EFFECT OF SUBSTRATE N/COD RATIO ON THE FORMATION AND CHARACTERISTICS OF AEROBIC GRANULES DEVELOPED IN SEQUENCING BATCH REACTORS

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ABSTRACT

With increasing demands on water quality, advanced and cost-effective techniques for nitrogen removal from wastewater become more and more important. Water industry has very strong demand for development of novel biotechnology for efficient removal of nitrogen from industrial and municipal wastewaters. This research work attempted to develop aerobic granules for simultaneous organic carbon and nitrogen removal. For this purpose, four sequencing batch reactors (SBR) were operated at different substrate N/COD ratios in the range of 5/100 to 30/100 by weight. Results showed that aerobic granules could be formed over the substrate N/COD ratios of 5/100 to 30/100, i.e. aerobic granulation would be independent of the substrate N/COD ratios applied, However, the substrate N/COD ratio showed a significant effect on microbial and physicochemical characteristics of aerobic granules. It was found that the activity of nitrifying bacteria was enhanced with the increase of substrate N/COD ratio. The cell hydrophobicity was improved significantly, while the production of extracellular polysaccharides showed a decreasing trend as the substrate NKOD ratio was increased.

Nitrifying, denitrifying and heterotrophic populations would co-exist in aerobic granules, and shifts in microbial populations in the granules were closely related to the substrate N/COD ratio. The relative abundance of heterotrophic, nitrifying and denitrifying populations in aerobic granules was a function of the substrate N/COD ratio and evolved until a balance among different species was achieved. Nitrifying and denitrifying populations were enriched with the increase of substrate NKOD ratio, while the quantity of heterotrophic populations showed a decreasing trend.

The results also revealed that the elemental compositions of aerobic granules were highly related to the substrate N/COD ratios, i.e. an increased substrate N/COD ratio resulted in an increased cell N/C ratio and a decreased cell O/C ra It was further shown that cell hydrophobicity of aerobic granules was inversely correlated to the cell O/C ratio.

Aerobic granules developed at high substrate N/COD ratios exhibited enhanced nitrification efficiency, while DO concentration and mixing power significantly influenced the efficiency of denitrification by aerobic granules. Complete denitrification was achieved at DO concentration of 0.5 mg l^{-1} with mixing that was necessary to ensure a sufficient contact between granules and soluble nitrate, otherwise denitrification by aerobic granules would slow down markedly.

The stability of aerobic granules is a key of long-term and stable operation of aerobic granular sludge bioreactor. As compared to anaerobic granules, aerobic granules had relatively low stability due to the fast growth of heterotrophic bacteria. In this work, a potential strategy to improve the stability of aerobic granules through selecting slow-growing nitrifying bacteria was proposed. The observed growth rate and mean size of mature aerobic granules were found to decrease as the substrate N/COD ratio increased, while nitrifying population was enriched markedly in aerobic granules developed at high substrate N/COD ratios. The enriched nitrifying population in aerobic granules was responsible for the observed low growth rate of aerobic granules. The substrate N/COD ratio is an important factor in selecting nitrifying bacteria in aerobic granules. Aerobic granules with low growth rates showed strong structure and good settleability in terms of specific gravity, SVI and cell hydrophobicity that further lead to high stability as compared to those having high growth rates. This work demonstrated that the selection of slow-growing nitrifying bacteria through controlling substrate N/COD ratio would be a useful strategy for improving the stability of aerobic granules.

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3. Yang, S.F., Tay, J.H. and Liu, Y. (2004). Inhibition of free ammonia to the formation of aerobic granules. *Biochemical Engineering Journal* 17, 41-48.
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LIST OF ABBREVIATIONS

BOD	biological oxygen demand (mg l^{-1})
COD	chemical oxygen demand (mg l^{-1})
DO	dissolved oxygen (mg l^{-1})
ECP	extracellular polymer
FA	free ammonia
HRT	hydraulic retention time (hour or day)
IA	image analysis
OLR	organic loading rate (kg COD m^{-3} day^{-1})
PUM	phosphate urea magnesium
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})
SS	suspended solid concentration (mg l^{-1})
SVI	sludge volume index (ml g^{-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})

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

The removal of nitrogenous matter in wastewater has increased significance in recent years as a means of protecting and preserving the environment. Many modifications and processes on conventional biological treatment had been developed and implemented for nitrogen removal from wastewater in order to meet the increasingly stringent environmental regulations. These include three-phase fluidised bed reactor, biofilm airlift suspension reactor, membrane bioreactor, and others (Hellinga et al., 1999; Strous, 2000; Bernet et al., 2000; Hao, 2001).

Recently, research efforts had turned to developing granules of activated sludge in aerobic sequencing batch reactor (SBR) systems (Beun *et al.*, 1999; Tay *et al.*, 2001). As compared to activated sludge flocs, the advantages of aerobic granular sludge are known as regular, denser and stronger microbial structure, good settling ability, high biomass retention, and ability to withstand high organic loading rate. Meanwhile, microbial diversity study revealed that aerobic granules were complex microbial consortia which contained microbes carrying different physiological functions (Tay et al., 2002; Jang et al. 2003; Yi et al., 2003). Many wastewater treatment plants need to be upgraded for nutrient removal, due to more and more stringent environmental regulations. Since wastewater often contains both organics and nitrogen, aerobic granules capable of simultaneously removing organics and nitrogen are highly desired. There is strong evidence that the presence of organic carbon can affect nitrification efficiency in both suspended and attached cultures, while the distribution of heterotrophic and nitrifying bacteria in biofilms is determined by the substrate N/COD ratio (Moreau et al., 1994; Ohashi et al., 1995; Ballinger et al., 2002). So far, limited information is available for the development of aerobic granules for simultaneous

organics and nitrogen removal, and effects of the substrate N/COD ratio on the formation, microbial distribution, elemental composition and characteristics of aerobic granules are not yet well studied.

The stability of aerobic granules is a key of long-term and stable operation of aerobic granular sludge bioreactor. It appears from previous research that the stability of aerobic granules is poorer than that of anaerobic granules developed in upflow anaerobic sludge blanket (UASB) reactor (Morgenroth et al., 1997; Peng et al., 1999; Zhu and Liu, 1999). It can be anticipated that the poor stability of aerobic granules would limit its application in wastewater treatment practice. To date, almost all research on aerobic granulation has been mainly focused on exploiting the feasibility of aerobic granulation in sequencing batch reactors. However, the question of how to improve the stability of aerobic granules remains untouched.

It is obvious that development of aerobic granules for simultaneous removal of organics and nitrogen may boost nitrogen removal technology. From the application point of view, a more compact bioreactor for carbon and nitrogen removal could be expected. However, the knowledge on aerobic granulation for simultaneous organics and nitrogen removal is very limited. Thus, an in-depth understanding of this topic is highly desired.

1.2 OBJECTIVES AND SCOPES

The main objective of this study is to develop aerobic granules for simultaneous removal of organics and nitrogen in sequencing batch reactors (SBR) run at different substrate N/COD ratios. The specific objectives are as follows:

- To develop aerobic granules grown at different substrate N/COD ratios, and further to investigate the characteristics of the gra es;

- To look into co-existence of heterotrophic, nitrifying and denitrifying populations in aerobic granules developed at different substrate N/COD ratios;

- To determine the elemental composition of aerobic granules and its effects on the characteristics of aerobic granules;

- To explore the feasibility of simultaneously removing organics and nitrogen by the developed aerobic granules, as wells as to investigate the effects of operation parameters on the performance of reactors;

- To exploit the strategy for improving the stability of aerobic granules.

1.3 ORGANIZATION OF THE THESIS

This thesis contains eight chapters. Chapter 1 is a brief introduction to the research, and Chapter 2 looks into the relevant literature information. Chapter 3 shows characteristics of aerobic granules developed at different substrate N/COD ratios. The microbial diversity of aerobic granules is investigated in Chapter 4, while Chapter 5 discusses elemental compositions of aerobic granules. Chapter 6 explores simultaneous removal of organics and nitrogen by the aerobic granules. The strategy for improving the stability of aerobic granules was developed in Chapter 7. Finally, the report was concluded by Chapter 8.

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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Rising nitrogen pollution, as indicated by the deterioration of water quality in different aquifers, eutrophication of receiving water bodies and rising N-related health problems, recently triggered stricter effluent standards. Implementation of stricter effluent standards requires not only removal of organic carbon but also nitrogen removal from wastewater. Biological process is generally more favourable for organic carbon and nitrogen removal than physico-chemical treatment methods as it is both environmental friendly and relatively inexpensive. There are two major groups of biological processes: aerobic process and anaerobic process. As compared to anaerobic process, aerobic process has many advantages, such as rapid start-up period, process stability, and high ability to withstand overload shocks or toxic events. In this chapter, a brief review on aerobic process for organic carbon removal and nitrogen removal is presented.

In biological process, the biomass (mainly microbial cells) has to be separated from the liquid after the transformation of pollutants. However, dispersed individual bacteria are stable in solution and extremely difficult to settle down due to their common features of tiny size, slightly-heavier-than-water specific gravity and negatively charged nature (Bitton, 1999). The immobilization of cells provides excellent separation ability of the biomass because of its compact microbial structure. Therefore, self-immobilization of the bacterial cells is particularly important and useful for biological wastewater treatment system. Cell immobilization technology, such as biofilm, anaerobic granulation and aerobic granulation, is also reviewed in this chapter.

2.2 AEROBIC PROCESS FOR ORGANIC CARBON REMOVAL

Major aerobic processes used for wastewater treatment include suspended-growth treatment process and attached-growth treatment process. Suspended-growth process is the biological treatment process in which the microorganisms responsible for the biological oxidation of organic pollutants are maintained in suspension within the liquid, while attached-growth process is the biological process in which the microorganisms are attached to some inert medium surfaces, such as rocks, slag, ceramic and plastic materials.

2.2.1 Aerobic metabolism

In wastewater biological treatment process, microorganisms use the organics in wastewater as a food supply and convert them into biological cells, or biomass. Wastewater may contain a wide variety of organics, thus a mixed culture is required for complete treatment. Each type of organisms in the mixed culture utilizes the food source most suitable to its metabolism. The biochemical reactions involved in metabolisms are extremely complicated and are not yet completely understood. Metabolic network of fixed bacteria basically includes interrelated catabolic and anabolic reactions. Microorganisms can obtain the energy necessary for the synthesis of new cells and the maintenance of other cell functions through catabolism, while anabolism provides the materials for cell growth. Once external food source is no longer available, organisms may obtain energy through endogenous respiration. Each type of microorganisms has its own metabolic pathway, from specific reactants to specific end products. A generalized concept of metabolic pathways of importance is shown in Fig. 2.1.

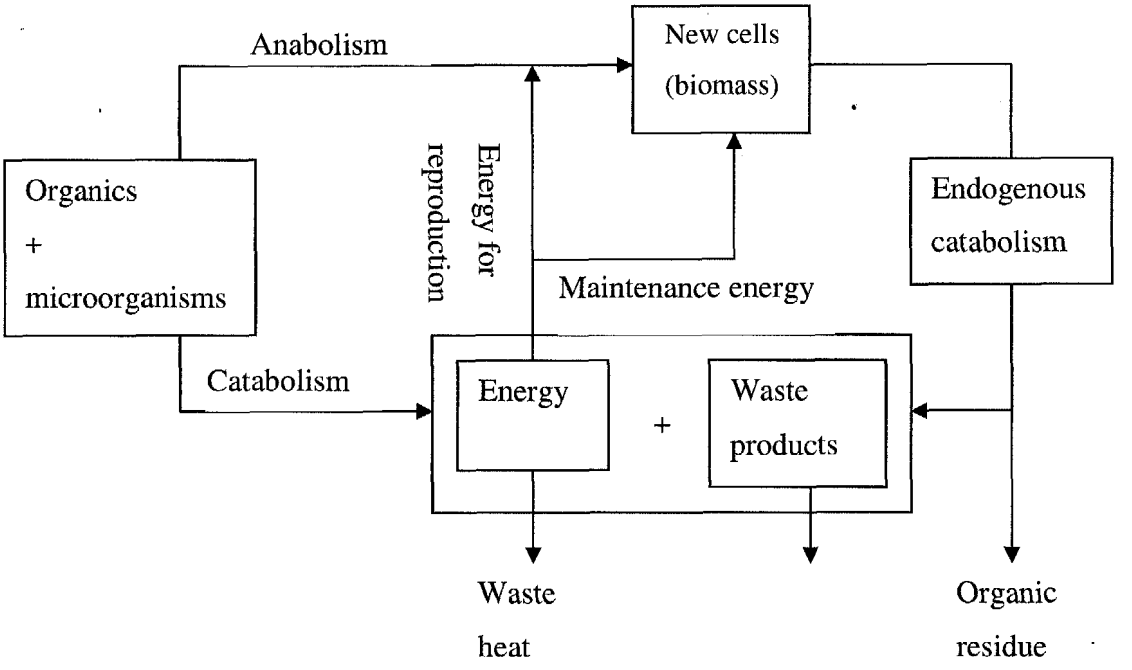

Figure 2.1 Generalized metabolic pathway (Peavy et al., 1985)

Energy is transferred from catabolic reaction to the anabolic reaction through high-energy phosphate bonds. The removal of hydrogen or the splitting of the carbon-carbon bond in the catabolic process will release energy. A sizable fraction 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 extra phosphate atom is removed, releasing the stored energy to the synthetic reaction. The resulting ADP is then transferred back to the carbolic reaction to be reenergized to ATP, and the cycle is repeated. This process is shown in Fig. 2.2.

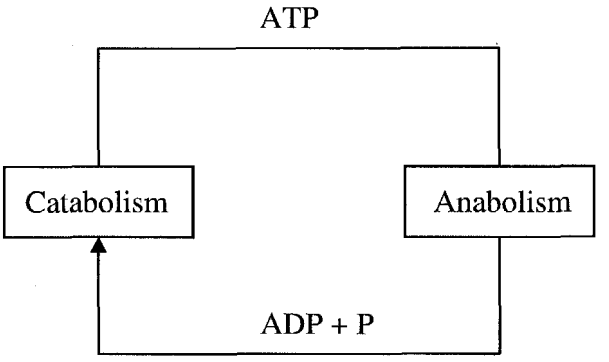

Figure 2.2 Energy transfer model (Peavy et al., 1985)

Catabolic processes involve either the oxidation or the reduction of material in the substrate (food supply). If free molecular oxygen is available, molecular oxygen is used as the electron acceptor in catabolism, the process is known as aerobic metabolism. In aerobic metabolism, free molecular oxygen will be added to the substrate and the waste products will be oxidized compounds. In the absence of free oxygen, bound oxygen may be removed from oxygen-bearing compounds and hydrogen added to elements of the substrate. The result is waste products composed of reduced compounds. Oxidation reactions are more efficient because they release greater amounts of energy. Consequently, aerobic metabolism predominates when oxygen is available. This is advantageous because the oxidized products of aerobic processes are less objectionable in natural water systems than the reduced products of anaerobic processes.

Like matter, energy can be neither created nor destroyed. Energy released in the catabolic process is transferred to the cellular material synthesized in the anabolic process, stored in the waste products of catabolism, or released as heat or mechanical energy. The relative quantities dispersed in these ways depend upon the nature of the reaction. The end products of aerobic catabolism are low-energy, stable compounds, with most of the energy being stored in the cellular material.

Under aerobic conditions, microorganisms assimilate and use organics in wastewater for subsistence, growth, and reproduction. The conversions carried out in general accordance with the stoichiometry are shown in Equations 2-1 and 2-2 (Metcalf & Eddy, 2003).

Oxidation and synthesis:

$$COHNS + O_2 + nutrients \xrightarrow{bacteria} CO_2 + NH_3 + C_5H_7NO_2 + other\ products \quad (2.1)$$

Endogenous respiration:

$$C_5H_7NO_2 + O_2 \xrightarrow{bacteria} 5CO_2 + 2H_2O + NH_3 + energy \quad (2.2)$$

In these equations, COHNS represents the organic matter in wastewater. From Equations 2.1 and 2.2, it can be seen that, to continue to reproduce and function properly, a source of energy, carbon for the synthesis of new cellular material, oxygen, and inorganic elements (nutrients) such as nitrogen, phosphorus, sulphur, potassium, calcium, and magnesium are the three major factors needed by microoranisms. It should be noted that organic nutrients may also be required for cell synthesis by some microorganisms. Although organic nutrients requirements differ from one microorganism to another, the major ones fall into the following three classes: (1) amino acids, (2) purines and pyrimidines, and (3) vitamins (Stanier et al., 1986).

2.2.2 Factors affecting organic carbon removal

Several external factors may affect the rate of biomass production and organics utilization. These include temperature, pH, and toxins. Biomass production and organics utilization increase with increasing temperature within the range of 0 to 55°C. Increases in reaction rates approximately follow the van't Hoff-Arrhenius rule of doubling with every 10°C increase in temperature (Schroeder et al., 1977) up to a maximum temperature. Excessive heat can destroy the microorganisms. The pH of the surrounding microorganisms is also an important factor. Microorganisms that degrade

wastewater organics function best near neutral pH, with a tolerance range of from pH 6 to pH 9.

Other factors such as toxicants, salt concentration, and oxidants influence microbial growth. Toxicants poison the microorganism, salt concentrations interfere with internal-external pressure relationships, and oxidants may destroy enzymes and cellular materials. In fact, microorganisms are capable of adjusting to a wide range of most environmental factors, provided changes occur gradually. Sudden changes, such as a rapid drop in pH or a slug of salt, may do irreparable damage to the culture.

2.3 BIOLOGICAL PROCESS FOR NITROGEN REMOVAL

The control of nitrogen is becoming increasingly important in water quality management and in the design of wastewater treatment plants, because nitrogen in treated effluent may accelerate the eutrophication of lakes and reservoirs, stimulate the growth of algae and rooted aquatic plants in shallow streams. Besides that, beneficial uses of the water resources, particularly when they are used for water supplies, fish propagation, and recreation, may be interfered by the presence of algae and aquatic plants. Concentrations of molecular or free ammonia above 0.5 mg l^{-1} can cause fish toxicity (Metcalf & Eddy, 2003). Significant concentrations of nitrogen in the effluent have other adverse effects, such as depleting dissolved oxygen in receiving waters, exhibiting toxicity toward aquatic life, affecting chlorine disinfection efficiency, presenting a public health hazard, and affecting the suitability of wastewater for reuse.

Industrial wastewaters typically have higher nitrogen content compared to domestic wastewater. The concentrations range between 100 and 4,100 mg l^{-1}. Fresh domestic wastewater also contains nitrogen, mainly in the form of ammonia and organic nitrogen. The total nitrogen present ranges from 20 to 85 mg l^{-1} and approximately 60% are in the ammonia form. The remaining is organically bound nitroge such as proteins, peptides urea etc., which can be slowly decomposed by bacteria to ammonia, carbon dioxide and water.

A number of biological processes have been used for nitrogen removal from both industrial and domestic wastewater. Nitrification-denitrification is the most common method for nitrogen removal from wastewater. The removal of nitrogen by nitrification-denitrification is a two-step process. In the first step, ammonia is converted aerobically to nitrate, which is known as nitrification. In the second step, nitrate is converted to nitrogen gas, which is called denitrification.

2.3.1 Biological nitrification

2.3.1.1 Nitrification process

Nitrification is an autotrophic process. In contrast to heterotrophs, nitrifiers use inorganic carbon rather than organic carbon for synthesis of new cells. Two bacteria genera are responsible for nitrification, *Nitrosomonas* and *Nitrobacter*. Table 2.1 gives some features of *Nitrosomonas* and *Nitrobacter* (Sharma and Ahlert, 1977). The estimated generation time for *Nitrosomonas* is 8 to 36 hours and 12 to 59 hours for *Nitrobacter*. The yield coefficient of *Nitrosomonas* is approximately three times that of *Nitrobacter*. This is consistent with the fact that more free energy is released during the oxidation of ammonia than the oxidation of nitrite to nitrate.

Table 2.1 Characteristics of nitrifying bacteria (Sharma and Ahlert, 1977)

Feature	Nitrosomonas sp.	Nitrobacter sp.
Cell shape	Ovid to rod-shaped	Ovid to rod-shaped
Cell size	1 × 1.5 μm	0.5 × 1.0 μm
Gram test	Negative	Negative
Generation time (hr)	8 to 36	12 to 59
Autotroph	Obligate	Facultative
Yield (VSS/g N oxidized)	0.04 to 0.13	0.02 to 0.07

Nitrification is a two-step process. In the first step, ammonia is converted to nitrite by *Nitrosomonas*; in the second step, nitrite is converted to nitrate by *Nitrobacter*.

Nitrfication in wastewater treatment process has been attributed primarily to *Nitrosomonas* and *Nitrobacter* although other autotrophic bacteria genera are capable of obtaining energy from the oxidation of ammonia.

According to Metcalf and Eddy (2003), the conversion can be described as follows:

First step,

$$NH_4^+ + \frac{3}{2}O_2 \xrightarrow{Nitrosomonas} NO_2^- + 2H^+ + H_2O \qquad (2.3)$$

Second step,

$$NO_2^- + \frac{1}{2}O_2 \xrightarrow{Nitrobacter} NO_3^- \qquad (2.4)$$

Equations 2.3 and 2.4 are energy yielding reactions. The energy from these reactions is used for the bacterial cell growth and maintenance. The following is the overall reaction:

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \qquad (2.5)$$

Along with obtaining energy, some of the ammonia ion is assimilated into cell tissue. The biomass synthesis reaction is presented in Equation 2.6:

$$4CO_2 + HCO_3^- + NH_4^+ + H_2O \longrightarrow C_5H_7O_2N(Bacteria) + 5O_2 \qquad (2.6)$$

Thus, the overall oxidation and synthesis reaction can be represented as follows:

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \longrightarrow$$
$$0.021C_5H_7O_2N + 0.98NO_3^- + 1.041H_2O + 1.88H_2CO_3 \qquad (2.7)$$

Equation 2.7 gives the following important information of the nitrification process:

(1) 1.83 moles of oxygen per mole of ammonia (4.18 g of oxygen per g of ammonia) oxidized to nitrate are needed.

(2) In the conversion process, a large amount of alkalinity is consumed: 8.64 g HCO_3^- per g of ammonia oxidized.

(3) Bacteria cell yields in the nitrification process are very low: 0.15 g VSS g^{-1} NH_4^+-N oxidized and 0.02 g VSS g^{-1} NO_2^--N oxidized.

2.3.1.2 Factors affecting nitrification process

Nitrifying bacteria are very sensitive to a number of factors, such as culture temperature, dissolved oxygen, pH, alkalinity, the presence of toxic and inhibitors (Metcalf and Eddy, 2003; Martinage and Paul, 2000; Mauret et al., 1996, 2001; Paul et al., 1998). Temperature exerts a tremendous influence on the growth of nitrifying bacteria, which produces two opposite effects: bacteria activation and free ammonia inhibition. Temperature, as in any other biological process, activates cellular metabolism. Temperature also influences free ammonia concentration in the wastewater, which may inhibit nitrifiers. An increase in temperature of 1°C, within a range of 10-29°C, brings about an increase of 2% in the nitrification rate, reaching the maximum at 28-29°C (Fdz-Polanco et al., 1994). In general, *Nitrobacter* is much more sensitive to free ammonia than *Nitrosomonas*, that is, for *Nitrobacter*, the free ammonia inhibition prevails against the activation effect. For both of nitrifying bacteria, no activity is detected below 5°C and above 40°C (Mitchell, 1974).

Dissolved oxygen (DO) concentration above 1 mg l^{-1} is essential for nitrification to occur (Metcalf and Eddy, 2003). Limiting amounts of dissolved oxygen inhibit nitrification and cause nitrite accumulation or nitrous and nitric oxide production (Goreau al., 1980; Painter, 1986; Bernet et al., 2001). It had b reported that the growth rate for *Nitrosomonas* was independent of the DO concentration greater than 1.0 mg l^{-1}, and above 2.0 mg l^{-1} for *Nitrobacter* (Stenstrom and Poduska, 1980; Princic

et al., 1998). There was no inhibition on nitrification process at DO concentrations of up to 60 mg l^{-1} (Haug and McCarty, 1972).

The pH is another important factor affecting nitrifying bacteria. The maximum reaction rates for both *Nitrosomonas* and *Nitrobacter* are in a narrow pH range of 7.5 to 8.6, with 90% of the maximum occurring at 7.8 and 8.9, and less than 50% of optimum below 7.0 and above 9.8 (Painter, 1970; Alleman, 1984). Watson et al. (1989) found that the pH range for growth of pure cultures of *Nitrosomonas* is 5.8 to 8.5, and the pH range for growth of *Nitrobacter* is 6.5 to 8.5. It had been reported that nitrification ceased completely at pH less than 5.5, and the activity of nitrifying bacteria could restore to its original rate when pH was subsequently increased back to above 7.0 (Haug and McCarty, 1972; Princic et al., 1998). This in turn indicates that lower pH is not toxic to nitrifying bacteria, but would only reduce the nitrification rates.

In nitrification process, alkalinity serves as inorganic carbon source for the growth of nitrifying bacteria, while it can also be used for neutralization of hydrogen ions produced by nitrification. Usually, insufficient alkalinity in influent wastewater would hinder the activity of nitrifying bacteria, leading to a low nitrification efficiency (Jun et al., 2000; Wett and Rauch, 2003).

A variety of organic and inorganic agents may inhibit the growth and action of nitrifying bacteria (Patureau et al., 2000; Rustrian et al., 1997). High concentrations of ammonia and nitrous acid can inhibit nitrification process (Mauret et al., 1996). Some research showed that *Nitrosomonas* was not inhibited by ammonia nitrogen concentration up to 3,000 mg l^{-1} (Princic et al., 1998), while the activity of *Nitrobacter* was inhibited by 40% at a nitrite nitrogen concentration of 1,400 mg l^{-1} and a complete inhibition occurred at 2,500 mg l^{-1} (Sharma and Ahlert, 1977). In fact, there is strong evidence that *Nitrobacter* population was quite sensitive to free ammonia, the inhibition threshold being between 0.1 and 1.0 mg N l^{-1}. However for *Nitrosomonas* population, this value was as high as 10 to 150 mg N l^{-1} (Anthonisen et al., 1976; Liu and Capdeville, 1994).

The presence of toxic substances, such as metals and phenol, also exerts a significant impact on the performance of nitrifying bacteria. A copper concentration of 30 mg l^{-1} inhibited the activity of nitrifying bacteria and, at least 94% ammonia oxidation inhibition occurred when the 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). Unlike heavy metals, several organic compounds are more toxic to *Nitrobacter* than to *Nitrosomonas*, such as potassium chlorate and sodium cyanate (Wood et al., 1981).

2.3.2 Biological denitrification

Nitrification converts ammonia into nitrite or nitrate, thereby eliminating problems of toxicity to fish and reducing the nitrogen oxygen demand in the water. For the aim of eutrophication control, it is necessary to remove nitrate from the wastewater. The removal of nitrogen in the form of nitrate by conversion to nitrogen gas can be accomplished biologically under anoxic conditions.

2.3.2.1 Denitrifying bacteria

Nitrite and nitrate are biologically reduced to gaseous nitrogen by a variety of facultative heterotrophs in anoxic conditions, these denitrifying bacteria include Achromobacter, Aerobacter, Alcaligenes, bacillus, Brevibacterium, Flavobacterium, Lactobacillus, Micrococcus, Proteus, Pseudomonas, Spirillum and so on (Payne, 1981). Most of denitrifying bacteria are facultative. For some species nitrous oxide may be the end products instead of nitrogen gas. For denitrifying growth, besides the essential requirement of an organic carbon source, mineral nutrients like sulfate, phosphate, chloride, sodium, potassium, magnesium and calcium are also required.

Denitrifying bacteria are heterotrophs capable of dissimilatory nitrate reduction. The reactions involved are as follows (Metcalf & Eddy, 2003):

$$6NO_3^- + 5CH_3OH \longrightarrow 3N_2 + 5CO_2 + 7H_2O + 6OH^- \quad (2.8)$$

$$2NO_2^- + CH_3OH \longrightarrow N_2 + CO_2 + H_2O + 2OH^- \quad (2.9)$$

Equations 2.8 and 2.9 show that an organic carbon source, such as methanol, is needed to act as an oxygen acceptors as well as carbon source for biological synthesis. Methanol is the most common carbon source used in the denitrification process in wastewater treatment because it is the least expensive synthetic compound available that can be applied without leaving a residual BOD in the processed effluent. Other inexpensive sources of organic carbon like ethanol and domestic sewage can also be used (Bernet et al., 1996b; Rustrian et al., 1999). It appears from the above equations that one mole of alkalinity per mole of nitrate nitrogen consumed is produced, which in turn reduces the alkalinity requirements in the nitrification-denitrification process.

2.3.2.2 Factors affecting denitrification

Denitrification occurs under a wide of range of environmental conditions. Factors affecting the process efficiency include:

(1) Substrate: Denitrification by heterotrophic bacteria occurs in the presence of available organic substrates. Denitrification rate is independent of the substrate concentration if the organic matter concentration is in excess of the amount required for nitrification.

(2) Dissolved oxygen (DO): In denitrifying process, DO concentration is a critical parameter. The presence of DO will suppress the enzyme system needed for denitrification (Metcalf and Eddy, 2003).

(3) pH: The optimal pH lies between 7 and 8 with different optimums for different bacterial populations. Denitrification may be seriously inhibited under acidic conditions. At pH values of less than 5.0, no denitrification occurs. Below pH

6.0, nitrogen gas production is inhibited and N_2O is the only product (Mitchell, 1974).

(4) Temperature: Temperature affects the removal rate of nitrate and the microbial growth rate. The optimum temperature is 25°C for denitrification process, but denitrifying bacteria have a wide temperature tolerance from 2 to 60°C (Loehr, 1984). Thus denitrification still can occur even in the winter.

