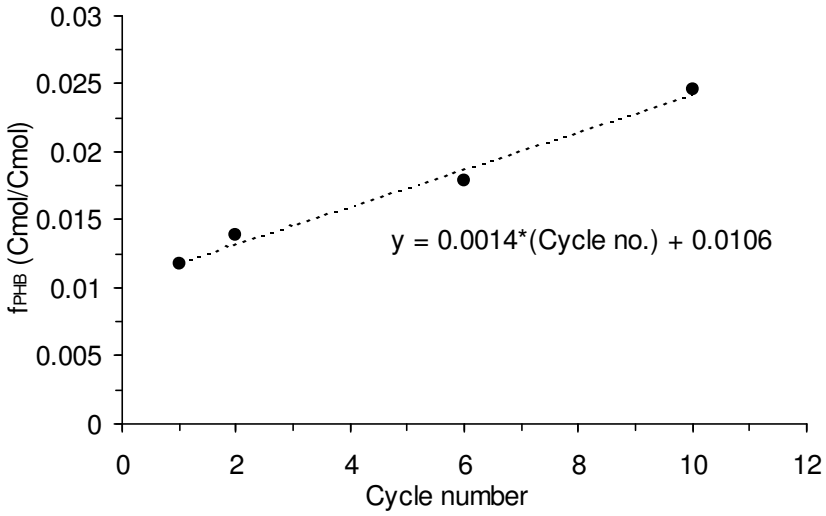

Figure 5.3: The PHB fraction of the biomass (f_{PHB}) measured at the end of the aerobic phase of different cycles during continuous SBR operation during SOUR-controlled aerobic phase length.

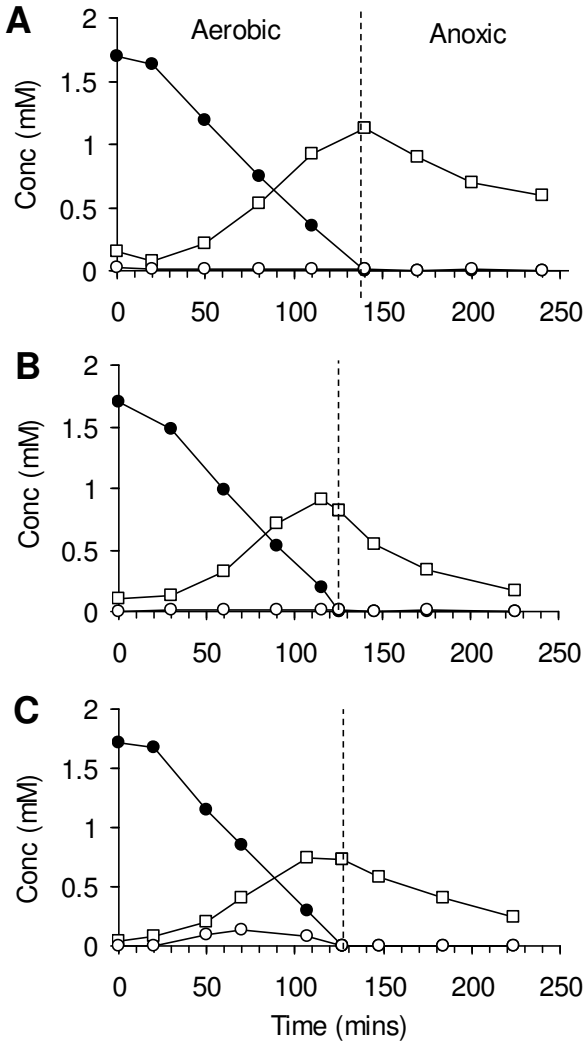

Figure 5.4: N-removal in three cycles during continuous SBR cycling over 2 days with SOUR-controlled aerobic phase length, A: Cycle 1, B: Cycle 5 and C: Cycle 12; NH_4^+ (●), NO_3^- (□) and NO_2^- (❍).

To visualise the use of PHB for oxygen versus nitrate reduction, the rates of electron flow to oxygen and nitrate were plotted for cycles 1, 5 and 12 (Fig 5.5). In accordance with previous observations (Fig 5.1B), the rate of PHB degradation decreased over time in each cycle as indicated by the decrease in total electron flow from PHB (Fig 5.5). In each cycle, electron flow to nitrate was highest during the initial hour of the aerobic famine phase, when the SOUR was also the highest (Fig 5.2). With each cycle of using SOUR-controlled aerobic phase length, not only the PHB level and SOUR increased, but also the electron flow to nitrate and hence SND increased (Figs 5.4, 5.5). As the purpose of PHB preservation is its use as an electron donor for denitrification, its aerobic oxidation can be considered "wastage". Despite the greater wastage rate of PHB when it had built up to higher concentrations, there was also a greater proportion of electron flow from PHB used for nitrate reduction.

Table 5.1: Characteristics of the tested SBR cycles during continuous SBR operation with SOUR-controlled aerobic phase length (Figs 5.2, 5.3, 5.4, 5.5).

SBR Cycle	**1**	**5**	**12**	**170**
Maximum SOUR during famine period (mg O_2 g X^{-1} h^{-1})	33	37	45	41
% N-removal via SND	34	47	52	97
% Total N removal	65	90	85	100
NH_4^+ oxidation rate (mmol $g^{-1}X$ h^{-1})	0.24	0.31	0.29	0.13
Rate of SND (mmol NO_3^- $g^{-1}X$ h^{-1})	0.07	0.11	0.14	0.13
PHB available at end of aerobic phase (CmM) (f_{PHB} Cmol/Cmol)	1.1 (0.007)	1.9 (0.013)	3.3 (0.022)	-
Total PHB oxidised (CmM)* (f_{PHB} Cmol/Cmol)	3.1 (0.033)	4.4 (0.037)	5.9 (0.043)	-

* Calculated from the integral of the electron flow diagrams (Fig 5.5).

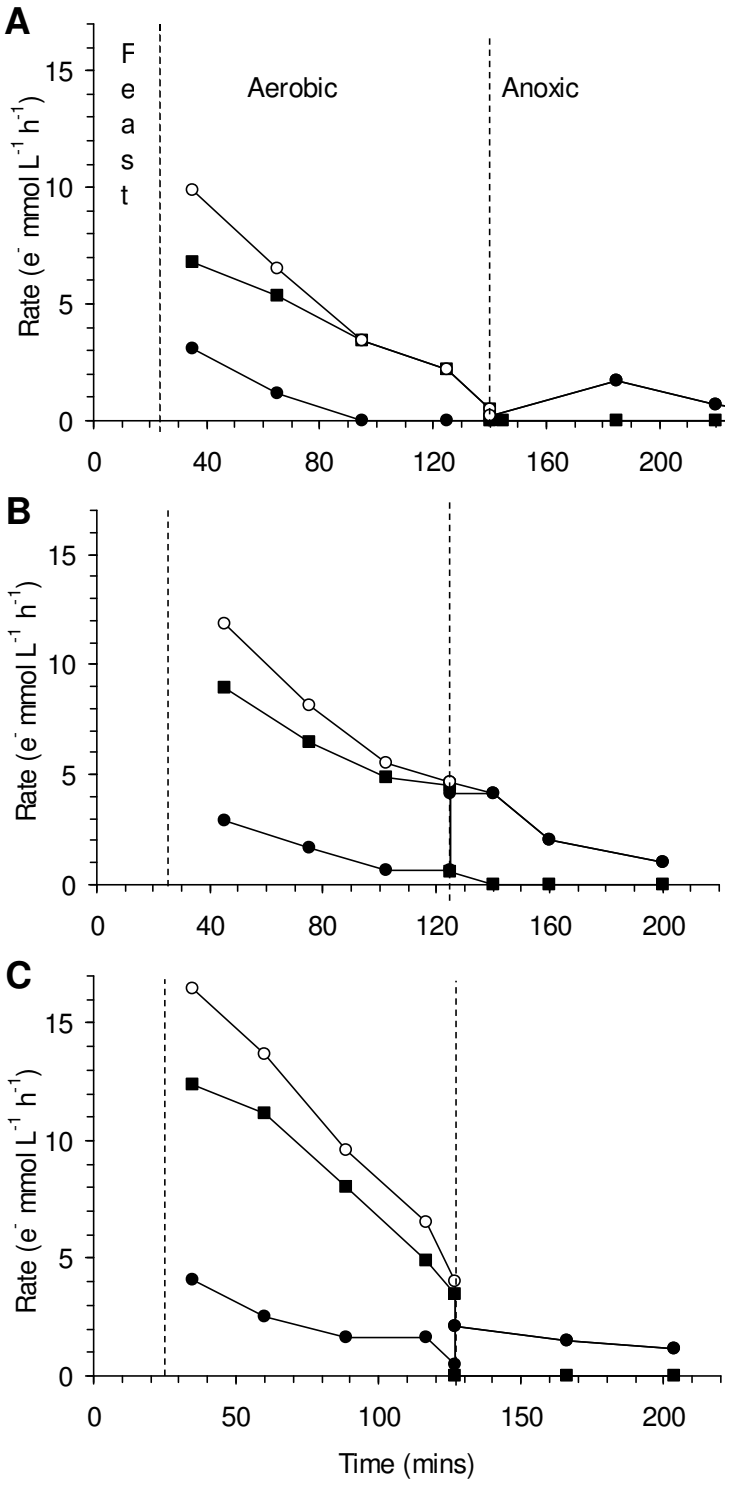

Figure 5.5: The rate of electron flow to both oxygen (♡) and nitrate (●) and the total e^- flow from PHB to O_2 and NO_3^- (❍) during, A: Cycle 1, B: Cycle 5 and C: Cycle 12.

There was therefore an apparent contradiction in the results: During progressive cycles more PHB was oxidised, yet the PHB level increased. To visualise how this might occur, the biochemical model of PHB metabolism developed in Chapter 2 was used. The model did not aim to reproduce the experimental values obtained but to visualise trends in the data. Simulated results showed that by terminating the aerobic phase before all PHB was depleted, PHB was preserved in the first cycle (Fig 5.6). However, due to the first order rate of PHB degradation (Beun et al., 2002, Chapter 3), the increased availability of PHB in the second cycle meant that the available PHB was oxidised faster, reducing the net quantity of PHB produced. The net amount of PHB preserved over progressive cycles decreased until finally the amount of PHB produced during one cycle (from conversion of the acetate feed) was equal to the amount of PHB oxidised, resulting in no further net increase in PHB. As a consequence, the biomass reached a higher steady-state PHB concentration than in the earlier cycles without SOUR-aeration control (close to zero) (Fig 5.1B). The PHB accumulation observed experimentally relied on the strict termination of aeration after ammonium oxidation, in order to preserve the small residual PHB remaining at the end of each cycle.

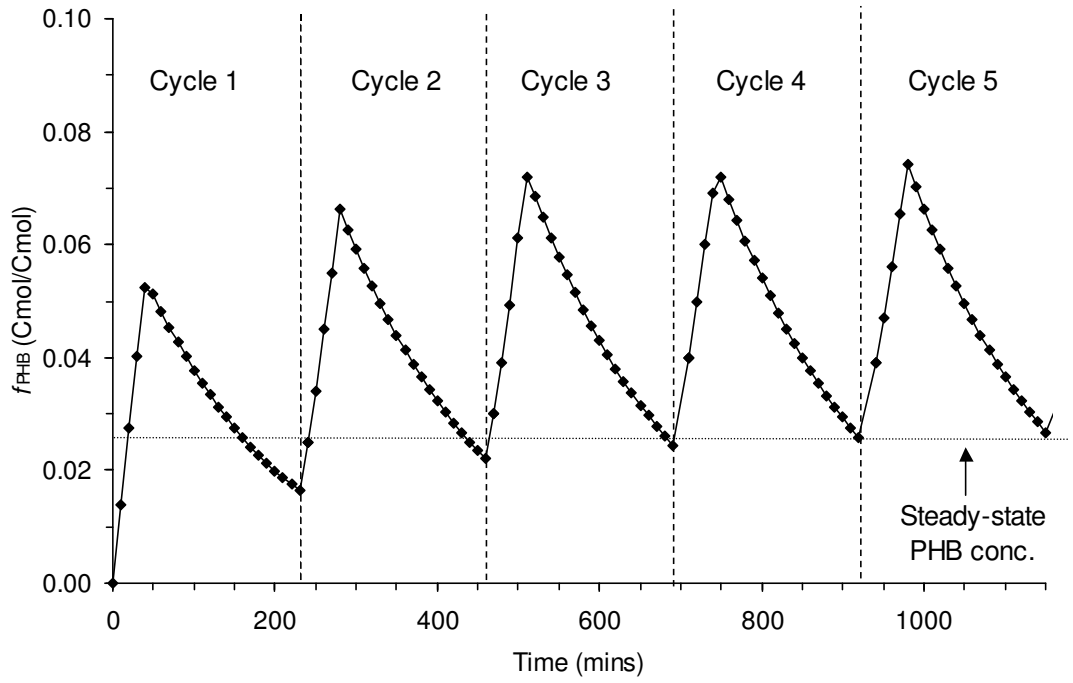

Figure 5.6: Simulated data showing the biomass PHB concentration (f_{PHB}) over consecutive SBR cycles during SOUR-controlled aerobic phase length.

Extended SBR Operation with SOUR-Controlled Aerobic Phase Length

The laboratory-SBR was run continuously with SOUR-controlled aerobic phase length for an extended period of time (1 month). It was observed macroscopically that the biomass had formed large flocs over the month of operation (floc size > 500 µm). The sludge volume index (SVI) decreased from 110 mL. $g^{-1}X$ originally, to less than 70 mL. $g^{-1}X$, indicating a significant improvement in sludge settling efficiency. The nitrogen removal capacity of the reactor was measured after 4 weeks (cycle 170) (Fig 5.7). Almost complete nitrogen removal via simultaneous nitrification and denitrification was achieved (97 %) during the aerobic phase. At the DO setpoint used (1 mg L^{-1}), the specific ammonium oxidation rate had slowed down to almost half its original rate (0.13 mmol NH_4^+ $g^{-1}X$ h^{-1}) (Table 5.1), which meant that the SOUR drop did not occur until 180 minutes, as opposed to 120 minutes in the earlier cycles. However, all nitrogen was removed by the end of the aerobic phase. Long-term operation with the SOUR-controlled aerobic phase length therefore had a significant impact on the nitrogen removal capacity of the biomass, showing that nitrogen removal can depend on the previous operation mode of the reactor.

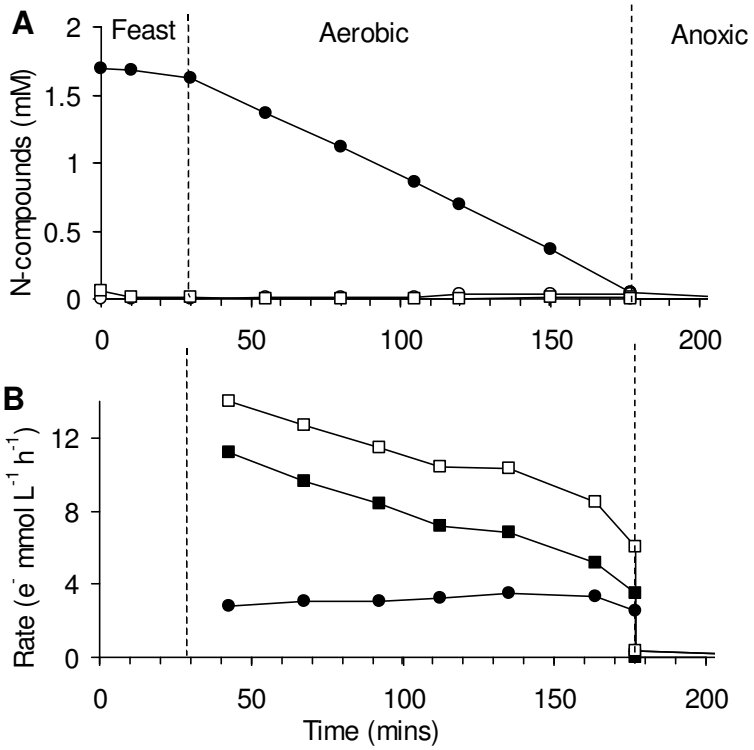

Figure 5.7: Nitrogen removal and electron flow rates after 1 month of SBR operation with SOUR-controlled aerobic phase length (cycle 170), A: Nitrogen removal (NH_4^+ (●), NO_3^- (□) and NO_2^- (❍), and B: Electron flow rates from PHB to oxygen (♡) and nitrate (●) and total electron flow from PHB (□).

Application of the SOUR-Aeration Control Technique to Raw Wastewater

It was shown previously that the nitrification end-point could be detected from SOUR data with raw wastewater (Chapter 4). To gain a preliminary insight into the feasibility of using long-term SOUR-aerobic phase control with raw wastewater, the SBR biomass was replaced with fresh activated sludge and was run continuously for several days with wastewater and automated aerobic phase termination (Fig 5.8). On-line monitored data showed that the SOUR again increased gradually over progressive cycles until around cycle 22. The longer time taken to reach an apparent steady state SOUR may have been due to the lower BOD content of the wastewater (200 mg L^{-1}) in comparison to the synthetic medium (300 mg L^{-1}). These preliminary results suggest this technique may be able to increase the SOUR of activated sludge in wastewater and may consequently improve SND.

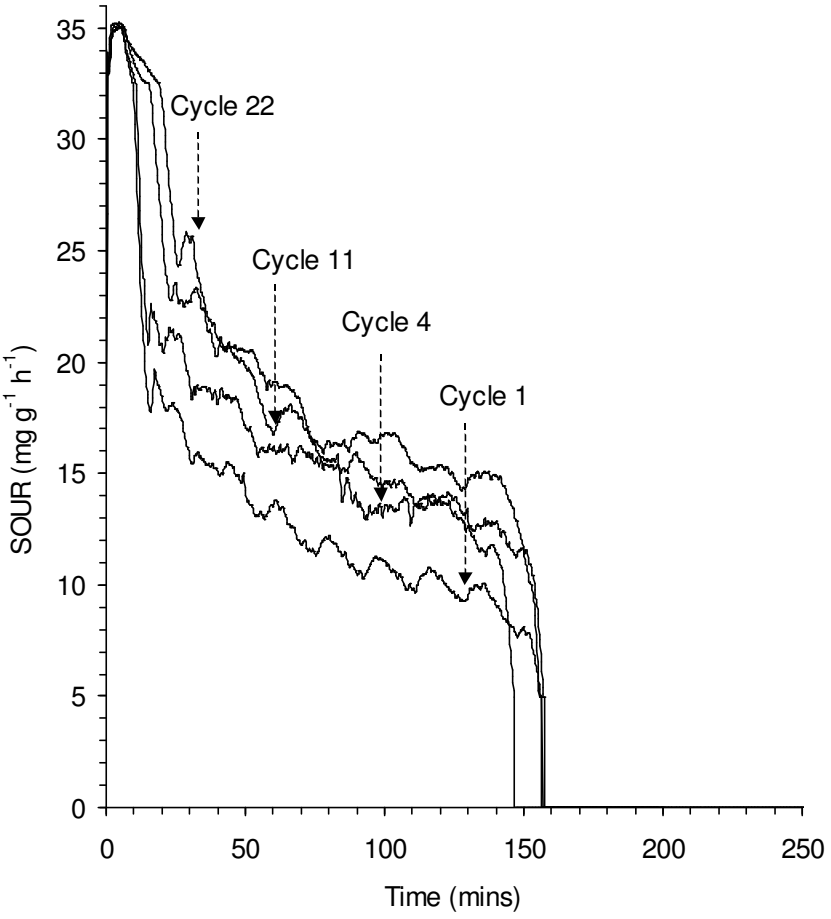

Figure 5.8: The SOUR over progressive cycles during feeding of the SBR with raw wastewater, while operating continuously with SOUR-controlled aerobic phase length.

