Removal of nitrogen and phosphorus from reject water of municipal wastewater treatment plant

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**REMOVAL OF NITROGEN AND PHOSPHORUS FROM
REJECT WATER OF MUNICIPAL WASTEWATER
TREATMENT PLANT**

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Removal of Nitrogen and Phosphorus from Reject Water of Municipal Wastewater Treatment Plant

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ABSTRACT

Reject water, the liquid fraction produced after dewatering of anaerobically digested activated sludge on a municipal wastewater treatment plant (MWWTP), contains from 750 to 1500 mg N /L of ammonium and up to 130 mg P/L phosphorus. It contributes from 10 to 30% of the nitrogen load and from 10% to 80% of phosphorus load on the main stream of MWWTPs. The removal of phosphorus and nitrogen from reject water could significantly reduce the nutrient load to the main stream of the MWWTP and improve the effluent quality.

The removal of phosphorus from reject water using anoxic bioreduction of iron ore particles of different sizes by iron reducing bacteria was studied in static and rotating reactors. In static reactors, the highest ferrous production rate (40 mg/L·d) was achieved with the smallest iron ore particles (0.6 mm), whereas in rotating reactors, the highest rate (44.5 mg/L·d) was observed with the largest particles (7.6 mm). The maximum phosphorus removal rate was 11 mg P/L·d and 25 mg P/L·d for static and rotating reactors, respectively. The parameters associated with ferrous production rate and phosphate removal rate were either the specific surface area of iron ore particles in static reactor or the production of fine particles from initially loaded coarse iron ore in rotating reactor.

The removal of nitrogen and phosphorus from reject water using the sequential biooxidation of NH_4^+ and bioreduction of NO_3^- combined with precipitation of phosphate by Fe^{2+} ions produced due to bioreduction of Fe^{3+} in iron ore was studied. The results showed that there was a competition between denitrifying and iron-reducing bacteria for electron donor during anaerobic treatment of reject water. The addition of acetate as electron donor increased both bioreduction rates of Fe^{3+} and NO_3^- but decreased the efficiency of phosphate removal from reject water due

to competition between acetate, nitrate, and phosphate anions for ferrous ions. As a conclusion, the stages of denitrification and ferric bio-reduction/phosphate precipitation should be performed sequentially.

The interactions between nitrate and ferric reductions during anaerobic treatment of reject water were further studied. Nitrate reduction significantly inhibited ferric reduction because denitrifying bacteria over competed iron-reducing bacteria for organic electron donors. The total Fe(II) concentration was diminished at presence of nitrate. Nitrite, the product of incomplete nitrate reduction, enhanced the oxidation of ferrous in reject water and also inhibited the activity of iron-reducing bacteria. Organic electron donors affected the extent and rate of nitrate reduction. Iron-reducing bacteria recovered after complete nitrate reduction by utilizing the remaining organic electron donors and iron ore reduction resumed. This recovery was associated with organic electron donors because the replacement of used reject water with fresh reject water effectively increased the maximum iron ore reduction rate from 45 mg/L·d to 116.5 mg/L·d. Ferrous was chemically oxidized in reject water. The protons produced from Fe(II) oxidation partially neutralized the alkalinity produced from the nitrate reduction process.

The interaction between ferrous oxidation and nitrate reduction during anaerobic treatment of reject water were further studied. It was showed that when reject water contained sufficient concentration of organic electron donors for complete nitrate reduction, ferrous ions inhibited this process. In the presence of 100 mg Fe(II)/L, the nitrate reduction rate was 7.9 mg N/L·h, as compared with 33.0 mg N/L·h in the control; the presence of 200 and 400 mg/L Fe(II) caused 6 h and 18 h lag-phase in nitrate reduction, and average reduction rate after the lag phase was 7.6 mg N/L·h and 3.0 mg N/L·h, respectively. The drop of pH due to ferrous hydrolysis together with the partial coverage of the surface of denitrifying bacterial by iron-hydroxides

precipitate caused the inhibition. When reject water did not contain sufficient concentration of organic electron donors for complete nitrate reduction, nitrate reduction was limited by the shortage of electron donors and the inhibition by ferrous was not significant. Nitrate-dependent ferrous oxidation was not involved in nitrate reduction process.

Biofilm was successfully developed on the rough iron ore surface in reject water under the moderate organic loading rate, short period of starvation, and high shear force. FISH showed that heterotrophic bacteria occupied the outer layer of biofilm, while ammonium oxidizers and nitrite oxidizers located in the inner layer, and denitrifying bacteria grew in the deepest layer of biofilm. The nitrification efficiency was 50% for reject water containing low TOC and 90% for reject water containing high TOC without addition of sodium bicarbonate as buffering substance, but the effluent pH was reduced to 4.05 due to the unsuccessful buffering capacity of iron ore. The addition of sodium bicarbonate as buffering substance increased the average and maximum nitrification efficiencies to above 95% and 99.76 % at nitrogen loading rate of 380-468 mg N/Ld, and maximum nitrification capacity was 464 mg N/L d. The average and maximum nitrification rates of 23.6 mg N/L h and 33.04 mg N/L h were achieved with optimum pH control. The average and maximum nitrate reduction rates of 21.96 and 30.4 mg N/L h were achieved by denitrifying bacteria under anaerobic conditions, respectively. The simultaneous removal of organic carbon and nitrogen from reject water under alternating aerobic and anaerobic conditions was achieved at average and maximum ammonium removal rates of 19.8 mg N/L h and 27.06 mg N/L h, respectively, and the average nitrite reduction rate and maximum nitrite reduction rate were 158.3 mg N/L h and 229.4 mg N/L h, respectively. Moreover, simultaneous nitrification and denitrification process for nitrogen and organic carbon removals was achieved also at DO concentration of 8 mg/L. The average and maximum nitrite reduction rates

were 97.8 mg N/L h and 119.8 mg N/L h, respectively. The biomass detached from iron ore biofilm could be developed into smooth, compact, and dense aerobic granule under moderate organic loading rate and high shear force.

The removal of nitrogen and organic carbon by the aerobic granules cultivated in reject water was studied. The aerobic granules cultivated in 5 L beaker fed with reject water under aerobic aeration had settling velocity of 45 m/h. Ammonium oxidizers located in the outer layer of the granules and achieved the average ammonium removal rate of 9.1 mg N/g VSS h by partial nitrification. Nitrite oxidizers were minority due to the inhibition by high concentrations of free ammonia and free nitrous acid, and located together with heterotrophic bacteria in the outer and middle layer. Denitrifying bacteria located in the deeper layer and achieved an average nitrate reduction and average nitrite reduction rates of 43.4 mg N/g VSS h and 72.4 mg N/g VSS h, respectively. The produced nitrogen gas negatively affected the granules structure. And ferrous ions slightly affected nitrification process by aerobic granules. The aerobic cultivated granules were successfully further cultivated under alternating aerobic and anaerobic conditions and achieved simultaneous nitrogen and organic carbon removal at nitrogen loading rate of 370 mg N/L d. Average ammonium removal rate of 6.3 mg N/g VSS h and average nitrite reduction rate of 32.7 mg N/g VSS h were achieved with microbial granules. Aerobic heterotrophic bacteria in the granules achieved the average TOC removal rate of 22.76 mg/g VSS h. Denitrifying bacteria of the granule achieved average nitrate reduction rate of 8.17 mg N/g VSS h under DO concentration of 8 mg/L. Moreover, the simultaneous nitrification and denitrification (SND) process for complete nitrogen removal could be achieved at DO concentration of 7-8 mg/L. By multiple addition of organic carbon at the late stage of each cycle in sequencing batch reactor, the maximum ammonium removal rate of 5.22 mg N/g VSS h and maximum nitrite removal rate of 30.3 mg N/g VSS h were achieved with a nitrogen

loading rate of 390 mg N/L d. The structure of granules was not affected by nitrate/nitrite reduction but the maintenance of pH was important for the microbial activity of granules.

PUBLICATIONS

Ivanov, V., Kuang, S. L., Stabnikov, V. and **Guo, C. H.** (2009), “The removal of phosphorus from reject water in a municipal wastewater treatment plant using iron ore”, Journal of Chemical Technology and Biotechnology, Vol. 84, pp. 78-82.

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LIST OF ABBREVIATIONS

Anammox	anaerobic ammonia oxidation
AOB	ammonium oxidizing bacteria
BABE	bio-augmentation batch enhanced
BOD	biological oxygen demand
BOD ₅	5 days biological oxygen demand
C	carbon
CANON	completely autotrophic nitrogen removal over nitrite
CLSM	confocal Laser Scanning Microscopy
COD	chemical oxygen demand
DIRB	dissimilatory iron reducing bacteria
DMRB	dissimilatory metal reducing bacteria
DO	dissolved oxygen
DOM	dissolved organic matter
DON	dissolved organic nitrogen
EPBR	enhanced biological phosphorus removal
EPS	extracellular polysaccharides
FA	free ammonia
FISH	fluorescence in situ hybridization
FNA	free nitrous acid
FRM	Fe(III)-respiring microorganisms
GAOs	glycogen accumulating organisms
HAP	calcium phosphate (hydroxyapatite)
HRT	hydraulic retention time
IOB	iron-oxidizing bacteria
IRB	iron- reducing bacteria
MAP	magnesium ammonium phosphate hexahydrate
MLSS	mixed liquor suspended solids
MLVSS	mixed liquor volatile suspended solids

MPN	most probable number
MWWTP	municipal wastewater treatment plant
N	nitrogen
NLR	nitrogen loading rate
NOB	nitrite oxidizing bacteria
OLAND	oxygen-limited autotrophic nitrification-denitrification
OLR	organic loading rate
ORP	oxidation/reduction potential
P	phosphorus
PAOs	phosphate-accumulating organisms
PHAs	poly-hydroxyalkanoates
RBCs	rotating biological contactors
SBR	sequencing batch reactor
SBBR	sequencing batch biofilm reactor
SEM	scanning electron microscope
SHARON	the single reactor system high activity ammonium removal over nitrite
SND	simultaneous nitrification and denitrification
SOUR	specific oxygen uptake rate
SRT	sludge retention time
SVI	sludge volume index
TDS	total dissolved solid
TKN	total kjeldahl nitrogen
TOC	total organic carbon
TSS	total suspended solids
UASB	upflow anaerobic sludge blanket reactor
VFA	volatile fatty acid
VSS	volatile suspended solids

Chapter 1

Introduction

1.1 Background

The discharge of phosphorus and nitrogen from municipal wastewater water treatment plant (MWWTP) to aquatic systems can cause eutrophication. The general emission limit of phosphorus varies from 0.05 to 2 mg P/L (Ivanov et al., 2005). Ammonium emission limit varies between countries and types of water body. European Union sets it from 10 to 15 mg N/L, while China establishes a wider range from 10 to 30 mg N/L. In the United States, the limit of nitrate and free ammonia in drinking water is 10 mg N/L and less than 1.0 mg N/L, respectively (U.S.EPA, 1993).

Reject water (also known as return liquor, digester supernatant, or sludge digester liquid) is the liquid fraction produced from dewatering stage, which is subsequent to anaerobic digestion of activated sludge. Reject water contains from 750 to 1500 mg N/L ammonium (Berends et al., 2005; Perez et al., 2007), up to 130 mg P/L phosphorus (Pitman, 1999) and 700 to 5400 mg/L COD (Laurich et al., 2004). Although the flow is relatively small, which is around 2% of total raw sewage flow (Janus and Van, 1997), it contributes from 10 to 30% of the nitrogen load and from 10 to 80% of phosphorus load in the activated sludge tank (van Loosdrecht and Salem, 2006). During the peak loading period, reject water stream may even cause overloading of wastewater treatment plant (Meyer and Wilderer, 2004). Therefore, the stream should be treated for removal of nitrogen, phosphorus, and organic carbon before being recycled to the main stream (Pitman, 1999). This could significantly reduce the nutrient loads to the main stream and diminish the concentrations of these nutrients in the plant effluent, eventually preventing eutrophication of aquatic systems and deterioration of water quality.

Phosphorus can be removed biologically and chemically. Chemical processes such as struvite or MAP (Magnesium ammonium phosphate hexahydrate, $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) are proposed (De-Bashan and Bashan, 2004; Münch and Barr, 2001; Schulze-Rettmer, 1991; Yoshino et al., 2003). The low crystallization rate of struvite and high chemical consumption, however, make the processes disadvantageous. The precipitation of phosphorous using ferric chloride (FeCl_3) or ferrous sulfate (FeSO_4) is faster, but costly in terms of iron salts. Another precipitant is biologically produced ferrous from the anaerobic reduction of iron ore/iron hydroxide using iron-reducing bacteria (IRB) (Ivanov et al., 2005; Stabnikov et al., 2004; Tay et al., 2008). Since iron ore is cheap and the ferrous-phosphate precipitate can be used as fertilizer (De-Bashan and Bashan, 2004), the proposed method is a potentially cost effective and environmental friendly technology. The application of this innovative treatment in reject water has not yet been studied in details.

Ammonium, another eutrophic contaminant in reject water, can be removed by various processes namely aerobic nitrification followed by denitrification; bio-augmentation batch enhanced (BABE) technology (Salem et al., 2004); the single reactor system high activity ammonium removal over nitrite (SHARON) process (Gali et al., 2006b; Hellinga et al., 1998); anaerobic ammonium oxidation (ANAMMOX) process (Fux et al., 2004; Jetten et al., 2001; Strous et al., 1997); SHARON process combined with ANAMMOX process (Van Dong et al., 2001); completely autotrophic nitrogen removal over nitrite (CANON) process (Szatkowska et al., 2007); oxygen-limited autotrophic nitrification-denitrification (OLAND) process (Kuai and Verstraete, 1998); and aerobic/anoxic deammonification process (Gut et al., 2006). Most of the methods, however, are sensitive to environmental conditions. SHARON process makes use of the different growth rates of ammonium oxidizers and nitrite oxidizers at high temperature to achieve partial nitrification, but it has limited capacity and can only decrease the

total nitrogen load by 30% (van Kempen et al., 2001). BABE technology achieves significant benefit only at low temperature. Anammox bacteria have low growth rate and need a very long start-up period. The CANON, OLAND, and aerobic/anoxic deammonification are unstable and require long start up time. Moreover, all of the biological removal methods for nitrogen in reject water do not remove phosphorous.

An alternative method is the sequential biooxidation of NH_4^+ and bioreduction of NO_3^- combined with the precipitation of phosphate by biologically produced Fe^{2+} as mentioned above. Therefore, the combination of anaerobic nitrate reduction and iron reduction for nitrogen and phosphorous removal from reject water becomes an innovative approach in environmental engineering.

There are plenty of literature reviews regarding the inhibition of iron reduction by nitrate reduction such as in the case of anaerobic sediment (Sorensen, 1982), pure cultures (DiChristina, 1992), soil systems (Acht nich et al., 1995), and bacteria associated with iron seeps in a sulfur-rich freshwater ecosystem (Haa ijer et al., 2008). The inhibition is explained by the competition for electron donors among nitrate reducers and iron-reducing bacteria. Also, nitrate tends to be more available to cells compared to the insoluble form of Fe(III) (Acht nich et al., 1995; DiChristina, 1992; Obuekwe et al., 1981; Sorensen, 1982). During nitrate reduction process, the biologically produced NO_2^- from NO_3^- abiotically re-oxidized Fe^{2+} to produce Fe(III) hydroxide, which coats the cell and inhibits the transport of electron acceptors into the cell (Coby and Picardal, 2005; Cooper et al., 2003). However, the reject water contains various components and the conditions are different from that of previous study, therefore, the occurrence of this inhibition in reject water, however, is still unclear.

The reoxidation of ferrous, however, can enhance nitrate reduction process in some cases, such as paddy soil (Ratering and Schnell, 2001) and activated sludge anoxic (denitrification) process (Nielsen and Nielsen, 1998a; 1998b). During the nitrate-dependent ferrous oxidation process, the anaerobic ferrous oxidation is coupled to nitrate reduction to produce nitrogen gas. The microbiological population involved is enrichment culture or pure culture of nitrate-reducing bacteria or mesophilic denitrifying bacteria (Benz et al., 1998; Straub et al., 1996). It is proposed that solid-phase Fe(II) compounds are good electron donors for biological nitrate reduction (Weber et al., 2001). However, when ferrous salts are used to precipitate phosphorous from municipal wastewater, ferrous can interact with nitrite or nitrate and show inhibitory effect on activated sludge (Clark et al., 2000; Philips et al., 2003). Therefore, the impact of biologically produced ferrous on nitrate reduction process during anaerobic treatment of reject water should be studied.