2.3.3 Description of conventional nitrification-denitrification process

Based on whether denitrification is accomplished in combined carbon oxidation nitrification-denitrification systems using internal and endogenous carbon sources, or in separate reactors using methanol or other suitable external carbon source, the nitrogen removal process can be classified into single-stage sludge and two-stage sludge systems. Nitrification and denitrification can be accomplished by suspended-growth or attached-growth systems.

2.3.3.1 Single-sludge system

In the single stage sludge process, carbon oxidation, nitrification-denitrification steps are accomplished in a single reactor (Metcalf and Eddy, 2003). In this process, nitrification and denitrification can be achieved by arranging an anoxic section in the aeration tank or through intermittent aeration (Bernet et al., 2000). The typical single stage sludge system is shown in Fig. 2.3.

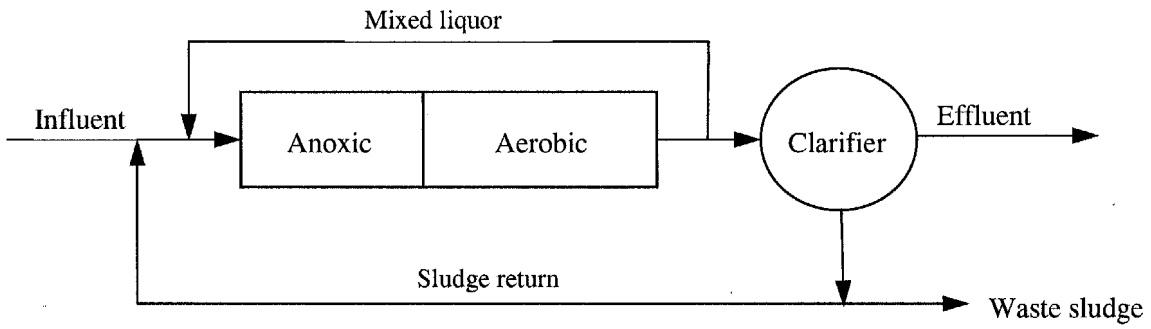

Figure 2.3 Single-stage sludge system for nitrogen removal (Metcalf and Eddy, 2003).

Wastewater initially enters an anoxic zone, in which denitrification is carried out. In the following aerobic zone, nitrification then occurs. The nitrified effluent is recycled to the head of the anoxic zone for denitrification. The single stage sludge system has the advantages of (i) reduction in the reactor volume, (ii) elimination of the need for supplemental organic carbon sources required for denitrification, and (iii) elimination of intermediate clarifiers. However, this system has poor protection against toxicants, meanwhile its operation stability strongly depends on the performance of secondary clarifier for biomass return.

2.3.3.2 Two-stage sludge system

The typical two-stage sludge system is illustrated in Fig. 2.4. An aerobic reactor for carbon oxidation and nitrification is followed by a separated anoxic reactor for denitrification (Metcalf and Eddy, 2003). Compared to the single-stage process, the two-stage sludge system has good protection against most toxicants, and low effluent ammonia is achievable.

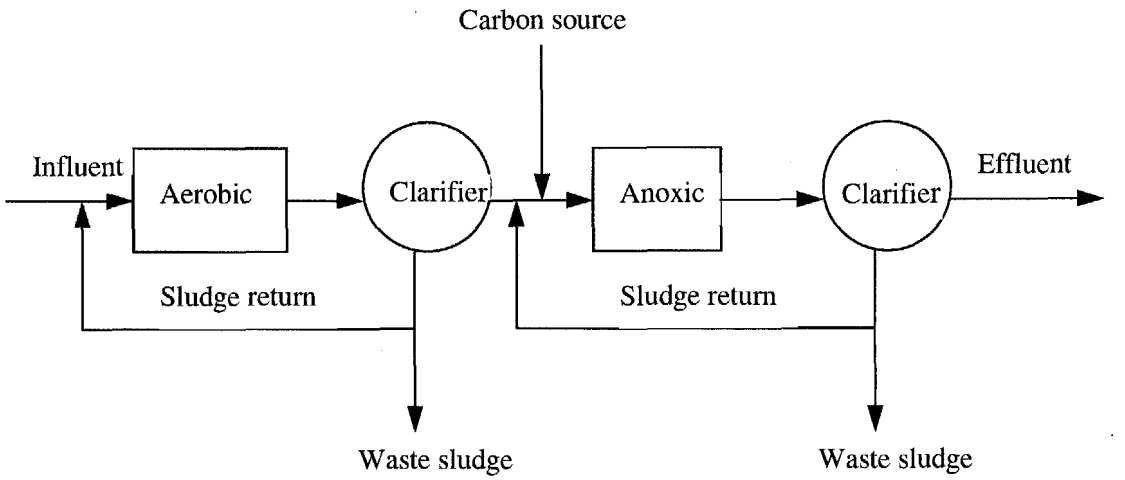

Figure 2.4 Separate-sludge system for nitrogen removal (Metcalf and Eddy, 2003).

Single stage sludge and two-stage sludge systems can be operated as suspended-growth or attached growth cultures. So far, a number of attached-growth systems have been developed for nitrogen removal, such as aerated biofilter, rotating biological contactor (RBC), fluidized-bed reactor and air-lift biofilm systems etc (Akunna et al., 1994; Delgenès et al., 1998; Rustrian et al., 1998). In the fluidised-bed reactors, the wastewater to be treated passes upward through a bed of fine-grained material, at sufficient velocity to suspend or fluidize the media. Under anoxic conditions, the fluidized-bed reactor can be used for denitrification (Pugh et al., 1987). Rotating biological contactors is a reactor, in which media in the form of large, flat disks mounted on a common shaft are rotated through specially contoured tank, in which wastewater flows on a continuous basis, and microorganisms growing on the medium surface remove food from the wastewater by uptaking oxygen from the air to sustain the metabolic processes. Rotating biological contactors can be used for both carbon removal and nitrogen removal. In the rotating biological contactor, nitrification proceeds only after carbon concentrations have been substantially reduced.

Sequencing batch reactors (SBR) have been successfully applied for nitrogen removal. As compared to conventional activated sludge systems, the SBR systems have many advantages including reduced operational costs, improved nitrogen and phosphorus removal, and less bulking (Akin and Ugurlu, 2004). Previous research showed that the removal efficiencies of organic carbon and nitrogen in SBR were strongly related to solids retention time, cycle time, hydraulic retention time and phase-length distribution (Chang and Hao, 1996; Colunga and Martines, 1996; Andreottola et al., 1997; Umble and Ketchum 1997; Chang et al., 2000; Kargi and Uygur 2002, 2004).

2.3.4 New biology for nitrogen removal

Conventional nitrogen removal processes are based on time or space separation of two phases: aerobic nitrification and anaerobic denitrification (Patureau et al., 1998a). Recent research showed new biology for nitrogen removal which would make nitrogen removal more sustainable and reduce organics requirements and energy consumption.

2.3.4.1 Nitrite route

Direct denitrification from the nitrite by performing a nitrate shunt has advantages of reduction of carbon requirements, lower energy consumption for aeration, reduced reactor volume due to a shortened reaction pathway, as well as significant reduction in plant operation costs. At least four factors have been found to influence nitrite build-up: (1) dissolved oxygen concentration; (2) temperature; (3) the relative initial ratio between *Nitrosomonas* and *Nitrobacter*, $(M_{ao})_{Ns}/(M_{ao})_{Nb}$; (4) the level of free ammonia, particularly at greater than 0.1 mg N l^{-1} that can be inhibitory to *Nitrobacter* (Liu and Capdeville, 1994; Bernet et al., 1996; Liu and Tay, 2001c).

SHARON is the process with single reactor system for high-rate ammonium removal over nitrite (Hellinga et al., 1999). In this process, nitrification is significantly enhanced since ammonium oxidizers have a higher relative growth rate than nitrite oxidizers

under proper conditions. Essentially, the SHARON is a chemostat system, in which the dilution rate is controlled at level higher than the maximum growth rate of nitrite oxidizing bacteria, but lower than the growth rate of ammonium-oxidizing bacteria. Such an operation strategy favours the accumulation of nitrite or partial nitrification occurring in the reactors. The SHARON process has been successfully operated in combination with denitrification to treat high-strength nitrogen-containing wastewater.

2.3.4.2 Aerobic denitrification

In a number of bacterial species, denitrification may proceed at a substantial rate under aerobic conditions (van Loosdrecht, 1998), and in particular cases even at DO close to 7 mg l^{-1} (Patureau et al., 1994; 1996a, b; 1997). In these bacteria, oxygen and nitrate could be consumed simultaneously in a phenomenon called co-respiration (Patureau et al., 1996a, b; 1998a). Such properties of co-respiration can allow nitrifiers and aerobic denitrifier (e.g. *Microvirgula aerodenitrificans*) to co-exist in a single aerated reactor under a continuous or sequencing batch reactor (Patureau et al., 1998b). The aerobic denitrifier can be maintained by the intermittent addition of organic carbon (Patureau et al., 1998a).

2.3.4.3 Autotrophic denitrification

Denitrification by nitrifiers had been proposed by Wrage et al. (2001), namely nitrifier denitrification. In this pathway of nitrification, ammonia (NH_3) is oxidized to nitrite (NO_2^-) followed by the reduction of NO_2^- to nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2). These transformations are carried out only by autotrophic nitrifiers. It appears that nitrifier denitrification differs from the coupled nitrification-denitrification, in which denitrifiers are responsible for the reduction of NO_2^- or NO_3^- produced nitrifiers to nitrogen gas. In fact, nitrifier denitrificati nainly occurs in soils, and low oxygen conditions coupled with low organic carbon contents of soils favor this pathway. Finally, it should be pointed out that nitrifier denitrification would

contribute to the production of the greenhouse gas N_2O, and also causes losses of fertilizer nitrogen in agricultural soils.

2.3.4.4 Heterotrophic nitrification

In addition to the autotrophic nitrification, many heterotrophic bacteria are able to produce oxidized nitrogen forms from ammonia (van Loosdrecht, 1998). In contrast to the autotrophic nitrification that is proportionally related to cell growth, heterotrophic nitrification is indeed independent of cell yield. This is due to the fact that most of the products of heterotrophic nitrification are formed during the stationary growth phase, and the heterotrophic nitrification reactions are not ATP-coupled. Castignetti et al. (1990) studied the proton translocation of heterotrophic nitrifiers, and found that heterotrophic nitrification did not conserve energy during the oxidation of nitrogenous substrates. Although heterotrophic nitrification has been demonstrated in soils, sewage treatment, rivers and lake waters, autotrophic nitrification was ten times more significant than heterotrophic nitrification in natural systems (Williams et al., 1992). When substances that are selectively inhibitory to autotrophic nitrifiers are added to soil or activated sludge, nitrification is usually completely inhibited. This in turn implies that autotrophic nitrification is the major oxidation pathway of ammonium, and heterotrophic nitrification does not appear to make a major contribution to the conversion of ammonia to nitrite and nitrate ions.

2.3.4.5 Anaerobic ammonium oxidation (ANAMMOX)

The anaerobic oxidation of ammonium by deep-branching *Planctomycetes*, which can be used for cost-effective and space-saving nitrogen removal from high-strength wastewater, had been widely reported in the environmental engineering field (Jetten et al., 2001; ›h et al., 2002; van Loosdrecht, 1998). In 1986, a sp‹ ıl reaction zone called ANAMMOX (anaerobic ammonium oxidation) was discovered in an anaerobic denitrifying fluidized bed reactor (Mulder et al., 1995). During the anaerobic

ammonium oxidation, ammonium and nitrite are directly converted to dinitrogen gas without requiring COD or the addition of an external carbon source as follows (van de Graaf et al., 1996): $NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O$. In this reaction, nitrite is the preferred electron acceptor, while hydroxylamine and hydrazine are identified as important intermediates. Under suboptimal conditions, the overall double time of microbial community responsible for the anaerobic ammonium oxidation was longer than 3 weeks (Jetten et al., 2001). The ANAMMOX activity is highly sensitive to oxygen, and even a trace amount of oxygen of 0.03 mg l^{-1} could inhibit this process (Strous, 2000). ANAMMOX can also be inhibited by nitrite but not ammonium and nitrate, i.e. the ANAMMOX process may be inactivated at a nitrite concentration greater than 100 mg N l^{-1} (Strous et al., 1999).

2.4 BIOFILM AND GRANULAR SLUDGE

Cell immobilization technology has been used in bioengineering and environmental engineering areas for decades. Biofilm and granular sludge technologies will be briefly reviewed in this section.

2.4.1 Biofilm

The biofilm reactor has been frequently used in wastewater treatment. The feasibility and efficiency of biofilm reactors for removing biodegradable organic matter, nitrogen and phosphate from municipal and industrial wastewater had been reported (Liu and Capdeville, 1996; Selivanovskaya et al., 1997; Rusten et al., 1998).

2.4.1.1 Biofilm formation

The form on of biofilms is a multiple-step process, and ph ochemical and biological factors are involved (Daniels, 1980; Calleja, 1984; van Loosdrecht et al.,

1987; Liu, 1995; Liu and Wang, 1996). According to Liu and Tay (2001b), the biofilm formation could be roughly described as follows:

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 follow:

(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 triplets.

Step 3 Microbial forces to make immobilized cells mature: production of expolymers, 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 biofilms would be finally shaped by hydrodynamic shear force to form a certain structured community, however, extremely high shear force would cause serious disagregation and inhibition of development of biofilms.

2.4.1.2 Major factors affecting the formation of biofilms

A number of factors can influence the formation of biofilms. In a biofilm culture growth and detachment forces are two key factors that highly influence the structure and performance of steady state biofilm (Trulear and Characklis, 1982; Speitel et al., 1987; van Loosdrecht et al., 1995; Beyenal et al., 2000; Liu and Tay, 2001b) The diffusion-reaction theory shows that the growth rate of biofilm is dependent on substrate loading rate applied to the biofilm system (Trulear and Characklis, 1982), thus the substrate loading rate may represent the capability of biofilm growth and can be regarded as the growth force of biofilm culture. It had been reported that under high surface loading conditions, the average biofilm thickness on individual media particles increased, resulting in a corresponding decrease in effective pore space (Rittmann, 1993). On the other hand, shear stress resulting from gas-liquid interaction or particle-particle collision may act as detachment force, which represses the development of biofilm (Chang et al., 1991; Ohashi and Harada, 1994; Liu and Tay, 2001a). High detachment force results in a compact and dense biofilm. On the contrary, biofilm tends to become a heterogeneous, porous and weaker structure when growth force is increased (Trulear and Characklis 1982; Chang et al., 1991; Rittmann et al., 1992; Liu et al., 2003b). An equilibrium biofilm thickness and density can be obtained at a given detachment force.

It has been demonstrated that exopolysaccharides (EPS) can promote adhesion of cells (Skillman et al., 1999; Lopes et al., 2000). Danese et al. (2000) used a genetic approach to examine the potential role of colanic acid, an exopolysacchride of *Escherichia coli* K-12 in biofilm formation, and they found that colanic acid production is critical for the formation of the complex three-dimensional structure and depth of E. coli biofilms. Tsuneda et al. (2001) found that addition of EPS substitute enhanced the formation of nitrifying biofilms. These clearly indicated that exopolysacchrides in biofilms play a key role in building up and maintaining the architecture of biofilms.

2.4.2 Anaerobic granulation

The feasibility and efficiency of anaerobic granulation technology in removing high-strength biodegradable organic wastes from industrial wastewater has been sufficiently demonstrated. To date, more than 900 upflow anaerobic sludge blanket (UASB) units are operating across the world (Alves et al., 2000).

2.4.2.1 Mechanisms and models for anaerobic granulation

A sound understanding of the mechanisms responsible for anaerobic granulation is essential for environmental engineer to design and operate granular sludge-based treatment systems. This section provides an overview of the mechanisms and models developed for anaerobic granulation in the past decades.

Thermodynamic models

The formation of anaerobic granules is a multiple-step process, and both physicochemical and biological forces are involved. It has been suggested that microbial adhesion can be defined only in terms of the energy involved in the formation of the adhesive junction. When a bacterium approaches another bacterium, the interaction between them includes repulsive electrostatic force, attractive van de Waals force, and repulsive hydration interaction (Parsegian and Rand, 1991). In order to interpret the role of physicochemical forces in anaerobic granulation process, some thermodynamic models have been developed.

1. Secondary minimum adhesion model

Based on theoretical calculations and experimental observations, the secondary minimum adhesion model suggests that reversible adhesion takes place in the secondary minimum of the DLVO (Derjaguin, Landau, Verwey and Overbeek) free energy curve. The Gibbs energy of reversible adhesion is relatively small, and there is

always a separation distance between two adhering bacteria. The reversible adhesion can be shifted to irreversible adhesion at the primary minimum by overcoming the energy barrier or by protruding fibrils or fimbriae, which bridge the gap between bacteria (van Loosdrecht and Zehnder, 1990). Microstructural analysis of the UASB granule supports this separation distance between bacteria at the secondary minimum.

2. Hydrophobic interaction and local dehydration models

Wilschut and Hoekstra (1984) proposed a local dehydration model when studying membrane fusion, and suggested that under the physiological conditions, the strong repulsive hydration interaction was the main force to keep the cells apart. The model shows that when bacterial surfaces are strongly hydrophobic, irreversible adhesion will occur. This indicates that hydrophobicity (or hydrophilicity) of the bacterial surfaces may be another important factor involved in irreversible adhesion. Hydrophobicity and hydrophilicity are usually used to describe a molecule or a structure having the feature of being rejected from an aqueous medium (i.e., hydrophobicity), or being positively attracted (i.e., hydrophilicity). Hydration interaction becomes significant at surface separations of 2 to 5 nm or less, depending on the nature of bacterial surfaces. According to the surface thermodynamics theory, increasing the hydrophobicity of cell surfaces would cause 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. Local dehydration of the short-distance-apart surfaces has been identified as the prerequisite for bacterial adhesion. It has been shown that the hydrophobicity of bacterial surface plays a crucial role in the initiation of both anaerobic and aerobic granules (Tay et al. 2000, 2001b).

3. Surface tension model

It was found that in the bulk solution with low surface tension, the relative hydrophilic bacteria in particular were favoured to adhere to one another, while with high surface tension the hydrophobic species were likely to adhere. In general, most acidogenic bacteria are hydrophilic, and methanogens appear to be hydrophobic (Daffonchio et al.,

1995). Surface tension model is based on the consideration of liquid surface tension in UASB reactors (Thaveesri et al., 1995). Depending on the liquid surface tension (γ) in the UASB reactor, bacterial cells may grow in rather loose associations, in multilayered granules (γ<50 mN m^{-1}) or in mixed conglomerates (γ>56 mN m^{-1}) (Thaveesri et al., 1995; Grootaerd et al., 1997). Thus, the adhesion of hydrophilic cells is enhanced at low liquid surface tension, while the opposite is true for hydrophobic cells.

Treating sludge granulation, only as a physical or physicochemical process is not adequate. Bacterial granulation involves too many changes and unidentified components to allow one to build a purely physical model with any confidence (Parsegian and Rand, 1991). The nature of bacteria makes them far from either "dead" chemicals or "ideal" particles. They have no rigidly definite surface boundary, simple geometry, or uniform molecular surface composition (Darnell et al., 1986; Rouxhet and Mozes, 1990). It seems unlikely that an understanding of the physical forces between bacterial cells alone can fully explain bacterial granulation. The physical phenomena of microbial granulation must eventually be related to the biological triggers that control them.

Structural models

In order to understand the mechanisms behind the sludge granulation process, physical, chemical and microbiological compositions of the granules, granular microstructure analysis, and the effects of many factors on sludge granulation have been examined. Several structural models for sludge granulation arising from these observations and investigations were proposed (Liu et al., 2003c). These models are mainly focused on the formation of initial/embryonic granules, which are inert matters in inert nuclei model; cation-bridged bacterial aggregates in divalent cation-bridge model; extracellu · polymer (ECP)-bound bacterial cells in ECP b··ding model and Capetown's model; filamentous bacterial aggregates in "spaghetti" theory and

crystallized nuclei formation model; syntrophic microcolonies in syntrophic microcolonies model.

1. Inert nuclei model

The inert matter content of a granule was thought to initiate the sludge granulation by acting as a precursor or nucleus, to which anaerobic bacteria could attach to form the initial embryonic granule (Lettinga et al., 1980). A large amount of these initially formed embryonic granules in an UASB reactor would facilitate the overall sludge granulation process. The use of inert matters in granulation is often claimed to provide a beneficial effect on anaerobic granulation (Yoda et al., 1989; Wirtz and Dague, 1996).

2. Divalent cation-bridge model

Divalent cations, both Ca^{2+} with concentrations of 2.0-5.0 mM and Mg^{2+} with 0.5-10.0 mM were found to exert a positive impact on anaerobic granulation (Mahoney et al., 1987; Grotenhuis et al., 1991; Schmidt and Ahring, 1993; Teo et al., 2000). Study on membrane fusion showed that Ca^{2+} might cause conformational changes of some surface proteins or polypeptide groups that could interact with two surfaces and bridge them together (Papahadjopoulos et al., 1990). Divalent cations were suggested either to stimulate granulation by neutralizing negative charges on bacterial surfaces as a result of relatively strong van der Waals attractive forces, or to function as cationic bridges between bacteria (Hulshoff Pol et al., 1983; Guiot et al., 1988; Grotenhuis et al., 1991; Schmidt and Ahring, 1993).

3. ECP bonding model

The accumulation of extra-cellular polymer (ECP) as capsular material and peripheral slime has been correlated with biological adhesion and aggregation processes (Costerton et al., 1981). The metabolic blocking of exopolysaccharides synthesis was found to prevent microbial adhesion (Cammarota and Sant'Anna, 1998). ECP in granules was hypothesized to bridge two neighboring bacterial cells physically to each

other as well as with other inert particulate matters, and settle out as floc aggregates (Ross, 1984; Shen et al., 1993; Schmidt and Ahring, 1994). It is not yet demonstrated whether the genes for ECP production are expressed before or after bacterial adhesion, i.e., if the bacteria initially make ECP and then adhere to each other, or first adhere and then produce ECP. In the former case, ECP production prior to adhesion, the appearance of polymer materials at the initial site of contact between microbial cells may be due to the migration of polymer molecules already on the cell surface. In the latter case, ECP production after adhesion, bacterial adhesion may provide a certain physiological condition for ECP excretion.

4. Cape Town's model

The Cape Town Group proposed that under the conditions of high hydrogen partial pressure and limited cysteine, the overproduction of amino acids (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 suggests that ECP (mainly long-chain polypeptides) is excreted by *Methanobacterium* strain AZ, a hydrogen-utilizing methanogen.

5. "Spaghetti" theory

In the initial development of granular sludge, the "spaghetti" theory suggests that filamentous *Methanothix* attached on precursors, and then through a branched-growth process formed a network, where other bacteria could be entrapped in its knots (Wiegant, 1988; Sanchez et al., 1994; Wu et al., 1996). This "spaghetti" structured aggregate would grow due to the bacterial growth and entrapment and becomes denser and spherically shaped by the hydrodynamic shear stress of the upflow liquid and biogas. This model is supported by the observation that the development of UASB granules corresponded to an increase in the relative number of *Methanothix* (Morgan et al., 1991).

6. Crystallized nuclei formation model

Similar to "spaghetti" model, the crystallized nuclei formation model suggests that the network or nucleus formation would initiate the sludge granulation process (Zhu et al., 1997). The thermodynamic energy change of the bacterial aggregate ecosystem would induce the growth of filamentous bacteria, and then the network formation, just like the crystallization of chemicals.

7. Syntrophic microcolony model

Many types of anaerobic bacteria are involved in the biodegradation process, thus they must live in a close synergistic relationship, where products such as H_2 and other intermediates can be efficiently transferred among the respective bacterial groups (Thiele and Zeikus, 1988). The syntrophic microcolony model suggests that the above requisites would eventually lead to the formation of stable microcolonies or consortia, i.e., initial granules (Hirsch, 1984).

The above structural models may explain some phenomena during the sludge granulation process under specific laboratory conditions. However, each model considers only the role of one or two leading factors involved in the anaerobic granulation process. These factors usually exert their influences under specific environmental conditions and in specific steps during the whole granulation process. Often experimental results contradict these models, e.g. it was reported that the granules could be developed without the addition of any inert materials (Thiele et al., 1990). Some studies showed that calcium ion did not induce sludge granulation (Guiot et al., 1988) and high concentrations of magnesium ion caused disaggregation of granules (Schmidt and Ahring, 1993). It is also not possible to explain certain experimental phenomena with these proposed models, e.g. no model is able to explain a spontaneous and sudden washout of the established sludge bed as a result of a change in wastewater composition. This is a common problem for all operating UASB systems. If a factor is not dependent on the wastewater composition and, on the other hand, can

initiate the formation of granules, a change in the wastewater composition should not lead to the washout of the entire granular sludge bed.

Proton translocation-dehydration theory

The previous works demonstrated the essential of proton translocation concept that (i) the hydrophobic interaction of a considerable extent was closely related to the initiation of bacterial adhesion; (ii) the proton conductance across a bacterial surface could induce surface dehydration; and (iii) the proton translocating activity could induce the protonation of bacterial 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., 2000; Teo et al.; 2000). The overall sludge granulation process was initiated by the dehydration of bacterial surfaces, followed by embryonic granule formation, granule maturation and post-maturation.

2.4.2.2 Major factors influencing the formation of anaerobic granules

The following factors may influence the formation and performance of anaerobic granules.

Upflow velocity and hydraulic retention time (HRT). A short HRT combined with a high liquid upflow velocity could cause washout of non-competent bacteria in granulation, and in turn promote sludge granulation.

Substrate loading rate (SLR). From the operational perspective, there was evidence to show that anaerobic granulation in UASB reactor was accomplished by gradually raising SLR during the start-up (Kosaric et al., 1990; Tay and Yan, 1996). However, it had been reported that as the SLR increased, the anaerobic granule tended to become weaker (Quarmby and Forster, 1995).

Characteristics of substrate. Characteristics of feed substrate have been considered a key factor influencing the formation, composition and structure of anaerobic granules. The presence of high-energy substrate in the influent would facilitate the overall process of sludge granulation in the UASB reactors, and the complexity of the substrate may exert a selection pressure on microbial diversity in anaerobic granules.

Characteristics of seed sludge. Microbial species would differ in their capacity for aggregation, and some species are more competent for aggregation, but some are less under the same operation conditions. It seems certain that anaerobic granulation can be expedited simply by manipulating the composition of seed sludge.

Reactor temperature. Any biological system is sensitive to temperature variation. When the reactor temperature is below 30°C, the growth of methanogens would be seriously inhibited. At extreme high temperature, most bacteria would lose their metabolic activity. Mesophilic UASB reactors are often operated at a temperature range of 30 to 35°C for their successful functioning.

Reactor pH. Most methane-producing bacteria can function optimally at a very narrow pH range of 6.7 to 7.4 (Bitton, 1999). Once the reactor pH drops to a very low value, and such a decline of activity in turn causes a serious operation problem and even results in the failure of system. Therefore, it is necessary to regularly monitor the reactor pH and pay much attention to its changes.

Flow pattern. The liquid flow pattern in UASB reactors can create a circular flow and microbial aggregates are constantly subject to such a circular hydraulic attrition. According to the thermodynamics, the circular flow could force microbial aggregates to be shaped as regular granules that have a minimum surface free energy, provided those aggregates could be kept in the reactors under given dynamic conditions (Liu and Tay, 2002).

2.4.3 Aerobic granulation

2.4.3.1 The formation of aerobic granules

The formation of aerobic granules in SBR was tracked by using advanced image analysis techniques, and was shown to be a gradual process. Dispersed seed sludge with a mean size of about 100 μm developed into small aggregates, which evolved into compact granular sludge, which finally matured into aerobic granules with a mean size greater than 0.25 mm (Tay et al., 2001a). Scanning electron micrographs (SEM) of aerobic granules grown on acetate as sole carbon source revealed a compact microbial structure in which individual cells were tightly linked up together (Tay et al., 2002a, b, c; 2003a, b; 2003). Sludge volume index (SVI) measurements showed that mature aerobic granules possessed significantly improved sludge settleability compared to the initial seed sludge.

2.4.3.2 Factors affecting aerobic granulation

Substrate composition

The essential role of carbon source in the formation of anaerobic granules had been demonstrated as discussed earlier. In the case of aerobic granulation, the experimental evidence suggests that aerobic granulation seemed to be insensitive to the nature of substrate carbon source, e.g., aerobic granules had been successfully cultivated with a wide variety of substrates, including glucose, acetate, ethanol, phenol, and synthetic wastewater (Tay et al., 2001a; Moy et al., 2002; Jiang et al., 2002, 2003a, b). However, granule microstructure and species diversity appeared to depend on the type of carbon source. The glucose-fed aerobic granules exhibited a filamentous structure (Tay et al., 2001a), while acetate-fed aerobic granules had a non-filamentous and very compact bacterial structure, in which a rod-like species was predominant (Tay et al., 2001a). It should be pointed out that aerobic granules could also be cultivated with nitrifying bacteria and an inorganic carbon source (Tay et al., 2002c). These nitrifying aerobic granules showed excellent nitrification ability.

Organic loading rate

The organic loading rate (OLR) is one of the most important parameters in the design and operation of biological wastewater treatment facilities. The essential role of organic loading rate in the formation of anaerobic granules had been commonly recognized. Relatively high organic loading rates facilitated the formation of anaerobic granules in UASB systems. In contrast to anaerobic granulation, accumulated evidence suggested that aerobic granules can form across a very wide range of organic loading rates, from 2.5 to 15 kg COD m^{-3} day^{-1}, i.e. aerobic granulation was less dependent upon the organic loading rate applied (Beun et al., 1999; Moy et al., 2002). This is probably due to the nature of aerobic bacteria. Compared to anaerobic oxidation, aerobic oxidation produces much more energy (Metcalf and Eddy, 2003). Therefore, aerobic bacteria could obtain sufficient energy for biomass yield and functioning even at a low organic loading rate.

