DEVELOPMENT OF MICROBIAL GRANULES UNDER ALTERNATING AEROBIC-ANAEROBIC CONDITIONS FOR CARBON AND NITROGEN REMOVAL

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ABSTRACT

Aerobic granulation is a novel environmental biotechnology recently developed for wastewater treatment. Nearly all research on aerobic granulation has been conducted in sequencing batch reactors (SBR); however, the effect of one of the most important operating parameters in SBR, i.e. settling time, is still unknown on the formation of aerobic granules. Thus, the first part of the present research investigated the effect of settling time on the development of aerobic granules. For this purpose, four SBRs were operated at different settling times of 5 to 20 minutes. Results showed that aerobic granules were successfully cultivated and became dominant only in the SBR operated at the shortest settling time of 5 minutes, while a mixture of aerobic granules and suspended sludge was observed in the reactors run at settling times of 20, 15 and 10 minutes, respectively. It was also found that a short settling time could significantly improve the production of extracellular polysaccharides, cell surface hydrophobicity and microbial activity. These seem to indicate that a short settling time would favour aerobic granulation. One may expect to manipulate the formation and characteristics of aerobic granules by properly controlling settling time.

There is an increased demand for nitrogen removal in wastewater treatment all over the world. Since microbial granules exhibit superior advantages over conventional activated sludge, processes using granules as medium to remove organic carbon and nitrogen through nitrification and denitrification are highly desired. In this case, the second part of the research looked into the development of microbial granules in alternating aerobic-anaerobic SBR without addition of external carbon source in anaerobic phase, and the SBR was operated at the optimal settling time of 2 minutes as predetermined. Microbial granules were successfully formed at various nitrogen loading rates (NLR) of 0.15-0.45 kg N m^{-3} day^{-1} and the same chemical oxygen demand (COD) loading of 2.0 kg COD m^{-3} day^{-1} during aerobic phase, which giving different COD/N ratios of 100/7.5, 100/12.5, 100/17.5, and 100/22.5 in respective reactor. Results showed that NLR would have a significant effect on the characteristics of microbial granules. The mean size of the microbial granules was

1.96, 1.53, 0.50, and 0.41 mm at respective NLR of 0.15, 0.25, 0.35, and 0.45 kg N m^{-3} day^{-1}. It was found that the SVI values of microbial granules were as low as 36 to 27 ml g^{-1}, which seemed to be lower than or comparable with those of aerobic granules cultivated under absolute aerobic conditions. Heterotrophic, nitrifying and denitrifying populations could coexist in the microbial granules. The activities of nitrifying and denitrifying populations in microbial granules could be enhanced by increasing NLR; however, the activity of heterotrophic populations tended to decrease in a significant way. Over 95% of COD removal and almost complete nitrification was achieved in the aerobic phase. However, only 12 to 27 mg l^{-1} NO_x-N produced in the aerobic phase was denitrified in the anaerobic phase, corresponding to a nitrogen removal efficiency of 24 to 50%, and the specific denitrification rates were relatively low, in the range of 1.9 to 3.5 mg N g^{-1} VS h^{-1} at various NLRs. Such a partial denitrification is mainly due to the fact that no external carbon source was added to the anaerobic phase in this part of the research.

In order to improve the nitrogen removal efficiency, ethanol as external carbon source was fed to the above alternating aerobic-anaerobic SBRs in the beginning of anaerobic phase. Denitrification metabolism was activated and greatly improved with sufficient supply of external carbon source. The COD removal efficiency was maintained as high as 95.1 to 97.2%, and the nitrogen removal efficiency was increased up to 99% at all the tested NLRs. The specific denitrification rates with addition of external carbon source fell into the range of 12.0 to 25.3 mg N g^{-1} VS h^{-1}, which were one-order magnitude higher than those obtained under conditions without addition of external carbon source. The respective activity of heterotrophic, nitrifying and denitrifying populations was found to be associated with the availability of external carbon source. With supply of external carbon source, the activities of both heterotrophic and denitrifying population were increased as compared to that determined under the conditions without supply of external carbon source, while the activity of nitrifying population did not change significantly.

The last part of the present research examined the feasibility of denitrification on poly-β-hydroxybutyrate (PHB) in microbial granular sludge SBR. To study the

potential role of PHB in denitrification process by microbial granules, a series of batch experiments with various availabilities of external carbon and nitrate sources were designed and conducted. Results showed that in the absence of external carbon source, pre-accumulated PHB in microbial granules could be utilized for cell maintenance and denitrification. With supply of external carbon, but no addition of nitrate, PHB accumulation accounted for the main mechanism of the organic removal. Under balanced growth conditions (with organic carbon and nitrogen supply), external organic carbon was consumed simultaneously for denitrification, PHB storage as well as for cell functions. It was revealed that the potential role of PHB for denitrification by microbial granules was very limited, i.e. less than 28 mg NO_3-N l^{-1} was found to be denitrified with internally accumulated PHB. This may indicate the limiting capacity of PHB as reducing power for denitrification by microbial granules.

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PUBLICATIONS

I. Papers produced from the Ph.D. study:

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II. Papers co-authored during the Ph.D. study:

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Liu Y., Yang S.F., Tay J.H., Liu Q.S., **Qin L.** and Li Y. (2004) Cell hydrophobicity is a triggering force of biogranulation. Enzyme and Microbial Technology 34: 371-379.

Liu Y., Yang S.F., Li Y., Xu H., **Qin L.** and Tay J.H. (2004) The influence of cell and substratum surface hydrophobicities on microbial adhesion. Journal of Biotechnology 110: 251-256.

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NOMENCLATURE

BOD	biological oxygen demand (mg l^{-1})
COD	chemical oxygen demand (mg l^{-1})
DO	dissolved oxygen (mg l^{-1})
ECP	extracellular polymer
EPS	extracellular polysaccharides
EPN	extracellular proteins
FA	free ammonia
HRT	hydraulic retention time (hour or day)
IA	image analysis
NLR	nitrogen loading rate (kg N m^{-3} day^{-1})
OLR	organic loading rate (kg COD m^{-3} day^{-1})
ORP	oxidation-reduction potential
PHB	poly-β-hydroxybutyrate
SBR	sequencing batch reactor
SEM	scanning electron microscope
SG	specific gravity
SOUR	specific oxygen utilization rate (mg O_2 g^{-1} dry weight h^{-1})
SRT	sludge retention time (hour or day)
SS	suspended solid (mg l^{-1} or g l^{-1})
SVI	sludge volume index (ml g^{-1})
TOC	total organic carbon (mg l^{-1})
TS	total solid (mg l^{-1})
UASB	upflow anaerobic sludge blanket
VS	volatile solid (mg l^{-1})
VSS	volatile suspended solid (mg l^{-1})

Chapter 1

Introduction

1.1 Background

Immediate removal of wastewater from its sources of generation followed by treatment is necessary to ensure protection of public health and the environment. Accompanied with increasing world population and industrial wastes, the demands for water quality have been increasing more significantly than ever. Health and environmental concerns also continuously stimulate the increasing required degree of treatment and thus require more advanced and cost-effective techniques for wastewater treatment. In recent years, based on conventional biological treatments, a variety of processes has been proposed and developed for organic matter and nutrients removal from municipal and industrial wastewater.

Recently, research has been focused on the development of aerobic granules of activated sludge in sequencing batch reactor (SBR) systems (Beun et al., 1999; Tay et al., 2001a, b). Aerobic granules represent a novel and promising biotechnology for high-performance industrial and domestic wastewater treatment. Previous research indicates that aerobic granules have several advantages over conventional activated sludge, e.g., high settling ability, high biomass retention, strong microbial structure and high resistance to inhibitory and toxic wastes (Beun et al., 1999; Tay et al., 2001b; Jiang et al., 2003). Aerobic granule-based bioreactor requires smaller reactor footprint, and can handle much higher organic loadings than activated sludge system. Consequently, aerobic granular sludge would be a promising aerobic biological treatment technology.

Factors involved in aerobic granulation in SBR include organic loading rate, hydrodynamic shear force, substrate composition, feast-famine regime, dissolved oxygen and reactor configuration (Peng et al., 1999; Tay et al., 2001a, b, 2003; Liu and Tay, 2002; Moy et al., 2002; McSwain et al., 2004). The effects of these parameters on aerobic granulation will be reviewed in Chapter 2. A unique feature of SBR is cycle operation. Each cycle consists of filling, aeration, settling and decanting. In the column-type SBR, settling time is a parameter that determines the hydraulic selection to sludge particles, i.e. at a shorter settling time, only those heavy and good settling sludge could be retained in the reactor, while the light and poor settling sludge would be washed out. However, how selection pressure in terms of settling time acts as a triggering force for aerobic granulation is still unknown. Previous research on anaerobic granulation showed that selection pressure in terms of upflow liquid velocity in the upflow anaerobic sludge blanket reactor (UASB) was a crucial factor in the formation of anaerobic granules (Hulshoff Pol et al., 1988; Liu et al., 2003a). So far, the role of selection pressure and how such pressure influences aerobic granulation have been hardly studied, and little information is available on the mechanisms responsible for aerobic granulation, which is essential for environmental engineers to design and operate granular sludge-based treatment systems.

As regulations on nutrient discharge become more and more stringent, many wastewater treatment plants are required to upgrade for nutrient removal (Cinar et al., 1998; Chudoba and Pujol, 2000). Conventional systems for nitrogen removal have encountered problems such as sludge bulking, sensitivity to shock loading, and low retention or washout of nitrifying biomass, which lead to low removal efficiency or even failure of the process (Eikelboom et al., 1998; Wanner et al., 2000). Thus, aerobic granules capable of removing organic carbon and nitrogen are greatly desired.

1.2 Objectives and Scopes

Selection pressure has been demonstrated to be a decisive factor in the formation of anaerobic granules (Hulshoff Pol et al., 1988; Liu et al., 2003a); however, it is still not clear that the role of hydraulic selection pressure on the development of aerobic granules in SBR. Therefore, the first objective of this study is to look into the effect of settling time as a selection pressure on aerobic granulation in SBR. The second main objective of this study is to develop microbial granules for simultaneous organic carbon and nitrogen removal in a single granular sludge-based aerobic-anaerobic SBR. The specific objectives of this study include the following aspects:

(1) To look into how the selection pressure in terms of settling time influences aerobic granulation in SBR.

(2) To develop microbial granules grown at different nitrogen loadings under alternating aerobic-anaerobic conditions and further to examine the physico-chemical properties of the cultivated granules.

(3) To explore the reactor performance for simultaneous organic carbon and nitrogen removal as well as the feasibility of simultaneous removal of organic carbon and nitrogen in single-sludge alternating aerobic-anaerobic SBR.

(4) To investigate the potentials of poly-β-hydroxybutyrate (PHB) as an alternative carbon source for denitrification by microbial granules.

1.3 Outline of the Thesis

This thesis contains seven chapters. The outline of the thesis is described below:

1) Chapter 1 gives a brief introduction to the research and outlines the main objectives of this work.
2) Chapter 2 presents a literature review relevant to this research. The review covers fundamentals of biological organic carbon and nitrogen removal as well as biogranulation. Studies of operational factors on aerobic granulation are also reviewed.
3) Chapter 3 shows the effect of settling time on aerobic granulation. It is demonstrated that settling time is a main driving force toward aerobic granulation in SBR.
4) Chapter 4 discusses the physicochemical and microbial properties of microbial granules cultivated under alternating aerobic-anaerobic conditions without supply of external carbon source.

5) Chapter 5 explores the performance of alternating aerobic-anaerobic SBR for the simultaneous removal of organic carbon and nitrogen in the presence of external carbon source.
6) Chapter 6 looks into the potentials of PHB as internal carbon source for denitrification by microbial granules.
7) Chapter 7 concludes the major findings from this study and some recommendations for future work are proposed as well.

Chapter 2

Literature Review

2.1 Introduction

The required degree of wastewater treatment has increased significantly with new directions and concerns for wastewater treatment in various specific areas, such as the changing nature of the wastewater to be treated, emerging health and environmental concerns, the problem of industrial wastes and the impact of new regulations. New technologies and processes are constantly introduced to solve contemporary problems, or make the process more stable and cost-effective. During the biological process, the separation of the treated supernatant from the biomass by sedimentation represents a critical link in the operation of treatment process. However, in the traditional activated sludge systems, it is extremely difficult for the dispersed sludge to settle down efficiently because of their quality of tiny size, low specific gravity and negatively charged surface (Bitton, 1999). Immobilized cells have demonstrated to exhibit excellent separation ability of the biomass from the treated effluent due to the compactness of their microbial structure. Technology of cell immobilization with carriers, like biofilms, as well as self-immobilization without carriers, such as anaerobic granules and more recently aerobic granules, is highly desired and applicable for biological wastewater treatment systems.

One major concern in the wastewater treatment industry is nitrogen removal. Nitrogen removal has great significance in environmental fields because of toxicity of ammonia and nitrite to aquatic life and human beings as well as its oxygen demands and eutrophication potentials in receiving waters (Sharma and Ahlert, 1977). Stricter regulations were thus proposed. As both organic carbon and nitrogen exist in the wastewater, process for simultaneous removal of organic carbon and nitrogen is required to implement stricter effluent standards. Biological process is generally more favorable for organic carbon and nitrogen removal than physico-chemical methods as it is environmental friendly and less expensive. Aerobic and anaerobic processes are two major divisions of biological processes, while their mechanisms are completely different. Due to the comparatively fast growth of cells, aerobic processes show inherent superiorities over anaerobic processes in several aspects, such as rapid start-up, applicable for nutrient removal, ability to withstand environmental shocks (overloads, pH fluctuation and temperature change) or toxic events. In this chapter, a brief review of aerobic processes for organic and nitrogen removal is presented, while cell immobilization technology including biofilms, anaerobic granulation and aerobic granulation is also discussed.

2.2 Biological Processes for Organic Carbon Removal

The biological processes for wastewater treatment consist of two main groups: suspended growth and attached growth processes. In suspended growth process, microorganisms are maintained in suspension within liquid by appropriate mixing methods. An important characteristic of suspended growth process is the formation of floc particles, which can be removed by settling, providing a relatively clear treated effluent. In attached growth process, microorganisms are attached to an inert packing material, including rock, gravel, slag, sand, redwood, plastics or synthetic materials. Both suspended and attached processes can be operated under aerobic or anaerobic conditions.

2.2.1 Process Metabolism

In biological processes for wastewater treatment, microorganisms consume organics as their food supply to produce energy for maintenance and synthesis of new cells (biomass), i.e., biomass is produced continuously while the organics in wastewater are consumed and oxidized simultaneously. The biological reactions involved in metabolisms are extremely complicated and are not yet completely understood. However, it is generally believed that two interrelated processes must exist, called catabolism and anabolism (synthesis). Catabolism is the biochemical process performed by microorganisms to yield energy for synthesis, motility and respiration to remain viable, while microorganisms process organic matter to create new cells through anabolism (Fig. 2.1).

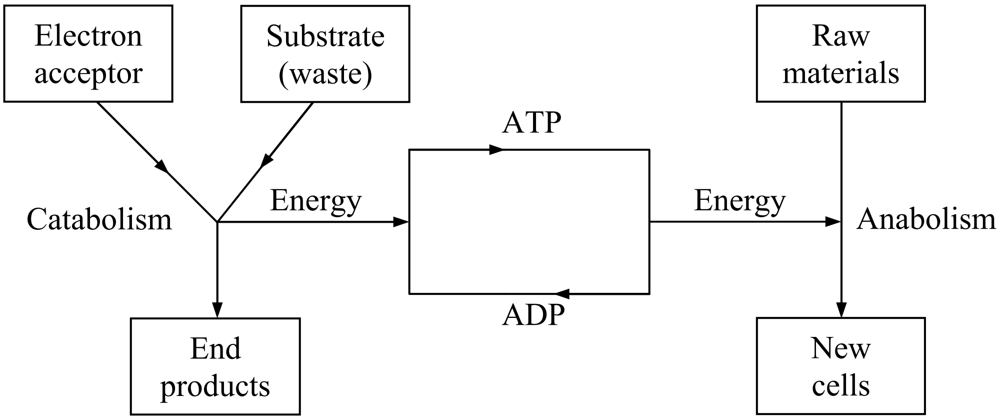

Figure 2.1 General scheme of metabolism (Davis and Cornwell, 1998)

Based on the nutritive requirements, bacterial can be classified into heterotrophic and autotrophic bacteria. In heterotrophic metabolism, organic compounds in wastewater are used as energy sources and carbon sources for synthesis.

$$Organics + O_2 \longrightarrow CO_2 + H_2O + energy \qquad (2.1)$$

Autotrophic bacteria utilize inorganic carbon as a carbon source and oxidize inorganic matter for energy. Autotrophic bacteria of greatest significance in wastewater engineering are the nitrifying, sulfur, and iron bacteria, which perform the following reactions:

$$NH_3 + O_2 \xrightarrow{Ammonia-oxidizing \quad bacteria} NO_2^- + energy \qquad (2.2)$$

$$NO_2^- + O_2 \xrightarrow{Nitrite-oxidizing \quad bacteria} NO_3^- + energy \qquad (2.3)$$

$$H_2S + O_2 \longrightarrow H_2SO_4 + energy \qquad (2.4)$$

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + energy \qquad (2.5)$$

Anabolism (synthesis) is the biological process of substrate utilization to form new protoplasm for growth and reproduction. The cellular protoplasm formed is a combination of hundreds of complex organic compounds, including proteins, carbohydrates, nucleic acids and lipids.

The primary product of metabolism is energy, and the chief use of this energy is for biosynthesis. Energy release and biosynthesis are coupled biological processes that cannot be separated. The maximum rate of biosynthesis occurs simultaneously with the maximum rate of energy release. Thus, in heterotrophic metabolism of wastewater organics, maximum removal rate of organics for a given population of microorganisms can be obtained at the maximum biological growth rate. Energy is transferred from the catabolic reaction to anabolic reaction through high-energy phosphate bonds. The removal of hydrogen or the splitting of the carbon-carbon bond in the catabolic process releases energy. A significant part of this energy is used to add a phosphate atom to adenosine diphosphate (ADP), converting it to adenosine triphosphate (ATP). The ATP is transferred to the anabolic reaction where the extra phosphate atom is removed, releasing the stored energy to the synthesis reaction. The resulting ADP is then transferred back to the catabolic reaction to be reenergized to ATP, and the energy cycle is repeated (Fig. 2.1).

Catabolic processes involve either the oxidation or the reduction of the substrate. If free molecular oxygen is available, molecular oxygen is used as the terminal electron acceptor, this process is known as aerobic metabolism. Aerobic oxidation of a wider spectrum of organic materials is more efficient than any other type of decomposition (e.g., anoxic and anaerobic reactions). This is due to the fact that large amount of energy can be released from the aerobic oxidation reactions. Also the final end products are oxidized to a very low energy level, leading to a more stable end product than can be achieved in the other oxidation systems.

The majority of organic matter in wastewater is in the form of large molecules that cannot penetrate the bacterial cell membrane. In order to metabolize those large molecules, bacteria must be capable of hydrolyzing complex carbohydrates into soluble sugar units, protein into amino acids, and insoluble fats into fatty acids. Under aerobic conditions, microorganisms further oxidize the reduced soluble organic matter into carbon dioxide and water as end products.

$$Organics + O_2 \longrightarrow CO_2 + H_2O + energy \tag{2.6}$$

2.2.2 Factors Affecting Organic Carbon Removal

The BOD removal by heterotrophic microorganisms is affected by several environmental factors including temperature, pH, dissolved oxygen (DO) concentration, nutrient availability and toxicity of substrate.

It has been shown that microbial growth rate is related to temperature by the Arrhenius law, which indicates that growth rate doubles for every 10°C temperature rise in the range between 5 and 35°C (Viessman and Hammer, 1998). Temperature may affect the overall performance of a biological system in two aspects: i) optimum temperatures for bacterial activity are in the range from 25 to 35°C, while aerobic digestion and nitrification would cease when temperature rises up to 50°C; ii) temperature has significant effect on sedimentation of biomass during separation phase. It has been reported that a 1°C differential between the influent wastewater and the wastewater in the sedimentation tank will result in a density current to occur (Metcalf and Eddy, 2003). These imply that excessive heat will destroy microorganisms, however cold wastewater can lower BOD removal efficiency.

Most aerobic systems exhibit good performance near a neutral pH, with a tolerable range of pH 6.0 to 9.0. Most bacteria cannot tolerate pH below 4.0 or above 9.0. pH affects the enzyme activity and the ionization of chemicals, thus plays a role in the transport of the nutrients into the cells. DO concentration above 2.0 mg l^{-1} is generally required in aerobic systems. It is reported that the growth rate of heterotrophs is independent of the DO concentration higher than 0.5 mg l^{-1}. Microbial growth will be inhibited at the low concentrations of nitrogen and phosphorus. Especially for industrial wastewater, care must be taken to assure that sufficient nutrients (nitrogen and phosphorus) are adequately available for the amount of organic carbon to be oxidized. Toxic substances, such as heavy metals and phenol, may adversely affect biological treatment systems. Fortunately, heterotrophic bacteria responsible for BOD removal can tolerate higher concentration of toxic substances than other species, such as methanogens, nitrifying and denitrifying bacteria.

2.3 Biological Nitrogen Removal

Excessive accumulation of various forms of nitrogen in surface and ground waters can lead to adverse economical and human health effects. Nitrogen removal is necessarily required before discharging treated wastewater to sensitive water bodies for several environmental concerns, such as eutrophication, toxicity of ammonia to aquatic life, DO deficit, and potential harmful effects of nitrite to humans. Another consideration for nitrogen removal is for groundwater recharge or water-reuse applications. Wastewater from industry and agriculture often has higher nitrogen content than municipal sewage, ranging from 100 to 4000 mg l^{-1}. Typical municipal sewage contains 20 to 85 mg l^{-1} nitrogen among which 60% are in the ammonia form (Metcalf and Eddy, 2003). The standard of drinking water for nitrate is 10 mg l^{-1} as nitrogen and for unionized free ammonia is 0.1 to 1.0 mg l^{-1} as nitrogen (U.S. EPA, 1993.).

2.3.1 Biological Nitrification

2.3.1.1 Nitrification process

Nitrification is an autotrophic process in which ammonia is converted to nitrate. Nitrification takes place in two consecutive steps, i.e. ammonia is firstly oxidized to nitrite by ammonia oxidizers, and subsequently nitrite is oxidized to nitrate by nitrite oxidizers. Nitrification in wastewater treatment process has been attributed primarily to *Nitrosomonas* and *Nitrobacter* even though other autotrophic bacteria genera are capable of generating energy from the oxidation of ammonia.

The conversion equation for the oxidation of ammonia to nitrite can be written as:

$$\begin{aligned} &NH_4^+ + 1.5O_2 \xrightarrow{Ammonia-oxidizers} NO_2^- + 2H^+ + H_2O \\ &\Delta G^o = -270kJ/mol \quad NH_4^+ - N \end{aligned} \tag{2.7}$$

The conversion reaction for the oxidation of nitrite to nitrate follows:

$$\begin{aligned} &NO_2^- + 0.5O_2 \xrightarrow{Nitrite-oxidizers} NO_3^- \\ &\Delta G^o = -80kJ/mol \quad NO_2^- - N \end{aligned} \tag{2.8}$$

The expression of overall oxidation is:

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \tag{2.9}$$

As shown in Equations 2.7 and 2.8, energy is derived from the oxidation of ammonia and nitrite respectively. The energy produced is then used for cell growth and maintenance of the bacteria. Biosynthesis of ammonia-oxidizer can be described by the following equation:

$$13NH_4^+ + 15CO_2 \longrightarrow 10NO_2^- + 3C_5H_7NO_2 + 23H^+ + 4H_2O \tag{2.10}$$

For nitrite-oxidizer, the corresponding growth expression is:

$$10NO_2^- + 5CO_2 + NH_4^+ + 2H_2O \longrightarrow 10NO_3^- + C_5H_7NO_2 + H^+ \tag{2.11}$$

Thus, the biosynthesis reaction can be represented as follows (Metcalf and Eddy, 2003):

$$\begin{aligned} &NH_4^+ + 1.863O_2 + 0.098CO_2 \longrightarrow \\ &\qquad 0.0196C_5H_7NO_2 + 0.098NO_3^- + 1.98H^+ + 0.0941H_2O \end{aligned} \tag{2.12}$$

Equation 2.12 shows that for each mol of ammonia nitrogen oxidized, 1.86 mol of oxygen is required, while 7.07 g of alkalinity as $CaCO_3$ are consumed and 0.08 g of inorganic carbon are utilized in the formation of new cells.

2.3.1.2 Microorganisms involved in nitrification

Nitrification is performed collectively by two distinct autotrophic groups (Laanbroek et al, 1994). As mentioned early, ammonia is oxidized to nitrite and then to nitrate. Nitrifying bacteria either oxidize ammonia to nitrite or nitrite to nitrate. So far, no autotrophic bacterium is known to oxidize ammonia directly to nitrate. Ammonia-oxidizing bacteria (AOB) obtain energy by catabolizing un-ionized ammonia to nitrite (Suzuki et al., 1974), while nitrite-oxidizing bacteria (NOB) further mineralize nitrite to nitrate. The yield coefficient of *Nitrosomonas* is 0.04-0.13 g VSS g^{-1} NH_4^+-N and 0.02-0.07 g VSS g^{-1} NH_4^+-N for *Nitrobacter* (Sharma and Ahlert, 1977). This is consistent with the fact that the free energy released per mole of nitrogen oxidized in Equation 2.7 is greater by comparison with Equation 2.8 (Gibbs and Schiff, 1960).

AOB are traditionally classified by cell morphology into the five different genera: *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* (Koops and Möller, 1992). Recently, on the basis of 16S rRNA sequence homology, *Nitrosospira*, *Nitrosovibro* and *Nitrosococcus* have been proposed to be combined into one common genus *Nitrosospira* (Head et al., 1993). NOB include the genera *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitrospina* (Watson et al., 1986; Hagopian and Riley, 2004). In general, ammonia-oxidizing bacteria are obligatory chemolithotrophs, although some of them can use organic compounds for mixotrophic growth. All members of the Nitrobacter-aceae family are gram-negative facultative chemoautotrophs, which are characterized by the ability to utilize an inorganic chemical substrate (NH_3) as a source of electrons for the immobilization of inorganic carbon (CO_2 or HCO_3^-) into biomass.

Some heterotrophic bacteria and fungi are also capable of nitrification, provided that an organic substrate is present (Focht and Verstraete, 1977; Castignetti et al., 1990). As the nitrogen oxidation is the only energy-yielding process in autotrophic nitrifiers, nitrification in heterotrophic organisms seemingly does not contribute significantly to their energy metabolism.

Nitrosomonas europaea is the most abundantly cited and extensively studied nitrifier (Watson, 1971). *Nitrosomonas europaea* has been identified as the dominant genus in environments where there are wide fluctuations in oxygen concentration (Smorczewski and Schimidt, 1991), while the presence of *Nitrosomonas sp.* has been confirmed as the dominant ammonium oxidizing group (Wagner et al., 1996). It has been shown that *Nitrosomonas europaea* and *Nitrosomonas eutropha* could simultaneously nitrify and denitrify wastewater when grown under oxygen limitation conditions (Bock et al., 1995). Another important ammonia-oxidizer, *Nitrosococcus mobilis*, was reported to be dominant in industrial nitrifying/denitrifying plant (Juretschko, 1998) and nitrifying sequencing batch biofilm reactors (Daims, 2001). In contrast, *Nitrosospira*-related ammonia-oxidizers were found to be dominant in situ in a laboratory scale fluidized bed reactor (Schramm, 1998). These may indicate that the ecology of ammonia-oxidizing bacteria is closely related to operation conditions.

2.3.1.3 Factors influencing nitrification

Temperature

The nitrification rate is strongly dependent on temperature. The nitrification process occurs over a range of approximately 4 to 45°C, with about 35°C optimum for *Nitrosomonas*, and 35 to 42°C for *Nitrobacter* (U.S.EPA, 1993). The optimal temperature range is the result of two interactive processes: the anticipated increase in reaction rate with increase in temperature and protein denaturation above a critical temperature. The optimal temperature for nitrification in pure cultures was reported to be in the range of 30 to 36°C (Alexander, 1965). Although temperature greatly affects the rate of nitrification, it cannot be easily controlled from a practical point of view.

DO

The DO concentration has a significant effect on the growth rates of nitrifiers and nitrification rate. The theoretical nitrogenous oxygen demand is 4.57 mg O_2 per mg of NH_3-N, which is obviously greater than carbonaceous oxygen demand. Although the actual ratio of oxygen consumed to nitrogen oxidized is often different from that predicted stoichiometrically, the growth rate of nitrifiers and nitrification rate is affected at low DO concentrations. Through modeling the growth of *Nitrosomonas* using the Monod equation, with DO as the growth-limiting substrate, its half saturation coefficient has been reported as 0.15-0.20 mg l^{-1} O_2 (U.S.EPA, 1993). The value for the coefficient increases with increasing temperature. Evidence shows that the growth rate of *Nitrosomonas* is not limited at DO concentration above 1.0 mg l^{-1}, but in practice DO concentration greater than 2.0 mg l^{-1} may be required.

pH

pH has a significant effect on the nitrification process through three aspects: i) activation-deactivation of nitrifying bacteria; ii) nutrition effect, associated with alkalinity; iii) inhibition through free ammonia and free nitrous acid, and through heavy metals. A range of pH between 7.0 and 8.0 was reported as optimal for nitrification (Jones and Paskins, 1982; Antoniou et al., 1990). The optimal pH may vary from 7.9 to 8.2 for *Nitrosomonas*, and 7.2 to 7.6 for *Nitrobacter* (Alleman, 1984). The availability of nutrition is determined by the chemical equilibrium ($CO_3^{2-} \rightarrow HCO_3^{-} \rightarrow CO_2$) which largely depends on the pH. At low pH, CO_2 will be stripped out of the reactor with aeration, resulting in alkalinity deficiency; however at high pH, carbon will be mineralized to carbonate, which is insoluble and hardly assimilable.

At certain pH, ammonia and nitrite would be transformed into un-ionized forms, i.e., free ammonia and nitrous acid, while both can inhibit the activities of nitrifying bacteria and their concentrations are highly pH-dependent (Anthonisen et al., 1976; Kowalski and Lewandowski, 1983; Suthersan and Ganczarczyk, 1986; Hellinga et al., 1997). Free ammonia concentration increases at high pH, while nitrous acid concentration rises at low pH (Ford, 1980). Evidence shows that free ammonia begins to inhibit *Nitrosomonas* at a concentration of 10-150 mg l^{-1} and *Nitrobacter* in the range of 0.1-1.0 mg l^{-1} (Anthonisen et al., 1976; Bae et al., 2001).