5.4. Discussion

A High SOUR Improves SND

Control of the aerobic phase length via the SOUR resulted in an increased biomass PHB fraction (Fig 5.3) and consequently a progressively higher SOUR over the initial 12 cycles (Fig 5.2). The increasing SOUR corresponded to an increasing amount of nitrogen removal via SND (Table 5.1). Plots of the electron flow to oxygen and nitrate during aeration (Fig 5.5) showed that, while an increasing amount of PHB was "wasted" by aerobic oxidation, the higher heterotrophic oxygen consumption was beneficial to SND. This finding is in accordance with the prediction by Newland (1998), whereby the proportion of SND in the aerated zone would be greatest when OUR values are highest, since a higher OUR implies a larger pool of reducing power, high oxidation rates and consequently reduced penetration of dissolved oxygen into the floc. Studies have reported that the amount of nitrogen removed via SND decreases towards the end of the aerobic period when the SOUR is lowest and hence dissolved oxygen penetration into the floc is likely to be highest (Beun et al., 2001, Chapter 3). The results presented here support the above prediction and findings, by showing that an increased SOUR does indeed result in increased SND during aeration.

PHB Accumulation during Continuous SBR Operation with SOUR-Controlled Aeration

During continuous SBR operation with SOUR-controlled aerobic phase length, the PHB content of the biomass increased by a small amount after each cycle. However, this small increment of preserved PHB resulted in a 3-fold increase in the biomass PHB content (f_{PHB}) over 12 cycles (Fig 5.3, Table 5.1). When compared to the maximum PHB-storing capacity of acetate-fed mixed cultures of between 0.6 – 0.8 Cmol/Cmol (Beun et al., 2002, Chapter 3), the amount of PHB accumulated at the end of the 12 cycles was relatively low (0.03 Cmol/Cmol). Yet, the extra preserved PHB (a total of 3.3 CmM between cycles 1 – 12) caused a directly observable increase in the respiration activity (O_2 and NO_3^- reduction) (Fig 5.5) and nitrogen removal (Fig 5.4). Experimental and simulated results showed that there is a limit to the amount of PHB that can be preserved during SOUR-controlled SBR operation. The limit is reached when a steady state is established between the PHB oxidation rate and the PHB accumulation rate. The addition of PHB-saturated biomass to a reactor would vastly improve nitrogen removal during subsequent cycles, but it would not be possible to maintain the high PHB

content. According to the findings here, the faster oxidation rate of PHB at high biomass PHB fractions would result in oxidation of excess PHB over progressive cycles, until it reaches a steady-state PHB content.

The exact steady-state biomass PHB concentration (f_{PHB}) finally reached will depend on some of the following factors: the acetate concentration in the influent, the percentage conversion of acetate to PHB (or $Y_{PHB/Ac}$), the anoxic phase length and the DO concentration, which will in turn affect ammonium and PHB oxidation rates. For example, PHB will only accumulate over cycles if the influent soluble COD is sufficiently high such that PHB remains after ammonium oxidation. PHB preservation also relies on a healthy nitrifying population. If ammonium oxidation is too slow, the aerobic period will be extended and most or all PHB will be oxidised during aeration, preventing PHB accumulation. The dissolved oxygen concentration should be maintained high enough such that the ammonium oxidation rate proceeds close to maximum rate (e.g. DO $\geq 2 \cdot k_{S,O2}$), while still being low enough to enable efficient SND.

The effect of the small amount of PHB preserved per cycle (less than 0.2 CmM) highlights the importance of careful aeration control during SBR operation. One single event of aeration control failure during continuous SOUR-controlled operation caused the immediate loss of the PHB accumulated over many cycles and took time to restore the biomass PHB content (data not shown). This may explain why one period of over-aeration in large-scale SBR plants can lose the "historical effects" of biomass performance (Newland, personal communication).

Effects of Long-Term Oxygen Management

In addition to the increased SND-capacity of the biomass after continuous SBR operation using SOUR-controlled aeration, two other changes were noted: The settleability of the biomass improved from an SVI of 110 to less than 70 mL $g^{-1}X$ and the specific ammonium oxidation rate had slowed down to half its original rate (Fig 5.7, Table 5.1). During SOUR-controlled operation, the total cycle time was reduced by 100 minutes (Fig 5.4) in comparison to cycles with fixed aeration length (Fig 5.1). This effectively resulted in a higher substrate loading rate (37 mg COD $g^{-1}X$ h^{-1}), compared to standard SBR operation (28 mg COD $g^{-1}X$ h^{-1}). The formation of

biomass with efficient settling characteristics (i.e. low SVI) in activated sludge reactors has been found to increase with an increased substrate loading rate, decreased shear rate and dynamic feeding strategy (Beun et al., 1999, Van Loosdrecht et al., 1995, Tijhuis et al., 1995). The combination of a low stirring speed used in this study (80 rpm), together with an increased substrate loading rate and dynamic feeding strategy may have caused the observed large floc formation. With regard to the decrease in the specific ammonium oxidation rate over the one-month SOUR-controlled operation period, the cause of the decreased nitrifying activity is unclear. It may have been simply due to oxygen diffusion limitation, caused by the high SOUR of the heterotrophic population. However, the decreased rate was not a negative aspect in terms of nitrogen removal (Table 5.1). The slower rate of ammonium oxidation meant that SND was not limited by denitrification at any stage and hence nitrate did not accumulate. The overall result was complete nitrogen removal within 3 hours of low oxygen supply (Fig 5.7), as opposed to incomplete nitrogen removal within 4 hours (Fig 5.4). This could imply that if reactors are operated optimally for SND by tight oxygen management, reactors of smaller volume could potentially treat the same influent throughput rate, achieving decreased operating costs together with improved N-removal.

Summary of Oxygen Management Strategy

For operation of sequencing batch reactors in which optimal SND is the ultimate aim, without the use of anoxic liquor recycling or the addition of an external carbon supplement, a very tight oxygen management strategy is necessary on 4 fronts: 1) A low dissolved oxygen concentration ($DO < k_{S,O2(HET)}$) during the feast phase is necessary for maximum conversion of acetate to PHB (Chapter 2), (2) A low dissolved oxygen concentration during the aerobic famine period (< 2 mg L^{-1}) increases the amount of nitrogen removed via SND (Beun et al., 2001, Pochana and Keller, 1999, Munch et al., 1996, Sen and Dentel, 1998, Chapter 3), (3) Termination of the aerobic phase upon ammonium depletion decreases the amount of PHB that is "wasted" aerobically and increases the PHB available for anoxic denitrification (Chapter 4), and (4) Long term use of controlled aerobic phase length can increase the PHB content of the biomass and increase its capacity for SND. In terms of aeration costs, the part of the aeration cycle that was avoided was the period of lowest SOUR after ammonium oxidation (Fig 5.1). A measure of the energy saved for aeration could be obtained by multiplying the required k_La (h^{-1}) by the aeration time (h), resulting in a dimensionless

measure of required "aeration intensity". According to this, the actual energy saved by omitting the end of the aerobic period may not be significant in the short term. However, the accumulated savings in time and energy during continuous operation could be considerable.

5.5. Conclusions

This study has highlighted both the short- and long-term advantages of continuous aerobic phase length control in an SBR, by showing that;

- Minimisation of the aerobic phase length over many cycles results in PHB accumulation in the short-term.
- An increased biomass PHB content results in an increased SOUR and an increased amount of nitrogen removal via SND during the aeration phase.
- Long-term oxygen management can achieve increased nitrogen removal in a shorter time interval.
- Changes in the nitrogen removal capacity of biomass can be significantly affected by the reactor operation "history".

In combination, the results of this study show that the nutrient removal efficiency of SBR reactors is not only affected by short-term process control measures, but there is also a significant mid-term effect (over a few SBR cycles) and long-term effect that is governed by the operation of previous cycles. According to our laboratory results, one event of over-aeration of the biosolids during one single cycle may have plant performance consequences over a number of successive cycles.

CHAPTER 6

The CANON System (Completely Autotrophic Nitrogen-removal Over Nitrite) under Ammonium Limitation: Interaction and Competition between Three Groups of Bacteria[1]

Abstract

The CANON system (Completely Autotrophic Nitrogen-removal Over Nitrite) can potentially remove ammonium from wastewater in a single, oxygen-limited treatment step. The usefulness of CANON as an industrial process will be determined by the ability of the system to recover from major disturbances in feed composition. The CANON process relies on the stable interaction between only two bacterial populations: *Nitrosomonas*-like aerobic and *Planctomycete*-like anaerobic ammonium oxidising bacteria. The effect of extended periods of ammonium limitation was investigated at the laboratory scale in two different reactor types (sequencing batch reactor and chemostat). The lower limit of effective and stable nitrogen removal to dinitrogen gas in the CANON system was 0.1 kg N m^{-3} day^{-1}. At this loading rate, 92 % of the total nitrogen was removed. After prolonged exposure (> 1 month) to influxes lower than this critical NH_4^+-influx, a third population of bacteria developed in the system and affected the CANON reaction stoichiometry, resulting in a temporary decrease in nitrogen removal from 92 % to 57 %. The third group of bacteria were identified by activity tests and qualititative FISH (Fluorescence In Situ Hybridisation) analysis to be nitrite-oxidising *Nitrobacter* and *Nitrospira* species. The changes caused by the NH_4^+-limitation were completely reversible, and the system re-established itself as soon as the ammonium limitation was removed. This study showed that CANON is a robust system for ammonium removal, enduring periods of up to one month of ammonium limitation without irreversible damage. The use of improved dissolved oxygen control would prevent problems caused by extended ammonium limitation.

[1] This chapter is published in Systematic and Applied Microbiology 24 (4) 588-596

6.1. Introduction

A co-operation between aerobic and anaerobic ammonium oxidising bacteria under oxygen limitation has been observed previously (Strous 2000, Sliekers et al., 2002a). This is a promising new principle for wastewater treatment, as only a single oxygen-limited step might be needed to remove ammonium from wastewater. The process relies on the interaction of two groups of autotrophic bacteria under oxygen-limiting conditions that perform two sequential reactions, simultaneously. Under oxygen limitation, ammonium is oxidised to nitrite by aerobic ammonium oxidisers, such as *Nitrosomonas* and *Nitrosospira* (Eq 1).

$$1\ NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \qquad \text{(Eq 1)}$$

The nitrite produced in this reaction can be used by planctomycete-like Anammox bacteria, which anaerobically oxidise ammonium using nitrite as electron acceptor (Strous, 2000) (Eq 2).

$$1\ NH_4^+ + 1.3\ NO_2^- \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2\ H_2O \qquad \text{(Eq 2)}$$

As the nitrite also serves as an electron donor for the formation of biomass from carbon dioxide, the formation of nitrate in the reaction is stoichiometrically coupled to growth. The combination of the above two reactions results in nitrogen removal according to (Eq 3):

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \qquad \text{(Eq 3)}$$

The interaction of aerobic and anaerobic ammonium oxidising bacteria under oxygen-limitation results in an almost complete conversion of ammonium to dinitrogen gas, along with small amounts of nitrate. A high loss of nitrogen has been reported in several systems with high ammonium loading and low organic carbon content of the wastewater (Helmer et al. 2001, Helmer and Kunst, 1998, Helmer et al. 1999, Hippen et al., 1997, Koch et al. 2000, Kuai and Verstraete, 1998, Siegrist et al., 1998). The autotrophic conversion of ammonium into dinitrogen gas was defined microbiologically (Strous, 2000) and the process has been named CANON, an acronym for Completely Autotrophic Nitrogen-removal Over Nitrite (Dijkman and Strous, 1999). If ammonium removal can be achieved in a single reactor, it would represent a very economical and efficient option for water treatment, especially for wastewater rich in ammonium but

devoid of organic carbon (COD). Ammonium removal from wastewater is traditionally performed using oxic nitrification to nitrate, involving high aeration demands, followed by anoxic denitrification of the nitrate to nitrogen gas, in a separate tank. The CANON process is completely autotrophic, therefore avoiding COD addition, which is often required for the heterotrophic denitrification step in traditional systems. In addition, the entire nitrogen removal can be achieved in a single reactor with very low aeration, greatly reducing space and energy requirements. The autotrophic process consumes 63 % less oxygen and 100 % less reducing agent than traditional nitrogen removal systems (Kuai and Verstraete, 1998). Unlike other autotrophic nitrogen removal systems, such as the SHARON-Anammox process where the nitrite is generated in a separate reactor (Jetten et al., 1997a), there is no requirement for nitrite addition in the CANON system. Thus, an ammonium-rich wastewater can be fed directly to a single oxygen-limited reactor at a suitable loading rate. Nitrogen removal rates of up to 1.5 kg N_{total} m^{-3} day^{-1} have been reported for the CANON process (Sliekers et al., 2002b).

If the CANON process is to be applied on a large scale, it is important to know the limitations of the system, and whether severe disturbances to the system are reversible. The CANON process relies on the harmonious and balanced interaction between only two groups of bacteria, the aerobic and the anaerobic ammonium oxidising bacteria. If under certain conditions the balance is disturbed, for example if other groups of bacteria, such as aerobic nitrite oxidisers develop, they may interfere with nitrogen removal. The following study investigated the effects of severe ammonium limitation, as may occur during periods of low nitrogen loading in wastewater treatment. The effect of ammonium limitation on the CANON system was studied in two different reactor types; the sequencing batch reactor (SBR) and the chemostat. The bacterial populations were monitored by Fluorescence In Situ Hybridisation (FISH) to investigate changes in the composition of the biomass under ammonium limitation. Together with activity measurements and mass balances, changes in the bacterial populations and hence in the nitrogen removal characteristics were monitored. The reversibility of the changes imposed by the limitation was investigated.

6.2. Materials and Methods

6.2.1. Reactor Systems and Control Equipment

The Sequencing Batch Reactor (SBR)

A glass vessel (height 0.21 m, diameter 0.14 m) without baffles, with a 2 L working volume and equipped with a water jacket (maintained at 30^{o} C) was used as the SBR. The glass vessel was fitted with a fermentor lid containing a turbine propellor stirrer, acid/base inflow tubes, feed inflow tube, dissolved oxygen probe, pH probe, outflow tube, level controller and a sampling port (Fig 6.1). The vessel was stirred at 100±10 rpm. Dissolved oxygen was measured using a Clark-type oxygen electrode (Ingold, Switzerland), which was calibrated every two weeks. The dissolved oxygen concentration was monitored using an Applikon 1030 biocontroller (Applikon, The Netherlands), which also controlled the pH at 7.8 using 0.5 M Na_2CO_3 and 1 M HCl. The gas-flow was maintained constant using a mass-flow controller (Brooks Instruments, The Netherlands). During anaerobic periods the reactor was flushed with Helium, and during aerobic periods air was used. The pumps and stirrer were controlled by timers.

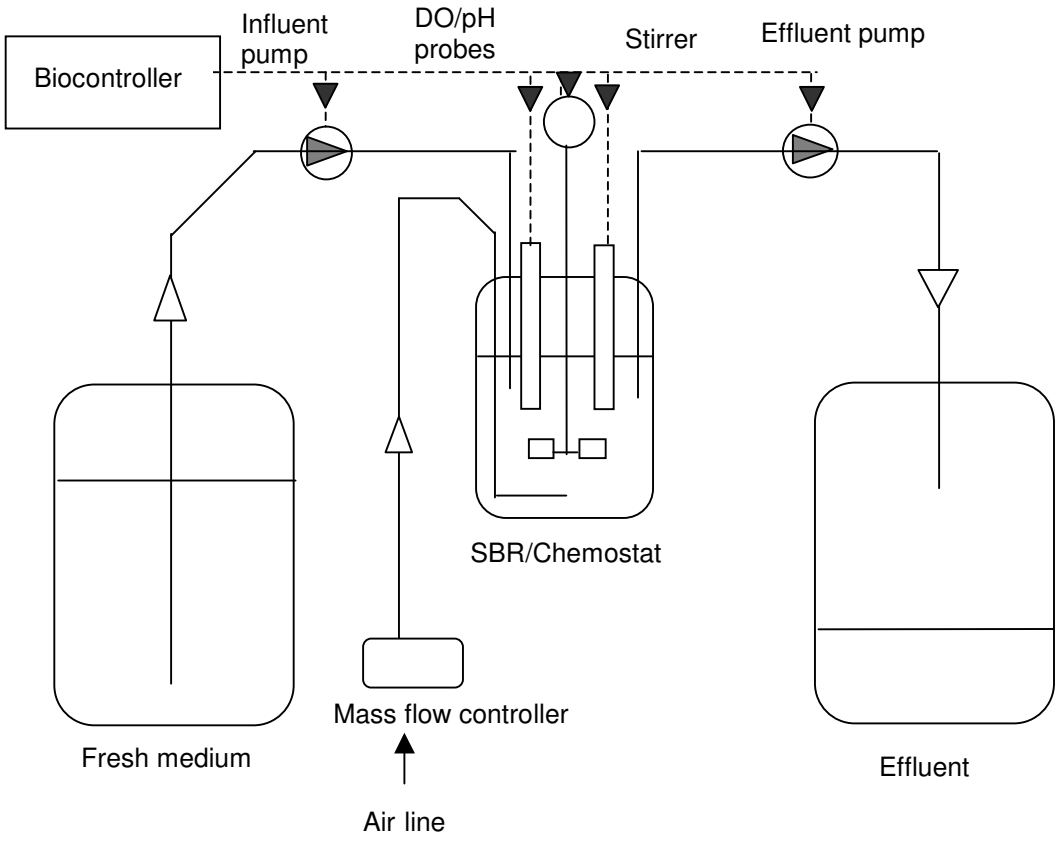

Figure 6.1: Experimental set-up of the reactor system.

The SBR was run as a continuously-fed sequencing batch reactor with a 12 hour cycle. It was filled continuously with fresh medium over 11.5 hours at a flow rate of 1.45 mL min^{-1}. The minimum volume was 1 L, and at the end of the cycle the final volume was 2 L. After the filling period, the stirrer and influent pump were stopped and the biomass aggregates were allowed to settle for 15 minutes. In the remaining 15 minutes of the cycle, 1 L of supernatant was removed by an effluent pump.

The Chemostat

The same 2 L vessel used for the SBR was converted to a chemostat and maintained at a constant volume (1.5 L) by a level controller. The feed flow rate started initially at 2.6 L day^{-1} (dilution rate 0.072 h^{-1}). To enable biomass retention the reactor was fitted with a settling device in the outflow tube, in which the upflow rate of the outflowing liquid was slower than the settling rate of the biomass. Hence, biomass settled in the tube (diameter around 5 cm) and was periodically returned to the reactor (every 2 – 3 days). The stirrer and inflow pump were turned off for half an hour each cycle, to mimic the SBR and to make a comparison possible.

6.2.2. Origin of Biomass

Anammox

The biomass was taken from the outflow tank of a 15 L Anammox-SBR-reactor in which 80% of the biomass consisted of planctomycete-like Anammox bacteria (Strous et al., 1999b). The biomass in the outflow tank was 2 – 3 months old. The biomass was never separated from its supernatant liquid before inoculation, as it has been shown to significantly decrease its initial activity.

Nitrifying Biomass

The nitrifying biomass was taken from a refrigerated sample from a previously oxygen-limited nitrifying chemostat, known to actively oxidise ammonium at a rate of 106 nmoles NH_4^+ mg $protein^{-1}$ min^{-1}.