Although the effect of organic loading rate on the formation of aerobic granules was insignificant, the physical characteristics of aerobic granules were organic loading rate-dependent (Toh et al., 2002). The mean size of aerobic granules increased from 1.6 to 1.9 mm with the increase of the organic loading organic loading from 3 to 9 kg COD m^{-3} day^{-1}. This was simply due to the rapid growth of aerobic bacteria at high organic loading rates. A similar trend was also observed in anaerobic granulation (Grotenhuis et al., 1991). It seems that the growth patterns of both aerobic and anaerobic granules under different organic loading rates are subject to the classical Monod model. The effect of organic loading rate on the morphology of mature aerobic granules in terms of roundness was found to be insignificant, while the aerobic granules developed at different organic loading rates exhibited comparable dry biomass density, specific gravity and SVI. On the other hand, the physical strength of aerobic granules decreased with the increase of organic loading rate (Liu et al., 2003a). Similarly, in anaerobic granulation process, it was also found that a high organic loading rate resulted in a reduced strength of anaerobic granules, i.e., partial loss of structural integrity and disintegration would occur at high organic loading rates (Morvai et al., 1992; Quarmby

and Forster, 1995). In fact, an increased organic loading rate may raise the biomass growth rate, and high growth rate of microorganisms in turn would reduce the strength of the three-dimensional microbial community structure. Consequently, organic loading rate plays an important role in maintaining the stability of aerobic granules.

Hydrodynamic shear force

The contribution of hydrodynamic shear to anaerobic granulation in UASB had been reported (Alphenaar et al., 1993; Arcand et al., 1994; O'Flaherty et al., 1997; Alves et al., 2000), while its essential role in biofilm process has attracted intense research attention (Chang et al., 1991; van Loosdrecht et al., 1995; Kwok et al., 1998; Liu and Tay, 2001a, 2002). A high shear force results in biofilms with a strong and compact microbial structure, while a weak shear force produces biofilms with a heterogeneous and porous structure. Shear force also plays a very important role in the formation of aerobic granules. A high shear force favors the formation of aerobic granules and granule stability (Shin et al., 1992; Tay et al., 2001b). It was found that aerobic granules could be formed only above a threshold shear force value in terms of superficial upflow air velocity above 1.2 cm s^{-1} in a column SBR, and more regular, rounder and compact aerobic granules were developed at higher hydrodynamic shear force (Tay et al., 2001b). It was found that the aspect ratio of acetate-fed aerobic granules increased with shear force in terms of superficial upflow air velocity, i.e., granules would become rounder when shear force increased, while the granule density and strength that represent the compactness of a microbial community were also proportionally related to the shear force applied. These may imply that the structure of aerobic granules is mainly determined by the hydrodynamic shear force present in the bioreactor. In fact, the effect of shear force on granule structure was similar to its effect on biofilm, i.e. higher shear force leads to a thinner and denser biofilm (Vieira et al., 1993; van Loosdrecht et al., 1995; Kwok et al., 1998; Wasche et al., 00; Liu and Tay, 2001b).

It is well-known that extracellular polysaccharides can mediate both cohesion and adhesion of cells and play a crucial role in maintaining structural integrity in a community of immobilized cells (Christensen, 1989; Sutherlan, 2001; Tay et al., 2001c; Liu and Tay, 2002). Tay et al. (2001b) reported that the production of extracellular polysaccharides (PS) was closely associated with the shear force. The ratio of extracellular polysaccharides content to proteins (PN) increased with the shear force in terms of superficial upflow air velocity, i.e., high shear force stimulated bacteria to secrete more extracellular polysaccharides. In fact, shear force-induced production of extracellular polysaccharides had been commonly observed in biofilm process (Trinet et al., 1991; Ohashi and Harada, 1994). Consequently, the enhanced production of extracellular polysaccharides at high shear can contribute to the compact and stronger structure of aerobic granules. The metabolic network of cells includes interrelated catabolic and anabolic reactions. The catabolic activity of microorganisms is directly correlated with the electron transport system activity, which can be described by the specific oxygen utilization rate (SOUR). Tay et al. (2001b) reported that the SOUR of aerobic granules increased with the increase of shear force. It is most likely that the shear force can stimulate microbial respiration activity. It appears that when the shear force is increased, much more energy generated by catabolism would be used for the production of extracellular polysaccharides rather than for microbial growth. This in turn indicates that when shear force exerted on granular sludge is high, the granules would have to regulate their metabolic pathway so as to maintain a balance with the external shear force, through consuming non-growth-associated energy, i.e. the microbial community may respond to shear force by metabolic changes and some biological events might be involved in shear-associated phenomena.

Settling time

In sequencing batch reactor (SBR) settling time acts as a major hydraulic selection pressure exerted on microbial community. 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. Qin et al. (2003) reported that aerobic granules were successfully cultivated and became dominant only in the SBR operated at a settling time of 5 minutes, while a mixture of aerobic granules and suspended sludge was observed in the SBRs run at settling times of 20, 15 and 10 minutes, respectively. It was found that the production of extracellular polysaccharides was stimulated, and cell surface hydrophobicity was improved significantly at short settling times. As discussed earlier, it appears that aerobic granulation is a process driven by selection pressure, and one may expect to manipulate the formation and characteristics of aerobic granules by controlling selection pressure.

Hydraulic retention time

By nature SBR is operated in cycles. Thus SBR cycle time can serve as a main hydraulic selection pressure imposed on microbial community in the system. Tay et al. (2002c) investigated the effect of hydraulic selection pressure on the development of nitrifying granules in column-type sequencing batch reactors. According to their results, no nitrifying granulation was observed in the SBR operated at the longest cycle time of 24 hours due to a very weak hydraulic selection pressure, while the washout of nitrifying sludge was found in the SBR run at the shortest cycle time of 3 hours, and led to a failure of nitrifying granulation. Excellent nitrifying granules were successfully developed in the SBR operated at cycle times of 6 hours and 12 hours. A short cycle time would stimulate microbial activity, production of cell polysaccharides and also improve the cell hydrophobicity, and these hydraulic selection pressure-induced microbial changes would favour the formation of nitrifying granules. It seems that nitrifying granules can be developed at a proper hydraulic selection pressure in term of SBR cycle time.

2.4.3.3 Characteristics of Aerobic Granule

Morphology

Compared to conventional bioflocs, aerobic granules have a more regular and rounder shape. The average roundness in terms of aspect ratio is higher than 0.6 for aerobic granules grown on different carbon sources. As discussed earlier, the roundness of aerobic granules is mainly influenced by the external shear force (Tay et al, 2001b). The mean diameter of mature aerobic granules varies largely; depending on the substrate composition, organic loading rate, shear force, and so on.

Settleability

The settling property of aerobic granules is a key operation factor that determines the efficiency of solid-liquid separation, and it is essential for the proper functioning of wastewater treatment systems. Both SVI and settling velocity are used to characterize the sludge settleability. The SVI of aerobic granules is much lower than that of conventional bioflocs. This implies that, from an engineering perspective, the settleability of sludge can be improved significantly through the formation of aerobic granules, and a more compact clarifier would be adequate. The settling velocity of aerobic granules is associated with granule size and structure. The settling velocity of aerobic granules is as high as 70 m h^{-1} (Moy et al., 2002; Tay et al., 2002b), which is comparable with that of the UASB granules, and is at least three times higher than that of activated sludge flocs, which have a typical settling velocity of around 8 to 10 m h^{-1} (Campos et al., 1999).

Granule density and strength

The spec c gravity of aerobic granules falls into a range of 1.004 1.065 (Tay et al., 2002b, c). The granules with high physical strength would have a strong ability to withstand high abrasion and shear. The physical strength, expressed as integrity

coefficient (%), which is defined as the ratio of residual granules to the total weight of the granular sludge after 5 minutes of shaking at 200 rpm on a platform shaker, was higher than 95% for the aerobic granules grown on glucose and acetate (Tay et al., 2002b). This indicates that the physical strengths of aerobic and anaerobic granules are comparable.

Cell surface hydrophobicity

Cell surface hydrophobicity is an important affinity force in cell self-immobilization and attachment processes (Kos et al., 2003; Marshall and Gruickshank, 1973; Pringle and Fletcher, 1983; Zita and Hermansson, 1997). The role of cell surface hydrophobicity in the formation of aerobic granules has not been clear. Recently, Liu et al. (2003c) investigated the role of cell surface hydrophobicity in the formation of aerobic heterotrophic and nitrifying granules in sequencing batch reactors, while the effects of shear strength, hydraulic selection pressure and organic loading rate on the cell surface hydrophobicity were also studied. It was found that the formations of heterotrophic and nitrifying granules were associated very closely with the cell surface hydrophobicity, and the hydrophobicity of granular sludge was nearly two-fold higher than that of conventional bioflocs. A high shear force or hydraulic selection pressure imposed on microorganisms resulted in a significant increase in the cell surface hydrophobicity, while the cell surface hydrophobicity seemed not to be sensitive to the changes in the organic concentrations or loading rates in the range of 500 to 3000 mg COD l^{-1}. Consequently, cell surface hydrophobicity could induce and further strengthen cell-cell interaction, and might be a main triggering force to initiate the granulation of heterotrophic and nitrifying bacteria.

2.4.3.4 Mechanisms of aerobic granulation

For bacteria to form aerobic granules, a number of conditions have to be fulfilled, and the contributions of physical, chemical and biological forces to the granulation process should be considered jointly.

It should be emphasized that the hydrophobicity of bacterial surface might play a crucial role in the initiation of aerobic granulation. According to the thermodynamics theory, increasing the hydrophobicity of cell surfaces would cause a corresponding decrease in the excess Gibbs energy of the surface, which in turn promotes cell-to-cell interaction and further serves as a driving force for bacteria to self-aggregate out of liquid phase (hydrophilic phase). It has been pointed out that hydrophobic binding has a prime importance for cell attachment (Marshall and Gruickshank, 1973; Pringle and Fletcher, 1983). A high hydrophobicity of the cell surface would result in a stronger cell-to-cell interaction and a denser structure. Cell polysaccharides can mediate both cohesion and adhesion of cells and play a crucial role in maintaining the structural integrity in a community of immobilized cells. The polysaccharide contents of aerobic granules are much higher than that of sludge flocs (Tay et al., 2001c). Cell polysaccharides would also contribute greatly to aerobic granulation.

It should be pointed out that the mechanisms for aerobic granulation as discussed above does not answer a basic question, i.e. what are triggering or inducing forces of aerobic granulation? More recently, Qin et al. (2003) found that aerobic granules were successfully cultivated in the SBRs operated at a settling time less than 15 minutes, while only bioflocs appeared in the reactor run at the longest settling time of 20 minutes, while they also observed that a short settling time could significantly improve the production of cell polysaccharide, cell surface hydrophobicity and microbial activity. The feature of SBR is cycle operation, and settling time acts as hydraulic selection pressure imposed on the microorganisms in SBR. It seem that these selection pressure-induced microbial changes favour the formation of aerobic granules. This in

turn may imply that the selection pressure may serve as a driving force of aerobic granulation in SBR.

2.5 SUMMARY

A number of aerobic processes have been developed for the treatment of a variety of wastewaters. Nitrogen in wastewater can be removed by traditional suspended- and attached-culture processes, but long detention times and large reactor volumes are usually required since the nitrifying and denitrifying bacteria are very slow-growing bacteria. Meanwhile, shock loading or other changes in environmental conditions often result in the low nitrogen removal efficiency due to the sensitivity of nitrifying and denitrifying bacteria. Water industry has a urgent demand for compact and efficient treatment technology for nitrogen removal. Aerobic granulation technology developed recently has a number of advantages over conventional aerobic treatment processes, such as a denser and stronger microbial structure, better settability, higher biomass retention and ability to withstand higher organic loading rate. The aerobic granulation technology appears to have the potential to respond to the challenges of nitrogen removal from wastewater. Therefore, hybrid aerobic granules capable of simultaneously removing organic carbon and nitrogen are highly desired since wastewater often contains both organics and nitrogen. However, it appears from this review that little information is currently available with respect to hybrid granules for simultaneous organic carbon and nitrogen removal. It is believed that the development of aerobic granules for simultaneous organic carbon and nitrogen removal would lead to a novel biotechnology that has potential applications in wastewater treatment.

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CHAPTER 3

CHARACTERISTICS OF AEROBIC GRANULES DEVELOPED AT DIFFERENT SUBSTRATE N/COD RATIOS

ABSTRACT

The effects of the substrate N/COD ratio on formation and characteristics of aerobic granules were studied in four sequencing batch reactors operated at different substrate N/COD ratios ranging from 5/100 to 30/100 by weight. Results showed that aerobic granules formed at the substrate N/COD ratios studied, and both nitrifying and heterotrophic activities of aerobic granules were governed by the substrate N/COD ratio. The nitrifying activity was significantly enhanced with the increase of the substrate N/COD ratio, while the heterotrophic activity decreased. The production of extracellular polysaccharides showed a decreasing trend as the substrate N/COD ratio increased. This is probably due to enriched nitrifying population with the high N/COD ratios. This study clearly demonstrated that an aerobic granule-based SBR would have a great potential for simultaneous organic oxidation and nitrification.

3.1 INTRODUCTION

As stricter environmental regulations are imposed, advanced and cost effective techniques for nitrogen removal from wastewater become more and more important. Many modifications and processes had been developed and implemented for nitrogen removal from wastewater, such as ANAMMOX, SHARON and CANON processes (Hellinga et al., 1999; Strous, 2000; Bernet et al., 2001; Hao, 2001). Basically these process can be classified as suspended or fixed-film cultures, which still suffer from sludge bulking, large reactor volume required, sensitivity to shock loading, biofilm-associated clogging and sloughing problems. In fact, due to the sensitivity of nitrifying

bacteria to environmental factors as well as their low growth rates, it is difficult to obtain and maintain sufficient nitrifying biomass in conventional suspended or fixed culture-based wastewater treatment systems (Moreau et al., 1994; Ballinger et al., 2002; Ochoa et al., 2002).

Recently, research efforts had turned to develop aerobic granules in sequencing batch reactor systems (Beun et al., 1999; Peng et al., 1999; Tay et al., 2001). Aerobic granules are the result of cell-to-cell immobilization under given conditions (Liu and Tay, 2002). Compared to conventional activated sludge flocs, aerobic granules have advantages of regular, compact and dense microbial structure, excellent settling ability, high biomass retention as well as ability to withstand shock loading rate (Beun, 1999; Peng et al., 1999; Tay et al., 2001; Moy et al., 2002). Previous research showed that it would be possible for nitrification and denitrification to occur in aerobic granular sludge sequencing batch reactors without optimisation of the process conditions (Beun et al., 2001; Peng et al., 2001). There is evidence that the presence of organic carbon can affect the nitrification (Moreau et al., 1994; Ohashi et al., 1995; Ballinger et al., 2002). Since wastewater often contains both organics and nitrogen, hybrid aerobic granules capable of simultaneously removing organic carbon and nitrogen are highly desired. So far, very limited information is available for the development of hybrid aerobic granules for simultaneous organics removal and nitrification. Therefore, the main objectives of this work were to investigate the feasibility of the development of hybrid aerobic granules at different substrate N/COD ratios, and further to look into the physical characteristics and metabolic behaviours of hybrid aerobic granules. It is expected that this study would be useful for the development of innovative hybrid granular sludge reactor for simultaneous carbon and nitrogen removal from wastewater.

3.2 MATERIALS AND METHODS

3.2.1 Experiment set-up and media

Four columns (80 cm in height and 6 cm in diameter) with a working volume of 2.4 l were used as sequencing batch reactors, and each reactor had the same geometrical configuration. Reactors 1 to 4 (R1, R2, R3 and R4) were supplied with an air flow rate of 4.0 l min^{-1}, equivalent to a superficial upflow air velocity of 2.4 cm s^{-1}. All reactors were operated in a sequential mode: 4 min of feeding, 230 min of aeration, 2 min of settling and 4 min of effluent withdrawal. Effluent was discharged from the middle port of the column reactor.

Reactors 1 to 4 were inoculated by 650 ml of fresh activated sludge taken from Jurong Water Reclamation Plant, Singapore. This gave an initial reactor biomass concentration of 2000 mg dry weight l^{-1}. Synthetic substrate mainly consists of ethanol as sole carbon source, NH_4Cl, $NaHCO_3$ and other necessary nutrients. The ethanol chemical oxygen demand (COD) concentration was fixed at 500 mg l^{-1}, while the NH_4^+-N concentration varied from 25 to 150 mg l^{-1} in R1 to R4, which gave a respective substrate N/COD ratio of 5/100 to 30/100 by weight. To satisfy the growth requirement of nitrifying bacteria, the ratio of HCO_3^-/ NH_4^+-N was kept constant at a value of 8.0 mg mg^{-1} for all reactors. The N uptake would be intricate if P concentration is limiting. Therefore, in this study the P/COD ratio was kept at 1/100 according to Metcalf and Eddy (2003). The micronutrients in the synthetic wastewater can be found elsewhere (Liu and Capdeville, 1994). The minimum dissolved oxygen (DO) concentrations detected in four reactors were above 2.0 mg l^{-1}, while the reactor pH fell into a range of 7.5 and 8.2. The experiments were conducted in a temperature control room of 25°C.

3.2.2 Analytical methods

3.2.2.1 Organics and nitrogen concentrations in solution

Ammonium, nitrite and nitrate concentrations were measured by using a flow injection analyser (QuikChem Method 10-107-06-1-I and QuickChem Method 10-107-04-1-F,

Lachat Instruments, Inc.), while COD concentration was determined by Standard Method 5220C (APHA, 1998).

3.2.2.2 Extracellular polysaccharides and protein

Extracellular polysaccharides and proteins of sludge were extracted by cold aqueous extraction techniques (Zhang et al., 1998). A 20-ml sample of the biomass was centrifuged at 12,000 rpm for 10 minutes. The supernatant was removed. The remaining pellet was re-suspended in an 8.5% NaCl solution containing 0.22% formaldehyde. The solution was chilled in ice and mixed in a homogenizer for 3 minutes, during which time the extracellular polysaccharides and protein were extracted into the solution. After removing the residual solids by high speed centrifugation (12,000 rpm) for 30 minutes the supernatant was used to determine the extracellular polysaccharides and protein by the methods suggested by Dubois et al. (1956) and Lowry et al. (1951). Bovie serum albumin and glucose were used as protein and polysaccharides standards, respectively.

3.2.2.3 Cell hydrophobicity

Cell hydrophobicity was measured using the method described by 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 min of partitioning.

3.2.2.4 Specific oxygen utilization rate

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 (nitritation) and nitrite oxidizers, were measured using Standard Method 2710B (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 in 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 $NaNO_2$, while the biomass, COD, NH_4-N, NO_2-N concentration was kept constant at 500 mg l^{-1}, 400 mg l^{-1}, 20 mg l^{-1} and 20 mg l^{-1}, respectively. The temperature was controlled at 25°C.

3.2.2.5 Physical characteristics of granule

The size of granular sludge was obtained by using laser particle size analysis system (Malvern Mastersizer Series 2600) or image analyser (Quantimnet 500 Image Analyzer, Leica Cambridge Instruments), while the microbial structure of granules was examined with a scanning electron microscope (Stereoscan 420, Leica Cambridge Instruments). Sludge volume index (SVI) and specific gravity of sludge were measured by using standard methods (APHA, 1998).

3.3 RESULTS

3.3.1 Characteristics of seed sludge

The seed sludge was taken from the aerobic unit of Water Reclamation Plant, Singapore. The characteristics of the seed sludge are shown in Table 3.1.

Table 3.1 Characteristics of the seed sludge

Characteristics	Values
VSS/SS	0.71
Specific oxygen utilization rate (SOUR)	41.5 mg O_2 g^{-1} h^{-1}
Mea particle size	90 μm
Sludge volume index (SVI)	265 ml g^{-1}
Specific gravity	1.002

3.3.2 Variation of pH and DO in one cycle operation

It has been known that nitrifying bacteria are sensitive to changes in pH and dissolved oxygen (DO). The pH in R4 operated at the substrate N/COD ratio of 30/100, decreased from 8.3 to 7.4 after one cycle of reaction. The pH variations in R2 and R3, operated at substrate N/COD ratios of 10/100 and 20/100, followed the similar pattern, while the pH had a slight drop in R1 operated at the substrate N/COD ratio of 5/100. It had been reported that over the range of pH 7.0 to 8.0, there was little effect on nitrification rate (Poduska and Andrews, 1975). Since the pH in the reactors fell within this range, thus the pH would not be an influencing factor in this study.

After the start-up, the reactor DO concentration was monitored regularly. The minimum DO concentration in all reactors was about 2.0 mg l^{-1}. It has been commonly agreed that when the DO concentration is above 2.0 mg l^{-1}, DO would not be a limiting factor for nitrification (Benefield and Randall, 1980). Thus, DO is not a factor that influences the activity of nitrifying bacteria and further nitrification efficiency in this study.

3.3.3 Formation of aerobic granules

Figs. 3.1 to 3.4 illustrate the evolution of morphology of microbial aggregates observed in R3 run at a substrate N/COD ratio of 20/100. Similar trends were also found in the other three reactors.

It appears from Figs. 3.1 to 3.4 that the formation of aerobic granules was a gradual process from the dispersed seed sludge to tiny aggregates, and finally to mature spherical granules with a dense structure. As shown in Table 3.1, the seed sludge had a mean floc size of 0.09 mm. After two weeks of operation, tiny granules appeared in four re: ors. Mature aerobic granules with a respective mean si of 1.9, 1.5, 0.5 and 0.4 mm became dominant in R1 to R4 at 3-week of operation, while the biomass concentrations in the reactors increased up to 10 g l^{-1} total solids (TS) at steady state.

Fig. 3.5 further shows IA images of the mature aerobic granules developed in R1 to R4. Compared to the seed sludge (Fig. 3.1), the aerobic granules had compact structure with a clear outer round shape.

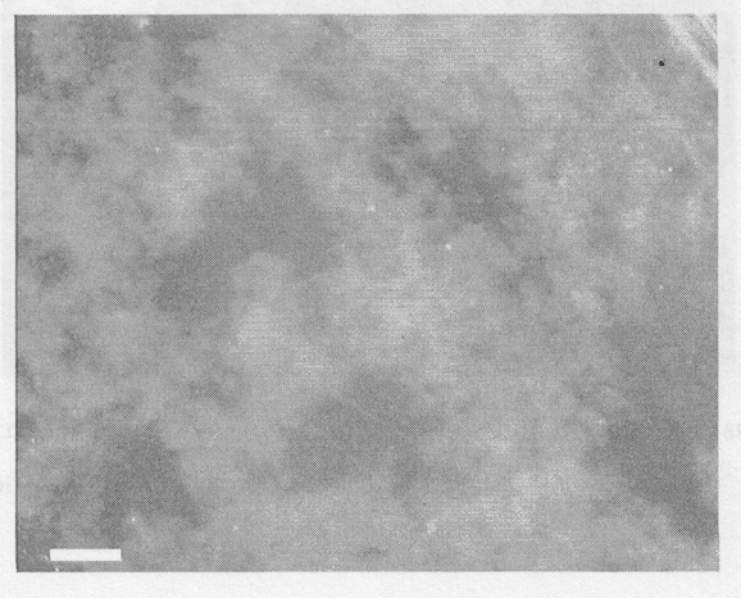

Figure 3.1 Seed sludge. Bar: 1 mm.

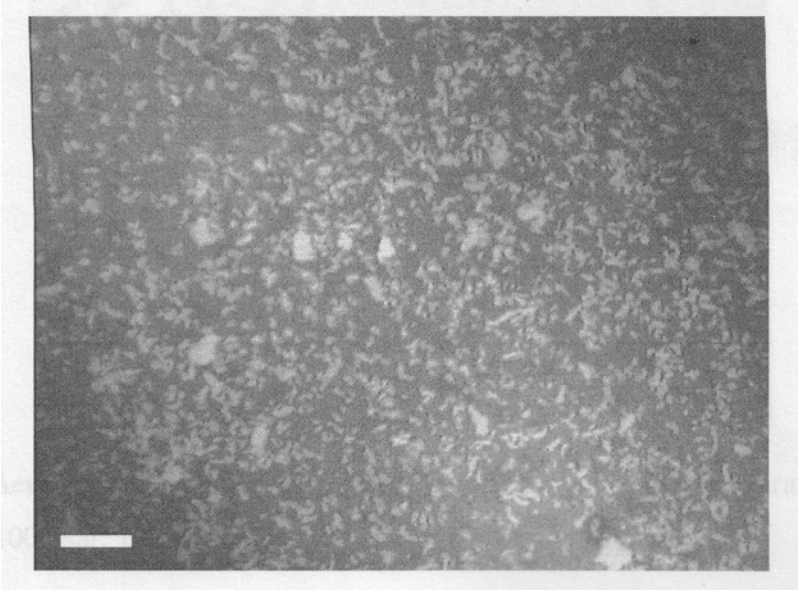

Figure 3.2 Microbial aggregates after 1-week operation in R3 run at the substrate N/COD ratio of 20/100. Bar: 1 mm.

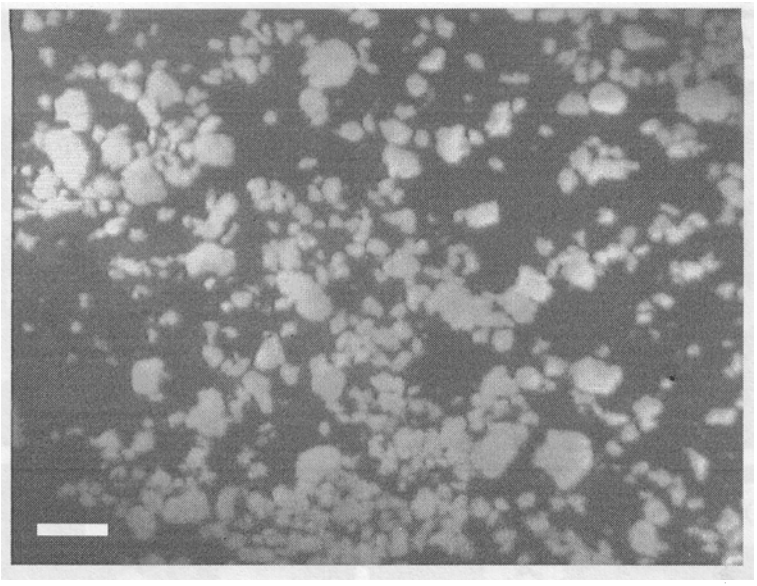

Figure 3.3 Microbial aggregates after 2-week operation in R3 run at the substrate N/COD ratio of 20/100. Bar: 1 mm.

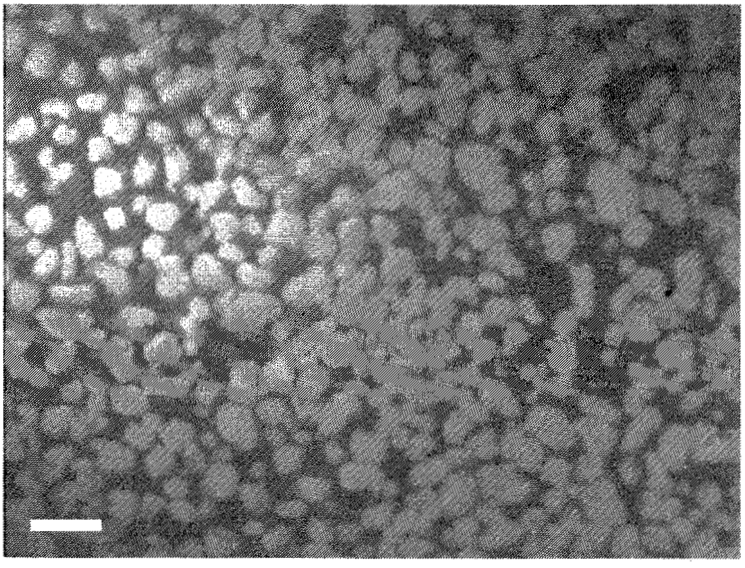

Figure 3.4 Aerobic granules after 3-week operation in R3 run at the substrate N/COD ratio of 20/100. Bar: 1 mm.