Ammonia and nitrite loading/concentration

High concentration of free ammonia and un-dissociated nitrous acid was suggested to inhibit the activity of nitrifying bacteria (Anthonisen, 1976; Sharma and Ahlert, 1977). Some research showed that *Nitrosomonas* was not inhibited by ammonia nitrogen up to 3000 mg l^{-1} (Prinčič et al., 1998). It is also reported the activity of *Nitrobacter* was inhibited by 40% at a nitrite nitrogen concentration of 1400 mg l^{-1} and a complete inhibition occurred at 2500 mg l^{-1} (Sharma and Ahlert, 1977). Evidence shows that free ammonia can seriously inhibit the activity of *Nitrobacter* at a concentration of 0.1-1.0 mg l^{-1} and would inhibit *Nitrosomonas* at a much higher concentration of 10-150 mg l^{-1} (Anthonisen et al., 1974). As the fraction of free ammonia in the total of ammonia and ammonium is pH and temperature-dependent (Anthonisen et al., 1976), the range of such species led to inhibition should also be associated with pH and temperature of the system. Likewise, the degree of inhibition by free nitrous acid depends upon the nitrite-nitrous acid equilibra, which is greatly influenced by pH. For domestic wastewater, ammonia and nitrite concentrations are not in the inhibition range; however, substrate and product inhibition are of significance in dealing with industrial and agriculture wastewater.

Toxin effect

Nitrification process can be inhibited by many different substances. These substances include certain inorganics (i.e., specific metals), some organic compounds, and certain forms of nitrogen. Chlorite ion strongly inhibits both steps of nitrification (Hyne and Knowles, 1983), whereas sodium chlorate has been proven effective for inhibition of the second step conversion (Belser and Mays, 1980). Metals can inhibit nitrification. A copper concentration of 30 mg l^{-1} could inhibit the activity of nitrifying bacteria, whereas about 94% inhibition of ammonia oxidation occurred when nickel concentration increased to 250 mg l^{-1} (Lee et al., 1997). In general, *Nitrosomonas* are more sensitive to some metals such as copper and nickel than *Nitrobacter* (Lee et al., 1997).

Some organic compounds, such as sulphur components, aniline components, phenols and cyanide, have an especially strong inhibiting effect. Some toxic substances, such as heavy metals and phenols, are found to cause some degree of nitrification inhibition. Such inhibition occurs through interference with the general metabolism of the cell or with the primary oxidative reaction. Unlike inhibition by heavy metals, some organic compounds, such as sodium cyanide are more toxic to *Nitrobacter* rather than to *Nitrosomonas* (Wood et al., 1981). As discussed early, un-ionized ammonia and nitrite at certain concentrations are known to inhibit nitrification, especially nitration, known as the second step of nitrification (Anthonisen et al., 1976; Charley et al., 1980; Both et al., 1992). It has been pointed out that nitrifiers can adapt themselves to many inhibitory compounds if inhibitors are constantly present rather than slug discharge to the influent.

2.3.2 Biological Denitrification

2.3.2.1 Denitrification process

Denitrification involves the microbial reduction of nitrate to nitrite, and ultimately to gaseous forms of nitrogen. This process is performed by various chemoorganotrophic, lithoautotrophic and phototrophic bacteria and some fungi, under oxygen-reduced or anoxic conditions (Focht and Chang, 1975). Electrons originated from organic matter, reduced sulfur compounds, or molecular hydrogen, are transferred to oxidized nitrogen compounds instead of oxygen in order to build up a proton motive force usable for ATP regeneration.

In the process of denitrification, nitrate and nitrite act as electron acceptor in the respiratory electron transport chain. The process involves transferring electrons from a reduced electron donor, e.g., an organic substrate, to the electron acceptor, nitrite and nitrate. Most denitrifying bacteria are facultative: they can use either oxygen or nitrate as their terminal electron acceptor. The switch from oxygen to nitrate is controlled by oxygen levels through inhibiting the synthesis of the enzymes required for denitrification or the activities of such enzymes. Concurrent oxygen and nitrate utilization was observed in activated sludge system operated at a high DO level (Kugelman et al., 1991; U.S.EPA, 1993).

Biological denitrification involves the biological oxidation of organic substrates using nitrate or nitrite as the electron acceptor. Reaction stoichiometry for methanol as carbon source is shown as follows (Metcalf and Eddy, 2003).

$$5CH_3OH + 6NO_3^- \longrightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \tag{2.13}$$

$$CH_3OH + 2NO_2^- \longrightarrow N_2 + CO_2 + H_2O + 2OH^- \tag{2.14}$$

As shown in Equations 2.13 and 2.14, an organic carbon source (e.g. methanol) is necessarily required to act as the electron donor as well as carbon source for biological synthesis. The selection of organic carbon source depends primarily on cost and local availability. Methanol is the most commonly used carbon source in the denitrification process in wastewater treatment as it is inexpensive and widely available as the product of fermentation. Other organic compounds that could be used include acetic acid, ethanol, and organics present in municipal and industrial wastewater.

2.3.2.2 Microorganisms involved in denitrification

Denitrifiers belong to a biochemically and taxonomically diverse group of facultatively anaerobic bacteria, characterized by the ability to use nitrogen oxides (nitrate and nitrite) as electron acceptors, producing mainly nitrogen gas as reduction products (Knowles, 1982). Nearly 130 denitrifying bacteria species have been found within more than 50 genera (Zumft, 1997). *Pseudomonas* species are the most common and widely distributed of all the denitrifiers and have been shown to use a wide array of organic compounds (Payne, 1981). Other genera of bacteria include *Micrococus*, *Archromobacter*, *Thiobacillus*, and *Bacillus* (Halling-Sørensen and Jørgensen, 1993). As denitrification is a multi-step process starting from nitrate via nitrite, nitric oxide, nitrous oxide to the dinitrogen gas, different species work together to carry out the whole process. Some bacteria reduce nitrate only to nitrite or N_2O as they possess nitrate reductase but lack N_2O reductase, whereas other species grow anaerobically with nitrite or N_2O as the sole respiratory electron acceptor because these organisms possess N_2O reductase but they cannot produce N_2O from nitrate or nitrite (Wilderer et al., 1987; Martienssen and Schöps, 1999).

2.3.2.3 Factors influencing denitrification

Dissolved oxygen

Denitrification is a respiratory process. Most denitrifying bacteria are facultative. They can transfer from aerobic respiration with the presence of oxygen to anoxic respiration with the absence of oxygen. The enzymes involved in denitrification are nitrate reductase, nitrite reductase, nitric oxide reductase, and finally nitrous oxide reductase (Zumft et al., 1988). DO appears to regulate synthesis of nitrate reductase enzyme and inhibits denitrification in pure culture of facultative denitrifying bacteria so that the substrate electrons flow to oxygen cytochromes (Stouthamer, 1988).

It seems there is some contradiction about the effect of DO on denitrification. On the one hand, some researchers suggested denitrification will be inhibited or even cease after the DO reach up to a critical concentration (Tiedje, 1988). The critical DO concentrations have been reported in a number of pure culture studies: 0.2 mg l^{-1} (Focht and Chang, 1975), 0.13 mg l^{-1} (Nelson and Knowles, 1978), and less than 0.1 mg l^{-1} (Krul, 1976). On the other hand, simultaneous nitrification and denitrification has been reported in activated sludge with DO concentration as high as 3 mg l^{-1} (Robertson et al., 1988; Munch et al., 1996).

It has been reported that pure strains of denitrifying bacteria can grow simultaneously using both oxygen and nitrate (Robertson and Kuenen, 1984; Patureau et al., 1994). Noticeably, activated sludge flocs can exist in anoxic regions and these micro-environments allow nitrification in the outer part in contact with bulk water DO, and denitrification in an inner part, especially if the floc particle diameter is greater than 100 μm (Mueller et al., 1968; Smith, 1984; Hanel, 1988). Oh and Silverstein (1999) investigated the effect of DO on denitrification by activated sludge over a range of 0 to 5.6 mg l^{-1} DO. Their results showed that DO inhibited denitrification at a very low DO concentration: the specific denitrification rate decreased over 35% with DO at 0.09 mg l^{-1} as compared to anoxic conditions. Some denitrification activity was observed with DO as high as 5.6 mg l^{-1} with the cost of the low denitrification rate, while decreasing DO concentration was found to enhance the denitrifying activity (Patureau et al., 2004).

pH

In general, denitrification will be much less sensitive to pH than nitrification. However, denitrification rates are influenced with the changes in pH, i.e. the rates are depressed when pH drops below 6.0 or rise above 8.0. As denitrification produces alkalinity, it may increase reactor pH while high concentration of nitrate is to be removed. Glass and Silverstein (1998) observed that at pH≤7.0, denitrification was completely inhibited with an high initial nitrate concentration, however as pH was increased from 7.5 to 9.0, the accumulation of nitrite also increased significantly, which suppressed the nitrite reduction rate by 63%. A low pH (<7.0) plays an important role for the end product resulting from the denitrification since an increased amount of nitric oxides will be produced when the pH declines.

Temperature

Denitrification can occur in the range of 5 to 60°C (Loehr, 1984). It was shown that the nitrate removal rate was very slow at 5 to 10°C (Dawson and Murphy, 1972) and the optimal temperature for denitrification was around 35°C (Chen and Lin, 1993).

Carbon source and C/N ratio

When wastewater lacking a suitable source of organic carbon and/or with a low COD/N ratio is treated for biological nitrogen removal, carbon from an external source must be added for a complete denitrification. Various organic carbons and organic loadings for denitrification have been studied, and they include ethanol (Schügerl, 1989; Chang et al., 1993), methanol (Nurse, 1980; Timmermans and van Haute, 1983; Nyberg et al., 1992), and acetate or acetic acid (Wilderer et al., 1987; Almedia et al., 1995; Rahmani et al., 1995; Mohseni-Bandpi et al., 1999). Other kinds of carbon source are also tested, such as waste sludge (Min et al., 2002), sludge hydrolysate (Kristensen and Jørgensen, 1990; Barlindhaug and Odegaard, 1996; Aravinthan et al., 2001) and industrial effluent (Bernet et al., 1996).

The specific denitrification rate and the growth rate of denitrifiers are dependent on the type of carbon source. Henze (1989) compared the denitrification rates using several organic carbon sources, and found that the denitrification rate with raw wastewater was about one third of the rate obtained with acetic acid or methanol. Tam et al. (1992) also reported that acetate gave the highest denitrification rate, followed by methanol and glucose. An immediate increase in denitrification rate was observed as an immediate response to acetate addition (Tam et al., 1992; Isaacs et al., 1994; Hallin et al., 1996). Christensson et al. (1994) carried out a comparison study using methanol and ethanol, and stated that ethanol was more readily available carbon source for denitrification than methanol. The growth rate of denitrifiers with ethanol was 2-3 times higher than that with methanol.

The amount of COD required to denitrify nitrate was lower for ethanol (3.85 g g^{-1} N) than for methanol (4.45 g g^{-1} N). Constantin and Fick (1997) also demonstrated that ethanol favors the growth of microorganisms, whereas the acetic acid provided a higher specific denitrification rate. The denitrification rate with hydrolysate obtained from biologically hydrolyzed sludge was similar to the rate obtained with acetate, but the hydrolysate generated from thermally or chemically hydrolyzed sludge gave the rates only half the acetate value (Kristensen and Jørgensen, 1990). dos Santos et al. (2004) found that ethanol was the most effective electron donor in the short period as compared to methanol and methane.

COD/N ratio is a critical parameter that determines denitrification rate and the extent of completeness of denitrification. Low COD/N ratios lead to poor or incomplete denitrification (Narkis et al., 1979). The influent with low COD/N ratio is deficient in organic carbon, and the low carbon source level can limit the overall denitrification process. To ensure complete denitrification a wide range of COD/N ratios can be found in the literature. In practice, the COD/N ratios required for satisfactory or complete denitrification are in the range of 4-15 g COD g^{-1} N and a minimum ratio of 3.5-4 is recommended (Henze et al., 1989; Nyberg et al., 1992; Christensson et al., 1994).

The accumulation of nitrate will occur due to incomplete denitrification caused by low COD/N ratio in the influent. An increase in the COD/N ratio would increase the efficiency of denitrification under the same oxygen partial pressure in the air phase (Watanabe et al., 1995). However, excessive addition of organic carbon or too high COD/N ratio results in increased operational cost and a high COD level in the effluent that causes the secondary pollution.

2.3.3 Nitrification-Denitrification Processes

A biological nitrogen removal process involves an aerobic zone in which biological nitrification occurs and anoxic zone or period to facilitate biological denitrification (Metcalf and Eddy, 2003). The suspended growth biological nitrogen-removal process can be categorized as single-sludge and two-sludge process.

2.3.3.1 Single-sludge nitrification and denitrification processes

Single-sludge systems for nitrogen removal basically combine carbonaceous removal, ammonia oxidation, and nitrate reduction within the same process. Single-sludge nitrification-denitrification processes were first developed and applied in the 1960s (U.S.EPA, 1993). Since then, as the stringent regulations on nitrogen discharge become more and more prevalent in various countries, increased efforts have been made to develop or modify versions of single-sludge process. Single-sludge systems have been developed with various combinations of single or multiple anoxic zones, oxidation ditches, sequencing batch reactors, and cyclical aeration systems. Nitrification and denitrification can be achieved in single-sludge system by arranging an anoxic zone in the aeration tank or through intermittent aeration (Bernet et al., 1996).

Single-sludge systems offer several advantages over multiple-sludge systems or separate-stage systems. Without intermediate clarifiers or separate denitrification units, there is potential cost advantage for single-sludge system. Single-sludge system has advantages such as less space requirements, reduction in alkalinity consumption, use of wastewater carbon as a carbon source for denitrification and low oxygen requirements. In addition, single-sludge systems are more readily used in retrofitting existing activated sludge plants for nitrogen removal. Potential limitations of single-sludge systems compared to separate stage systems include greater sensitivity to toxicity or inhibition without a separate upstream step, lower nitrogen removal efficiency, and high energy usage.

According to whether the anoxic zone is located before, after or within the aerobic nitrification zone, single-sludge systems are classified into preanoxic, postanoxic and simultaneous nitrification-denitrification (SND) processes (Metcalf and Eddy, 2003). In the preanoxic configuration, nitrate produced in the aerobic zone is recycled to the preanoxic section (Fig. 2.2a). Postanoxic process can be operated with or without an external carbon source (Fig. 2.2b). The denitrification rate would be slow without an external carbon source, with the process relying on the endogenous respiration of the activated sludge to provide electron donor. In this process, a long retention time would be required for high nitrate-removal efficiency. Single-tank reactor has also been used in which nitrification and denitrification occur simultaneously (Fig. 2.2c). The SND applications require DO control or other type of control methods to assure that both nitrification and denitrification occur in a single tank.

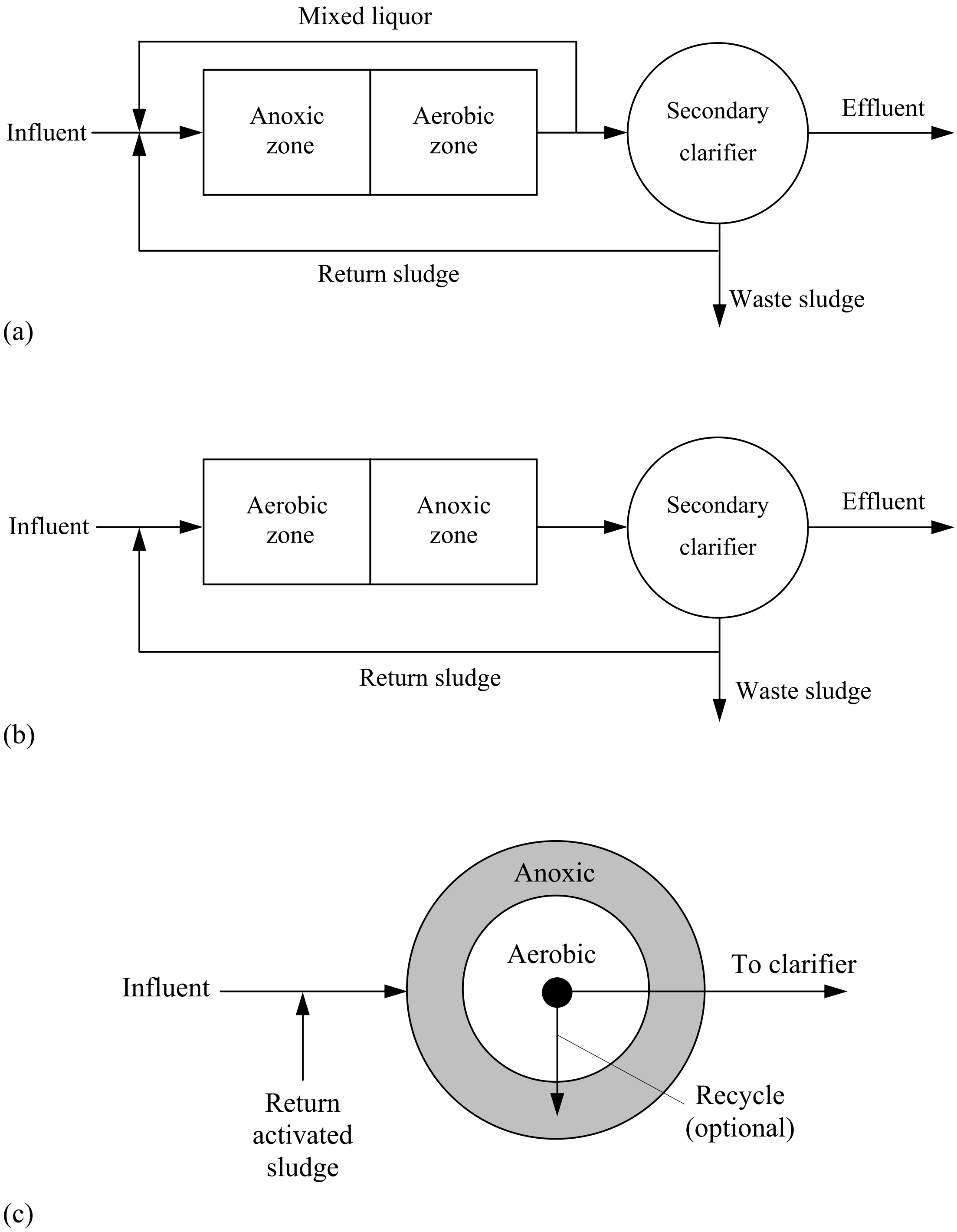

Figure 2.2 Single-stage sludge systems for nitrogen removal: (a) preanoxic, (b) postanoxic, (c) simultaneous nitrification and denitrification (Metcalf and Eddy, 2003)

Intermittent/Cyclical aeration process

Alternating anoxic/aerobic sequences are achieved by cycling the aerators on and off. This type of intermittent aeration in an activated sludge facility is termed cyclical nitrogen removal (CNR). CNR process can be effectively applied at existing plants, requiring only minor process modification. Long SRT system, like oxidation ditch processes may apply intermittent aeration to accomplish both nitrification and denitrification in a single tank. During the aeration-off period, the aeration tank operates essentially as an anoxic reactor as nitrate is used instead of DO for organic carbon removal. During the anoxic period, the tank operation is similar to a preanoxic tank because influent COD is added to drive the denitrification. The time for the anoxic and aerobic period is important to the system's performance and this intermittent operation can be controlled using oxidation-reduction potential (ORP) measurements.

Sequencing Batch Reactor (SBR) process

In the SBR process, nitrate removal can be accomplished by three methods: i) nitrate reduction by using a mixed non aerated fill period, ii) cycling aeration on/off during the react period, and iii) operating at low DO concentration to stimulate SND (Metcalf and Eddy, 2003). Denitrification can also occur during the settle and draw period (U.S.EPA., 1993). One of the primary features of SBR technology is the flexibility to exercise control as a function of time rather than space. SBR systems have many advantages compared to conventional activated sludge systems such as reduced operational costs, improved nitrogen removal and less bulking, and the flexibility in adjustment of sequence of treatment cycles (Wilderer et al., 2001).

2.3.3.2 Separate-stage nitrification and denitrification processes

Compared with single-stage process, a main feature of separate-stage system is the good protection against toxicants, thus effluent with low ammonia concentration is achievable. Separate system might be more desirable when effluent filtration is necessary to meet stringent effluent criteria.

2.4 Poly-β-hydroxybutyrate (PHB)

Activated sludge processes are highly dynamic with respect to the feed regime especially when use is made of SBR process. Biomass in SBR is subject to unbalanced conditions, such as substrate feast and famine as well as alternating aerobic and anaerobic periods (as for nutrient removal). Under these transient conditions, a survival strategy adopted by microbial population is to store excess substrate as internal polymers, mainly poly-β-hydroxybutyric acid (PHB), when external substrate is available.

Microorganisms that are able to store substrate and consume the stored substrate exhibit a competitive advantage over organisms without capacity of storage. The stored PHB can be used for the growth and the maintenance when no external substrate is available (Mino et al., 1987; Majone et al., 1998; Beun et al., 2000). Storage of PHB has also been reported in nitrification and denitrification system where microbial population is subject to dynamic conditions with respect to the availability of external substrate, nitrogen and oxygen. In the aerobic period, microorganisms accumulate PHB internally with external substrate, and in the anaerobic period, when there is no external substrate, they use it as internal energy and carbon source for growth and denitrification (Majone et al., 1998; Beun et al., 2000).

In the past years, a number of studies have focused on the storage of soluble substrate as PHB and its subsequent degradation in activated sludge culture (van Niekerk et al., 1987; Mino et al., 1987; Majone et al., 1998; Beun et al., 2000; Dircks et al., 2001).

2.4.1 PHB Storage

Intracellular storage and degradation of PHB may occur under dynamic conditions. In activated sludge culture, it has been reported that 70% of the amount of substrate consumed in the feast period is used for synthesis of PHB, and the remainder is used for growth processes (Beun et al., 2000). A mixed culture selected under aerobic conditions and intermittent feed was able to take up acetate (92 mg COD g^{-1} COD h^{-1}) and store it as PHB (37 mg COD g^{-1} COD h^{-1}) under anoxic conditions (Majone et al., 1998). Furthermore, Dinoisi et al. (2001) showed that the growth response was very little when PHB storage was prevailing. It appears that the storage phenomenon would play a role in the design and control of processes for carbon and nitrogen removal, like pre/post-denitrification with external addition of readily biodegradable COD.

Third et al. (2003a) have investigated the effect of oxygen on microbial conversion of COD to PHB. They found that oxygen limitation was necessary for optimum PHB yields with minimum biomass growth, while an oversupply of oxygen could compromise PHB production. It is accepted in general that limiting the microbial growth rate can enhance PHB synthesis. Limited oxygen supply offers a possibility of slowing down the growth and enhancing PHB synthesis. By slowing down these reactions that compete with PHB synthesis for acetyl-CoA, a larger portion of acetyl-CoA will flow into PHB.

The storage phenomenon is also related to the substrate fed to the systems (Majone et al., 2001; Beccari et al., 2002; Dionisi et al., 2004a, b). According to Beccari et al. (2002), the PHB storage was an important removal mechanism when the substrate was acetate or ethanol, while no appreciable formation of storage compounds was detected using glutamic acid, while only negligible amount of PHB was found with raw and filtered wastewater. On the contrary, Dionisi et al. (2004a) reported the PHB storage from glutamic acid as the sole carbon source, e.g. the average rates of glutamic acid uptake and PHB storage was 53 and 5.4 mg COD g^{-1} COD h^{-1} with a corresponding storage yield of 0.10 (mg COD mg^{-1} COD) and PHB accounted for 16% of overall solids formed from glutamic acid.

2.4.2 PHB Degradation

Shift from a readily biodegradable substrate to the slowly degradable PHB (36-16 mg COD g^{-1} COD h^{-1}) was associated with a sharp decrease of nitrate uptake rate under anoxic conditions (Majone et al., 1998). It has been believed that the degradation rate of PHB is dependent on the level of PHB initially stored/obtained in the cells at the end of feast phase (Meinhold et al., 1998; Dircks et al., 2001). Among the kinetics of PHB degradation, multiple order kinetics gives a good description of the rate of PHB degradation (Dircks et al., 2001).

2.4.3 PHB as an Internal Carbon Source for Denitrification

Khan and Hiraishi (2001) identified a new denitrifying bacterium capable of degradation of PHB aerobically and anaerobically. A high denitrification rate of 19 mg NO_3^--N g^{-1} h^{-1} was found with poly(3-hydroxybutyrate-co-hydroxyvalerate) (PHBV) as the electron donor. Beun et al. (2001) also looked into the effect of storage and degradation of PHB on the denitrification process by performing a simulation in which the PHB storage and degradation was inactive for one full cycle in the steady-state SBAR, and they found that no denitrification occurred in the process without PHB storage and degradation, and denitrification occurred in the simulation with PHB process.

Mergaert et al. (2001) isolated a collection of heterotrophic bacteria from a continuous-upflow fixed-bed reactor for the denitrification of drinking water in which PHBV granules acted as biofilm carrier, carbon source and electron donor. They found that the majority of the isolates were Gram negative bacteria, and most of them were able to utilize 3-hydroxybutyrate as sole carbon source for denitrification. In addition, Majone et al. (1998) also reported a denitrification rate of 3-10 mg N g^{-1} VSS h^{-1} on the stored PHB, which is compared to the denitrification rate of 20 mg N g^{-1} VSS h^{-1} with acetate obtained in anoxic batch experiments. Studies by Third et al. (2003b) have shown PHB metabolism in mixed cultures and its relation to SND and nitrogen removal.

These seem to indicate that the nature of PHB slow-degradation makes it a suitable substrate for SND since ammonia oxidation can proceed at a similar rate to PHB oxidation (for denitrification) at low DO less than 1.5 mg l^{-1}. However, the reported amount of NO_3^--N denitrified with internally stored PHB is less than 2 mmol N l^{-1} (Majone et al., 1998; Third et al., 2003b; Zeng et al., 2003).

2.5 Biofilms and Granular Sludge

Cell immobilization technology has been developed for industrial and municipal wastewater treatment. Biofilm and granular technologies are briefly reviewed in the following section.

2.5.1 Biofilms

Biofilms have been successfully used in water and wastewater treatment for over a century (Atkinson, 1981). The main advantages of biofilm processes are their ability to increase biological reaction rates through accumulation of high concentration of active biomass and the high resistance of this attached biomass to overloading and toxic compounds (Lazarova and Manem, 1994). Numerous laboratory, bench scale, and industrial studies have consistently illustrated the technical advantage of biofilms (Heijnen et al., 1991; Liu and Capdeville, 1996; Rusten et al., 1997).

2.5.1.1 Biofilm formation

The formation of biofilms is a multiple-step process, and physicochemical and biological factors are involved (Daniels, 1980; Calleja, 1984; van Loosdrecht, 1987; Liu, 1995; Liu and Wang, 1996). The biofilm formation could be roughly described as a four-step process (Liu and Tay, 2001b):

Step 1 Physical movement to transport cells from liquid to carrier surface. The following forces are generally involved in this step: hydrodynamics; diffusion; gravity; thermodynamic force, such as Brownian movement and cellular mobility.

Step 2 Initial attractive forces to retain cells on the support surface and further to promote stable multicellular contacts. Those attractive forces are as follows:

(a) Physical forces: van der Waals forces; opposite charge attraction; thermodynamic forces including free energy of surface; surface tension and hydrophobicity.

(b) Chemical forces: hydrogen bonds; formation of ionic pairs; formation of ionic triplet.

Step 3 Microbial forces to make immobilized cells mature; production of exopolymers, such as exopolysaccharides etc; growth of cellular cluster; metabolic changes and genetic competence induced by the environment, which facilitate and further strengthen the cell-cell interaction, and result in the high density of adhering cells.

Step 4 Steady state three-dimensional structure of immobilized cells shaped by hydrodynamic conditions. The biofilm would be finally shaped by hydraulic shear force to form a certain structured community, however extreme high shear force would result in serious disaggregation and inhibition of development of biofilms.

Tijhuis et al. (1994) reported the hydraulic retention time should be shorter than the inverse of the maximum growth rate of the suspended bacteria in order to obtain a predominant formation of stable biofilms with high biomass concentration, i.e. at a long hydraulic retention time, a low amount of attached biomass as patchy biofilm can be observed on the carrier material. It appears that a higher surface loading determines only the amount of biomass on the biofilm particles, i.e. increasing the thickness of biofilms.

It had been proposed that biofilm structure would be regulated by the interaction of growth and detachment forces (van Loosdrecht et al., 1995; Liu et al., 2003c). The higher detachment forces on the biofilm lead to thinner and denser biofilms, while a higher substrate loading rate leading to a higher biomass production rate more easily balances the detachment forces resulting in a thicker biofilms (Kwok et al., 1998; Liu and Tay, 2001b). Both increases of biomass production rate and decreases of detachment forces would decrease the biofilm density and biofilm shape factor. It seems that smooth and stable biofilms will be obtained with the right balance between the biofilm surface loading and detachment forces.

2.5.1.2 Factors affecting the formation of biofilms

The specific substrate loading rate directly determines the growth rate of biofilm (van Loosdrecht et al., 1995). Increasing the substrate loading rate on the biofilm would lead to a heterogeneous biofilm with large protuberances. Consequently, the biofilm thickness is expected to increase because of an increase in surface substrate loading (Tijhuis et al., 1996). At higher detachment forces, the biofilm would become denser and more concentrated. Simultaneously, protuberances would be removed by high shear leading to a smoother biofilm. If detachment forces are reduced, the biofilm becomes more heterogeneous with numerous pores and protuberances (Rittmann et al., 1992; van Loosdrecht et al., 1995; Kwok et al., 1998; Liu et al., 2003c). Although substrate concentration and applied shear force has been shown to be perhaps the most important determinants, nutrient composition can affect biofilm structure as well. Ohashi et al. (1995) has shown that the prevailing C/N ratio influences the structure of a multispecies biofilm composed of nitrifying bacteria and heterotrophs. The proportion of nitrifiers in biofilms decreased with increasing the substrate C/N ratio.

2.5.2 Anaerobic Granulation

The feasibility and efficiency of anaerobic granulation technology for removing high-strength wastewater has been substantially reported (Lettinga et al., 1980, 1984; Alphenaar et al., 1993; Fang and Chui, 1993; Schmidt and Ahring, 1996; Tay and Yan, 1996). In several decades, anaerobic granulation technology has been successfully applied to treatment of a wide variety of industrial and domestic wastewater. Anaerobic granulation is a complex process in which a number of physicochemical and biological parameters are involved. Thus, a comprehensive understanding of mechanisms responsible for anaerobic granulation is necessary for environmental engineer to design and operate granular sludge-based treatment systems.

2.5.2.1 Mechanisms and models for anaerobic granulation

An overview of the mechanisms and models proposed for anaerobic granulation in the past decades is provided in this section.

Physico-chemical models

In a thermodynamic sense, when one bacterium approaches another, the interaction between them includes repulsive electrostatic force, attractive van de Waals force, and repulsive hydration interaction (Parsegian and Rand, 1991). Based on the thermodynamics, some thermodynamic models have been developed which are also reviewed in the following.

1. Inert nuclei model

Lettinga et al. (1980) proposed that the inert microparticles in an UASB reactor, to which anaerobic bacteria could attach to form the initial embryonic granules, could initiate the sludge granulation by acting as a precursor or nucleus. The mature granules can be further developed through the growth of these attached bacteria. Experiments demonstrated that addition of such particles into inoculated sludge effectively promotes the formation of anaerobic granules (Hulshoff Pol, 1989; Wirtz and Dague, 1996).