6.2.3. Synthetic wastewater

Ammonium and nitrite were added to a mineral medium in the required amounts (concentrations are specified in the Results section) in the form of $NaNO_2$ and $(NH_4)_2SO_4$. The composition of the mineral medium was (g L^{-1}): $KHCO_3$ 1.25,

KH_2PO_4 0.025, $CaCl_2.2H_2O$ 0.3, $MgSO_4.7H_2O$ 0.2, $FeSO_4$ 0.00625, EDTA 0.00625 and 1.25 mL L^{-1} of trace elements solution (as described in Chapter 2). The separate components of the medium were autoclaved at 120 ^oC to avoid bacterial growth in the feed vessels, although the reactors were not run aseptically.

6.2.4. Activity measurements

The maximum *aerobic* NH_4^+ and NO_2^--oxidizing activity of the biomass were measured under oxygen saturation conditions. A 10 mL sample of biomass suspension was taken from the reactor and put into a 250 mL Erlenmeyer flask. Nitrite and ammonium were added (if not already in the medium) to final concentrations of about 2 mM. The flask was then incubated at 30^o C on a rotary shaker. Ammonium, nitrate and nitrite were measured every half an hour for up to two hours. To measure the maximum *anaerobic* ammonium oxidation activity, biomass was taken out of the reactor and put into a serum bottle with a gas-tight rubber stopper. The flask was made anaerobic by flushing with Argon. Nitrite and ammonium were added to the flasks to final concentrations of about 2 mM. Nitrite and ammonium consumption and NO_3^- production were measured over time during 4 hours. All activities were calculated from the rate of substrate disappearance or product appearance per concentration of biomass present (i.e. nmoles mg $protein^{-1}$ min^{-1}).

6.2.5. Dry weight determinations

A sample of biomass suspension (2 or 5 mL) was taken from the reactor and filtered through 0.2 μm filter paper. The filter paper was then dried in a microwave for 25 minutes at a low temperature (180^o C), and the weight of the dried biomass was determined. Protein measurements of the biomass were performed according to the method of Lowry (Lowry et al., 1951) and showed that the protein concentration was consistently approximately half of the measured dry weight. Protein concentrations were thus calculated as half of the measured dry weight.

6.2.6. Fluorescence in situ hybridization (FISH)

Fixation of cells for in situ hybridisation

The method was adapted from Amann (1995). Cells were washed in 10 mM phosphate buffer (pH 7.2, containing 130 mM NaCl) and resuspended in the same buffer. One volume of the cell suspension was mixed with 3 volumes of the same buffer (freshly prepared and containing 4 % paraformaldehyde) and kept on ice for 1 – 3 hours. The fixative was then removed by centrifugation and the cells were resuspended in 0.5 mL phosphate buffer. Finally, 0.5 mL of absolute ice-cold ethanol was added and the cells stored at -20^{o} C until used.

Immobilisation of fixed cells on microscope slides

The immobilisation step was adapted from Juretschko et al. (1998), whereby 5-10 µL aliquots of cell suspension were loaded in each gelatine-coated well of a teflon-printed slide (Nutacon, the Netherlands). After loading, the slide was dried for 10 min at 46 ^{o}C. The cells were then dehydrated by successive passage through 50, 80 and 90 % ethanol (3 minutes each) and air dried.

Hybridization of immobilised cells

This step was also adapted from Juretschko et al. (1998). To each well on the teflon-printed slide 10 µL of freshly prepared hybridisation buffer (pH 8, 20 mM Tris/HCl, 180 mM NaCl, formamide 35%), and 1 µL probe working solution containing 5 ng of each of the respective dye-labelled probes was added. The 16S rRNA gene probes used were Amx820 for planctomycete-like Anammox cells (Strous et al., 1999a), Neu 653 for *Nitrosomonas*-like aerobic ammonium-oxidizng bacteria and NIT and Nspa for *Nitrobacter* or *Nitrospira*-like aerobic nitrite-oxidizing bacteria (Juretschko et al., 1998). Probes were synthesized and directly labelled with the hydrophilic sulphoindocyanine flourescent dye Cy3 or the flourochrome 5(6)-carboxy-fluorescin-N-hydroxysuccinimide ester (FLUOS) (Interactiva, Germany).

The slides were incubated at 46 ^{o}C for 90 min in 50 mL Falcon tubes, containing a tissue paper moisturised with 2 mL of hybridisation buffer. After hybridisation, the slides were washed rapidly with 20 mM Tris/HCl (pH 8, 48 ^{o}C and containing 5 mM EDTA and NaCl concentrations depending on the applied formamide concentration in

the buffer. For 35 % formamide, 80 mM NaCl was added. The slides were rapidly immersed in the same buffer and incubated at 48 ^{o}C. After 20 min the slides were rinsed with cold MilliQ water, air dried rapidly and stored at –20 ^{o}C until studied under the microscope.

Epifluorescence microscopy and documentation

The slides were embedded in vectashield (Vector laboratories, USA) and analysed with a Zeiss Axioplan 2 imaging fluorescent microscope (Zeiss, Weesp, the Netherlands). Photomicrographs were taken using LeicaQFluoro imaging software (Leica, the Hague).

6.2.7. Chemical Analyses

Nitrate, nitrite and ammonium were measured colorimetrically as described previously (Strous et al. 1998, Sliekers et al. 2002a). Gas measurements were performed by gas chromatography (Interscience, Breda, Netherlands) equipped with two columns (Molsieve 5A and a Hayesep column), an Electron Capture Detector and a Hot Wire Detector (Interscience, Netherlands).

6.3. Results

The Sequencing Batch Reactor (SBR) for Investigation of CANON under NH_4^+-Limitation

Start-Up of the CANON System (Non-Limiting NH_4^+ Supply)

To start the CANON process, nitrifying biomass (0.01 g L^{-1}) and oxygen (7.9 mL min^{-1} air) were introduced into an Anammox culture, which had been anaerobically oxidising ammonium with nitrite as electron acceptor (feed concentrations 18 mM NO_2^- and 25 mM NH_4^+). The feed was changed to contain NH_4^+-N only (12 mM) and was fed at a rate of 1.0 mmole NH_4^+ L^{-1} h^{-1}. The change in gas flow from helium, during anammox operation, to air in the CANON system resulted in a rapid increase of the dissolved oxygen to around 3 % of air saturation (0.24 mg L^{-1}). Within a few hours the oxygen concentration was reduced to below the detection limit (< 0.1 % sat.), indicating immediate activity of the added nitrifying biomass.

The concentrations of ammonium, nitrite and nitrate were followed over time for 2 weeks. Ammonium was always present in excess, while nitrite levels gradually reached zero (Fig 6.2a). This indicated that the anammox bacteria were consuming all the available nitrite produced by the aerobic ammonium oxidisers and that a cooperation had been established between the aerobic and anaerobic ammonium oxidizers. The molar ratio of $NO_{3\ produced}^-/NH_{4\ consumed}^+$ was about 0.13, corresponding to the overall CANON stoichiometry (Eq 3). The nitrogen removal characteristics during the period where ammonium was in excess are shown in Table 6.1. Nitrogen was removed in the SBR at a rate of 0.11 kg N m^{-3} day^{-1}. The nitrogen removal characteristics of the SBR were similar to the removal rates in the chemostat investigated later (± 10%), hence the values given in Table 6.1 are an average of the rates observed in both the SBR and the chemostat. No nitrite oxidising activity could be detected in the aerobic activity tests in the SBR or chemostat when ammonium was in excess (Row 8, Table 6.1).

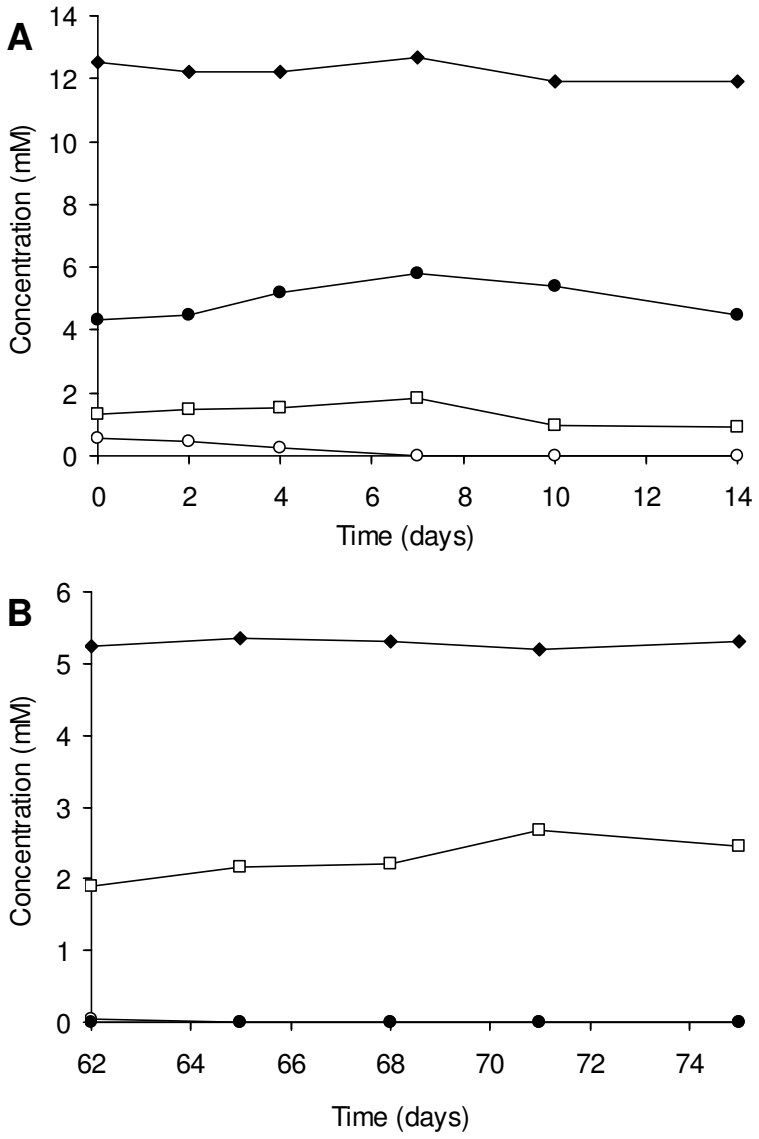

Figure 6.2: Concentrations of nitrogen compounds over time in the CANON reactor with A: Non-limiting ammonium (12 mM) in the feed supply and, B: Prolonged NH_4^+-limitation (◆ feed NH_4^+, ❍ NO_2^-, □ NO_3^- and ● NH_4^+).

CANON under NH_4^+-Limitation in the SBR

The ammonium influx was decreased stepwise to investigate the effect of ammonium limitation on the stability of the CANON system. At an ammonium influx of 0.36 mmoles NH_4^+ L^{-1} h^{-1} (feed concentration 5.2 mM NH_4^+), true ammonium limitation was achieved. At the beginning of the SBR cycle the dissolved oxygen increased to values greater than 25 % air saturation and then fell slowly, reaching 0 – 3 % half way

through the cycle (Fig 6.3). This was due to the changing oxygen transfer capacity (k_La) of the reactor, caused by the changing volume over the 12-hour cycle (i.e. filling from 1 to 2 L). The presence of high oxygen concentrations during the initial part of the cycle caused a reversible inactivation of anaerobic ammonium oxidation during this period, resulting in accumulation of nitrite to around 1.2 mM (Fig 6.3). The recovery of Anammox activity towards the end of the cycle resulted in removal/reduction of all of the nitrite that had accumulated.

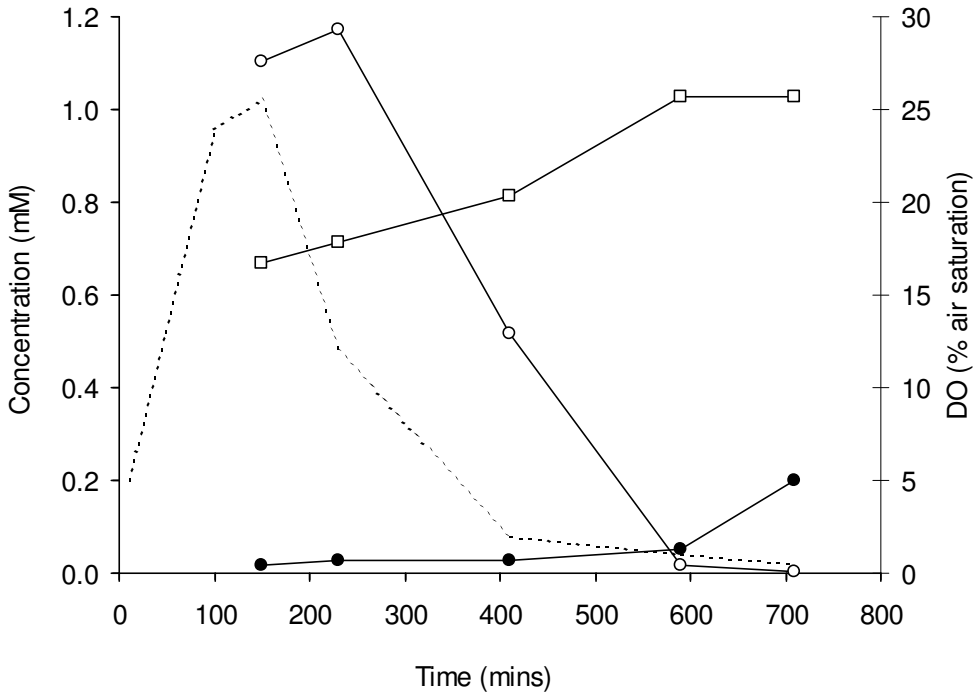

Figure 6.3: Profile of nitrogen compounds in one SBR cycle during prolonged ammonium limitation, showing large fluctuations during one cycle: ● NH_4^+, ❍ NO_2^-, □ NO_3^- and DO concentration (dotted line).

Biomass samples were taken 14 days after the onset of NH_4^+-limitation to examine the composition of the bacterial community. Qualitative FISH analysis showed that planctomycete-like Anammox bacteria and *Nitrosomonas*-like aerobic ammonium oxidisers dominated the biomass (Fig 6.4A). The analysis also showed the presence of very small amounts of *Nitrospira* sp. (Fig 6.4B). The presence of these nitrite-oxidising bacteria was surprising, as aerobic activity tests failed to detect any nitrite oxidising activity (Row 8, Table 6.1).

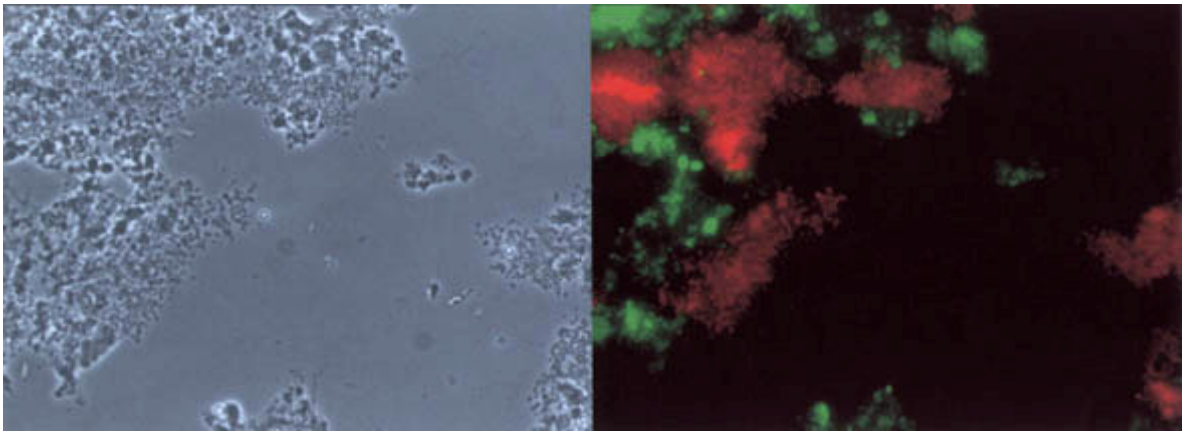

Figure 6.4A: FISH analysis of CANON SBR biomass under NH_4^+-limitation at day 14 (Left: Phase contrast x 630 magnification. Right: The same section of biomass showing *Nitrosomonas* like aerobic ammonium-oxidising cells stained with probe FLUOS-Neu 653 (green) and anammox cells stained with probe CY3-Amx820 (red).

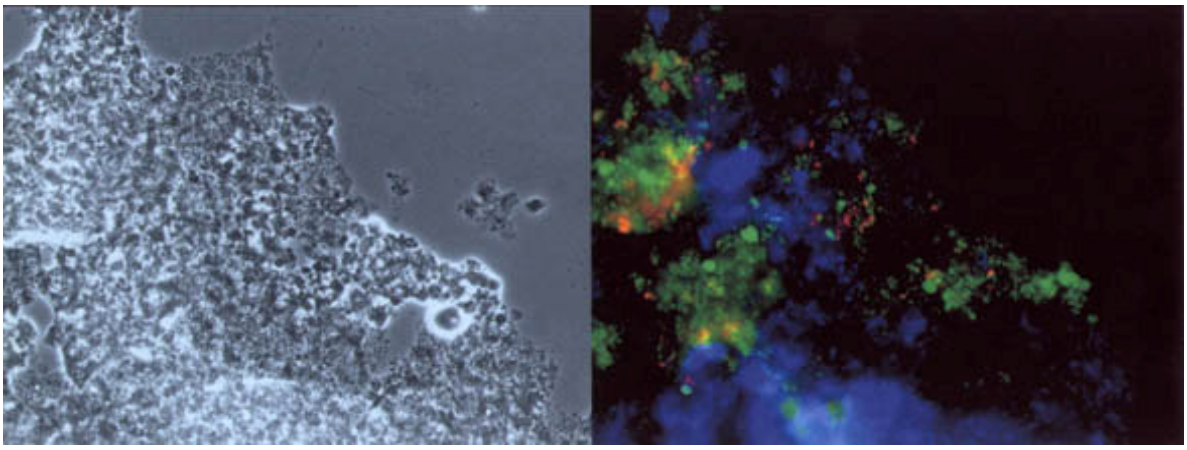

Figure 6.4B: FISH analysis of biomass in the CANON-SBR under NH_4^+-limitation at day 14; Left: Phase contrast at x 630 magnification. Right: *Nitrospira*-like nitrite-oxidising cells stained with CY3-NITSP (red), *Nitrosomonas*-like cells stained with probe Fluos-NIT (green) along with anammox cells stained with probe CY5-Amx820 (blue).

CANON with Prolonged NH_4^+-Limitation

The SBR was maintained for a period of two months with a constant NH_4^+-influx of 0.36 mmoles NH_4^+ L^{-1} h^{-1} (NH_4^+-limitation). The concentrations measured daily in the reactor over time (Fig 6.2B) showed there were particularly high concentrations of nitrate present in the reactor. The molar ratio of $NO_3^-{}_{produced}/NH_4^+{}_{consumed}$ in the CANON system with NH_4^+ in excess had been around 0.13, as expected for the CANON system

(Eq 3). However under prolonged NH_4^+-limitation this molar ratio had increased to around 0.40, indicating increased nitrate production by aerobic nitrite-oxidising bacteria.

Activity tests conducted during the period of NH_4^+-limitation indeed indicated a gradual increase in the aerobic nitrite oxidising activity of the biomass. When ammonium had been in excess, up to 100 % of the nitrite produced by aerobic NH_4^+-oxidation was reduced to dinitrogen gas by Anammox-like bacteria. After two months of NH_4^+-limitation, the percentage of nitrite reduced anaerobically had decreased to only 31 % of the total nitrite produced, and the remaining 69 % NO_2^- was oxidised aerobically to nitrate (activities are given in Table 6.1, rows 6 – 8). The growth of aerobic nitrite-oxidising bacteria in the system due to the ammonium limitation resulted in a reduction of nitrogen removal capacity from 92 % to 57 % N-removal (row 4, Table 6.1).