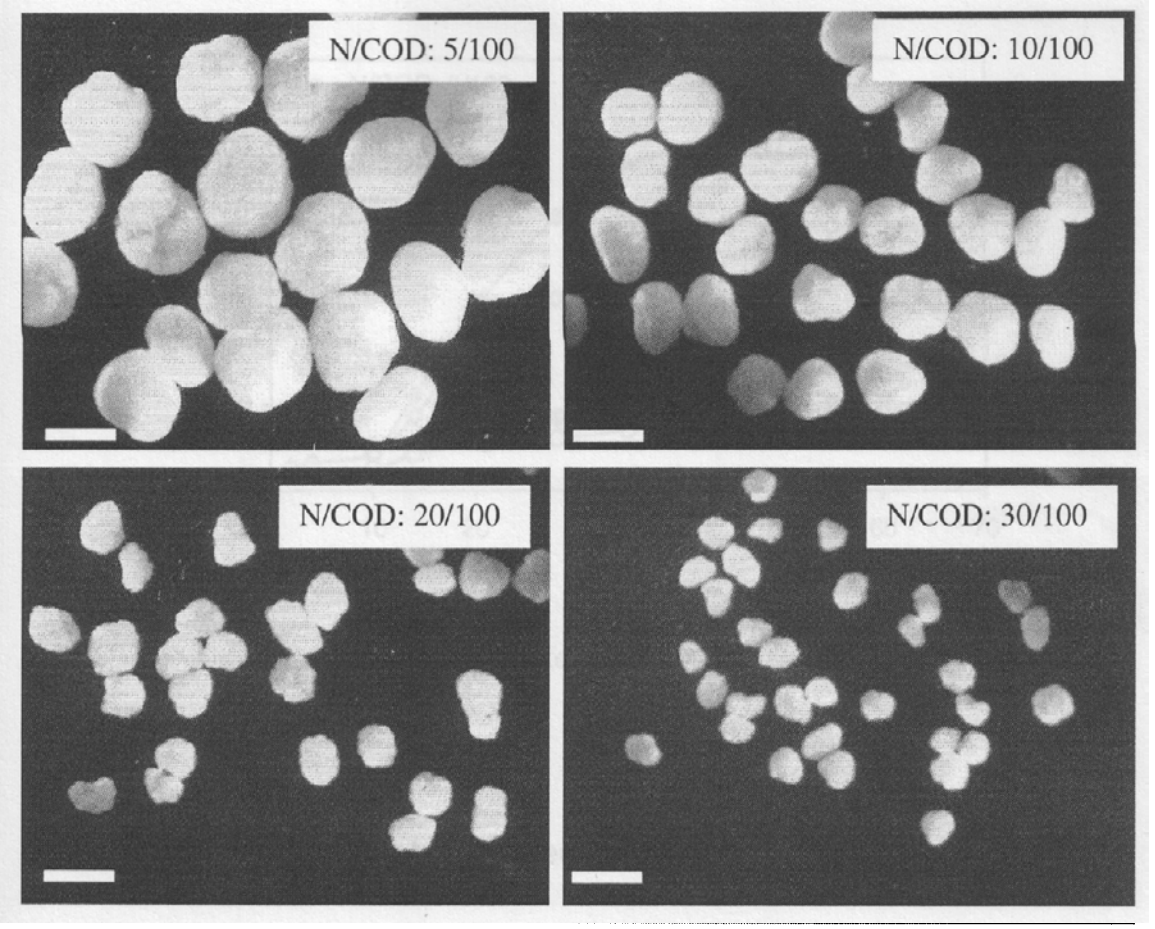

Figure 3.5 Morphology of mature granules taken on day 40 in four reactors operated at different substrate N/COD ratios. Bar: 1 mm.

3.3.4 Change in size of microbial aggregates in the course of operation

Changes in size of microbial aggregates observed in the courses of operation in four reactors are shown in Fig. 3.6. It can be seen that the mean sizes of microbial aggregates developed at different substrate N/COD ratios gradually increased and stabilized, while small aerobic granules were cultivated at high substrate N/COD ratios.

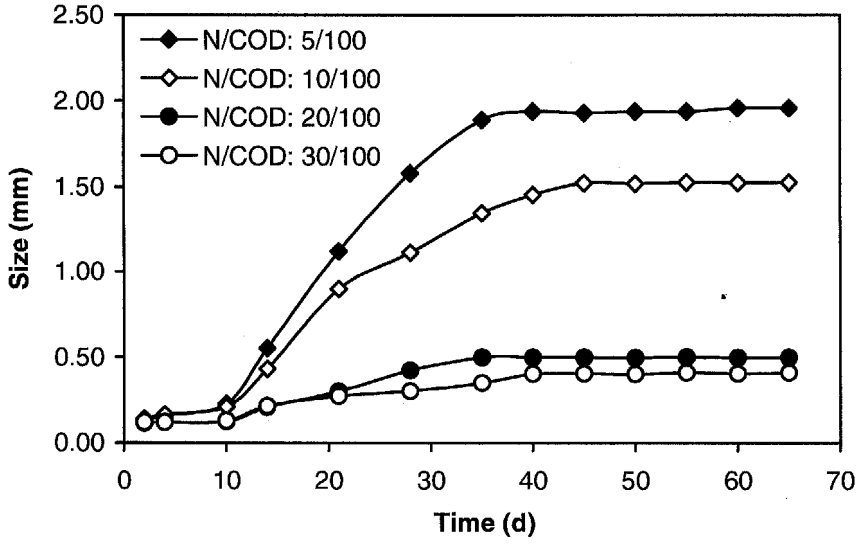

Figure 3.6 Evolution of aggregate size in four reactors operated at different substrate N/COD ratios.

3.3.5 Settleability of aerobic granules

In the environmental engineering field, sludge settleability has been commonly described by sludge volume index (SVI). Fig. 3.7 shows the profiles of SVI observed in R1 to R4. Obviously, after the formation of aerobic granules on day 20 onwards, the SVI dropped to as low as 50 ml g^{-1} in all four reactors. Fig. 3.7 together with Fig. 3.6 seem to indicate that smaller and denser aerobic granules would form at higher substrate N/COD ratios. Such information is important because one may expect to manipulate the structure of aerobic granules by controlling substrate N/COD ratios. Fig. 3.8 further displays the effect of substrate N/COD ratio on the SVI of mature aerobic granules. The SVI exhibits a decreasing trend with the increase of substrate N/COD ratio. The lowest SVI of 51 ml g^{-1} was found at the highest substrate N/COD ratio of 30/100. ıese results imply that the substrate N/COD ratio has a ˙gnificant effect on the structure of microbial granules, i.e. a more compact microbial structure could be

expected at higher substrate N/COD ratio. Compared to the seed sludge with a SVI of 265 ml g^{-1}, the settleability of aerobic granules was improved markedly.

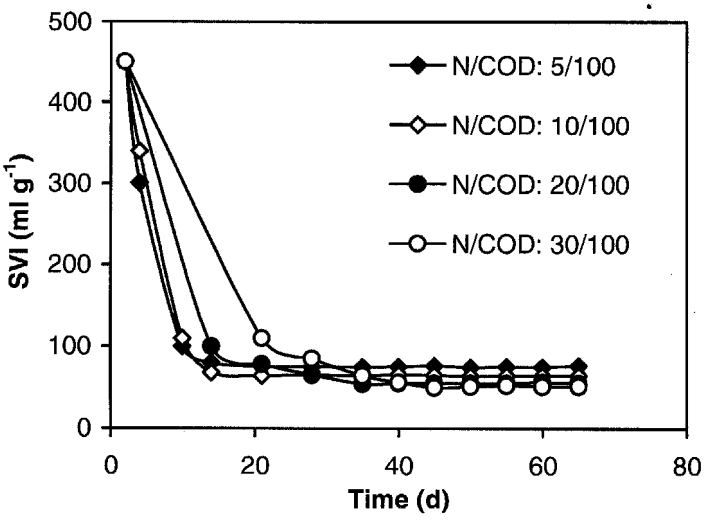

Figure 3.7 Change of SVI versus operation time in four reactors operated at different substrate N/COD ratios.

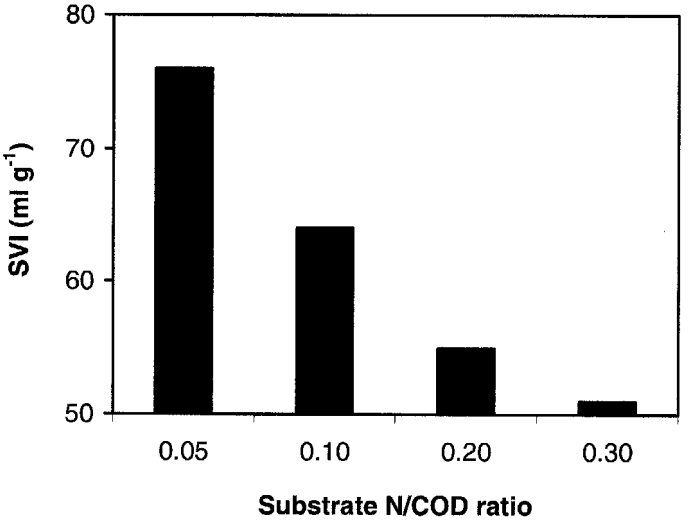

Figure 3.8 SVI of aerobic granules developed at different substrate N/COD ratios.

3.3.6 Specific gravity of aerobic granules

The specific gravity of microbial association represents the compactness of a microbial community. Fig. 3.9 shows the specific gravity of the mature aerobic granules developed at different substrate N/COD ratios. It appears that the specific gravity of granules increases with the increase of substrate N/COD ratio, i.e. increasing the substrate N/COD ratio results in a much more compact structure of aerobic granules. Such a trend is indeed consistent with the values of SVI as shown in Fig. 3.8. Compared to the seed sludge with a specific gravity of 1.002 (Table 3.1), the aerobic granules had a much denser and more compact microbial structure.

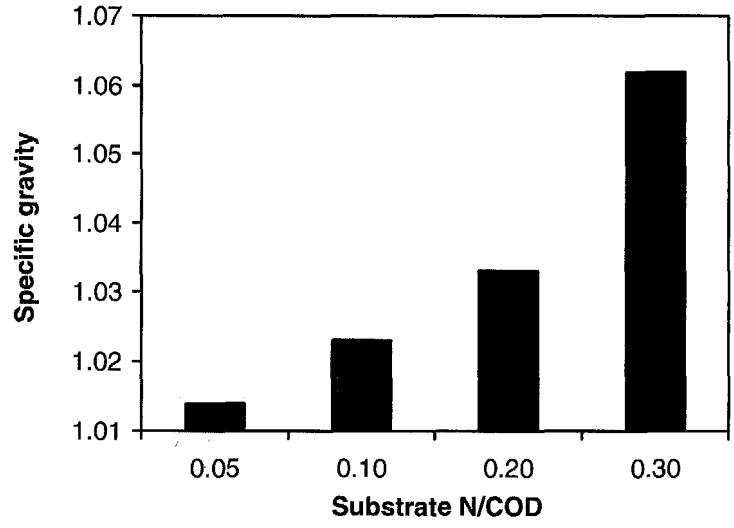

Figure 3.9 Specific gravity of aerobic granules developed at different substrate N/COD ratios.

3.3.7 Cell hydrophobicity

It has b n recognized that cell hydrophobicity plays a crucial rc in the formation of biofilm and anaerobic granules (Mahoney et al., 1987; Rouxhet and Mozes, 1990; Tay et al., 2000). The profiles of cell hydrophobicity of microbial aggregates cultivated at

different substrate N/COD ratios are shown in Fig. 3.10. These results indicated that the cell hydrophobicity gradually increased until a stable value after 40-day operation, while the cell hydrophobicity at steady state exhibits an increasing trend with the increase of the substrate N/COD ratio.

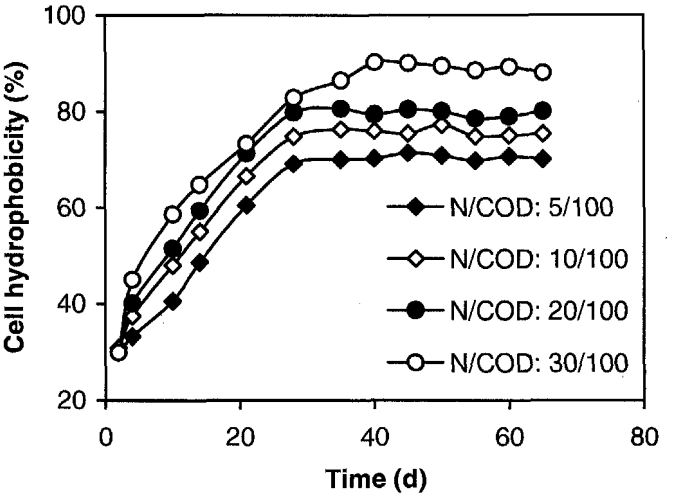

Figure 3.10 Profiles of cell hydrophobicity in four reactors operated at different substrate N/COD ratios.

3.3.8 Production of extracellular polysaccharides

Extracellular polysaccharides can mediate both cohesion and adhesion of cells, and play a crucial role in building and maintaining structural integrity in a community of immobilized cells (Fletcher and Floodgate, 1973; Lopes et al., 2000; Schmidt and Ahring, 1996). Fig. 3.11 shows the profiles of the ratio of extracellular polysaccharides (PS) to proteins (PN) in four reactors. It was found that the PS/PN ratio increased in a very significant way with the formation of aerobic granules, e.g. the PS/PN ratio increased from an initial value of 0.57 for the seed sludge to 4.0 – 5.0 for the aerobic granules. These results seem to suggest that cell aggregation would be partially related to the production of extracellular polysaccharides. On the other hand, with increasing

the substrate N/COD ratio, the PS/PN ratio shows a decreasing trend. It had been reported that a reduced substrate N/COD ratio stimulated the production of extracellular polysaccharides, resulting in improved bacterial attachment to solid surfaces (Schmidt and Ahring, 1996; Durmaz and Sanin, 2001). In addition, Tsuneda et al. (2003) found that extracellular polysaccharides exhibited good correlation with cell adhesion, while no protein was related to cell adhesion.

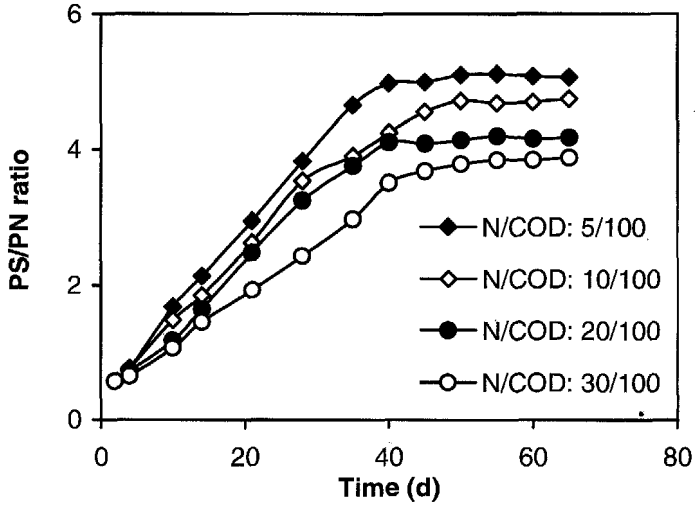

Figure 3.11 Profiles of the PS/PN ratio in four reactors operated at different substrate N/COD ratios.

3.3.9 Heterotrophic and nitrifying activities in aerobic granules

The respective activity of ammonium oxidizers and nitrite oxidizers was described by the specific ammonium oxygen utilization rate $(SOUR)_{NH4}$, and the specific nitrite oxygen utilization rate $(SOUR)_{NO2}$, while the activity of heterotrophic bacteria was quantified by its specific heterotrophic oxygen utilization rate $(SOUR)_H$. $(SOUR)_{NH4}$, $(SOUR)_{NO2}$ and $(SOUR)_H$ of aerobic granules measured on day are shown in Fig. 3.12. Both $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$ increase markedly with the increase of the substrate N/COD ratio, while the $(SOUR)_H$ exhibits a decreasing trend. Similar

phenomena were also observed in biofilm reactors (Moreau et al., 1994; Ochoa et al., 2002).

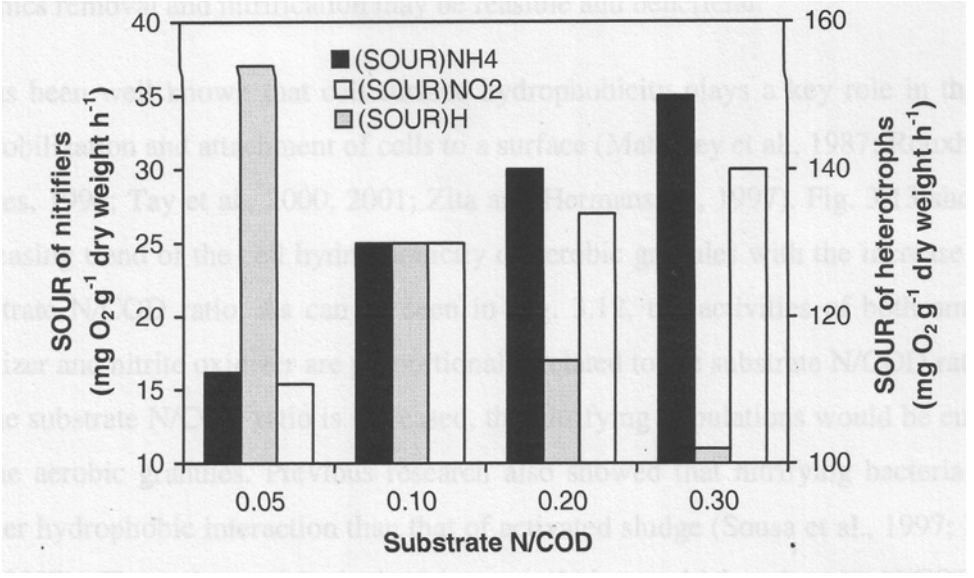

Figure 3.12 Activities of heterotrophic and nitrifying populations measured on day 40 in four reactors operated at different substrate N/COD ratios.

3.4 DISCUSSION

Fig. 3.3 shows that aerobic granules can be formed in a wide range of substrate N/COD ratios from 5/100 to 30/100. It should be mentioned that the settling velocity of the aerobic granules cultivated in four reactors was greater than 60 m h^{-1}, while the biomass retention reached about 10 g VS l^{-1} in all reactors. The settling velocity of conventional activated sludge was generally less than 10 m h^{-1} (Campos et al., 1999). Compared to the conventional bioflocs, the excellent settleability of aerobic granules can ensure easy and effective separation of biosolids from the effluent, while the high biomass concentration implies that a compact and small aerobic granular sludge reactor could be developed for simultaneous organic removal and nitrification. Fig. 3.1 clearly shows that aerobic granular sludge reactors can be started up within four weeks. In this

study, the aerobic granular sludge reactors had been run stably over a period of one year before the experiments were terminated. It seems certain that the use of aerobic granules for upgrading the existing wastewater treatment plants towards simultaneous organics removal and nitrification may be feasible and beneficial.

It has been well known that cell surface hydrophobicity plays a key role in the self-immobilization and attachment of cells to a surface (Mahoney et al., 1987; Rouxhet and Mozes, 1990; Tay et al., 2000, 2001; Zita and Hermansson, 1997). Fig. 3.13 shows an increasing trend of the cell hydrophobicity of aerobic granules with the increase of the substrate N/COD ratio. As can be seen in Fig. 3.12, the activities of both ammonia oxidizer and nitrite oxidizer are proportionally related to the substrate N/COD ratio, i.e. as the substrate N/COD ratio is increased, the nitrifying populations would be enriched in the aerobic granules. Previous research also showed that nitrifying bacteria had a higher hydrophobic interaction than that of activated sludge (Sousa et al., 1997; Kim et al., 2000). Thus, the enriched nitrifying population at high substrate N/COD ratio would be responsible for the higher cell hydrophobicity. Del Re et al. (2000) reported that autoaggregation of bacteria was associated with a good degree of hydrophobicity of bacterial surface.

In a thermodynamic sense, reducing cell hydrophobicity would simultaneously cause an increase in the excess Gibbs energy of the surface, which in turn favours the self-aggregation of bacteria from liquid phase to form a new solid phase, namely microbial aggregates (Liu and Tay, 2002). In fact, there is evidence that the hydrophobicity of bacteria is an important affinity force in cell immobilization process (Bossier and Verstraete, 1996; Rouxhet and Mozes, 1990; Zita and Hermansson). It seems certain that hydrophobic binding force has a prime importance for the cell-to-cell approach and interaction, and the hydrophobicity of bacterial surface can act a driving force for the initiation of cell-to-cell aggregation, which is the first step towards aerobic granulation, and further keep the aggregated bacteria tightly together.

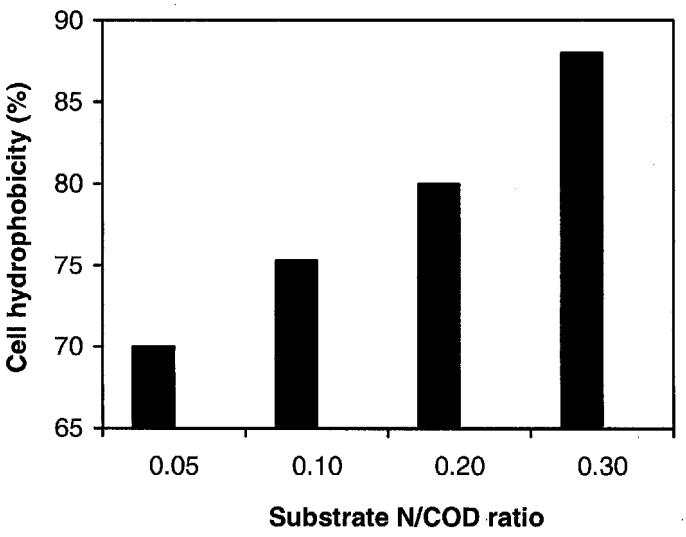

Figure 3.13 Effect of the substrate N/COD ratio on the cell hydrophobicity.

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 surfaces (Sutherlan, 2001). The PS/PN ratios of aerobic granules tend to decrease with the increase of the substrate N/COD ratio (Fig. 3.14 and Table 3.2). As shown in Fig. 3.12, the nitrifying populations in aerobic granules were greatly sustained at high substrate N/COD ratios. Thus, the lower production of extracellular polysaccharides at higher substrate N/COD ratio can be easily understood because nitrifying bacteria cannot utilize organic carbon for microbial growth, and only 11% to 27% of energy generated goes to biosynthesis (Laudelout et al., 1968).

Fig. 3.15 further indicates that the production of cell polysaccharides is quasi-linearly dependent on the respiration activity of heterotrophic bacteria present in the aerobic granule i.e. a high catabolic activity favours the production of cell polysaccharides. These are consistent with the previous research showing that the production of extracellular polysaccharides was energy-dependent (Robinson et al., 1984; Wuertz et

al., 1998). In fact, there is evidence that cell carbohydrate content increases and protein content decreases in a very significant way as the substrate N/COD ratio decreases (Durmaz and Sanin, 2001). It seems reasonable to consider that nitrifying bacteria would produce much less extracellular polysaccharides than heterotrophic bacteria. Recently, Tsuneda et al. (2001) used extracellular polysaccharides produced by heterotrophic bacteria to enhance the formation of nitrifying biofilm. As shown in Fig. 3.14, the content of aerobic granule-polysaccharides at steady state is at least 3-fold higher than that of proteins. This implies that cell proteins would less contribute to the structure and stability of aerobic granules. Vandevivere and Kirchman (1993) also found that the content of exopolysaccharides was 5-fold greater for attached cells than for free-living cells.

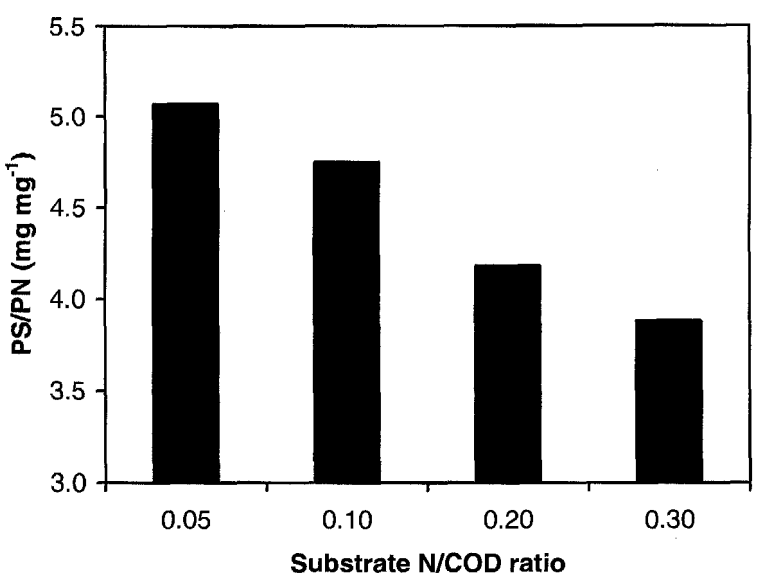

Figure 3.14 Effect of the substrate N/COD ratio on PS/PN ratio.

Table 3.2 PS and PN values in four reactors

	R1	R2	R3	R4
PS (mg g^{-1}SS)	242.35	158.63	114.32	96.71
PN (mg g^{-1}SS)	47.80	33.40	27.35	24.93

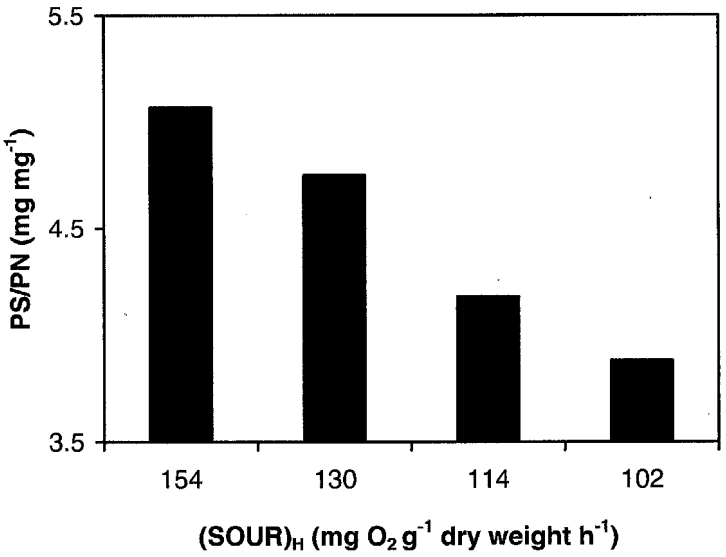

Figure 3.15 Relationship between $(SOUR)_H$ and PS/PN ratio.

3.5 CONCLUSIONS

Aerobic granules were successfully developed at different substrate N/COD ratios in the range of 5/100 to 30/100 by weight. It was found that the substrate N/COD ratio had significant impact on the microbial and physicochemical characteristics of aerobic granules. The activities of nitrifying bacteria were enhanced by the increased substrate N/COD ratio. The cell hydrophobicity showed an increasing trend, while the production of extracellular polysaccharides decreased with increasing the substrate N/COD ratio.

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CHAPTER 4

MICROBIAL DIVERSITY OF AEROBIC GRANULES DEVELOPED AT DIFFERENT SUBSTRATE N/COD RATIOS

ABSTRACT

By measuring respective respirometric activities of heterotrophic, ammonia-oxidizing and nitrite-oxidizing bacteria, it was found that the relative abundance of nitrifying bacteria over heterotrophs in aerobic granules was closely related to the substrate N/COD ratios. Results showed that the populations of both ammonia and nitrite oxidizers were significantly enriched at high substrate N/COD ratio, but a decreasing trend of heterotrophic populations was observed as the substrate N/COD ratio was increased. These seem to indicate that high substrate N/COD ratio favors the selection of nitrifying bacteria in the aerobic granules. It was further found that the relative activity of nitrifying populations against heterotrophic populations evolved until a balance between two populations was reached in the aerobic granular sludge community. Denitrifying populations in aerobic granules was proportionally related to the substrate N/COD ratio or nitrifying population, i.e. the denitrifying activity was enhanced with the increase of the substrate N/COD ratio. However, an increased DO could reduce the denitrifying activity. These results showed that nitrifying, denitrifying and heterotrophic populations can co-exist in aerobic granules developed at different substrate N/COD ratios.

4.1 INTRODUCTION

To remove organics and nitrogen from wastewater, nitrifying, denitrifying and other heterotrophic populations should co-exist in aerobic granules. It appears from previous research that different substrate N/COD ratios would lead to significant populations shift in both suspended and attached cultures (Ballinger et al., 2002; Moreau et al.,

1994; Ohashi et al., 1995; Prinčič et al., 1998). It was reported that a variation of the relative substrate composition in the bulk fluid might result in rapid and drastic changes of the relative abundance and spatial distribution of organisms in biofilms (Fruhen et al., 1995). Zhang et al. (1995) found that heterotrophs, supported by soluble microbial products or metabolic products, could exist in nitrifying biofilms. Nitrifiers, however, have difficulty to survive in heterotrophic biofilms. These observations could be due to the competition for dissolved oxygen and space between heterotrophs and nitrifiers at different substrate N/COD ratios. Inhibition or elimination of nitrifying populations by interspecies competition usually leads to a decline in nitrification efficiency, or even a failure of the process. Thus, an understanding of the effects of substrate N/COD ratio on the dynamic changes of microbial species is essential for optimizing removal of organics and nitrogen from wastewater. Therefore, the objective of this chapter is to look into microbial diversity and dynamic changes in aerobic granules in terms of the activities of nitrifying, denitrifying and heterotrophic populations.

4.2 MATERIALS AND METHODS

Mature aerobic granules as characterized in Chapter 3 were used to study microbial diversity-substrate N/COD relationship. Details on set-up, media and analytical methods can be found in Chapter 3.

4.3 RESULTS

The performance of four reactors reached steady state after 2 months of operation. Mature and stable aerobic granules were therefore sampled from 60 days onwards in this study.

4.3.1 Evolution of heterotrophic activity

The activity of heterotrophic bacteria was quantified by its specific heterotrophic oxygen utilization rate $(SOUR)_H$. Fig. 4.1 shows the activities of heterotrophic populations in aerobic granules developed at different substrate N/COD ratios, on day 60, 67, 86, 162 and 334. It can be seen that the activity of heterotrophs slightly decreased with operation time in R2 to R4, which were operated at the respective substrate N/COD ratios of 10/100, 20/100 and 30/100, while the activity of heterotrops in R1 operated at the substrate N/COD ratio of 5/100 seemed unchanged.