Beside the practical approaches to remove nitrogen and phosphorous from reject water, microbial activity involved in the treatment is also of concern. The aggregation of bacteria to survive in toxic reject water under different conditions can be vividly seen in the specific cases of biofilm and aerobic granule.

Biofilm is a complex coherent structure of microbial cells and cellular products, like extra-cellular polymers (Van Loosdrecht et al., 1995b). Favorable conditions for the formation of biofilm include roughness of carrier surface (Van Loosdrecht et al., 1988), the balanced moderate substrate loading rate and high shear force (Kwok et al., 1998; Liu et al., 2003b; Tjihuis et al., 1995a). In biofilm reactor, microorganisms are immobilized or attached to static biofilm carrier (Liu and Tay, 2002) or suspended carriers (Nicoletta et al., 2000) to accumulate high biomass concentration and achieve high biodegradation rate. The coexistence of different

microbial populations in the biofilm allows various biological reactions to be performed simultaneously. Specifically, the coexistence of heterotrophic bacteria in the outer layer, nitrifiers in the inner layer, and denitrifiers in the deepest layer of the biofilm induces simultaneous nitrification and denitrification (SND). The simultaneous process is reported in trickling biofilm filter (Biesterfeld et al., 2003; Mehlhart, 1994) as well as fixed biofilm hybrid system (Rong et al., 2007). The biofilm processes are applied to remove nitrogen and organic carbon from wastewater at the same time (Liu et al., 2010). Wastewater that contains high N/C ratio would enhance the development of nitrifying bacteria in biofilm (Ohashi et al., 1995). Combining all the facts, it seems to be promising to develop biofilm on the rough iron ore surface using reject water containing high ratio of N/COD. If successful, the developed biofilm can be tested for nitrogen and organic carbon removal under different operating conditions.

Aerobic granulation is a phenomenon of cell-to-cell self-immobilization to form a compact and dense structure (Liu, 2008). Granulation can be regarded as a special case of biofilm (Liu and Tay, 2002). Aerobic granules have unique advantages such as better settling ability, mass transfer efficiency and bioactivity (Su and Yu, 2005a); strong microbial structure; ability to achieve high biomass concentration and high biodegradation rate in the reactor; durability to withstand high organic loading rate (OLR) and shock loading; tolerance to toxic chemicals and heavy metals in wastewater (Liu and Tay, 2004). Aerobic granules have been cultivated using various substrates including synthetic wastewater, toxic wastewater, real municipal and industrial wastewater. The removal of nutrients and organic carbon in wastewater using aerobic granules has also been studied extensively. Yet granulation in reject water has not been researched. Therefore, a study involving the cultivation, the characteristics, and the ability to remove nitrogen and organic carbon of aerobic granules in reject water will be conducted.

1.2 Objectives and Scopes

This study focuses on all aspects of the removal of nitrogen and phosphorus from reject water. The following topics were studied and discussed:

1. The removal of phosphorus from reject water using anoxic bioreduction of iron ore by iron reducing bacteria in static and rotating reactors. The ferrous production rate, phosphorus removal rate, and removal efficiency were examined. The mechanisms of iron reduction and phosphorus removal in both systems were studied.
2. The possibility of combining anaerobic nitrate reduction and iron reduction to remove both nitrogen and phosphorus in reject water. The basic interactions between NO_3^- , NO_2^- , NH_4^+ , Fe^{3+} , Fe^{2+} , HPO_4^{2-} , and CH_3COO^- ions during the combined process were studied and discussed, followed by technological recommendations.
3. The interactions between nitrate reduction, iron ore reduction, and organic carbon oxidation during anaerobic treatment of reject water. The mechanisms behind the inhibition of iron reduction by nitrate reduction, the effect of the inhibition on iron concentration, the recovery of iron reduction after complete nitrate reduction were studied. At the same time, the effects of organic carbon on iron ore reduction, nitrate reduction, and iron reduction recovery were examined.
4. The effects of ferrous addition on nitrate reduction in model reject water and the interactions of ferrous oxidation and nitrate reduction during anaerobic treatment of reject water. The mechanism of the inhibition of nitrate reduction by ferrous, the mechanism of ferrous oxidation in reject water, and the contribution of nitrate dependent ferrous oxidation on nitrate reduction were studied.
5. The development of biofilm on iron ore surface in reject water. The distribution of bacteria in the biofilm, nitrification of ammonium by the biofilm with and without addition of sodium bicarbonate as buffering substance, nitrate reduction by denitrifying bacteria in the biofilm under different conditions, the complete removal

of nitrogen from reject water under alternating aerobic-anaerobic conditions as well as simultaneous nitrification and denitrification (SND) process for complete nitrogen removal by biofilm at high DO concentration were studied.

6. The cultivation of aerobic granules in reject water using different strategies. The characteristics of the granules, the distribution of bacteria in the granules, the effects of ferrous on nitrification by aerobic granules in reject water, nitrate reduction by denitrifying bacteria in the granules under different conditions, the simultaneous removal of organic carbon and nitrogen from reject water under alternating aerobic-anaerobic conditions and finally simultaneous nitrification and denitrification (SND) process for complete nitrogen removal at high DO concentration were studied.

1.3 Outline of Thesis

The thesis contains 9 chapters, including:

- Chapter 1 Introduction of the research background and the scopes.
- Chapter 2 Literature review on nitrogen and phosphorus removal, iron reduction and BioIronTech process, iron and nitrogen interactions, aerobic biofilm and aerobic granulation.
- Chapter 3 The removal of phosphorus from reject water using anoxic bioreduction of iron ore by iron reducing bacteria.
- Chapter 4 The removal of nitrogen and phosphorus from reject water using ferric and nitrate bioreductions.
- Chapter 5 The interactions between nitrate reduction, iron ore reduction, and organic carbon oxidation during anaerobic treatment of reject water from MWWTPs.
- Chapter 6 The interactions between ferrous oxidation and nitrate reduction during anaerobic treatment of reject water from MWWTPs.
- Chapter 7 The removal of nitrogen from reject water using iron ore as biofilm

carrier.

Chapter 8 The removal of nitrogen and organic carbon from reject water using microbial granules.

Chapter 9 Conclusions and recommendations.

Chapter 2

Literature Review

2.1 Introduction

The reject water discharged from the sludge treatment process contains high concentration of nutrients thus imposing significant loading on the main process. The adverse effects of reject water can be controlled or eliminated by proper planning, design, and operation. Nutrient removal and recovery can provide a major solution for nutrient pollution control and should be widely implemented (Randall, 2004).

To provide a full picture of the removal of ammonium and phosphorus from reject water, literature review introduces all the related aspects including the removal of nitrogen from reject water, the removal of phosphorus from reject water, iron reduction by iron-reducing bacteria and BioIronTech process for nutrient removal, the interactions among nitrogen and iron cycles; the perspectives of aerobic biofilm and aerobic granulation for nitrogen removal from reject water.

2.2 Reject water as a source of nutrient load on the main stream of MWWTP

The sludge treatment process (sludge thickening, digestion, and dewatering) may cause the release of the accumulated N and P. Ammonium is produced from the break-down of proteins and bacteria in the anaerobic digestion stage. Phosphorus that is incorporated into bacteria biomass in the enhanced biological phosphorus removal process is released during anaerobic digestion. Therefore, reject water, the liquid fraction produced in dewatering stage after anaerobic digestion of activated sludge (Fig. 2.1), carries very high contents of ammonium and phosphorous. The removal of ammonium and phosphorus from the reject water can reduce the

nitrogen loading in the aerobic tank, diminish its oxygen consumption, and cut costs for the tertiary treatment for nutrient removal. It can be considered as a cost effective solution and a practical alternative for enlargement of the main wastewater treatment stages (Meyer and Wilder, 2004).

Fig. 2.1 The generation of reject water in MWWTP.

2.3 Removal of ammonium from reject water

2.3.1 Air stripping process and steam stripping process

Air stripping removes ammonia by volatilization of gaseous ammonia at high pH (10.5-11.5) with the addition of lime (Metcalf & Eddy, 1991). In actual applications, pH range of 9.5-10.5 is sufficient for effective ammonium removal (Thorndahl, 1993). This process is disadvantageous in the requirement for large air volume and lime, together with high operating cost. Steam stripping is a distillation process that takes place at temperature close to the boiling point of water for the recovery of ammonium from reject water (Zeng et al., 2006). However, there are problems of high operating cost and clogging in the heat exchanger (Water Environment Federation, 2006).

2.3.2 MAP or struvite process

The precipitation of ammonium, phosphate, and magnesium produces struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) as indicated in Equation 2-1 and 2-2 (Celen and Turker, 2001; Water Environment Federation, 2006):

The suitable conditions for MAP process include a NH_4 : Mg: PO_4 molar ratio of 1:1:1 and pH ranging from 8 to 10 (Water Environment Federation, 2006). MAP process has been applied to remove/recover ammonium and phosphorus from reject water (Celen and Turker, 2001; Wedi and Konig, 1993). However, the rate of crystallization is low and chemical consumption cost is high.

2.3.3 Nitrification process and denitrification process

2.3.3.1 Nitrification process

The conventional system for the removal of nitrogen from wastewater is a two-steps biological process, i.e. nitrification followed by denitrification. Under aerobic conditions, ammonium is oxidized to nitrite by ammonium oxidizers then nitrite is oxidized to nitrate by nitrite oxidizers. The stoichiometry associated with nitrification reactions is as follows:

Ammonium oxidizers: Ammonium oxidizers belong to the β - and γ - subdivision of proteobacteria (Purkhold et al., 2000). Up to now, there are 25 known cultured species of ammonia-oxidizing bacteria, in which the most commonly recognized genus is *Nitrosomonas* (Bitton, 1999). The nitrifying bacteria from this genus are chemolithoautotrophic ammonium-oxidizing bacteria, which can use carbon dioxide as carbon source to gain energy from ammonium oxidation under aerobic conditions.

Nitrite oxidizers: The most common nitrite oxidizers are bacteria from genus *Nitrobacter* (e.g., *N. agilis*, *N. Winogradski*). *Nitrobacter* sp are closely related to the α -subdivision of the proteobacteria, while others are from genera *Nitrospira* and *Nitrococcus* (Rittmann and McCarty, 2001). Members of *Nitrospira* are obligatory chemolithotrophic, whereas members of *Nitrobacter* can grow in organic compounds for energy generation (Bock, 1976). Members of *Nitrospira* are the most abundant nitrite oxidizers in wastewater treatment plant (Daims et al., 2001; Koops and Pommerening-Roser, 2001). Currently there are eight known pure cultures of nitrite-oxidizing bacteria (Koops and Pommerening-Roser, 2001).

Factors affecting nitrification process: An oxygen requirement of 4.57 mg O₂/mg NH₄⁺-N oxidized to nitrate is often recommended for design calculation. Dissolved oxygen should be above 1 mg/L for nitrification to occur and maintained above 2 mg/L for it to proceed. The process might slow down or cease if DO drops below 1 mg/L (Metcalf & Eddy, 1991).

The optimum pH for *Nitrosomonas* and *Nitrobacter* is between 7.5 and 8.5 (U.S.EPA, 1975). Nitrification will stop at or below pH 6.0 (Painter, 1970). The mechanism of pH affecting nitrification rate is based on that ammonia-ammonium and nitrite-nitrous acid equilibrium depend on pH, and both free ammonia and free

nitrous acid inhibit nitrifiers (Anthonisem, 1974). As 7.14 mg/L alkalinity as CaCO_3 is required for 1 mg N/L NH_4^+ -N oxidized (U.S.EPA, 1975), the addition of alkalinity such as lime is required to neutralize the acid produced by nitrification.

In addition to oxygen concentration and pH, other factors such as ammonia/nitrite concentration, temperature, BOD_5/TKN ratio, concentration of nitrifiers, sludge age, organic loading, detention time, and toxic inhibition should also be considered (Bitton, 1999; Metcalf & Eddy, 1991; Water Environment Federation, 2006).

2.3.3.2 Denitrification process

Denitrification process involves the biological reduction of nitrate and /or nitrite to nitrogen gas in the absence of dissolved oxygen (anoxic). In the process, the nitrate and/or nitrite serve as terminal electron acceptor(s) and organic matters serve as carbon and energy sources (Bitton, 1999):

Overall equation is:

Denitrifying bacteria: The heterotrophic denitrifying bacteria are commonly found in typical wastewater treatment processes, which might not be designed to remove nitrogen. These bacteria are facultative, i.e. they can use both oxygen and nitrate/nitrite as terminal electron acceptors (Water Environment Federation, 2006). The most widespread genera are *Pseudomonas* (*P. fluorescens*, *P. aeruginosa*, *P. denitrificans*) and *Alcaligenes*, which are commonly found in soils, water, and wastewater (Painter, 1970). Denitrification is carried out according to the following

sequences (Metcalf & Eddy, 1991; Bitton, 1999):

Factors affecting denitrification process: In denitrifying systems, the dissolved oxygen concentration is the critical parameter. DO competes effectively with nitrate to become the final electron acceptor and suppresses the enzyme needed for denitrification. Denitrification rate may be reduced with dissolved oxygen as low as 0.2 mg/L in the anoxic zone (Water Environment Federation, 2006).

Substrate is another limiting factor. Different substrates have been studied and recommended, including domestic/industrial wastewater, methanol, ethanol, acetic acid, and food processing organic waste materials (sugars) (Water Environment Federation, 2006). In the case of methanol, Equation 2-8 indicates the dosage required for complete denitrification is 1.91 mg methanol/mg nitrate denitrified. Some researchers have suggested that maximum removal of nitrate can be achieved when the mass ratio $\text{CH}_3\text{OH}/\text{N-NO}_3^-$ is approximately 2.5 (Water Environment Federation, 2006). U.S. EPA (1975) similarly suggested a value of 3.0 for complete denitrification.

Optimal pH for denitrifiers is between 7 and 8 (Metcalf & Eddy, 1991). Theoretically, 3.57 mg/L alkalinity as $\text{CaCO}_3/\text{mg N/L NO}_3^-$ denitrified can be produced (Equation 2-8) at the end of the process. Therefore, denitrification can compensate half of the alkalinity consumed by nitrification. In addition, nitrate concentration, temperature, presence of toxic chemicals, and trace metals can also affect the process (Bitton, 1999).

2.3.3.3 The removal of ammonium from reject water by nitrification and denitrification processes

Reject water has an unfavourable COD:N ratio for denitrification. Therefore, the addition of an external carbon source such as methanol is required. The alkalinity available in the reject water is about 50-60% of the amount needed for completed nitrification (Fux and Siegrist, 2004; Water Environment Federation, 2006), hence alkalinity (lime, soda) must also be supplied or can be gained from denitrification process. The high ammonium concentration in reject water requires 16-36 hours aerobic detention time for nitrification (Water Environment Federation, 2006). In terms of reactor, SBR is widely used in reject water treatment (Arnold et al., 2000; Mossakowska et al., 1997; Wett et al., 1998).

2.3.4 Partial nitrification

Partial nitrification is the oxidation of ammonium to nitrite instead of nitrate and the further nitrite oxidation is prevented. The combined partial nitrification and conventional denitrification (so called “nitrite route”) can save 25% oxygen requirement for nitrification (Equation 2-3) and 40% carbon source (Equation 2-7) for denitrification. Partial nitrification can also achieve higher denitrification rate and lower biomass production (Turk and Mavinic, 1989; Abeling and Seyfried, 1992). It is cost-effective to denitrify the reject water that contains low C/N ratio; the emission of CO₂ to atmosphere will be reduced (Schmidt et al., 2003).

2.3.4.1 Strategies for successful partial nitrification

Some researchers have studied the inhibition of nitrite oxidizers by changing pH to control the concentrations of free ammonia and free nitrous acid (Anthonisen et al.,

1976; Turk and Mavinic, 1989; Abeling and Seyfried, 1992). The free ammonia begins to inhibit *Nitrosomonas* at the concentration between 10 and 150 mg N/L, whereas the activity of *Nitrobacter* is already seriously reduced at the concentration between 0.1 and 1 mg N/L. *Nitrobacter* is inhibited by free nitrous acid at the concentration higher than 0.2-2.8 mg N/L HNO_2 (Anthonisen et al., 1976). Abeling and Seyfried (1992) reported that the ammonia concentration of 1 to 5 mg N/L could inhibit *Nitrobacter* and the free nitrous acid inhibition limit for *Nitrobacter* was 0.13 mg N/L. Park and Bae (2009) reported that the inhibition constant (K_i) of nitrite oxidizers was 46 μM for FA and 1.7-6.8 μM for FNA, and nitrite oxidizers were inhibited more seriously under the simultaneous inhibition by FA and FNA.