2. Selection pressure model

The basis of anaerobic granulation had been thought to be a continuous selection of sludge particles occurred in the reactors, i.e. light and dispersed particles would be washed out, while heavier components remained in the system (Hulshoff Pol et al., 1988). The microbial aggregation in UASB reactors is suggested to be an effective protection response against the selection pressure, which is represented generally by upflow flow pattern. A certain intensity of hydraulic selection pressure seems to be necessary for the formation and development of anaerobic granules and a relatively higher selection pressure may be in favor of fast production of granules (Alphenaar et al., 1993; Arcand et al., 1994; O'Flaherty et al., 1997).

3. Multi-valence positive ion-bonding model

It is found that introducing multi-valence positive ions, such as calcium, ferric, aluminum or magnesium ions, into seed sludge would expedite processes of anaerobic granulation due to reduction of the electrostatic repulsion between negatively charged bacteria. Addition of Ca^{2+} with concentration of 80-200 mg l^{-1}, Mg^{2+} of 12-120 mg l^{-1} or Al^{3+} of 300 mg l^{-1} was reported to promote anaerobic granulation in UASB reactors (Schmidt et al. 1993; Teo et al. 2000; Yu et al. 2001a). However, extremely high calcium concentration of over 500 or 600 mg/l was proved to be detrimental to the process (Guiot et al. 1988; Thiele et al. 1990; Yu et al. 2001b).

4. ECP bonding model

The extracellular polymers (ECPs) can mediate both cohesion and adhesion of cells, and act a crucial role in maintaining structural integrity of microbial matrix, and the metabolic blocking of exopolysaccharides synthesis would prevent microbial aggregation (Schmidt and Ahring, 1994; Cammarota and Sant'Anna, 1998). It had been found that ECPs could modify the surface negative charge of the bacteria, and further bridge two neighbor cells physically as well as with other inert particulate matters (Shen et al., 1993; Schmidt and Ahring 1994, 1996).

5. Synthetic and natural polymer-bonding model

The synthetic polymers can be applied to expedite the development of UASB granules. In fact, freely moving polymeric chains may form a bridge between cells, and this would facilitate the formation of initial microbial nuclei, which is the first step towards granulation. It appears that both synthetic (El-Mamouni et al., 1998) and natural polymers (Kalogo et al., 2001) can assist anaerobic bacteria to aggregate together and further form granules rapidly.

6. Secondary minimum adhesion model

This model is based on the well-known DLVO theory for colloidal particles, which shows that reversible adhesion takes place in the secondary minimum of the DLVO free energy curve. The Gibbs energy of reversible adhesion is relatively small, and there is always a separation distance between two adhering bacteria. Consequently, this reversible adhesion can be converted to irreversible adhesion at the primary minimum by overcoming the energy barrier or by protruding fibrils or fimbriae, which bridge the gap between bacteria (Rouxhet and Mozes, 1990). Microstructural analysis of the UASB granules supports this separation distance between bacteria at the secondary minimum.

7. Local dehydration and hydrophobic interaction model

It has been believed that under normal physiological conditions strong hydration repulsion is the main force to keep the cells apart. This model as proposed by Wischut and Hoekstra (1984) shows that when bacteria are strongly hydrophobic, irreversible adhesion will occur. Theoretically, increasing the hydrophobicity of cell surface would lead to a corresponding decrease in the excess Gibbs energy of the surface, which in turn promotes cell-to-cell interaction and further serves as driving force for cell self-separation from liquid phase (van Loosdrecht et al., 1987; Rouxhet and Mozes, 1990). There is experimental evidence showing that the hydrophobicity plays a crucial role in initiating anaerobic granulation (Lettinga et al., 1980; Hulshoff Pol et al., 1988; Wu et al., 1991; Tay et al., 2000a, b).

8. Surface tension model

According to the thermodynamic theory, microbial granulation is a process to create a new interface, granule-liquid by disrupting preexisting individual bacteria-liquid interface, and a molecular contact between the two adhering bacteria surface is involved. The free energy of adhesion (ΔG_{adh}) can be expressed as follows (Rouxhet and Mozes, 1990):

$$\Delta G_{adh} = 2(r_c^{1/2} - r_l^{1/2})(r_l^{1/2} - r_s^{1/2}) \qquad (2.15)$$

in which r_c is the surface free energy of bacteria, r_1 is the surface free energy of liquid and r_s is the surface free energy of inert particle. This equation shows that if the surface free energy of bacteria is lower than that of the liquid, the free energy of aggregation decreases and aggregation is favored with decreasing surface energy of the inert carrier. The opposite trend would occur if the surface energy of bacteria is greater than that of the liquid. This thermodynamic equation is a theoretical basis of the surface tension model.

In an UASB reactor, it was found that the aggregation of hydrophilic cells was enhanced at low liquid surface tension, while the opposite was true for hydrophobic cells (Thaveesri et al., 1995). Depending on the liquid surface tension (γ) in the UASB reactor, bacteria may grow in rather loose associations, in multi-layered granules ($\gamma < 50$ mN m^{-1}), or in mixed agglomerates ($\gamma > 56$ mN m^{-1}) (Thaveesri et al., 1995; Grootaerd et al., 1997)

The above physico-chemical models only contribute one or two factors to the initial granulation process in UASB reactor. However, granulation process involves too many changes and unidentified components to allow one to build up a pure thermodynamic model with confidence (Parsegian and Rand, 1991). There were some reported observations conflicting with the above models. Opposite to the inert nuclei model, anaerobic granules could be developed even without the existence of any inert materials (Thiele et al., 1990). As to the multi-valence positive ion-bonding model, some studies showed that calcium ion did not make any contribution to sludge granulation (Guiot et al., 1988) and a high concentration of magnesium ion would cause disaggregation of granules (Schmidt and Ahring, 1993).

In the local dehydration and surface tension models, bacterial granulation is oversimplified to a purely thermodynamic process, and such a simple description is usually not adequate since microbial aggregation is a very complex biological phenomenon. The physico-chemical forces alone do not appear to interpret the overall microbial process of granulation thoroughly.

Structural models

1. Capetown's model

Similar to the polymer-bonding model as reviewed previously, the Capetown model suggests that under the conditions of high hydrogen partial pressure and limited cysteine, the overproduction of amino acid (except cysteine) would induce the ECP formation to enmesh *Methanobacterium* strain AZ and other bacteria, thus leading to the formation of granules (Palns et al., 1987; Sam-Soon et al., 1988). This model suggested that ECP (mainly long chain polypeptides) is secreted by *Methanobacterium* strain AZ, a hydrogen-utilizing methanogen.

2. Spaghetti model

This model shows that in the initial development of the UASB granules, filamentous Methanosaeta firstly attach on precursors, and then through a branched-growth process form a three dimensional network, where other bacteria could be easily entrapped in this network (Wiegant, 1998; Sanchez et al., 1994; Wu et al., 1996). The structured aggregates would further grow due to cellular multiplication of the entrapped bacteria, and become denser and spherically shaped by the hydrodynamic shear force created by upflow liquid and biogas. It must be emphasized that in the spaghetti model, the formation of the structured aggregate is a decisive step in the overall granulation process.

3. Syntrophic microcolony model

Many types of anaerobic bacteria are involved in the biodegradation process, thus they have to live in a close synergistic relationship where products such as hydrogen and other intermediates can be efficiently transferred among the respective bacteria groups. The syntrophic model suggests that the above requisites would eventually lead to the formation of stable microcolonies or consortia, i.e. initial granules (Hirsh, 1984). The close packing of bacteria in granule architecture definitely facilitates the exchange of metabolites.

4. Multi-layer model

According to a multi-layer model proposed by MacLeod et al. (1990) and Guiot et al. (1992), the microbiological composition of granules differs within each layer. The inner layer mainly consists of methanogens that may act as nucleation centers necessary for the initiation of granules. Hydrogen-producing and hydrogen-utilizing bacteria are dominant in the middle layer, and a mixed species including rods, cocci, and filaments takes major part in the outmost layer. The layered structure was supported by the experimental work of Arching et al. (1993) and Lens et al. (1995).

All the above structure models may explain some phenomena during the sludge granulation process under specific laboratory conditions. However, similar to the physico-chemical models, each structural model considers only the role of one or two leading factors usually exerting their impact under specific environmental conditions and in specific steps during the whole granulation process. The experimental results often contradict these models. According to the Capetown's model, anaerobic granulation would not occur in UASB reactors treated with acetate, propionate, or butyrate because of inadequate hydrogen partial pressure. However, there is experimental evidence that anaerobic granules formed in UASB systems fed with those above-mentioned organics (Arching et al., 1993; van Lier et al., 1995). Contrary to the multi-layer model, anaerobic granules with non-layered structure were also reported (Grotenhuis et al., 1991; Fang et al., 1995; Wu et al., 2001). Any individual structural model cannot explain a spontaneous and sudden washout of the established granular sludge bed as a result of a change in wastewater composition, which is commonly encountered in the operation of the UASB systems.

Proton translocation-dehydration model

The previous works demonstrated the essentials of proton translocation concept that (1) the hydrophobic interaction of a considerable extent was closely related to the initiation of bacteria adhesion; (2) the proton conductance across a bacterial surface could induce surface dehydration; and (3) the proton translocating activity could induce the protonation of bacteria cell surfaces. Based on these observations and a consideration of the proton translocating activity on bacterial membrane surfaces, a new proton translocation-dehydration theory for molecular mechanism of sludge granulation was proposed and proved by experiments (Tay et al., 2000b; Teo et al., 2000). The overall sludge granulation process is initiated by the dehydration of bacteria surfaces, followed by embryonic granule formation, granule maturation, and post-maturation.

2.5.2.2 Major factors affecting the formation of anaerobic granulation

The granulation processes were affected by a number of environmental conditions that are essential for enhanced and fast formation of anaerobic granules, thus efficiency and good performance of the whole system.

Upflow liquid velocity and hydraulic retention time (HRT)

Hydraulic stress is crucial for the development of anaerobic granulation. A short HRT combined with a high liquid upflow velocity could cause washout of non-competent bacteria in granulation and further promote sludge granulation (Alphenaar et al., 1993). In contrast, a long HRT accompanied with a low upflow liquid velocity may allow dispersed bacterial growth and be less favorable for granulation. Hulshoff Pol et al. (1988) have explained the effects of upflow liquid velocity on anaerobic granulation by the selection pressure theory.

Organic loading rate (OLR)

In a microbial sense, a low OLR means that the microorganisms in the reactor are starved, while a high OLR would ensure a fast microbial growth. Practically, granulation is accomplished by gradually raising the OLR during start-up period (Holshoff Pol et al., 1989; Kosaric et al., 1990; Tay and Yan, 1996). Conversely, a high OLR tends to reduce granular strength and weaken the structural integrity of granules (Quarmby and Forster, 1995).

Characteristics of substrate/wastewater

Characteristics of the substrate have been considered a key factor determining the formation, composition and microstructure of anaerobic granules. In UASB reactors the existence of high-energy substrate in the influent would benefit process of sludge granulation, and the complexity of substrate may exert a selection pressure on microbial diversity in anaerobic granules.

Characteristics of seed sludge

The quality of particular seed sludge can be judged in terms of ash content, the SMA and the settleability. Microbial species differ in their capability of aggregation, and some species are more competent for aggregation, while others are less under the same operation condition. It is reasonable that anaerobic granulation could be expedited simply by manipulating the composition of the seed sludge.

Reactor pH

Microbial species involved in anaerobic reaction could be roughly divided into three categories: (1) bacteria responsible for hydrolysis; (2) acid-producing bacteria; (3) methane-producing bacteria (Guiot et al., 1992; Bitton, 1999). Acid-producing bacteria can tolerate a low pH with an optimal pH of 5.0-6.0, but most methane-producing bacteria function optimally within a quite narrow pH range of 6.7-7.4 (Bitton, 1999). Researches indicated that the granular structure was relatively stable at pH between 5.5 and 8.0, and beyond this range, either high or low pH condition would cause a decrease in granule strength and result in a failure of system.

Reactor temperature

Any biological system is sensitive to changes in temperature because involved bacteria and reaction are closely related to certain temperature. The growth of methanogens would be seriously slowed down when the temperature is below 30°C. However, at extremely high temperature most bacteria would lose their metabolic activity. Thus, mesophilic UASB reactors are generally operated at a temperature range of 30-35°C.

2.5.3 Aerobic Granulation

The anaerobic granulation technology has some drawbacks, such as a relative long start-up period, high operation temperature, inapplicable for low-strength organic wastewater and nutrient removal. To overcome these weakness, research has been devoted to the development of aerobic granulation technology. After Mishima and Nakamuar (1991) first cultivated aerobic granules with diameter of 2 to 8 mm in a continuous aerobic upflow sludge blanket (AUSB) reactor, many researchers have put their interests on aerobic granulation in sequencing batch reactors (SBR) with respect to fundamentals as well as applications (Morgenroth et al., 1997; Beun et al., 1999; Peng et al., 1999; Etterer and Wilderer, 2001; Tay et al., 2001a, b, c; Liu and Tay, 2002).

Aerobic granules have been studied to treat high-strength wastewater, to remove organics and nutrients, to clarify recalcitrant toxic compounds (phenol, etc.) and to treat wastewater with particulate organic matter (Moy et al., 2002; Tay et al., 2002a, c; Lin et al., 2003; Jiang et al., 2004a, b; Schwarzenbeck et al., 2004; Tay et al., 2004b; Yang et al., 2004a; Tay et al., 2005a, b). Aerobic granulation is a gradual process during which a number of environmental factors or operational conditions are required. The main operational parameters involved and characteristics of aerobic granules are reviewed here based on current knowledge so far.

2.5.3.1 The influences of operational factors on aerobic granulation

Hydrodynamic shear force

In a bubble column or airlift SBR, hydrodynamic shear force is mainly created by aeration that can be described with the upflow air velocity. A high shear force appears to favor the formation of aerobic granules (Shin et al., 1992; Beun et al., 1999; Tay et al., 2001a). Tay et al. (2001a) found that only fluffy flocs were observed in the SBR operated at a low superficial air upflow velocity of 0.8 cm s^{-1}, while regular-shape granules were successfully developed at a high shear superficial air velocity of 2.5 cm s^{-1}. Beun et al. (1999) also observed that a low superficial air velocity did not lead to the formation of aerobic granules in SBR; however, at a relatively high superficial air velocity, smooth, dense and stable granules formed due to the high shear strength. The granule density and strength were also proportionally related to the shear force applied (Tay et al., 2004c). Shin et al. (1992) stated that the granulation was governed by the physical stress on granular sludge. These results may imply that the structure of aerobic granules is mainly determined by the hydrodynamic shear force present in a bioreactor.

Organic loading rate

Similar to the formation of biofilms, high organic loading rate (OLR) would be unfavorable for aerobic granulation process because of excessive growth in suspended forms. Aerobic granules can form when the COD loading rates ranged from 2.5 to 15 kg COD $m^{-3}\cdot day^{-1}$ (Moy et al., 2002; Liu et al., 2003b). It seems the effect of OLR is not significant on the formation of aerobic granules, but the physical characteristics of aerobic granules are closely related to the OLR (Toh et al., 2003) The mean size of aerobic granules increased from 1.6 to 1.9 mm with the increase in OLR from 3 to 9 kg COD $m^{-3}\cdot day^{-1}$. Morphology in terms of roundness, dry biomass density, specific gravity and sludge volume index (SVI) has not shown close relationship with the applied OLR. In contrast, the physical strength of aerobic granules decreased with the increase of OLR. The comparable low OLR of 0.42-2.1 kg COD $m^{-3}\cdot day^{-1}$ has also been applied to form aerobic granules (Mishima and Nakamura, 1991; Morgenroth et al., 1997). In fact, a higher OLR can raise the biomass growth rate and high growth rate which in turn would reduce the strength of the 3-dimentional structure of the microbial community (Liu et al., 2003c).

Substrate composition

Aerobic granules have so far been cultivated with a variety of substrates including glucose, acetate, ethanol, phenol, yeast extract and other synthetic wastewater (Shin et al., 1992; Peng et al., 1999; Beun et al., 1999; Tay et al., 2001b, 2004c; Moy et al., 2002; Jiang et al., 2003, 2004a; Yang et al., 2004a). Nitrifying and phosphorus-accumulating granules have also been developed (Lin et al., 2003; Tsuneda et al., 2003, 2004a, b). These, in turn, indicated that the granulation process seemed to be less sensitive to the characteristics of substrate fed to the system; however, the type of carbon source had a profound impact on the selection of microbial diversity, microstructure and elemental composition of mature aerobic granules (Tay et al., 2001b; Liu et al., 2003b). Comparison study on aerobic granules cultivated with glucose and acetate found that the glucose-fed granules had a filamentous structure, while acetate-fed granules exhibited a compact microstructure with no filamentous structure (Tay et al., 2001b, 2004a). More recently, aerobic granules were also successfully developed in lab-scale SBR for treating particulate organic matter-rich wastewater (Schwarzenbeck et al., 2004). It is noted that recalcitrant compounds such as phenol can also be used as sole carbon source to develop aerobic granules, which imply the aggregation of microbial cells into compact aerobic granules might provide an effective protection against toxicity (Jiang et al., 2004a, b; Tay et al., 2004b, 2005a, b).

Hydraulic retention time

During the formation of aerobic granules in SBR, light and dispersed sludge is washed out and heavy granules are retained in the reactor. The SBR cycle time represents the frequency of solid discharge and is related to the hydraulic retention time (HRT) at a given volume exchange ratio. In other words, short SBR cycle time lead to short HRT, vice versa. A short cycle time would suppress the growth of dispersed sludge as it would be washed out frequently. However, extremely short cycle time would result in great sludge loss through hydraulic washout which could not be compensated with the normal bacterial growth rate. In that case, a complete washout of sludge blanket occurs and leads to a failure of microbial granulation. Pan et al. (2004) has investigated the effect of HRT on the stability of mature aerobic granules. They found that the seeded aerobic granules kept stabilized with HRT from 2 to 12 hours, however, a too short HRT of 1 hour triggered complete biomass washout and led to reactor failure. When the long HRT of 24 hours was applied, the seeded aerobic granules were gradually substituted by bioflocs due to low frequency of volumetric exchange. It seems that aerobic granules can be developed at a proper selection of HRT.

Dissolved oxygen

Dissolved oxygen (DO) concentration is an important parameter in the operation of aerobic wastewater treatment system. Aerobic granules can form at the DO concentration as low as 0.7 to 1.0 mg l^{-1} in a SBR (Peng et al., 1999), while aerobic granules were also successfully developed at DO concentrations higher than 2.0 mg l^{-1} (Moy et al., 2002; Tay et al., 2002b).

Feast-famine regime

SBR is operated in a sequencing cycle of feeding, aeration, settling and discharging. In SBR, the aeration period indeed consists of two phases, a degradation phase in which the substrate is depleted to a minimum, followed by an aerobic starvation phase in which the external substrate is no longer available. Thus, it is likely that microorganisms in the SBR are subject to a periodical feast and famine regime, called periodical starvation. Under periodical feast-famine conditions, bacteria would become more hydrophobic, and high cell hydrophobicity in turn facilitates the microbial adhesion and aggregation (Tay et al., 2001b; Bossier and Verstraete, 1996).

McSwain et al. (2004) recently developed an operating strategy to enhance aerobic granulation by intermittent feeding, i.e. different filling times were applied to SBR reactors, resulting in the different degrees of feast-famine period to microorganisms. When bacteria are subject to a periodical feast-famine regime, microbial aggregation would be an effective strategy of cells against starvation. In fact, the periodical feast-famine regime in SBR can be regarded as kind of microbial selection pressure that may alter the surface properties of cell. However, more recent research showed that aerobic granules could not successfully developed if settling time in SBR was not properly controlled even though the periodical feast-famine regime was present (Qin et al., 2004). In addition, when the starvation time in SBR was reduced from 3 hours to about 30 minutes, no significant impact on aerobic granules was observed. These may imply that the periodical feast-famine regime could favour aerobic granulation, but it is unlike to act as a driving force of aerobic granulation. Consequently, the essential role of the periodical feast-famine regime in aerobic granulation is still debatable.

Trace element

Tsuneda et al. (2004a) found Fe added as a trace element to the inorganic wastewater accumulated at the central part of nitrifying granules in an aerobic upflow fludized bed (AUFB) reactor. Their results shows nitrifying granules could be promoted by pre-aggregated sludge using hematite or 5 g m^{-3} of Fe addition to the feed substrate. 1.1 and 1.9% g/g MLSS of Fe was accumulated in their cultivated nitrifying granules.

Furthermore, the accumulation of Fe was observed at the central part of the granule, whereas no accumulation was detected at the surface of the granule. Based on these results, they proposed a nitrifying granulation mechanism. 1) As a first step, nitrifying bacteria aggregate to form the cores of granules; 2) As a second step, the aggregates grow up to a spherical or elliptical form due to multiplication of the nitrifying bacteria and moderate shear stress in the reactor, and then mature nitrifying granules are produced.

Reactor configuration

So far, almost all aerobic granules were produced in column SBR. The flow pattern of liquid and microbial aggregates in the reactors was determined by reactor configuration (Beun et al., 1999; Liu and Tay, 2002). Obviously, hydrodynamic flow pattern in column-type reactor are completely different from flow pattern in completely mixed tank reactor (CMTR). In column SBR air flow is subject to an upflow pattern. The air or liquid upflow pattern in column reactor can create a relatively homogenous circular flow and localized vortex along the reactor height, and microbial aggregates are constantly subject to such a circular hydraulic attrition. The circular flow could force microbial aggregates to be shaped as regular granules that have a minimum surface free energy (Liu and Tay, 2002). In column SBR a higher ratio of reactor height to diameter (H/D) can ensure a circular flowing trajectory, which in turn creates a more effective hydraulic attrition to microbial aggregates. On the other hand, a high H/D ratio would facilitate selection of microbial aggregates by the difference in settling velocities of microbial aggregates.

2.5.3.2 Characteristics of aerobic granules

Aerobic granules are very much different from the fluffy, irregular and loose-structured conventional activated sludge flocs in following aspects:

- Round and regular shape with a clear and smooth outer surface;
- Dense and compact microbial structure;
- Large enough to be visible as separate entities in the mixed liquor during mixing and settling phase;

- Excellent settleability to ensure a fast and easy liquid-solid separation;
- High biomass retention;
- Ability to withstand high organic loading rates;
- Ability to resist the toxicity of recalcitrant chemicals and heavy metals in wastewater.

Size and morphology

The morphology of aerobic granules is completely different from the floc-like sludge as shown by microscopic examination. Aerobic granules exhibit regular and nearly spherical shape with a clear outline (Peng et al., 1999; Tay et al., 2001b; Moy et al., 2002). The size and roundness are two important parameters used to describe the magnitude and morphology of aerobic granules. In general the average diameter of aerobic granules varies in the range of 0.2 to 5 mm and the roundness in terms of aspect ratio is above 0.6. Both average diameter and roundness are the balanced outcomes between growth and abrasive attachment generated from hydrodynamic shear force (Liu and Tay, 2002). Thus they vary greatly with respect to substrate composition, organic loading rate, shear force and other operational conditions.

Settleability

The settleability is a prominent quality distinguishing aerobic granules from conventional activated sludge. The settleability can be quantified by sludge volume index (SVI) and the settling velocity. The SVI of aerobic granules mostly falls in the range of 50 to 100 ml g^{-1}, sometimes even lower than 50 ml g^{-1}, which is much lower than that of conventional bioflocs (Mishima and Nakamura, 1991; Peng et al., 2001; de Kreuk and van Loosdrecht, 2004). From an engineering point of view, the improvement of settleability upon the formation of aerobic granules makes it possible that solid-liquid separation can finish in a more compact clarifier with ease and efficiency. According to Stokes equation, granule size, specific gravity and structure determine the settling velocity of aerobic granules. The settling velocity of aerobic granules can be as high as 30 to 70 m h^{-1}, which is comparable with that of anaerobic granules in UASB, and is several times higher than that of activated sludge flocs (typical settling velocity of around 8 to 10 m h^{-1}) (Shin et al., 1992; Etterer and Wilderer, 2001; Tay et al., 2002b; Toh et al., 2003).

Density and strength

The specific gravity of aerobic granules typically ranges from 1.004 to 1.065 (Etterer and Wilderer, 2001; Tay et al., 2001b; Tsuneda et al., 2003). The granules with a high physical strength withstand high abrasion and shear. The physical strength, expressed as integrity coefficient (i.e., the ratio of residual granules to the total weight of the granular sludge after 5 min of shaking at 200 rpm on a platform shaker, expressed as percentage), is higher than 95% for the glucose and acetate-grown granules (Tay et al., 2002b). The physical strengths of aerobic and anaerobic granules are comparable. Small aerobic granules tend to be more compact than large ones (Toh et al., 2003).

Cell surface hydrophobicity

Cell surface hydrophobicity is an important affinity force in cell self-immobilization and attachment processes (Pringle and Fletcher, 1983; Liu et al., 2003d). The role of cell surface hydrophobicity in the formation of aerobic granules has not been clear. Liu et al. (2003d) have investigated the role of the cell surface hydrophobicity in the formation of aerobic granules. Their study found that the hydrophobicity of granular sludge is two time higher than that of conventional bioflocs. A high shear force resulted in an increase in the cell surface hydrophobicity (Tay et al., 2001a). The specific surface hydrophobicity was found to increase as granule size increased (Toh et al., 2003), however the changes in influent substrate concentration from 500 to 3000 mg COD l^{-1} seems not to have much influences on the cell surface hydrophobicity of aerobic granules (Liu et al., 2003b).

2.6 Summary

Aerobic granulation process is a novel biotechnology recently developed for wastewater treatment. Some research had been done in this area; however, it is still lacking a sound understanding of the mechanisms responsible for aerobic granulation, which is a prerequisite for environmental engineers and scientists to design large scale aerobic granules-based bioreactors. The experiences from biofilms and anaerobic granulation process imply that selection pressure would play a crucial part in aerobic granulation as well. So far, little is known about the role of selection pressure in the formation of aerobic granules. An understanding of the essential role of the settling time on the development of aerobic granules should be helpful for the application of this technology.

A number of aerobic processes have been developed for the treatment of wastewater containing organic carbonaceous and nitrogenous compounds. In traditional nitrogen removal processes, long detention time and large reactor volume are required because of the low growth rate of nitrifying bacteria. As nitrifying bacteria are sensitive to the environmental conditions, shock loadings or other environmental changes often result in reducing nitrogen removal efficiency or even process failure. High biomass retention of nitrifying and denitrifying bacteria is required for stable performance for the nitrogen removal systems. For simultaneous occurrence of carbon oxidation, nitrification and denitrification, alternating aerobic/anoxic/anaerobic condition should maintained to enable heterotrophic, nitrifying and denitrifying bacteria to coexist in the aerobic granular sludge systems. Since aerobic granulation technology shows great advantages over conventional aerobic processes, this technology might be an alternative to meet the challenge of nitrogen control in the wastewater. Aerobic granules for removing carbonaceous and nitrogenous compounds are greatly expected, however, little information is available with respect to carbon oxidation and nitrogen removal by aerobic granules. Microbial granules for carbon and nitrogen removal need to be thoroughly investigated in the lab-scale reactors before it can be applied in the industrial level.

Chapter 3

Effect of Settling Time on Aerobic Granulation in Sequencing Batch Reactor

3.1 Introduction

Previous research showed that the selection pressure in terms of upflow liquid velocity was a driving force of anaerobic granulation in UASB reactors (Hulshoff Pol et al., 1988; Alphenaar et al., 1993). Aerobic granulation technology has received great research attention as it is likely to overcome the weakness of anaerobic granulation process. As aerobic granulation is a gradual process, for cells to aggregate a number of environmental conditions have to be satisfied. Although SBR is now extensively used for aerobic granulation, it is not yet clear that how the aerobically grown granules would be formed in SBR.

The main feature of SBR is its cycle operation, each cycle consisting of filling, aeration, settling and discharging. In SBR, the settling time is likely to exert a selection pressure on the sludge particles, i.e. only particles that can settle down below the discharge point within the given settling time would be retained in the reactor; otherwise they would be washed out of the system. Therefore, this work aimed to investigate the effect of settling time on aerobic granulation in SBR. Possible mechanism of aerobic granulation was also discussed in this chapter. It is expected that this study will help to set up a practical guideline for successful aerobic granulation in SBR, meanwhile the study presented in this chapter will further provide the theoretical basis for the development of microbial granules with both organic and nitrogen removal capability as discussed in the following chapters.

3.2 Materials and Methods

3.2.1 Reactor Set-up and Operation

Four columns (127 cm in height and 5 cm in diameter), each with a working volume of 2.5 l, were operated as sequencing batch reactors, namely R1, R2, R3 and R4. The configuration of the reactor is shown in Fig. 3.1. R1 to R4 were run at a respective settling time of 20, 15, 10 and 5 min, while the other operation conditions were kept the same (4 h of total cycle time, 5 min of filling, and 5 min of effluent withdrawal). The remaining time in each cycle was the aeration period. Fine air bubbles were introduced at an air flow rate of 3.0 l min^{-1} through a dispenser located at the bottom of each reactor, resulting in a dissolved oxygen concentration of above 50% saturation. Effluent was discharged at the middle point of each SBR. A hydraulic retention time of 8 h was maintained for all the reactors. The sequential operation of the reactors was automatically controlled by timers, while two peristaltic pumps were employed for influent feeding and effluent withdrawal. In order to look into the effect of settling time on aerobic granulation, in the first phase of the study, R1 to R4 were run at respective settling time of 20, 15, 10, and 5 minutes. After the stabilization of four reactors, the settling times in R1-R3 were further shortened from 20 to 5, 15 to 2, and 10 to 1 min in the second phase of the study.

3.2.2 Media

R1-R4 were inoculated by 2000 mg dry weight l^{-1} of fresh activated sludge taken from a local municipal wastewater treatment plant. The composition of synthetic wastewater consisted of CH_3COONa, 2560 mg l^{-1}, NH_4Cl, 200 mg l^{-1}, K_2PO_4, 45 mg l^{-1}, $CaCl{\cdot}2H_2O$, 30 mg l^{-1}, $MgSO_4{\cdot}7H_2O$, 25 mg l^{-1}, $FeSO_4{\cdot}7H_2O$, 20 mg l^{-1} and micronutrient, 1.0 ml l^{-1}. The initial sodium acetate concentration was fixed at 1000 mg l^{-1} chemical oxygen demand (COD) in all four reactors. The composition of the micronutrient solution included H_3BO_3, 50 mg l^{-1}; $ZnCl_2$, 50 mg l^{-1}; $CuCl_2$, 30 mg l^{-1}; $MnSO_4$ mg l^{-1},50 mg l^{-1}; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 50 mg l^{-1}; $CoCl_2{\cdot}6H_2O$, 50 mg l^{-1}; $NiCl_2$, 50 mg l^{-1}.