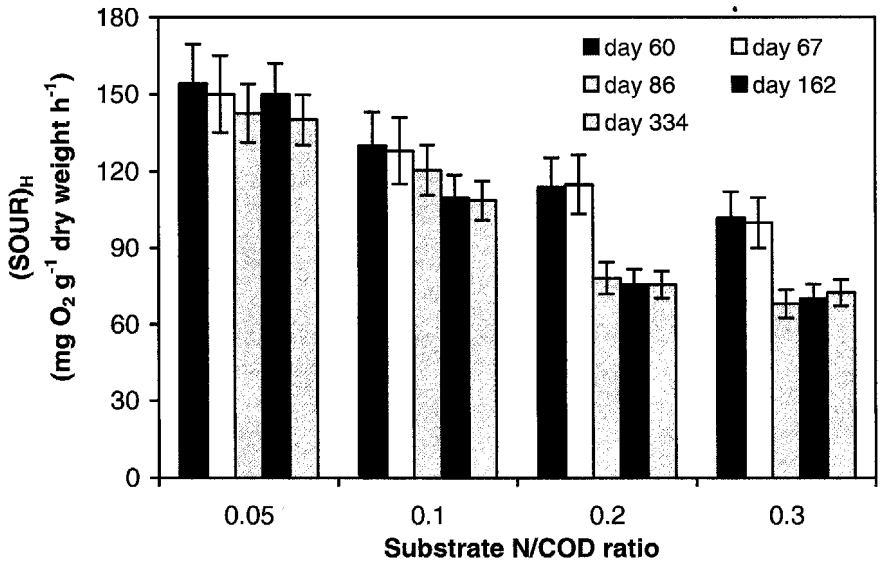

Figure 4.1 Respirometric activities of heterotrophs in aerobic granules sampled on day 60, 67, 86, 162 and 334.

4.3.2 Evolution of nitrifying activity

The respective respirometric activity of ammonia oxidizer and nitrite oxidizer was describe(ɔy the specific ammonium oxygen utilization rate $(SOUR)_{NH4}$, and the specific nitritation oxygen utilization rate $(SOUR)_{NO2}$. The acuvity evolutions of ammonia oxidizer and nitrite oxidizer are shown in Figs. 4.2 to 4.6. It can be seen that the activity of both ammonia oxidizers and nitrite oxidizers increased with operation

time. It is reasonable to consider that the sum of $(SOUR)_{NH4}$ and $(SOUR)_{NO2}$, namely $(SOUR)_N$ represents the overall activity of nitrifying populations. The values of $(SOUR)_N$ on day 60, 67, 86, 162 and 334 are also presented in Figs. 4.2 to 4.6. The overall activity of nitrifying populations increased in the course of operation and reached a stable value on day 86 onwards.

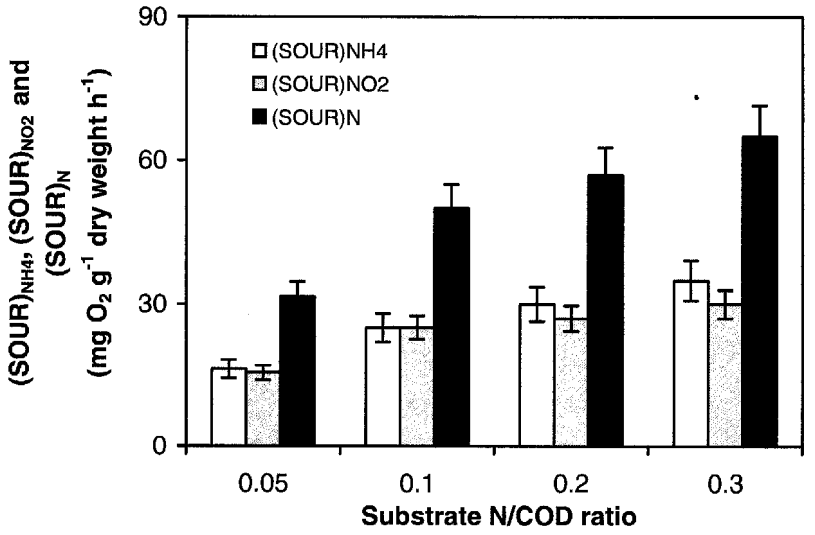

Figure 4.2 Respirometric activities of ammonium oxidizers, nitrite oxidizers and nitrifying populations in aerobic granules measured on day 60.

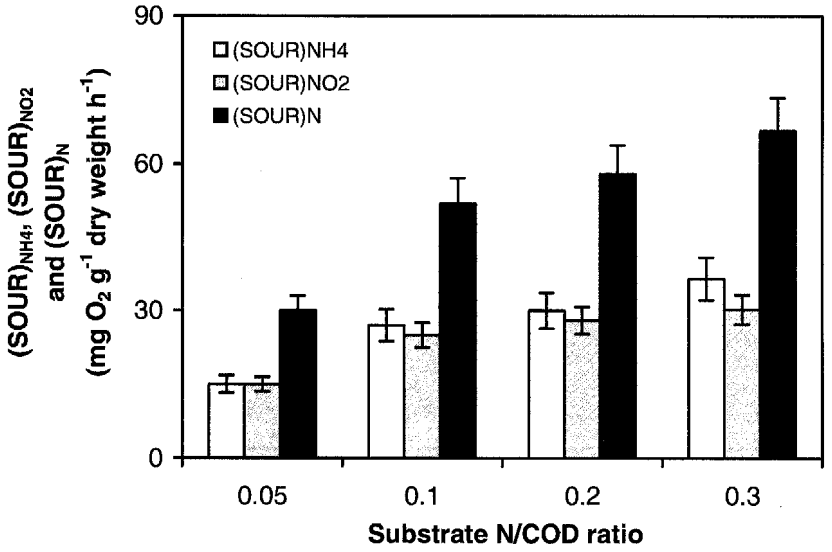

Figure 4.3 Respirometric activities of ammonium oxidizers, nitrite oxidizers and nitrifying populations in aerobic granules measured on day 67.

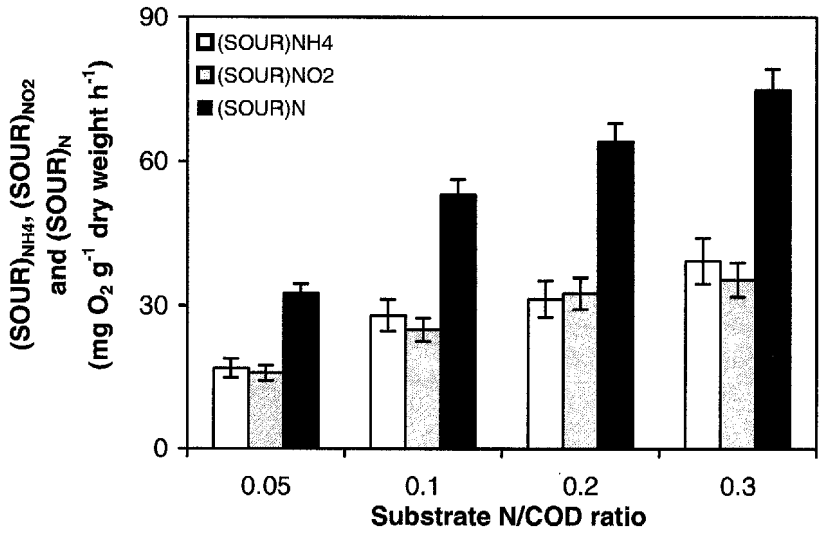

Figure 4.4 Respirometric activities of ammonium oxidizers, nitrite oxidizers and nitrifying populations in aerobic granules measured on day 86.

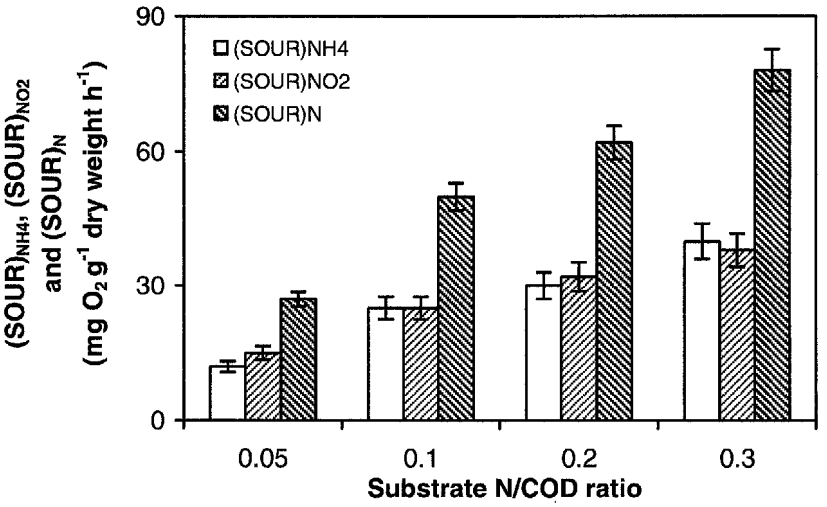

Figure 4.5 Respirometric activities of ammonium oxidizers, nitrite oxidizers and nitrifying populations in aerobic granules measured on day 162.

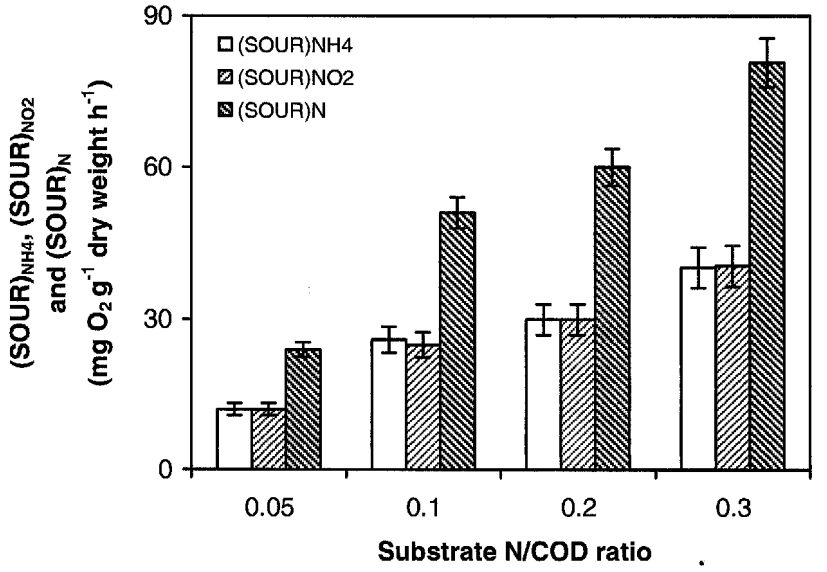

Figure 4.6 Respirometric activities of ammonium oxidizers, nitrite oxidizers and nitrifying populations in aerobic granules measured on day 334.

4.3.3 Interaction between heterotrophic and nitrifying populations

The fraction of active biomass in a culture would be proportionally related to the respirometric activity (Ochoa et al., 2002). Thus, the relative abundance of nitrifying populations over heterotrophic populations can be proportionally represented by $(SOUR)_N/(SOUR)_H$. Fig. 4.7 shows the relative abundance of nitrifying populations over heterotrophic populations in the aerobic granules sampled on day 60, 67, 86, 162 and 334. The value of $(SOUR)_N/(SOUR)_H$ in R4 run at the substrate N/COD ratio of 30/100 was 0.6 on day 60 and further increased to about 1.1 on day 86 onwards. Interactions between heterotrophic and nitrifying populations in R2 and R3 operated at the substrate N/COD ratios of 10/100 and 20/100 followed the similar pattern, i.e. $(SOUR)_N/(SOUR)_H$ gradually stabilized at a certain level. These seem to imply that a balance between two populations could be achieved finally. However, $(SOUR)_N/(SOUR)_H$ in R1 operated at the substrate N/COD ratio of 5/100 almost remained constant.

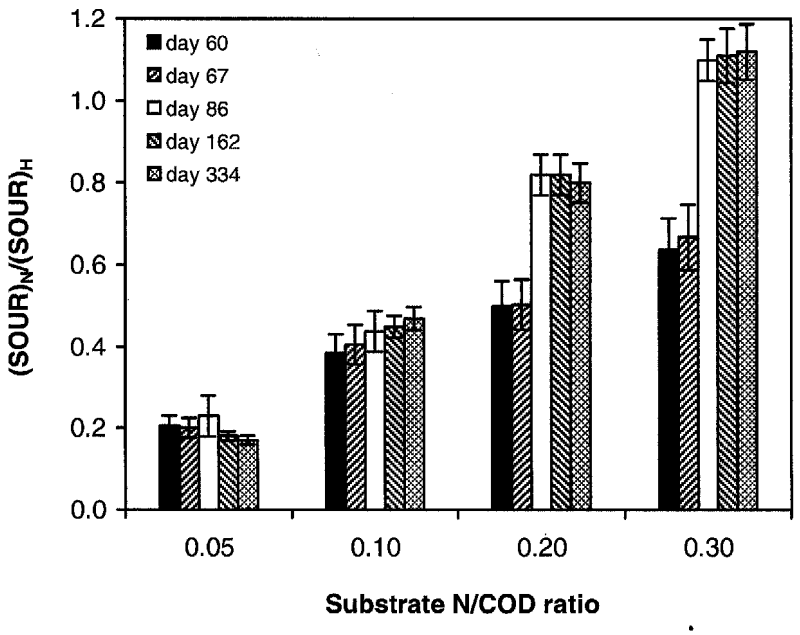

Figure 4.7 Evolution of relative abundance of nitrifying populations over heterotrophic populations in aerobic granules.

4.3.4 Activity of denitrifying populations in aerobic granules

The activity of denitrifying populations could be represented by the specific nitrogen reduction rate (q_{obs}). Fig. 4.8 shows the q_{obs} values of aerobic granules developed at different substrate N/COD ratios at 0.5 mg l^{-1} of DO. It appears that denitrifying populations in aerobic granules is proportionally related to the substrate N/COD ratio.

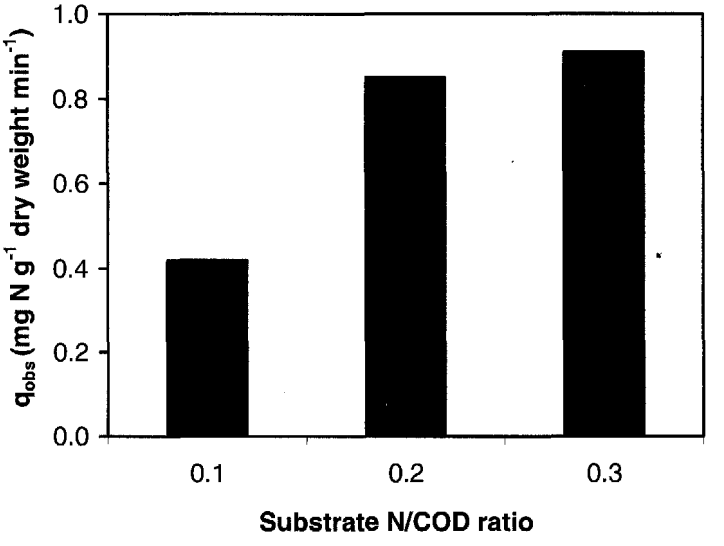

Figure 4.8 Denitrifying activity of aerobic granules developed at different substrate N/COD ratios.

4.4 DISCUSSION

Fig. 4.1 shows that aerobic granules in R1 kept a stable heterotrophic activity, while the activities of hetertrophs in R2 to R4 operated at substrate N/COD ratios of 10/100, 20/100 and 30/100, decreased with the operation time, while it appears from Figs. 4.2 to 4.6 that the activities of both ammonium oxidizers and nitrite oxidizers in R2 to R4 increased with operation time. However, the activities of ammonium oxidizers and nitrite oxidizers in R1 run at the substrate N/COD ratio of 5/100 almost remained unchanged. These results imply that the substrate N/COD ratio would have remarked

effect on the activity distribution of ammonium-oxidizing and nitrite-oxidizing bacteria in the aerobic granules, i.e. both ammonium-oxidizing and nitrite-oxidizing activities were significantly increased with the increase of the substrate N/COD ratio, while the heterotrophic activity in the aerobic granules tended to decrease. Figs. 4.2-4.6 show that at high substrate N/COD ratios, heterotrophs became less and less dominant, and nitrifying populations would be able to compete with heterotrophs, and became an important component of the aerobic granules. Similar results were also reported in the study on biofilm (Moreau et al., 1994; Ohashi et al., 1995; Ochoa et al., 2002).

As shown in Fig. 4.7 that nitrifying populations continued to build up over heterotrophic population in the aerobic granules until a balance between heterotrophic and nitrifying populations was reached on day 86 onwards. Nitrifying populations are commonly found in activated sludge and biofilms, while their quantity is generally insufficient because they would be out-competed by heterotrophs (Moreau et al., 1994). Aerobic granules can provide a protective matrix for nitrifying bacteria to grow peacefully without the risk of being washed out from the system. It may be expected that aerobic granule-based compact and efficient bioreactor for simultaneous organic removal and nitrification would be developed in near future.

Fig. 4.8 shows the effect of substrate N/COD ratio on the overall activity of denitrifying populations in microbial granules. The respective specific total nitrogen reduction rate by microbial granules in R2 to R4 operated at the respective substrate N/COD ratios of 10/100, 20/100 and 30/100 were 0.42, 0.85 and 0.91 mg N g^{-1} dry weight min^{-1}, at DO concentration of 0.5 mg l^{-1}, which are comparable with the activity data obtained from conventional denitrification processes (Glass and Silverstein, 1998; Foglar and Briški, 2003). It should be realized that q_{obs} tends to increase with the increase of substrate N/COD ratio, while Fig. 4.7 shows that the increased substrate N/COD ratio results in an enriched nitrifying populations in microbial granules, i.e. the nitrate concentrations in the reactors also increases with the substrate N/COD ratio as shown in Figs. 5.5 to 5.8. Batchelor (1982) used Eq. 4.1 to describe the effects of DO and nitrate concentration on the activity of denitrifying bacteria:

$$q_{NO3} = q_{NO3,\max} \times \frac{S_{NO3}}{k_{NO3} + S_{NO3}} \times \frac{S_c}{k_c + S_c} \times \frac{k_o}{k_o + DO} \quad (4.1)$$

in which, q_{NO3} is the specific total nitrogen reduction rate (mg N g^{-1} dry weight min^{-1}), $q_{NO3,\ max}$ is the maximum specific total nitrogen reduction rate, S_{NO3} is NO_3-N concentration, mg l^{-1}, S_c is concentration of organic substrate, mg l^{-1}, k_{NO3} is half-saturation constant for NO_3-N, mg l^{-1}, k_c is half-saturation constant for organic substrate, mg l^{-1}, and k_o is half-saturation constant for oxygen, mg l^{-1}. According to Eq. 4.1, the increase of nitrate concentration will lead to the increase of the specific nitrate reduction rate. The experimental data are consistent with the prediction of this model. Consequently, it appears from this study that nitrifying, denitrifying and other heterotrophic populations can co-exist in aerobic granules, and a novel high-efficiency granules-based bioreactor could be expected for simultaneous organics and nitrogen removal.

4.5 CONCLUSIONS

Nitrifying, denitrifying and other heterotrophic populations can co-exist in the granules, and shifts in microbial population in granules were closely related to the substrate N/COD ratio. It seems that different species may co-exist in the same microbial matrix of granules, which provides a platform for bacteria to function synergically. The aerobic granules developed at high substrate N/COD ratios exhibited enhanced nitrifying and denitrifying activities, while the activity of heterotrophic bacteria in granules showed a decreasing trend. The relative activity of nitrifying populations against heterotrophic populations evolved until a balance between two populations was reached in the aerobic granular sludge community. These seem to indicate that high substrate N/COD ratio favours the selection of nitrifying bacteria in the aerobic granules. It is expected that a more compact bioreactor for carbon and nitrogen removal would be designed in near future.

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CHAPTER 5

ELEMENTAL COMPOSITIONS OF AEROBIC GRANULES

ABSTRACT

In this chapter, elemental compositions of aerobic granules developed at different substrate N/COD ratios were determined. Results showed that the substrate N/COD ratio had a direct and profound effect on the elemental compositions of aerobic granules. The respective ratios of cell oxygen, nitrogen and calcium to cell carbon were determined by the substrate N/COD ratio. It was found that cell hydrophobicity of aerobic granules was inversely related to the ratio of cell oxygen normalized to cell carbon. Since the cell calcium contents in the aerobic granules developed at different substrate N/COD ratios were even lower than that in the seed sludge, the cell calcium would not contribute to aerobic granulation significantly. This study demonstrated that the elemental composition, microbial distribution and characteristics of aerobic granules were related to the substrate N/COD ratio applied.

5.1 INTRODUCTION

As shown in Chapter 3, characteristics of the aerobic granules are substrate N/COD ratio-dependent. It has been known that changes in characteristics are usually related to the changes in chemical compositions of microorganisms (Pitryuk et al., 2002), while in mixed culture, chemical compositions of microorganisms may reflect the changes of microbial community and growth conditions (Duboc et al., 1995; Heldal et al., 1996). The microorganisms are found to differ significantly in their relative contents of C, H, N, O, and other elements when they experience the shift of microbial community and the change of growth conditions (Duboc et al., 1995; Pitryuk et al., 2002). Obviously, information on chemical composition of microorganisms is essential for a sound understanding of the behaviours of microbial community. Thus, this chapter aimed at

the elemental compositions of aerobic granules developed at different substrate N/COD ratios, and the relationships between elemental compositions and characteristics of aerobic granules were also investigated.

5.2 MATERIALS AND METHODS

5.2.1 Experiment set-up and media

The experiment set-up and media were detailed in Chapter 3.

5.2.2 Analytical methods

In order to analyze the elemental composition of aerobic granules, granule samples were dried to a constant weigh at 105 °C, then pulverized (APHA, 1998) and 1.0 mg of the prepared sample was used to determine the cell C, H, N, S and O content by CHNS/O analyzer (CHNS/O 2400II, PerkinElmer). Then, 0.1 g of the above-prepared sample was digested with nitric acid (APHA, 1998) and multi-elemental analysis was performed, using an inductively coupled plasma emission spectrometer (PerkinElmer Optima 2000).

5.3 RESULTS

5.3.1 Elemental composition of aerobic granules

Table 5.1 summarizes the elemental formulas of the aerobic granules sampled on days 60, 67 and 86, and Table 5.2 shows the elemental compositions of aerobic granules determined on day 60. These data indicate that aerobic granules mainly comprise six major elements, i.e. C, H, O, N, S and P. Figures 5.1 and 5.2 further display the effect of substrate N/COD ratio on the respective ratio of cellular oxygen, nitrogen and calcium normalized to cellular carbon. It can be seen that an increased substrate N/COD ratio results in an increased cell N/C ratio, while the cell O/C ratio exhibits a decreasing trend as the substrate N/COD ratio increases. These imply that the substrate

N/COD ratio would have a profound effect on the elemental compositions of aerobic granules. Vrede et al. (2002) also reported that elemental composition of bacterioplankton was closely related to the substrate carbon and nitrogen and the lowest cell carbon content was found in carbon-limited cells. In addition, Heldal et al. (1996) observed a marked reduction in $(O/C)_{cell}$ level when the conditions changed from nitrogen-limitation to carbon-limitation.

The accumulation of calcium in anaerobic granules had been reported (Yu et al., 2001). However, Figure 5.2 suggests that no accumulation of cell calcium occurs in aerobic granules cultivated at different substrate N/COD ratios. In fact, the cell Ca/C ratio of aerobic granules is even lower than that of the seed sludge (7.5 mmol mol^{-1}).

Table 5.1 Elemental formula of aerobic granules

Sampling day		Day 60	Day 67	Day 86
Substrate N/COD ratio	5/100	$C_{5.8}H_{12.2}O_{4.0}NP_{0.04}$	$C_{5.6}H_{11.4}O_{3.9}NP_{0.04}$	$C_{5.4}H_{8.3}O_{3.3}NP_{0.04}$
	10/100	$C_{5.4}H_{10.8}O_{3.7}NP_{0.04}$	$C_{5.5}H_{10.9}O_{3.8}NP_{0.04}$	$C_{5.2}H_{7.7}O_{3.1}NP_{0.05}$
	20/100	$C_{5.3}H_{10.9}O_{3.4}NP_{0.04}$	$C_{5.1}H_{10.3}O_{3.4}NP_{0.04}$	$C_{5.1}H_{7.3}O_{3.2}NP_{0.04}$
	30/100	$C_{5.3}H_{10.8}O_{3.3}NP_{0.04}$	$C_{5.1}H_{9.9}O_{3.1}NP_{0.04}$	$C_{4.8}H_{7.1}O_{2.9}NP_{0.04}$

Table 5.2 Elemental composition of aerobic granules sampled on day 60, in terms of grams of element in 100 g dry weight of granules

Element	Substrate N/COD ratio			
	5/100	10/100	20/100	30/100
C	41.960	42.820	41.890	43.023
H	7.380	7.020	7.160	7.300
O	38.770	38.740	36.540	36.200
N	8.490	9.080	9.220	9.480
P	0.750	0.810	0.850	0.830
S	1.010	0.890	0.900	0.990
Ca	0.420	0.230	0.290	0.540
Fe	0.180	0.043	0.049	0.160
Mg	0.130	0.070	0.110	0.140
Al	0.022	0.003	0.042	0.250
Mn	0.005	0.002	0.003	0.002
Co	0.001	0.000	0.000	0.000
Cu	0.150	0.008	0.019	0.098
Ni	0.011	0.011	0.075	0.055
Zn	0.093	0.010	0.016	0.034
Na	0.330	0.110	0.480	0.430
K	0.300	0.160	0.360	0.480
Formula	$C_{5.8}H_{12.2}O_{4.0}NP_{0.04}$	$C_{5.4}H_{10.8}O_{3.7}NP_{0.04}$	$C_{5.3}H_{10.9}O_{3.4}NP_{0.0}$	$C_{5.3}H_{10.8}O_{3.3}NP_{0.04}$

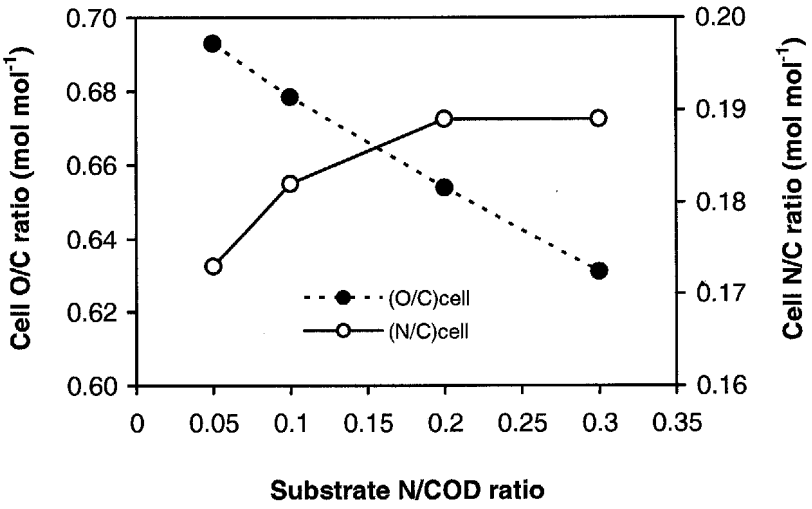

Figure 5.1 Effects of substrate N/COD ratios on the cell O/C and N/C ratios of aerobic granules.

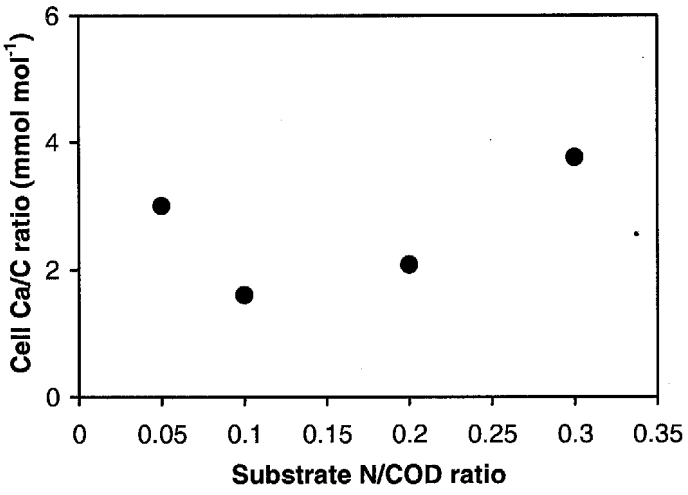

Figure 5.2 Effect of substrate N/COD ratios on the cell calcium of aerobic granules.

5.3.2 Relationship between cell composition and the ratio of $(SOUR)_N/(SOUR)_H$

The ratios of $(SOUR)_N$ to $(SOUR)_H$ in aerobic granules sampled on day 60 are shown in Fig. 5.3. It can be seen that the $(SOUR)_N/(SOUR)_H$ ratio almost linearly increases with the increase in substrate N/COD ratio. Similar phenomena were also observed in biofilm reactors (Moreau et al., 1994; Ochoa et al., 2002). Figure 5.4 further displays the relationships between cell O/C ratio, cell N/C ratio and $(SOUR)_N$ to $(SOUR)_H$ ratio.

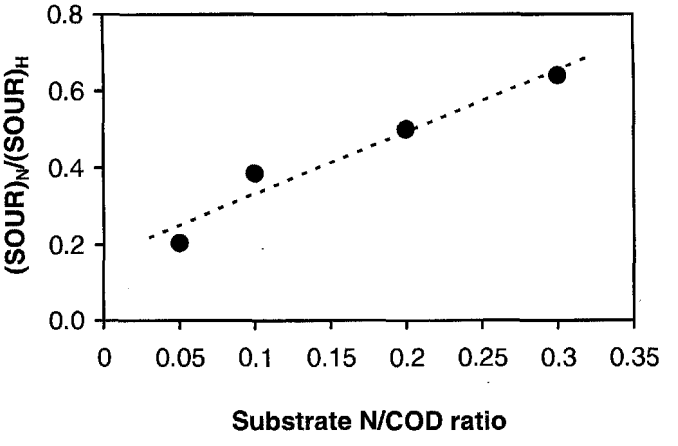

Figure 5.3 $(SOUR)_N/(SOUR)_H$ versus substrate N/COD ratio determined on day 60.