Nitrosomonas has higher growth rate than *Nitrobacter* at higher temperature (more than 20 °C) (Hellenga et al., 1998). The partial nitrification in SHARON process is achieved by the careful control of sludge age to wash out the *Nitrobacter* and achieve partial nitrification (Hellenga et al., 1998; Mulder et al. 2001; Van Kempen et al., 2001). The low DO concentration (less than 0.4 mg/L or 5% air saturation) with surplus ammonium can inhibit the growth of *Nitrobacter* due to their lower oxygen affinity (0.3 mg/L) as compared to that of *Nitrosomonas* (1.1 mg/L) (Schmidt et al., 2003). Ruiz et al. (2003) achieved the inhibition of nitrite oxidizers in the DO range of 0.7-1.7 mg/L. The competition for oxygen and nitrite occur when partial nitrification and nitrite reduction are coupled, which significantly decreases the growth of *Nitrobacter*. The creation of anoxic conditions inside the floc or the rapid circulation between aerobic and anoxic conditions is an effective approach (van Loosdrecht and Salem, 2006). It was reported that periodic aerobic/anoxic process inhibited nitrite oxidizers and achieved partial nitrification (Katsogiannis et al., 2002; 2003), such inhibition was due to the deactivation of NOB enzyme under anoxic conditions (Kornaros et al., 2010); and the application of such theory in the pilot scale for municipal wastewater treatment was demonstrated (Kornaros et al.,

2008). Park et al. (2010) recommended the good combination of pH, DO, and total ammonium nitrogen achieved stable partial nitrification.

2.3.4.2 Partial nitrification in sequencing batch reactors (SBR) for reject water treatment

Cultivation of microorganisms in sequencing batch reactor (SBR) is a variable volume suspended-growth treatment technology that uses time sequence to perform the various treatment operations; the four basic phases of SBRs (fill, react, settle, and decant) replace four different tanks in continuous reactors (Water Environment Federation, 2006).

As reject water contains high concentration of ammonium, it is easy to obtain partial nitrification in SBR for the removal of ammonium from reject water (Dosta et al., 2007; Gali et al., 2006b; Lai et al., 2004). Nitrite reduction requires less organic carbon and the alkalinity produced from this process can compensate the requirement in partial nitrification. As a result, the combination of partial nitrification and nitrite reduction in SBR is a promising alternative for reject water treatment.

2.3.5 SHARON process

SHARON process is an acronym for Single reactor system for High activity Ammonium Removal Over Nitrite. It was first studied in Technical University of Delft in 1994 to treat reject water in a single reactor without sludge retention (Hellenga et al., 1998). The SHARON system is basically a continuous nitrification system operated as a chemostat ($SRT = HRT = 1$ day) at relative high temperature (30-40 °C) and pH (7-8) (Hellenga et al., 1998). The process can save 25% aeration

energy required for nitrification and 40% carbon source required for denitrification; reduce 30% sludge production and 20% CO₂ emission (van Kempen et al., 2005). But it has limited capacity; the total nitrogen load of the effluent can only be decreased by 30% (van Kempen et al., 2001).

2.3.6 BABE technology

BABE is an acronym for Bio-Augmentation Batch Enhanced (Salem et al., 2004). The concept is augmented with the endogenous nitrifying bacteria present in the return sludge from the main stream. The cultivation is done in a BABE reactor, which is located in the side stream line, using reject water and small amount of return sludge (Salem et al., 2003). The SRT in the BABE reactor should be optimized to achieve maximum inoculation. The best performance can only be achieved at low temperature; hence the implementation of the BABE technology in Singapore is limited due to the hot weather.

2.3.7 Anammox process

Anammox process is an acronym for the ANaerobic AMMonia OXidation. It is a lithoautotrophic biological conversion process mediated by planctomycete bacteria (Strous et al., 1999). This process consists of partial nitrification of 55 to 60% of the ammonium and the subsequent conversion of the remaining ammonium in the presence of nitrite to nitrogen gas under anoxic conditions (Mulder et al., 1995); the molar ratio of ammonium to nitrite must be approximately from 1 to 1.3 (Equation 2-9) (Strous et al., 1998).

Anammox process has been widely used for reject water treatment in both laboratory scale (Furukawa et al., 2009; Vazquez-Padin et al., 2009a) and full scale (Joss et al., 2009). It helps to save over 50% oxygen without any external organic carbon source for denitrification process (Fux et al., 2002; 2004). However, it has long start-up time due to the slow grow rate of Anammox biomass (Van Dongen et al., 2001). Moreover, the Anammox bacteria are reversibly inhibited by oxygen and irreversibly inhibited by nitrite (at concentration in excess 70 mg N/L for several days) and phosphate (> 60 mg P/L for several days) (Strous et al., 1997), it will be inhibited if reject water contains too much phosphate.

2.3.8 The SHARON/Anammox process

The combination of stable partial nitrification in first reactor (SHARON) with the anaerobic ammonium oxidation in second reactor (Anammox) is an effective option for the removal of ammonium from reject water.

The combined SHARON/Anammox process for ammonium removal does not require external carbon source and can be operated independently. Hence, it is possible to optimize the COD and nitrogen removal separately (van Dongen et al., 2001). It has been successfully applied in both lab scale (Fux et al., 2002; van Dongen et al., 2001) and full-scale facilities for reject water treatment (Fux and Siegrist, 2004). Yet it is difficult to maintain a constant 1:1.3 molar ratio of ammonium to nitrite due to the variable nitrogen load. The low growth rate of Anammox bacteria and the low biomass yield make the start-up period taking longer (several months) even under favorable conditions (Fux and Siegrist, 2004).

2.3.9 CANON process

CANON is an acronym for Completely Autotrophic Nitrogen removal Over Nitrite. This concept is the combination of partial nitrification and Anammox in a single-aerated reactor (Third et al., 2005). The ammonium is removed in a single reactor by *Nitrosomonas*-like aerobic bacteria and *Planctomycete*-like anaerobic bacteria (Sliekers et al., 2002).

The mechanism of CANON process is based on biofilm technology. CANON has been successfully performed for reject water treatment (Szatkowska et al., 2007; Vazquez-Padin et al., 2009b). The main challenge is the long start-up period of the Anammox step, which take even half a year, and the CANON process has quite sensitive operational characteristics such as DO, ammonium surface load, biofilm thickness, and temperature, etc (Ahn, 2006; Third et al., 2005).

2.3.10 OLAND process

OLAND is an acronym for Oxygen-Limited Autotrophic Nitrification-Denitrification. The active enrichment cultures of autotrophic nitrifiers are first cultivated in Laboratory of Microbial Ecology at Ghent University (Belgium) (Verstraete and Philips, 1998). The main difference between the OLAND and CANON process is that the OLAND process makes good use of the denitrifying activity of conventional nitrifying bacteria, whereas the CANON incorporates the Anammox process in one single reactor (Ahn, 2006). However, Pynaert et al. (2003) showed that the Anammox bacteria were responsible for the denitrification in the OLAND process.

OLAND process is simple and reliable for nitrogen removal from ammonium-rich wastewater such as reject water at suitable loading rate (Kuai and Verstraete, 1998). The removal of nitrogen from digested black water (> 1000 mg N/L ammonium) by

OLAND rotating biological reactor can achieve nitrogen removal rate of 700 mg N/L d (Vlaeminck et al., 2009). However, the mechanism of the OLAND process has not been thoroughly understood (Ahn, 2006).

2.3.11 Aerobic/anoxic deammonification process

The aerobic/anoxic deammonification process is termed by the University of Hanover (Germany) (Hippen et al., 1997). The process can be defined as a combination of preceding nitrification and succeeding anaerobic oxidation of the remaining ammonium with nitrite as electron acceptor (Helmer-Madhok et al., 2002; Hippen et al., 2001). The mechanism is based on CANON concept and the nitrifiers and Anammox bacteria can cooperate under oxygen limiting conditions (Schmidt et al., 2003). It is a highly promising method for treatment of ammonium-rich wastewater. The process has been applied in pilot scale of moving-bed biofilm reactor (Gut et al., 2006) and SBR (Jeanningros et al., 2010) in reject water treatment. However, the long start-up period of the Anammox step and sensitive operational characteristics are disadvantageous.

2.4 Removal/recovery of phosphorus

Phosphorus, a multivalent nonmetal element, is commonly found in the form of apatite - $\text{Ca}_{10}(\text{PO}_4)_6\text{X}_2$ (X being F in fluorapatite, OH in hydroxyapatite, or Cl in chlorapatite). This type of inorganic rock contains about 95% of all P in the Earth's crust (Smil, 2000). The resource is nonrenewable and can only support the global P consumption for another 80 to 250 years (Smil, 2000). Therefore, the recovery of phosphorous from reject water might compensate partially the deficiency. More importantly, the recovery helps to prevent eutrophication in aquatic systems as phosphorous content in MWWTP discharge will be lowered to acceptable level.

2.4.1 Chemical precipitation

The main pathway of phosphate removal is through a combination of precipitation, adsorption, exchange, and agglomeration. Extremely low orthophosphate concentration (< 0.02 mg P/L) can be achieved over a wide range of pH (5.7 to 8) by chemical precipitation (Takacs et al., 2006). In chemical precipitation, a divalent or trivalent metal salt is added to precipitate insoluble metal phosphates in wastewater. The most staple metals are iron (both ferric and ferrous) and aluminum in the form of chloride or sulphate salt (De-Bashan and Bashan, 2004). The usage of lime is also common. Some polymers may be used effectively in conjunction with alum and lime to aid sedimentation and solid separation. The selection criteria for precipitation chemical include influent phosphorus level, wastewater suspended solids, alkalinity, chemical cost, reliability of chemical supply, sludge handling facilities, final disposal methods, and compatibility with other treatment processes (De-Bashan and Bashan, 2004).

2.4.1.1 Precipitation of phosphorus by aluminum salt

The most commonly used aluminum compound in phosphorus removal is alum-aluminum sulfate. The reaction is as follows (Hammer and Hammer, 2004):

The theoretical molar ratio of Al to P is 1 to 1. Coagulation studies suggest a greater alum dosage to account for a competing reaction with natural alkalinity:

Optimum removal of phosphorus is conditioned with pH value in the range of 5.5 to 6.5. Aluminum compounds cannot be used as soil amendment due to its phytotoxicity.

2.4.1.2 Precipitation of phosphorus by iron salts

Commercial availabilities of iron salts are ferric chloride, ferric sulfate, and ferrous sulfate. Both ferrous and ferric ions can form the precipitates with phosphates as follows (Hammer and Hammer, 2004):

Though the theoretical Fe:P molar ratio is 1:1 for ferric salt and 1.5:1 for ferrous salt, the excess amounts of iron (especially ferrous) will be added to wastewater as a result of competing hydroxide and sulfide reactions (Takacs et al., 2006; Fytianos et al., 1998). When the residual P concentration in secondary effluent is about 0.5 mg P/L, dosage of iron salts usually falls in the range of 1 to 3 Fe/P on a molar basis or higher (Water Environment Federation, 2006). However, iron-phosphate precipitation will consume the alkalinity of wastewater and suppress the pH of process water. Iron compounds are most effective for P removal at pH values between 7 and 8. The addition of lime or sodium hydroxide might be necessary to compensate the lost alkalinity. There will be a significant quantity of sludge produced (Water Environment Federation, 2006).

The iron precipitate produced is potential fertilizer. Johnston and Richards (2003) suggested that part of the precipitate was beneficial to plants. The drawback is that the surplus iron ions in the sludge will bond to soluble phosphate in the soil. High cost of iron salt is another burden of this method.

2.4.1.3 Phosphorus precipitation by lime

When lime, in the form of either hydrated lime $\text{Ca}(\text{OH})_2$ or quicklime CaO , is used, both calcium and hydroxide will react with orthophosphates to form insoluble hydroxyapatite (De-Bashan and Bashan, 2004).

Typical quantity of lime needed to precipitate phosphate in wastewater is from 1.4 to 1.5 times of the total alkalinity as CaCO_3 (Metcalf & Eddy, 2004). Lime–phosphate precipitation is a common method for phosphorus removal from wastewater due to the low cost. However, it increases sludge quantity by 50% and volume by up to 150%. The sludge furthermore cannot be used for land application; high operation and maintenance costs in association with problematic handling, storage, and feeding of lime further discourage users from this application (Water Environment Federation, 2006).

2.4.2 Crystallization

Crystallization is based on seed crystal-induced phosphate deposition on mineral surfaces (Smil, 2000). This simple technology is free from precipitants and can avoid the increase in sludge volume. The product obtained can either be used directly as fertilizer or indirectly as a raw phosphate substitute in fertilizer industry.

Both magnesium ammonium phosphate (MAP or Struvite) and calcium phosphate (hydroxyapatite, HAP) could be the recovered product depending on the seed material used and wastewater composition. HAP is more preferable than MAP with respect to the application in phosphate production industry. The crystallization of HAP and MAP for phosphorus removal from reject water follows the equations

below (Momborg and Oellermann, 1992):

The removal/recovery of phosphorus from wastewater by MAP process (Uludag-Demirer and Othman, 2009) and HAP process has been successfully achieved by some researchers (Berg et al., 2006; Song et al., 2006). However, both processes have low crystallization rate and high chemical cost. And MAP is affected by high molar ratio of $\text{Ca}^{2+}/\text{PO}_4^{3-}$ -P (Moerman et al., 2009).

2.4.3 Biological phosphorus removal

Phosphorus is removed in biological treatment by means of incorporating orthophosphate, polyphosphate, and organically bound phosphorus into cell tissues. Enhance biological phosphorus removal (EBPR) or luxury uptake of phosphorus is the result of microbial action in the activated sludge process (Bitton, 1999). It relies on the selection and proliferation of phosphate-accumulating organisms (PAOs), which are capable of storing orthophosphate in excess of their biological growth requirements. PAOs can grow and function under special design and operational conditions. In anaerobic zone, PAOs convert organics to energy-rich carbon polymers called poly-hydroxyalkanoates (PHAs). The energy is derived through breakdown of polyphosphate molecules to orthophosphate, resulting in the increase of phosphate concentration in this stage. In the aerobic stage, PAOs can oxidize previously stored PHAs to obtain energy for the growth and maintenance, concurrently uptake and polymerize the phosphorus. Therefore the effluent from the EBPR reactors is low in phosphorus. The excessive accumulation of orthophosphate in the microorganisms of mixed liquor, which should be adequately wasted, results

in the removal of P as much as 80-95% (De-Bashan and Bashan, 2004; Water Environment Federation, 2006).

The main microbial populations in EBPR process are bacteria of the beta-2 subclass of *Proteobacteria* (De-Bashan and Bashan, 2004). Another common organisms found in EBPR process with poor phosphorus removal are Glycogen accumulating organisms (GAOs). EBPR process will fail if GAOs are dominant. Therefore, the competition between the PAOs and GAOs should be carefully controlled. Other than that, wastewater composition, volatile fatty acids, cations, temperature, DO concentration, nitrate concentration in anaerobic stage, pH, etc can also affect EBPR operation (Mulkerrins et al., 2004). And some optimal operation factors are listed in Table 2.1 (Water Environment Federation, 2006).

Table 2.1 Some optimal operational parameters for EBPR system

Operational parameters	Optimal value
Total COD:TP	45
Solid retention time (SRT)	1.5-2 days
Hydraulic retention time (HRT)	1-2 hours in anaerobic zone
Temperature	20 °C
pH	5.0-6.6

In terms of categorization, a variety of EBPR systems have been reported (Metcalf & Eddy, 1991). Anaerobic/oxic (A/O) process and combined biological-chemical process (Phostrip process) are designed for phosphorus removal. The combined nitrogen and phosphorus removal system such as anaerobic/anoxic/aerobic (A²/O) system, five-stage Bardenpho system, University of Cape Town (UCT) system, Virginia Initiative Process (VIP) system, and SBR system are also demonstrated. The EBPR system can reduce TP effluent concentration to below 1 mg P/L.

However, the bacteria in EBPR process are sensitive to environmental conditions; therefore, the stability and reliability of EBPR process are fluctuated. The high chemical cost for precipitation/crystallization contributes to the huge operating cost. Therefore, the optimum solution for phosphorus removal from reject water is chemical precipitation of phosphorus using biologically produced precipitant. To fulfill such requirement, BioIronTech process, which will be studied in details in Chapter 3, is proposed. Simultaneously, nitrogen in reject water can be removed by combining this BioIronTech process with the sequential biooxidation of NH_4^+ and bioreduction of NO_3^- . Detailed description of this process will be presented in Chapter 4.