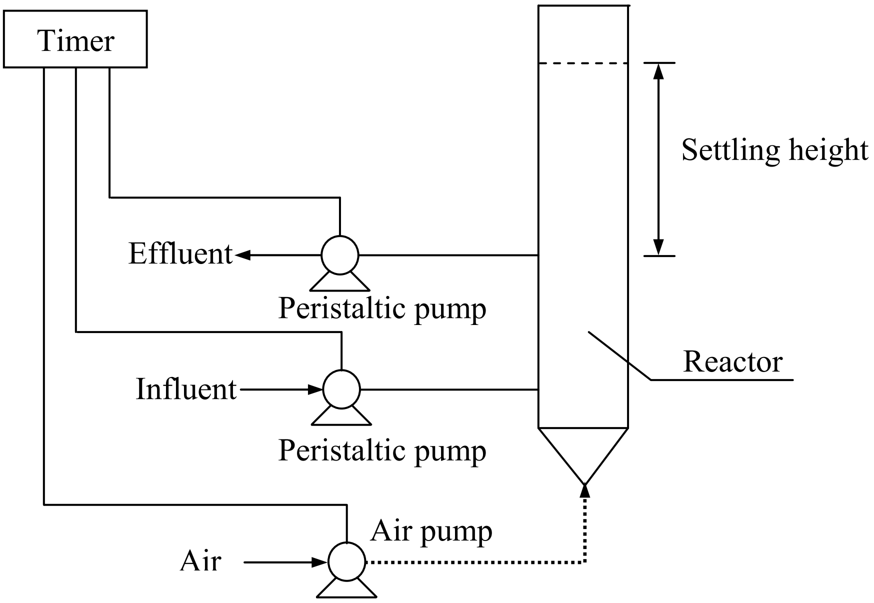

Figure 3.1 Schematic diagram of experimental system.

3.2.3 Characteristics of Seed Sludge

Activated sludge taken from a local municipal wastewater treatment plant was precultured with sodium acetate as sole carbon source in a batch mode for one week. The precultured sludge as seed was then used to inoculate R1 to R4. The characteristics of the seed sludge are summarised in Table 3.1.

Table 3.1 Characteristics of seed sludge

Parameter	Value
MLSS (mg l^{-1})	2700
MLVSS (mg l^{-1})	2232
MLVSS/MLSS	0.83
SVI (ml g^{-1})	230
Mean particle size (mm)	0.11

3.2.4 Analytical Methods

Total solids (TS), volatile solids (VS) of sludge, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI) and specific oxygen utilization rate (SOUR) were determined using standard methods (APHA, 1998). The size of the granular sludge was measured by a laser particle size analysis system (Malvern Mastersizer Series 2600, Malvern), or an image analysis (IA) system (Image-Pro Plus, v 4.0, Media Cybernetics). The morphology of granules in terms of aspect ratio was analyzed by IA techniques. Aspect ratio was defined as the ratio between minor axis and major axis of ellipse equivalent to the granule (0 = line, 1 = circle). Cell surface hydrophobicity was determined using the method of Rosenberg et al. (1980). Hexadecane (0.25 ml) was used as the hydrophobic phase. Hydrophobicity was expressed as the percentage of cells adhering to the hexadecane after 15 minutes of partitioning. Extracellular polysaccharides (EPS) and proteins (EPN) of sludge were extracted by cold aqueous extraction techniques (Zhang et al., 1998), and quantified by the methods of Dubois et al. (1956) and Lowry et al. (1951), respectively. In order to analyze the contents of polyvalent ions of sludge, sludge samples were dried until constant weight at 105ºC, and then were pulverized. 0.1 g of the above prepared sample was digested with nitric acid (APHA, 1998), and multi-elemental analysis was performed using inductively coupled plasma (ICP) emission spectrometer (PerkinElmer Optima 2000). In this study, the fraction of granules was estimated by measuring the volume of granules with a mean size above 0.35 mm in the total volume of sample. For this purpose, granule was considered as sphere. Thus, the fraction of granules (f) in terms of percentage was calculated from Eq. (3.1) as follows:

$$f = \frac{n\frac{\pi}{6}d^3}{V_t} \times 100\% \tag{3.1}$$

in which V_t is the total volume of sample, and d and n is the mean diameter of granules and number of granules with a diameter above 0.35 mm in the sample, respectively.

3.3 Results

A unique feature of SBR is its cyclic operation. In the present operation scheme, the rate of sludge washout from SBR is directly determined by the settling time of sludge, i.e. sludge that could not settle down to below the discharge port of the reactor would be washed out. Therefore, the settling time of SBR may represent the major hydraulic selection pressure exerted on microbial community (Li and Yuan, 2002). At a given settling time or selection pressure, light and dispersed sludge will be easily washed out, and only those heavier particles with a good settleability will remain in the reactor.

3.3.1 Effect of Settling Time on the Formation of Aerobic Granules

The seed sludge had a mean floc size of 0.11 mm, and an SVI value of 230 ml g^{-1}. After 7 days of operation, aerobic granules were first observed in R4 operated at the settling time of 5 minutes. On day 10, tiny aggregates appeared in R1 to R3 run at a respective settling time of 20, 15 and 10 minutes. After three-week operation, four reactors reached steady state. The respective biomass concentration in steady state R1- R4 was 5.3, 4.9, 5.5 and 5.4 g l^{-1}. Fig. 3.2 shows that the aerobic granules have a very regular and spherical outer shape, and the size of aerobic granules seems to increase gradually with the decrease of the settling time.

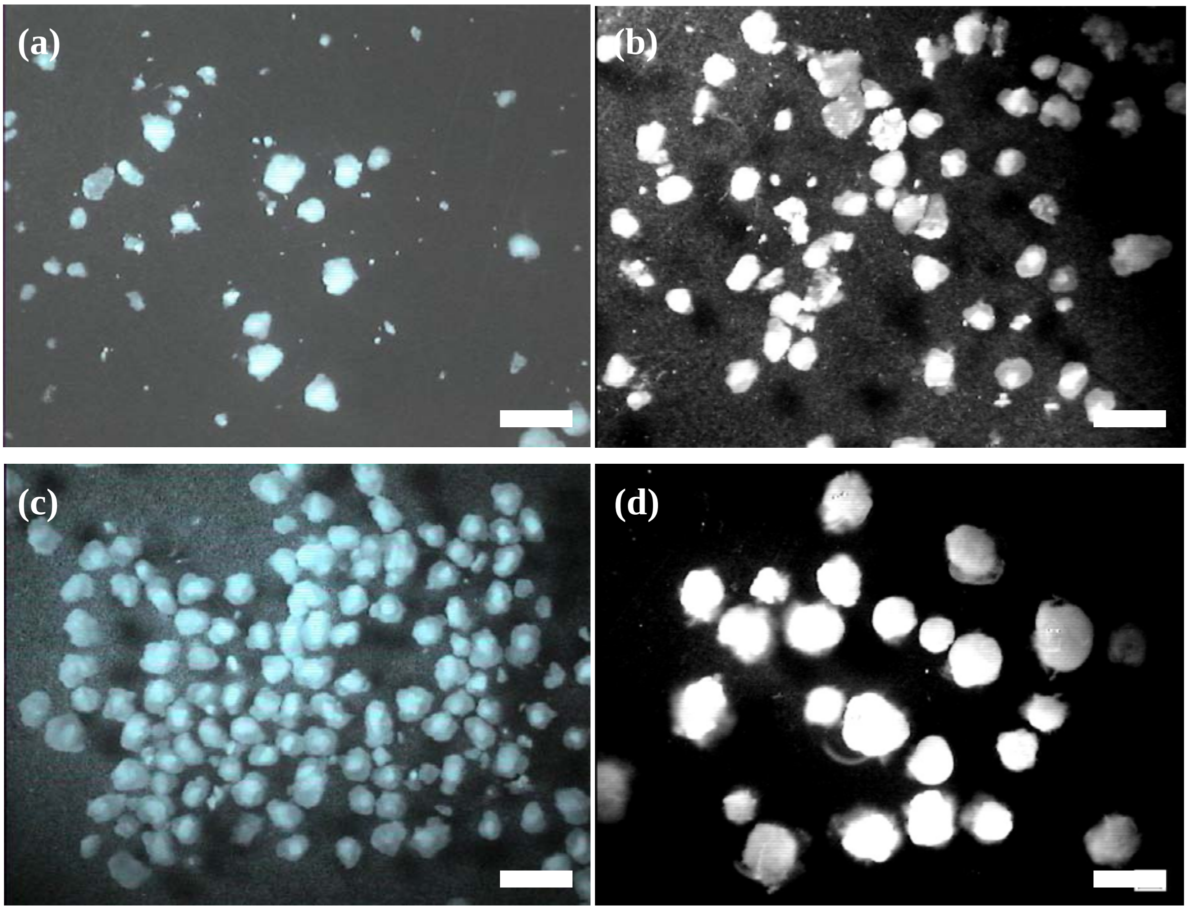

Figure 3.2 Morphology of aerobic granules developed in R1 (a), R2 (b), R3 (c), and R4 (d). Bar: 2 mm.

Fig. 3.3 further shows the fraction of aerobic granules with a mean size above 0.35 mm in steady state R1 to R4, respectively. It is obvious that only in R4 run at the shortest settling time of 5 minutes aerobic granules were dominant form of growth; whereas the fraction of aerobic granules was about 10% in R1, 15% in R2 and 35% in R3. These indicate that a mixture of aerobic granules and suspended sludge were developed in R1 to R3 instead of dominant aerobic granules as observed in R4. The fraction of aerobic granules in the reactors seems to be related to the settling time.

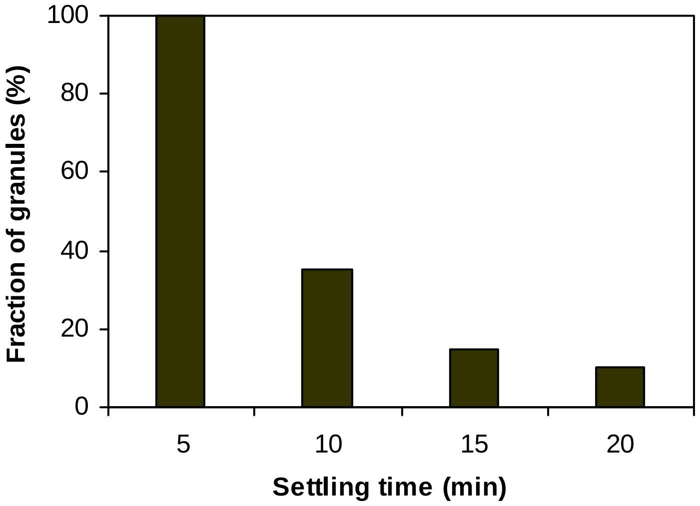

Figure 3.3 Fraction of aerobic granules at different settling times.

3.3.2 Effect of Settling Time on Settleability of Sludge

SVI has been commonly used to describe the settleability and compactness of activated sludge in the environmental engineering field. Fig. 3.4 shows the relationship between the settling time and SVI observed in steady state R1 to R4. It was found that the SVI was closely related to the settling time, i.e. a more compact microbial structure could be expected at a shorter settling time. The SVI decreased from 230 ml g^{-1} in seed sludge to 49 ml g^{-1} in R4 after the formation of aerobic granules. However, in partial aerobic granulation systems (R1-R3), the SVI was much higher than that in R4. In consideration of the portion of aerobic granules in each reactor, it is reasonable to consider that the SVI is determined by the degree of aerobic granulation in the reactors.

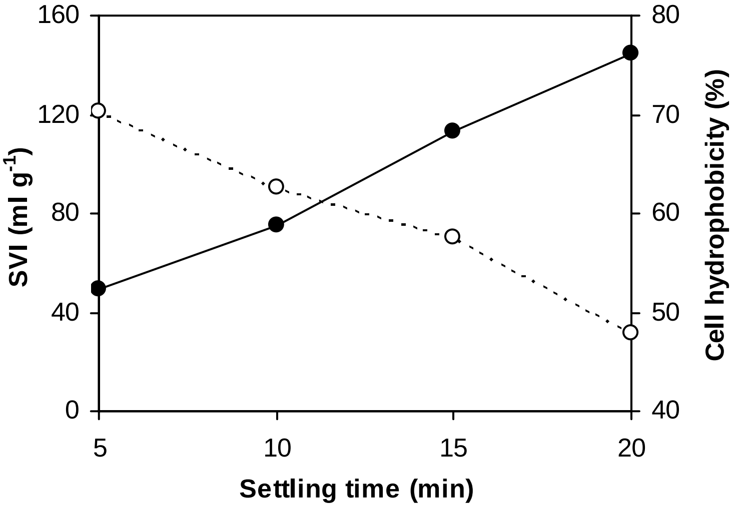

Figure 3.4 Effect of settling time on SVI (●) and cell surface hydrophobicity (○).

3.3.3 Effect of Settling Time on Cell Hydrophobicity

The effect of settling time on cell hydrophobicity is shown in Fig. 3.4. A low cell hydrophobicity was associated with a long settling time. The hydrophobicity increased from 20% in the seed sludge to a stable value of 48% in R1, 58% in R2, 63% in R3, and 72% in R4. These imply that the cell surface hydrophobicity would be inversely related to the settling time, i.e. microbial community developed at short settling time exhibits a high cell surface hydrophobicity. As shown in Fig. 3.3, the partial aerobic granulation was observed in R1, R2 and R3, while aerobic granules were dominant in R4. It appears that the selection pressure-induced change in cell hydrophobicity would contribute to cell-to-cell aggregation. In fact, it has been well known that cell surface hydrophobicity highly contributes to the formation of biofilm and anaerobic granules (Mahoney et al., 1987; Tay et al., 2001; Liu et al., 2003a, 2004).

3.3.4 Effect of Settling Time on the EPS/EPN Ratio

It has been generally believed that extracellular polysaccharides (EPS) can mediate both bacterial cohesion and adhesion and act as a decisive role in building and maintaining structural integrity of a microbial community (Schmidt and Ahring, 1996; Sutherland, 2001a; Liu et al., 2003a). It seems that a shortened settling time would stimulate the production of EPS, from 60.0 to 166.2 mg g^{-1} VS in the mature granules with the decrease of settling time in R1 to R4, while the production of extracellular protein (EPN) is not significantly influenced by the settling time. Fig. 3.5 shows the effect of settling time on the ratio of EPS to EPN. The EPS/EPN ratio was inversely correlated to the settling time, i.e. a shorter settling time would stimulate cells to produce more polysaccharide. Together with Fig. 3.3, this seems to suggest that extracellular polysaccharides would play an essential role in the formation and further maintaining the structure and stability of aerobic granules.

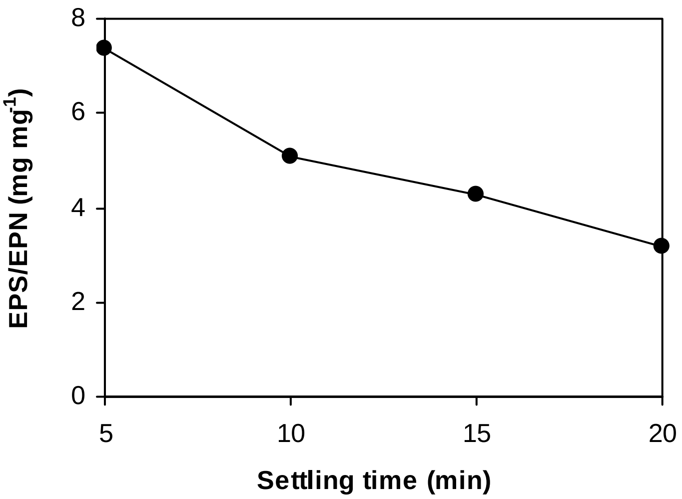

Figure 3.5 Effect of settling time on EPS/EPN ratio.

3.3.5 Effect of Settling Time on Microbial Activity

In this study, microbial activity was quantified by the specific oxygen utilization rate (SOUR) in terms of mg oxygen consumed per mg volatile suspended solids per hour. The correlation between the SOUR and settling time is presented in Fig. 3.6. The SOUR was inversely related to the hydraulic selection pressure in terms of the settling time, i.e. a shorter settling time would significantly stimulate the respirometric activity of microorganisms. These may imply that bacteria may regulate their energy metabolism in response to the changes in hydraulic selection pressure exerted on them.

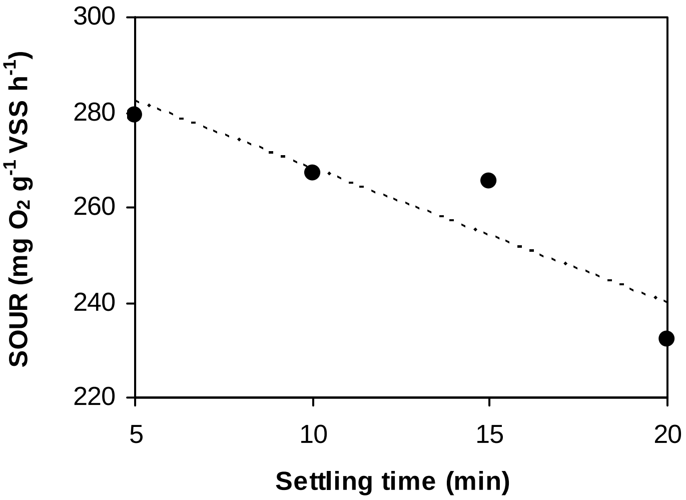

Figure 3.6 Effect of settling time on microbial activity in terms of SOUR.

3.3.6 Effect of Settling Time on the Accumulation of Polyvalent Cations in Aerobic Granules

The contents of poly-valence cations (Ca, Mg, Fe and Al) in microorganisms cultivated in R1 to R4 are shown in Table 3.2. It was found that the microbial calcium content increased significantly at the shorter settling times, while the total content of Mg, Fe and Al in granules did not show much differences at various settling times (Fig. 3.7). The increased granule calcium content results in a decrease of the ratio of volatile solids (VS) to total solids (TS) from 0.88 to 0.53. It appears that microorganisms tend to selectively accumulate calcium that could play a part in the initiation and development of aerobic granules. In fact, it has been generally accepted that multi-valence positive ions, especially calcium, can favour both anaerobic and aerobic granulation (Schmidt and Ahring, 1996; Teo et al., 2000; Yu et al., 2001; Jiang et al., 2003).

Table 3.2 Metal content in aerobic granules in % by dry weight

	R1	R2	R3	R4	AS[a]	AG[b]
Ca	2.039	2.130	7.287	18.757	0.975	14.556
Mg	0.256	0.264	0.238	0.262	0.187	1.904
Fe	0.3813	0.3261	0.3533	0.4826	1.4420	0.087
Al	0.0041	0.0053	0.0049	NA[d]	0.4440	NA[d]
Microelement[c]	0.0446	0.0481	0.0408	0.0439	0.9038	NA[d]

[a] Activated sludge

[b] Anaerobic granules (data from Fukuzaki et al., 1991)

[c] Microelement including Co, Cu, Mn, Ni, Zn

[d] Not available

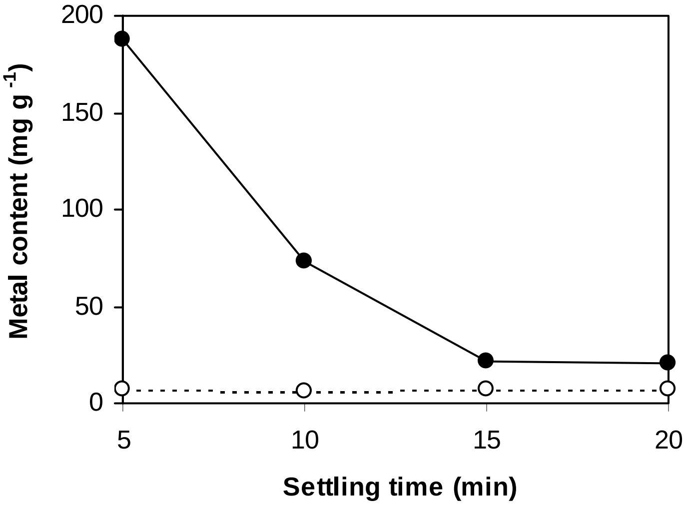

Figure 3.7 The accumulation of poly-valence cations in aerobic granules developed at various settling times, Ca (●) and total Mg, Fe and Al (○).

3.3.7 Effect of Shift of Settling Time on Aerobic Granulation

As shown in Fig. 3.3, the fraction of aerobic granules is in the range of 10-35% in R1 to R3 operated at respective settling time of 20, 15 and 10 minutes. In order to confirm the effect of settling time or hydraulic selection pressure on aerobic granulation, the settling time in steady state R1, R2 and R3 was shifted from 20 to 5, 15 to 2 and 10 to 1 minutes on day 60 accordingly. This led to immediate washout of the light and dispersed sludge from the reactors, while only heavier components remained. Two weeks after the shift of settling time, R1 to R3 gradually restabilized, and aerobic granules completely replaced suspended sludge and became dominant in R1 to R3. Fig. 3.8 shows comparison of cell surface hydrophobicity and microbial settleability before and after shift of settling time in R1 to R3. It is obvious that both cell surface hydrophobicity and microbial settleability were improved significantly after the settling time was shortened. The production of extracellular polysaccharides was increased after the settling time was shortened. Moreover, the bed volume of aerobic granules in R1-R3 reached 100% since the sludge bed had been converted from partial to complete aerobic granulation after the settling time was shortened respectively.

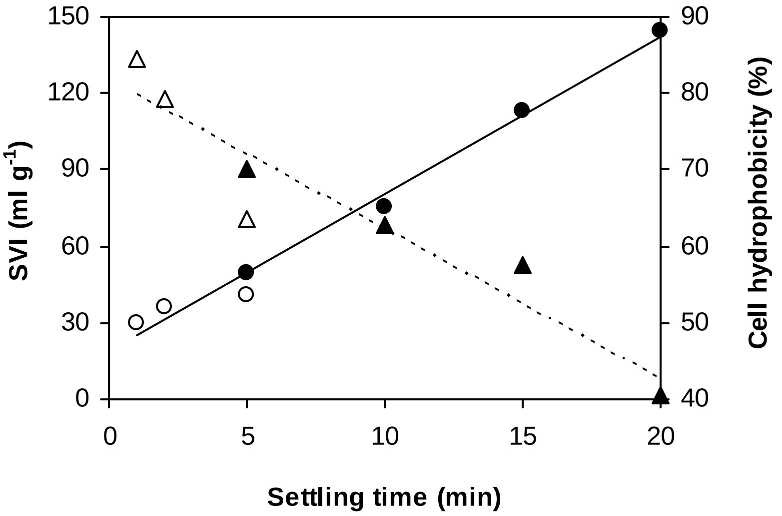

Figure 3.8 SVI before (●) and after (○) shift of settling time; cell surface hydrophobicity before (▲) and after (△) shift of settling time.

3.4 Discussion

To date, the mechanisms behind the formation of aerobic granules have been not fully understood yet. Fig. 3.3 shows that aerobic granules could be dominant only when the settling time is as short as 5 minutes, and a mixture of aerobic granules and suspended sludge could be developed at longer settling times. In SBR, a short settling time preferentially selects for the growth of good settling bacteria, and the sludge with a poor settleability would be washed out. Obviously, the selection of good settling sludge is crucial for aerobic granulation. In fact, absence of anaerobic granulation in the UASB reactors was observed at very weak hydraulic selection pressure in terms of liquid upflow velocity (O'Flaherty et al., 1997). As compared to the operation of aerobic SBR, it is extremely difficult to quantitatively describe the hydraulic selection pressure in the UASB reactor due to the eventual production of biogas.

Evidence shows that bacteria can change their surface hydrophobicity under some stressful conditions (Bossier and Verstraete, 1996; Tay et al., 2001). The cell surface hydrophobicity of the seed sludge was about 20%. After the appearance of aerobic granules in R1 to R4, the cell surface hydrophobicity was greatly improved (Fig. 3.4). In R4 dominated by aerobic granules, the cell surface hydrophobicity is much higher than those in R1 to R3. The settling time seems to induce changes in cell surface hydrophobicity, and a shorter settling time or a stronger hydraulic selection pressure results in a more hydrophobic cell surface. Previous research on anaerobic granulation also showed that anaerobic granular sludge in UASB reactors was more hydrophobic than the non-granular sludge washed out (Mahoney et al., 1987). It seems that microbial association has to adapt its surface properties to resist being washed out from the reactors through microbial self-aggregation at short settling time.

Extracellular polysaccharides are produced by most bacteria out of cell wall with the purpose of providing cells with the ability to compete in a variety of environments, providing a mode for adhesion to surface or self-immobilization (Christensen, 1989; Schmidt and Ahring, 1996; Tay et al., 2001). As shown in Fig. 3.5, a high selection pressure in terms of settling time could stimulate the production of extracellular polysaccharides in a very significant way. It had been reported that high shear force could induce both aerobic biofilms and granules to secrete more extracellular polysaccharides leading to a balanced structure of biofilm or granules under given hydrodynamic conditions (Ohashi and Harada, 1994; Tay et al., 2001; Liu and Tay, 2002). In addition, it should be realized that the content of extracellular polysaccharides in aerobic granules is much higher than the content of extracellular proteins.

The EPS/EPN ratios in the aerobic granules cultivated in R2 to R4 are much higher than that in the seed sludge (about 0.5 mg mg^{-1}). Vandevivere and Kirchman (1993) found that the content of exopolysaccharides for attached cells was five-time higher than for free-living cells. Previous research showed that aerobic granulation failed in SBR in which the production of extracellular polysaccharides was inhibited (Yang et al., 2004b), while the disappearance of aerobic granules in SBR was found to be tightly coupled to a drop of extracellular polysaccharides (Tay et al., 2001). These indicate that the production of extracellular polysaccharides seems essential for the formation of aerobic granules. In fact, the enhanced production of extracellular polysaccharides means that microbial community may metabolically respond to changes in environmental conditions.

The catabolic activity of microorganisms is directly correlated to the electron transport system activity, which can be described by SOUR. As shown in Fig. 3.6, the SOUR was closely related with the hydraulic selection pressure in terms of settling time, e.g. a shorter settling time results in a remarkable increase of SOUR. This may indicate that the microbial community could respond metabolically to changes in hydraulic selection pressure. As pointed out earlier, shorter settling time may trigger the production of extracellular polysaccharides. The correlation between the EPS/EPN ratio and SOUR is further shown in Fig. 3.9. The production of extracellular polysaccharides was greatly stimulated at high SOUR. It is most likely that when the microbial community is exposed to an increased hydraulic selection pressure, much energy produced through the catabolism would go for the synthesis of extracellular polysaccharides rather than for growth, i.e. under a high selection pressure, microbial community would have to regulate its metabolic pathway in order to maintain a balance with the external forces through consuming non-growth-associated energy for the production of polysaccharides and the improvement of cell hydrophobicity.

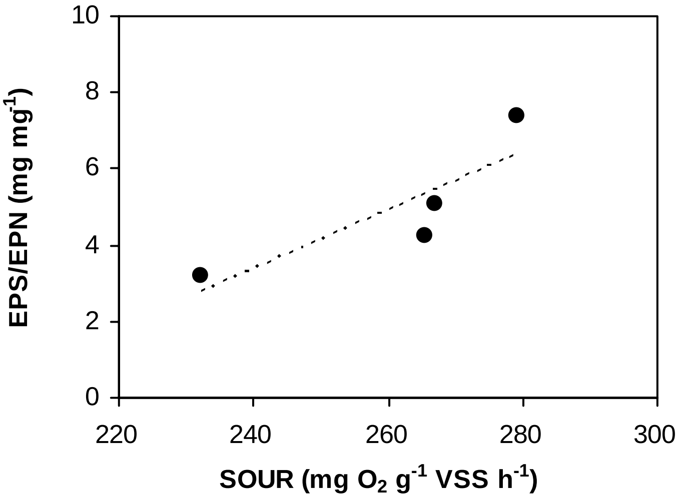

Figure 3.9 Relationships between EPS/EPN and SOUR.

It appears from Fig. 3.7 that the calcium content of granules in R4 operated at shortest settling time of 5 minutes is about 18% of dry weight, which is much higher than those in the granule-suspended sludge mixtures cultivated in R1 to R3. However, the total content of iron, magnesium and aluminium in aerobic granules is minor and independent of the selection pressure as compared to the calcium, i.e. microbial community prefers to accumulate calcium instead of iron, magnesium and aluminium. In this study, the accumulation of calcium was accompanied with a rapid increase in granule size, while a nucleus was observed in the aerobic granule with high calcium content.

The selective accumulation of calcium would be a defensive strategy of microbial community to selection pressure to increase their settleability to resist washout from the reactor. According to the proton translocation-dehydration theory developed for anaerobic granulation, Teo et al. (2000) proposed a biological explanation for the selectively calcium accumulation in anaerobic granulation, and they considered that the positive effect of calcium on anaerobic granulation was probably due to the calcium-induced dehydration of bacterial cell surfaces, which was observed by Xu et al. (1993), i.e. the calcium-induced cell fusion might initiate the formation of cell cluster, which acted as microbial nuclei of further granulation.

It had been reported that the calcium content in anaerobic granules was about 14.6% by dry weight (Fukuzaki et al., 1991). In fact, calcium is a constituent of extracellular polysaccharides and/or proteins, which are used as adsorbing and linking materials in anaerobic granulation process (Morgan et al., 1991). However, it should be pointed out that some researchers considered that calcium could not induce granulation, and the contribution of calcium to anaerobic granulation was overestimated (Guiot et al., 1988; Thiele et al., 1990). In this study, the VS/TS ratio in aerobic granules declined from 88% to 53% when the calcium content in aerobic granules increased from 20.4 to 187.6 mg g^{-1} TS. It is obvious that calcium and calcium-related compounds would be mainly responsible for the reduced VS content in aerobic granules. As a result, aerobic granules are substantially mineralized at high calcium contents.

Fig. 3.3 shows that the fraction of aerobic granules is less than 35% in R1 to R3 operated at settling times of 10, 15 and 20 minutes. After the reactors reached steady states, the respective settling time in R1, R2 and R3 was shifted from 20 to 5 minutes, 15 to 2 minutes and 10 to 1 minutes on day 60. Two weeks after shift of settling time, R1 to R3 stabilized. It was found that aerobic granules completely replaced bioflocs and became dominant in the reactors. Fig. 3.8 summarises the data of cell surface hydrophobicity and SVI before and after the shift of settling time in R1 to R3. As can be seen in Fig. 3.8, cell surface hydrophobicity was increased significantly after the settling times were shortened in the reactors, while microbial settleability in terms of SVI was highly improved due to the formation of aerobic granules. It should be noted that microbial responses to the settling time in terms of cell hydrophobicity and settleability are subject to the parallel patterns no matter how the settling time was manipulated. This might mean that the state of microbial community in a reactor is mainly determined by the external selection pressure exerted on it. Fig. 3.8 also implies that the initial state of seed sludge would less influence the final granulation process as well as physical characteristics of mature granules; in other words, the characteristics of mature granules is highly dependent on the selection pressure applied. It is reasonably considered that self-aggregation of bacteria would be a natural response of microbial community to strong selection pressure in order to resist to be washed out from the systems. These results point to a fact that aerobic granules would be driven by hydraulic selection pressure in terms of settling time.

3.5 Conclusions

It was demonstrated in this chapter that hydraulic selection pressure in terms of settling time was a decisive parameter in the formation of aerobic granules in SBR. Results showed that aerobic granules were successfully cultivated and became dominant only in the SBR operated at the settling time no longer than 5 minutes, while a mixture of aerobic granules and suspended sludge was observed in the reactors run at settling times of 20, 15 and 10 minutes, respectively. Very weak selection pressure did not favour aerobic granulation, and relatively strong selection pressure was essential for the development of aerobic granules in SBR. It was also found that microbial community could metabolically respond to hydraulic selection pressure through the effective regulation of its metabolism, which in turn led to the improved cell surface hydrophobicity and enhanced production of extracellular polysaccharides. It is most likely that aerobic granulation would be an effective defensive or protective strategy of microbial community against external selection pressure. This study offers a deeper insight into the mechanisms behind aerobic granulation.