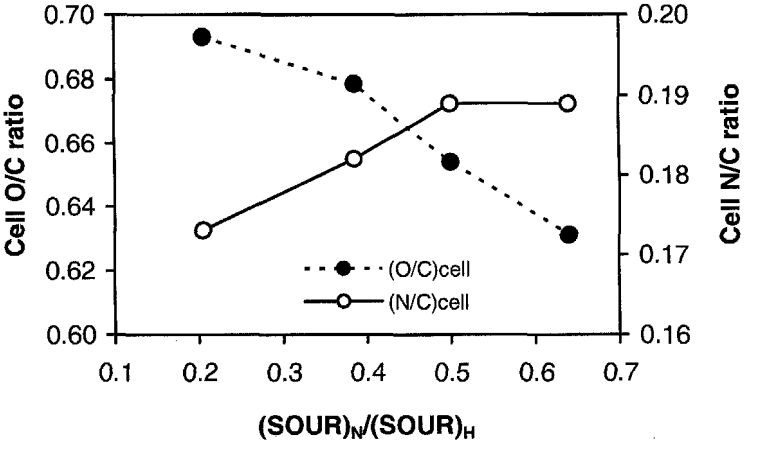

Figure 5.4 Effect of $(SOUR)_N/(SOUR)_H$ ratio on cell O/C and cell N/C.

5.3.3 Relationship between cell composition and hydrophobicity

It has been recognized that cell hydrophobicity plays an important role in microbial aggregation and flocculation (Bossier and Verstraete, 1996; Del Re et al., 2000; Liu et al., 2003). Figure 5.5 shows that the cell hydrophobicity is inversely related to the ratio of cell oxygen content normalized to cell carbon content $(O/C)_{cell}$, while the $(O/C)_{cell}$ decreases with the increase in substrate N/COD ratio as shown in Fig. 5.1. In fact, previous research on biofilms also found that a higher $(O/C)_{cell}$ resulted in a lower cell hydrophobicity (Changui et al., 1987; Rouxhet and Mozes, 1990).

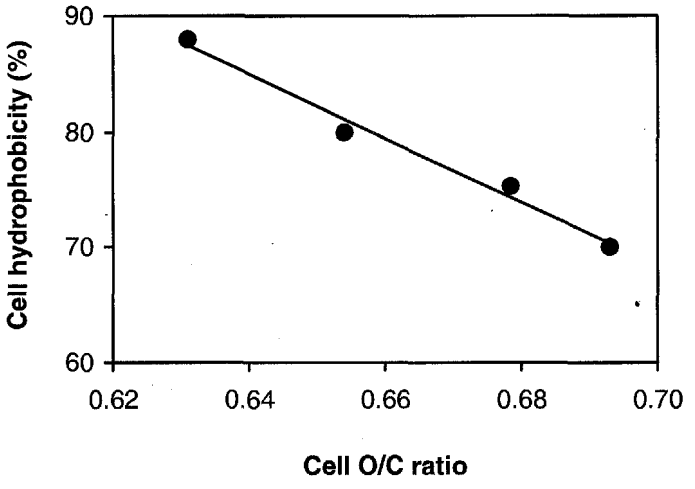

Figure 5.5 Effect of cell O/C ratio on cell surface hydrophobicity.

5.4 DISCUSSION

Figure 5.1 shows a profound impact of the substrate N/COD ratio on both the ratio of cell oxygen content to cell carbon content, $(O/C)_{cell}$, and the ratio of cell nitrogen content to cell carbon content, $(N/C)_{cell}$. Alteration of the elemental compositions of a microbial community may imply that microbial diversity distribution in the aerobic granules would vary. This is consistent with the results in Fig. 5.3, showing that the $(SOUR)_N/(SOUR)_H$ ratio linearly increases with the substrate N/COD ratio. Compared

with heterotrophic activity, nitrifying activity in the aerobic granules was enhanced in a very significant way as the substrate N/COD ratio increased (Figure 5.3). According to the elemental compositions of the aerobic granules cultivated at different substrate N/COD ratios, the corresponding empirical formulas of the aerobic granules were determined (Table 5.1). These empirical formulas of the aerobic granules are similar to those found in suspended nitrification processes, e.g. $C_5H_9O_{2.5}N$ for SHARON sludge and $C_5H_{13.3}O_{3.3}N$ for ANAMMOX sludge (Hao, 2001). In fact, as Elser et al. (2000) noted, the variation in cell elemental compositions may imply how species-interactions develop in ecosystems under different conditions of energy input and nutrient supply.

The seed sludge used had a ratio of cell calcium to carbon of 7.5 mmol mol^{-1}. It appears from Fig. 5.2 that the cell calcium content in the aerobic granules cultivated at different substrate N/COD ratios fluctuated at around 3 mmol mol^{-1}, which is two-fold lower than that in the seed sludge. These may imply that the cell calcium is unlikely to contribute to aerobic granulation. It was reported that Ca^{2+} could facilitate the formation of anaerobic granules (Teo et al., 2000; Yu et al., 2001). Teo et al. (2000) proposed a biological explanation for the calcium-enhanced anaerobic granulation; and they considered that the positive effect of calcium addition on anaerobic granulation was probably due to the calcium-induced dehydration of bacterial cell surfaces, leading to high cell hydrophobicity. However, the cell hydrophobicity is likely independent of the cell calcium contents in the aerobic granules.

On contrary, a close correlation of cell hydrophobicity to $(O/C)_{cell}$ ratio was observed instead (Figs. 5.2 and 5.5). Jiang et al. (2002) studied the effect of substrate calcium concentrations in the range of 10 to 300 mg l^{-1} on the formation of aerobic granules in sequencing batch reactors and found that the contribution of substrate calcium to aerobic granulation was not significant. As shown in Fig. 5.6, a higher cell hydrophobicity results in a more compact and stable microbial structure of aerobic granules in term of SVI. In fact, there is evidence that autoaggr tion of bacteria is associated very closely with bacterial surface hydrophobicity (Rouxhet and Mozes, 1990; Bossier and Verstraete, 1996; Del Re et al., 2000).

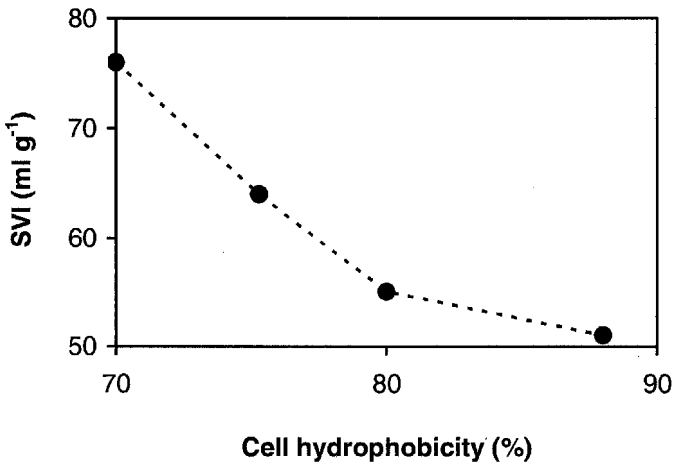

Figure 5.6 Relationship between cell hydrophobicity and SVI.

The $(SOUR)_N/(SOUR)_H$ ratio could represent the distribution of nitrifying bacteria over heterotrophic bacteria in aerobic granules. Figure 5.4 shows that the cell N/C ratio increases, while a decrease in the cell O/C ratio is observed as the $(SOUR)_N/(SOUR)_H$ ratio increases. This can be attributed to possible carbon storage under transient conditions or population shift in aerobic granules developed at different substrate N/COD ratios. However, a study on carbon fluxes in the growth of aerobic granules cultivated at different substrate N/COD ratios clearly showed that the carbon storage in the granules were negligible. Thus, alteration of elemental compositions of aerobic granules may imply the changes of microbial diversity distribution in the aerobic granules, i.e., enriched nitrifying populations in the aerobic granules lead to a lower cell O/C and higher cell N/C. Mulyukin et al. (2002) proposed that the elemental composition could be used to analyze the species distribution and physiological state of microorganisms. Consequently, it appears from this study that the elemental compositions of aerobic granules seem to be closely related to the substrate N/COD ratios.

Cell hydrophobicity is related to the chemical characteristics of cell surface. From Fig. 5.1, the ratio of cell oxygen content to cell carbon content $(O/C)_{cell}$ was found to

increase as the substrate N/COD ratio was increased, while an inverse correlation of cell hydrophobicity to the $(O/C)_{cell}$ ratio was observed (Fig. 5.5). Previous research showed that a higher $(O/C)_{cell}$ would resulted in a lower cell hydrophobicity (Changui et al. 1987; Rouxhet and Mozes, 1990). The $(O/C)_{cell}$ ratio of the seed sludge is 0.77, higher than those of aerobic granules. It seems certain that the cell surface charge should be related to the $(O/C)_{cell}$ ratio. According to the DLVO (Derjaguin, Landau, Verwey, Overbeek) theory, when the two surfaces carry a charge of the same sign, there exists a free energy barrier between surfaces, which would act a repulsive force. On the other hand, recent study showed that the cell surface hydrophobicity was inversely correlated to the quantity of surface charge of microorganisms (Liao et al., 2001). Reduced surface negative charge had been shown to promote the immobilization of nitrifying bacteria (Liu, 1995; Teixeira and Oliveira, 1998; Hibiya et al., 2000). These provide plausible explanation for the results in Fig. 5.5.

5.5 CONCLUSIONS

It was found that the substrate N/COD ratio had a direct and profound effect on the elemental compositions and characteristics of the aerobic granules. The respective ratio of cell oxygen, nitrogen and calcium to cell carbon were closely related to the substrate N/COD ratio. Cell hydrophobicity of aerobic granules was inversely correlated to the ratio of cell oxygen to cell carbon. Since the cell calcium content in aerobic granules developed at different substrate N/COD ratios was pretty low, the cell calcium would not contribute to aerobic granulation in a significant way.

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CHAPTER 6

SIMULTANEOUS REMOVAL OF ORGANICS AND NITROGEN BY AEROBIC GRANULES

ABSTRACT

The feasibility of simultaneous removal of organics and nitrogen by aerobic granules was investigated. Results showed that nitrification efficiency was proportionally related to the substrate N/COD ratios, while dissolved oxygen (DO) concentration had a significant effect on the efficiency of denitrification by aerobic granules. It was found that a certain mixing power would be provided to ensure mass transfer between liquid and granules during denitrification. Complete organics and nitrogen removal can be achieved in a single granule-based SBR with high efficiency and stable performance.

6.1 INTRODUCTION

Biological nitrogen removal involves nitrification and denitrification. It is a feasible and effective strategy for complete nitrogen removal from municipal and industrial wastewater. Conditions required for denitrification are quite different from those of nitrification. Nitrification occurs under high dissolved oxygen (DO) and low organic carbon concentrations. There is evidence that presence of organic carbon raises competition for oxygen between autotrophic nitrifying bacteria and carbon removing aerobic heterotrophic bacteria (Steinquist, 1974; Parker and Richards, 1986; Dangcong et al., 2000; Peng et al., 2001). Nitrifying bacteria have less affinity for oxygen and much lower maximum specific growth rate than that of heterotrophic bacteria (Sharma and Ahlert, 1977). Heterotrophs can dominate the nitrifying bacteria under conditions that are conducive to both groups of microorganisms.

The rate of denitrification depends on the availability of organic carbon, DO, nitrite and nitrate concentrations and the density of denitrifying bacteria. Most denitrifiers are facultative. It had been well established that most denitrifying enzymes have first to be induced by growth of the organisms in the presence of nitrate and absence of oxygen (Wainwright and Nevill, 1956; Delwiche, 1956; Kornaros and Lyneraos, 1998). Anoxic conditions were initially considered to be most favorable for biological denitrification. Kornaros and Lyberatos (1998) studied the transient growth characteristics of *Pseudomonas dinitrificants* resulting from a shift from anoxic to aerobic conditions, and found that each step of the denitrification pathway was affected differently by DO concentration. It was observed that 0.5 mg l^{-1} of DO was a critical value for denitrfication in the wastewater treatment plant (Li and Bishop, 2003).

As discussed in previous chapters, nitrifying, denitrifying and heterotrophic bacteria can co-exist in the aerobic granules cultivated at different substrate N/COD ratios. Therefore, this study is to further explore the feasibility of complete removal of organics and nitrogen by aerobic granules developed previously. Since aerobic granules have very high specific gravity and excellent settleability (see Chapter 3), mixing would play an important role in the denitrification process in order to ensure good contact between soluble substrate and biomass. Therefore, the effect of DO and mixing on denitrification efficiency by aerobic granules was also studied.

6.2 MATERIALS AND METHODS

In order to look into the denitrification capability of microbial granules developed at different substrate N/COD ratios, the SBR cycle time was increased to 6 h comprising 4 min of feeding, 230 min of aeration, 2 h of anaerobic or anoxic period, 2 min of settling and 4 min of effluent withdrawal, from day 340 onwards. Following three series of experiments were then carried out. (i) The DO concentrations in all reactors were decreased to 0.8 mg l^{-1} by lowering the air flow rate to 1.0 l m... $^{-1}$ from day 342. (ii) Starting from day 350, the reactor DO was further lowered down to 0.5 mg l^{-1} by

reducing aeration rate to 0.5 l min^{-1}. (iii) A DO free condition was created in all reactors by stopping aeration from day 355 onwards. At the beginning of anaerobic or anoxic phase, ethanol as external carbon source for denitrification was fed to the reactors at a concentration of 600 mg COD l^{-1}.

The denitrification capability of aerobic granules was represented by the specific total nitrogen reduction rate, which was determined by the slope of the drop in NO_x-N in the reactors during anaerobic or anoxic period.

Details on geometry of the reactors, stirring devices, media and analytical methods can be found in Chapter 3.

6.3 RESULTS

6.3.1 Effect of substrate N/COD ratio on nitrification efficiency

Figures 6.1 to 6.8 show the profiles of COD concentration and nitrification in R1 to R4 observed on day 40 and day 340. The salient points of the data are that (i) almost all influent COD is removed in the first hour; (ii) no nitrite and nitrate are produced in R1 run at a substrate N/COD ratio of 5/100, while typical nitrification profiles were observed in R2 to R4 operated at a respective substrate N/COD ratio of 10/100, 20/100 and 30/100; (iii) a complete nitrification occurred after the COD removal in R2 to R4; (iv) ammonium-nitrogen removal in the first hour of the cycle was the result of microbial growth requirement for nitrogen source instead of nitrification because neither nitrite or nitrate was produced in this period; (v) no lag nitrate production with respect to nitrite formation was observed.

Nitrification is completed by two kinds of bacteria, ammonia oxidizers responsible for nitrite formation, and nitrite oxidizers for converting nitrite to nitrate. The biological oxidation sequence can be simplified to two consecutive reactions:

$$NH_4^+ + 3O_2 \longrightarrow 2NO_2^- + 4H^+ + 2H_2O \qquad (6.1)$$

$$2NO_2^- + O_2 \longrightarrow 2NO_3^- \qquad (6.2)$$

It should be realized that nitrite is an intermediate of nitrification process. The complete nitrification observed in Figs. 6.1 to 6.8 indicates that that both ammonia oxidizer and nitrite oxidizer present sufficiently in the aerobic granules. According to Liu and Tay (2001), at least three factors would influence the nitrification profiles: (a) the relative specific growth rate of ammonia oxidizer and nitrite oxidizer in the aerobic granules; (b) the relative ratio between ammonia oxidizers and nitrite oxidizers in the aerobic granules and (c) the level of free ammonia. It appears that the ammonia oxidation rate is proportionally related to the substrate N/COD ratios, i.e. a higher substrate N/COD ratio, a faster fall of ammonium-N concentration is.

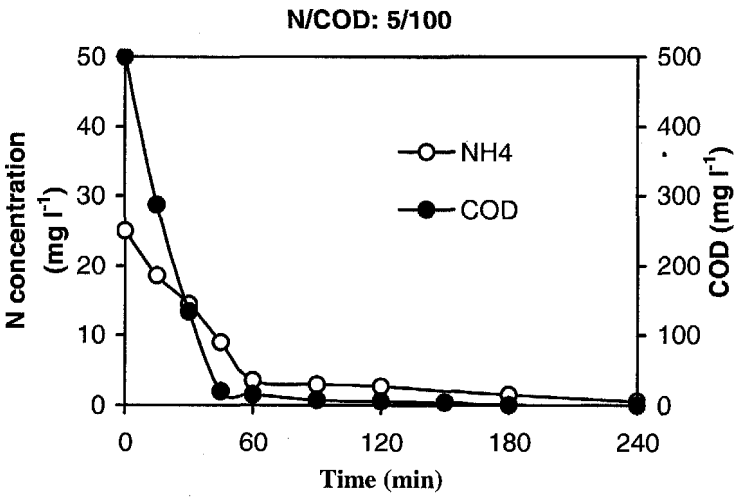

Figure 6.1 COD and nitrification profiles in R1 observed on day 40.

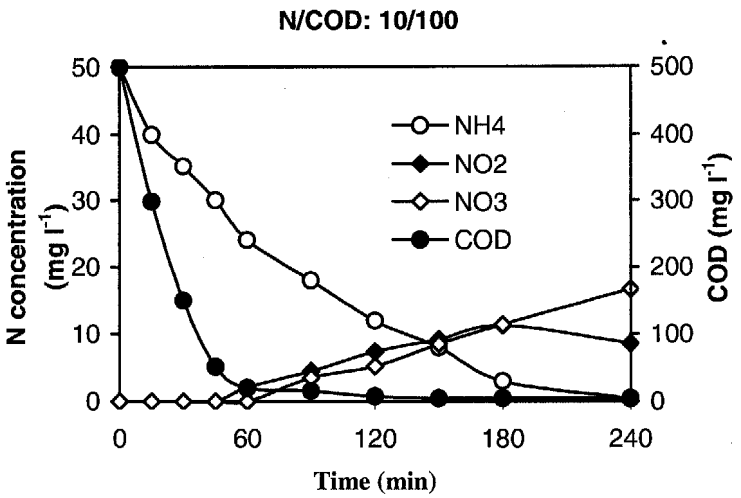

Figure 6.2 COD and nitrification profiles in R2 observed on day 40.

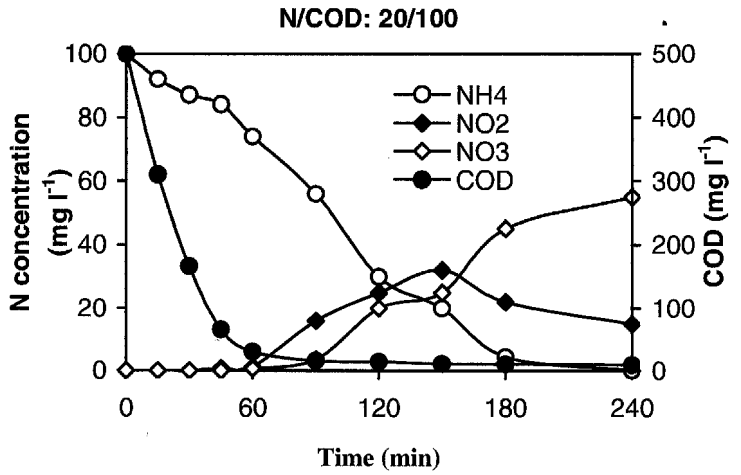

Figure 6.3 COD and nitrification profiles in R3 observed on day 40.

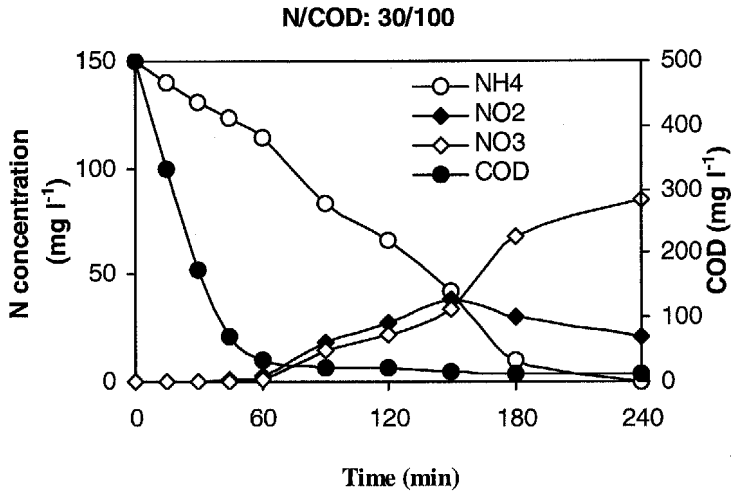

Figure 6.4 COD and nitrification profiles in R4 observed on day 40.

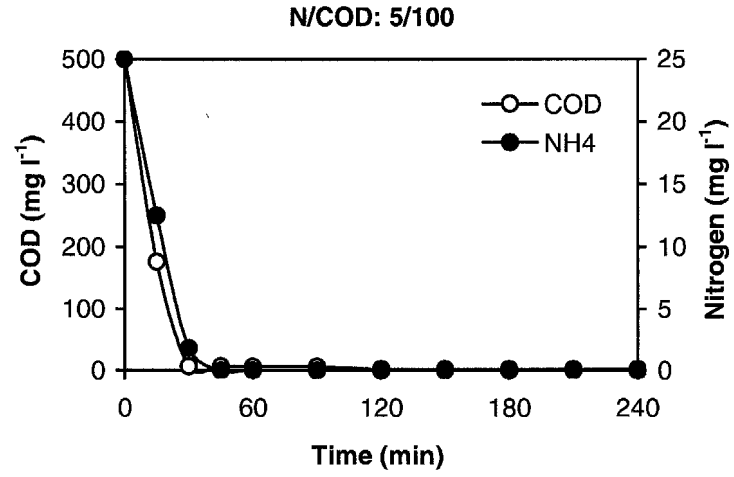

Figure 6.5 COD and nitrification profiles in R1 observed on day 340.

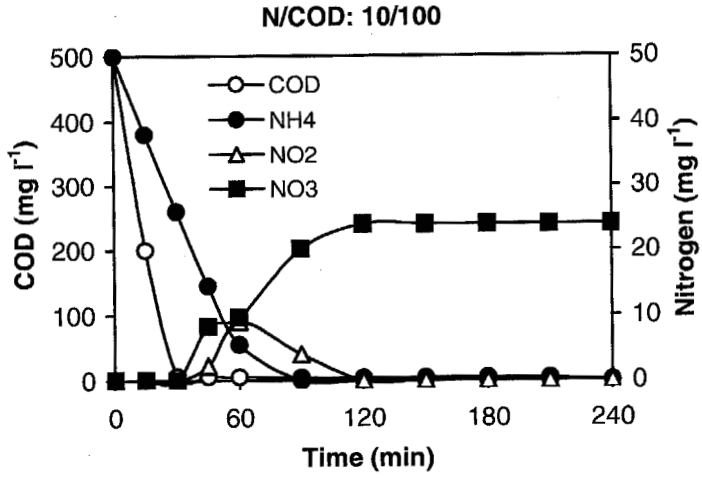

Figure 6.6 COD and nitrification profiles in R2 observed on day 340.

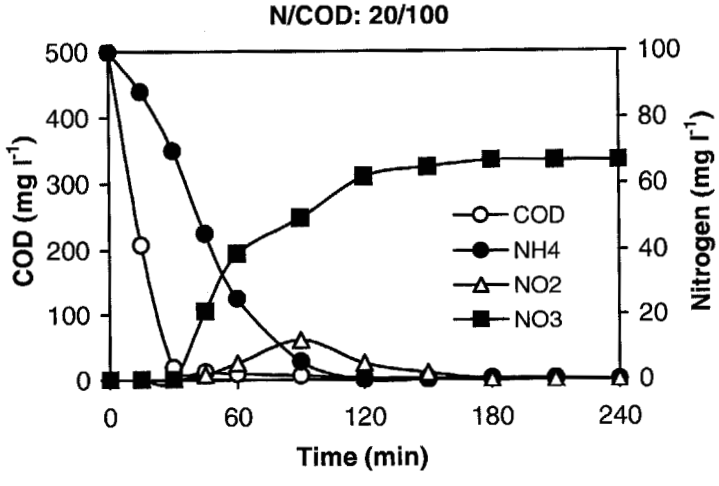

Figure 6.7 COD and nitrification profiles in R3 observed on day 340.

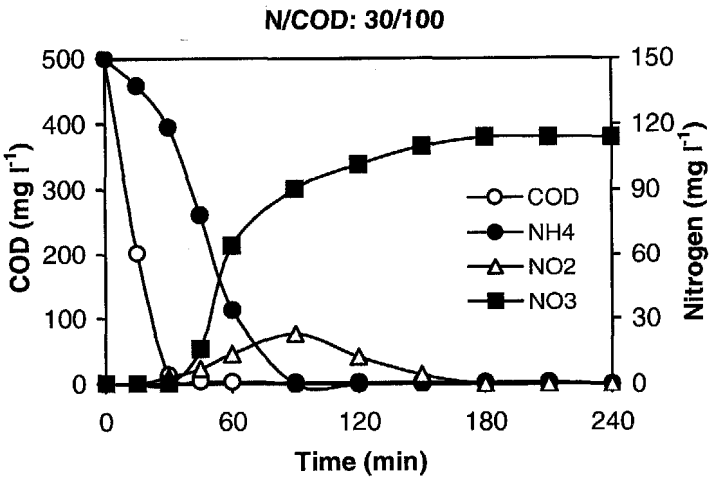

Figure 6.8 COD and nitrification profiles in R4 observed on day 340.

6.3.2 Denitrification under DO-free condition without mixing

The COD and denitrification profiles in R2 to R4 run at the substrate N/COD ratio of 10/100, 20/100 and 30/100, respectively under DO free condition are shown in Figs. 6.9 to 6.11. It can be seen that only slight denitrification occurred. The respective total nitrogen removal efficiencies are 21%, 24% and 26% in R2 to R4, while the external COD removal efficiencies are also very low under such operation conditions. As shown in Chapter 3, the specific gravity of aerobic granules was as high as 1.065. Typical activated sludge flocs had a specific gravity of around 1.002 (Zhu and Liu, 1999). It is clear that aerobic granules are much heavier than conventional bioflocs. As a result, they would settle down to the bottom of the reactor in the case where no sufficient mixing was provided. This in turn results in a poor contact between granules and substrate solution, i.e. mass transfer efficiency was much lowered. Consequently, the mass transfer limitation due to the lack of mixing would be responsible for the observed low denitrification efficiency (Figs. 6.9 to 6.11).

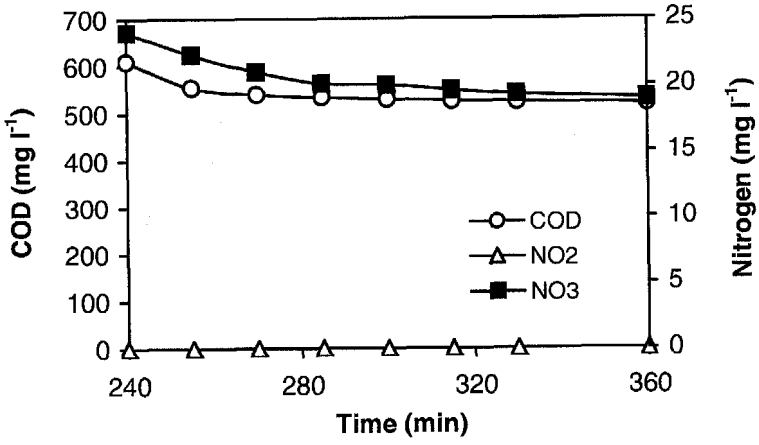

Figure 6.9 COD and denitrification profiles under DO-free condition without mixing in R2 operated at substrate N/COD ratio of 10/100.

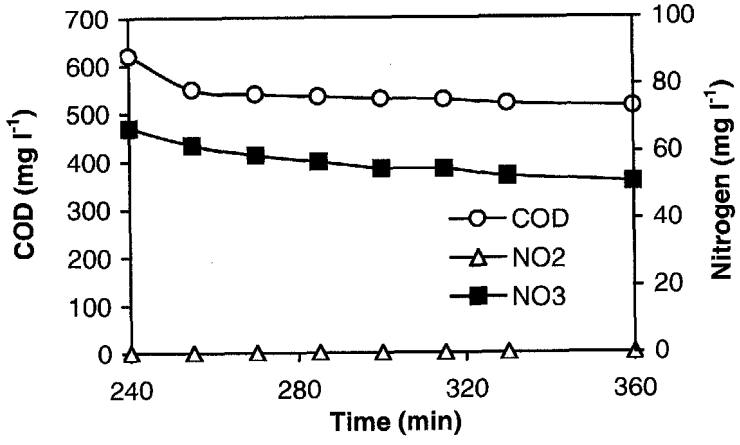

Figure 6.10 COD and denitrification profiles under DO-free condition without mixing in R3 operated at substrate N/COD ratio of 20/100.

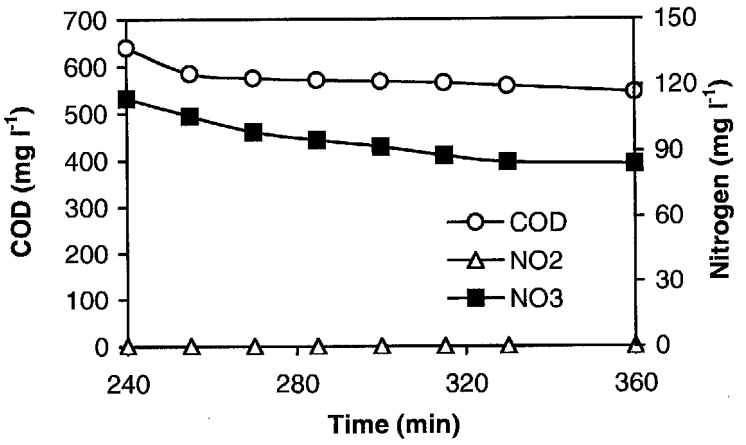

Figure 6.11 COD and denitrification profiles under DO-free condition without mixing in R4 operated at substrate N/COD ratio of 30/100.