2.5 Iron reduction by iron reducing bacteria and the BioIronTech process

2.5.1 Iron reduction

Iron ranks the fourth in most abundant elements in the earth's crust. In nature, the dominant redox states of iron are Fe(II) and Fe(III). The redox cycle between the two can be achieved by chemical reaction or biological reaction. The dissimilatory Fe(III) reduction refers to the process in which microorganisms conserve energy from the coupling of organic compounds and/or H_2 oxidation to ferric reduction under anaerobic conditions (Fedrickson and Gorby, 1996; Ivanov and Hung, 2010; Lovley and Anderson, 2000). The microorganisms are often called dissimilatory iron reducing bacteria (DIRB), dissimilatory metal reducing bacteria (DMBR) or Fe(III)-respiring microorganisms (FRM) (Lovley, 2000). DIRB are believed to play an important role in the geochemical cycling of iron as well as other elements such as carbon, sulfur, phosphorous, manganese, and other trace metals.

2.5.2 Iron reducing bacteria

Iron reducing bacteria are increasingly being recognized as an ecologically and environmentally important group of microorganism. The phylogenetically diverse IRB have been isolated from various sources (Luu and Ramasy, 2003) including sediments, soils, deep terrestrial areas, and hot springs (Straub et al., 2001). *Geobacter metallireducens* is the first pure culture discovered to completely oxidize acetate using Fe(III) as the electron acceptor (Fredrickson and Gorby, 1996; Luu and Ramasy, 2003). The genera *Geobacter*, together with *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* belong to the delta subdivision of the *Proteobacteria*, members of these genera which comprise the *Geobacteraciae* share a common Fe(III)-reducing ancestor (Fredrickson and Gorby, 1996).

Yet the ability to reduce Fe(III) is not limited to *Geobacteraciae*. In the gamma subclass of *Proteobacteria*, organisms such as *Shewanella putrefaciens*, *Shewanella alga*, and *Ferrimonas balearica* can also achieve dissimilate ferric reduction (Lovley, 2000). The Fe(III)-reducing bacteria sometimes do not originate from the *Proteobacteria*. *Geovibrio ferrireducens* isolated from hydrocarbon-contaminated surface sediments and *Geothrix fermentans* isolated from petroleum-contaminated aquifers are two phylogenetically distinct IRB. The phylogenetically diverse placement of these novel organisms indicates that the ability to reduce Fe(III) is spread throughout the *Bacteria* and *Archaea* domains (Luu and Ramasy, 2003).

2.5.3 The mechanism of microbial iron reduction

Understanding the mechanism of electrons transfer to Fe(III) is important in determining the rate and extent of iron reduction. To reduce Fe(III), iron reducing bacteria must be able to either (i) attach to the iron substrate and directly transfer electrons to it, or (ii) solubilize the iron and deliver it to the electron transport chain, or (iii) use available/produce their own electron shuttling compounds. Due to the

phylogenetic diversity of DIRB, it is likely that more than one strategy for Fe(III) bioreduction exist.

Direct contact: Iron reducing bacteria can directly contact with the insoluble Fe(III) mineral and transfer electrons to it. However, the relation between Fe(III) reduction rate and the amount of cells contacted with Fe(III) mineral is not straightforward; in some cases, the bacteria adhesion to iron ore is not a prerequisite for Fe(III) reduction (Caccavo et al., 1997). It was reported that iron reducing bacteria *Shewanella oneidensis* MR-1 reduced Fe(III) in the hematite (Fe_2O_3) nanoparticles by direct and indirect transfer of electrons to Fe(III)-oxide nanoparticles (Bose et al., 2009). Yan et al. (2008) also demonstrated the reduction of hematite nanoparticles by iron reducing bacteria *Geobacter sulfurreducens* through direct contact of bacteria and hematite, and the reduction rate was associated with the bacteria-hematite contact area. It was also known that iron reducing bacteria *Geobacter metallireducens* synthesized lateral flagella and pili to contact with insoluble Fe(III) oxides (Childers et al., 2002). Reguera et al. (2005) reported that iron reducing bacteria *Geobacter sulfurreducens* reduced Fe(III) oxides by direct attachment through the pili, which was highly conductive and served as nanowire to transfer the electrons from bacteria to Fe(III) oxides.

Electron transport chain: The studies of *Shewanella spp.*, Myers and Myers (1992) suggested that DIRB could transfer electrons by localizing c-type cytochromes in its outer membrane, the contact area with Fe(III) oxides. DiChristina et al. (2002) found that a 91 kDa heme-containing protein associated with the outer membrane fraction of *Shewanella putrefaciens* strain 200 was related to Fe(III) reduction. Other researchers also agree that Fe(III) reductase is membrane bound enzyme that participate in the generation of proton gradient and enabling contact with insoluble Fe(III) oxides, the terminal electron acceptors (Gaspard et al., 1998; Magnuson et

al., 2000).

Humic substances electron shuttling compounds: Humic substances (humics), derived from the degradation of organisms, act as electron-shuttling compounds between DIRB and insoluble Fe(III) oxides. DIRB oxidize organics by transferring electrons to humic substances. The reduced humics then abiotically transfer electrons to Fe(III), regenerating the oxidized form of the humics (Scott et al., 1998). The electron-shuttling by humics alleviates the necessity for direct contact between DIRB and Fe(III) oxides and accelerates Fe(III) reduction (Lovley and Woodward, 1996; Nevin and Lovley, 2000). Roden et al. (2010) further demonstrated that solid-phase humic substances accepted electrons from iron reducing bacteria and shuttled electrons from bacteria to oxide surface to significantly accelerate iron (III) oxide reduction.

2.5.4 Factors affecting microbial iron reduction process

Some factors such as crystal structure, morphology, particle aggregation, free energy (Luu and Ramasy, 2003), surface area, pH, nutrient supply, and Fe(II) sorption to mineral and cell surface (Fredrickson and Gorby, 1996) were reported to influence the rate and extent of iron reduction.

The structure of Fe(III) compounds: Fe(III) hydroxide minerals are common in oxidized terrestrial subsurface sediments (Fredrickson and Gorby, 1996). Typical minerals in aerobic aquifer solids include ferric hydroxide ($(\text{Fe}(\text{OH})_3)$), lepidocrocite ($\text{FeO}(\text{OH})$), maghemite ($\gamma\text{-Fe}_2\text{O}_3$), magnetite (Fe_3O_4), hematite (Fe_2O_3), and goethite ($\alpha\text{-FeO}(\text{OH})$) (Fredrickson and Gorby, 1996). The form of Fe(III) has an important effect on its bioavailability. In general, the more crystalline the Fe(III) oxide is, the less susceptible it is to microbial reduction. IRB can reduce amorphous

and poorly crystalline iron oxide easier than that in crystalline phase (Lovley, 1991). Previous studies also confirmed that DIRB reduced only amorphous and poorly crystalline iron oxides in natural sediments (Kostka et al., 1999). The slow reduction rate of crystalline Fe(III) oxide by IRB in nature prevents them from competing with sulfate-reducing or methanogenic microorganisms for electron donors. Therefore, the poorly crystalline Fe(III) oxide, typically present as coating on clays or other surfaces, is the primary source of Fe(III) for the IRB (Luu and Ramasy, 2003). However, the crystalline Fe(III) oxides, such as goethite and hematite, can be reduced under controlled experimental conditions (Bose et al., 2009; Roden and Zachara, 1996; Royer et al., 2004; Yan et al., 2008).

The surface area of Fe(III) mineral in relation to the adsorption of produced ferrous: The surface area of Fe(III) mineral controls its bioavailability hence the rate of reduction. Higher reaction rate is expected with larger surface area. The surface area of amorphous Fe(III) is greater than that of crystalline Fe(III), thus IRB prefer to reduce the amorphous one. Roden and Zachara (1996) suggested that the initial rate and long-term extent of reduction reaction were linearly correlated with oxide surface area. Royer et al. (2004) concluded that bioreduction rate of powder hematite, the main component in iron ore, increased with improved mass transport condition. However, Yan et al. (2008) suggested that the reduction rate of hematite nanoparticles by *Geobacter sulfurreducens* was not directly affected by the total hematite surface, but was proportional to the bacteria-hematite contact area.

Clearly, the reactive surface area of iron oxide affects the initial rate of iron ore reduction by IRB (Roden and Zachara, 1996; Roden, 2003). The accumulated ferrous in the system has negative effects on iron reduction (Royer et al., 2004). The adsorption/precipitation of Fe(II) on iron reducing bacteria inhibits their activities (Liu et al., 2001a; Urrutia et al., 1999). The surface-bound Fe(II) to Fe(III) oxide

also negatively affects crystalline iron(III) oxide reduction in the same manner (Roden and Zachara, 1996). As a solution, aqueous Fe(II)NTA complexant is used to prevent/delay Fe(II) sorption to oxide/iron reducing bacteria (Urrutia et al., 1999). The removal of produced ferrous via aqueous-phase transport similarly improves the situation (Roden et al., 2000; Roden and Urrutia, 1999). Yan et al (2008) suggested that the formation of vivianite to sequester Fe(II) positively affected the higher area-normalized iron reduction rate of hematite nanoparticles.

The concentrations of electron donors and acceptors: Iron reducing bacteria anaerobically reduce Fe(III) by consuming H_2 or organic substrate. Obviously the rate and extent of iron reduction are deemed to be closely related with the concentrations of electron acceptors and donors. Liu et al. (2001b) observed the enhancement in both rate and extent of Fe(III) reduction when goethite concentration or lactate concentration was increased. Fredrickson et al. (2003) reported that the increasing HFO:lactate ratio resulted in decreasing Fe(III) reduction rate. Similarly, the impact is shown in the coexistence of iron reduction and nitrate/nitrite reduction. Since nitrate reducers out-compete iron reducers for electron donors, the iron reduction process is usually inhibited (Dichristina, 1992; Sørensen, 1982). Therefore, it can be concluded that the availability of organic carbon as electron donors affects the success of iron reduction process.

2.5.5 BioIronTech process

BioIronTech process: BioIronTech process is an innovative technology combining biological and chemical processes. The principle of it has been described in the US patent (Tay et al., 2008). The process includes anaerobic and aerobic steps. At first, the low-cost Fe^{3+} source (iron ore) is anaerobically reduced to Fe^{2+} by iron reducing bacteria using organic substances present in wastewater:

The ferrous ions produced would precipitate phosphate in wastewater according to the following equation:

In the second step, ferrous phosphate is chemically or biologically oxidized by oxygen:

The application of BioIronTech process in environmental engineering: The feasibility of this application has been tested in: 1) anaerobic treatment of fat-containing wastewater due to precipitation of long chain fatty acids with ferrous ions (Ivanov et al., 2002); 2) the enhancement of anaerobic treatment of sulfate-containing wastewater due to competitive inhibition of sulfate reduction by ferric reduction (Ivanov et al., 2004a; Stabnikov and Ivanov, 2006); 3) treatment of food-processing wastewater (Ivanov et al., 2004b); 4) the enhanced removal of phosphate from reject water of MWWTP (Ivanov et al., 2005); 5) The improvement of sludge quality (Ivanov et al., 2004a); 6) Biodegradation and complete removal of xenobiotics such as diphenylamine, m-cresol, 2, 4-dichlorophenol, and p-phenylphenol (Ivanov et al., 2005; 2010).

Other potential applications could be the bio-restoration of eutrophicated lakes and ponds; the maintenance of water quality in aquaculture ponds; the production of ferrous coagulant for pretreatment of industrial wastewater, desalination of sea water, and desilting of storm water; the biocementation of dams, slopes and land reclamation sites; the microbially-enhanced oil recovery; the treatment of landfill leachate; the removal of arsenic and other heavy metals as well as radionuclides from groundwater (Ivanov and Hung, 2010; Tay, et al., 2008).

The perspectives of applying BioIronTech process to remove phosphorus from reject water: 1 ton of Fe in iron ore costs approximately US\$140, while 1 ton of Fe in FeCl_3 is 7.2 times more expensive (De-Bashan and Bashan, 2004). Moreover, reject water contains a lot of volatile fatty acids, which are good electron donors and sufficient for iron reducing bacteria; the addition of external carbon source is normally not necessary. The iron ore supplied from China contains 60% of Fe, which is equal to 0.6 ton Fe/ ton iron ore, based on the equations (2-17 & 2-18), one ton iron can remove about 571 kg P and the cost of iron ore for P removal is estimated about US\$ 245 per ton of P removed. Therefore, it is very cost effective to precipitate phosphorus in reject water using ferrous produced from the coupling of anaerobic iron reduction to organic carbon oxidation. Though crystalline hematite (Fe_2O_3) in iron ore is relatively recalcitrant to iron reducing bacteria (Lovley, 1991), the successful bioreduction of powder hematite (Royer et al., 2004) and hematite nanoparticles (Bose et al., 2009; Yan et al., 2008) was demonstrated. The numerous amounts of powders covering iron ore particles create large surface areas as well as large reactive areas in iron reduction. This characteristic of iron oxides considerably affects the initial rate of iron ore reduction by IRB (Roden and Zachara, 1996; Roden, 2003). Therefore, in order to increase the reduction rate, coarse iron ores should be crushed into smaller particles.

2.6 The potential interactions between nitrogen and iron cycles in reject water treatment

The potential interactions between nitrogen and iron cycles during reject water treatment are shown in Fig. 2.2. The interaction can be either competitive or collaborative.

2.6.1 Competitive interaction

The competitive interaction is due to the inhibition of Fe^{3+} reduction by nitrate and nitrite. The inhibitory effect has been reported in anaerobic sediment (Sørensen, 1982) and laboratory culture of *Shewanella putrefaciens* (Dichristina, 1992). It was reported that the presence of iron oxide also partially inhibited NO_3^- and NO_2^- reduction (Cooper et al., 2003; Coby and Picardal, 2005).

The mechanisms of partial inhibition of iron reduction by nitrate are summarized: 1) denitrifiers out-compete iron reducing bacteria for electron donors due to the different redox potentials; 2) the biologically produced NO_2^- inhibites the transfer of electrons to ferric iron (Dichristina, 1992); 3) the biologically produced NO_2^- abiotically reacts with cell surface-adsorbed Fe^{2+} to produce N_2O and $\text{Fe(III)(hydr)oxide}$, the produced $\text{Fe(III)(hydr)oxide}$ can coat the cell and inhibit the transport of soluble electron acceptors into the cell (Cooper et al., 2003; Coby and Picardal, 2005).

However, the inhibitory effect can be relieved by complete removal of nitrate or nitrite (Sørensen, 1982) or the removal of biogenic Fe(II) to reduce the effects of Fe(II) binding to the iron(III) surface (Roden and Urrutia, 1999; Roden et al., 2000). In the continuous anaerobic reactor, the removal of Fe(II) through aqueous-phase transport is important.

Fig. 2.2 schematic representations of potential interactions between iron and nitrogen cycles: Bold arrows show the proposed process for nitrogen removal from return liquor; 1: volatilization; 2 and 3: nitrification; 2 and 7: partial nitrification and denitrification or SHARON process; 4 and 6: Anammox process; 2 and 6 and 4: combined SHARON/Anammox process, CANON process, OLAND process, aerobic/anoxic deammonification process; 4 and 8: anaerobic ammonium oxidation coupled to nitrate reduction; 9-12: denitrification; 5 and 20: nitrate dependent ferrous oxidation coupled to nitrate reduction; 11 and 20: nitrite dependent ferrous oxidation coupled to nitrite reduction; 19: iron (III) reduction by iron reducing bacteria; 16 and 21: ammonium anaerobic oxidation coupled to iron reduction (ferric ammonium oxidation process); 15, 22, 25 and 17: the ferrous wheel hypothesis; 13, 23, 14 and 24: BioDeNO_x process; 18: aerobic iron oxidation process (can be chemically oxidized or biologically oxidized mediated by iron-oxidizing bacteria); 20: anaerobic/anoxic ferrous oxidation (can be abiotically oxidized with catalyst or biologically oxidized by anaerobic phototrophic bacteria at light or by nitrate/nitrite dependent ferrous oxidizing denitrifiers); 26: nitrate abiotic reduction with (Fe(II)₄Fe(III)₂(OH)₁₂SO₄·yH₂O) as reductant.