Chapter 4

Microbial Granulation under Alternating Aerobic-Anaerobic Conditions – Partial Denitrification without External Carbon Source

4.1 Introduction

As noted in Chapter 2, the increasingly stricter regulations on wastewater discharges require advanced and economical techniques for nitrogen removal from wastewater. For biological nitrogen removal, it is difficult to obtain and maintain sufficient nitrifying biomass in the aeration tank due to the sensitivity of nitrifying bacteria to environmental factors as well as their extremely low growth rate (Moreau et al., 1994; Liu and Capdeville, 1996; Ballinger et al., 2002; Ochoa et al., 2002). Large reactor volume and frequent sludge bulking are the general shortcomings of existing biological nitrogen removal systems. Aerobic granules are dense microbial communities that typically include millions of organisms per gram biomass. None of the individual species in this microecosystem is capable of completely degrading influent wastes. Complete degradation of industrial and municipal wastewater involves complex interactions between the resident species. Thus, granular sludge reactors are desirable for biological wastewater treatment because a very high number of organisms can be maintained substantially in the bioreactor through forming granules.

Complete nitrogen removal generally involves nitrification and denitrification as reviewed in Chapter 2. So far, there is very limited information on simultaneous organic and nitrogen removal by microbial granules. In this study, four lab-scale SBRs were operated under alternating aerobic-anaerobic conditions to develop microbial granules for simultaneous removal of organics and nitrogen. The physicochemical characteristics and metabolic behaviors of the microbial granules developed as well as the efficiencies of carbon and nitrogen removal were investigated. In addition, it appears from Chapter 3 that settling time is a critical parameter in the development of microbial granules in SBR and the settling time for successful aerobic granulation should be less than 5 min. Thus, a settling time of 2 minutes was selected for successful granulation in the present study.

4.2 Materials and Methods

4.2.1 Reactor Set-up and Operation

Four columns (127 cm in height and 5 cm in diameter), each with the same configuration and a 2.5 l working volume, were used as SBRs (namely R1, R2, R3 and R4). The configuration of reactor is illustrated in Fig. 4.1. The SBRs were operated with 4 cycles per day, and each cycle consisted of 5-min feeding, 230-min aerobic reaction, 119-min anaerobic reaction, 2-min for sludge settling and 4-min effluent discharge. Synthetic wastewater was fed into the SBRs at the beginning of each cycle. Air was supplied at a gas flow rate of 3.0 l min^{-1} in the aerobic phase, which gave a dissolved oxygen concentration of above 50% saturation. Pure nitrogen gas was purged in the subsequent stage at a gas flow rate of 1.0 l min^{-1} to maintain anaerobic condition as well as to provide sufficient mixing in the reactor. After the settling phase, 1.25 l of supernatant was discharged from the middle port of the reactors. The sequential operations of the SBRs were automatically controlled by programmable timers (Theben, TR 644 S), solenoids valves, and peristaltic pumps.

4.2.2 Media

Each SBR was inoculated with 650 ml of fresh activated sludge taken from a local municipal wastewater treatment plant with nitrification-denitrification capacity. Synthetic wastewater used in this study mainly consisted of ethanol as sole carbon source, ammonia chloride, sodium bicarbonate and other necessary nutrients. The ethanol chemical oxygen demand (COD) concentration in R1 to R4 was kept constant at 500 mg COD l^{-1}, giving an organic loading rate of 2 kg COD m^{-3} d^{-1}. The ammonium-nitrogen concentration was fixed respectively at 37.5, 62.5, 87.5 and 112.5 mg N l^{-1} in R1 to R4, which gave corresponding nitrogen loading rates (NLR) of 0.15, 0.25, 0.35 and 0.45 kg N m^{-3} d^{-1}, respectively. The nutrient solution contained $MgSO_4 \cdot 7H_2O$, 20 mg l^{-1}; $CaCl_2 \cdot 2H_2O$, 15 mg l^{-1}; $FeCl_3 \cdot 3H_2O$, 8 mg l^{-1}; KH_2PO_4, 17.5 mg l^{-1} and Na_2HPO_4, 37.5 mg l^{-1}. The composition of micronutrients in the synthetic wastewater can be found Chapter 3.

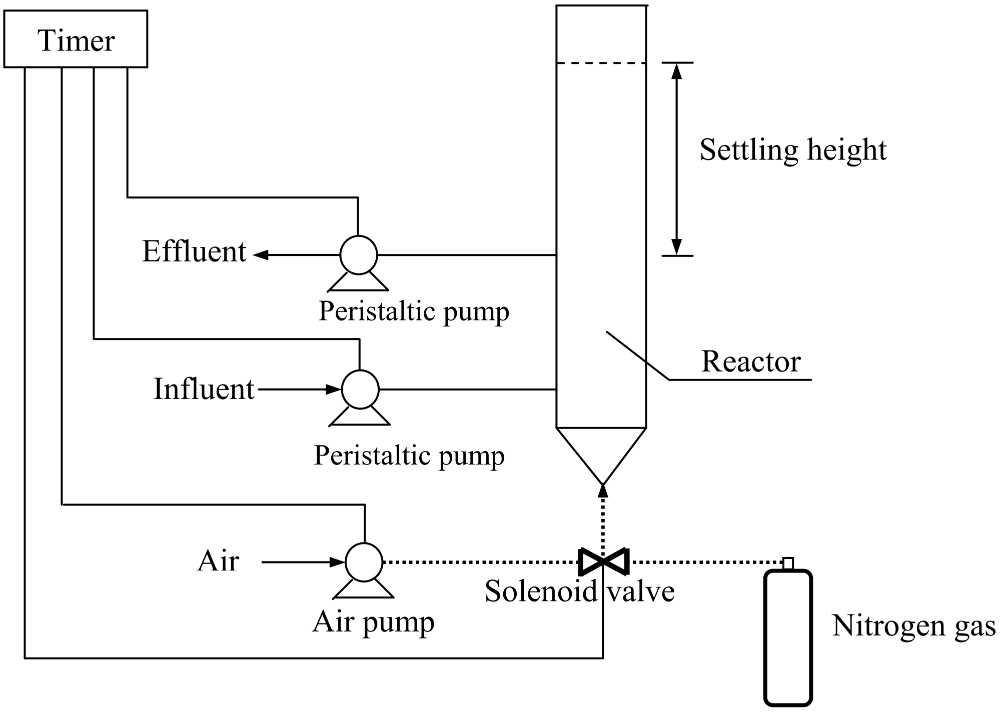

Figure 4.1 Schematic diagram of experimental system.

4.2.3 Analytical Methods

Mixed liquor samples were immediately filtered with 0.45 µm cellulose acetate filter paper (Sartorius AG) to remove suspended solids and prevent further reaction before analysis. NH_4^+, NO_2^- and NO_3^- concentrations were determined using a flow injection analyzer (QuikChem Method 10-107-06-1-I, Lachat Instruments, Inc.), while COD concentration was measured by standard methods (APHA, 1998). The specific oxygen utilization rate $(SOUR)_H$ by heterotrophic bacteria and specific ammonium and nitrite oxygen utilization rates, $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ by ammonium oxidizers and nitrite oxidizers, were measured using standard methods (APHA, 1998). A certain amount of granule sample was carefully washed with tap water, and was put in a pre-cleaned BOD bottle. Then, the BOD bottle was fully filled with the pre-aerated nutrient and substrate solution, and the oxygen sensing probe with stirring mechanism was immediately inserted into the BOD bottle. The decrease of DO was recorded at an interval of 15 seconds. Specific oxygen utilization rate was calculated according to the DO concentration recorded over time. The respective substrate used for determination of $(SOUR)_H$, $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ was ethanol, NH_4Cl and $NaNO_2$ at the concentration of 400 mg COD l^{-1}, 20 mg N l^{-1} and 20 mg N l^{-1}, respectively. The biomass concentration for each test was kept at 500 mg SS l^{-1}. Details on other analytical methods can be found in Chapter 3.

4.3 Results

4.3.1 Formation of Microbial Granules under Alternating Aerobic-Anaerobic Conditions

The seed sludge used had a mean floc size of 90 μm with loose and irregular structure (Fig. 4.2). Microbial granules formed in all reactors after 40 days of operation at different nitrogen loading rates under alternating aerobic-anaerobic conditions. Figs. 4.3 to 4.5 illustrate the evolution of microbial granulation in R2 operated at a NLR of 0.25 kg N m^{-3} day^{-1}. Similar phenomena were also observed in the other three SBRs operated at various NLRs. Microscopic morphology of the granules developed in four SBRs is shown in Fig. 4.6(a-d). It can be seen that the microbial granules had spherical outer shape with compact structure. The aspect ratios of the granules fell into a range of 0.72-0.79. Figs. 4.7 and 4.8 further show the respective macrostructure and microstructure of microbial granules developed at the NLR of 0.25 kg N m^{-3} day^{-1}, while similar observations were also recorded in the granules developed at the other NLRs. The micrograph by SEM shows the outer layer of granules mainly consists of tightly interconnected cocci, with bacilli as subsequent dominant species (Fig. 4.8). The biomass concentration gradually increased from 2 g VS l^{-1} at the beginning to 3.0

in R1, 4.4 in R2, 4.6 in R3 and 5.5 g VS l^{-1} in R4, i.e. the granulation can improve the biomass retention in the reactors.

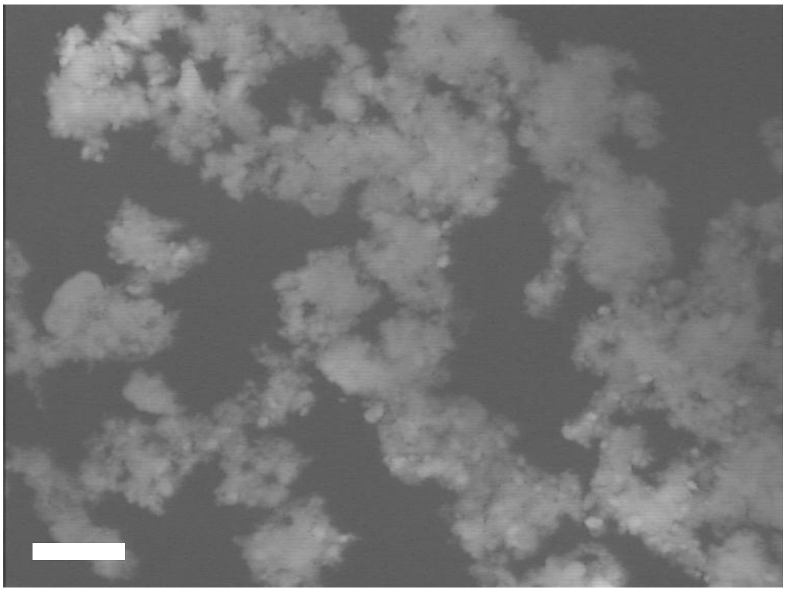

Figure 4.2 Morphology of seed sludge. Bar: 2 mm.

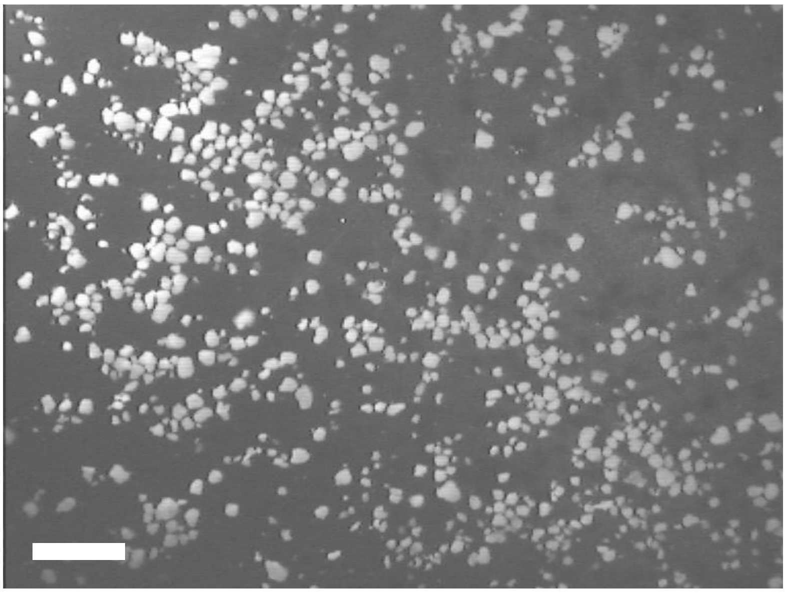

Figure 4.3 Morphology of microbial aggregates after one week operation in R2 run at NLR of 0.25 kg N m^{-3} day^{-1}. Bar: 2 mm.

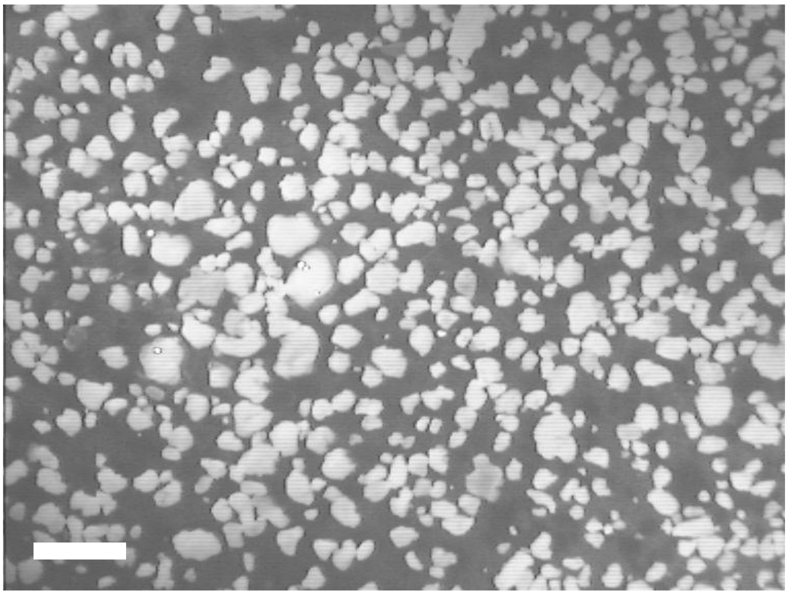

Figure 4.4 Morphology of microbial aggregates after one month operation in R2 run at NLR of 0.25 kg N m^{-3} day^{-1}. Bar: 2 mm.

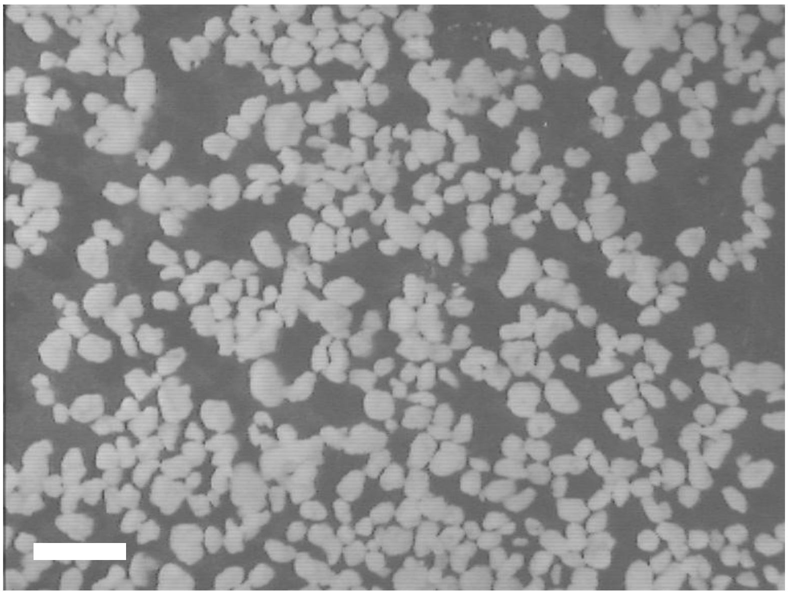

Figure 4.5 Morphology of microbial granules after two months operation in R2 run at NLR of 0.25 kg N m^{-3} day^{-1}. Bar: 2 mm.

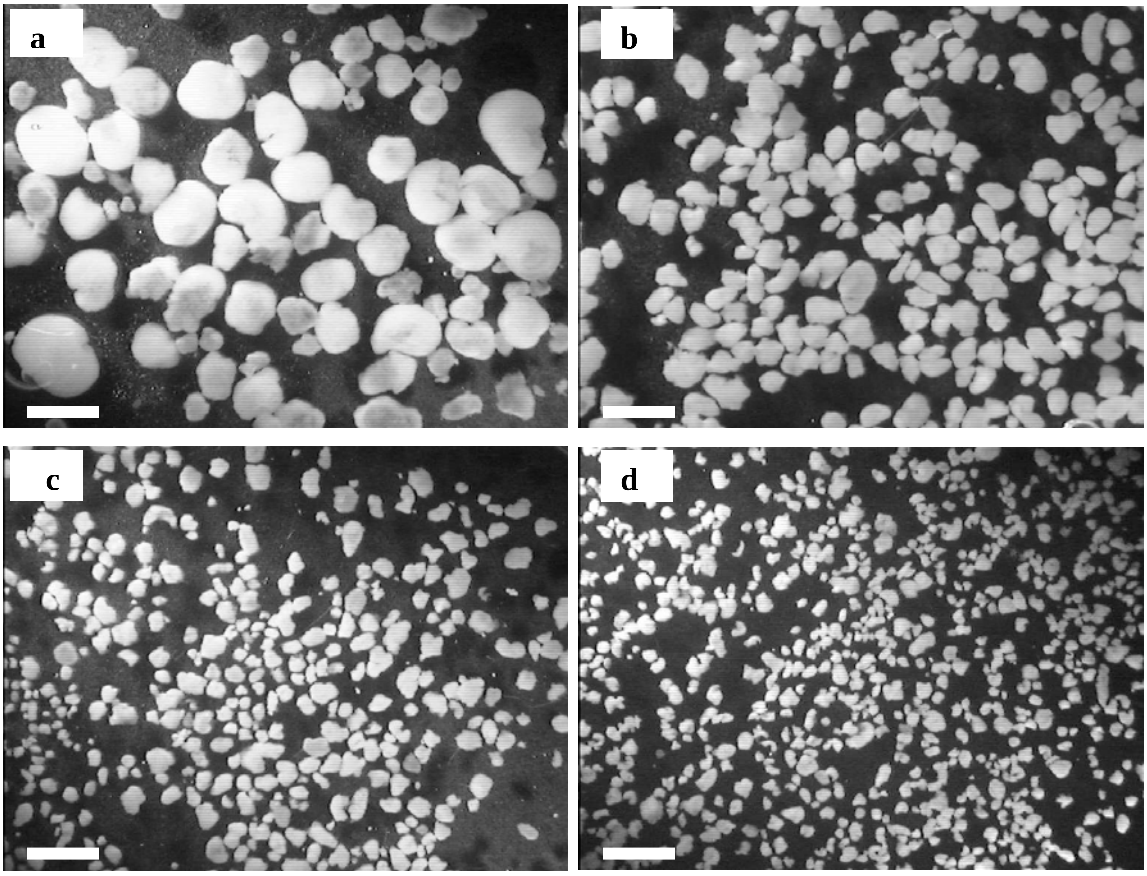

Figure 4.6 Morphology of microbial granules cultivated under aerobic-anaerobic conditions: (a) NLR = 0.15; (b) 0.25; (c) 0.35; (d) 0.45 kg N m^{-3} day^{-1}. Bar: 2 mm.

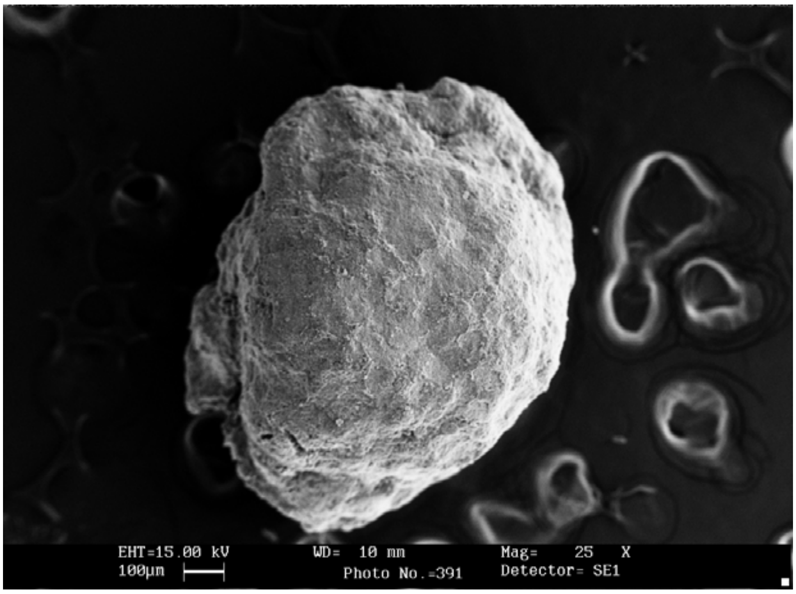

Figure 4.7 Overview of mature granules in R2 run at NLR of 0.25 kg N m^{-3} day^{-1}.

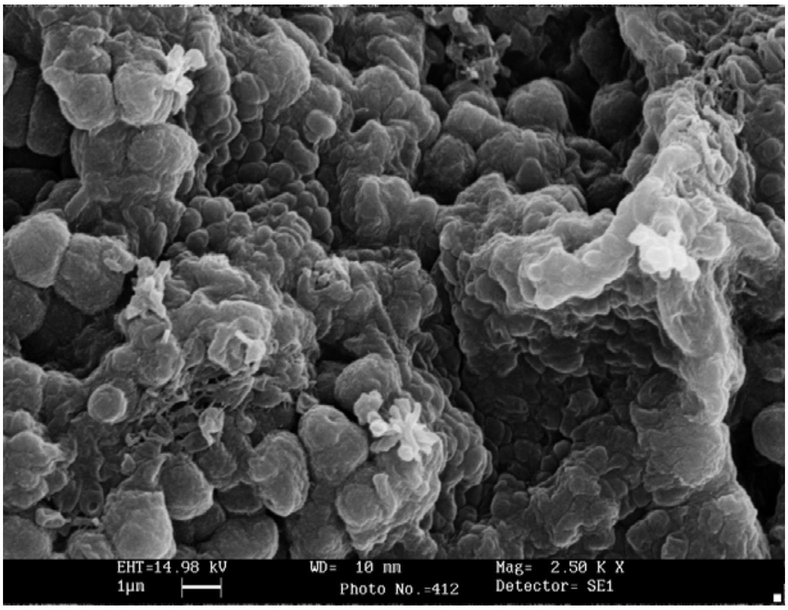

Figure 4.8 Closeview of mature granules in R2 run at NLR of 0.25 kg N m^{-3} day^{-1}.

4.3.2 Physical Characteristics of Microbial Granules

Size

Upon microbial granulation, the mean size of microbial granules increased gradually and stabilized at 1.96, 1.53, 0.50, and 0.41 mm developed at respective NLR of 0.15, 0.25, 0.35, and 0.45 kg N m^{-3} day^{-1}. It seems that the mean size of microbial granules tends to decrease with the increase in NLR, i.e. smaller granules were developed at higher NLR applied.

Settleability

Settling ability of microbial aggregates can be described by SVI and zone settling velocity (ZSV). Fig. 4.9 shows the profiles of SVI of microbial granules developed at different NLRs. Along with the formation of microbial granules, the SVI decreased from 230 ml g^{-1} for the seed sludge to 36 to 27 ml g^{-1} after successful granulation in R1 to R4 (Fig. 4.9). It seems the SVI of the microbial granules was even lower than the typical SVI of granules cultivated under absolute aerobic conditions, i.e. 50-100 ml g^{-1}. The settling velocities of the granules cultivated in R1 to R4 are greater than

60 m h^{-1}, which is much higher than that of conventional activated sludge with a typical value of about or less than 10 m h^{-1} (Campos et al., 1999).

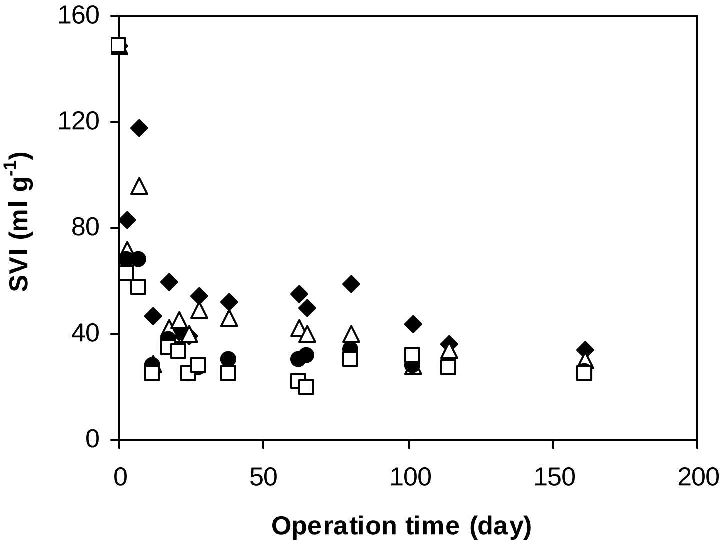

Figure 4.9 SVI of microbial granules developed under alternating aerobic-anaerobic conditions. ◆ NLR = 0.15; △ 0.25; ● 0.35; □ 0.45 kg N m^{-3} day^{-1}.

Cell Hydrophobicity

As discussed in Chapter 3, cell surface hydrophobicity plays a significant role in the formation of biofilms, anaerobic and aerobic granules. The profiles of cell hydrophobicity of microbial aggregates cultivated at different NLRs are shown in Fig. 4.10. With the formation of granules, the cell surface hydrophobicity gradually increased to a stable value. It appears that the cell hydrophobicity at steady state increases from 67.3% to 85.0% with the increase in NLR from 0.15 to 0.45 kg N m^{-3} day^{-1}.

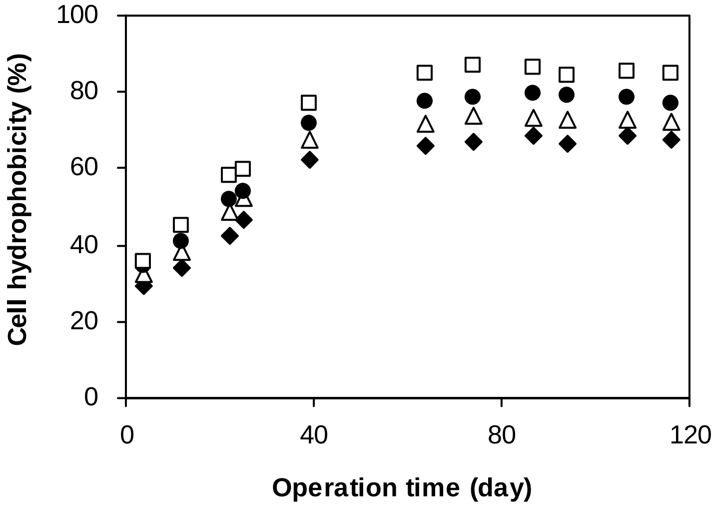

Figure 4.10 Cell hydrophobicity of microbial granules under alternating aerobic-anaerobic conditions. ◆ NLR = 0.15; △ 0.25; ● 0.35; □ 0.45 kg N m^{-3} day^{-1}.

4.3.3 Chemical Properties of Microbial Granules

Extracellular Polysaccharides and Proteins

Extracellular polysaccharides (EPS) are believed to mediate both cohesion and adhesion of cells, and play a crucial role in building and maintaining structure integrity in a community of immobilized cells. Chapter 3 showed that the EPS production would be essential for the formation of aerobic granules. Under the alternating aerobic-anaerobic conditions, the EPS concentration increased from the initial value of 9.6 mg g^{-1} VS for the seed sludge to 30-40 mg g^{-1} VS with the formation of microbial granules. This implies that EPS would act a positive role in microbial granulation under the alternating aerobic-anaerobic conditions. In the meantime, as shown in Fig. 4.11, the EPS content in microbial granules has a decreasing trend with the increase of NLR from 0.15 to 0.45 kg N m^{-3} day^{-1}.

Extracellular proteins (EPN) are the other major components of extracellular matrix in microbial granules. In contrast to EPS, the content of extracellular proteins exhibits an increasing trend with the increases in NLR (Fig. 4.11). It was found that the contents of polysaccharides and proteins in the mature granules fell into a similar range of 20-40 mg g^{-1} VS. These results show that the EPS content in microbial granules is higher than in the seed sludge.

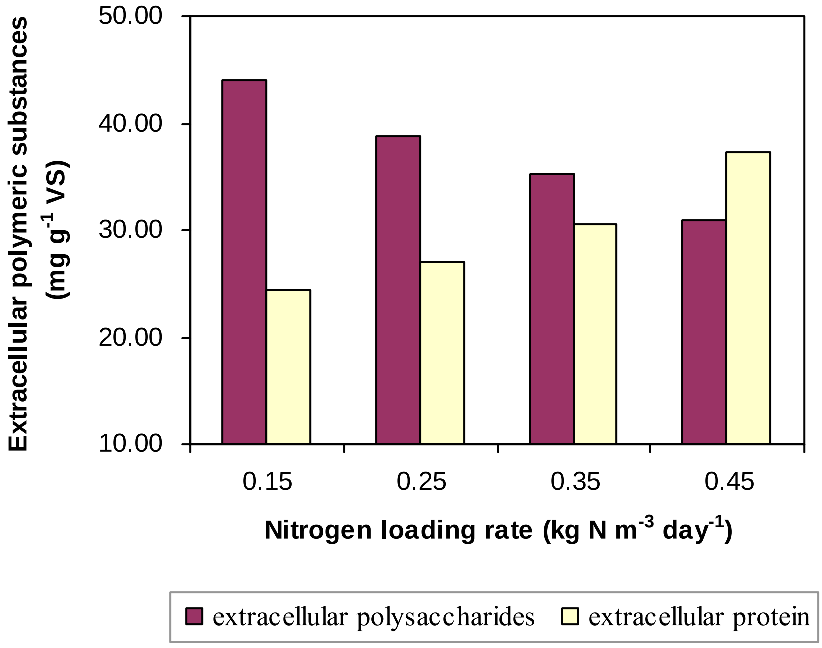

Figure 4.11 EPS and EPN in the microbial granules at various NLRs.

Elemental Compositions

The elemental composition of microorganisms can reflect the energy and nutrients flow within the microorganisms under certain culture conditions. In this study, the contents of carbon (C), nitrogen (N), oxygen (O), hydrogen (H), sulphur (S) of microbial granules developed at various NLRs were analyzed and shown in Table 4.1. These results indicate that microbial granules are composed of six major elements, i.e. C, H, O, N, P and S. It can be seen that the C, N and H contents in the granules varied significantly with the NLR applied and the resulting cellular C:N ratio decreased from 5.18 to 4.19 with the increase in NLR from 0.15 to 0.45 kg N m^{-3} day^{-1}. The observed cellular C:N ratios of microbial granules are consistent with those reported for natural and cultured bacteria (Fukuda et al., 1998; Vrede et al., 2002). It appears that the cell C:N ratio is closely related to the substrate carbon and nitrogen loading, e.g. high cell C:N ratio was recorded in the cells cultivated at relative low NLR.