After the 3 months of prolonged ammonium limitation, the feed was changed back to an excess of ammonium (12 mM feed, influx 1 mmole L^{-1} h^{-1}). Within one week, the molar ratio of NO_3^-/NH_4^+ gradually decreased from a ratio of 0.4 to its original ratio of 0.1, indicating that aerobic and anaerobic ammonium oxidising bacteria had re-established their co-operation, leaving no oxygen or nitrite for the aerobic nitrite oxidising bacteria.

The Chemostat for Investigation of CANON under NH_4^+-Limitation

The oxygen transfer rate in the CANON-sequencing batch reactor fluctuated during each cycle due to the changing volume over the 12 hour cycle (continuous filling from 1 to 2 L). The changing oxygen transfer rate resulted in large changes in the dissolved oxygen concentration during one cycle (Fig 6.3), which made it difficult to determine changes in bacterial activity and similarly in mass balances over time. To eliminate these "non steady-state" fluctuations, a chemostat reactor system with biomass retention was run to investigate effects of ammonium limitation on the CANON system. After 30 days, a steady-state with ammonium in excess (0.57 mmoles L^{-1} h^{-1}) at a dilution rate of 0.072 h^{-1} was established. A stable interaction between the aerobic and anaerobic ammonium oxidising bacteria had been reached, as indicated by the molar ratio of $NO_3^-{}_{produced}/NH_4^+{}_{consumed}$ of 0.1 (Eq 3). The ammonium consumption rate at steady-state was 0.4 mmoles NH_4^+ L^{-1} h^{-1} (or 0.13 kg N m^{-3} day^{-1}). Interestingly, the ammonium conversion rate was very similar to that observed in the SBR with NH_4^+ in excess (0.11

kg N m^{-3} day^{-1}). From GC measurements it was calculated that around 0.82 moles of O_2 were being used for every mole of NH_4^+, as expected according to CANON nitrogen removal (Eq 3).

Table 6.1: Characteristics of CANON N-removal in the chemostat and SBR (N-removal characteristics for the two reactor types were similar (± 10%), hence an average is given).

Reactor characteristic	**NH_4^+-excess** (1 mmole NH_4^+ L^{-1} h^{-1})	**NH_4^+-saturation**[1] (0.40 mmoles NH_4^+ L^{-1} h^{-1})	**NH_4^+-limitation** (≤0.36 mmoles NH_4^+ L^{-1} h^{-1})
1. Nitrogen load (kg N m^{-3} day^{-1})	0.22	0.12	0.07
2. Nitrogen consumption (kg N m^{-3} day^{-1})	0.11	0.12	0.07
3. Nitrogen removal (kg N m^{-3} day^{-1})	0.08	0.11	0.04
4. % Nitrogen removal	36	92	57
5. Dry weight conc. (g/L)	1.0	0.92	0.75
Activity Tests			
6. Max. aerobic NH_4^+-oxidising activity[2]	72	74	33
7. Max. anaerobic NH_4^+-oxidising activity	38	27	7
8. Max. aerobic NO_2^- oxidising activity**	0	0	15

From day 48 onwards, the NH_4^+-influx was decreased gradually to impose NH_4^+-limitation on the system (Fig 6.5). The NH_4^+-influx was decreased from 0.40 to 0.21 mmoles L^{-1} h^{-1} on day 52 by increasing the hydraulic retention time from 13.8 hours to 22.8 hours. The decrease in the NH_4^+-influx caused an immediate increase in the oxygen concentration (up to 3.5 %), in turn causing an increase in NO_2^- concentration from 0 to 1.6 mM, due to partial inactivation of the Anammox bacteria.

[1] "NH_4^+-saturation" refers to the situation where ammonium was neither limiting nor in excess.
[2] Maximum aerobic activity tests were performed in separate shake flask tests with optimum aeration. All activity measurements are given in nmoles mg $protein^{-1}$ min^{-1}.

The biomass was exposed to nitrite and oxygen for 5 days. The nitrite gradually disappeared after 5 days in the presence of oxygen, together with an increase in NO_3^- levels, indicating growth of aerobic nitrite-oxidising bacteria. The HRT of 23 days was maintained for two weeks. At day 70, the HRT was increased further to 30 days, lowering the NH_4^+-influx even further to 0.16 mmoles L^{-1} h^{-1}. It was interesting to note that the further lowering of the NH_4^+-influx directly increased the oxygen concentration, and in turn increased the nitrate production due to increased aerobic nitrite oxidation (Fig 6.5). However, when the ammonium limitation was removed (day 85), the nitrate concentration in the reactor decreased accordingly and within one week the cooperation between the aerobic and anaerobic ammonium oxidisers had been re-established, leaving no nitrite or oxygen for the nitrite oxidisers. The shift in bacterial populations caused by the ammonium limitation was completely reversible.

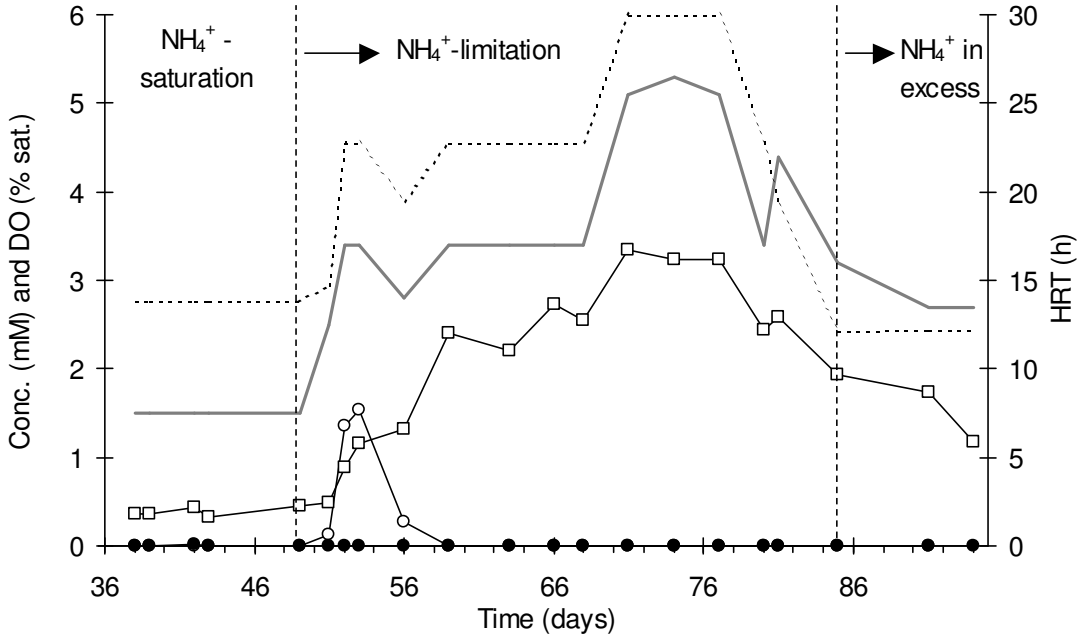

Figure 6.5: Change in nitrogen compounds (● NH_4^+, ❍ NO_2^- and □ NO_3^-), O_2 concentration (grey line) and hydraulic retention time (HRT, dotted line) during operation of the CANON chemostat before, during and after NH_4^+-limitation.

Aerobic activity tests with optimum aeration were conducted regularly during operation of the chemostat before and after the onset of ammonium limitation (Fig 6.6). When ammonium was in excess, no aerobic nitrite-oxidising activity was detected in the chemostat, as indicated by the negligible nitrate-production rate. After the onset of NH_4^+-limitation, the aerobic nitrate-production rate increased steadily over time to

25 nmoles NO_3^- mg protein^{-1} min^{-1}. At day 85 in the chemostat, both the aerobic ammonium- and nitrite-oxidising activities were high (Fig 6.6), and it was suspected that the anaerobic ammonium-oxidising bacteria had been outcompeted from the system. The anaerobic ammonium oxidising activity of Anammox had decreased from 27 nmoles NH_4^+ mg protein^{-1} min^{-1} (ammonium not limiting) to 7 nmoles NH_4^+ mg protein^{-1} min^{-1} (row 7, Table 6.1). The Anammox bacteria were still present and active, albeit at a low rate. FISH analysis of biomass from the chemostat at day 65 showed the presence of all three bacterial populations (Figs 6.7).

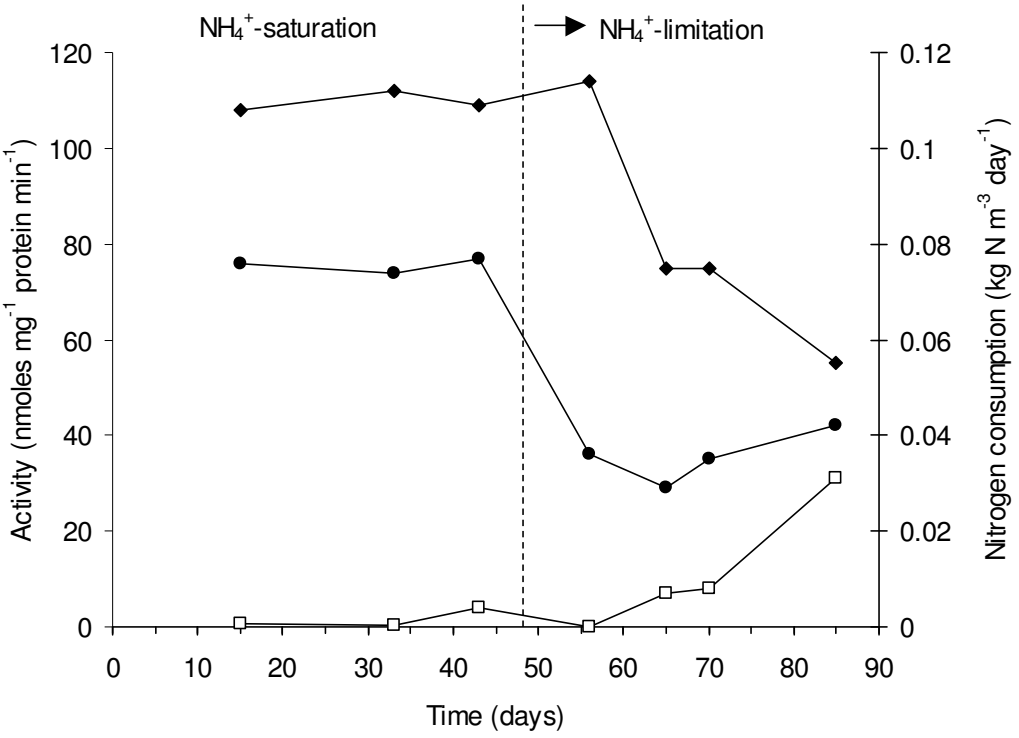

Figure 6.6: Changes in nitrification activity in samples of biomass taken from the CANON chemostat, before and during NH_4^+-limitation. Activities were measured in shake flask tests with optimum aeration (● NH_4^+ consumption rate, □ NO_3^- production rate) and total nitrogen consumption (◆).

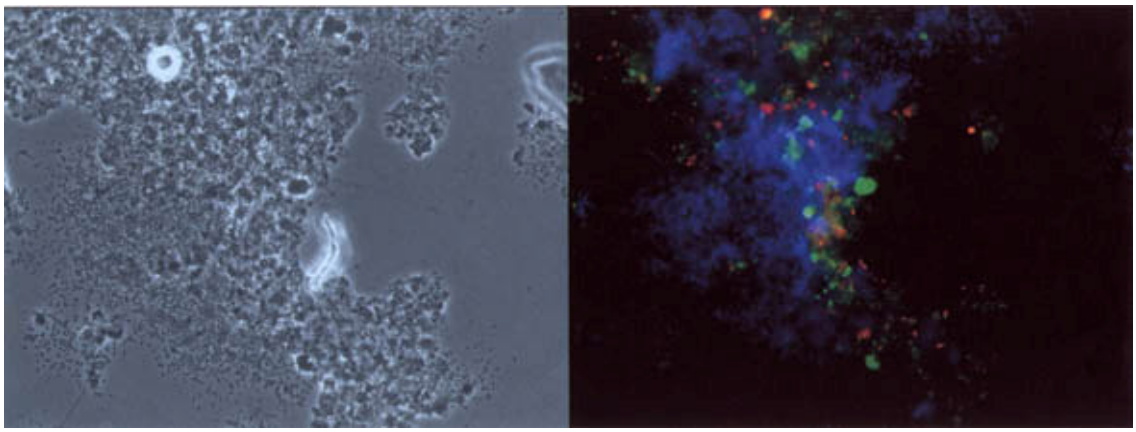

Figure 6.7A: FISH analysis of CANON chemostat biomass under NH_4^+-limitation (day 65); Left: Phase contrast x 630 magnification, Right: *Nitrosomonas*-like aerobic ammonium-oxidising cells stained with probe FLUOS-Neu 653 (green), *Nitrospira*-like nitrite-oxidising cells stained with CY3-NITSP (red) and anammox cells stained with CY5-Amx820 (blue).

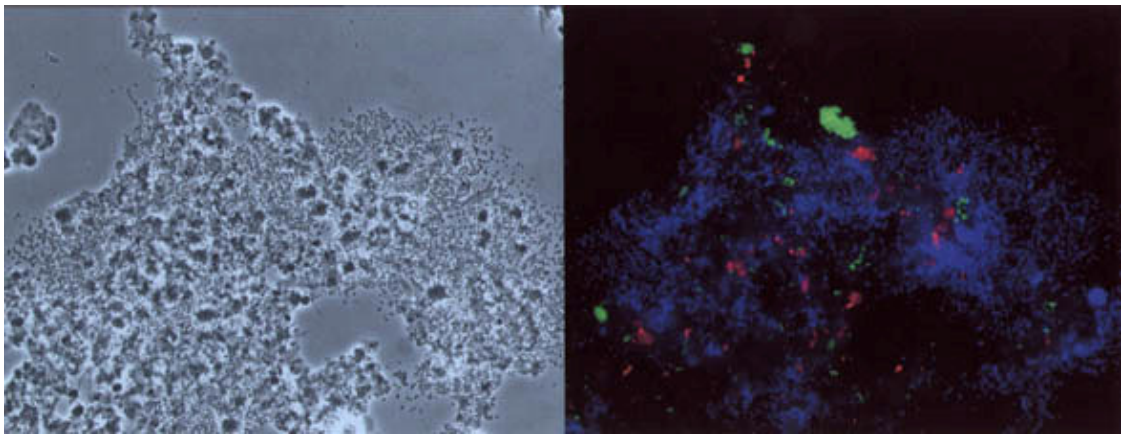

Figure 6.7B: FISH analysis of chemostat biomass (day 65); Left: Phase contrast x 630 magnification. Right: *Nitrospira*-like nitrite-oxidising cells stained with CY3-NITSP (red), *Nitrobacter*-like nitrite-oxidising cells stained with FLUOS-NIT (green) and Anammox cells stained with CY5-Amx820 (blue). Samples in both Figs 6.7A and 6.7B were prepared with 40% formamide concentration.

6.4. Discussion

The CANON system could effectively remove nitrogen in a single oxygen-limited treatment step. The lower limit for stable nitrogen removal to dinitrogen gas was 0.4 mmoles L^{-1} h^{-1} (0.12 kg N m^{-3} day^{-1}). At and above this ammonium influx, a stable interaction existed between aerobic and anaerobic ammonium oxidising bacteria under oxygen limitation. The system was easy to maintain during the test period (3 months) both in a Sequencing Batch Reactor (SBR)-type as in a chemostat with biomass retention. Retention of the biomass, in the form of flocculated material, is required in the first place by the unusually low growth rate of the anaerobic ammonium oxidisers. At the same time the presence of dense biomass-flocs creates (a)biotic physical and chemical gradients allowing coexistence and (co)performance of microbial processes and populations with complementary and/or opposed environmental requirements.

During the normal ammonium-saturating operation of the CANON system, the aerobic nitrite oxidising bacteria in the flocculated material experience a double limitation of nitrite and oxygen. They compete with the *Nitrosomonas*-like bacteria for oxygen, and with the Anammox-like bacteria for nitrite. The aerobic and anaerobic ammonium oxidisers, on the other hand, experience only a single limitation. The *Nitrosomonas*-like bacteria are limited by their oxygen supply, and the Anammox-like bacteria are dependent on the aerobic ammonium oxidisers for their nitrite supply (nitrite-limited). As long as the single limitation for each of these two groups of bacteria can be maintained, the CANON system can run effectively, and the nitrite oxidisers will be out-competed. However, during ammonium limitation both aerobic and anaerobic ammonium oxidisers are limited by their respective electron donor (NH_4^+) *and* their acceptors (O_2 and NO_2^-), due to competition with the nitrite oxidisers.

During ammonium-limiting operation, an initial increase in the oxygen and nitrite concentrations occurred in both the chemostat and the SBR. The ammonium influx was not enough for the aerobic ammonium oxidisers to consume all the available oxygen. The remaining oxygen inhibited the Anammox bacteria, and nitrite accumulated temporarily (Figs 6.3, 6.5). The simultaneous presence of excess nitrite and oxygen were perfect for the survival and growth of the nitrite oxidising bacteria. Their competition for nitrite with anammox and for oxygen with ammonium-oxidising

bacteria was relieved, and the cells could start to produce nitrate. The interaction between aerobic and anaerobic ammonium oxidising bacteria was interrupted, and nitrogen removal efficiency was reduced from 92 % to 57 % (Row 4, Table 6.1).

The opportunity for nitrite-oxidisers to develop in the system occurred when the NH_4^+-limitation caused an increase in the dissolved oxygen concentration. This could be prevented in a plant situation by the inclusion of dissolved oxygen-controllers, which control the airflow and prevent the increase in dissolved oxygen if the incoming ammonium concentration decreases. If the dissolved oxygen concentration does not increase, then there is no room for the nitrite oxidisers to proliferate. Similarly, if the oxygen could be limited until all the nitrite has been anaerobically reduced by anammox-bacteria, the nitrogen removal efficiency could be maintained during low-loading periods. Control of aeration would therefore play an important role at full scale, and is currently being investigated.

Both the sequencing batch reactor and the chemostat achieved very similar nitrogen removal rates. In the "non-steady state" conditions in the SBR during NH_4^+-limitation, the bacteria were exposed to fluctuating availabilities of electron donors and acceptors (Fig 6.3), however the overall efficiency compared to the chemostat was not affected. Anammox-bacteria were reversibly inhibited by the fluctuating oxygen conditions, yet their activity resumed as soon as the oxygen was no longer inhibiting. Prolonged exposure to oxygen in such systems might lead to the loss of Anammox bacteria from the reactor. However, anaerobic activity tests showed that there was still anaerobic NH_4^+-oxidation activity after one month of ammonium limitation and oxygen excess, albeit at a low rate (7 nmoles/mg protein/min; Table 6.1). FISH analysis confirmed that Anammox cells were still present in significant numbers (Fig 6.7). Furthermore, the interactive operation between the aerobic and anaerobic ammonium oxidising bacteria could be re-established within one week as soon as the ammonium limitation had been relieved.