2.6.2 Concomitant and collaborative interactions

The concomitant interaction between nitrate reduction and iron(III) reduction occurs during anaerobic mineralization of organic substrates (such as phenol) (Broholm et al., 2000). The collaborative interactions between nitrate/nitrite reduction and iron reduction/oxidation can enhance autotrophic removal of nitrate. At neutral anoxic environment (pH 7), all the redox pairs of the nitrate reduction pathway have higher potential than that of ferric/ferrous redox pairs (Straub et al., 2001). Therefore, nitrate, nitrite, nitric oxide, and nitrous oxide are sequentially favorable electron acceptors for ferrous iron oxidation. Under the catalysis of Cu^{2+} at pH of 8, Fe(II) produced from anaerobic reduction of Fe(III) can be abiotically re-oxidized to Fe(III) by coupling with the reduction of nitrite/nitrous oxide to N_2 (Moraghan and Buresh, 1977). The same coupling can be conducted biotically by the anaerobic nitrate/nitrite-dependent denitrifiers in the dark (Straub et al., 1996; Benz et al., 1998). Trace amount of N_2O is produced in this process as byproduct. When light exists, the biological oxidization is additionally contributed by the anaerobic, phototrophic, purple bacteria (Widdel et al., 1993; Ennenreich and Widdel, 1994). The denitrifiers are not inhibited in this case (Straub et al., 2001).

Oxidation of ferrous by phototrophic anaerobic purple bacteria needs sunlight, which may limit the combination of these bacteria with iron reducing bacteria in anaerobic treatment of reject water. Therefore, the nitrate/nitrite-dependent denitrifiers should be more dominant in the bioreactor. The idea has been confirmed by Staub et al. (1996) and Benz et al. (1998). Hauck et al. (2001) reported that nitrate-dependent ferrous oxidizing bacteria made up to 58% of the total cultivable denitrifiers. One facultative autotrophic pure culture of nitrate-dependent Fe(II)EDTA oxidizer has been isolated from denitrifying bioreactor and named as *Paracoccus ferrooxidans* strain BDN-1. This bacteria can use nitrate, nitrite, N_2O ,

Fe(II)EDTA, and oxygen as electron acceptors (Kumaraswamy et al., 2006). Ferrous mediated denitrification can be important during reject water treatment.

The application of nitrate/nitrite-dependent ferrous oxidation process to remove nitrate has been demonstrated in paddy soil (Ratering and Schnell, 2001) and activated sludge anoxic (denitrification) process (Nielsen and Nielsen, 1998a; 1998b). The highest nitrate removal rate of 0.31 mmol $\text{NO}_3^-/\text{g VSS} \cdot \text{h}$ has been achieved at the optimum pH of 8 with nitrogen gas and Fe(III) as final products (Nielsen and Nielsen, 1998b). Ferrous-dependent nitrate reduction has also been used in BioDeNO_x process. NO_x is absorbed in FeEDTA solution to form FeEDTA(NO), then FeEDTA(NO) is regenerated by denitrification to produce Fe(III)EDTA and nitrogen gas; finally, the Fe(III)EDTA is reduced by iron reducing bacteria to Fe(II)EDTA (Maas et al., 2004). Fe(II)citrate has also been applied in the BioDeNO_x process (Xu and Chang, 2007).

Fig. 2.3 The potential collaborative interactions between iron reduction/oxidation and nitrate reduction.

During the collaborative interaction (Fig. 2.3), ferrous produced from ferric reduction can serve as electron donor for denitrification, while ferric hydroxide produced from ferrous oxidation can be as good electron acceptor for iron reduction. In short, the nitrate in reject water may be removed both heterotrophically and autotrophically.

2.6.3 The interactions between ammonium oxidation and iron/nitrate reduction

Under anaerobic conditions, ammonium oxidation could be coupled to ferric reduction to produce nitrite (ferric ammonium oxidation process) (Clément et al., 2005) or coupled to nitrate reduction to produce nitrogen gas (Thamdrup and Dalsgaard, 2002). Moreover, nitrate can also be abiotically reduced by green rust ($\text{Fe(II)}_4\text{Fe(III)}_2(\text{OH})_{12}\text{SO}_4 \cdot y\text{H}_2\text{O}$) to produce ammonium and magnetite (Fe_3O_4) (Hansen et al., 1996) (Fig. 2.4).

Fig. 2.4 The interactions between ammonium anaerobic oxidation and iron/nitrate reduction.

2.6.4 The ferrous wheel hypothesis

During the ferrous wheel hypothesis, Fe(II) reduces nitrate to nitrite, and the reduced form of carbon (such as alcohol) regenerates Fe(II) to produce Fe(III) . Nitrite reacts with dissolved organic matters (DOM) to produce dissolved organic nitrogen (DON); both biotic and abiotic processes are involved in the ferrous cycle (Davidson et al., 2003) (Fig. 2.5).

Fig. 2.5 The ferrous wheel hypothesis.

2.6.5 The potential interactions between carbon source utilization, phosphorus removal, iron reduction/oxidation, and nitrate removal in reject water treatment

Under anaerobic conditions, the carbon source is not only the electron donor but also partially the driving force in iron and nitrogen cycles. The carbon source affects the rate of heterotrophic denitrification, and also supplies electrons to iron reducing bacteria. The removal of phosphorus from reject water using anaerobic reduction of Fe(III) by iron reducing bacteria has also been demonstrated (Ivanov et al., 2005). The complicated potential interactions between carbon source utilization, phosphorus removal, iron reduction/oxidation, and nitrate removal during anaerobic treatment of reject water would be discussed in chapter 5 and 6 (Fig. 2.6).

Fig. 2.6 The potential interactions between organic carbon oxidation, phosphorus removal, iron reduction/oxidation, and nitrate reduction during anaerobic treatment of reject water.

2.7 The removal of nitrogen and organics from reject water in Biofilm process

2.7.1 Biofilm process

Biofilm is a complex coherent structure of microbial cells and cellular products, like extra-cellular polymers (van Loosdrecht et al., 1995b). In the biofilm, microorganisms are immobilized /attached to static solid face (Liu and Tay, 2002) or suspended solid particles (Nicolella et al., 2000). They are coated with glycocalyx that sequestered and protected them from harm and toxicants (Bishop, 1997). Therefore, the bacteria in biofilm are more tolerant to the changes of environmental conditions such as temperature, pH, nutrient condition, and toxic substances. In comparison to activated sludge process, biofilm has certain advantages such as the coexistence of different microbial populations that enable it

to perform various biological reactions to degrade a variety of substrates and the glycocalyx coating layer that protect microorganisms in biofilm from harm and toxicants (Bishop, 1997). Moreover, the high biomass concentration in biofilm ensures high volumetric loading and fast biodegradation rate.

2.7.2 The formation of biofilm

The biofilm formation involves physical, chemical, and biological processes. Jefferson (2004) proposed 4 driving forces for biofilm formation: 1) a stress response and the protection of bacteria from harmful conditions in the host (defense); 2) to remain in a favorable niche (colonization); 3) utilization of cooperative benefits (community); 4) a default mode of growth. Simoes et al. (2010) listed 9 processes in biofilm formation including: 1) pre-conditioning of the biofilm carrier surface by molecules in the bulk liquid; 2) the deposition of biomass on the carrier surface; 3) adsorption of bacteria at the carrier surface; 4) desorption of reversibly adsorbed bacteria from surface; 5) irreversible adsorption of bacteria at the carrier surface; 6) production of cell-cell signaling molecules; 7) transport of substrates and oxygen to the biofilm; 8) biofilm bacteria utilization of substrates for cell growth, EPS production, and transport of products out of the biofilm. 9) biofilm removal by the detachment or sloughing.

To cultivate biofilm successfully, some operating conditions should be fulfilled. It was known that the surface roughness of carrier enhanced the development of biofilm (Heijnen et al., 1992). The detachment of biomass from biofilm is mainly influenced by shear force (Gjaltema et al., 1995). The surface roughness protects the adhering cells from detachment by shear force to maintain long enough on the surface for irreversible adhesion and biofilm development, therefore, it is one of the dominating factors for biofilm growth (van Loosdrecht et al., 1988).

Other than carrier roughness, the operational conditions affecting the biofilm formation and development substantially include:

Dilution rate: The high dilution rate of system could prevent the biomass growth in suspension. High dilution rate is applied as a selection pressure for biofilm development; the hydraulic retention time should be shorter than the inverse of the maximum growth rate of the suspended bacteria to obtain stable biofilm with high biomass concentration, but should be long enough for the slow growth rate of nitrifying biofilm to avoid the complete wash out of the suspended nitrifying biomass (Tijhuis et al., 1994a).

Shear force: Shear force, resulting from hydraulics and /or particle-particle collision, is considered as one of the most influencing factors in the formation, structure, and stability of biofilm (van Loosdrecht et al., 1995a; Liu and Tay, 2002). The certain shear force enhances the formation of proper and stable biofilm; higher shear force helps to form thin, dense, and compact biofilm (Kwok et al., 1998; van Loosdrecht et al., 1995a). Low shear force on the other hand leads to the formation of heterogeneous porous and thick biofilm despite the high substrate loading rate (Tijhuis et al., 1995a; van Loosdrecht, 1995a). Similarly, Massol-Deya et al. (1995) observed that biofilm formed heterogeneous porous structure under low shear force, hence caused instabilities and sloughing phenomena

Substrate loading rate: Biofilm thickness increases with substrate loading rate and decreases with shear force, while biofilm density increases with the high shear stress (Chang et al., 1991; Wijeyekoon et al., 2004). Peyton (1996) noticed that biofilm roughness, measured by the standard deviation of the thickness, increased with the thickness, and area mass density increased with substrate loading rate instead of shear force. Therefore, it is clear that substrate loading strongly

influences the biofilm morphology.

Obviously, the combination of moderate substrate surface loading and high shear force is important for formation of stable biofilm and the good balance between substrate loading rate and shear force results in smooth and strong biofilm (Tijhuis et al., 1995a; Kwok et al., 1998; Van Loosdrecht et al., 1995a). Similarly, Liu et al. (2003b) also suggested that a proper balance between growth force, which resulted from substrate loading rate, and detachment force, which resulted from shear force, was crucial for development of a compact and stable biofilm. And Rochex et al. (2008) reported that high shear force slowed down the biofilm maturation and tended to maintain a young biofilm.

Nutrient composition: Nutrient composition also affects biofilm structure and development. The heterotrophic biofilm development is affected by the COD surface loading (van Benthum et al., 1997). And a high N/COD ratio favors the growth of nitrifying population in the biofilm (Moreau et al., 1994; Ochoa et al., 2002). Ohashi et al. (1995) also demonstrated that the prevailing C/N ratio affected the structure of multispecies biofilm composed of nitrifying bacteria and heterotrophic bacteria; the portion of nitrifying bacteria in biofilm decreased with increasing substrate C/N ratio.

2.7.3 The biofilm structure and microbial population

The structure of biofilm is the net result of biomass growth and detachment, and the formation of biofilm structure is dependent on external shear force, internal diffusion profile and microbial competition (van Loosdrecht et al., 1995a).

In the mixed cultures biofilm, the distribution of bacteria is significantly affected by

the particular microenvironment around them, i.e. bacteria try to reach the microenvironment that is best for their survival (Watnick and Kolter, 2000). Costerton et al. (1981) showed that the mixed bacterial communities in the biofilms formed different thick-layered structures. The substrate concentration gradient in the biofilm induces the growth rate gradient of bacteria and leads to layered (or stratified) structure, in which fast growing heterotrophic bacteria are in the outer layer, and slow growing autotrophic bacteria are in the inner layer (Nicolella et al., 2000). Specifically, concentration of heterotrophic bacteria is 4 or 5 times higher than that of nitrifying bacteria (Zhang et al., 1994; 1995). Wimpenny and Colasanti (1997) found the change of diffusion rate in the biofilm could form the mushroom-like structure and concluded that biofilm structure was significantly affected by the nutrient concentration. Tijhuis et al. (1995b) compared the biofilm of nitrifying and heterotrophic bacteria in his work, in which the former formed denser biofilm than the latter under similar conditions (temperature, shear).

Oxygen diffusion coefficient in biofilm is only 40-80% of the value in water (Siegrist and Gujer, 1987). Aerobic heterotrophic organisms usually have higher growth rate (approximately 0.3-0.5/h at 30°C) than nitrifying bacteria (approximately 0.08/h at 30°C) (Wiesmann, 1994). Obviously, the heterotrophic and autotrophic bacteria compete for substrate and space in biofilm; the increased organic loading and nitrogen loading result in the competition for oxygen between the bacteria and the heterotrophs out-compete nitrifiers for oxygen and space. As a result, nitrifiers are located in the deeper layer of biofilm, where oxygen still could penetrate, to avoid the space competition with the fast grower heterotrophs (Bishop, 1997). Okabe et al. (1996) confirmed an interesting correlation between the C/N mass ratio and microbial distribution. In the biofilm with vertical stratified structure, heterotrophic and nitrifying bacteria coexisted in the outer layer of biofilm when the C/N mass ratio was 0, and nitrifying bacteria would be pushed into the inner layer

when the ratio was 1.5.

The inhibition of nitrification under high organic loading rate has been reported (Elenter et al., 2007; Nogueira et al., 2002; Okabe et al., 1996). Bovendeur et al. (1990) confirmed that biofilm removed organic matters at a rate that was directly proportional to the COD loading rate; high organic loading rate suppressed nitrification by suppressing the transfer of oxygen into the biofilm; and the reduction of nitrification rate by simultaneous COD oxidation was quantified as $0.015 \text{ g/m}^2 \text{ d}$ $\text{NH}_4\text{-N}$ nitrified per $\text{g/m}^2 \text{ d}$ COD removed. Therefore, in order to achieve stable nitrifying activity, sufficient oxygen penetration to the deep layer of biofilm must be ensured by regulating the surface COD loading (van Loosdrecht et al., 1995b). The high DO oxygen in the bulk solution is also beneficial for nitrifiers as it reduces the oxygen competition and oxygen diffusion limitation in the biofilm. The heterotrophic bacteria in the out layer of biofilm prevent the diffusion of nutrients into the biofilm but at the same time protect the inner layer of nitrifying biomass from detachment (Furumai et al., 1994). The inhibited nitrifiers can be fully recovered when the biofilm is solely fed with ammonia wastewater (Lee et al., 2004).

2.7.4 The removal of nitrogen and organic carbon in biofilm process

Nitrogen removal: The high microbial activity of nitrifying bacteria in biofilm ensures the successful removal of nitrogen from wastewater. The maximum specific activity of nitrifying biofilm is attained with a film thickness in the range of 15-25 μm ; and the specific activity begins to decrease with additional accumulation of biofilm (Liu and Capdeville, 1996). Welander et al. (1997) obtained a maximum nitrification rate of $40 \text{ g (NH}_4\text{-N)/(m}^3 \text{ reactor h)}$ in the treatment of landfill leachate. Rodgers et al. (2006) achieved the average ammonium removal rate of 0.30 ± 0.10

kg/m³d at ammonium loading rate of 0.70 ± 0.02 kg/m³d in a vertically moving biofilm reactor. And high nitrification efficiency has also been reported in fluidized-bed biofilm nitrification reactor (Aslan and Dahab, 2008).

Simultaneous removal of nitrogen and organic carbon: The coexistence of nitrifying and heterotrophic bacteria in biofilm makes the simultaneous removal of ammonium and COD possible. When organic loading is low or the oxygen concentration in bulk solution is high, the competition for oxygen is not severe and nitrification is not significantly affected, nitrifying bacteria are protected from shear stress and less biomass are detached thank to the cover layer of heterotrophic bacteria. Obviously, COD/N ratio plays an important role in this process in which a ratio of 5 results in simultaneous removal of nitrogen and organic carbons whereas a ratio of 6 deteriorates it (Liu et al., 2010).

Simultaneous nitrification and denitrification (SND) in single biofilm reactor: In thick mixed biofilm, the oxygen gradient creates the aerobic and anaerobic microenvironments; hence denitrifying bacteria can exist in the inner layer (Biesterfeld et al., 2003; Mehlhart, 1994; Revsbech et al., 1989). The denitrifiers utilize the carbon sources from the wastewater and convert nitrate/nitrite, the products of the nitrification, to nitrogen gas. Therefore, nitrification and denitrification can be integrated in the biofilm system.

Rong et al. (2007) achieved more than 94% removal efficiency for both COD and TN in coke plant wastewater through simultaneous nitrification and denitrification in fixed biofilm hybrid system with the supply of sufficient carbon sources. Xia et al. (2008) achieved 90% COD removal efficiency and 83.3% SND efficiency in different zones of suspended carrier biofilm reactor with a DO concentration of 2 mg/L. Walters et al. (2009) achieved 75% of nitrogen removal from reject water

through SND process in biofilm airlift suspension (BAS) reactor. The SDN process has also been demonstrated in membrane-aerated biofilm reactor with a C/N ratio range from 3.0 to 5.25 (Matsumoto et al., 2007).

However, it should be pointed out that the layered distribution of bacteria in the biofilm is disadvantage for SND process, as most COD is consumed in the outer aerobic layer. Moreover, fast growing heterotrophic organisms out-compete the slow growing autotrophic organisms for oxygen, so the nitrifying capacity is inhibited.