Table 4.1 Elemental composition of microbial granules in % by dry weight

Element	NLR (kg N m^{-3} day^{-1})			
	0.15	0.25	0.35	0.45
C	39.98	38.25	37.92	37.08
H	5.40	6.13	6.04	6.95
O	36.49	38.29	38.97	38.48
N	7.72	8.16	8.60	8.86
P	0.78	0.82	0.87	0.90
S	1.41	0.73	0.91	0.63
Formula	$C_{6.0}H_{9.8}O_{3.9}NP_{0.04}$	$C_{5.5}H_{10.5}O_{3.9}NP_{0.04}$	$C_{5.1}H_{9.8}O_{3.8}NP_{0.04}$	$C_{4.9}H_{11.0}O_{3.8}NP_{0.04}$

4.3.4 Activity Distribution in Microbial Granules

Since molecular oxygen serves as final electron acceptor in aerobic oxidation process, the metabolic activity of a microbial community is directly related to the respiratory activity of cells. In this study, the overall activity of heterotrophic populations in microbial granules was quantified by its specific oxygen utilization rate $(SOUR)_H$. Fig. 4.12 shows the profiles of $(SOUR)_H$ versus operation time observed at different NLRs. It can be seen that the activity of heterotrophic bacteria decreased over the operation time, while the microbial granules cultivated at low NLR exhibits slightly higher heterotrophic activity (Fig. 4.12).

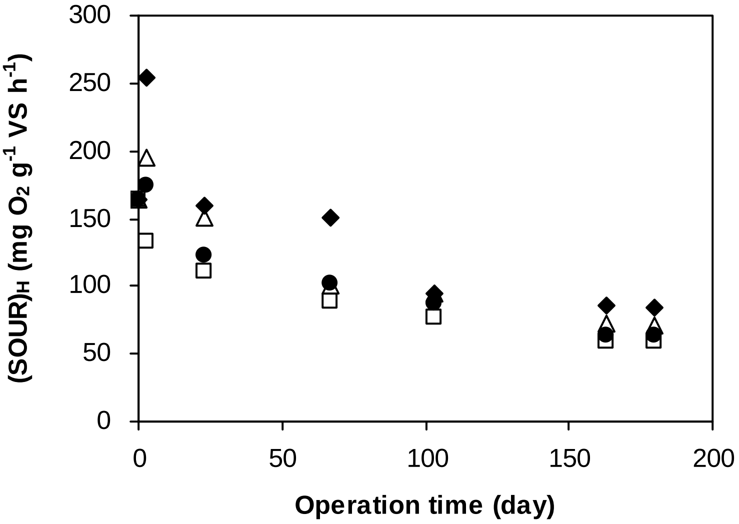

Figure 4.12 $(SOUR)_H$ versus operation time. ◆ NLR = 0.15; △ 0.25; ● 0.35; □ 0.45 kg N m^{-3} day^{-1}.

The activity distribution of heterotrophic populations in the microbial granules cultivated at different NLRs on day 180 is shown in Fig. 4.13. The results indicate that the $(SOUR)_H$ decreases with the increase of the NLR. Similar phenomena had been observed in biofilm processes (Moreau et al., 1994; Ochoa et al., 2002).

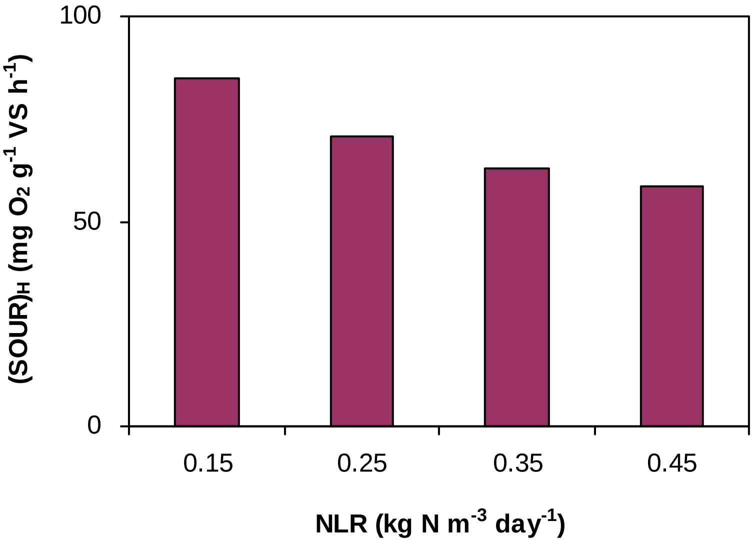

Figure 4.13 Heterotrophic SOUR at different NLRs on day 180.

Nitrification is mainly carried out by ammonia oxidizer for conversion of ammonia to nitrite, and nitrite oxidizer responsible for further oxidation of nitrite to nitrate. In this study, the respective respirometric activity of ammonia oxidizer and nitrite oxidizer was described by the specific nitritation oxygen utilization rate $(SOUR)_{NH4}$, and the specific nitratation oxygen utilization rate $(SOUR)_{NO2}$. The overall activity of nitrifying bacteria, $(SOUR)_N$, can be represented by the sum of $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$. Fig. 4.14 shows changes in $(SOUR)_N$ in the course of operation. Obviously, the overall nitrifying capability was built up over the operation period and gradually stabilized.

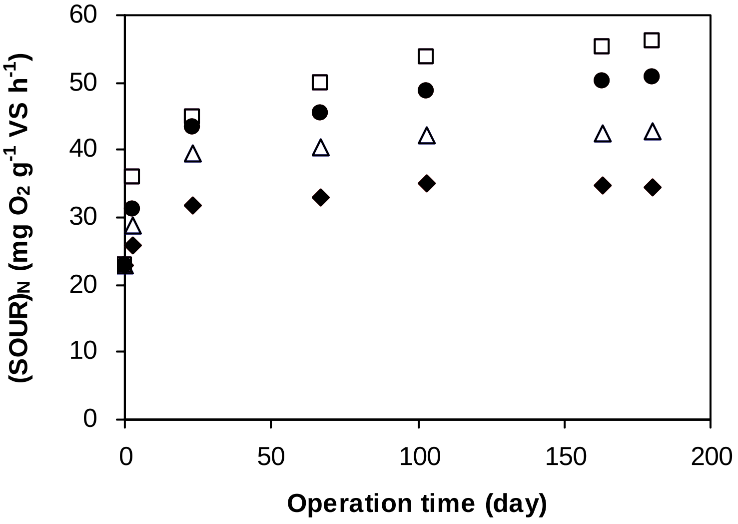

Figure 4.14 $(SOUR)_N$ versus operation time. ◆ NLR = 0.15; △ 0.25; ● 0.35; □ 0.45 kg N m^{-3} day^{-1}.

Fig. 4.15 further shows the changing trend of $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ with the NLRs on day 180. It is obvious that $(SOUR)_{NH4}$ increases with the increase of the NLR, whereas $(SOUR)_{NO2}$ seems not change very much at different NLRs. The rule of thumb currently applied to wastewater biological treatment processes is that the ratio of influent degradable organic matter expressed on a COD basis to nitrogen should be COD/N of 100/5 (Droste, 1997). In R1 run at the NLR of 0.15 kg N m^{-3} day^{-1}, corresponding to COD/N of 100/7.5, ammonia would be mainly used as nutrient for heterotrophic growth. In this case, heterotrophic bacteria are likely to out-compete nitrifying bacteria for ammonia. Consequently, a low nitrification rate would result. This is supported by Fig. 4.15, showing a high nitrifying activity of microbial granules at high nitrogen loading rate. It appears that the COD/N ratio is a key towards a deep understanding of the nitrogen dynamics in microbial granules cultivated at different NLRs. Similar phenomena had been observed in suspended and attached processes (Moreau et al., 1994; Satoh et al., 2000; Strauss and Lamberti, 2000; Ochoa et al., 2002). It has been reported that increased substrate C/N ratio would induce the competition between ammonia-oxidizing and heterotrophic bacteria and resulted in a decrease in a lowered ammonia oxidation rate and a decrease in ammonia-oxidizing population (Satoh et al., 2000). Strauss and Lamberti (2000) also found that at an increased C/N ratio heterotrophic bacteria would outcompete nitrifying bacteria for available NH_4^+, thereby leading to a reduced nitrification rate.

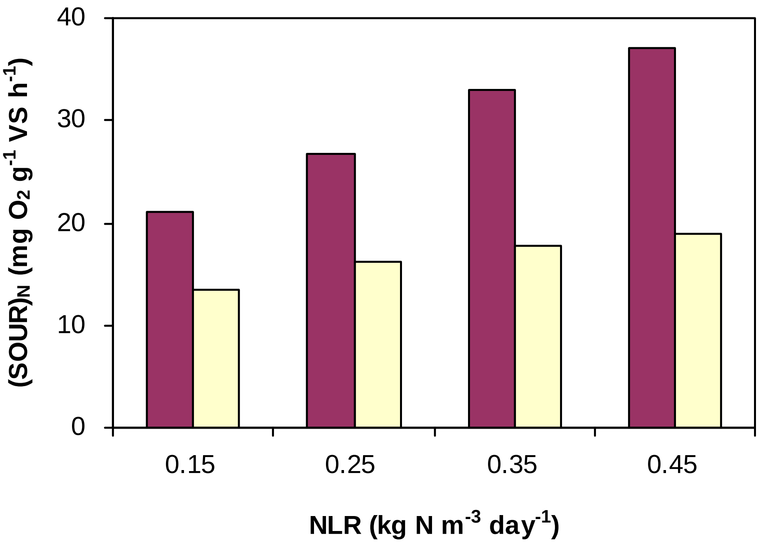

Figure 4.15 SOUR of nitrifiers at different NLRs on day 180. ■: ammonia oxidizer; □: nitrite oxidizer.

4.3.5 Organic Carbon and Nitrogen Removal under Conditions without Supply of External Carbon

Fig. 4.16 shows the typical concentration profiles of COD and different forms of nitrogen observed during an aerobic-anaerobic cycle in R4 operated at the NLR of 0.45 kg N m^{-3} day^{-1} with no supply of external carbon source in the anaerobic phase. Similar profiles were also obtained in other reactors. Over 95% of influent COD was removed within the first hour of aerobic reaction, which was accompanied by the slow consumption of ammonium used for microbial growth. After the COD depletion, ammonium concentration started to decline fast mainly due to nitrification. At the end of aerobic phase, ammonia was entirely converted to nitrite and nitrate. In the subsequent anaerobic stage, slight denitrification was observed in R1 to R4, i.e. about 12 to 27 mg l^{-1} NO_x^--N was denitrified under the conditions without the supply of external carbon source. COD removal efficiency of over 95% was achieved in all the reactors, whereas nitrogen removal efficiency was 50%, 44%, 24%, and 26% in R1, R2, R3, and R4, respectively. The specific denitrification rate fell into the range of 1.9 to 3.5 mg N g^{-1} VS h^{-1} at various NLRs.

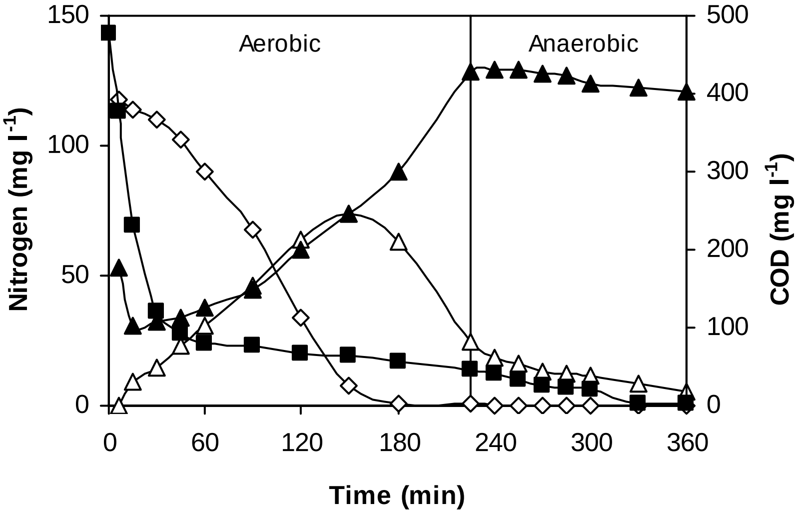

Figure 4.16 Typical organics oxidation, nitrification and partial denitrification profiles in one cycle with no addition of external carbon source (in R4 run at NLR of 0.45 kg N m^{-3} day^{-1}). ■ COD; ◇ NH_4; △ NO_2; ▲NO_3.

4.4 Discussion

Microbial granules can form under the alternating aerobic-anaerobic conditions in SBR (Figs. 4.2-4.6). The SVI of the granules decreased from 230 ml g^{-1} for the seed sludge to 36-27 ml g^{-1} after microbial granulation in R1 to R4. These seem to indicate that the settleability of the granules developed under alternating aerobic-anaerobic conditions is not only much better than conventional activated sludge, but also superior than granules cultivated under absolute aerobic conditions with a typical SVI of 50-100 ml g^{-1} (Tay et al., 2001; Moy et al., 2002; Liu et al., 2003e; McSwain et al., 2004). Obviously, a more compact structure of microbial granules can be expected under alternating aerobic-anaerobic condition (Figs. 4.2-4.8). It has also been noticed that the SVI of microbial granules decreased with the increase in NLR. It is likely that microbial granules developed at high NLR would have a more compact and denser structure.

The loose structure of activated sludge may cause relatively poor settleability, which is often associated with the growth of filamentous bacteria (Metcalf and Eddy, 2003), whereas the anoxic or anaerobic phase designed for biological nutrient removal can serve as a metabolic and/or kinetic selector of denitrifying bacteria, meanwhile suppress the growth of filamentous bacteria which can not utilize nitrate and nitrite as an electron acceptor (Kim and Pagilla, 2000). Alternating aerobic-anoxic conditions were found to promote growth of good settling bioflocs and contribute to an increase of denitrifying fraction in the bioflocs (Bilanovic et al., 1999). The settling velocity of microbial granules cultivated in R1 to R4 was greater than 60 m h^{-1}, whereas the settling velocity of conventional activated sludge is often less than 10 m h^{-1} (Campos et al., 1999). Sludge bulking is a common problem encountered in almost all biological nutrient removal processes (Eikelboom et al., 1998; Wanner et al., 2000). It seems that microbial granulation would be a feasible option to overcome this problem as well as to upgrade existing biological treatment plants for efficient nitrification.

As discussed in Chapter 3, cell surface of granular sludge is more hydrophobic than non-granular sludge and a shorter settling time or a stronger selection pressure leads to a more hydrophobic cell surface. It seems that microbial association has to adapt its surface properties to resist being washed out from reactors through microbial self-aggregation at shorter settling time. Fig. 4.10 shows under the alternating aerobic-anaerobic conditions, cell surface hydrophobicity of microbial granules was distinctly higher than that of seed sludge, while the cell hydrophobicity of microbial granules increased with the increase of the NLR applied. Previous research showed that the formation of nitrifying and denitrifying biofilm was also correlated with surface hydrophobicity (Kim and Pagilla, 2000; Kim et al., 2004). As nitrifying bacteria grow very slowly, the washout of nitrifying bacteria from bioreactor is a common headache problem occurring in many conventional nitrification units. However, the formation of microbial aggregates, like biofilms and granules through bacteria adhesion can be a protective strategy for sensitive nitrifying biomass against washout. In fact, there is evidence that the hydrophobicity of bacteria is an important affinity force in the cell immobilization process (Bossier and Verstraete, 1996; Zita and Hermansson, 1997; Liu et al., 2004). It seems certain that increased cell surface hydrophobicity favor cell-to-cell adhesion, and can act as an initial driving force for cell-to-cell aggregation, which is the first step towards microbial granulation.

EPS is produced by most bacteria to provide a mode for adhesion to surfaces or self-immobilization, and plays a crucial role in building and further maintaining 3-dimensional structure and the integrity of immobilized-cell communities (Schmidt and Ahring, 1996; Sutherland, 2001b; Liu and Tay, 2002). EPS can exist in any form of microbial aggregates, such as bioflocs, biofilms, anaerobic and aerobic granules. Seviour and Kristensen (1983) found that the EPS yield dropped as the initial nitrogen concentration in the medium increased. Fig. 4.11 shows that the EPS decreases as the NLR increases.

Figs. 4.13 and 4.15 further indicate that the overall respirometric activity of nitrifying population tends to increase, while the activity of heterotrophic population shows a decreasing trend with the increase in NLR, i.e. nitrifying population in the microbial granules would be enriched at high NLR and heterotrophic population would become less dominant. There is evidence that the production of EPS was energy-dependent

(Wuertz et al., 1998). This may imply that heterotrophic bacteria are capable of producing much more EPS than nitrifying bacteria. Thus, the lower EPS content in microbial granules cultivated at higher NLR can be reasonably explained in a way such that nitrifying bacteria can not utilize organic carbon for microbial growth, and only 11% to 27% of energy generated goes to biosynthesis (Laudelout et al., 1968).

In contrast to EPS, the production of EPN exhibits an increasing trend with the increases in NLR (Fig. 4.11). In general, nitrogen in the cells is associated with proteins and nucleic acids (Norland et al., 1995). Bura et al. (1998) reported an obvious decrease in protein content when the molar ratio of C:N:P decreased from 100:5:1 to 100:1:1 in the fed medium. Similarly, Durmaz and Sanin (2001) also found a remarkable decrease in the EPN content as the C:N ratio was increased from 17.5 to 40. The results in Fig. 4.11 are consistent with the previous findings in the literature.

The growth conditions and differences in the bacteria community can cause the variability of elemental composition (Vrede et al., 2002). This means that variability of the elemental composition of microbial granules (Table 4.1) would reflect the difference of microbial diversity distribution in the microbial granules. These are supported by the results presented in Figs. 4.13 and 4.15, indicating that the nitrifying activity was considerably enhanced with the increase in NLR. The empirical formulas of microbial granules developed at different NLRs can be obtained according to their elemental compositions (Table 4.1). The derived formulas are comparable with those reported in suspended nitrification process, e.g. $C_5H_9O_{2.5}N$ for SHARON process and $C_5H_{13.3}O_{3.3}N$ for ANAMMOX process (Hao, 2001).

As noted by Elser (2001), the variation in cell elemental compositions may imply how species-interactions develop in ecosystems under various conditions of energy input and nutrient supply. Reeslev et al. (1996) also found that ammonium:glucose ratio in the growth medium had significant impact on the microbial composition and nitrogen content of the cells. Therefore, the variation of elemental composition in microbial granules implies the variations in microbial distribution. In other words, culture conditions like NLR in this study would have significant effect on the microbial composition and species distribution in the granules, which further led to distinct metabolic pathways and resulted in variations of elemental composition in microbial granules.

The stoichiometric amount of ethanol required for nitrate reduction can be derived from the following equation (Mohseni-Bandpi and Elliott, 1996):

$$97NO_3^- + 50C_2H_5OH \rightarrow 5C_5H_7O_2N + 75CO_2 + 84H_2O + 46N_2 + 97OH^- \quad (4.1)$$

This equation shows that the amount of COD required for denitrification with ethanol is 3.5 g ethanol-COD per gram NO_3-N denitrified. As the nitrate reduction during the anaerobic phase was about 10 mg NO_3-N l^{-1} in all reactors, i.e. the COD required for the observed denitrification should be around 35 mg COD l^{-1}. However, it appears from Fig. 4.16 that the COD concentration remaining after the aerobic phase was as low as 10 mg l^{-1} that is insufficient for the observed denitrification in the anaerobic phase. These may imply that in the observed denitrification, some other unidentified carbon source should be involved, such as internally stored carbon compounds. This point will be further investigated in the following chapter.

4.5 Conclusions

Microbial granules were successfully developed under alternating aerobic-anaerobic conditions in SBR. The results showed that the physical and chemical properties of microbial granules would be closely related to the NLR applied. It was found that the SVI values of microbial granules were as low as 36 to 27 ml g^{-1}, which were lower than or comparable with those of aerobic granules cultivated under absolute aerobic conditions. The NLR could influence the relative distribution of extracellular polymers, i.e. at low NLR, a high polysaccharides content and a low protein content was observed. Heterotrophic, nitrifying and denitrifying populations could coexist in the microbial granules, while the activities of nitrifying and denitrifying populations in granules could be enhanced by increasing NLR and the activity of heterotrophic populations decreased in a significant way. The relative abundance of nitrifying population over heterotrophic population increased with the increase in NLR. Over 95% of COD removal and complete nitrification was achieved in the aerobic phase for all the reactors, whereas only partial dentrification occurred in the subsequent anaerobic phase due to lack of external carbon source. The obtained nitrogen removal efficiency was 24% to 50% and the specific denitrification rate was 1.9 to 3.5 mg N g^{-1} VS h^{-1} at various NLRs. This study showed that microbial granules subject to the alternative aerobic and anaerobic conditions are capable of removing organics and nitrogen from wastewater and coexistence of different species in the same microbial matrix provides a protective strategy for sensitive nitrifying populations to overcome the limitations of the conventional nutrient removal systems.

Chapter 5

Microbial Granulation under Alternating Aerobic-Anaerobic Conditions – Complete Denitrification with External Carbon Source

5.1 Introduction

As nitrogen pollutants are responsible for promoting the eutrophication in receiving water, the removal of nitrogen compounds from wastewater is of increasing importance. Stringent standards have been set accordingly by different countries and organizations, including the U.S., Canada, the EEC and the WHO (Sayre, 1988). The standard set by the U.S. EPA (1993) for nitrate in drinking water is 10 mg N l^{-1} and by the EEC (1980) and WHO (1993) 50 mg l^{-1} as nitrate (about 11.3 mg N l^{-1}). Biological nitrogen removal can be accomplished by nitrification and denitrification under alternating aerobic and anoxic conditions in single-sludge reactors. Organic carbon is required as the electron donor for the reduction of nitrate and nitrite to nitrogen gas. The COD/N ratio for the complete denitrification may range from 3.5 to 15 g COD g^{-1} N associated with type of organic carbon (Henze, 1991). Chapter 4 has demonstrated a partial denitrification by microbial granules under the conditions with no supply of external carbon source. In order to achieve complete nitrogen removal, this chapter further reported results obtained from the granular sludge SBR fed with ethanol as an external carbon source.

5.2 Materials and Methods

Ethanol, as external carbon source for denitrification, was supplied to SBRs operated at different NLRs on day 182 onwards. Ethanol was fed to each reactor at the beginning of anaerobic phase and the dosage was based on a ratio of ethanol to NO_x^--

N of 2:1 by weight. This operation lasted for 180 days. Details on the reactor set-up, operating conditions, media and analytical methods were presented in Chapter 4.

5.3 Results

5.3.1 Organics and Nitrogen Removal

The concentration profiles of COD and different forms of nitrogen under conditions without addition of external carbon source were shown in Figs. 5.1 to 5.4. As discussed in Chapter 4, it can be seen from Figs. 5.1 to 5.4 that only less than 28 mg l^{-1} NO_x^--N was denitrified under the condition without supply of external carbon source. In order to achieve complete denitrification, at the beginning of the anaerobic stage, ethanol as the external carbon source was supplied to R1 to R4, based on a stoichiometric ratio of ethanol to NO_x^--N of 2:1 by weight. With the addition of ethanol, almost all nitrate and nitrite produced in the aerobic phase were removed rapidly in the first half hour in four reactors run at different nitrogen loadings, i.e. nitrogen removal efficiency of over 99% was achieved in all four reactors operated at different NLRs (Figs. 5.5 to 5.8). It seems that denitrification metabolism is activated by the addition of external carbon source (Table 5.1).

The specific denitrification rates in R1 to R4 with addition of external carbon source ranged from 12.0 to 25.3 mg N g^{-1} VS h^{-1}, which are one-order magnitude higher than the specific denitrificaition rates of 1.9 to 3.5 mg N g^{-1} VS h^{-1} obtained under conditions without addition of external carbon (Figs. 5.1 to 5.4 and Chapter 4). These indicate that the denitrification activity of microbial granules is closely related to the availability of external carbon source in anaerobic phase. It had been reported that denitrification occurred with a low rate when a slowly biodegradable organic matter was provided as the carbon source (van Haandel et al., 1981), whereas the addition of a readily biodegradable carbon instantaneously increased the denitrification rate (Isaacs and Henze, 1995).

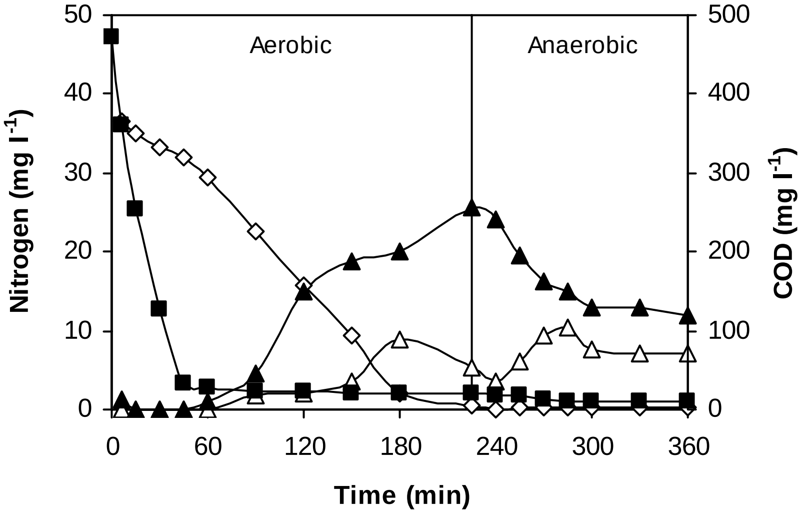

Figure 5.1 COD and nitrogen profiles at NLR of 0.15 kg N m^{-3} day^{-1} with no addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

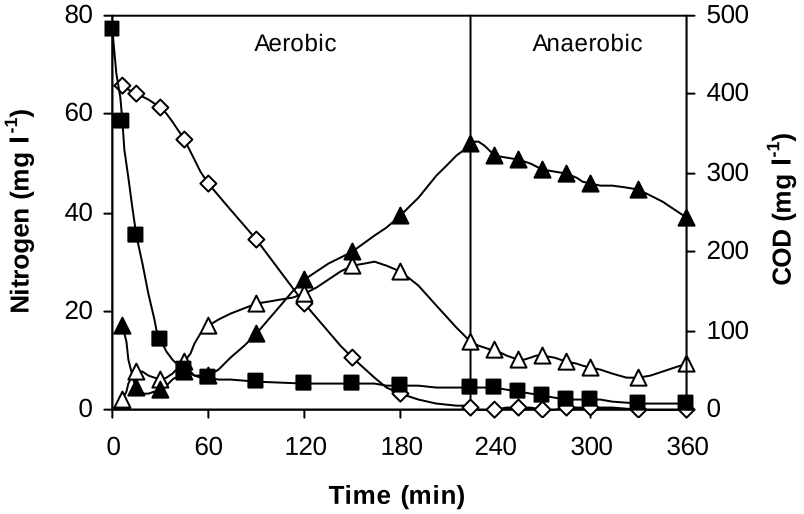

Figure 5.2 COD and nitrogen profiles at NLR of 0.25 kg N m^{-3} day^{-1} with no addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

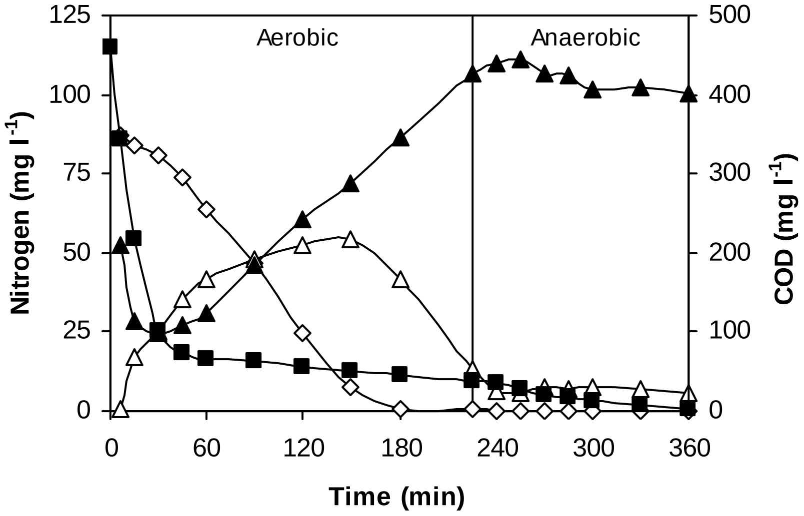

Figure 5.3 COD and nitrogen profiles at NLR of 0.35 kg N m^{-3} day^{-1} with no addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

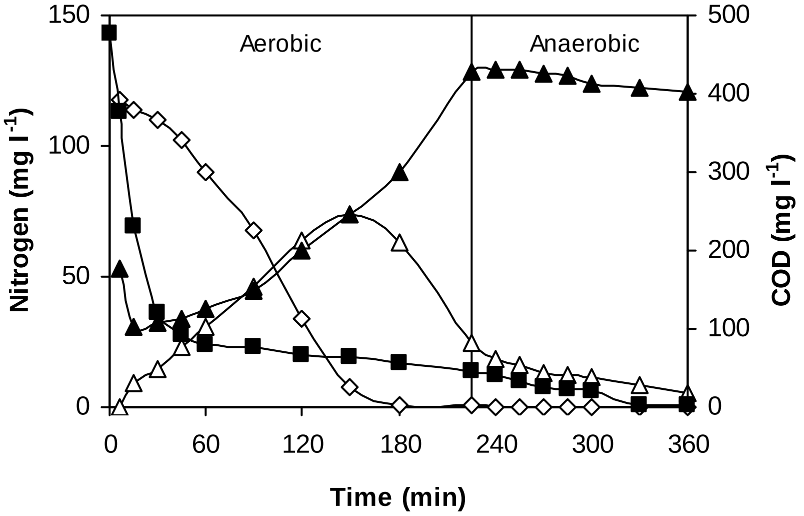

Figure 5.4 COD and nitrogen profiles at NLR of 0.45 kg N m^{-3} day^{-1} with no addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

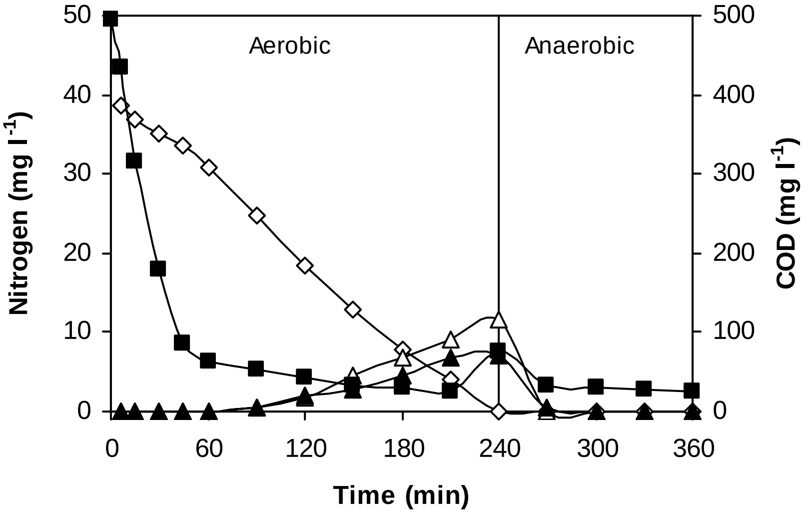

Figure 5.5 COD and nitrogen profiles at NLR of 0.15 kg N m^{-3} day^{-1} with addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