6.3.3 Denitrification at DO of 0.5 mg l^{-1} with mixing

In this phase of study, the reactor DO was maintained at 0.5 mg l^{-1} by regulating the aeration rate, which in turn provided solid-liquid mixing. Figures 6.12 to 6.14 show the observed COD and denitrification profiles in R2 to R4. It is obvious that complete denitrification occurred in R2 to R4. All nitrate converted to gaseous nitrogen in 2-hour anoxic period. The respective specific total nitrogen reduction rates were 0.42, 0.85 and 0.91 mg N g^{-1} dry weight min^{-1} in R2 to R4. These values indeed are comparable to those obtained in conventional biological processes (Glass and Silverstein, 1998; Foglar and Briški, 2003).

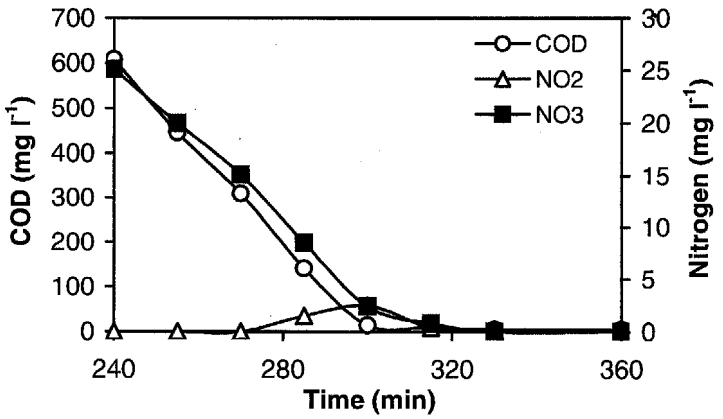

Figure 6.12 COD and denitrification profiles at DO concentration of 0.5 mg l^{-1} with mixing in R2 operated at substrate ratio of 10/100.

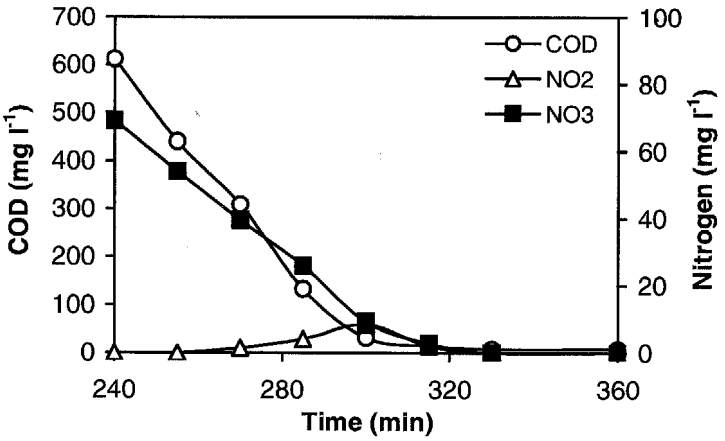

Figure 6.13 COD and denitrification profiles at DO concentration of 0.5 mg l^{-1} with mixing in R3 operated at substrate N/COD ratio of 20/100.

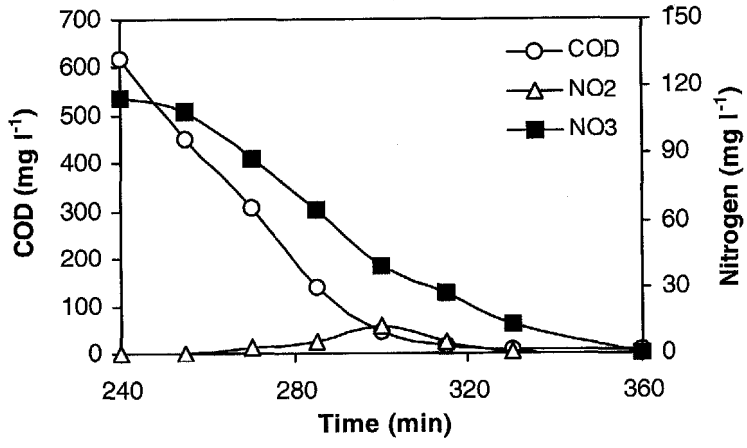

Figure 6.14 COD and denitrification profiles at DO concentration of 0.5 mg l^{-1} with mixing in R4 operated at substrate N/COD ratio of 30/100.

6.3.4 Denitrification at DO of 0.8 mg l^{-1} with mixing

In order to investigate the effect of DO on denitrification by aerobic granules, the DO concentrations in the reactors was decreased to 0.8 mg l^{-1} by raising aeration rate. COD and denitrification profiles at DO concentration of 0.8 mg l^{-1} in R2 to R4 operated at the respective substrate N/COD ratio of 10/100, 20/100 and 30/100 are shown in Figs. 6.15 to 6.17. The nitrogen removal efficiency was about 40%, leading to high effluent nitrate concentration in all reactors. These show a partial denitrification as compared to the results obtained at the DO concentration of 0.5 mg l^{-1} (Figs. 6.12-6.14). It seems that the activity of denitrifying populations in aerobic granules could be inhibited by high DO concentration. In fact, there is evidence that the DO acts as an inhibitor to the activity of the denitrifying reductases rather than a repressor of their synthesis, and denitrification can be ignored when dissolved oxygen concentration is greater than 1.0 mg l^{-1} (Eckenfelder and Argaman, 1991; Koranaros and Lyberatos, 1998).

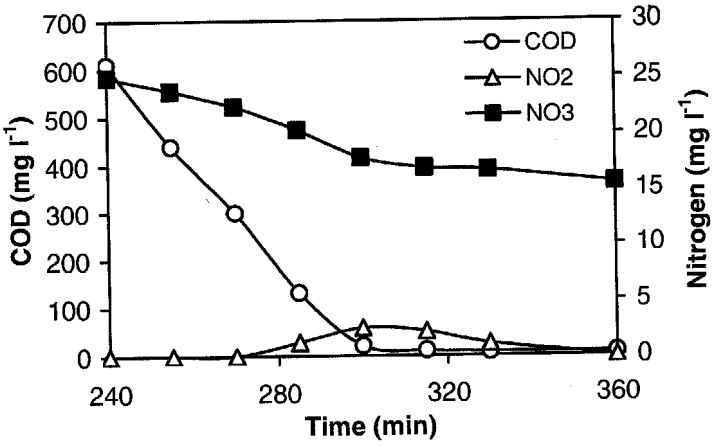

Figure 6.15 COD and denitrification profiles at DO concentration of 0.8 mg l^{-1} with mixing in R2 operated at substrate N/COD ratio of 10/100.

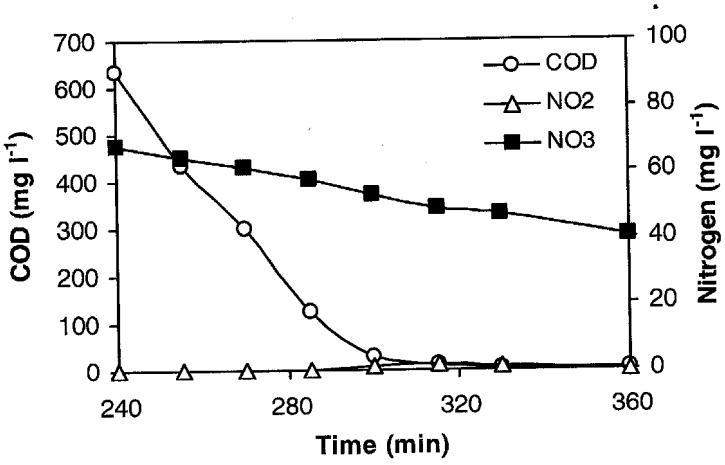

Figure 6.16 COD and denitrification profiles at DO concentration of 0.8 mg l^{-1} with mixing in R3 operated at substrate N/COD ratio of 20/100.

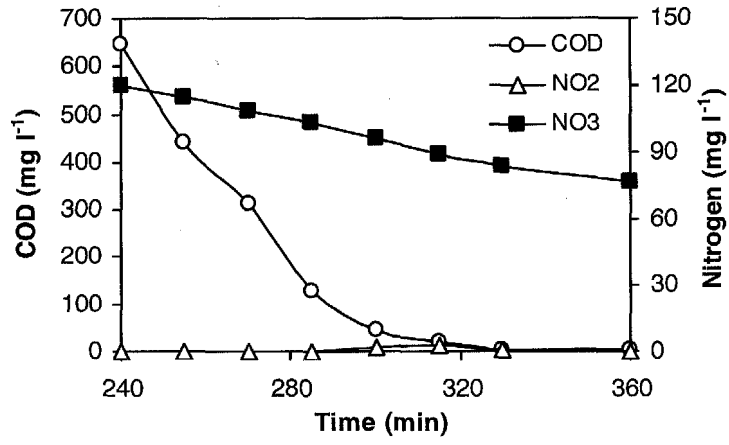

Figure 6.17 COD and denitrification profiles at DO concentration of 0.8 mg l^{-1} with mixing in R4 operated at substrate N/COD ratio of 30/100.

6.4 DISCUSSION

According to Figs. 6.1 to 6.8, the observed specific ammonium-N utilization rate by aerobic granules on day 340 was 40.3, 99.4 and 142.6 mg N g^{-1} d^{-1} in R2 to R4 operated at a respective substrate N/COD ratio of 10/100, 20/100 and 30/100. These values are comparable to those obtained in nitrifying biofilm reactor (Liu and Capdeville, 1996). These results also imply that the nitrification efficiency is dependent on the substrate N/COD applied. In fact, it had been widely reported that the substrate N/COD ratio was a critical factor associated with the performance of nitrification systems (Surampali et al., 1995; Beg et al., 1997a, b, c).

In this study, it was observed that aerobic granules developed would settle down to the reactor bottom within one minute in the case where no sufficient mixing power was provided. This in turn leads to extremely poor contact between granules and soluble nitrate, consequently denitrification would not occur efficiently as shown in Figs. 6.9 to 6.11. It therefore suggested that in order to achieve efficient nitrification in the granule-based bioreactor, a certain mixing power is essential to ensure a mass transfer between granules and soluble nitrate.

Under the mixing conditions, it can be seen in Figs. 6.12 to 6.17 that DO less than 0.5 mg l^{-1} will be advantageous for denitrification, while denitrification by aerobic granules would be hindered at a DO concentration of 0.8 mg l^{-1} and above. Denitrifying bacteria may use the route of denitrification as an alternative to normal aerobic respiration. When oxygen is available, aerobic respiration is a major metabolism of denitrifying bacteria. The synthesis of nitrate reductases would be repressed by oxygen, and nitrate and nitrite may act as electron acceptors in the respiratory electron transport chain after depletion of oxygen. Both processes are accomplished through use of cytochromes in an electron transport chain (Ballard et al., 1988). However, in case where oxygen is present, different cytochromes are needed for the reduction of oxygen to water, and dissimilatory nitrate reduction is inhibited (Hernandez and Rowe, 1987). In fact, it was found that the denitrifcation enzymes, once synthesized, would be maintained by the cells under aerobic conditions, but their function was inhibited by the high DO concentration (Kornaros and Lyneratos, 1998). On the other hand, it has been widely reported that dissolved oxygen inhibited each step of the denitrification pathway to some extent (Wild et al., 1995; Kornaros and Lyneratos, 1998).

6.5 CONCLUSIONS

Aerobic granules developed at different substrate N/COD ratios in SBRs are capable of simultaneously removing organics and nitrogen. The aerobic granules developed at high substrate N/COD ratios exhibited enhanced nitrification efficiency. It was demonstrated that DO concentration and mixing power were two factors influencing the efficiency of denitrification by aerobic granules. Complete denitrification was achieved at DO concentration of 0.5 mg l^{-1}, while a certain mixing is necessary to ensure a sufficient contact between granules and soluble nitrate, otherwise denitrification by aerobic granules would be slowed down markedly. This study opens a door for environmental engineers to further develop novel compact and high-efficiency granules-based biological process for removing organic and nitrogen from wastewater.

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CHAPTER 7

IMPROVED STABILITY OF AEROBIC GRANULES BY SELECTING SLOW-GROWING NITRIFYING BACTERIA

ABSTRACT

A potential strategy to improve stability of aerobic granules through selecting slow-growing nitrifying bacteria was proposed. The observed growth rate and mean size of mature aerobic granules were found to decrease as the substrate N/COD ratio increased, while nitrifying population was enriched markedly in aerobic granules developed at high substrate N/COD ratios. The enriched nitrifying population in aerobic granules was responsible for the observed low growth rate of aerobic granules. It seems certain that the substrate N/COD ratio is an important factor in selecting nitrifying bacteria in aerobic granules. Aerobic granules with low growth rates showed strong structure and good settleability in terms of specific gravity, SVI and cell hydrophobicity that further lead to high stability as compared to those having high growth rates. This study demonstrated that the selection of slow-growing nitrifying bacteria through controlling substrate N/COD ratio would be a useful strategy for improving the stability of aerobic granules.

7.1 INTRODUCTION

Microbial immobilization is a process of cell-to-cell interaction, or cell-to-carrier surface attachment that involves biological, physical and chemical actions. There are three main members in the family of microbial immobilization, i.e. anaerobic, aerobic granule and biofilms. Among these three members, biofilm has the longest research history, while the first serious technical report on anaerobic granulation appeared in 1980 (Lettinga et al., 1980). Compared to the development of biofilms and anaerobic granulation technologies, aerobic granulation is a recently described phenomenon

(Morgenroth et al., 1997; Beun et al., 1999; Peng et al., 1999; Tay et al., 2001b; Moy et al., 2002). It is believed that aerobic granulation would be a novel and promised biotechnology for handling industrial and municipal wastewater.

It appears from previous research that the stability of aerobic granules is poorer than that of anaerobic granules developed in upflow anaerobic sludge blanket (UASB) reactor (Morgenroth et al., 1997; Peng et al., 1999; Zhu and Liu, 1999). This is probably due to the fact that aerobic bacteria have a much higher growth rate than anaerobic bacteria. It can be anticipated that the poor stability of aerobic granules would limit its application in wastewater treatment practice. In fact, it was found that the stability of biofilm is closely related to the growth rate of bacteria, i.e. higher growth rate of bacteria resulted in a weaker structure of biofilm (Tijhuis et al., 1995; Kwok et al., 1998; Liu, 1997). To date, almost all research on aerobic granulation has been mainly focused on exploiting the feasibility of aerobic granulation in sequencing batch reactors. However, the question of how to improve the stability of aerobic granules remains untouched. Therefore, this study attempted to look into microbial selection-based strategy for improving the stability of aerobic granules, and further contributed to a sound understanding of cell-to-cell immobilization. These would be very useful for the development of full-scale aerobic granules-based bioreactor for wastewater treatment.

7.2 MATERIALS AND METHODS

Details on set-up, media and analytical methods can be found in Chapter 3.

7.3 RESULTS

7.3.1 The growth of aerobic granules by size

Under hydrodynamic conditions, the growth of aerobic granules after the initial cell-to-cell attachment is the net result of interaction between bacterial growth and detachment, while the balance between growth and detachment processes in turn leads to an

equilibrium or stable granule size (Liu and Tay, 2002). Thus, size evolution of the microbial aggregates can be used to describe the growth of granular sludge. Figure 7.1 shows the evolution of microbial aggregates in terms of size observed at different substrate N/COD ratios. It can be seen that the size of microbial aggregates increased gradually and finally stabilized. According to the granular growth curves in Fig. 7.1, the aerobic granulation process can be divided into three phases, i.e. acclimation or lag phase, granulation and followed by maturation indicated by a stable granule size in the four reactors. The specific growth rate (μ_d) by size of microbial aggregates can be defined as

$$\mu_d = \frac{dD/dt}{D} \tag{7.1}$$

in which D is mean size of microbial aggregates, and t is operation time. Integrating Eq. 7.1 gives

$$\ln D = \mu_d t + \text{constant} \tag{7.2}$$

The observed size-dependent specific growth rate of microbial aggregate can be determined from the slope of the straight line described by Eq. 7.2. It should be pointed out that this approach had been successfully employed to estimate the growth rates of biofilms and anaerobic granules (Liu, 1997; Yan and Tay, 1997). Figure 7.2 shows the effect of substrate N/COD ratio on μ_d. It is obvious that a higher substrate N/COD ratio had resulted in a lower specific growth rate of aerobic granules.

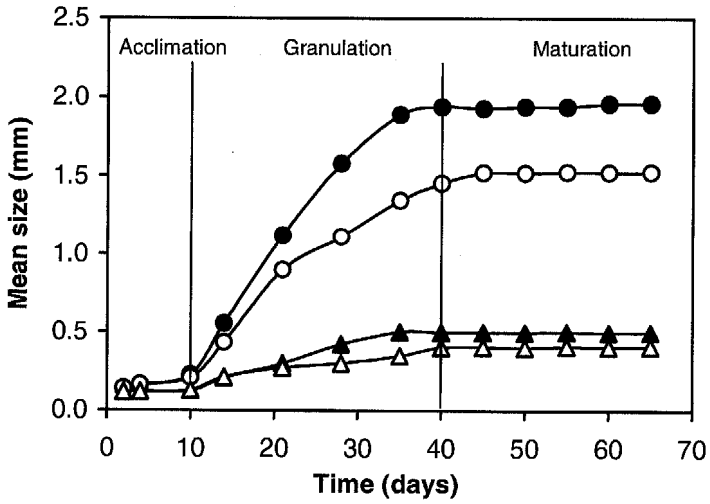

Figure 7.1 Changes in size of microbial aggregates in the course of aerobic granulation. •: substrate N/COD ratio of 5/100; ○: 10/100; ▲: 20/100; Δ: 30/100.

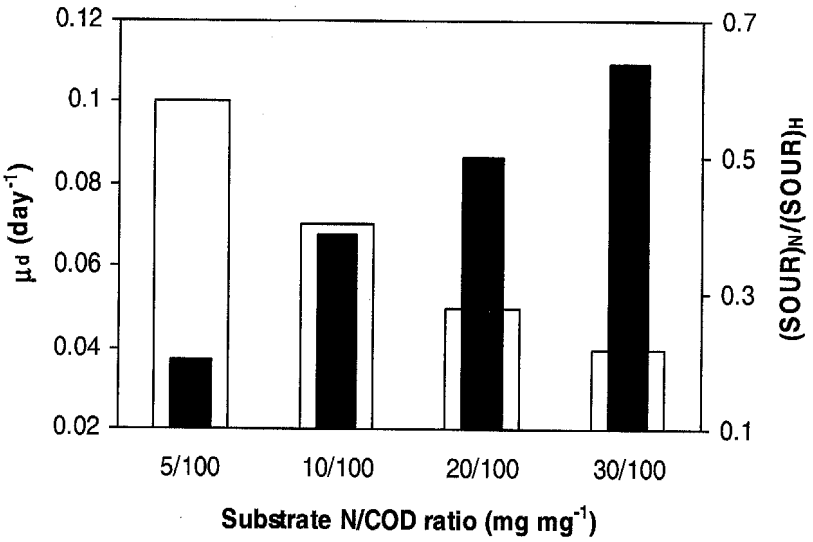

Figure 7.2 Effect of substrate N/COD ratio on μ_d (□) and $(SOUR)_N/(SOUR)_H$ (■) of aerobic g nules.

As discussed in Chapter 4, the $(SOUR)_N/(SOUR)_H$ ratio exhibits an increasing trend with the increase of substrate N/COD ratio (Fig. 7.2). Moreau et al. (1994) reported that the activity distribution of nitrifying population over heterotrophic population in biofilms was proportionally related to the relative abundance of two populations under given conditions. Figure 7.3 further indicates that the increased $(SOUR)_N/(SOUR)_H$ ratio would result in a lower observed growth rate of aerobic granules and an improved cell hydrophobicity.

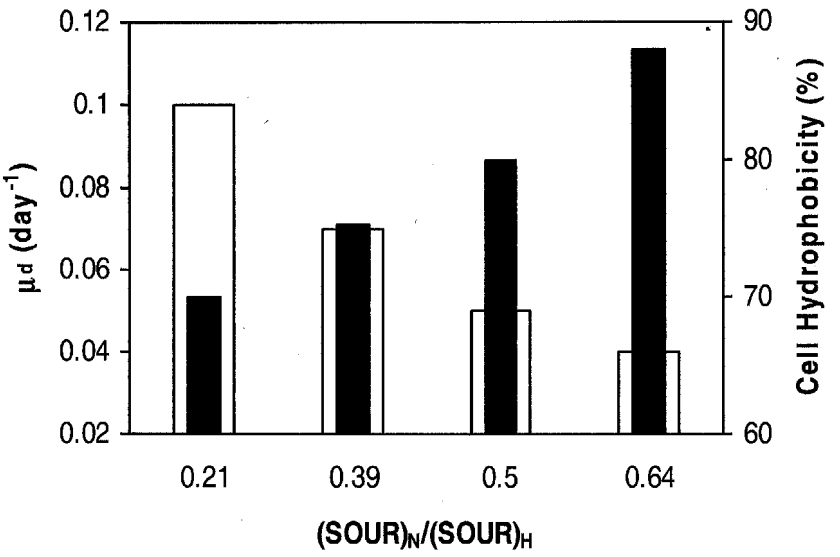

Figure 7.3 Effect of $(SOUR)_N/(SOUR)_H$ on μ_d (□) and cell hydrophobicity (■) of aerobic granules.

7.3.2 Effect of μ_d on the mean size of aerobic granules

Figure 7.4 shows the effect of the observed growth rate of granules on the stable mean size of aerobic granules. It can be seen that aerobic granules with low growth rate have smaller size.

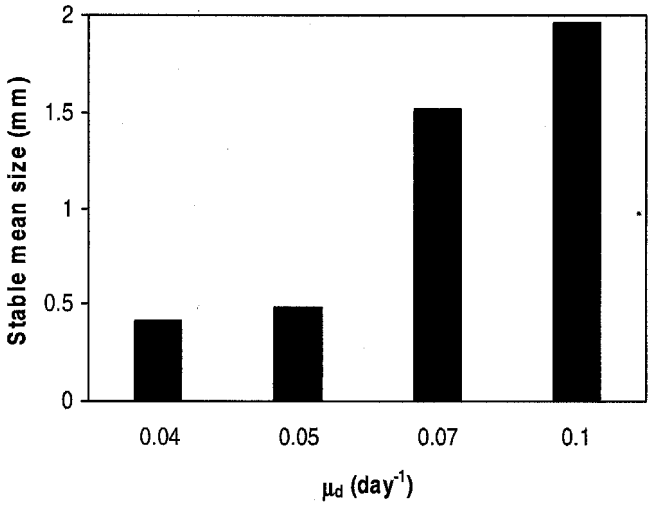

Figure 7.4 Effect of μ_d on stable granule size.

7.3.3 Effect of μ_d on specific gravity of aerobic granules

The specific gravity represents the compactness of a microbial community. The specific gravity of aerobic granules increased with the decrease of μ_d (Fig. 7.5). Heterotrophic granular sludge grown on glucose and acetate was found to have a specific gravity of 1.004 to 1.008 (Tay et al., 2001a). It appears from Fig. 7.5 that aerobic granules developed for simultaneous organics and nitrogen removal seem to have a much more compact structure than heterotrophic granules. This is supported by the fact that slow-growing microorganisms can form compact and dense biofilms as compared to fast-growing microorganisms (Liu and Tay, 2002; Kwok et al., 1998; Oga et al., 1991; Tijhuis et al., 1995).

7.3.4 Effect of μ_d on SVI of aerobic granules

The effect of μ_d on SVI of aerobic granules is shown in Fig. 7.5. With the decrease of μ_d, the SVI value of aerobic granules dropped to as low as 50 to 60 ml g^{-1}. Compared to

the seed sludge with a SVI of 265 ml g^{-1}, the settleability of aerobic granules was improved significantly. Figure 7.5 seems to indicate that aerobic granules with low growth rate have more compact structure.

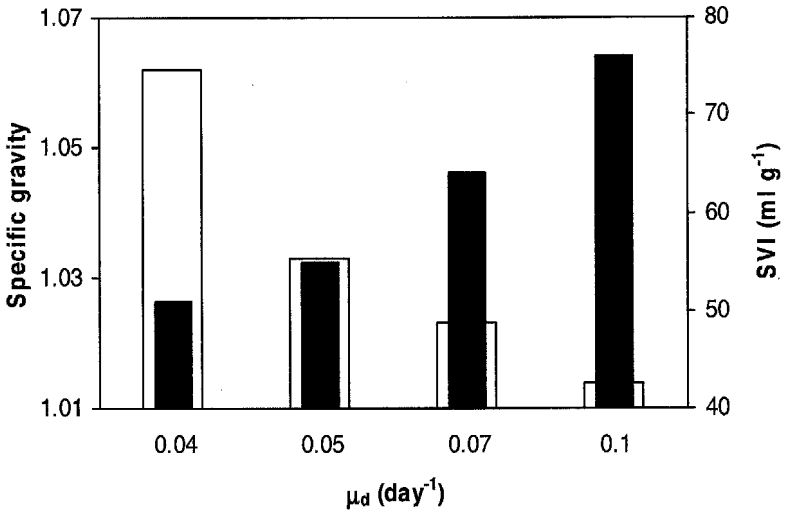

Figure 7.5 Effect of μ_d on specific gravity (□) and SVI (■) of aerobic granules.

7.3.5 Relationships between specific gravity, SVI and cell hydrophobicity

Figure 7.6 further shows the relationship between the specific gravity, SVI and cell hydrophobicity. It appears that both specific gravity and SVI of aerobic granules are closely correlated to the cell hydrophobicity, i.e. high cell hydrophobicity favoured a compact structure of aerobic granules.

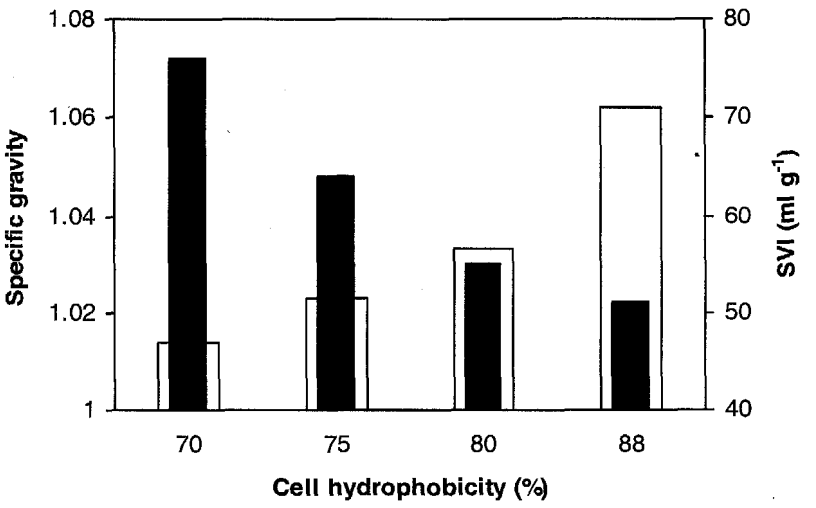

Figure 7.6 Relationships of specific gravity (□) and SVI (■) to cell hydrophobicity of aerobic granules.

7.4 DISCUSSION

Aerobic granulation is a gradual rather than instant process from dispersed sludge to mature aerobic granules with a stable size (Fig. 7.1). The acclimation phase observed in Fig. 7.1 implies that a newly inoculated culture does not begin growing immediately, and a period of about 10 days is required for bacteria to acclimate to the new living conditions instead of growth. The observed growth rate by size and mean size at equilibrium of aerobic granules are closely related to the substrate N/COD ratio, i.e. higher substrate N/COD ratio results in smaller granules with lower growth rate (Fig. 7.2). Meanwhile, Fig. 7.2 also shows that nitrifying population in aerobic granules is enriched significantly with increasing substrate N/COD ratio. As the result, the heterotrophs in aerobic granules became less and less dominant at high substrate N/COD ratio. It seems that the high substrate N/COD ratio is an important factor in microbial selection with a predominantly nitrifying population. Since the growth of nitrifying bacteria is much slower than heterotrophs (Sharma and Ahlert, 1977), aerobic granules may offer a protective matrix for nitrifying population to grow without the

risk of being washed out from the system. Similar phenomena had been reported in biofilm and suspended cultures (Moreau et al., 1994; Ochoa et al., 2002).

Figure 7.3 shows that the specific growth rate by size of aerobic granules is highly dependent on the distribution of nitrifying population over heterotrophic population in aerobic granules. The enriched nitrifying population in aerobic granules is mainly responsible for the lowered growth rate of aerobic granules observed at high substrate N/COD ratios. In study of anaerobic granulation, Yan and Tay (1997) proposed that if granulation is purely the result of bacterial aggregation and growth and granule formed is ideal, a relationship between specific growth rate by size and that by biomass could be derived as follows:

$$\mu_g = \frac{1}{X}\frac{dX}{dt} = \frac{1}{\frac{\pi}{6}\rho D^3}\frac{d(\frac{\pi}{6}\rho D^3)}{dt} = 3\frac{1}{D}\frac{dD}{dt} = 3\mu_d \qquad (7.3)$$

in which μ_g is specific growth rate by biomass (g biomass g^{-1} biomass d^{-1}), X is biomass concentration of granules, and ρ is density of granules. According to Eq. 7.3, the specific growth rate by size can be converted to the specific growth rate by biomass. The respective μ_g value of aerobic granules developed at substrate N/COD ratios of 5/100, 10/100, 20/100 and 30/100 are 0.3, 0.21, 0.15 and 0.12 d^{-1}. The μ_g values of aerobic granules developed at high substrate N/COD ratios in which nitrifying population is dominant are comparable with those of nitrifying biofilms (Oga et al., 1991).