2.7.5 The formation of granules from detached biofilm biomass

During the development of biofilm, the high shear force applied make the bacteria in the biofilm have good characteristic for adhesion and aggregation, therefore, the biomass detached from biofilm can be easily cultivated into granules. DiIaconi et al (2005) explained the biomass evolution from biofilm to granules in sequencing batch biofilm (SBBR) system: 1) the formation of thin biofilm all over the carrier; 2) the increase of biofilm thickness; 3) the detachment of biofilm with released biofilm particle; 4) the high shear force promoted the rearrangement of biofilm particles in smooth granules.

In summary, the successful development of biofilm can be achieved with proper control of operating conditions; and the biofilm structure and the distribution of microbial population in biofilm are affected also. The coexistence of heterotrophs and nitrifiers in biofilm, their different growth rates and oxygen diffusion limitation induce the competitions for substrate, oxygen, and space. The presence of denitrifying bacteria in deepest part of biofilm is possible. The application of biofilm in separate and simultaneous nutrient removal has been extensively studied.

Yet biofilm using iron ore as carrier in reject water has not been reported, hence this study presented in chapter 7 would focus on the development of biofilm on iron ore surface together with its performance in removing nitrogen and organic carbon from reject water.

2.8 Aerobic granulation

Aerobic granule, a special case of biofilm, is a phenomenon of cell to cell self immobilization instead of attaching to solid surface, to form a compact and dense structure (Liu, 2008). Compared to conventional activated sludge, aerobic granules have better settling ability, mass transfer efficiency, and bioactivity (Su and Yu, 2005a). The good settling property of granular sludge promotes more biomass to retain in reactor; the resulting high biomass concentration favors the performance and stability of granulation reactor, which can withstand high organic loading rate (OLR) and shock loading (Liu and Tay, 2004).

2.8.1 Factors affecting aerobic granulation

Aerobic granulation is a gradual process from seed sludge to compact aggregates, granular sludge, and finally to mature granules (Tay et al., 2001a). Apparently a number of environmental factors or operational conditions have to be fulfilled during the process. The main factors affecting aerobic granulation are discussed below.

Substrate composition: Different substrates produce different aerobic granules. Various types of synthetic wastewater, toxic wastewater, real municipal wastewater, and industrial wastewater have been used. The organic carbon source affects the granule microstructure and species diversity. The glucose-fed granules are

dominated by filamentous bacteria, while the acetate-fed granules are populated with rod bacteria (Tay et al., 2002b). The increased substrate N/COD ratio decreases the mean size of mature aerobic granules and sludge volume index (SVI), favors more compact and denser granule and improves the stability (Liu et al., 2004; Yang et al., 2005). The formation of aerobic granules in SBR does not depend on the characteristics of organic substrate (Tay et al., 2001a).

Organic loading rate: Aerobic granules have been successfully cultivated with a wide range of organic loading rates (OLR), from 2.5 to 15 kg COD/m³d (Moy et al., 2002). Wide range of N/COD ratio (from 5/100 to 30/100) is similarly possible for granule development (Yang et al., 2005). And nitrifying granules and P-accumulating granules are also cultivated at a wide range of ammonium-nitrogen and phosphate loading (Lin et al., 2003; Liu et al., 2008). Although Tay et al. (2004a) considered granule formation was unfavored when OLR was lower than 2 kg COD/m³d, Liu et al. (2003a) proved that organic loading rate was not decisive factor in the formation but granule physical characteristic was affected. High organic loading rate enhances the growth rate of biomass and the mean size of aerobic granules (Liu et al., 2003a; Moy et al., 2002); while reduces their physical strength (Liu et al., 2003a).

Hydrodynamic shear force: Aerobic granules can only be formed above threshold shear force of 1.2 cm/s in column SBR (Tay et al., 2001b); high hydrodynamic shear force favors the granule density and strength (Iaconi et al., 2006; Tay et al., 2004b).

Settling time: Settling time is the main factor deciding granule development and hydraulic pressure on microbial community (Beau et al., 1999; Liu and Tay, 2004). A short settle time is required to wash out free living and floc forming

microorganisms; and apply selective pressure for granule formation (Morgenroth et al., 1997). The shorter the settling time, the better granulation is formed (Qin et al., 2004a; 2004b; Wang et al., 2004). Similarly, Adav et al. (2009) suggested that short settling times at initial stage determined the efficiency of subsequent granulation process. And the mature aerobic granules tend to settle within 1 minute, leaving clear supernatant in the reactor (Tay et al., 2001a).

Hydraulic retention time: SBR cycle time represents the frequency of solids discharge and is closely related to the hydraulic retention time (HRT) at a given exchange ratio (Qin, 2005). The short HRT represents high frequency of solids discharge, and suppresses the growth of suspended solids. However, too short HRT in SBR causes sludge loss hence granulation failure since bacteria growth can not compensate the discharged biomass. As a result, HRT should be optimized to suppress the growth of suspended biomass and ensure the accumulation of good settleability sludge (Liu and Tay, 2004). Tay et al. (2002a) reported that the cultivation of nitrifying granulation was achieved at cycle times of 6 to 12 h. Tay et al. (2005a) reported an exception when the cultivation of aerobic granules using tert-butyl as substrate in SBR at a cycle time of 24 h and aerobic granules was observed after 180 days of operation. As a conclusion, it is important to optimize the HRT in aerobic granule cultivation.

Feast-Famine regime: Microorganisms are subjected to a periodic feast-famine regimen called periodic starvation (Liu and Tay, 2004) during the aeration stage of SBR operation. Tay et al. (2001a) found that bacteria became more hydrophobic during this starvation. The high hydrophobicity facilitates the microbial adhesion and aggregation, which seems to be the strategy of bacteria against starvation. Therefore, starvation plays a role in bacterial aggregation process and contributes to the formation of stronger and denser granules (Liu and Tay, 2004); and the periodic

feast-famine regimen can be regarded as microbial selection pressure that alters the surface properties of cell (Qin, 2005). Liu and Tay (2008) added that aerobic granules developed faster with shorter starvation time, but the starvation time should be optimized as too short starvation time negatively affected the long term stability. Moreover, the short term C-, N-, P-, starvation negatively affects the aerobic granules (Wang et al., 2006a). Thus, there is no solid evidence showing if periodic starvation can trigger aerobic granulation in SBR.

Feeding strategy: Both intermittent and pulse feeding enhance aerobic granulation in SBR (McSwain et al., 2004). Despite feeding strategy influences the characteristics of aerobic granules in SBR, it is not a decisive factor (Liu and Tay, 2004).

Dissolved oxygen: Aerobic granules can be formed at both low DO (0.7 to 1.0 mg/L) (Peng et al., 1999) and high DO. Therefore, if the aerobic condition is maintained, DO is not a limiting factor in the formation of aerobic granules.

Reactor configuration: Aerobic granules are mostly cultivated in column SBR, in which the air flow pattern is upward. The high ratio of reactor height (H) to diameter (D) improves the oxygen transfer and ensures a circular flow trajectory, which creates a more effective hydraulic attrition for microbial aggregates and shapes regular granules (Qin, 2005). However, Pan (2003) suggested that aerobic granules could be developed in SBR with various H/D ratios. And H/D ratio does not affect granule formation, physical characteristics, microbial community structure, and reactor performance (Kong et al., 2009).

Exchange ratio: The exchange ratio is defined as the volume of effluent withdrawn at the end of the given settling time divided by the working volume of the SBR.

High volume exchange ratio facilitates the formation and development of aerobic granules thus can serve as an effective selection pressure for aerobic granulation in SBR (Wang et al., 2006c).

Discharge time: Prolonged discharge time can delay or prevent the formation of aerobic granules even though both settling time and volume exchange ratio are properly controlled. Therefore, the discharge time of effluent from the SBR is as critical as the exchange ratio (Wang, 2007).

Trace elements: Jiang et al. (2003) reported that the addition of Ca^{2+} accelerated the aerobic granulation process, and the cultivated granules had better settling and strength characteristics as well as high polysaccharides content. And Fe in the substrate can be accumulated in the central part of nitrifying granules and promotes nitrifying granules (Tsuneda et al., 2004).

Inhibition of aerobic granulation: Yang et al. (2004) reported that aerobic granule formed only when free ammonia concentration was less than 23.5 mg N/L and high free ammonia concentration reduced the cell hydrophobicity as well as the production of extracellular polysaccharide, which were responsible for the failure of aerobic granulation. Therefore, the free ammonia concentration should be controlled during the cultivation of aerobic granulation.

In summary, settling time, volume exchange rate, and discharge time constitute the three main selection pressure parameters in aerobic granulation in SBR (Liu et al., 2005). No matter how other factors are monitored, aerobic granules can not be successfully developed without proper control of these three main selection parameters in the SBR (Liu, 2008).

2.8.2. Mechanism of aerobic granulation

Aerobic granulation can be regarded as the net result of the interaction between the bacterial growth and detachment (Liu et al., 2003b). It is a gradual process from seed sludge to dense aggregates, then granular sludge, and finally to compact mature granules (Tay et al., 2001a). Normally, the granulation process completes in 1 to 2 weeks or even more, depending on the process control. A longer time is needed for slow growing bacteria such as nitrifying bacteria (Tsuneda et al., 2003) and for toxic wastewater (Tay, et al., 2005a; Yi et al., 2006). Granulation process can be divided into 3 phases: acclimation, granulation, and granule maturation (Liu and Tay, 2004). Tsuneda et al. (2004) proposed the core formation mechanism for nitrifying granulation. They demonstrated that Fe ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) added as trace element (5 mg/L) in the feed was accumulated at the central part of the nitrifying granules and promoted nitrifying granulation; the pre-aggregation of seed sludge using hematite (Fe_2O_3) promoted core formation and led to rapid production of nitrifying granules. They proposed 3 stages for nitrifying granulation (Fig. 2.7):

- 1) Nitrifying bacteria aggregated along with Fe precipitation to form the cores of granules, the Fe precipitate assisted in the aggregation process.
- 2) The aggregates grew up to a spherical or elliptical form due to multiplication of the nitrifying bacteria and moderate shear stress in the reactor.
- 3) Finally the mature nitrifying granules were developed.

Fig. 2.7 Schematic illustration of the proposed formation mechanism of nitrifying granules with addition of Fe in the feed (Tsuneda et al., 2004).

As activated sludge flocs are commonly negatively charged, the mechanism of positive charge particles as core of phosphorus accumulation granules has been proposed by Wu et al. (2010), they suggested that the positive charged particle formed with the release of phosphorus in the anaerobic stage served as the cores of granules and stimulated the formation of phosphorus accumulating microbial granules.

However, Liu and Tay (2002) described aerobic granulation process in 4 steps: (1) physical movement to initiate bacterium to bacterium contact or bacterial attachment onto nuclei; (2) initial attractive forces to keep stable multicellular contacts; (3) microbial forces to make attached bacteria or aggregated bacteria mature; (4) shaping of the steady state three-dimensional structure of microbial aggregate by hydrodynamic shear force. Identically, Su and Yu (2005a) successfully cultivated aerobic granules in SBR fed with soybean-processing wastewater and concluded the formation of granule consisted of 4 phases: acclimating, shaping, developing, and matured.

In terms of characteristics, cell surface hydrophobicity plays a crucial role in enhancing the initiation of aerobic granulation; high cell surface hydrophobicity promotes strong cell-cell interaction and the formation of a denser structure (Liu et al., 2003c). Extracellular polysaccharides mediate the cohesion and adhesion of cells; help to maintain the integrated granular structure and contribute greatly to aerobic granulation (Tay et al. 2001c).

2.8.3 Characteristics of aerobic granules

Settling velocity: The typical settling velocity of conventional activated sludge is less than 5 m/h (Giokas et al., 2003). The settling velocity of aerobic granules can be as high as 30-70 m/h (Liu and Tay, 2004). The sludge volume index (SVI) can be less than 50 mL/g, which is much lower than that of activated sludge (Qin et al., 2004a, Wang et al., 2007b). The high settling velocity of granules ensures the high biomass loading in the reactor, high pollutant degradation rate, and compact reactor design.

Size: The mean size of aerobic granules is balanced by the microbial growth due to organic loading and the detachment due to hydrodynamic shear force (Liu and Tay, 2002). The mean size can be in the range of 0.3 to 9 mm. Shi et al. (2009) reported that the size of aerobic granules with high carbon- and ammonium- oxidizing activities was 0.85 mm. Various sizes of aerobic granules have been cultivated using different substrates, such as: 1-2 mm granules from 2, 4-dichlorophenol (Wang et al., 2007a), 0.37-2.07 mm granules from soybean-processing wastewater (Su and Yu, 2005a), 1.7 mm granules from abattoir wastewater (Cassidy and Belia, 2005), 2-7 mm granules from brewery wastewater (Wang et al., 2007b), and 1-1.3 mm granules from synthetic wastewater (Jang et al., 2003).

Surface hydrophobicity: The hydrophobicity of granular sludge is nearly twofold of that of conventional bioflocs (Liu and Tay, 2004), which enhances substantially the cell self-immobilization and attachment processes (Liu et al., 2003c).

Growth of filamentous bacteria: The overgrowth of filamentous bacteria under stress conditions appears to be major cause of instability of aerobic granular sludge in SBR. The growth of filamentous can be due to long retention time of solids; low substrate concentration in the liquid phase; high substrate gradient, lack of dissolved oxygen, and lack of nutrient inside the granules (Liu and Tay, 2004). During the cultivation, various stresses may simultaneously present and result in progressive filamentous growth (Liu and Liu, 2006).

Specific oxygen utilization rate (SOUR): The microbial activity of granules can be characterized by the specific oxygen utilization rate (SOUR). The high shear force in the granulation increases the respiration activities of microorganisms (Tay et al., 2001b), as the high hydrodynamic shear increases the oxygen diffusion rate. The shorter settling time stimulates the respiratory activity of the microorganisms and the SOUR of granules (Qin et al., 2004a).

Storage stability: The stability and activity of aerobic granules are affected by the storage period and temperature. Detailed studies have been conducted with multiple types of aerobic granules. Typically, Tay et al. (2002b) observed that when aerobic granules were stored in tap water for 4 months at 4 °C, the glucose-grown aerobic granules reduced 60% of the initial metabolism activity, acetate-fed granules lost about 90% metabolic activity and the strength of granules was decreased by 7-8%. Zhu and Wilderer (2003) reported that after 7 weeks' storage of aerobic granules in ambient environment, aerobic granules did not lose integrity and metabolic potential. Their granules remained intact even without substrate and oxygen supply.

The metabolic activity of the stored granules could be recovered within a week with the supply of substrate and oxygen. Wang et al. (2008) reported the granules size and structure integrity remained during 7 months' storage at 4 °C, the reactivation made the activities of heterotrophic and nitrifying bacteria fully recovered with 16 and 11 days, respectively. Pijuan et al. (2009) found that aerobic granules could be stored under anaerobic or intermittent aerobic/anaerobic starvation for at least 4 weeks without losing their structure integrity. The activity of nitrifying bacteria was reduced by 20 and 36% during this period and fully recovered after 3 weeks of growing in wastewater.

Dissolved oxygen (DO) diffusion: DO has substantial effects on the distribution of microbial populations in aerobic granules, the conversion rate of different components, hence on overall nutrient removal efficiency (de Kreuk et al., 2007). In general, substrates can penetrate deeper into aerobic granules than oxygen. And the depth to which oxygen and substrate can penetrate into the granules has been of great interest. Li and Liu (2005) suggested that oxygen and substrate could diffuse into the whole granule when the size was less than 0.40 mm; dissolved oxygen faced diffusion limitation when granule size was greater than 0.5 mm. Jang et al. (2003) estimated a DO diffusion distance from the surface into the granule to be 0.3 mm. Li et al. (2008) further confirmed that the DO penetration distance from the granules surface to be 500 µm under substrate sufficient condition, and no limitation with a granule radius less than 2.2 mm under substrate free condition. Su and Yu (2005b) suggested that oxygen could fully penetrate into granules smaller than 0.5 mm, partially penetrate into granules larger than 1.5 mm, and could not penetrate into granules larger than 2 mm. However, Chiu et al. (2007) reported that oxygen was already limited by granules with size over 0.25 mm, and most of it was consumed in the surface layer with a thickness of 125-375 µm.