Other autotrophic systems for removing ammonium from wastewater include the SHARON-Anammox process (Van Dongen et al. 2001) and the OLAND (oxygen limited autotrophic nitrification-denitrification) process (Kuai and Verstraete, 1998). The nitrogen-removal rate observed in this study (0.12 kg N m^{-3} day^{-1}) compares well

with the rates reported in the other autotrophic systems (0.8 and 0.05 kg N m^{-3} day^{-1}, respectively). Under ammonium-saturated operation of CANON in the SBR and chemostat, the process was limited principally by the oxygen transfer rate to the aerobic ammonium-oxidisers. A recent investigation looked at increasing the oxygen transfer rate by using a gas-lift reactor for the CANON system. Nitrogen removal rates of up to 1.5 kg N m^{-3} day^{-1} have been reported when the gas-transfer rate is optimised (Sliekers et al., 2002b), showing that the CANON could be a very useful nitrogen removal process for very high strength ammonium wastewaters.

The ability of the CANON system to withstand ammonium limitation for up to one month without irreversible damage shows that the CANON system could be a robust and effective industrial system to remove ammonium from wastewater with a very low organic load.

CHAPTER 7

Enrichment of Anammox from Activated Sludge and its Application in the CANON Process[1]

Abstract

A microbial culture capable of actively oxidising ammonium to dinitrogen gas in the absence of oxygen, using nitrite as the electron acceptor, was enriched from local activated sludge (Western Australia) in less than 14 weeks. The maximum anaerobic ammonium oxidation (i.e. Anammox) activity achieved by the anaerobic culture was 0.26 mmol NH_4^+ g X^{-1} h^{-1} (0.58 kg total-N m^{-3} day^{-1}). Qualitative FISH analysis (Fluorescence In Situ Hybridisation) confirmed the phylogenetic position of the enriched microorganism as belonging to the Order *Planctomycetales,* in which all currently identified Anammox strains fall. Preliminary FISH analysis suggests the Anammox strain belongs to the same phylogenetic branch as the *Candidatus* Brocadia anammoxidans strain discovered in the Netherlands. However, there are quite some differences in the target sites for the more specific probes of these organisms and it is therefore likely to represent a new species of Anammox bacteria. A small amount of aerobic ammonium oxidising biomass was inoculated into the Anammox reactor (10 % v/v) to initiate completely autotrophic nitrogen removal over nitrite (the CANON process) in chemostat culture. The culture was always under oxygen-limitation and no organic carbon was added. The CANON reactor was operated as an intermittently aerated system with 20 minutes aerobiosis and 30 minutes anaerobiosis, during which aerobic and anaerobic ammonium oxidation were performed in sequential fashion, respectively. Anammox was not inhibited by repeated exposure to oxygen, allowing sustained, completely autotrophic ammonium removal (0.08 kg N m^{-3} day^{-1}) for an extended period of time.

[1] This chapter has been submitted to Microbial Ecology

7.1. Introduction

In conventional nitrogen removal from wastewater where nitrification is generally followed by denitrification, complete nitrogen removal can be limited by the availability of reducing power in the form of organic substrates. Complete nitrogen removal can be achieved from wastewaters with a carbon to nitrogen (TCOD:TKN) ratio as low as 7:1, provided effective process control is applied (Randall et al., 1992). However, several types of wastewater contain a high ammonium concentration and low COD content, resulting in a very low TCOD:TKN ratio. Examples of such wastewaters are industrial wastewater, sludge digester effluent and groundwater (Jetten et al., 1999). As an alternative to the costly addition of carbon substrates to anoxic reactors (Puznava et al. 1998), the CANON process (Completely Autotrophic Nitrogen-removal Over Nitrite) offers an opportunity to remove ammonium in a single, completely autotrophic, oxygen-limited process (Sliekers et al., 2002a, 2002b, Third et al., 2001). The autotrophic CANON process relies on the conversion of approximately half of the ammonium in the wastewater to nitrite under oxygen-limitation, which provides the substrates for anaerobic ammonium oxidation (Anammox), in which ammonium is oxidised using nitrite as the electron acceptor (Strous et al., 1999). The combination of aerobic and anaerobic ammonium oxidising activity results in almost complete nitrogen removal (87 %), using 63 % less oxygen and 100 % less reducing agent than in conventional processes (Kuai and Verstraete, 1998, Van Dongen et al., 2001) (Eq 1).

$$1\ NH_4^+ + 0.85\ O_2 \rightarrow 0.435\ N_2 + 0.13\ NO_3^- + 1.3\ H_2O + 1.4\ H^+ \qquad \text{(Eq 1)}$$

A current limitation to the widespread application of autotrophic N-removal processes is the difficulty associated with growing large quantities of Anammox biomass, due to the very low growth rate (0.003 h^{-1}) and biomass yield (0.07 Cmol/mol substrate) of Anammox bacteria (Strous, 2000). While molecular probing techniques have allowed the detection of Anammox biomass in wastewater treatment plants in the Netherlands, Germany, Switzerland, UK, Australia and Japan (Jetten, 2001), there are currently few reports of their successful enrichment from these sources (Van de Graaf et al., 1996, Strous et al., 1998, Toh et al., 2002a, Egli et al., 2001). Current cultivation techniques are not designed well to deal with slow-growing microorganisms such as Anammox. In addition to the fluidised bed reactor, the sequencing batch reactor (SBR) has been

proven to be a successful experimental setup for Anammox enrichment due to its very efficient biomass retention (> 90 %) (Strous et al., 1998). In addition, a homogeneous distribution of substrates, products and biomass aggregates over the reactor can be achieved. Reliable SBR operation for more than 2 years can be achieved under substrate-limiting conditions (Strous et al., 1998). The persisting stable and strongly selective conditions of the SBR can lead to a high degree of enrichment of Anammox activity (Jetten et al., 1999). In this study, it was aimed to apply the SBR for the enrichment of an Anammox culture from local activated sludge (Perth, Western Australia), and to assess the feasibility of establishing a completely autotrophic nitrogen-removal (CANON) reactor system in our laboratory for further optimisation.

7.2. Materials and Methods

7.2.1. Reactor System for Anammox Enrichment

A glass 1 L sequencing batch reactor (SBR) was used for enrichment of Anammox from activated sludge (Fig 7.1). The reactor was fitted with a water jacket and fermentor lid containing a feed inflow tube, a dissolved oxygen (DO) probe (Mettler Toledo, Australia), pH probe, acid and base inflow tubes for pH control, a gas-line with attached bubble diffuser and an effluent withdrawal line. Temperature was maintained constant at 35 – 36 ^{o}C and the pH was controlled to 7.8. Anaerobiosis was maintained by continuously bubbling an N_2/CO_2 (95 %/5 %) gas mixture through the liquid (4 mL min^{-1}). The stirring speed was maintained as low as possible to keep the biomass suspended (50 rpm) and a stirring paddle designed for minimum shear was used. The reactor and feed vessels were covered to protect them from light and algal growth. The SBR was operated as a continuously filling SBR of 12 hour cycles. One cycle consisted of an 11.5-hour filling period at the standard flow rate of 1.45 mL min^{-1}, after which the stirrer and feed pumps were stopped and the biomass was allowed to settle for 15 minutes. In the following 15 minutes, 500 mL of liquid was decanted, withdrawing finely dispersed and suspended material. The resulting hydraulic retention time (HRT) was 1 day, except where lower loading was required due to nitrite accumulation. The N-loading rate was increased by varying the concentration of NH_4^+ and NO_2^- in the feed vessel (as specified in the Results section). The pumps, stirrer and gas-flow solenoid valves were controlled by manual timers.

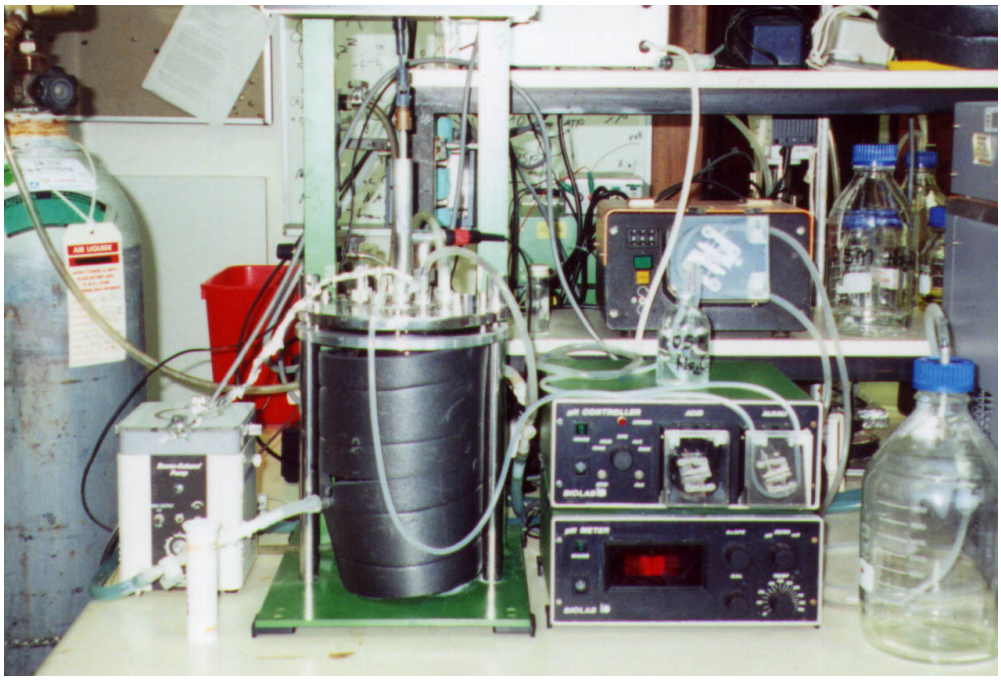

Figure 7.1: Photograph of the experimental setup of the Anammox enrichment SBR

7.2.2. Reactor System for CANON

For CANON operation, the same reactor set-up (1 L) was changed from an SBR to an intermittently-fed chemostat by removing the settle phase and maintaining constant volume through synchronised operation of the inflow and outflow pumps. Both pumps were turned on for 13 seconds (100 mL min^{-1}) in every 50 minutes, resulting in a dilution rate of 0.025 h^{-1}. It was ensured that there was always an excess of ammonium present (> 2 mM) such that the culture never became ammonium-limited, causing nitrite accumulation. Full biomass retention was achieved by fitting the outflow tube with a biomass settling device as described in Chapter 6. The CANON reactor was completely automated with all pumps, stirrer speeds, gas-flow solenoid valves and phase lengths controlled by National Instruments instrumentation control software LabView™ (USA). Dissolved oxygen and pH were monitored continuously on-line and the data were logged into a spreadsheet. During CANON operation, the gas supply was automatically alternated between air and the N_2/CO_2 mixture. The stirrer speed was controlled at a higher speed during aerobic phases (160 rpm) and at a lower speed for anaerobic phases (20 rpm).

7.2.3. Origin of Biomass

Activated sludge from a conventional plug-flow wastewater treatment plant (Subiaco, Western Australia) was used as inoculum for the Anammox enrichment. The same activated sludge was used as a source of aerobic nitrifiers, which was added to the enriched Anammox culture to start the CANON process (10 % v/v inoculum).

7.2.4. Synthetic Wastewater

Ammonium and nitrite were added to a mineral medium in the required amounts in the form of $NaNO_2$ and $(NH_4)_2SO_4$, as specified in the Results section. The composition of the mineral medium is described in Chapter 6.

7.2.5. Chemical Analyses

Nitrate, nitrite and ammonium were measured colorimetrically as described in Chapter 2.

7.2.6. Flourescence In Situ Hybridisation (FISH)

Cell fixation and FISH analysis were performed as described in Chapter 6. The 16 S rRNA gene probes used were: Pla46 for cells of the Order *Planctomycetales* (Neef et al., 1998); KST223 specific for Anammox cells *Candidatus* 'Kuenenia stuttgartiensis' (Schmid et al., 2000); Amx820 specific for *Candidatus* 'Brocadia anammoxidans' Anammox cells (Strous et al. 1999, Schmid et al. 2000); Amx223, 432 and 1015 which target other known specific strains within Brocadia anammoxidans. 2002) and Neu 653 for *Nitrosomonas*-like aerobic ammonium-oxidising bacteria (Juretschko et al. 1998). All hybridisations of Anammox probes only were performed with 20 % formamide and hybridisations containing probes for *Nitrosomonas* and anammox cells used 35 % formamide.

7.3. Results

Enrichment of Anammox Bacteria from Activated Sludge

The SBR was inoculated with concentrated activated sludge biomass (500 mL) from a conventional plug-flow wastewater treatment plant and the reactor was filled to 1 L with mineral medium (initial biomass concentration 5.4 g L^{-1}). To avoid possible toxicity of nitrite accumulation at any stage, the feed initially contained ammonium as the electron donor (1 mM) and nitrate (10 mM) as the electron acceptor (Van Dongen et al., 2001). The idea of supplying nitrate was that, since the inoculum contained an active denitrifying population, nitrite would be produced in the culture in low amounts by nitrate reduction, using remaining organics in the inoculum. The low amount of nitrite production should be sufficient for any Anammox bacteria present in the sludge. Small spikes (0.1 mM) of hydrazine (N_2H_4) and hydroxylamine (NH_2OH) were added to the reactor daily in attempt to kick-start the Anammox reaction. Due to the cyclic nature of the Anammox mechanism, cells need to invest reducing power to start their catabolism by producing NH_2OH from nitrite. This initial energy barrier can be overcome by the direct addition of NH_2OH or N_2H_4 (Strous et al., 1999). The reactor was monitored daily to test for ammonium consumption. After 47 days of feeding with NH_4^+ and NO_3^- only, the biomass showed the first signs of ammonium consumption. At this point, the feed composition was changed to contain 2 mM NH_4^+ and NO_2^- and spiking with hydroxylamine and hydrazine was stopped. The feed flow rate was initially dropped to half its value (0.7 mL min^{-1}) to prevent possible nitrite accumulation. As long as nitrite was undetectable in the liquid, the flow rate was increased gradually each day. When the feed flow rate reached its maximum value of 1 L day^{-1} (1.45 mL min^{-1}), the feed concentrations were increased. This resulted in a step-wise increase in the nitrogen load over time (Fig 7.2). The total nitrogen consumption rate increased from 2.1 to 42 mM day^{-1} (0.03 to 0.6 kg N m^{-3} day^{-1}) within 45 days (92 days after start-up). Significant wall growth occurred during the enrichment period, which was not removed at any stage in case activity was affected. This prevented accurate measurement of the biomass concentration. The maximum specific NH_4^+ consumption rate achieved (0.26 mmol NH_4^+ g X^{-1} h^{-1}) is thus expressed in terms of the suspended biomass only at the end of the enrichment period (2.9 g L^{-1}).

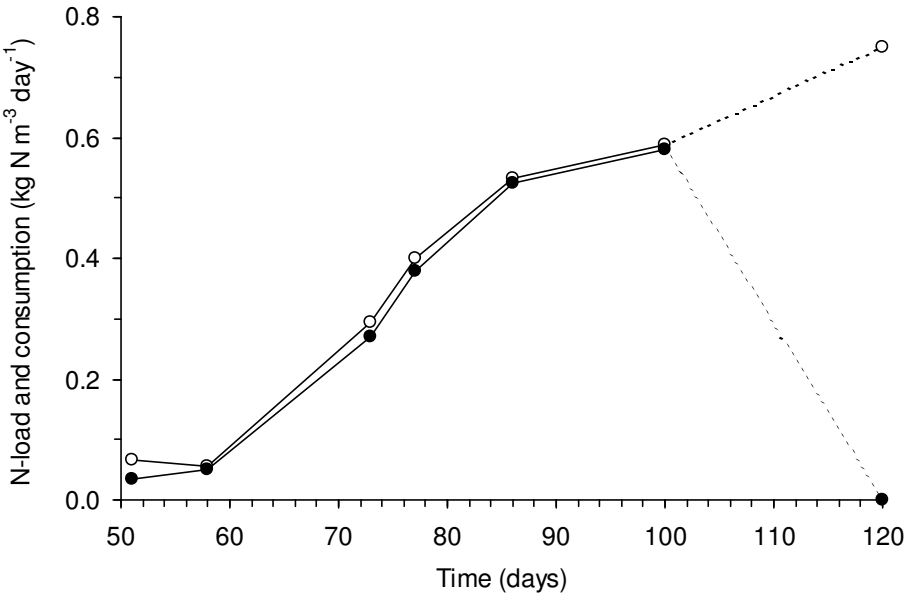

Figure 7.2: Increase in nitrogen load (❍) and nitrogen consumption (●) during enrichment of Anammox bacteria from activated sludge.

For the industrial use of slow-growing bacteria it is not only their specific activity but also their specific growth rate that is important. As biomass measurements were compromised by attached growth, the doubling time was estimated from the increase in their activity. A doubling of the nitrogen consumption rate was achieved over approximately 10 – 12 days (Fig 7.2). However, as the nitrogen loading rate was similar to the nitrogen consumption rate it is possible that the culture was substrate-limited and not growing at maximum rate, resulting in an under-estimation of their growth rate. From the data it can be inferred that the Anammox doubling time was not longer than 12 days (> 0.004 h^{-1}). After 100 days, the nitrogen load was increased suddenly to 0.75 kg N m^{-3} day^{-1} (Fig 7.2). This caused a temporary accumulation of nitrite (> 5 mM) for more than 12 hours and loss of all Anammox activity. Anammox activity did not resume again until all nitrite had been removed from the reactor and a small amount of hydrazine (0.1 mM) was added. Resumption of activity was detected by formation of gaseous bubbles in the liquid and nitrite consumption. However, the nitrogen removal capacity after the nitrite accumulation was reduced by about half to 21 mM day^{-1} (0.3 kg N m^{-3} day^{-1}), indicating a significant portion of the biomass had died or become inactive. It took several weeks to regain the same maximum N-consumption as before the nitrite accumulation (42 mM day^{-1}).

Characterisation of Anammox Biomass

The presence of Anammox bacteria in the enrichment culture was verified by qualitative FISH analysis. Positive hybridisation of the biomass with the probe Pla46 confirmed the presence of bacteria of the Order *Planctomycetales* (Fig 7.3A) and the culture also reacted positively with an oligonucleotide probe found to specifically detect *Candidatus* B. anammoxidans (Amx820) (Figs 7.3B, C). A negative result was obtained with the probe KST223, developed for Anammox strain Kuenenia stuttgartiensis (Schmid et al. 2000). Several other oligonucleotide probes specific for *Candidatus* B. anammoxidans (Amx223, Amx 432, Amx1015) did not react with the enriched organism. The probe pattern therefore indicated that the Anammox organisms branch with Brocadia anammoxidans, but are at least different in the target sites for the more specific probes for these organisms and may therefore represent a novel strain. FISH analysis clearly showed the tight cluster formation characteristic of Anammox cells (Fig 7.3).

Unlike other Anammox cultures enriched from activated sludge that have been reported to change from a brown to pinkish-red colour after a long enrichment period, the macroscopic appearance of the culture in this study did not change significantly over the 1.5-year enrichment period. The biomass remained a light brown colour, indicating no significant increase in cytochrome content as reported with other cultures (Van de Graaf et al., 1996, Strous, 2000). The biomass also did not form compact granules as previously reported with other Anammox enrichments, but consisted visibly of small, fine agglomerates (< 50 µm diameter, measured by haemocytometer). Despite this, the settling efficiency of the biomass was very effective (sludge volume index 110 mL g^{-1}X).