There is evidence that aerobic granules had relatively low stability as compared to anaerobic granules cultivated in UASB reactors (Morgenroth et al., 1997; Zhu and Liu, 1999; Ng, 2002). The poor stability of aerobic granules, to a great extent, limits its applicatio in real wastewater treatment and would make the rea r scaling-up and operation much more unstable and even difficult. The cause behind the poor stability of aerobic granules would be due to the fast growth of heterotrophic bacteria that

dominate aerobic granules. It has been known that nitrifying population grows much slower than heterotrophs, while the physical structure of nitrifying biofilms was much stronger than that heterotrophic biofilms (Oga et al., 1991). Figure 7.3 reveals that the observed growth rate of aerobic granules can be significantly lowered by enriching slow-growing nitrifying population, and this can be achieved through proper control of substrate N/COD ratio.

In the environmental engineering field, specific gravity has been commonly used to describe the structural compactness and stability of a microbial community. As can be seen in Figs. 7.4 and 7.5, the lowered growth rate in turn results in a smaller size of aerobic granules, but with a higher specific gravity indicating a compact and strong microbial structure. The direct relationship between the specific gravity and stable mean size of aerobic granules is further shown in Fig. 7.7, indicating that large granules have loose structure. Such observation is consistent with those found in biofilms, i.e. the compactness of biofilm would be lowered as biofilms became thick (Kwok et al., 1998; Liu and Tay, 2002). It seems certain that the aerobic granules with low growth rate have much stronger structure, i.e. the structural stability of aerobic granules can be significantly improved through selecting slow-growing nitrifying bacteria.

Aerobic granulation is known as a microbial self-immobilization process that should be similar to the growth of biofilm (Liu and Tay, 2002). In the study of biofilms, there is evidence that strength of biofilms is negatively related to the growth rate of microorganisms (Tijhuis et al., 1995). Kwok et al. (1998) reported that the biofilm density decreased as the growth rate increased, while the density of nitrifying biofilm was much higher than that of heterotrophic biofilm (Oga et al., 1991). These are consistent to the results reported in Figs. 7.4 and 7.5. Similarly, in anaerobic granulation process, it was also found that a high biomass growth rate led to a reduced strength of anaerobic granules, i.e., partial loss of structural integrity and disintegration would occur at high biomass growth rate (Morvai et al., 1992; Q mby and Forster 1995). It was observed in Fig. 7.4 that the high observed growth rate encouraged the outgrowth of aerobic granules leading to a rapid increase in the size of granule, further

a loose structure with low biomass density. It appears that high growth rate of microorganisms would reduce the strength of the three-dimensional structure of microbial community. As discussed earlier, high substrate N/COD ratio would favour the selection of nitrifying bacteria in aerobic granules, thereby one possible operation strategy that can be applied to improve the stability of aerobic granules is to select slow-growing nitrifying bacteria in aerobic granules by controlling the feed N/COD ratio. In fact, microorganisms may evolve strategies in a spatially explicit environment that will increase population densities of favourable microbial types under given conditions.

Figures 7.8-11 show the macro- and micro-structures of aerobic granules developed at different substrate N/COD ratios. The mushroom-like structure was observed in the aerobic granules cultivated at the substrate N/COD ratios of 20/100 (Fig. 7.10a), while similar structure was also observed in the granules developed at the substrate N/COD ratio of 30/100 (Fig. 7.11a). Confocal laser scanning microscopy (CLSM) images further revealed that nitrifying populations were dominant in the clusters. Figure 7.10b further shows that the top layer mainly consists of cocci-shape bacteria, while rod-shape bacteria are dominant subsequently. Tay et al. (2002) also reported that the nitrifying population was mainly located at a depth of 70 to 100 μm from the surface of the granule.

Previous research showed that biofilm of mixed bacterial communities formed thick layers consisting differentiated mushroom-like structures, which are very similar to that observed in Fig. 7.10a (Costerton et al., 1994). There is a strong evidence that bacteria may sense and move towards nutrients (Prescott et al., 1999). Figure 7.2 shows that the relative abundance of nitrifying population over heterotrophic population in the aerobic granules grown at the substrate N/COD ratio of 5/100 was very low as compared to the granules de loped at high substrate N/COD ratios. At high substrate N/COD ratio, competition between nitrifying and heterotrophic populations on nutrients would be significant.

It had been demonstrated that biofilm could form the mushroom-like structure by simply changing the diffusion rate, i.e. biofilm structure is largely determined by nutrient concentration (Wimpenny and Colasanti, 1997). Since nitrifying bacteria are slow-growing species, the mushroom-like structure would result from the demand of nitrifying population on nutrients, and it in turn ensures that nitrifying population in aerobic granules can maximize access to nutrients. As Watnick and Kolter (2000) noted, in mixed biofilms, bacteria distribute themselves according to who can survive best in the particular microenvironment, and high complexity of microbial community would be beneficial to its stability. These seem to indicate that the mushroom-like structure of densely slow-growing nitrifying bacteria would contribute to the stability of aerobic granules developed at high substrate N/COD ratios. In a recent study of activated sludge floc stability, similar remark was also made (Wilen et al., 2003). Consequently, the organization of different microbial populations may have an effect on the stability of aerobic granules.

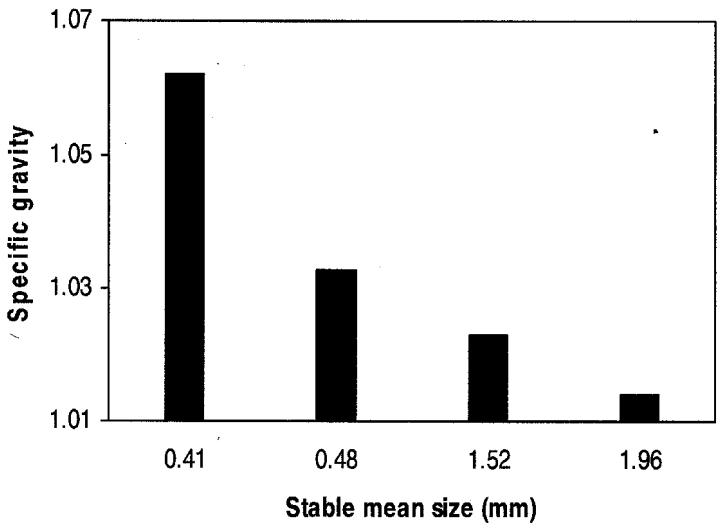

Figure 7 Relationship between stable mean size and specific gravity of aerobic granules.

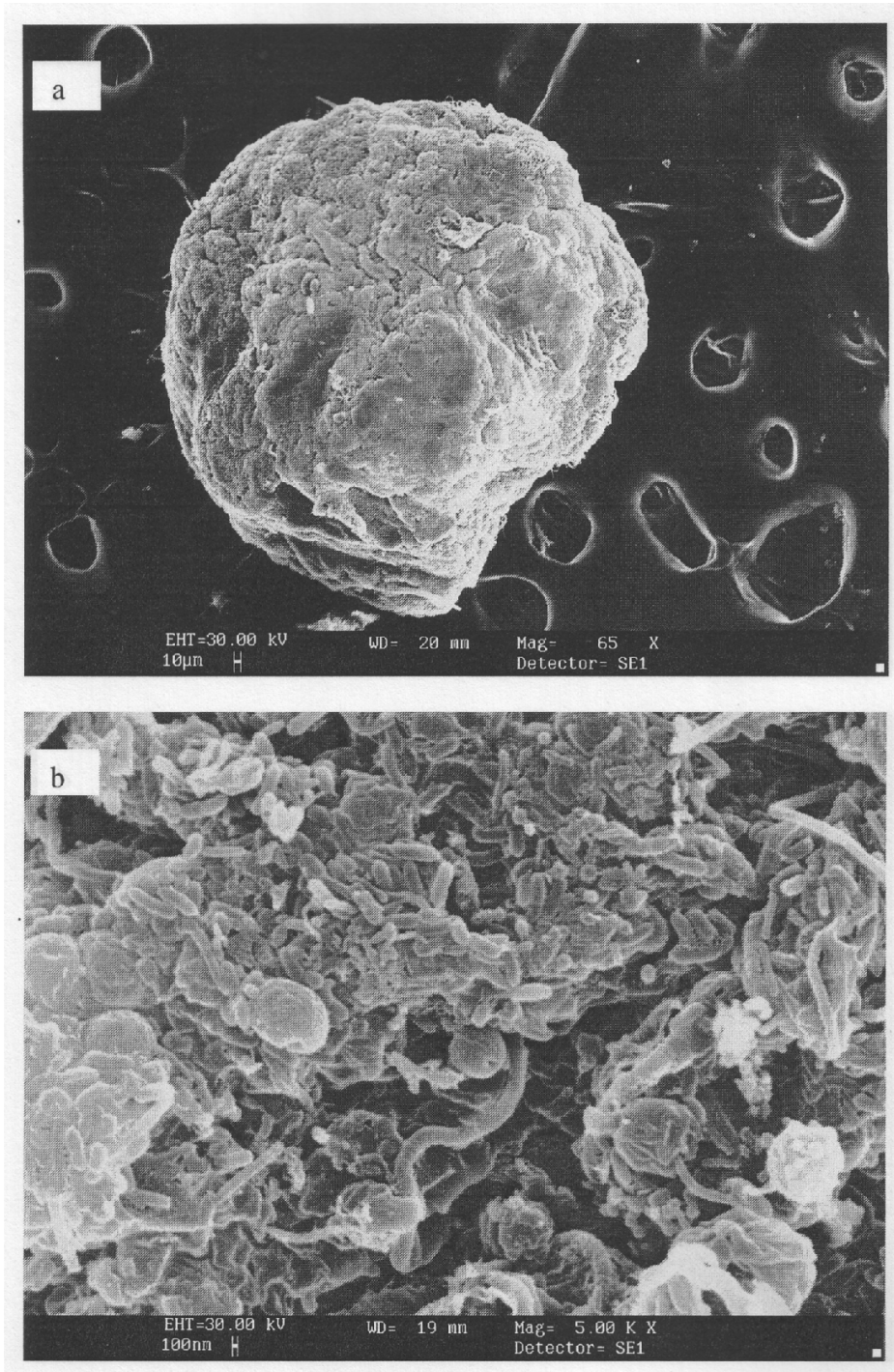

Figure 7.8 SEM images of aerobic granules developed at substrate N/COD ratio of 5/100 (a) macrostructure (b) microstructure.

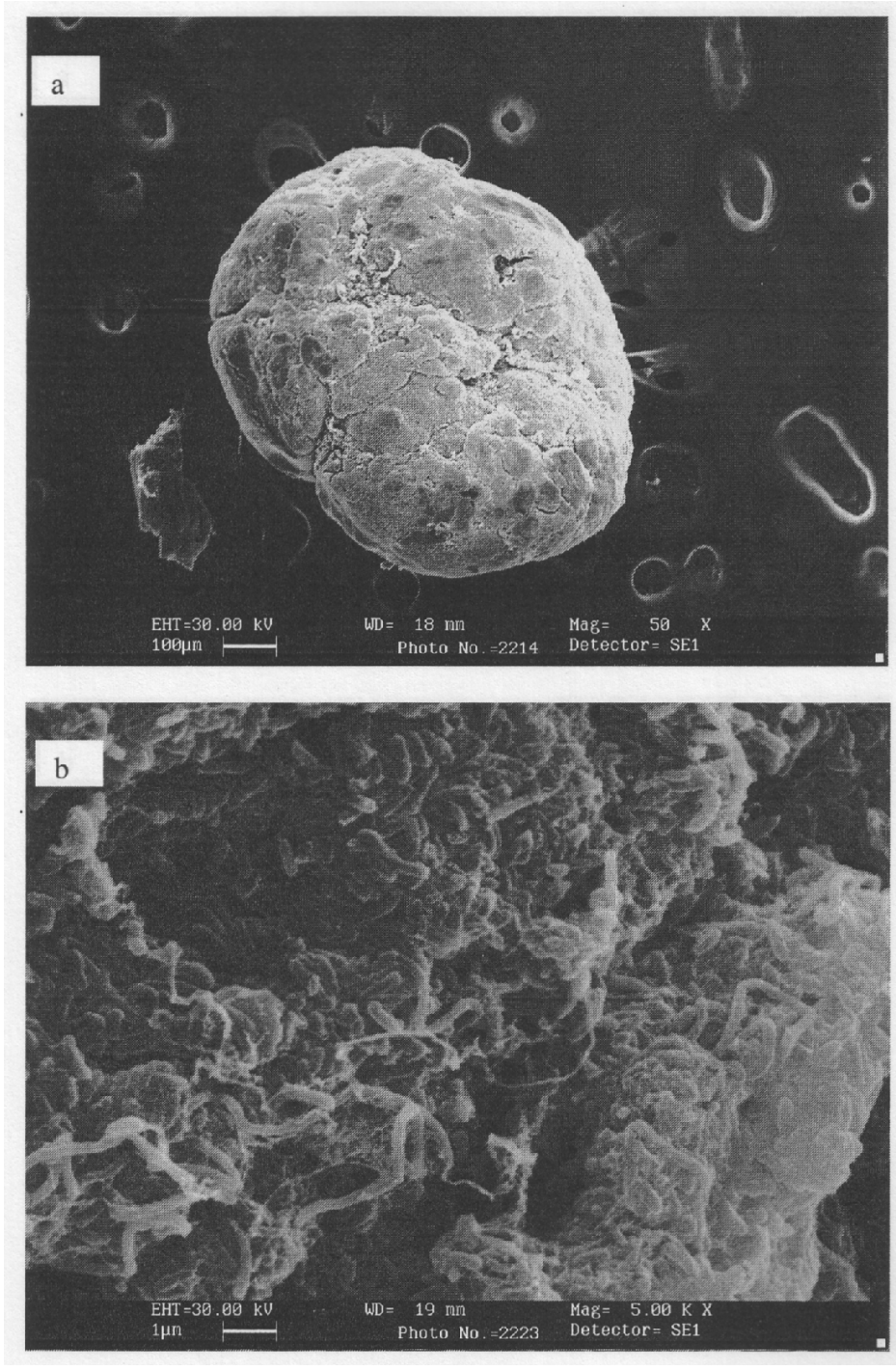

Figure 7.9 SEM images of aerobic granules developed at substrate N/COD ratio of 10/100 (a) macrostructure (b) microstructure.

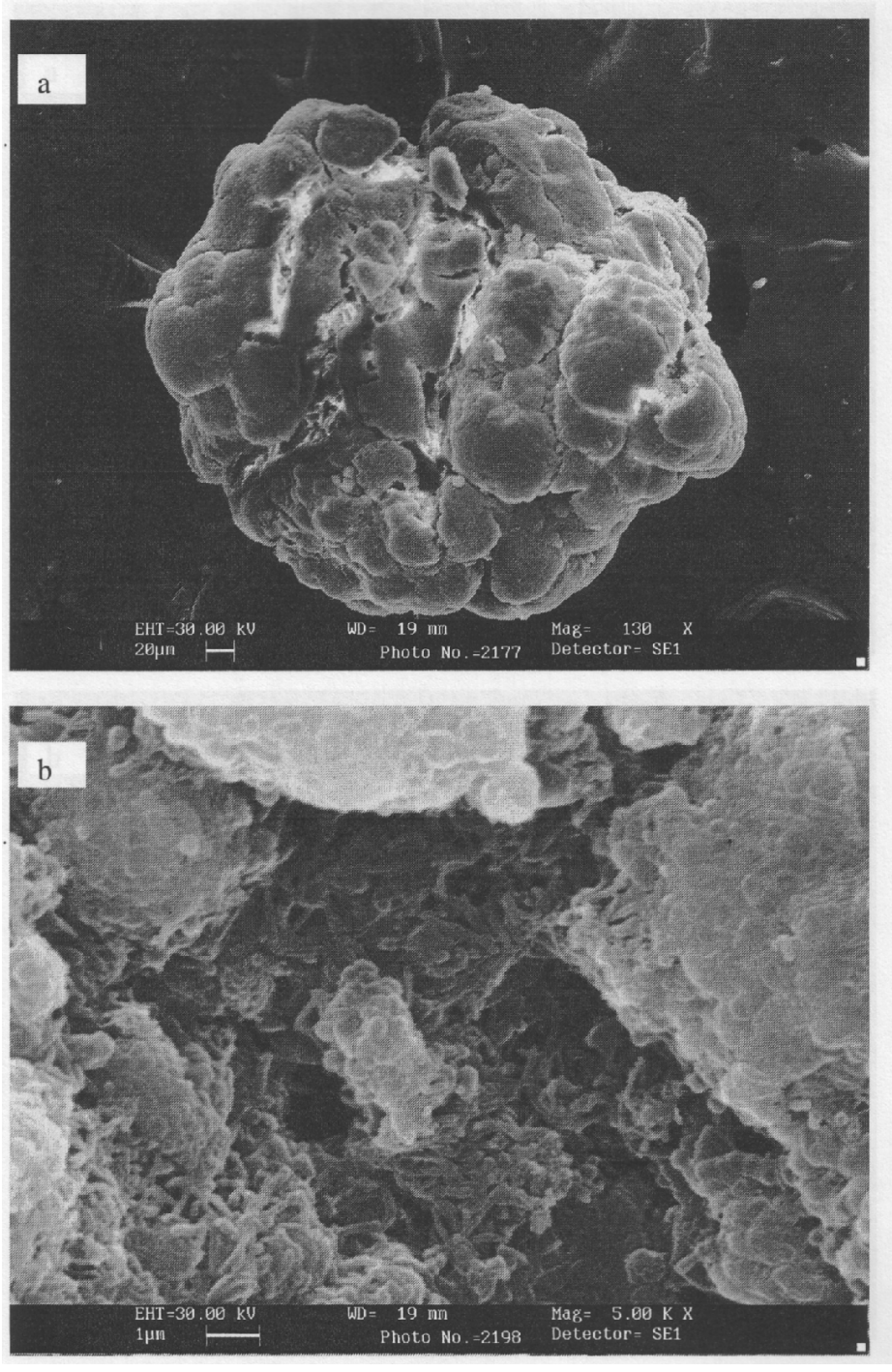

Figure 7.10 SEM images of aerobic granules developed at substrate N/COD ratio of 20/100 (a) macrostructure (b) microstructure.

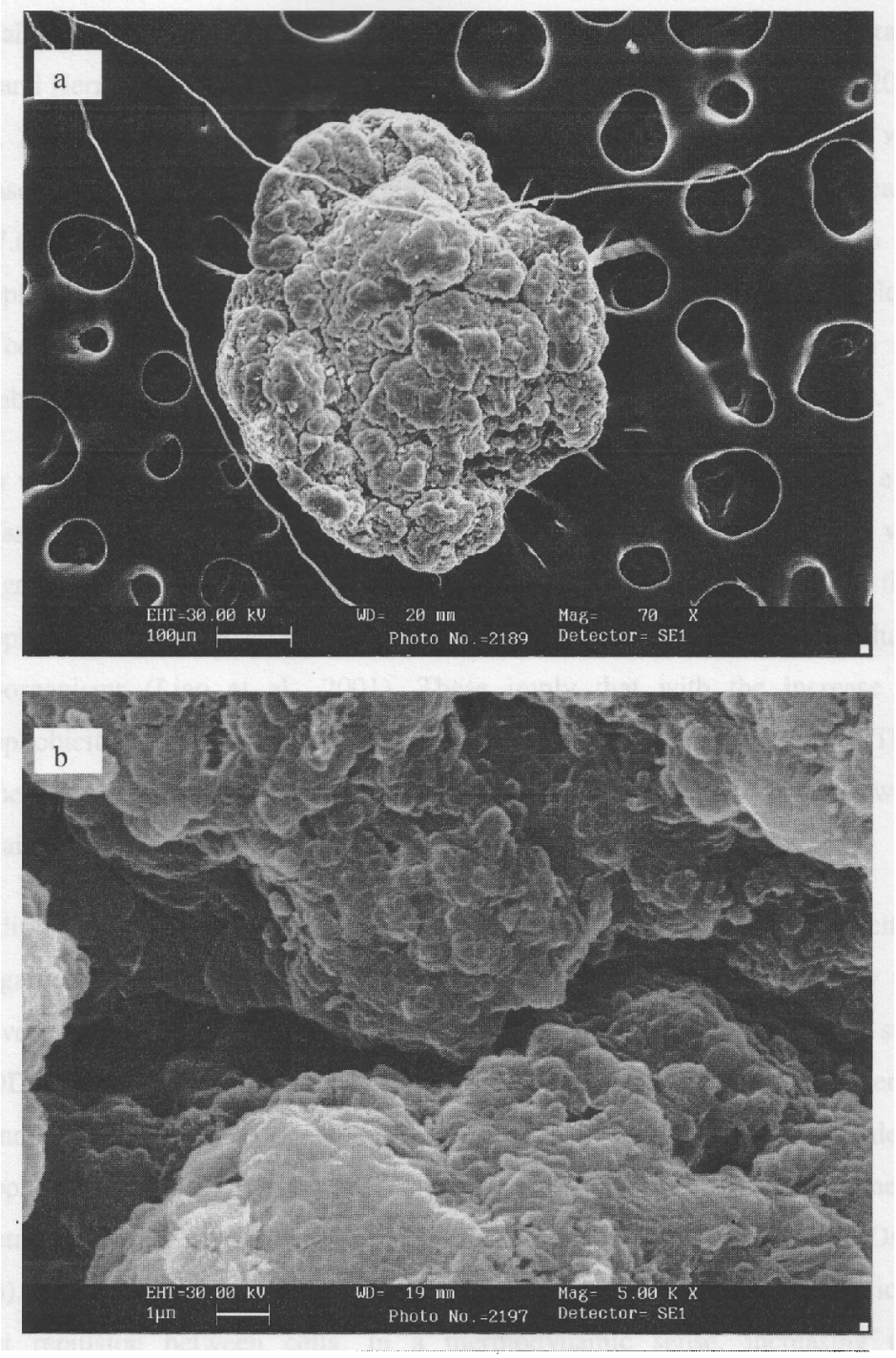

Figure 7.11 SEM images of aerobic granules developed at substrate N/COD ratio of 30/100 (a) macrostructure (b) microstructure.

It had been well documented that cell hydrophobicity is an important inducing and maintaining force for cell-to-cell immobilization and cell-to-carrier surface attachment (Zita and Hermansson, 1997; Del Re et al., 2000; Kim et al., 2000; Kos et al., 2003; Liu et al., 2003). As shown in Fig. 7.3, the cell hydrophobicity was improved with the increase of the $(SOUR)_N/(SOUR)_H$ ratio of aerobic granules. Further, it appears from Fig. 7.6 that the specific gravity of aerobic granules is positively related to the cell hydrophobicity, while the SVI is inversely dependent on the cell hydrophobicity, i.e. high cell hydrophobicity may promote cell-to-cell interaction leading to a good settleability, and favours a more stable structure of granular sludge community.

Under usual pH conditions, bacterial surface is negatively charged. There is evidence that fast-growing bacteria had a greater level of negative charge compared with the slow-growing bacteria (Shingaki et al., 1994), and it was also reported that cell hydrophobicity was inversely correlated to the quantity of surface charge of microorganisms (Liao et al., 2001). These imply that with the increase of cell hydrophobicity, repulsive force between cells would be weaker and weaker. Thus, the enriched slow-growing nitrifying population at high substrate N/COD ratio would be the main cause of the improved cell hydrophobicity as observed in Fig. 7.3.

In addition, Asconcabrera and Lebeault (1995) found that the cell hydrophobicity could be regarded as a key factor in the selection of microorganisms. It is likely that the observed mushroom-like structure of aerobic granules cultivated at high substrate N/COD ratio would be partly due to hydrophobic interaction of nitrifying bacteria (Figs. 7.3 and 7.6). The stability of aerobic granules is clearly related to a good degree of hydrophobicity of bacterial surface (Fig. 7.6). In fact, these are in agreement with expectations. According to the extended DLVO theory developed by Van Oss et al. (1986), cell hydrophobicity represents an attractive force, while cell hydrophilicity may reflect r ılsion between cells. In a thermodynamic sense, increasing the cell hydrophobicity simultaneously causes a decrease in the excess Gıubs energy of the surface, which results in an enhanced cell-to-cell interaction that further keeps the aggregated bacteria tightly together as Fig. 7.6 shows. It is most likely that microbial

selection-induced high cell hydrophobicity would enhance the structure and further stability of aerobic granules.

7.5 CONCLUSIONS

The stability of aerobic granules is a key of long-term and stable operation of aerobic granular sludge bioreactor. This study showed that the stability of aerobic granules can be significantly improved by selecting slow-growing nitrifying bacteria. The selection of nitrifying bacteria in aerobic granules was achieved by controlling the substrate N/COD ratio. The nitrifying population in aerobic granules developed at high substrate N/COD ratio was enriched markedly. The enrichment of slow-growing nitrifying bacteria led to the lower overall growth rate of aerobic granules with smaller size. The aerobic granules with low growth rates exhibited high stability in terms of specific gravity, SVI and cell hydrophobicity compared to those having high growth rates. This study showed that the substrate N/COD ratio would be a parameter that can be manipulated to improve the stability of aerobic granules by selecting the slow-growing nitrifying bacteria.

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CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1 CONCLUSIONS

Aerobic granules for simultaneous organics and nitrogen removal were successfully developed in the SBRs operated at the different substrate N/COD ratios. Following conclusions can be drawn from this study.

1. Aerobic granules could form at a wide range of substrate N/COD ratios.
   Aerobic granules were successfully developed at the substrate N/COD ratios ranging from 5/100 to 30/100 by weight. These indicated that aerobic granulation was less dependent on the substrate N/COD ratios in the range studied. Compared to the seed sludge, aerobic granules developed had excellent properties in terms of settleability and specific gravity. The settling velocity of aerobic granules cultivated was at least 6 times higher than that of conventional activated sludge. The specific gravity of aerobic granules fell into the range of 1.014 to 1.065, indicating that the aerobic granules developed had a much denser and more compact microbial structure than conventional bioflocs with a typical specific gravity of 1.002.

2. The characteristics of aerobic granules were determined by the substrate N/COD ratio.
   The microbial and physicochemical characteristics of aerobic granules were closely associated with the substrate N/COD ratio applied. Aerobic granules developed at higher substrate N/COD ratio had a more compact microbial structure, and high substrate N/COD ratio enhanced the activity of nitrifying populations. The cell hydrophobicity increased with the increasing substrate

N/COD ratio, while the production of extracellular polysaccharides decreased with the substrate N/COD ratio.

3. Heterotrophic, nitrifying and denitrifying populations could co-exist in the aerobic granules.

   By measuring the respirometric activities of heterotrophic, nitrifying and denitrifying populations, it was found that heterotrophic, nitrifying and denitrifying populations could co-exist in aerobic granules, and shifts in microbial populations in granules were closely associated with the substrate N/COD ratio applied. Nitrifying and denitrifying populations were significantly enriched by raising substrate N/COD ratio, while a decreasing trend of heterotrophic populations was observed in aerobic granules. The relative abundance of nitrifying population against heterotrophic population evolved until a balance between two populations was reached in aerobic granules. The activity of denitrifying population was proportionally correlated to that of nitrifying population in aerobic granules, while an increased DO would render the denitrifying activity of granules.

4. Elemental compositions of aerobic granules were associated with the substrate N/COD ratio.

   Elemental analyses of aerobic granules showed that high substrate N/COD ratio resulted in a high cell N/C ratio, but a low cell O/C ratio. It was further shown that cell hydrophobicity of aerobic granules was inversely related to the cell O/C ratio. The cell calcium contents in the aerobic granules developed at different substrate N/COD ratios were very low, thus it was most likely that the cell calcium would not contribute to aerobic granulation significantly.

5. Simultaneous organics and nitrogen removal could be achieved.

   This study showed that that complete and efficient organics and nitrogen removal was achievable in single granules-based SBR if the operating conditions could be properly controlled. COD and nitrogen removal efficiency

were larger than 95% even at high NH_4-N concentration of 150 mg l^{-1}. High system stability as well as organics and nitrogen conversion capacity could be maintained in the granules-based SBR.

6. DO and mixing power were two factors influencing denitrification by aerobic granules.

   Complete denitrification was achieved at DO concentration of 0.5 mg l^{-1}, while denitrification by granules would be hindered at a DO of 0.8 mg l^{-1} and above. Moreover, a certain mixing power should be provided to ensure a sufficient contact between granules and soluble nitrate and nitrite, otherwise denitrification rate by the aerobic granules would decline due to limitation of mass transfer.

7. The stability of aerobic granules could be improved by selecting slow-growing nitrifying bacteria.

   A strategy to improve the stability of aerobic granules through selecting the slow-growing nitrifying bacteria in aerobic granules was developed. Results showed that the selection of nitrifying bacteria in aerobic granules could be achieved by controlling the substrate N/COD ratio. The enrichment of slow-growing nitrifying bacteria at high substrate N/COD ratios led to the lower growth rate of aerobic granules with smaller size. The aerobic granules with low growth rates exhibited high stability in terms of specific gravity, SVI and cell hydrophobicity compared to those having high growth rates.

In summary, this study sheds lights into the development, characteristics and population distribution of aerobic granules in SBRs operated at different substrate N/COD ratios, and the feasibility of simultaneous organics and nitrogen removal by microbial granules was demonstrated. This work opens a door for environmental engineers to develop a novel compact and high-efficiency granules-based biological process for removing organics and nitrogen from wastewater.

8.2 RECOMMENDATIONS FOR FUTURE STUDY

This work provided deeper insights into the development of aerobic granules for simultaneous organics and nitrogen removal. Future study needs to look into the spatial structure and locations of different microbial populations in aerobic granules using modern techniques of molecular biology. It can be expected that such information is essential in understanding the ecology of aerobic granules. Future study is also suggested to develop novel aerobic granules-based bioreactor for simultaneous organics and nitrogen removal. It should be noted that ethanol, used as the sole carbon source in this study, is readily degradable. If some recalcitrant substances are used as the carbon source, the BOD/COD ratio would have significant impact on the removal of COD and nitrogen.