Microbial structure: The aerobic granule has a layered structure comprising aerobic and anaerobic zones from the surface to the center. The growth of heterotrophic and autotrophic bacteria in granule is simultaneous. The limited penetration of oxygen results in the distribution of autotrophs in the outer layer. The obligate aerobic ammonium-oxidizing *Nitrosomonas* spp. mainly locate at a depth of 70 to 100 μm from the granule surface (Tay et al., 2002c); channels and pores contained in the granules penetrate into the depth of 900 μm below the granule surface, the high porosity locates at a depth of 300 to 500 μm (Tay et al., 2002c; 2003). The anaerobic bacteria *Bacteroids* spp. locate at depth of 800 to 900 μm from the granule surface (Tay, et al., 2002d). The anaerobic biomass may produce biogas, which negatively affect the long-term stability of granules or even destroy them (Liu and Tay, 2004).

For high N/COD substrate, the mushroomlike structure is developed in the granules (Liu et al., 2004). Prescott et al. (1999) demonstrated that bacteria sensed and moved toward nutrients. Therefore, the mushroomlike structure facilitates the access of the slow growing nitrifying bacteria toward nutrients (Liu and Tay, 2004).

Ammonium oxidizing bacteria mainly locate in the upper and middle layers of the granule (Jang et al., 2003); and the main active part of nitrifying granule is in the upper 150 μm layer (Ni et al., 2008). However, autotrophic bacteria may accumulate in the inner zone of granules at high oxygen concentration (de Kreuk et al., 2007; Xavier et al., 2007). Obviously, heterotrophic, nitrifying, and denitrifying bacteria can co-existed in the granules, the increased substrate N/COD ratio causes significant shift among them (Yang et al., 2003). The high N/COD ratio increases the activities of nitrifiers and denitrifiers, at the same time decreases the heterotrophic populations in granules.

2.8.4 The applications of aerobic granules

Aerobic granules have been widely studied and used to remove nutrients and organics from various wastewaters.

Nitrifying granules for nitrogen removal: Various types of nitrifying granule have been cultivated for nitrogen removal, since they have excellent nitrification ability (Tay et al., 2002a). After 300 days of cultivation, Tsuneda et al. (2003) achieved an ammonium removal rate of $1.5 \text{ kg N/m}^3\text{d}$. Liu et al. (2008) developed the nitrifying granules in 21 days of SBR operation with a nitrogen loading rate from 0.72 to $1.8 \text{ kg/m}^3\text{d}$.

Aerobic granules for simultaneous removal of organic and nitrogen: Simultaneous removal of organic and nitrogen has been achieved with high efficiency in the treatment of a variety of wastewaters. Liu et al. (2007b) used acetate-fed aerobic granules to treat low-strength domestic wastewater and achieved complete COD removal together with an above 90% ammonium removal with a loading rate of $0.3 \text{ kg N/m}^3\text{d}$. Arrojo et al. (2004) cultivated the aerobic granule using industrial wastewater with $7 \text{ kg COD/m}^3\text{d}$ and $0.7 \text{ kg N/m}^3\text{d}$, the COD and nitrogen removal efficiencies were 70% and 80%, respectively. Wang et al. (2007b) developed aerobic granules from brewery wastewater and achieved 88.7% total COD and 88.9% ammonium removal at the loading rates of $3 \text{ kg COD/m}^3\text{d}$ and $0.24 \text{ kg N/m}^3\text{d}$.

The removal of nitrogen and COD under alternating aerobic and anaerobic conditions: At high N/COD ratio, the competitions for nutrients and oxygen between nitrifiers and heterotrophs are significant (Liu, 2008). Under alternating aerobic and anaerobic conditions in SBR, the aerobic granules containing

heterotrophs, nitrifiers, and denitrifiers have been successfully cultivated to remove organics and nitrogen with the supply of external carbon sources (Jang et al., 2003; Qin and Liu, 2006; Yang et al., 2003).

Simultaneous nitrification, denitrification, and COD removal: The DO concentration has strong effects on the N-removal as well as the coexistence of an outer aerobic zone and inner anaerobic zone in granules. In simultaneous nitrification and denitrification (SND) process, DO concentration should be adjusted to be high enough for oxygen to penetrate in the outer layer of granule and low enough to protect the denitrifier population. Beun et al. (2001; 2002) accomplished simultaneous nitrification, denitrification, and COD removal by aerobic granulation in sequencing batch airlift reactor at the optimum DO concentration of 3.2 mg/L. Wan and Sperandio (2009) demonstrated that significant denitrification occurred in the aerobic granules at low oxygen concentration of 1.8 mg/L. Mosquera-Corral et al. (2005) on the contrary concluded that the granules stability was negatively affected at low oxygen concentration; the granules could be disintegrated and biomass was washed out at DO concentration of 3.2 mg/L.

P removal by granule: The P uptake by granules is usually in the range of 1.9% to 9.3% by weight; the high substrate P/COD ratio decreases the granules size, promotes the compact and dense granules structure (Lin et al., 2003). Wu et al. (2010) obtained average phosphorus removal efficiency higher than 94.3% and the effluent phosphorus concentration was lower than 0.50 mg P/L after 300 days of operation.

Simultaneous removal of phosphorus, nitrogen, and COD: Aerobic granules cultivated in abattoir wastewater by Cassidy and Belia (2005) could remove more than 98% of COD and P and over 97% of N from abattoir wastewater with a DO

concentration of 3.2 mg/L. The similar result was achieved by De Kreuk et al. (2005) at a DO concentration of 1.8 mg/L.

Heavy metals removal: The biosorption of Zn^{2+} and Cd^{2+} by aerobic granules has been demonstrated (Liu et al., 2002; 2003d). The maximum biosorption capacity for Zn^{2+} was 270 mg/g of granule (Liu et al., 2002) and the sorption capacity for Cd^{2+} was 566 mg/g of granule (Liu et al., 2003d).

Treatment of wastewater containing toxic substrates: It is believed that cells immobilization is a useful strategy for bacteria to overcome the substrate inhibition. Aerobic granules are powerful bioagents for the removal of inhibitory or toxic compounds present in industrial wastewater (Liu, 2008). Aerobic granules have been used to remove phenol (Jiang et al., 2006; Moussavi et al., 2010; Tay et al., 2005b), tert-butyl alcohol (Tay et al., 2005a; Zhuang et al., 2005), 2, 4-dichlorophenol (Wang et al., 2007a), p-nitrophenol (Yi et al., 2006), and 4-chlorophenol (Carucci et al., 2009) from various wastewaters.

In summary, the aerobic granules have been successfully cultivated in various substrates; the characteristics and mechanisms of aerobic granulation have been widely studied; the applications of aerobic granule for the removal of nitrogen, phosphorus, organics, and toxic substrates from various wastewaters have also been demonstrated. However, the idea of cultivating aerobic granule in beaker fed with reject water is original. Similarly, researchers have not paid attention to removal of nutrients from reject water using aerobic granules. Therefore, one of the objectives of this study was to cultivate aerobic granules in reject water and use them to remove nitrogen and organics from the same medium. The description would be presented in chapter 8.

Chapter 3

The removal of phosphorus from reject water in municipal wastewater treatment plant using anoxic bioreduction of iron ore

3.1 Introduction

In Singapore, the reject water stream with a phosphorous concentration (mainly in the form of orthophosphate) of up to 150 mg P/L contributes about 4% to the total raw sewage flow and up to 40% phosphorus load in the activated sludge tank. Therefore, it is significantly important to remove phosphorus from reject water.

As discussed in Chapter 2, the removal of phosphorus can be obtained biologically and chemically. The disadvantages of MAP (Magnesium ammonium phosphate hexahydrate, $\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) process are the low crystallization rate of struvite and high chemical consumption. And the high cost of iron salt, which is about US\$1015/ton of iron in FeCl_3 (De-Bashan and Bashan, 2004), make iron salt not practically affordable to precipitate phosphate.

An alternative approach which can possibly solve the weaknesses of chemical treatment is to produce the precipitant biologically using BioIronTech process (Ivanov et al., 2005; Stabnikov et al., 2004; Tay et al., 2008). Under anaerobic conditions, the reduction of Fe(III) to Fe(II) by iron reducing bacteria is coupled to the oxidation of organic compounds and/or hydrogen gas (Fredrickson and Gorby, 1996; Ivanov, 2010; Lovley, 1991). The produced ferrous ions chemically precipitate phosphate, which can be used as fertilizer after settling (De-Bashan and Bashan, 2004). Iron ore/iron hydroxide can be the abundant sources of electron acceptors for iron-reducing bacteria (IRB) under anaerobic conditions (Ivanov et al.,

2005; 2009a; 2009b; Ivanov and Hung, 2010; Stabnikov et al., 2004; Tay et al., 2008). Economically, 1 ton of Fe in iron ore costs approximately US\$140, which is 7.2 times cheaper than that in iron salts (Ivanov et al., 2009a). Therefore, the purely anaerobic BioIronTech process can decrease remarkably the cost of iron salt reagents in phosphorus removal. This study tried to examine the process of phosphorus removal from reject water using anoxic bioreduction of iron ore by iron reducing bacteria in static and rotating reactors. Previous studies about BioIronTech process mainly focus on the improvement of sludge quality (Ivanov et al., 2004a), treatment of fat-containing wastewater (Ivanov et al., 2002), removal of xenobiotics such as diphenylamine, m-cresol, 2, 4-dichlorophenol, and p-phenylphenol (Ivanov et al., 2005; 2010). Hence the idea of removing/recovering phosphorus from reject water using IRB and iron ore is totally original. The aim of this research was to study the process of phosphorus removal from reject water using anoxic bioreduction of iron ore by iron reducing condition in the static and rotating reactors.

3.2 Materials and methods

3.2.1 Composition of reject water, anaerobic sludge, and iron ore

Reject water and anaerobic sludge were collected from a local MWWTP. Anaerobic sludge was used as a source of iron-reducing bacteria (Nielsen, 1996; Nielsen et al., 2002). They were stored at 4 °C before being used for the experiments. The characteristics of reject water and anaerobic sludge were determined in this study (Table 3.1). The details of analytical methods and procedures used are described in section 3.2.3.

Iron ore was delivered from JuYuan Trading PTE LTD (PingXiang City, GuangXi,

China). According to specification, iron ore contained 60% (w/w) iron and the major mineral content was hematite (Fe_2O_3). The porosity of iron ore particles was determined in this study and ranged from 45 to 55% (v/v) depending on the particle size. Five sets of iron ore particles with the sizes of 35 ± 5.0 mm, 17.0 ± 3.0 mm, 7.6 ± 1.9 mm, 2.4 ± 0.4 mm, and 0.6 ± 0.1 mm (as shown in Fig. 3.1) were used in the static cultivation experiments; whereas, three sets with the sizes of 7.6 ± 1.9 mm, 2.4 ± 0.4 mm, and 0.6 ± 0.1 mm were used in the rotating cultivation experiments. Iron ore particles with the sizes of 0.6 ± 0.1 mm and 2.4 ± 0.4 mm were selected by sieving after crushing; and iron ore particles with the sizes of 35 ± 5.0 mm, 17.0 ± 3.0 mm, and 7.6 ± 1.9 mm were manually selected.

Table 3.1 Characteristics of reject water and anaerobic sludge

Parameter	Concentration (mg/L)	
	reject water	anaerobic sludge
Dissolved total organic carbon (TOC)	450-600	1900
Total suspended solids (TSS)	430-670	5240
Volatile suspended solids (VSS)	380-560	4000
Dissolved phosphate, PO_4^{3-}	64-110	80
Total concentration of Fe^{2+} ions	4-7	167
Ammonium (NH_4^+ -N)	640-690	-
pH	7.60-7.70	7.8-7.9
Alkalinity as CaCO_3	2500-2596	-
NO_3^- -N	0.6-0.7	-
NO_2^- -N	0.03-0.04	-

Fig. 3.1 Iron ore particles with different sizes used in the experiments.

3.2.2 Experimental setup

The experiment on the removal of phosphorus in the static reactors (as shown in Fig. 3.2.) was performed in batch culture with five different sizes of iron ore. A mixture of 1 kg iron ore particles and 4.5 L reject water was added into each 5 L experimental bioreactor. An aliquot of 0.5 L anaerobic sludge contained iron reducing bacteria was added as inoculum to each experimental reactor. The reactors were purged with nitrogen gas for 5 minutes and sealed to ensure anaerobic conditions. No iron ore or anaerobic sludge was added in the control reactor. All the reactors were maintained at 35 °C for 28 days.

Fig. 3.2 The static reactors used in the static experiment.

The experiment on the removal of phosphorus in the rotating reactors (as shown in Fig. 3.3.) was performed in batch culture with iron ore particles of 3 different sizes. A mixture of 400 g iron ore particles and 1.8 L reject water was added in each 2 L bioreactor. An aliquot of 200 mL anaerobic sludge contained iron reducing bacteria was added as inoculum to each experimental reactor. The reactors were purged with nitrogen gas for 5 minutes and sealed to ensure anaerobic conditions. All the reactors were maintained at 35 °C and put on the VELP® OVERHEAD MIXER rotax 6.8 (Velp Scientifica) at 5 rpm/min for 18 days.

Fig. 3.3 Rotating reactors on the VELP OVERHEAD MIXER in rotating mixing experiment.

Abiotic controls were used to evaluate the phosphate adsorption onto the surface of iron ore particles in both types of reactors. Iron ore particles and a mixture of reject water with anaerobic sludge were used after autoclaving at 121 °C for 15 minutes. Other conditions were kept the same with the experimental reactors.

To identify the mechanism behind the production of fine iron ore particles in rotating reactors, an additional experiment was conducted. 160 g of iron ore of 3 different sizes (7.6 ± 1.9 mm, 2.4 ± 0.4 mm, and 0.6 ± 0.1 mm) were washed several

times with distilled water initially. The washed iron ore particles were mixed with 800 mL of distilled water in 1L plastic bottles and put on the VELP® OVERHEAD MIXER rotax 6.8 (Velp Scientifica) at 5 rpm/min for 14 days. The well mixed samples were tested in every 2 days for the concentration of total suspended solids using filtration of the liquid samples through 0.45 µm membrane filter. The particle sizes of the produced fine iron ores were measured by the particle size analyzer (Malvern Instrument). The images of the produced floating fine iron ore particles due to mechanism impulse were taken by the Olympus LG-PS2 microscope (Olympus DF Plapo, Japan).

To evaluate the removal of phosphorus from reject water by chemical precipitation, ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (GR grade, Merck) of different quantities was added to the same volume of reject water. In each of the six 2 L-bioreactors, 2 L of reject water adjusted to pH 9.1 using 6 N NaOH was purged with nitrogen gas for 10 minutes to ensure anaerobic conditions. Six different calculated amounts of ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) were added immediately to six reactors to achieve the Fe/P molar ratios of 0, 0.5, 1, 2, 3, and 4, respectively. All the reactors were kept at 35 °C on the VELP® OVERHEAD MIXER rotax 6.8 (Velp Scientifica) at 5 rpm/min for one day.

Finally, the production of soluble ferric coagulants from iron ore in reject water using chemical process was studied. In three separate 2 L experimental reactors, 2 L of reject water was adjusted to the pH of the reject water's original pH value, 4, and 5 using 5N HCl, respectively. For the control reactor, 2 L DI water adjusted to pH 5 was used instead of reject water. All reactors were added with 40 g of iron ore (< 106 µm) before putting on the VELP® OVERHEAD MIXER votex 6.8 (Velp Scientifica) at 5 rpm/min for 4 hours.

3.2.3 Analytical methods

Total suspended solids (TSS), volatile suspended solids (VSS), pH, alkalinity, concentrations of ammonium, nitrate, nitrite, total ferrous ions, phosphate, and dissolved total organic carbon (TOC) were measured in well-mixed samples using standard methods (APHA, 2005). The concentration of total ferrous ions was measured according to a modified phenanthroline method (Ivanov et al., 2005). The samples were acidified immediately after collection with the addition of 2 mL concentrated HCl (GR grade, Merck) per 100 mL sample. For dissolved orthophosphate, ammonium, nitrite, and nitrate tests, the samples were firstly centrifuged and filtered through a 0.45 μm nylon membrane. A modified vanadomolybdophosphoric acid colorimetric method (APHA, 2005) was then applied for dissolved orthophosphate sample; whereas ammonium, nitrite, and nitrate followed the standard methods APHA 4500-NH₃F, APHA 4500-NO₂⁻B, and APHA 4500-NO₃⁻I (APHA, 2005), respectively. The spectrophotometer UV-1201V (Shimadzu, Kyoto, Japan) was used to measure the total ferrous ions, ammonium, nitrite, and phosphate concentration. TOC concentration was tested by TOC analyzer C-Vcsh (Shimadzu, Kyoto, Japan) according to the standard methods (APHA, 2005). pH was measured by the EUTECH pH meter (Cyberscan PCD 6500) after calibration. The porosity of iron ore particles was determined based on the mass difference between 1L of iron ore filled with DI water in the pore and 1L iron ore without any DI water. The All analytical determinations were performed at least in triplicate. Mean values and standard deviations are shown.