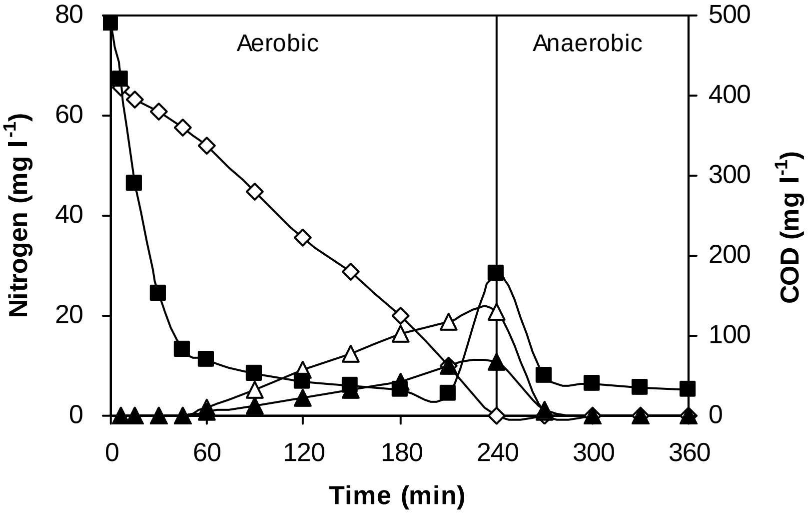

Figure 5.6 COD and nitrogen profiles at NLR of 0.25 kg N m^{-3} day^{-1} with addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

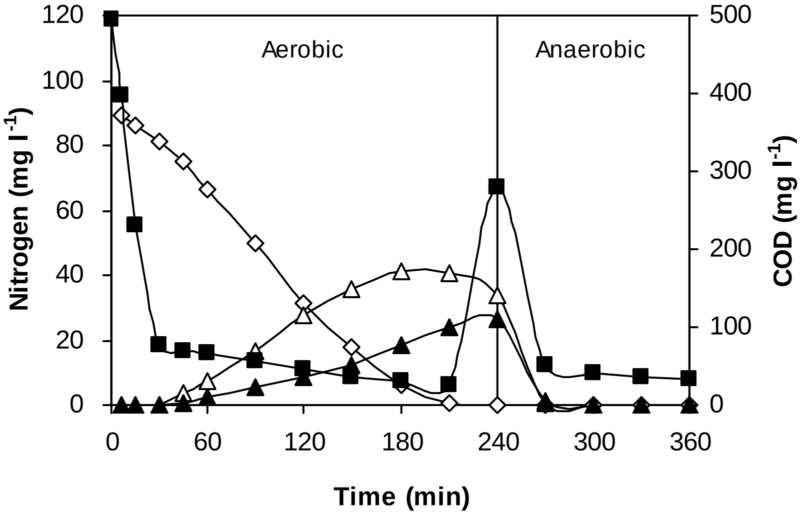

Figure 5.7 COD and nitrogen profiles at NLR of 0.35 kg N m^{-3} day^{-1} with addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

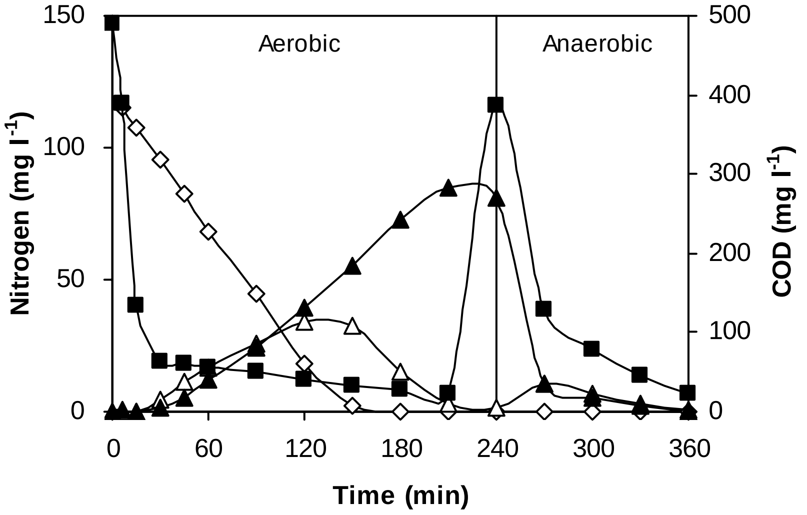

Figure 5.8 COD and nitrogen profiles at NLR of 0.45 kg N m^{-3} day^{-1} with addition of external carbon source. ■ COD; ◇ NH_4; △ NO_2; ▲ NO_3.

5.3.2 Characteristics of Microbial Granules Cultivated with Supply of External Carbon Source

The characteristics of microbial granules developed under conditions with supply of ethanol are summarized in Table 5.1. After the supply of external carbon source, the mean size of microbial granules increased and stabilized at 4.57, 2.19, 1.72, and 0.83 mm at the respective NLR of 0.15, 0.25, 0.35, and 0.45 kg N m^{-3} day^{-1}. The comparison of granule size between without and with addition of external carbon source was shown in Fig. 5.9. In fact, the thickness of biofilm has been postulated to be an important factor for nutrient removal in alternating biofilm systems (Pochana et al., 1999; Choi et al., 2001). Thin biofilm or small size granules would be advantageous for nitrification to provide sufficient oxygen flux into the deep layer of biofilm or granules, whereas a better denitrification can be expected in a thicker biofilm or a bigger granule.

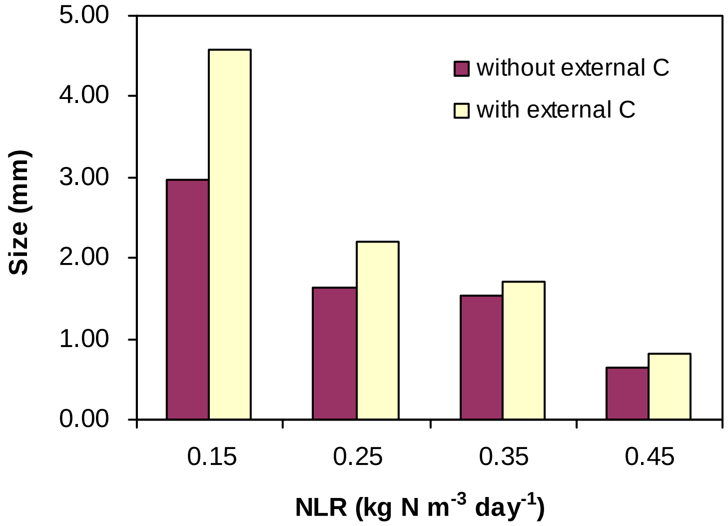

Figure 5.9 Size of microbial granules developed under conditions without and with supply of external carbon source.

Table 5.1 Sludge characteristics and reactor performance

	NLR (kg m^{-3} day^{-1})			
	0.15	0.25	0.35	0.45
Mean size (mm)	4.57	2.19	1.72	0.83
Aspect ratio	0.79	0.75	0.76	0.71
SVI (ml g^{-1})	30	25	21	12
VS/TS ratio	0.93	0.90	0.88	0.85
Biomass in reactor (g l^{-1})	3.0	4.4	4.6	5.5
Effluent COD (mg l^{-1})	26	31	33	23
COD removal efficiency (%)	95.1	95.2	95.5	97.2
Effluent N (mg l^{-1})	0.34	0.29	0.22	0.45
N removal efficiency (%)	99.0	99.5	99.7	99.6

It was found in Table 5.1 that with the addition of external carbon, the SVI of microbial granules decreased to the level as low as 30 to 12 ml g^{-1}. The aspect ratios of the granules fell into a range of 0.71 to 0.79. The biomass concentration retained in R1 to R4 was 3.0, 4.4, 4.6 and 5.5 g VS l^{-1}, respectively.

5.3.3 Activity Distributions of Heterotrophic, Nitrifying and Denitrifying Populations under Conditions with Supply of External Carbon Source

As discussed in Chapter 4, the respirometric activity of heterotrophic population decreased, while the activity of nitrifying population increased as the NLR was increased under the conditions without supply of external carbon source. For comparison purpose, the heterotrophic and nitrifying activities of granules on day 160 without external carbon source are illustrated in Fig. 5.10. It can be seen that the activity of ammonia oxidizer increased with the increase in the NLR, whereas the activity of nitrite oxidizer in the granules seems not change very much at various NLRs. However, Fig. 5.11 shows that in the case where external carbon source is available, the activity of ammonia oxidizer increased with the increase in NLR which is similar to the case before external carbon source was supplied, while $(SOUR)_H$ increased with the increases in NLR. This implies the activity of heterotrophic population was related with both NLR and the availability of external carbon source.

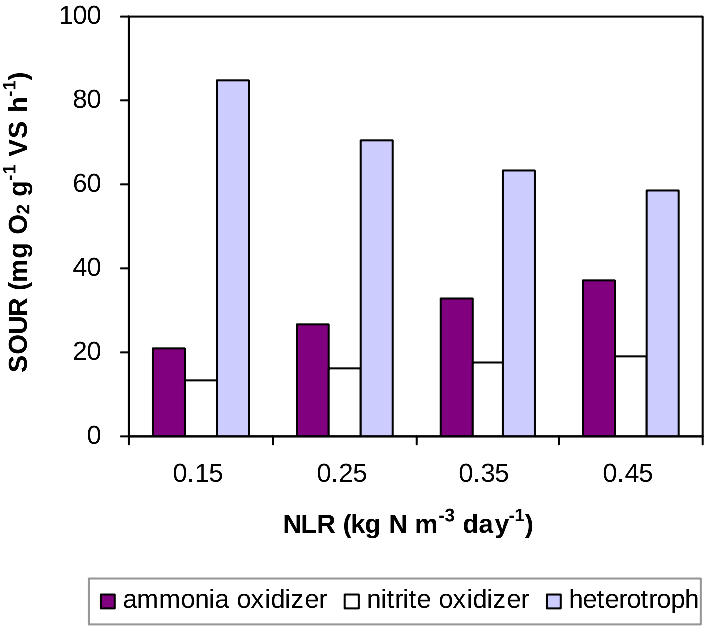

Figure 5.10 Respirometric activities of heterotrophs, ammonia oxidizer, and nitrite oxidizer in microbial granules developed in SBR without supply of external carbon source.

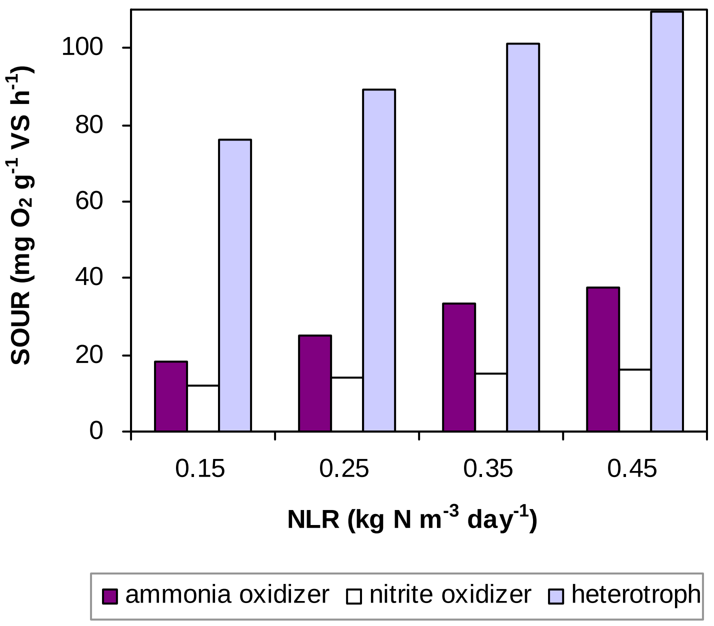

Figure 5.11 Respirometric activities of heterotrophs, ammonia oxidizer, and nitrite oxidizer in microbial granules developed in SBR with supply of external carbon source.

With the supply of external carbon source, denitrification was completely accomplished in all four reactors. The activities of denitrifying bacteria in microbial granules were determined in terms of specific total nitrogen (nitrate and nitrite) reduction rate in terms of mg NO_x-N reduced per gram granule per hour, namely q_{dn}. Fig. 5.12 shows comparison of the activity of denitrifying population, q_{dn}, with and without supply of external carbon source. It seems that the activity of denitrifying populations in microbial granules is markedly related to the availability of external carbon source as well as NLR. Denitrifying bacteria exhibit much higher activity in the presence of external carbon source than in the absence of external carbon source at all tested NLRs. It is also noticed that the activity of denitrifying bacteria increased considerably with the increase in NLR under conditions with addition of external carbon source; however, this activity slightly varies with NLR under conditions without external carbon addition.

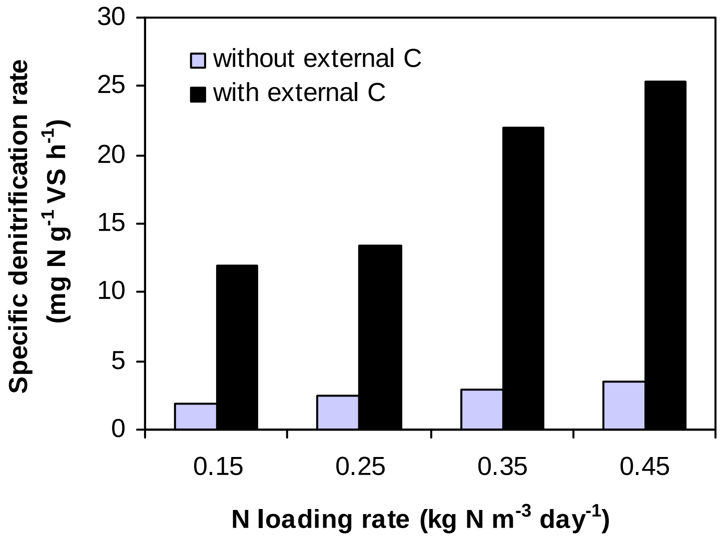

Figure 5.12 Activity of denitrifying population under conditions with and without addition of external carbon source.

5.3.4 Nitrite Accumulation in Aerobic Phase after Supply of External Carbon Source

As presented in Chapter 4, without supply of external carbon source, transient nitrite accumulation was observed in aerobic phase at all NLRs studied, i.e. at the end of the aerobic phase, almost all influent ammonia was converted to nitrate. On the contrary, Figs. 5.5 to 5.7 show that NO_2^--N was accumulated up to 65% of total nitrite and nitrate in R1-R3 with supply of external carbon source. As both ammonia oxidizers and nitrite oxidizers are involved in the complete oxidation of ammonia, nitrite accumulation presumably results from a lower rate of nitrite oxidation than that of ammonia oxidation (Liu and Tay 2001c). The ammonia and nitrite oxidation rates were shown in Fig. 5.13. In R1-R3 operated at the NLRs of 0.15-0.35 kg N m^{-3} day^{-1}, the ammonia oxidation rate was 1.3 to 3.4 mg N g^{-1} VS h^{-1}, which was 1.5-2 times higher than the nitrite oxidation rate (e.g. 0.86 to 1.7 mg N g^{-1} VS h^{-1}). In R4, it was found that the nitrite oxidation rate was higher than the ammonia oxidation rate, and thus no nitrite accumulation occurred. In fact, nitrite accumulation has been commonly reported in BNR system with or without control strategy (Rols et al. 1994; Rhee et al., 1997; Bernet et al., 2001).

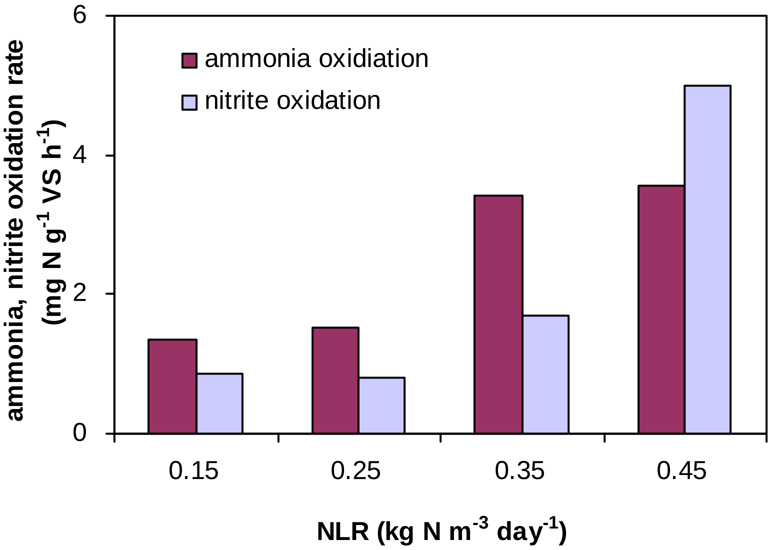

Figure 5.13 Ammonia and nitrite oxidation rate versus NLR

5.4 Discussion

Biological nitrogen removal involves two consecutive steps, i.e. nitrification and denitrification. Figs. 5.5 to 5.8 show that the complete nitrogen removal can be achieved in the alternating aerobic-anaerobic granular sludge SBR. As compared to the results obtained under conditions with no supply of external carbon source (Figs. 5.1 to 5.4), a complete denitrification was accomplished under conditions with supply of external carbon source. Therefore, denitrification efficiency of microbial granules is mainly determined by the availability of external carbon source. In fact, most denitrifying bacteria are facultative, and they use organic carbon as the electron donor for energy generation and growth. Methanol, acetate, and ethanol are the common choices as external carbon source for denitrification in wastewater industry because of their relative low cost and high resultant denitrification efficiency. In this study, ethanol was chosen as external carbon source for denitrification by microbial granules. The stoichiometric amount of ethanol required for nitrate reduction can be derived from the following equation (Mohseni-Bandpi and Elliott, 1996):

$$97NO_3^- + 50C_2H_5OH \rightarrow 5C_5H_7O_2N + 75CO_2 + 84H_2O + 46N_2 + 97OH^- \quad (5.1)$$

Equation 5.1 shows that the amount of ethanol in terms of COD required for denitrification is 3.5 g ethanol-COD g^{-1} NO_3-N denitrified. As presented in Chapter 4, when the external carbon was not supplied in the anaerobic phase, only less than 10% of NO_x-N can be denitrified and relatively high concentration of nitrate would remain in the effluent due to the incomplete denitrification (Figs. 5.1 to 5.4). In contrast, it appears from Figs. 5.5 to 5.8 and Table 5.1 that when the external ethanol is supplied at the beginning of the anaerobic phase, denitrification metabolism is activated in a short time period, and over 99% of nitrogen is removed. In partial denitrification under conditions without supply of external carbon source (see Chapter 4), total nitrogen removal efficiency was as low as 24 to 50% at the tested NLRs. However, the total nitrogen removal efficiency can reach up to 99% under conditions with supply of external carbon in all four reactors. This demonstrates that the elimination of organic carbon and total nitrogen can be perfectly achieved in alternating aerobic-anaerobic granules-based SBRs.

As reviewed in Chapter 2, there are several common problems involved in the conventional biological nitrogen removal (BNR) systems, such as sludge bulking, process stability, difficulties in maintaining sufficient nitrifying biomass, etc (Wanner and Grau, 1988; Ekama et al., 1996; Eikelboom et al., 1998). However, these problems would not occur in the granules-based system developed in this study, e.g. there is no generation of filamentous or bulking sludge in granular sludge system, which is the most notable problem met in typical nutrient removal activated sludge systems, causing seriously deteriorated effluent quality with excessive washout of sludge. Upon the formation of microbial granules in the reactors, only compact-structured microbial granules could be retained and their good settling properties lead to an easy settling phase and nitrifying bacteria could be effectively protected within the matrix of granules.

Another disadvantage of conventional BNR systems is large space requirement due to a relatively long sludge retention time (SRT). Uncoupling the sludge age from the nitrification requirement will significantly reduce bioreactor volumes and land area of secondary settling tanks. To uncouple sludge age from the requirement to nitrify, the suspended activated sludge systems can be modified in two ways: i) internal fixed media and ii) external fixed media for attached nitrifier growth. In the developed granules-based system, nitrifying, denitrifying and heterotrophic bacteria are co-existed in the microbial matrix of a single microbial granule which provides an effective protection to those bacteria. Subsequently, the reactor volume and the space requirement are greatly reduced in comparison with conventional activated sludge processes.

The specific denitrification rates in R1 to R4 ranged from 12.0 to 25.3 mg N g^{-1} VS h^{-1} with the addition of external carbon source, which was comparable with or greater than those obtained in conventional activated sludge systems (Henze, 1991; Nyberg et al., 1996; Hassleblad and Hallin, 1998). Compared to the denitrification rates obtained under the conditions without the addition of external ethanol in the anaerobic phase (1.9 to 3.5 mg N g^{-1} VS h^{-1}), it appears that the denitrification activity of microbial granules is highly dependent upon the availability of external carbon source in the anaerobic phase. It has been reported that denitrification would occur with a low rate when a slowly biodegradable organic matter was provided as the carbon source (van Haandel et al., 1981), whereas the addition of an easily degradable carbon instantaneously increased the denitrification rate (Isaacs and Henze, 1995).

Fig. 5.9 shows that the mean diameter of microbial granules increased after the supply of external carbon source at all tested NLRs. In study of biofilms, Choi et al. (2001) found that a thicker biofilm would benefit denitrification. This is consistent with Fig. 5.9. In fact, to provide sufficient oxygen into the deep layer of biofilm or granules for nitrification, a thin biofilm or small granules would be advantages for oxygen transfer. However, for denitrification to be carried out, thick biofilm or large-size granules would be desired. In simultaneous nitrification and denitrification process, the efficiency of SND was related to the floc size of activated sludge, and it was found that the SND activity decreased when the floc size was reduced because only large flocs would have the potential to develop anoxic zones internally which is essential for denitrification (Pochana et al. 1999). Actually, the microscopic observations have showed that microbial population responsible for denitrification is more likely to distribute at the inner part of microbial granules. A more recent research showed that for large size granules, an anoxic or anaerobic zone would exist at certain depth from the granule surface due to the diffusion limitation of oxygen (Li and Liu, 2005). The SVI values of microbial granules seemed to decrease with the supply of external carbon source, i.e. settleability of granules is improved.

The fraction of active biomass in a suspended or fixed microbial community was found to be proportional to the respirometric activity or specific substrate utilization rate (Moreau et al., 1994; Droste, 1997; Prescott et al., 1999; Ochoa et al., 2002). These may imply that relative abundance of microbial population should be correlated to the respirometric activity or specific substrate utilization rate (Trevors, 1984; Liu and Capdeville, 1996; Ruklisha and Ionina, 2000). Figs. 5.10 to 5.12 show that heterotrophic, nitrifying and denitrifying populations can co-exist in microbial granules developed under the alternating aerobic-anaerobic conditions with different NLRs. Nitrifying and denitrifying populations in the microbial granules were gradually built-up and stabilized with the increase of the NLR. High NLR seems to favour the selection of nitrifying bacteria in the microbial granules. Similar trends were also found in the biofilm systems (Ohashi et al., 1995; Fdz-Polanco et al., 2000; Ballinger et al., 2002).

The distribution of heterotrophic population was closely associated with the NLR as well as the availability of external carbon source. In the absence of external carbon source, heterotrophs became less dominant in the microbial granules as NLR increased. However, the supply of external carbon source would enhance the accumulation of heterotrophic population in microbial granules, especially at high NLRs. It can be seen in Fig. 5.12 that denitrifying bacteria exhibit much higher activity in the presence of external carbon source than in the absence of external carbon source, i.e. the availability of external carbon source may influence the activity of denitrifying bacteria in microbial granules. Consequently, the deficiency of external carbon source would result in a low denitrification rate in microbial granules.

Complex interactions between heterotrophic, nitrifying and dentrifying populations are found in the microbial granules at different NLRs. A positive correlation between $(SOUR)_N$ and q_{dn} was shown in Fig. 5.14. These results exhibit a synergetic relationship between nitrifying and denitrifying populations in the microbial granules cultivated at different NLRs. This is understandable because the increase in NLR results in enriched nitrifying populations and more nitrite and nitrate produced (Figs. 5.5 to 5.8), while nitrite and nitrate generated from nitrification process are the primary food for denitrifying bacteria.

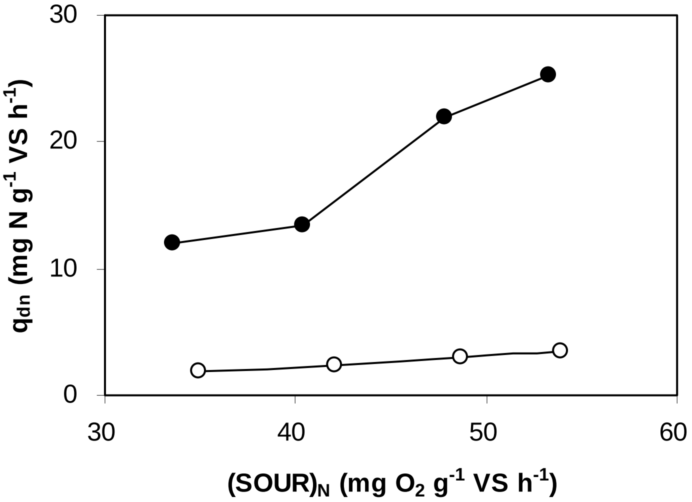

Figure 5.14 Interactions between nitrifying and denitrifying populations in microbial granules. (●) with supply of external carbon source; (○) without supply of external carbon source.

A positive correlation between $(SOUR)_H$ and $(SOUR)_N$ was obtained under conditions with external carbon source (Fig. 5.15). Under conditions with addition of external carbon source, the observed positive correlation between nitrifying and heterotrophic bacteria can be explained in a way such that most denitrifying bacteria are facultative heterotrophs, thereby external carbon required for denitrifying population would also facilitate the growth of heterotrophs. External ethanol-COD was supplied to the reactors proportional to the applied ammonia-N loadings. This may mean that the activities of heterotrophs and denitrifiers were also enhanced at higher nitrogen loading rates.

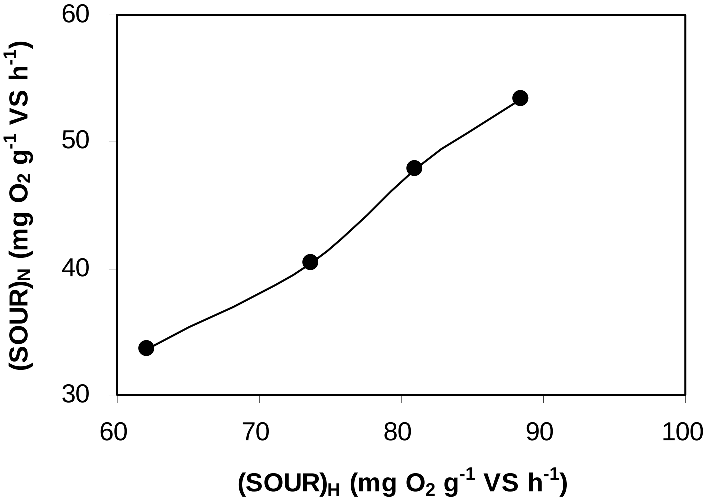

Figure 5.15 Interactions between heterotrophic and nitrifying populations in microbial granules (with supply of external carbon source).

In contrast, an inverse correlation between $(SOUR)_H$ and $(SOUR)_N$ was found under conditions without addition of external carbon source (Fig. 5.16). This inverse relationship of heterotrophic to nitrifying populations shows the competition between the two populations. As the growth rate of nitrifying bacteria is much lower than that of heterotrophic bacteria, the interspecies competition for dissolved oxygen and other nutrients between two populations should be considered (Fig. 5.16). Under conditions without supply of external carbon source, the increase in NLR would encourage the growth of nitrifying bacteria rather than heterotrophic bacteria, whereas at low NLR the interspecies competition leads to a decrease in the nitrifying population. In fact, it had been reported in biofilm culture that higher influent COD/N ratio retarded accumulation of nitrifying bacteria, especially nitrite-oxidizers due to competition for dissolved oxygen and space in the biofilm (Okabe et al., 1996; Atlas and Bartha, 1998; Fdz-Polanco et al., 2000; Nogueira et al., 2002). Consequently, the complex interactions between the heterotrophic, nitrifying and denitrifying populations in the microbial granules seem to be related to the applied NLR and the availability of external carbon source.

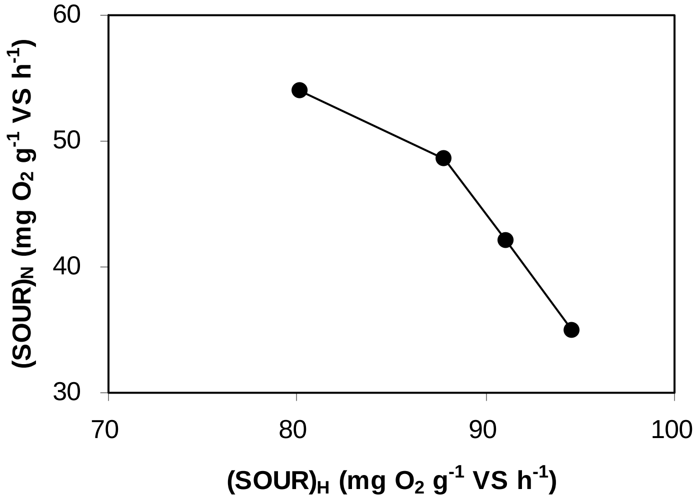

Figure 5.16 Interactions between heterotrophic and nitrifying populations in microbial granules (without supply of external carbon source).

As shown in Figs. 5.5 to 5.7, nitrite accumulation occurred in R1-R3 operated at the NLRs of 0.15-0.35 kg N g^{-1} VS h^{-1} with addition of external carbon source. This is mainly due to the differences between the growth rates of ammonia oxidizer and nitrite oxidizer as discussed earlier. The shortened nitrification pathway via nitrite instead of nitrate has been intensively studied (Turk and Marvinic, 1989; Rols et al. 1994; Rhee et al., 1997; Bernet et al., 2001). Nitrification and denitrification via nitrite have advantages of lowered oxygen requirement for the oxidation of nitrite to nitrate and reduced requirement on organic carbon for denitrification, and furthermore, denitrification rate with nitrite is often 1.5 to 2 times greater than that with nitrate (Turk and Marvinic, 1986; Abeling and Seyfried, 1992). In this study, nitrite accumulation occurred without application of any control strategies. It should be realized that nitrification and denitrification via nitrite is of great interests in the nitrogen removal systems and optimal conditions for nitrite build-up in granular sludge system need further investigation.