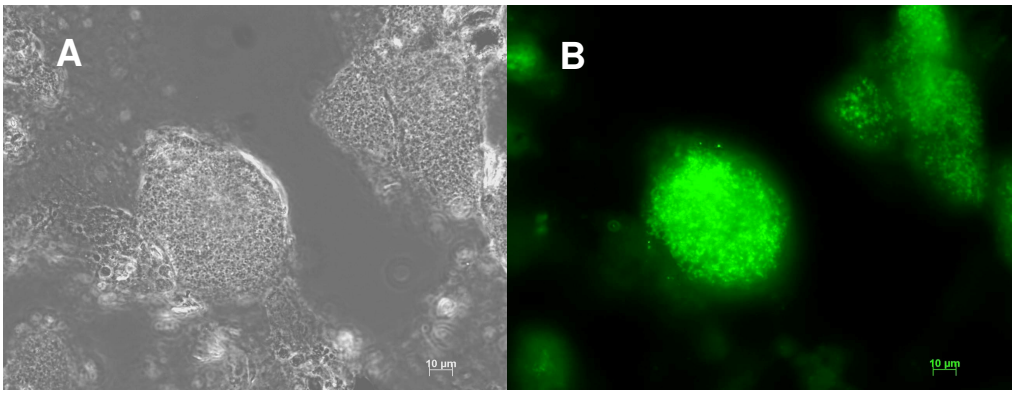

Figure 7.3A: Phase contrast (A) and epiflourescence image (B) of enriched Anammox biomass (day 120) hybridised with FLUOS labelled probe PLA46 for detection of general *Planctomycetales* cells.

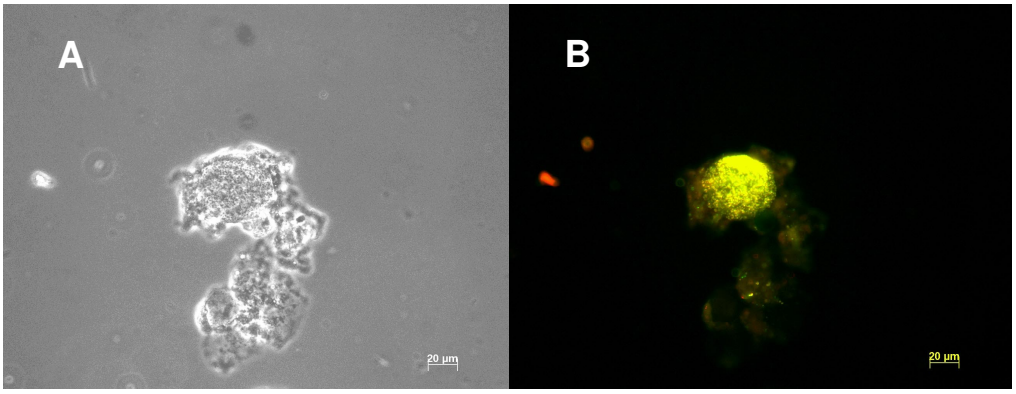

Figure 7.3B: Phase contrast (A) and epiflourescence image (B) of enriched Anammox biomass (day 120) hybridised with FLUOS labelled probe PLA46 (green) and Cy-3 labelled AMX 820 (red) for detection of Brocadia anammoxidans cells. The combined hybridisation with both probes resulted in a yellow image.

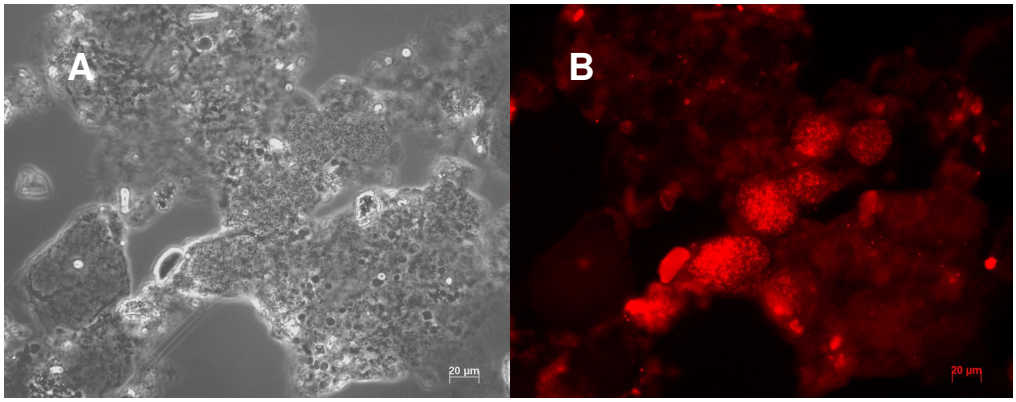

Figure 7.3C: Phase contrast (A) and epiflourescence image (B) of enriched Anammox biomass (day 120) hybridised with Cy3 labelled AMX 820 (red).

Initiation of the CANON Process

As it is not economically feasible to supply nitrite during treatment of low-COD wastewaters, the CANON system was set up to evaluate N-removal under more realistic conditions of feeding with ammonium only. By supplying limited oxygen, incomplete ammonium oxidation to nitrite can provide the substrates required for simultaneous occurrence of the Anammox reaction. The process control requirements of the CANON process are not yet well established. Previous studies on the CANON system have used granular Anammox biomass (granule diameter > 200 µm) enriched in Delft, the Netherlands (Sliekers et al., 2002a, 2002b, Chapter 6). In those studies, effective cooperation between the aerobic and ammonium oxidisers was enabled at constant, low airflow rates (8 mL min^{-1}), due to oxygen diffusion limitation imposed by the biomass granules. The smaller flocs formed in this study required a more careful DO supply to prevent oxygen diffusion inside the floc, as preliminary results showed that the application of constant low airflow rates (< 8 mL min^{-1}) resulted in complete (reversible) inactivation of the Anammox biomass (data not shown). The objective was to set up a computer-controlled CANON process that could achieve sustained autotrophic ammonium removal using non-granular biomass over a long period.

CANON Efficiency during Intermittent Aeration

The SBR was converted into a chemostat and the reactor was inoculated with a small amount of activated sludge (10 % v/v) to provide rapid start-up of aerobic ammonium oxidising activity. The feed was changed to contain ammonium only (10 mM) and was fed at a rate of 0.25 mmol NH_4^+ L^{-1} h^{-1}. It was initially unclear how much aeration would be required to obtain sufficient rates of both aerobic and anaerobic ammonium oxidising activity. As a continuous, low airflow supply to the CANON bioreactor had been found to completely inactivate the Anammox biomass, the effect of intermittent air supply (as opposed to continuous air supply) on anaerobic ammonium oxidising activity was investigated. Air was supplied (8 mL min^{-1}) for 10 minutes in every 90 minutes (Fig 7.4). The supply of air enabled aerobic ammonium oxidation (0.1 mmol NH_4^+ g X^{-1} h^{-1}) for 20 – 30 minutes. Ammonium measurements showed that anaerobic ammonium oxidation was minimal during aerobic periods and only began 40 minutes after the aeration supply was turned off, when the oxygen had fallen below 0.1 mg L^{-1}. Anammox occurred at a reduced rate (0.03 mmol NH_4^+ g X^{-1} h^{-1}), compared to

the anaerobic rate in the original enrichment culture (0.26 mmol NH_4^+ g X^{-1} h^{-1}). The nitrite concentration in the reactor increased over time (Fig 7.4), which showed that the autotrophic nitrogen removal process was limited by the Anammox reaction. The overall specific ammonium removal rate during intermittent aeration was 0.04 mmol g X^{-1} h^{-1} (equivalent to 0.04 kg N_{total} m^{-3} day^{-1} at the biomass concentration of 2.9 g X L^{-1}). At 260 minutes, the culture was flushed with N_2/CO_2, which caused an immediate increase in the rate of anaerobic ammonium oxidation to 0.08 mmol NH_4^+ g X^{-1} h^{-1}. The results showed clearly that Anammox activity was significantly slowed down during intermittent aeration and experienced long lag times after the air supply air was turned off. Flushing with N_2/CO_2 caused an immediate increase in Anammox activity. It was therefore decided to run the reactor with alternating periods of aeration and N_2/CO_2 flushing.

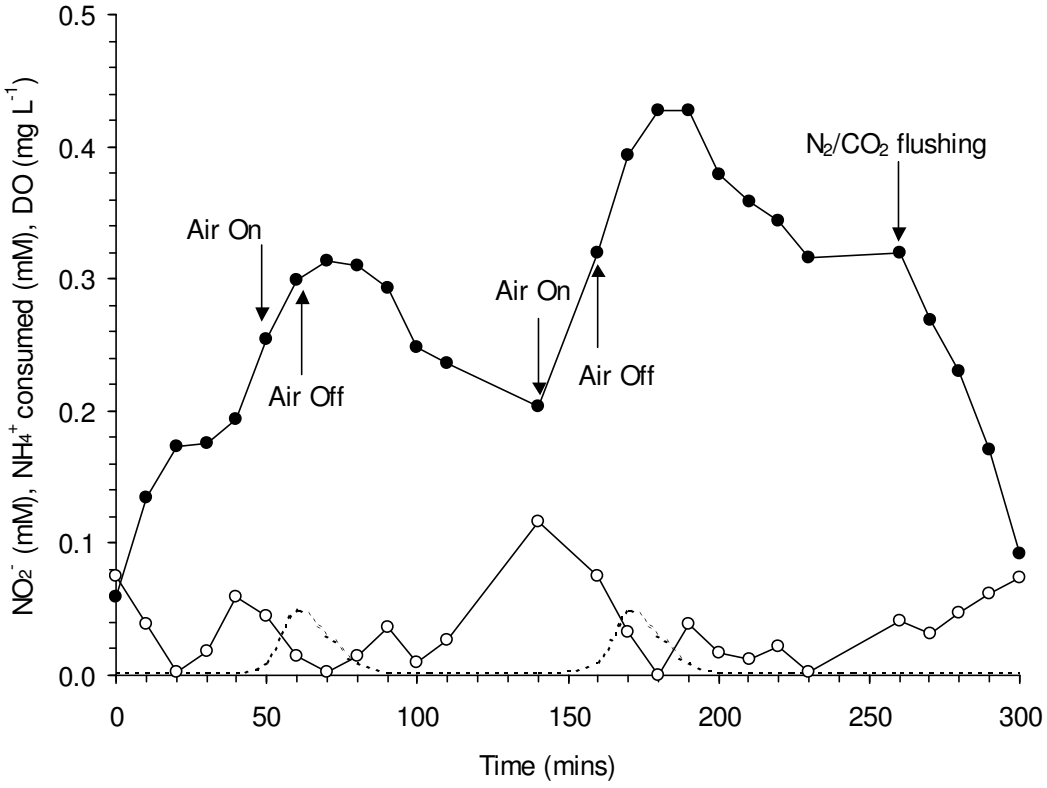

Figure 7.4: Operation of the CANON reactor with intermittent aeration, showing NO_2^- (●), NH_4^+ consumed (❍) and DO (dotted line). Air was supplied for 10 minutes in every 90 minutes, as indicated by the arrows. The culture was flushed with N_2/CO_2 to create strictly anaerobic conditions from 260 minutes onwards.

Control of CANON by Intermittent Aeration and N_2/CO_2 flushing

The computer-controlled reactor was automatically operated with alternating aerobic periods of 20 minutes (8 mL min^{-1} air) and anaerobic periods of 30 minutes (8 mL min^{-1} N_2/CO_2). In this manner it was attempted to achieve increased rates of aerobic and anaerobic ammonium oxidation in a sequential fashion, as opposed to simultaneous fashion as in previously reported CANON systems (Sliekers et al., 2002a, Chapter 6). During aerobic periods, nitrite was produced (0.08 mmol g X^{-1} h^{-1}) and it was again consumed during anaerobic periods, at a similar rate (Fig 7.5). This resulted in small oscillations in the nitrite level in the reactor over time, and resulted in completely autotrophic ammonium removal at an overall rate of around 0.08 mmol NH_4^+ $g^{-1}X$ h^{-1}. This was improved from the rate achieved with supply of air only (0.04 mmol NH_4^+ $g^{-1}X$ h^{-1}), but was still around 3 times lower than the specific ammonium removal rate achieved in the anaerobic Anammox enrichment culture (0.26 mmol NH_4^+ $g^{-1}X$ h^{-1}). This ammonium removal rate was sustained for an extended period (> 4 weeks) and showed that both the aerobic and anaerobic ammonium oxidising activity could occur during intermittent aerobiosis and anaerobiosis. The activity of neither group of bacteria was negatively affected by the alternating conditions over time, as confirmed by nitrogen measurements. Qualitative FISH analysis confirmed the presence of both aerobic ammonium oxidising *Nitrosomonas*-type cells and Anammox cells in the CANON reactor (Fig 7.6).

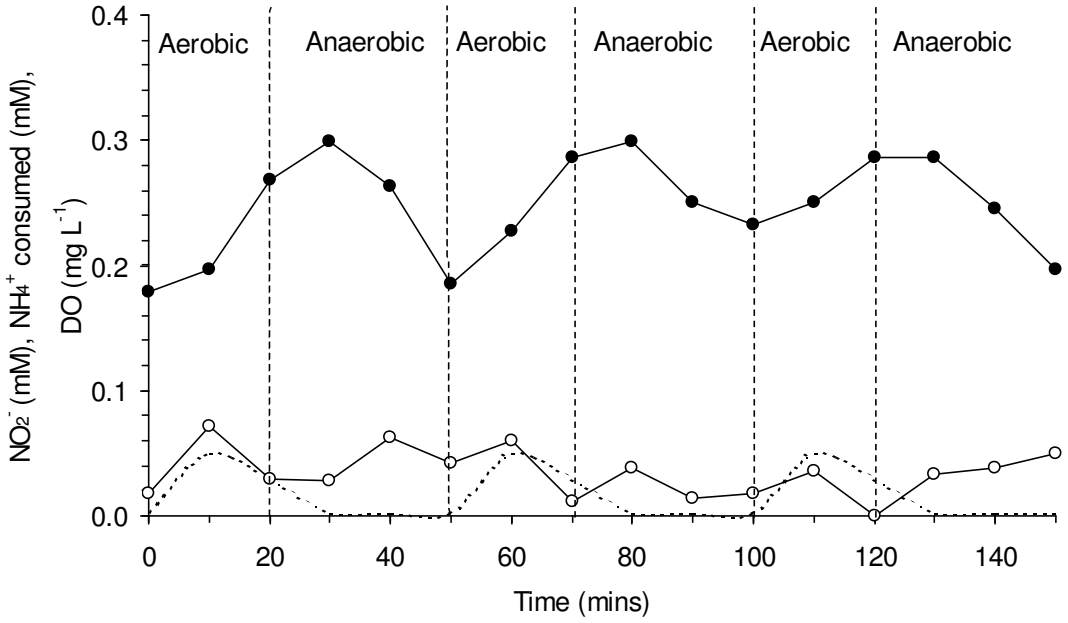

Figure 7.5: Changes in NO_2^- (●), NH_4^+ consumed (❍) and DO (dotted line) during operation of the CANON reactor with 20 minute aerobic and 30 minute anaerobic intervals.

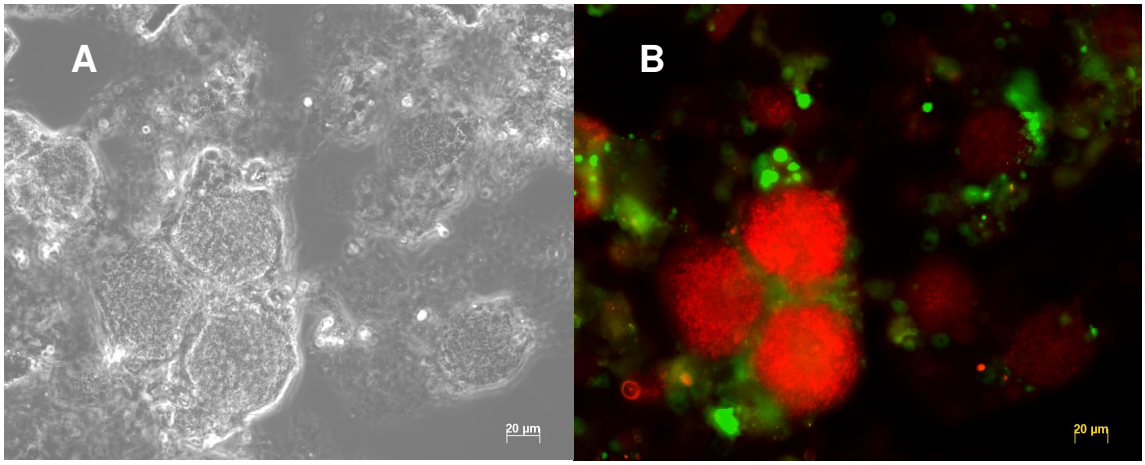

Figure 7.6: Phase contrast (A) and epiflourescence image (B) of CANON biomass (day 30 after CANON initiation) hybridised with Cy3 labelled AMX 820 (red) and FLUOS-labelled Neu 653 (green).

7.4. Discussion

Anammox Enrichment from Activated Sludge

An active bacterial culture capable of removing ammonium in the absence of oxygen, using nitrite as the electron acceptor, was enriched from local activated sludge during an enrichment period of 3.5 months (14 weeks). In comparison to other studies, the time required for enrichment was relatively short. Strous et al. (1998) enriched a high-purity Anammox culture with a nitrogen consumption rate of 0.9 kg N m^{-3} day^{-1} within 21 weeks (compared to 0.58 kg N m^{-3} day^{-1} in this study), although from an already enriched Anammox culture. Egli et al. (2001) required 6 months to enrich an Anammox culture of 88 % purity from a rotating biological contactor, which had also shown significant Anammox activity before the start of the enrichment period. Toh et al. (2002a) reported biofilm growth after one year in a fixed bed reactor that demonstrated Anammox activity, although at quite low N-removal rates (0.06 kg N m^{-3} day^{-1}). Toh et al. (2002b) detected Anammox activity after 15 months of adaptation and enrichment in a fixed bed reactor when treating synthetic coke oven wastewater (0.14 kg N m^{-3} day^{-1}). In comparison to these studies, the final activity achieved in this study was high in the short enrichment time frame. The difference may have been due to the use of the sequencing batch reactor for enrichment, as opposed to a rotating biological contactor or fixed bed reactor, although Strous et al. (1998) also used the SBR for enrichment. The enrichment time may have been shortened by the addition of hydrazine and hydroxylamine during the initial enrichment period, however the exact effect of these intermediates on Anammox growth still needs to be quantified.

The final nitrogen removal capacity of the microbial population enriched here was comparable to other Anammox enrichment cultures (Table 7.1). FISH analysis (Fig 7.3) revealed that the responsible microorganism branches phylogenetically with the strain *Candidatus* Brocadia anammoxidans, discovered in the Netherlands (Strous et al., 1999). The estimated slow doubling time of our Anammox culture (12 days) is similar to previously reported growth rates for Anammox of 14 days (Strous et al., 1998). At a microscopic level, the enriched biomass formed large, homogeneous clusters characteristic of Anammox cells (Figs 7.3, 7.6). Growth in clusters has been observed by many cultures of nitrifiers and Anammox and is thought to help with overcoming the

loss of catabolic and anabolic intermediates due to the passive diffusion over the biological membranes (Mobarry et al., 1996, Schramm et al. 1997, 1998). The intermediate electron donors in Anammox metabolism hydroxylamine and hydrazine have been detected experimentally outside Anammox cells (Strous, 2000). Small losses of these compounds would have a severe impact on the biomass yield due to the subsequent need to invest energy in generation of endogenous electron donors. Therefore, growth in clusters could decrease the impact of those losses, as each cell would benefit from its neighbours losses (Strous, 2000). As reported in other studies (Strous et al., 1999), the enriched Anammox culture in this study was severely affected by an accumulation of nitrite for more than 12 hours (Fig 7.2) and required several weeks to recover from the disturbance. This highlights one important requirement of process control for both CANON and Anammox: the prevention of nitrite accumulation. This might be accomplished by oxidation-reduction potential (ORP) and/or DO-control and is currently being investigated in our laboratory.