5.5 Conclusions

Microbial granules are capable of removing organic carbon and nitrogen through nitrification and denitrification in alternating aerobic-anaerobic SBR. The COD removal efficiency fell into the range of 95.1% to 97.2%. The nitrogen removal efficiency of over 99% was achieved at all the tested NLRs and the specific denitrification rates ranged from 12.0 to 25.3 mg N g^{-1} VS h^{-1} under conditions with the supply of external carbon source. The mean size of microbial granules stabilized at 4.57, 2.19, 1.72, and 0.83 mm at the respective NLR of 0.15, 0.25, 0.35, and 0.45 kg N m^{-3} day^{-1}. Heterotrophic, nitrifying and denitrifying populations can co-exist in the microbial matrix of granules. The respective activity of heterotrophic, nitrifying and denitrifying populations was highly related to the NLR and the availability of external carbon source. The activity of heterotrophic and denitrifying population was increased with the supply of external carbon, while the activity of nitrifying population did not show significant changes. The coexistence of heterotrophic, nitrifying and denitrifying populations with different specific biochemical functions in the microbial granules may provide a more efficient way for organic carbon and nitrogen removal. It is expected that a novel microbial granules-based biological process for organic carbon and nitrogen removal with high efficiency would be envisaged for industrial application in the near future.

Chapter 6

Denitrification on PHB by Microbial Granules

6.1 Introduction

Nitrogen removal from wastewater is often limited by the availability of reducing power for denitrification. SBR has been intensively applied for nitrogen removal due to its flexible operation, easy shifts among aerobic, anoxic and anaerobic conditions, and efficient selection of desired microbial population. In SBR biomass is subject to unbalanced conditions, such as substrate feast/famine and alternating aerobic-anaerobic periods. Under these dynamic conditions, a survival strategy adopted by microbial population is to store excess substrate as internal polymers, mainly poly-β-hydroxybutyric acid (PHB) from incoming substrate. The stored PHB will be used subsequently for the growth and the maintenance when no external substrate is available (Mino et al., 1987; Majone et al., 1998; Beun et al., 2000).

Storage of PHB can occur in nitrification and denitrification system in which microbial populations are subject to dynamic conditions with respect to the availability of external substrate, nitrogen and oxygen. In the aerobic period, microorganisms tend to accumulate PHB internally with external substrate, and in the anaerobic period, when there is no external substrate, microorganisms use the stored PHB as internal energy and carbon source for growth and denitrification (Majone et al., 1998; Beun et al., 2000). Although it has been known that the stored PHB can be metabolized to provide energy and carbon for biosynthesis of new cells; however, very limited information is available on the role of PHB as an alternative carbon source for denitrification in nitrogen removal process.

Chapters 4 and 5 showed partial denitrification in granular sludge SBR without the supply of external carbon, and a complete denitrification in the reactors supplied with external carbon source. As pointed out above, the storage of PHB has been observed in pure and mixed culture as an important mechanism of response to dynamic conditions. So far, little information is available on the PHB storage and subsequent utilization in microbial granules culture, and it is not clear about whether PHB could serve as internal carbon source for denitrification by microbial granules. Therefore, this chapter looked into the PHB storage behavior in microbial granules developed under alternating aerobic-anaerobic conditions and further to evaluate its potential role in denitrification process by microbial granules.

6.2 Materials and Methods

6.2.1 Reactor Set-up and Operation

The experimental set-up and operation conditions were detailed in Chapter 4.

6.2.2 Design of Batch Tests

Five batch experiments with various availabilities of external carbon and nitrate sources were designed and conducted under anaerobic conditions (Table 6.1). Batch 1: no external carbon and nitrate were supplied; Batch 2: nitrate was provided, but external carbon source was not available; Batch 3: external carbon source is supplied, while no nitrate present in the culture medium; Batch 4: nitrate was provided, but carbon source was supplied at a limiting level compared to nitrate; Batch 5: both carbon and nitrate were available, while carbon was provided in excess over nitrate. The microbial granules used in the above batch experiments were harvested at peak PHB concentration from the parent SBR (R3), and were gently washed with distilled water, and were then added to 1 L batch reactor. Nitrogen gas was purged into the batch reactor to keep anaerobic condition in all tests. Liquid samples from the batch reactors were analyzed for total organic carbon, nitrate-nitrogen, nitrite-nitrogen, and solid samples for measuring PHB content in granules. All batch experiments lasted for 5 hours, except for Batch 4 that was prolonged to 25 hours.

Table 6.1 Composition of the medium used for the batch tests (mg l^{-1})

		Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
Substrate	$CH_3CH_2OH^a$	–	–	60	60	120
	KNO_3^b	–	80	–	80	80
Nutrient	Na_2HPO_4	30	30	30	30	30
	KH_2PO_4	14	14	14	14	14
	$MgSO_4 \cdot 7H_2O$	16	16	16	16	16
	$CaCl_2 \cdot 2H_2O$	12	12	12	12	12
	$FeCl_3 \cdot 6H_2O$	6.4	6.4	6.4	6.4	6.4

[a] CH_3CH_2OH in mg C l^{-1}

[b] KNO_3 in mg N l^{-1}

6.2.3 Analytical Methods

Soluble ammonium-N, nitrite-N and nitrate-N concentrations were determined by a flow injection analyzer (QuikChem Method 10-107-06-1-I, Lachat Instruments, Inc.). Soluble total organic carbon (TOC) was measured by a TOC analyzer (Shimasu 5000), while soluble COD concentration was determined by standard methods (APHA, 1998). Biomass and PHB content in microbial granules were determined using the gravimetric method proposed by Marchessault et al. (1990) and Ramsay et al. (1990). To measure cell dry mass, 10 ml of culture sample was harvested by centrifugation at 10,000g for 10 min, and was washed with deionized water, and was further dried to constant weight at 90°C for 24 hours. For PHB measurement, 10 ml of sodium dodecyl sulfate solution (1% w/v sodium dodecyl sulfate at pH 10) was added to 0.1 g of the biomass sample, and the mixture was then incubated on an orbital shaker at 200 rev/min and 37°C. After one-hour incubation, the solids were recovered by centrifugation and washed with a sodium hypochlorite solution (5.64% w/v sodium hypochlorite, 1 ml) that had been diluted to 20 ml. The pellet was centrifuged at 7,000g for 4 minutes, and was then washed with 20 ml of deionized water, and was centrifuged again. The weight of PHB was measured as the final pellet after drying at 90°C for 24 hours to constant weight in a pre-weighed aluminium dish. PHB content was expressed as the mass fraction of cell dry weight. Specific ethanol-carbon uptake rate (q_s), specific nitrate uptake rate (q_{NO3}) and specific PHB production rate during feast period (q_p^{prod}) was calculated by performing linear regression on curves of ethanol-carbon degradation, nitrate uptake and PHB production against time, respectively. As nitrate uptake rates would differ significantly between two cases: with external carbon and without external carbon, to calculate the nitrate uptake rate the profiles of nitrate uptake were divided into two parts according to the availability of external carbon. Thus, the nitrate uptake rates were described with nitrate uptake rate during feast period (q_{NO3}^{feast}) and nitrate uptake rate during famine period (q_{NO3}^{famine}). The linear correlations between the parameters were evidenced by high correlation coefficients of 0.97 to 0.99. Thus, the specific rates of TOC, PHB and nitrate-nitrogen can be calculated and are reported in the results section.

6.3 Results

6.3.1 PHB Degradation without Supply of External Carbon and Nitrate

Fig. 6.1 shows time profile of PHB utilization obtained in Batch 1 without supply of external ethanol and nitrate. It can be seen that the PHB pre-accumulated in microbial granules gradually declined from an initial level of 18.4 to 13.8% by dry weight at the end of the 5-hour anaerobic cultivation. The observed decrease of the PHB pool in microbial granules is mainly due to anaerobic endogenous respiration of granule as the external carbon and nitrate sources are not available in Batch 1.

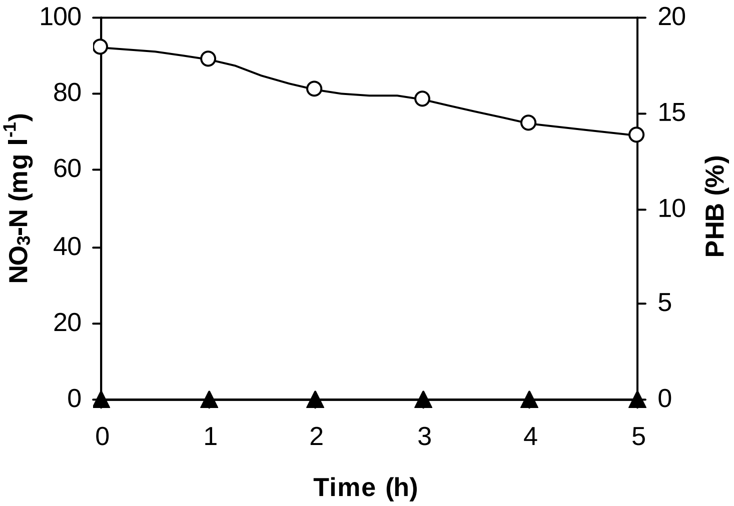

Figure 6.1 Nitrate-nitrogen and PHB profiles observed in Batch 1 with no carbon and nitrate. (▲) NO_3-N; (○) PHB.

6.3.2 Denitrification on PHB without Supply of External Carbon

As a reducing power, PHB can serve as an internal carbon source for denitrification (Majone et al., 1998). In order to examine the potential of PHB as alternative carbon source for denitrification in the absence of external carbon source, nitrate was added in Batch 2 at an initial nitrate-N concentration of about 80 mg l^{-1}, while no external carbon source was provided (Table 6.1). When external carbon source was not available under denitrifying condition, only about 10 mg NO_3^{-}-N l^{-1} was denitrified in 5-hour anaerobic period (Fig. 6.2). This amount of denitrified nitrate-N was consistent with that observed in the parent SBR (Fig. 5.1) as well as with those reported in the literature (Third et al., 2003a; Zeng et al., 2003). The specific nitrate uptake rate was estimated as 1.2 mg N g^{-1} VSS h^{-1} with PHB as internal carbon source for denitrification (Table 6.2).

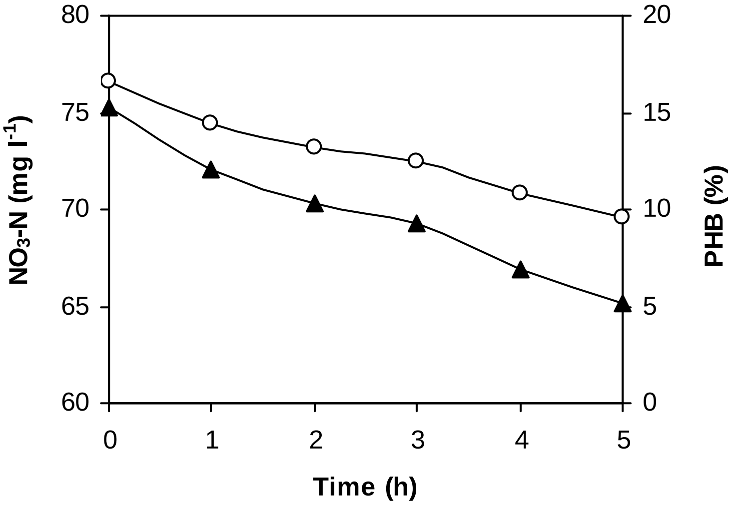

Figure 6.2 Nitrate-nitrogen and PHB profiles observed in Batch 2 with no carbon but with nitrate. (▲) NO_3-N; (○) PHB.

Table 6.2 Specific conversion rates in the batch tests

Parameter	Dimension	Batch 1	Batch 2	Batch 3	Batch 4	Batch 5
q_s	mg C g^{-1} C h^{-1}	–	–	46.50	45.52	52.35
q_p^{prod}	mg C g^{-1} C h^{-1}	–	–	21.39	15.17	14.72
q_p^{prod}/q_s	–	–	–	0.46	0.33	0.28
$q_{NO_3}^{fe}$	mg N g^{-1} VS h^{-1}	–	–	–	9.1	13.9
$q_{NO_3}^{fa}$	mg N g^{-1} VS h^{-1}	–	1.2	–	4.7	2.5

6.3.3 PHB Storage and Degradation with External Carbon

The intracellular accumulation of PHB as a reserve material is a common phenomenon observed in the presence of an external carbon source (Doi, 1990). Batch 3 was designed and conducted to look into the PHB accumulation within microbial granular sludge in the N-free medium, but with supply of external carbon at a concentration of 60 mg ethanol-carbon l^{-1}. Fig. 6.3 shows that ethanol-carbon was consumed gradually from 69.3 to 4.0 mg C l^{-1} (specific ethanol-carbon uptake rate decreased from 46.5 to 4.7 mg C g^{-1} C h^{-1}), accompanied with an increase of the PHB content from 17.6 to 22.8% dry weight. No net PHB degradation was observed during 5-hour anaerobic reaction.

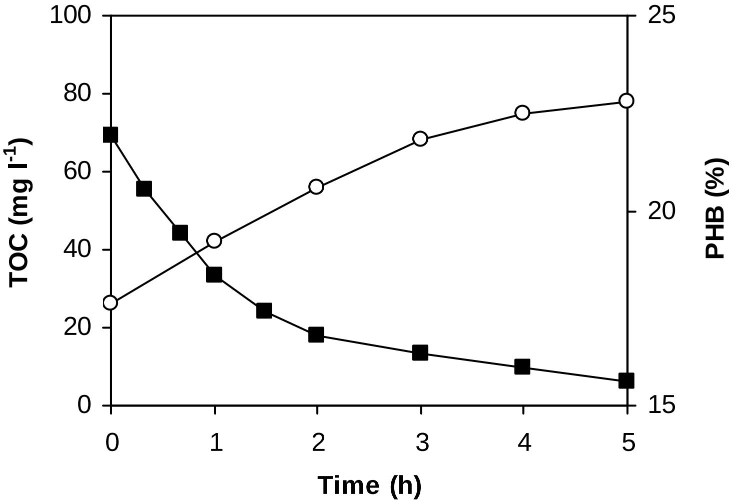

Figure 6.3 TOC and PHB profiles observed in Batch 3 with no nitrate but with carbon. (■) TOC; (○) PHB.

6.3.4 Denitrification Behaviour with Insufficient Carbon Source

In Batch 4, an initial nitrate-N concentration of 80 mg l^{-1} was supplied and ethanol-carbon was added at a concentration of 60 mg l^{-1}. According to the stoichiometry of denitrification, the added ethanol-carbon is insufficient with respect to the present nitrate. Fig. 6.4 shows time profiles of ethanol-carbon utilization, nitrate uptake, and PHB accumulation and subsequent degradation. It can be seen that ethanol was removed at a relative constant rate in the first two hours, which was accompanied with a PHB accumulation from 16.4 to 18.7% by dry weight as well as a nitrate reduction by 30 mg N l^{-1}. Upon the depletion of external ethanol carbon, a decline in PHB occurs, which is associated with a slower nitrate reduction. At the end of 5-hour anaerobic period, a nitrate removal efficiency of 64% was recorded. Respective nitrate uptake rate of 9.1 and 4.7 mg N g^{-1} VSS h^{-1} was obtained with carbon supplied externally and internally.

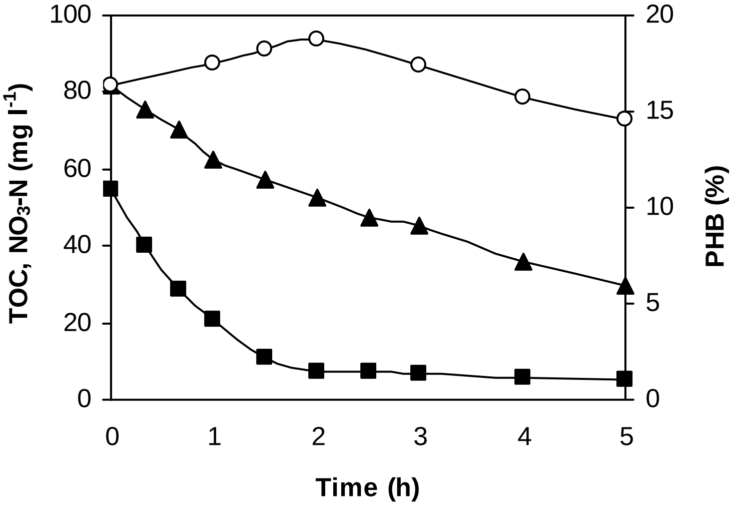

Figure 6.4 TOC, nitrate-nitrogen and PHB profiles observed in Batch 4 with nitrate and insufficient carbon. (■) TOC; (▲) NO_3-N; (○) PHB.

6.3.5 Denitrification Behaviour with Excessive Carbon Source

Compared to Batch 4, in Batch 5, excessive ethanol-carbon was supplied at a concentration of 120 mg l^{-1} (twice of that provided in Batch 4), while initial nitrate-N concentration was kept the same as that in Batch 4, i.e. 80 mg l^{-1} (Table 6.1). Time profiles of ethanol-carbon utilization, nitrate uptake, and PHB accumulation and subsequent degradation are shown in Fig. 6.5. The uptake of TOC was coupled with the accumulation of PHB from 16.8 to 20.2% and 70 mg l^{-1} of nitrate reduction until all TOC was depleted after the three-hour anaerobic reaction. Nitrate was removed linearly without nitrite accumulation as the external ethanol carbon was present in excess (Fig. 6.5). The specific nitrate uptake rate was estimated as 13.9 mg N g^{-1} VSS h^{-1} in the feast phase before the external carbon was depleted. In the last 2 hours of Batch 5, ethanol-carbon was completely eliminated, and a famine period appeared. During such an anaerobic-famine period, the stored PHB was consumed with a lower specific nitrate utilization rate of 2.5 mg N g^{-1} VSS h^{-1} (Table 6.1). The overall ratio between ethanol-COD and nitrogen uptake was 5.5 mg COD mg^{-1} N which is comparable with those reported for denitrification with external carbon supply (Isaacs and Henze, 1995).

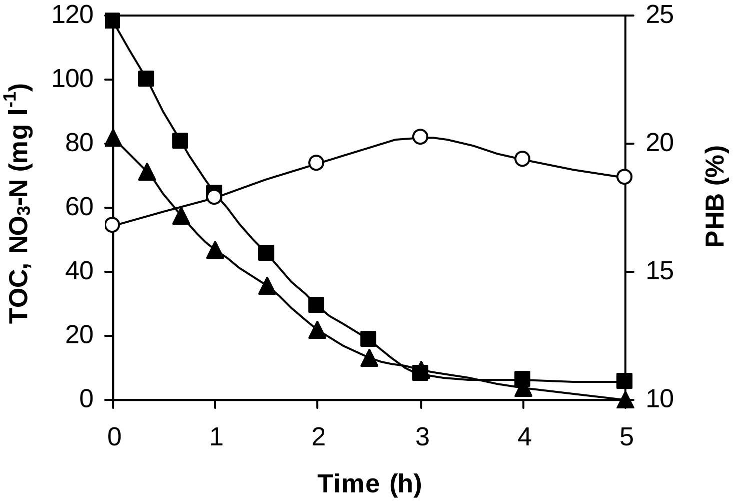

Figure 6.5 TOC, nitrate-nitrogen and PHB profiles observed in Batch 5 with nitrate and excess carbon. (■) TOC; (▲) NO_3-N; (○) PHB.

6.4 Discussion

As pointed out in Chapters 4 and 5, there might be other unidentified carbon source involved in the denitrification process especially under conditions without supply of external carbon source. The storage of PHB by heterotrophic bacteria has been widely reported (Mino et al., 1987; Majone et al., 2001; Wang and Yu, 2001). However, no information is available on the role of PHB in denitrification by microbial granules so far. In the absence of both external organic carbon and nitrate in the anaerobic phase, PHB was utilized at a constant rate. This could be attributed to the anaerobic endogenous respiration of microbial granules (Fig. 6.1). Fig. 6.1 reveals that about 4.6% of PHB was anaerobically degraded in 5 hours, which gives a specific PHB consumption rate of 12.5 mg C g^{-1} C h^{-1}. This amount of the degraded PHB could be considered as a baseline for other batch studies. When nitrate was supplied, but no external organic carbon was added to the medium, about 7% of PHB by dry weight was consumed at a higher degradation rate of 17.3 mg C g^{-1} C h^{-1} associated with a simultaneous nitrate reduction, i.e. 10 mg l^{-1} nitrate-nitrogen was denitrified (Fig. 6.2). The specific denitrification rate observed in Fig. 6.2 is close to that attained in the parent SBR (Fig. 5.3). These results seem to indicate that PHB can be utilized as carbon and energy source for microbial functions and denitrification.

A continuous accumulation of PHB in microbial granules was observed in the batch experiments operated with external carbon supply until all the external carbon was depleted, in the case of with or without addition of nitrate (Figs. 6.3 to 6.5). In the three cases with carbon supply, the PHB production rate and storage yield are high when nitrate-nitrogen is absent in the medium with respect to its presence in the medium (Table 6.2). In fact, the PHB production has been thought to be a response of microorganisms to nutrient limitation in the presence of excess carbon source (Anderson and Dawes, 1990; Dawes, 1990; Third et al., 2003b). When the medium contained excessive organic carbon and limited nitrogen or even without nitrogen, cell growth was unlikely to occur and external carbon was utilized to synthesize PHB as stored intracellular energy for cell maintenance (Fig. 6.3). As shown in Table 6.2, the observed storage yield of PHB (q_p^{prod}/q_s) for nitrogen-free batch culture (Batch 3) was 0.46, which is higher than the observed storage yields (0.38 and 0.28) in the nitrogen-rich batch cultures (Batches 4 and 5).

In fact, a PHB storage yield of 0.40 was reported in suspended activated sludge culture under anoxic conditions (Majone et al., 1998; Beun et al., 2000). It was found that under unbalanced growth conditions (no nitrogen, but in the presence of organic carbon), PHB production would be a relatively main mechanism for removing organic carbon as microbial growth was suppressed by nitrogen limitation (Fig. 6.3), whereas under balanced growth conditions (with organic carbon and nitrogen supply), external organic carbon was consumed simultaneously for denitrification, PHB storage as well as for cell growth and maintenance (Figs. 6.4 and 6.5).

Table 6.2 shows rate data of ethanol-carbon utilization, denitrification and PHB production under various conditions. It appears that PHB can serve as carbon and energy source only when external carbon is no longer available. The specific denitrification rate on PHB is 2 to 5 times lower than that on ethanol, a readily biodegradable carbon source (Table 6.2). Meanwhile, Figs. 6.1-6.5 indicate that less than 28 mg l^{-1} NO_3^--N could be denitrified using PHB as internal carbon source in the batch experiments as well as in the parent SBR. In fact, this value is consistent well with the literature results showing that the amount of NO_3^--N denitrified with internally stored PHB is less than 2 mmol N l^{-1} (Third et al., 2003a; Zeng et al., 2003).

In order to confirm the results reported in Fig. 6.2, the batch experiment was further prolonged from 5 to 25 hours in Batch 4. The nitrate and PHB profiles in the prolonged anaerobic period are illustrated in Fig. 6.6. It can be seen that the accumulation of PHB is coupled with the oxidation of TOC until all TOC is depleted after the two-hour anaerobic reaction. Afterwards reduction in nitrate is associated with the degradation of PHB until 8 hours of anaerobic reaction. However, during the period of 8 to 25 hours, nitrate was no longer removed, while PHB was slowly degraded solely for anaerobic endogenous respiration.

Figs. 6.1-6.6 seem to indicate that the potential of PHB for denitrification would be limited. In fact, the minimum PHB pool would exist, and a minimal fraction of PHB of 3.6% in cell mass had been reported in activated sludge cultures subjecting a prolonged degradation period (van Aalst-van Leeuwen et al., 1997). It is most likely that microorganisms would preferentially utilize PHB as internal carbon and energy source for cell functions rather than for denitrification when the PHB reservoir decreases to below a critical level. In this case, anaerobic degradation of PHB is dissociated from denitrification. In the sense of energy metabolism, a certain PHB reservoir is essential for microbial granules to survive under unbalanced or starvation condition. Thus, the capacity of PHB as reducing power for denitrification should not be overestimated.

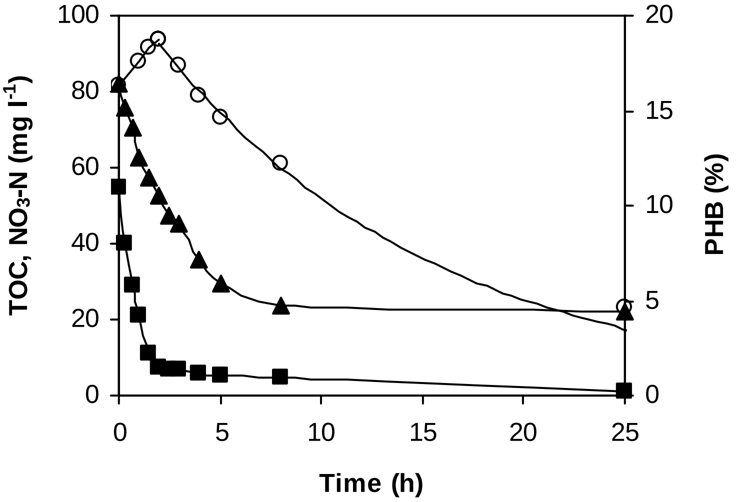

Figure 6.6 TOC, nitrate-nitrogen and PHB profiles in the prolonged Batch 4 under anaerobic condition. (■) TOC; (▲) NO_3-N; (○) PHB.

6.5 Conclusions

The potential role of PHB for denitrification was evaluated in five well-designed batch experiments. Without supply of external carbon and nitrate, PHB was degraded only for cell maintenance. With the addition of nitrate but no external carbon, PHB was utilized for both denitrification and cell maintenance. In the cases with supply of external carbon and nitrate, the external carbon mainly goes for PHB accumulation, denitrification and cell functions. The accumulated PHB can be used as energy and carbon source for denitrification and maintenance when external carbon is no longer available. A noticeable observation in the prolonged study is that anaerobic degradation of PHB is not necessarily coupled with denitrification. The utilization of PHB for cell maintenance was prior to its uptake for denitrification when PHB decreased to a critical level. In the absence of external carbon under denitrifying condition, only limited amount of nitrate (< 2 mmol NO_3^--N l^{-1}) can be reduced with internally stored PHB. These may imply that internally stored PHB in microbial granules can act as a feasible alternative carbon source for denitrification only in the case of low nitrogen concentration in the influent to be treated. However, if high nitrogen concentration is present in the influent, supply of an external carbon for a complete denitrification is necessary and indispensable.

Chapter 7

Conclusions and Recommendations

7.1 Conclusions

The study firstly explored the essential role of hydraulic selection pressure in terms of settling time in the formation of aerobic granules, which is found to be the driving force of aerobic granulation. This study offers good insight into the mechanisms behind aerobic granulation and provides a better understanding and practical operating strategies for enhanced aerobic granulation in SBR. Based on these findings, the selection pressure of successful aerobic granulation was optimized and applied to the subsequent studies on organics and nitrogen removal by microbial granules developed in alternating aerobic-anaerobic SBR. The conclusions drawn from this study are summarised as follows.

1. Effect of settling time on aerobic granulation in SBR

The hydraulic selection pressure in terms of settling time is a decisive parameter in the formation of aerobic granules in SBR. Very weak selection pressure did not favour aerobic granulation, and relatively strong selection pressure was essential for the development of aerobic granules in SBR. The settling time required for successful aerobic granulation would not be longer than 5 minutes for the lab-scale SBR used in this study. The microbial community was found to metabolically respond to hydraulic selection pressure through the effective regulation of its energy metabolism, which in turn led to enhanced production of extracellular polysaccharides and improved cell hydrophobicity. It is most likely that aerobic granulation would be an effective defence or protective strategy of microbial community against external selection pressure. This study shows good insight into the mechanisms behind aerobic granulation.

2. Microbial granulation under alternating aerobic-anaerobic conditions – partial denitrification without external carbon source

Under alternating aerobic-anaerobic conditions, microbial granules were successfully developed with comparable or better settleability as compared to granules cultivated under absolute aerobic conditions. The SVI of microbial granules was as low as 36 to 27 ml g^{-1}. The NLR showed a significant effect on the physico-chemical properties of microbial granules, while the NLR also influenced the relative distribution of extracellular polymers. Extracellular polysaccharide content decreased and protein content increased with the increase in NLR. Elemental analyses showed that the cell C:N ratio of microbial granules decreased with the increase in NLR. NLR also affected the distribution of heterotrophic, nitrifying and denitrifying populations in microbial granules. Nitrifying population was enriched in microbial granules at higher NLR. This ensured complete nitrification in the aerobic phase. Partial denitrification was observed due to insufficiency of electron donor when no external organic carbon was fed. The removal efficiency obtained was over 95% for carbon and 24 to 50% for nitrogen at various NLRs without the supply of external carbon source.

3. Microbial granulation under alternating aerobic-anaerobic conditions – complete denitrification with external carbon source

It was found that with the supply of external carbon source, microbial granules could efficiently remove organic carbon and nitrogen in alternating aerobic-anaerobic SBR. Complete denitrification was achieved at all the tested NLRs. The COD removal efficiency was maintained as high as 95.1 to 97.2%, and 99% of the nitrogen removal was achieved at all the tested NLRs. The specific denitrification rates ranged from 12.0 to 25.3 mg N g^{-1} VS h^{-1} at various NLRs. Heterotrophic, nitrifying and denitrifying populations can co-exist in the microbial matrix of granules. The activity of respective population was highly related to the applied nitrogen loading rates and the availability of external carbon source. The activity of heterotrophic and denitrifying population increased after the supply of external carbon, whereas the activity of nitrifying population did not show significant changes as compared to the cases without supply of external carbon.

4. Denitrification capacity on PHB by microbial granules

The potential role of PHB as internal carbon source was evaluated in the well-designed batch experiments. Under anoxic condition with supply of external carbon and nitrate, the external carbon mainly went for PHB accumulation, denitrification and cell functions. The accumulated PHB can be used as energy and carbon source for denitrification and maintenance when external carbon is not available. It was found that anoxic or anaerobic degradation of PHB was not necessarily coupled with denitrification. The utilization of PHB for cell maintenance may be prior to its uptake for denitrification when PHB decreased to a critical level. In the absence of external carbon under denitrifying condition, only limited amount of nitrate (<2 mmol NO_3^--N l^{-1}) can be reduced with internally stored PHB. These may imply that PHB can act as an alternative carbon source for denitrification only in the case of low nitrogen concentration in the influent to be treated. For treating wastewater with high concentration of nitrogen, an external carbon for complete denitrification is necessarily supplied. It is expected that this study is helpful for better understanding microbial granulation and its application for organics and nitrogen removal.

7.2 Recommendations for Future Study

This study has provided insights into both fundamentals and applications of microbial granules for wastewater treatment. Further study still need to look into the following aspects. It is suggested that quantitative investigation of individual populations, like ammonia oxidizing bacteria, nitrite oxidizing bacteria, and their spatial distribution within the microbial granules should be conducted. A key point for single-sludge nutrient removal system is to maintain selected population with respective function sufficiently and steadily. The distribution of nitrifying, denitrifying and heterotrophic communities may also be affected by other operating parameters, such as dissolved oxygen, and the ratio of aerobic period to anoxic period in every cycle. Further study is suggested to quantitatively investigate the influence of such parameters on selected nitrifying and denitrifying communities and to track the dynamics of reactor performance and microbial composition with the changes in these operating conditions. Further study should also be conducted to optimize operating conditions for simultaneous nitrification and denitrification (SND) via nitrite. Real-time control using the oxidation-reduction potential (ORP) is also suggested to be applied in the future study for nitrogen removal by microbial granules.

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