Despite the similarities to other known Anammox cultures, the macroscopic properties of the culture enriched in this study were strikingly different. Anammox biomass generally changes from brown to a deep red colour after 2 – 3 months of enrichment, when similar specific activities to those reported in this study are reached (Egli et al., 2001, Toh et al., 2002a, Strous et al., 1998, Van de Graaf et al., 1996). After longer enrichment periods in an SBR the formation of dense, red Anammox granules occurs (Strous, 2000). Even after 18 months of continuous enrichment in our SBR, the Anammox culture showed no signs of colour change. The red colour is attributed to the presence of many hemes present in the enzyme hydroxylamine oxidoreductase, which is essential for the reduction of hydrazine – an essential step of the anammox reaction (Jetten et al., 1999). It is therefore surprising that such essential cytochromes might be lacking in our enrichment culture, which shows similar Anammox activity to other cultures. However, the absence of the cytochromes still needs to be confirmed by spectrophotometry.

Table 7.1: Overview of total nitrogen conversions and specific ammonium consumption rates by different processes in various reactor set-ups.

Reactor Type	**Total N-conversion** (kg N m^{-3} day^{-1})	**Specific NH_4^+-consumption**[1] (nmol. mg^{-1} protein $.min^{-1}$)	**Reference**
Anammox (Anaerobic cultures)			
SBR	0.60	19	This study
RBC[2]	1.4	27	Egli et al. 2001
Fluidised Bed	0.9	7	Mulder et al. 1995
SBR	0.32	24	Sliekers et al. 2002a
Gas-Lift	8.7	97	Sliekers et al. 2002b
SBR	1.0	45	Strous et al. 1998
SBR	0.45	38	Chapter 6
Fluidised Bed	4.8	108	Van de Graaf et al. 1996
OLAND[3] (Microaerophilic)			
SBR	0.05	1.6	Kuai and Verstraete, 1998
(SHARON)[4]**-Anammox** (Aerobic-Anaerobic)			
CSTR-SBR	1.2	18	Van Dongen et al. 2001
CANON (Microaerophilic)			
SBR	0.08	2.3	This study
SBR	0.16	10	Sliekers et al. 2002a
Gas-Lift	1.5	37	Sliekers et al. 2002b
SBR	0.12	4	Chapter 6
Nitrification-Denitrification (Microaerophilic)			
SBR	0.22	8	Chapter 5

[1] Where rates were originally expressed as g or mg biomass, they were converted into units of protein by the authors assuming that the protein content of biomass is 0.5 g protein/g biomass.
[2] Rotating biological contactor
[3] Oxygen-Limited Autotrophic Nitrification-Denitrification
[4] Single reactor High activity Ammonia-Removal Over Nitrite

CANON Operation

Results of this study and others have shown that the activity, but not the viability, of Anammox is negatively affected by very low concentrations of oxygen (< 0.1 mg L^{-1}) (Strous et al., 1997a, Van de Graaf et al., 1996). Oxygen inhibition of Anammox activity in the CANON reactor was completely reversible and the activity resumed as soon as all oxygen was removed from the liquid (Figs 7.4, 7.5). This is a similar to finding to Strous et al. (1997a), who exposed an Anammox culture to alternating periods of aerobiosis (2 hours) and anaerobiosis (2 hours). The reversibility of the oxygen inhibition allowed relatively constant ammonium removal during intermittent aeration at a rate of 0.08 kg N m^{-3} day^{-1} in this study. The lower rate of ammonium removal observed in this study in comparison to other CANON reactors (Table 7.1) may be due to the lack of granular biomass formation characteristic of other systems. This prevented the possibility of supplying a constant, low airflow rate as in previous studies (Sliekers et al., 2002a, 2002b, Chapter 6). When using granular biomass, oxygen consumption by the aerobic nitrifiers in the outer layer of the granule prevents oxygen diffusion inwards, protecting Anammox cells from exposure to oxygen. In this manner, both aerobic and anaerobic ammonium oxidation can occur simultaneously, close to maximum rate. The rate-limiting step of CANON in granular systems is the rate of oxygen transfer to the aerobic cells and the highest rate has been reported in a gas-lift reactor (1.5 kg N m^{-3} day^{-1}), where the oxygen transfer rate was maximised (Sliekers et al., 2002b).

In contrast to granular systems, the operation of CANON with intermittent aeration results in significant downtime for both the aerobic and anaerobic ammonium oxidisers and only one group of bacteria is active at any one time, resulting in oscillations of the key intermediate, nitrite, over time (Fig 7.5). Instead of relying on oxygen diffusion limitation by granules, the reactor needed to be intermittently flushed with nitrogen gas in order for Anammox to proceed at a sufficient rate. In order to achieve similar rates as in granular CANON systems, an increased biomass concentration would be required in intermittently aerated CANON systems. While the CANON reactor in this study achieved sustained, autotrophic nitrogen removal for up to 4 weeks, the process requires further optimisation.

In summary, this study has shown that an active Anammox culture could be enriched from a local source of activated sludge in less than four months. While confirmed as belonging to the same phylogenetic branch as Brocadia anammoxidans, the different macroscopic properties of the biomass suggest significant differences between the Australian and Dutch strains. Comparative sequence analysis of the 16S rDNA sequence should reveal the genetic similarity between the two and is currently under way. By inoculating the enrichment culture with a small amount of aerobic nitrifiers and supplying intermittent periods of aeration, a co-existence of aerobic and anaerobic ammonium oxidisers was established, capable of removing ammonium at a constant rate over the measured 4 weeks, without the addition of an organic carbon substrate. While still in the early stages of optimisation, the process shows promise as an alternative treatment for wastewaters high in ammonium but low in COD content.

Conclusions and Outlook

The purpose of this section is to summarise the most important contributions of the research presented in this thesis and to identify limitations of the application of the knowledge generated to large-scale nitrogen removal processes. The conclusions are summarised briefly below;

PHB metabolism

- The conversion of reducing equivalents from acetate into PHB is maximised under O_2-limitation. This means that minimal aeration may be recommended during the feast phase at the beginning of the SBR ($DO < k_{S,O2(Het)}$).
- The observed effect of oxygen on PHB production made biochemical sense, as it was reproducible by a structured metabolic model. PHB production is stimulated by conditions of high NADH + H^+ and acetyl-CoA, which occurs when the respiratory chain is limited by O_2-limitation. High oxygen levels remove the limitation of the respiratory chain (and thus ATP production), allowing biomass growth at the expense of PHB production.
- The maximum storage capacity of the activated sludge was 0.6 Cmol/Cmol, around 15 times higher than the level reached during normal feeding conditions (i.e. influent COD 75 mg COD. g^{-1} X). It is therefore unlikely that the biomass ever approaches saturation during normal plant conditions, or even under temporarily high loading conditions.

Simultaneous Nitrification and Denitrification

- SND was critically dependent on the DO concentration: Lower DO concentrations (< 1 mg L^{-1}) increased the percentage of SND but decreased the rate at which it was achieved. Multiplication of the specific rate of SND by the fraction of SND (mol $NO_{3\ denitrified}^-$/mol $NH_{4\ oxidised}^+$) is a potentially suitable performance indicator to assess the optimal DO concentration for both rate and quantity of SND.
- The amount of SND achieved increases directly with an increased concentration of PHB and a corresponding increase in the SOUR. Higher PHB levels increase the PHB degradation rate and hence drive SND faster. A high SOUR reduces

penetration of oxygen into the floc and creates larger anoxic zones for denitrification.

Recommended Oxygen Management Strategy

Management of the oxygen supply throughout the entire SBR cycle can significantly increase the reactor nitrogen removal capacity from 45 % to 100 % (Appendix 3). The following points are recommended for optimal N-removal in an SBR;

- Limit the oxygen supply during the feast phase (DO < $k_{S,O2(Het)}$) to increase the availability of reducing power for the subsequent famine phase (Appendix 3).
- Control the dissolved oxygen concentration during the aerobic famine phase to around twice the apparent half saturation constant for oxygen of the autotrophic population ($k_{S,O2(Aut)}$). This allows nitrification to proceed at close to maximum rate, while achieving significant SND. Higher DO concentrations decrease both the rate and quantity of SND achieved.
- Control the length of the aerobic period to the exact time required for complete ammonium oxidation. A simple process control strategy to minimise the length of the aerobic phase can preserve reducing power, causing accumulation of intracellular PHB over time. Increased PHB levels caused by control of the oxygen supply can directly increase the amount of SND achieved. The "history" of the sludge has a direct impact on its capacity for nitrogen removal, with starved cultures having a lower capacity for SND.

Conclusions of the first research theme showed that the effective build up of PHB and its degradation are instrumental for maximum nitrogen removal. By relatively small changes to the operation of an SBR, nitrogen removal can be increased by up to 55 %, while also improving aeration economics. To make use of this technology, some on-line activity monitoring is necessary. Providing oxygen at unmeasurable concentrations during the feast phase, limiting the oxygen supply to strike a good compromise between nitrification and denitrification and precisely controlling the length of the aeration phase can enable optimal SND. However, it is not only the immediate process control but also the medium-term "historical" effects that are responsible for effective SND. It may be useful for full-scale WWTPs to consider such historical effects. Carefully accumulated reducing power (PHB) for denitrification may be inadvertently wasted by unnecessary over-oxidation.

CANON

- Dissolved oxygen control is necessary also during the CANON process. Nitrite oxidisers can develop in the system when the dissolved oxygen concentration increases temporarily, which negatively affects N-removal.
- The CANON process relies on oxygen diffusion limitation and the creation of anoxic zones for Anammox, to achieve simultaneous aerobic and anaerobic NH_4^+ oxidation. Large flocs enable coexistence of the two microbial populations that have complementary and opposed environmental requirements. Non-flocculated systems can be run with intermittent aeration, but involve significant lag-times in both Anammox and nitrification activity. Immobilisation of CANON biomass on support material may improve its performance.
- CANON has the lowest COD and oxygen requirement of all nitrogen removal processes and can potentially achieve competitive rates of N-removal (Appendix 4). However, its specific niche remains the treatment of low-COD, high-NH_4^+ wastewaters.

Limitations to Current Research

This research has helped to identify limitations to the SND process and ways in which they can be overcome in an ideal SBR, by simple process control measures. However, there are still many limitations and questions to be answered before the concepts can be applied on large-scale. For example, the reactor setup used in this study was an "ideal" SBR, in which the wastewater was fed during a short time interval (3 minutes), resulting in a true "feast" phase. Such rapid filling is not possible in large-scale reactors due to the large liquid volumes required. Consequently, most large-scale SBRs are operated as continuous-fill sequencing batch reactors (Mark Newland ESI Ltd, *personal communication*).

The biochemical model developed (Chapter 2) showed that PHB production relies on the intracellular accumulation of acetyl-CoA. This is highest under conditions of rapid filling when the substrate to microorganism ratio (F:M ratio) is highest. During continuous-fill processes, the F:M ratio at any particular time would be much lower, resulting in lower intracellular acetyl-CoA concentrations and consequently less PHB production. The effect of the feeding pattern on the amount of soluble substrate that can be stored is a question that needs to be clarified in future studies. As such, the amount

of COD that could be preserved as PHB in this study (77 %) is a theoretical maximum that is achievable only in a rapid-filling acetate-fed SBR. If a high F:M ratio can not be applied in the real situation by use of a relatively fast filling period, the application of aerobic conditions during the filling phase could result in bulking sludge, due to the growth of filamentous microorganisms at both low oxygen and low organic substrate conditions. Aerobic conditions should only be applied during filling if a high F:M ratio can be maintained during this period.

The use of a rapid fill phase in this study meant that all ammonium was oxidised at the same time, allowing easy on-line detection of its depletion. In a continuous-fill SBR, the point of NH_4^+-depletion would be obscured by incoming ammonium in influent, making it more difficult to use on-line respiration measurement as a means to terminate the aeration phase. Therefore, a more advanced process monitoring and control system would be required. Finally, all soluble substrate in the feed in this study (acetate) was directly "storable". To achieve similar rates and percentages of PHB-driven SND in large-scale processes, a similar amount of soluble COD is needed, that can be directly stored as a storage polymer (i.e. > 75 mg COD. g^{-1} X).

Future Work

The limitations of this research have provided direction for future research needed in this area. One of these requirements would be to define how "SND-able" a wastewater is. The composition of wastewater will also play a major role for effective SND. It is not only the COD:N ratio but also the extent to which soluble COD can be converted to PHB that is important. A useful technique for evaluating wastewater according to its potential for SND and its oxygen requirement for COD to PHB conversion should be developed.

It would be desirable to establish the difference between SND using stored substrates as the electron donor versus SND using particulate matter as the electron donor. Does the presence of storage products allow increased SND? During rapid fill phases with a short, high F:M period, cells in the centre of the floc have an opportunity to store soluble substrate as PHB. These microbes can then denitrify in the centre of the floc when nitrate becomes available. When particulates are used as the substrate, the amount

of particulate COD that reaches the centre of the floc and is available for SND may affect the amount of SND that can be achieved with particulates.

The potential for SND via nitrite has not been explored in this research, but is a promising option, as it results in a 60 % reduction in COD demand and 25 % reduction in the oxygen demand (Appendix 4). It would be desirable to establish to what extent SND via nitrite is already occurring in our system and how to encourage further SND via nitrite, to achieved increased savings in COD and oxygen. One possible research direction would be to investigate and model long-term "history" effects, such as changes in bacterial populations during SND via nitrite. It is likely that nitrite oxidisers would eventually be diluted from the biomass, enabling effective SND even when conditions are not controlled appropriately.

Future work is needed in the development of process control strategies to apply oxygen management to large-scale SBRs in which a continuous filling regime is used. A method for detecting the end-point of NH_4^+ oxidation and an appropriate control method are required, such that the aeration time can be minimised according to ammonium depletion. Finally, the use of low dissolved oxygen concentrations during denitrification has been reported to increase the levels of nitrous oxides produced (NO, N_2O). As these gases are extremely powerful greenhouse gases, future studies should aim at determining if the emission of these gases is significant in low-DO SND processes.

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Appendix 1: Calculation of the "real k_La" during oscillating DO conditions

The dissolved oxygen concentration in the reactor at any stage is the net result of oxygen being transferred into solution (OTR) minus oxygen being consumed by the biomass (OUR), i.e.

$$\frac{dc_L}{dt} = OTR - OUR \qquad \text{(Eq A1)}$$

Thus, the slope of the dissolved oxygen concentration (dc_L/dt) at any point can be used to determine the oxygen transfer rate and hence the k_La (Eq A2), if the oxygen uptake rate is known. The actual OUR of the biomass was measured several times and showed that it did not change significantly for periods up to 10 mins. This allowed the use of one OUR value for calculation of the OTR over a 2 – 3 min time interval.

$$OTR(mg.L^{-1}.h^{-1}) = k_La.(c_S - c_L) \qquad \text{(Eq A2)}$$

Measurement of the "Real OUR"

The undulating nature of the dissolved oxygen about the setpoint during the aeration period was interrupted several times by allowing the culture to aerate until steady state was reached. The air was then turned off and the decrease in DO was measured several times, while flushing the headspace with nitrogen gas to avoid oxygen transfer due to stirring (Figs A3 – A6).

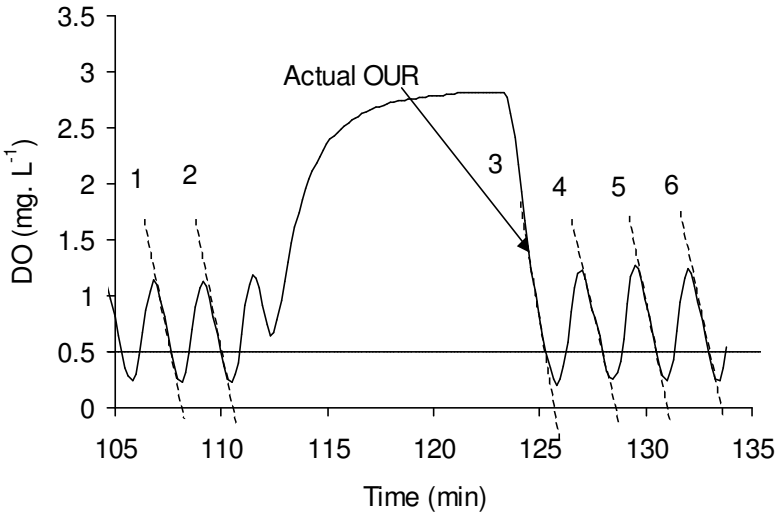


Figure A1: Calculation of the negative derivative of the DO as it approaches the setpoint both before and after the test to calculate the actual OUR.

Table A1: Calculation of the negative slopes during oscillating DO conditions to calculate the real OUR, in comparison to the downward slopes before and after interruption of the air supply, when the OUR is underestimated, due to residual oxygen transfer.

Time	Derv. No.	OUR (mg/L/min)	OUR (mg/L/h)	% difference
107	1	0.815	48.9	25
110	2	0.889	53.3	18
125	**3**	**1.080**	**64.8**	**0**
127	4	0.773	46.4	28
130	5	0.912	54.7	16
134	6	0.877	52.6	19

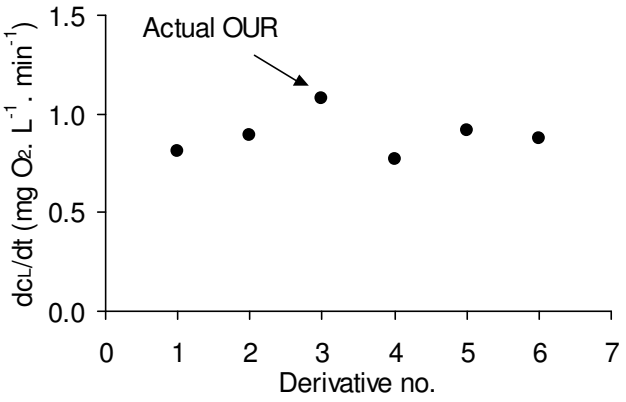


Figure A2: The actual OUR compared to the OUR as measured from the derivatives of the downward-sloping DO during the DO undulations.

The real OUR was consistently higher than the OUR values calculated from the downward slope of the DO undulations about the setpoint. This indicated that there was residual oxygen transfer after the air was switched off. This in turn implied that the k_La assumption, used in on-line calculation of the SOUR, was not accurate as oxygen transfer still continued even after the air supply was switched off. To determine the real nature of the k_La, the DO data (Fig A3) and the OUR value were used to calculate the OTR (Eq A1). The assumed k_La was assumed to be zero as soon as the air was off, and at its full value when the air was on. The corresponding assumed OTR was also calculated according to Eq A2.

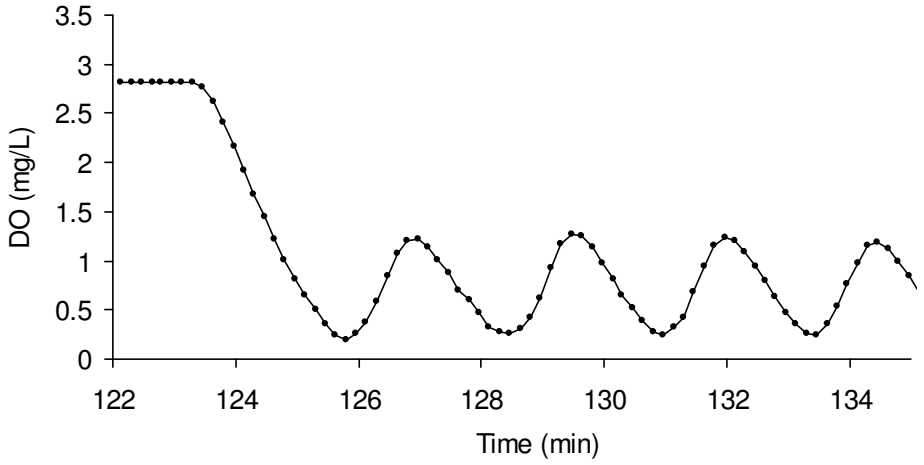


Figure A3: Dissolved oxygen data measured on-line used for calculation of the OTR during oscillating DO conditions.

Table 1: Data for calculation of the actual oxygen transfer rate and k_La compared to the assumed OTR and k_La.

Time (min)	DO (mg/L)	dDO/dt (mg/L/min)	dDO/dt (mg/L/h)	Actual OTR (mg/L/h)	Actual kLa (h-1)	kLa assumed (h-1)	Assumed OTR (mg/L/h)
123.22	2.81	0.000	0.00	65.00	14.81	0	0
123.39	2.81	-0.220	-13.19	51.81	11.81	0	0
123.55	2.78	-0.900	-53.98	11.02	2.49	0	0
123.71	2.62	-1.225	-73.49	-8.49	0.60	0	0
123.89	2.42	0.000	0.00	0.00	0	0	0
124.05	2.17	0.000	0.00	0.00	0	0	0
124.21	1.92	0.000	0.00	0.00	0	0	0
124.38	1.67	0.000	0.00	0.00	0	0	0
124.55	1.47	0.000	0.00	0.00	0	0	0
124.71	1.22	-1.223	-73.40	-8.40	-1.40	0	0
124.88	1.02	-1.177	-70.62	-5.62	-0.91	0	0
125.04	0.82	-1.007	-60.40	4.60	1.00	0	0
125.22	0.65	-0.893	-53.55	11.45	2.00	0	0
125.22	0.57	-0.861	-51.64	13.36	3.00	20	133
125.39	0.50	-0.829	-49.73	15.27	2.28	20	134
125.56	0.36	-0.701	-42.08	22.92	3.35	20	137
125.70	0.25	-0.283	-16.97	48.03	6.91	20	139
125.88	0.20	0.332	19.91	84.91	12.14	20	140
126.04	0.26	0.712	42.70	107.70	15.52	20	139
126.21	0.37	1.179	70.75	135.75	19.88	20	137
126.21	0.44	1.381	82.85	147.85	21.86	0	0
126.38	0.50	1.583	94.96	159.96	23.87	0	0
126.54	0.85	1.323	79.37	144.37	22.74	0	0
126.71	1.07	0.771	46.27	111.27	18.16	0	0
126.89	1.20	0.122	7.34	72.34	12.06	0	0
127.06	1.22	-0.524	-31.42	33.58	5.62	0	0

127.21	1.14	-0.841	-50.45	14.55	2.40	0	0
127.37	1.00	-0.775	-46.49	18.51	2.99	0	0
127.54	0.87	-0.829	-49.76	15.24	2.41	0	0
127.71	0.80	-0.884	-53.03	11.97	1.87	0	0
127.89	0.64	-0.900	-54.00	11.00	1.68	0	0
127.89	0.57	-0.871	-52.25	12.75	1.92	20	133
128.04	0.50	-0.842	-50.50	14.50	2.16	20	134
128.20	0.32	-0.269	-16.11	48.89	7.11	20	138
128.39	0.28	-0.108	-6.49	58.51	8.45	20	138
128.55	0.26	0.308	18.47	83.47	12.02	20	139
128.73	0.31	0.720	43.18	108.18	15.70	20	138
128.87	0.42	1.165	69.92	134.92	19.89	20	136
128.87	0.46	1.433	85.96	150.96	22.39	0	0
129.04	0.50	1.700	102.00	167.00	24.93	0	0
129.21	0.93	1.457	87.40	152.40	24.32	0	0
129.38	1.18	0.565	33.90	98.90	16.42	0	0
129.55	1.27	-0.160	-9.61	55.39	9.34	0	0
129.71	1.25	-0.676	-40.54	24.46	4.11	0	0
129.89	1.13	-0.943	-56.56	8.44	1.39	0	0
130.05	0.97	-0.969	-58.12	6.88	1.10	0	0
130.22	0.81	-1.055	-63.32	1.68	1.26	0	0
130.38	0.65	-0.796	-47.74	17.26	2.63	0	0
130.38	0.58	-0.790	-47.43	17.57	2.65	20	132
130.54	0.50	-0.785	-47.11	17.89	2.67	20	134
130.70	0.38	-0.617	-37.00	28.00	4.11	20	136
130.88	0.28	-0.191	-11.47	53.53	7.73	20	138
131.05	0.25	0.489	29.34	94.34	13.57	20	139
131.22	0.33	0.625	37.48	102.48	14.91	20	137
131.38	0.43	1.484	89.06	154.06	22.75	20	135
131.38	0.55	1.525	91.50	156.50	23.55	0	0
131.55	0.68	1.566	93.94	158.94	24.38	0	0
131.72	0.94	1.231	73.85	138.85	22.19	0	0
131.89	1.15	0.507	30.44	95.44	15.78	0	0
131.99	1.24	0.000	0.00	65.00	10.90	0	0
132.05	1.24	-0.195	-11.72	53.28	8.93	0	0
132.22	1.20	-0.733	-44.00	21.00	3.50	0	0
132.37	1.09	-0.888	-53.30	11.70	1.91	0	0
132.54	0.94	-0.897	-53.80	11.20	1.79	0	0
132.71	0.79	-0.958	-57.47	7.53	1.18	0	0
132.87	0.63	-0.897	-53.80	11.20	1.70	0	0
132.87	0.57	-0.822	-49.34	15.67	2.36	20	133
133.05	0.50	-0.748	-44.87	20.13	3.00	20	134
133.22	0.35	-0.565	-33.87	31.13	4.55	20	137
133.39	0.26	-0.052	-3.10	61.90	8.92	20	139
133.55	0.25	0.620	37.18	102.18	14.70	20	139
133.71	0.35	1.095	65.70	130.70	19.09	20	137
133.89	0.54	1.521	91.27	156.27	23.46	20	133
133.89	0.65	1.396	83.74	148.74	24.90	0	0
134.04	0.77	1.270	76.21	141.21	23.60	0	0
134.21	0.98	0.988	59.25	124.25	21.00	0	0

134.38	1.15	0.251	15.04	80.04	13.23	0	0
134.55	1.19	-0.369	-22.11	42.89	7.14	0	0
134.72	1.13	-0.766	-45.94	19.06	3.14	0	0
134.89	1.00	-0.893	-53.55	11.45	1.85	0	0
135.05	0.85	-0.973	-58.36	6.64	1.05	0	0
135.22	0.69	-0.858	-51.45	13.55	2.08	0	0
135.39	0.54	-0.938	-56.25	8.75	1.31	0	0
135.39	0.47	-0.794	-47.61	17.39	2.58	20	135
135.53	0.40	-0.650	-38.98	26.02	3.83	20	136
135.71	0.29	-0.302	-18.09	46.91	6.79	20	138
135.88	0.24	0.411	24.64	89.64	12.88	20	139
136.04	0.31	0.987	59.21	124.21	18.02	20	138

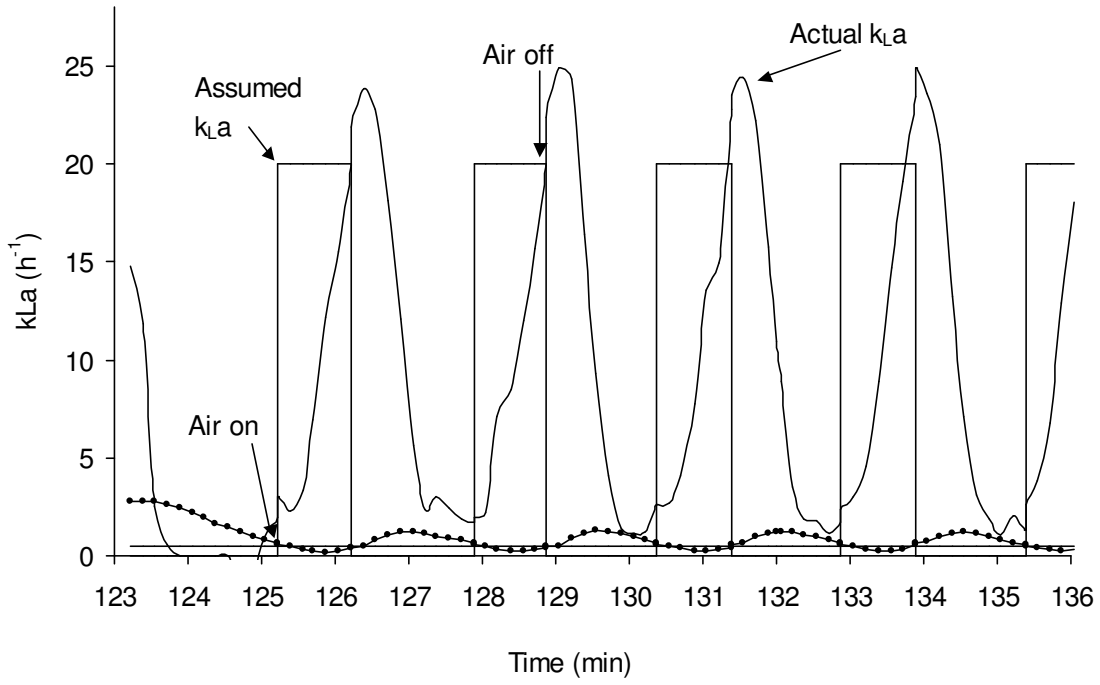


Figure A4: The real k_La calculated from DO data compared to the k_La assumed for all on-line calculations of the SOUR. The dissolved oxygen is also shown (•).

To quantify the error associated with the simplified assumption of the k_La compared to the real k_La (Fig A4), the oxygen transfer rates (mg L^{-1} h^{-1}) under the two different scenarios were plotted and integrated in order to find the difference in total oxygen transferred (mg L^{-1}).

Simplified assumption of the k_La

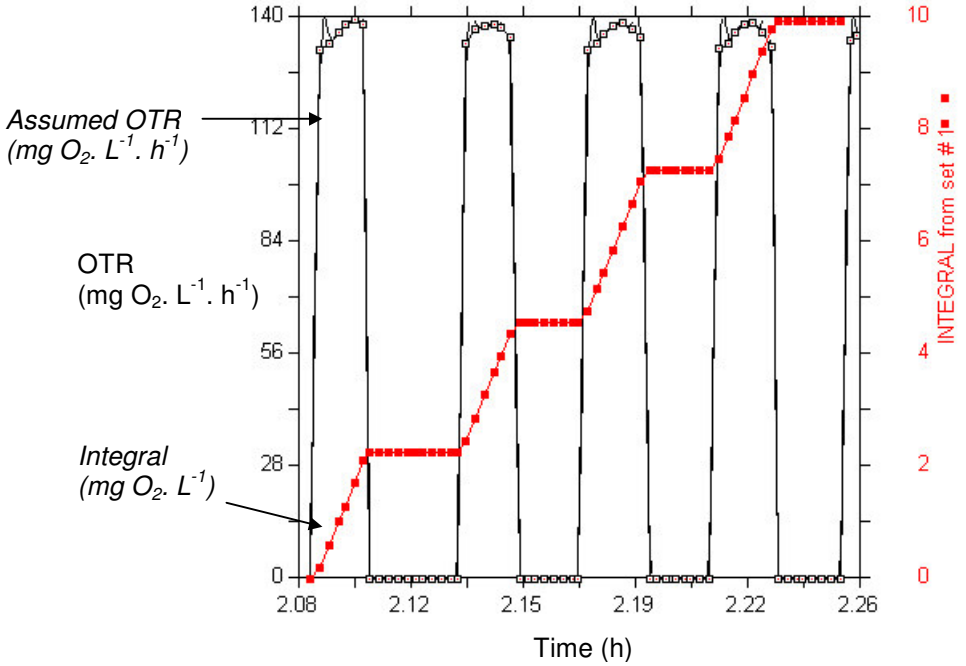


Figure A5: Plot of the assumed oxygen transfer rate and the total amount of oxygen transferred into solution over time under the simplified k_La assumption.

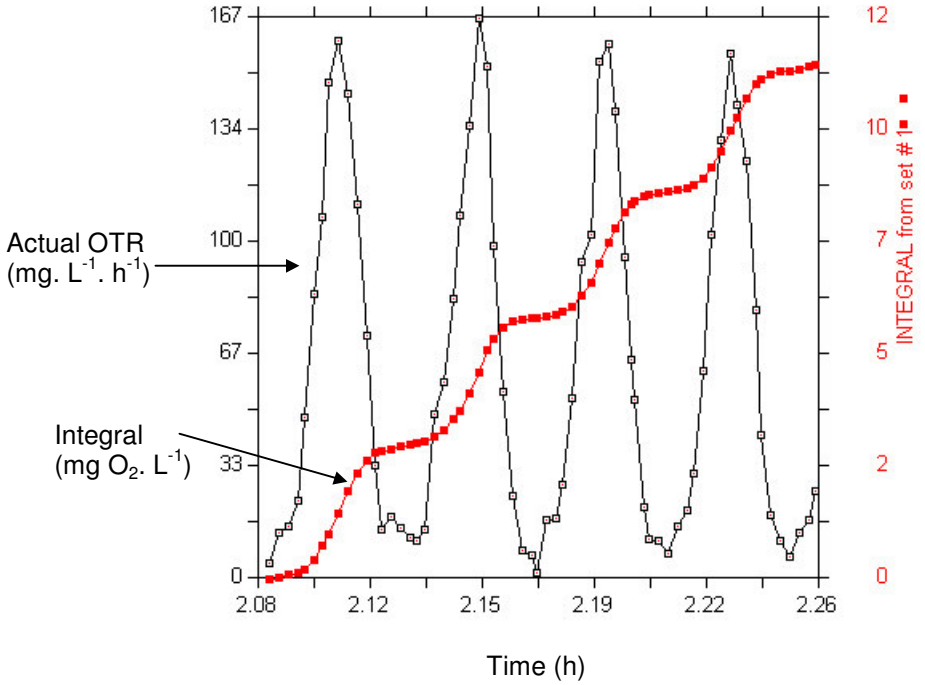


Figure A6: Plot of the real oxygen transfer rate and the total amount of oxygen transferred into solution.

The total amount of oxygen transferred into solution over the 10-minute interval under the assumed k_La dynamics was 9.98 mg O_2 L^{-1}, compared to 11.6 mg O_2 L^{-1} in the real situation. Therefore, the error associated with the simplified k_La assumption results in a 14 % under-estimation of the SOUR.

Appendix 2: The fraction of PHB used for denitrification vs aerobic oxidation. Absolute values of e^-mmol were calculated by integrating the electron flow rates curves (Figs 3.2, 3.8, 5.5).

	Figure	Electron flow to O_2 (e^- mM)	Electron flow to NO_3^- (e^- mM)	% e^- flow to NO_3^-
No DO control	**3.2**	**10.5**	**2.3**	**22**
DO setpoint 1 mg L^{-1}	3.8A	9.4	5.5	37
	3.8B	7.7	5.8	39
	3.8C	**8.6**	**6.9**	**41**
	5.5A	7	2.9	29
	5.5B	9	5	36
	5.5C	14.5	5.5	27
	5.7	18	8.5	31

Bold values are used in Appendix 3 as the minimum and maximum fractions of PHB used for nitrate reduction, without DO control and with DO control, respectively.

Appendix 3: Comparison of nitrogen removal possible with and without DO control, based on maximum and minimum conversion rates established from experimental data.

Feast Phase	**O_2-limitation** (Specific OTR < 0.04 mol O_2. Cmol X. h^{-1})	**O_2-excess** (Specific OTR > 0.06 mol O_2. Cmol X. h^{-1})
Available organic substrate (Ac) (mg COD L^{-1})	300	300
Available NH_4^+ (mg N L^{-1})	29	29
Max conversion Ac → PHB (%)	77	54
PHB available at end of feast phase (mg COD L^{-1})	231	162
Biomass production during feast phase[1] (CmM)	1.4	3.3
NH_4^+ left after growth of X_{HET}[2] (mg N L^{-1})	25	20
Final COD:N ratio	9 : 1	8 : 1

[1] Based on: Biomass yields under O_2-limitation and excess of 0.15 and 0.35 $Cmol_X/Cmol_{Ac}$, respectively (Table 2.1) and 1 CmM PHB = 36 mg COD L^{-1}

[2] Assuming consumption of 0.2mM NH_4^+ per Cmol biomass ($CH_{1.8}O_{0.5}N_{0.2}$)

Appendix 3 (continued): Comparison of nitrogen removal possible with and without DO control, based on maximum and minimum conversion rates established from experimental data.

Famine Phase	DO control and SOUR-controlled aeration	No DO control
PHB at start of famine phase (mg COD L^{-1})	231	162
PHB available as reducing power[1] (mg COD L^{-1})	116	81
NH_4^+ consumption due to growth of X_{HET}[1] (mg N L^{-1})	9	6
NH_4^+ remaining for oxidation (mg N L^{-1})	16	14
NO_x^- to be removed (mg N L^{-1})	16	14
COD:N ratio in famine phase	10 : 1	6 : 1
COD required (SND via NO_3^-) (mg COD L^{-1})	44	40
COD required (SND via NO_2^-) (mg COD L^{-1})	26	24
COD available[2]	48 (41 % PHB used for denit.)	18 (22 % PHB used for denit.)
Maximum N-removal possible (%)	100	45

[1] Assuming heterotrophic biomass yield on PHB 0.5 $Cmol_X/Cmol_{PHB}$

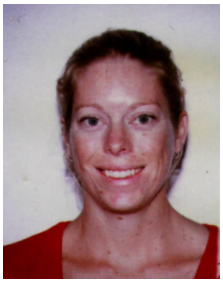
[2] Percentages of PHB used for NO_3^- reduction vs O_2 reduction are shown in Appendix 2

Appendix 4: Maximum rates, COD and O_2 requirement of the different N-removal processes.

Process	COD requirement (mg COD/mg N)	O_2 requirement (mg O_2/mg N)	Maximum rate (kg N m^{-3} day^{-1})	Reported in
CANON/SHARON -Anammox	0	3.8	1.5	Sliekers et al. 2002
SND via nitrite	1.7	3.4	-	-
SND via nitrate	2.9	4.6	0.22	Chapter 5
Nitrification-Denitrification	2.9	4.6	0.05	Strous et al. 1997

Curriculum Vitae

- Katie Third -



1999 – 2002	Ph.D. Student (Environmental Biotechnology), Murdoch University, Western Australia
2000	Visiting researcher, Delft University of Technology, the Kluyver Laboratory, Microbiology Department, The Netherlands (6 months)
1998 – 1999	BHP Minerals, Centre for Minerals Technology, Reno, Nevada (USA): Metallurgical research assistant in the bacterial leaching of mineral ores.
1998	Industry Practicum: BacTech (Australia) Ltd., Belmont, Western Australia in field of microbial leaching of sulphide ores.
1994 – 1998	Bachelor of Science with First Class Honours, Murdoch University. Thesis title: "Optimisation of Bacterial Leaching of Chalcopyrite"
1993	A.F.S Exchange Student, Paraguay, South America (12 months)
1988 – 1992	All Saints' College, Western Australia: Graduated year 12

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- Third KA, Newland M, Cord-Ruwisch R (2002) The effect of dissolved oxygen on PHB accumulation in activated sludge cultures. Biotechnology and Bioengineering. 82 (2) 238-250.
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