3.3 Results and discussion

3.3.1 Fe(II) production

Ferrous production was successfully achieved under both cultivation processes. In the static reactors, Fe(II) production rate increased with the decrease of the size of iron ore particles. Fe(II) production rate was low when the size of iron ore particles was larger than 2.4 mm. The bioreactor with smallest size iron particles (0.6 mm) had the highest mean ferrous production rate, which was 39.6 mg/L·d (0.7 mmol Fe/L·d), after 14 days of cultivation (Fig. 3.4). The corresponding maximum Fe(II) concentration was 554 mg/L. Total Fe(II) concentration in the abiotic control was unchanged. However, in the rotating reactors, Fe(II) production rate increased with the increase of the size of iron ore particles; the highest ferrous concentration of 490 mg/L was achieved in the reactor with mean iron ore particles size of 7.6 mm after 11 days of cultivation (Fig. 3.5). A highest ferrous production rate of 44.5 mg/L·d (0.79 mmol Fe/L·d) was achieved in the same reactor.

In the static experiment (Fig. 3.4), it was noted that the Fe(II) concentration in the bioreactor with 0.6 mm iron ore particles first increased and achieved the maximum concentration then decreased significantly. Similar phenomenon was found in the bioreactor with 2.4 mm iron ore particles. The phenomena could be explained hypothetically by the adsorption of ferrous ions on iron ore surface. The ferrous ions produced from iron ore reduction were probably adsorbed on the surface of iron ore particles. When Fe^{3+} reduction was stopped, concentration of Fe^{2+} ions dropped because of this adsorption. Iron ore particles were not sampled to determine the Fe(II) concentration, consequently, the adsorbed ferrous on the surface of iron ore particles was not tested and the determined Fe(II) concentration was decreased. Iron ore particles with the size of 0.6 mm had larger surface area and adsorbed more ferrous ions.

Fig. 3.4 Production of total ferrous ions in static reactors with different sizes of iron ore particles: ◆ control; ■ 0.6 mm; ▲ 2.4 mm; x 7.6 mm; ▽ 17 mm; ◻ 35 mm.

The results showed that reject water contained 450-600 mg/L TOC, most of which were volatile fatty acids (Table 3.1). The production of soluble ferric coagulants from iron ore in reject water by chemical process was studied and the results are shown in Table 3.2. During 4 hour of rotating cultivation (5 rpm/min), the total dissolved iron was not produced from 40 g of iron ore ($< 106 \mu\text{m}$) in reject water with different pH values (pH = 4, 5, and 7.6) and in DI water with pH value of 5. The volatile fatty acids contained in reject water did not actually dissolve the crystalline iron ore to form soluble ferric ions, a state that was more favorable for bacteria to reduce. The formation of soluble ferric ions due to the chemical reactions between iron ore and components of reject water was not observed. Therefore, crystalline iron ore did not dissolve in reject water and iron reducing bacteria reduced crystalline hematite of iron ore under anaerobic conditions.

Fig. 3.5 Production of total ferrous ions in rotating reactors with different sizes of iron ore particles: ■ 0.6 mm; ▲ 2.4 mm; x 7.6 mm.

Table 3.2 Total dissolved iron in reject water during the rotating mixing process (5 rpm/min) (average $\pm$ s.d.)

	Total dissolved iron (0 h) (mg/L)	Total dissolved iron (4 h) (mg/L)
Reject water (pH = 4)	0.52 ± 0.02	0.46 ± 0.02
Reject water (pH = 5)	0.49 ± 0.05	0.48 ± 0.02
Reject water (pH = 7.6)	0.39 ± 0.03	0.41 ± 0.06
DI water (pH = 5)	0.39 ± 0.02	0.41 ± 0.00

Although iron reducing bacteria reduced crystalline hematite of iron ore under anaerobic conditions, the maximum ferrous production rates in this study for both static reactors with iron ore particles of 0.6 mm (39.6 mg/L·d) and rotating reactor with iron ore particles of 7.6 mm (44.5 mg/L·d) were higher than the maximum ferrous production rate achieved (27 mg/L·d) in previous research, where ferric hydroxide was used as the Fe(III) source (Ivanov et al., 2005). The higher Fe(II) production rate achieved in this study could be explained hypothetically by the

relatively high experimental temperature (35°C) in this experiment, which was higher than the temperature in previous experiment (27°C); therefore, the enrichment culture of iron reducing bacteria used in this study was more active than enrichment and pure cultures of iron reducing bacteria used in the previous study.

In terms of correlation, since diffusion was the major mechanism of mass transfer in the static cultivation process, the ferrous production rate was associated with the size and specific surface of loaded iron ore particles. The ferrous production rate in static reactors had a statistically reliable correlation with the specific surface area of the iron ore particles. Assuming that the iron ore particles are spherical shape, the specific surface area (surface-to-volume ratio) can be calculated as follows:

$$S/V = (\pi D^2)/(\pi D^3/6) = 6/D \quad (3-1)$$

where D is the iron ore particle diameter; D can be replaced approximately by the mean size of the iron ore particle.

The linear relationship between the mean ferrous production rates in 28 days of static cultivation and the calculated specific surface area of the iron ore particles is shown in Fig. 3.6. It seemed that the ferrous production rate statistically reliably and positively correlated with specific surface area and negatively correlated with the size of iron ore particles; therefore, the specific surface area of iron ore particles appears to be the key factor affecting the iron reduction rate by iron reducing bacteria in the anaerobic static reactor.

$$y = 0.55x + 0.46 \quad (R^2 = 0.986) \quad (3-2)$$

Fig. 3.6 The relationship between ferrous production rate and calculated specific surface area of the iron ore particles.

The application of fixed bed reactor, fluidized bed reactor, or rotating bed reactor instead of static reactor to mix iron ore in solution will increase the mass transfer rate and promote the iron ore bioreduction rate. Due to the high risk of clogging caused by ferrous phosphate and ferrous hydroxide precipitated in the fixed bed reactor as well as the high energy consumption in the fluidized bed reactor when high density iron ore (4045 ± 3 g/L for 2.4 mm size particles) is used, the rotating reactor is more preferable system to mix iron ore in solution and avoid the clogging of the pores between iron ore particles.

In the rotating system, the highest ferrous production rate was obtained in the reactor with iron ore particle size of 7.6 mm. In the static system, however, the ferrous production rate negatively correlated with the iron particle size. The contradiction between the two systems can be explained by the formation of fine iron powder in the rotating reactor. The strong mechanical impulses and friction between iron particles created fine iron ore particles. The formation of these fine particles was more dominant for initially larger iron ore size (Fig. 3.7). It was probably due to the stronger interactive forces between larger particles. Fine

particles with high specific surface were bio-reduced faster than big particles. The production rates and sizes of fine iron ore particles produced in rotating reactors with initial iron ore sizes of 0.6 mm, 2.4 mm and 7.6 mm are shown in Table 3.3. The images of the floating fine iron ore particles are shown in Fig. 3.8.

Fig. 3.7 The production of fine iron ore particles in rotating reactors with different initial iron ore sizes: ♦ 0.6 mm; ■ 2.4 mm; ▲ 7.6 mm.

Table 3.3 Production rates and sizes of fine iron ore particles produced in rotating reactors with different initial iron ore sizes

Iron ore initial size (mm)	Sizes of produced fine particles (μm)			Production rate of fine particles(g/L·d)	
	d (10)	d (50)	d (90)	Maximum production rate	Average production rate
0.6	0.136	0.191	1.595	3.66	0.78
2.4	0.630	2.103	4.307	8.61	3.87
7.6	0.135	0.190	1.615	15.25	7.64

Fig. 3.8 The microscope images of the produced suspended fine particles in rotating reactors with different initial iron ore sizes: (a) 0.6 mm; (b) 2.4 mm; (c) 7.6 mm.

pH is important for activity of iron reducing bacteria. The pH of reject water, 7.6, was optimum for the activity of iron reducing bacteria. The results showed that reject water contained 2500 mg/L alkalinity as CaCO_3 (Table. 3.1), thus its pH slightly fluctuated in static reactors (Fig. 3.9a), and slightly increased in rotating reactors (Fig. 3.9b). The final pH in static reactors after 28 days of cultivation was 7.4-7.6; and pH in rotating reactors after 18 days of cultivation was 8.1. Therefore, the activity of iron reducing bacteria was not affected.

Fig. 3.9 The pH of reject water in reactors with different iron ore sizes: (a) static reactor; (b) rotating reactor; $\blacklozenge$ 0.6 mm; $\blacksquare$ 2.4 mm; $\blacktriangle$ 7.6mm; $\times$ 17 mm; $\square$ 35 mm.

3.3.2 Phosphorus removal

The concentration of dissolved phosphorus decreased during both cultivation processes (Fig. 3.10 & Fig. 3.11). During 28 days of static cultivation, the reactor with the largest iron ore size (35 mm) had the lowest mean phosphorus removal rate (1.27 mg P/L·d) and lowest removal efficiency (46.2%). The reactor with iron ore particles of 17 mm also had low mean phosphorus removal rate (1.46 mg P/L·d) and low removal efficiency (64.2%). The reactors with smaller iron ore particles (7.6 mm, 2.4 mm, and 0.6 mm) achieved about 90% removal efficiency and similar mean phosphorus removal rate (2.18 mg P/L·d). The maximum phosphorus removal rate, 11 mg P/L·d (0.36 mmol P/L·d), was obtained by the iron ore particles with mean size of 7.6 mm. Dissolved phosphate concentration in the control (without iron ore) remained the same during 28 days of cultivation.

After 4 days of rotating cultivation, phosphorus removal efficiencies in the reactor with iron ore particles mean sizes of 0.6 mm, 2.4 mm, and 7.6 mm were 79%, 88%, and 92%, respectively. After 8 days of rotating cultivation, they increased to 91%, 93%, and 97%, the bioreactor with larger iron ore particles had higher phosphorus removal rate. The reactor with mean iron ore size of 7.6 mm obtained the highest phosphorus removal rate of 25 mg P/L·d (0.81 mmol P/L·d) and final phosphate concentration of 1.7 mg P/L, which was lower than the permitted discharge level of municipal wastewater treatment plant (MWWTP) in many countries.

Fig. 3.10 Concentration of dissolved phosphorus in static reactors with different iron ore sizes: △control; ◆ 0.6mm; ■ 2.4 mm; ▲ 7.6mm; x 17mm; □ 35mm.

Fig. 3.11 Concentration of dissolved phosphorus in the rotating reactors with different iron ore sizes: ◆ 0.6mm; ■ 2.4 mm; ▲ 7.6mm.

The highest phosphorus removal efficiencies achieved in this study are comparable with those obtained in previous studies in which ferric hydroxide was used as the Fe(III) source (Ivanov et al., 2005; Stabnikov et al., 2004). The efficiencies are also similar or even higher than that of the traditional metal salt precipitation technology

((De-Bashan and Bashan, 2004; Morse et al., 1998). This was proven by the experiment of the chemical precipitation of phosphorus using $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ salt. The comparable result is demonstrated in Fig. 3.12. In terms of removal rate, the maximum values obtained in this study were 11 mg P/ L·d in static reactor and 25 mg P/ L·d in rotating reactor, which are higher than the removal rate of 2.3 mg P/ L·d achieved in previous study in which ferric hydroxide was used as the Fe(III) source (Ivanov et al., 2005). It can be suggested from the experimental data on ferrous production rate and phosphorus removal rate that iron ore could replace amorphous ferric hydroxide as a source of ferric for iron reducing bacteria.

Fig. 3.12 Phosphorus removal efficiencies using BioIronTech process with different iron ore particles sizes in two cultivation processes (first 8 columns) and using ferrous salt chemical precipitation with different molar ratios of Fe/P (last 4 columns).

During static cultivation process, there was no significant difference in the mean phosphorus removal rate between the reactors with iron ore particles of 35 mm and 17 mm, or among the reactors with iron ore particles of 2.4 mm, 7.6 mm, and 0.6 mm (Table 3.4). The relationship between mean phosphorus removal rate and iron ore particles specific area during 28 days of static batch cultivation is represented

by the saturation curve (Fig. 3.13). The phosphorus removal rate in static cultivation process was not associated with the specific area of iron ore particles when the mean size of the iron ore particle was less than 7 mm.

Fig. 3.13 The relationship between mean phosphorus removal rate and calculated specific surface area of iron ore particles.

It is known that chemical removal of phosphate is accompanied by the adsorption of phosphate ions and organic dissolved phosphorus on ferric hydroxide flocs (Takacs et al., 2006). The contribution of phosphate adsorption on iron ore particles of different sizes was estimated by abiotic controls in this study. Significant phosphate removal in static reactor was due to adsorption of phosphate on the surface of iron ore particles (Fig. 3.14). 83%, 87%, 63%, 68%, and 58% of the total phosphate removal were removed by adsorption in static reactors with iron ore particles of size 0.6 mm, 2.4 mm, 7.6 mm, 17 mm, and 35 mm, respectively. Apparently the removal of phosphate by adsorption in static reactors was prone to smaller iron ore particles as they had larger surface area.

Fig. 3.14 Phosphate removal efficiencies in static reactors with different iron ore sizes after 28 days of cultivation: ■ removal of phosphate by adsorption on iron ore particles surface and by precipitation with ferrous; □ removal of phosphate by adsorption on iron ore particles surface.

Table 3.4 Phosphorus removal and ferrous production rates associated with iron ore particles size in static reactors (ave $\pm$ s.d.)

Mean size of iron ore particles (mm)	Total P removal rate (mg P/ L·d)	P removal rate by adsorption (mg P/ L·d)	P removal rate by precipitation (mg P/ L·d)	Fe ²⁺ production rate (mg / L·d)	P removed by precipitation/ Fe ²⁺ produced (mol/mol)
35	1.27 $\pm$ 0.04	0.73 $\pm$ 0.01	0.54	0.37 $\pm$ 0.00	2.64
17	1.46 $\pm$ 0.06	1.04 $\pm$ 0.03	0.42	0.72 $\pm$ 0.03	1.05
7.6	2.18 $\pm$ 0.02	1.36 $\pm$ 0.04	0.82	1.27 $\pm$ 0.02	1.17
2.4	2.18 $\pm$ 0.04	1.89 $\pm$ 0.03	0.29	1.51 $\pm$ 0.06	0.35
0.6	2.19 $\pm$ 0.06	1.82 $\pm$ 0.02	0.37	5.99 $\pm$ 0.08	0.11

During rotating cultivation process, phosphate removal by adsorption was not dependent on the size of initially loaded iron ore particles. The removal of phosphate by adsorption was similar and was $62.0 \pm 0.5\%$, $62.3 \pm 0.4\%$, and $67.8 \pm 0.8\%$ of total phosphorus removal in the rotating reactors with iron ore particles of 0.6 mm, 2.4 mm, and 7.6 mm, respectively. This effect can be hypothetically explained by the formation of fine particles during the mechanical mixing of iron ores in the rotating reactor. In this case, the removal of phosphate by adsorption was associated with the size distribution of the fine particles. The distribution was approximately the same in all rotating reactors with the size of the fine particles ranged from 0.2 to 2 μm .

The theoretical molar ratios of P to Fe^{2+} in chemical compounds $\text{Fe}_3(\text{PO}_4)_2$ and FeHPO_4 are 0.67 and 1.0 (mol/mol), respectively. Chemical precipitation of phosphate by ferrous salts had a P/Fe molar ratio from 0.33 to 1, which could hypothetically correspond to precipitation of the $\text{Fe}_3(\text{PO}_4)_2$ and FeHPO_4 mixture (Takacs et al., 2006). The ratios of phosphate removal by precipitation to Fe^{2+} produced in static reactors with iron ore particles sizes of 2.4 mm, 7.6 mm, and 17 mm were 0.35, 1.17, and 1.05, respectively (Table 3.4). In rotating reactors with iron ore particles sizes of 0.6 mm, 2.4 mm, and 7.6 mm, the ratios were 1.20, 0.39, and 0.14 as shown in Table 3.5. The ratio was high (2.64) in static reactor when iron ore particles with a mean size of 35 mm were used. This could be explained hypothetically by the chelation of phosphate with ferrous ions. The highest ferrous production rate induced low P/Fe ratios, which were 0.11 in static reactor (with 0.6 mm iron ore particles) and 0.14 in rotating reactor (with 7.6 mm iron ore particles) (Table 3.6). This might be the consequence of the excess of ferrous ions for phosphorus removal and the formation of insoluble ferrous iron hydroxide binding less phosphate than ferrous ions.

Table 3.5 Phosphorus removal rate and ferrous production rate associated with iron ore particles size in rotating reactors (ave $\pm$ s.d.)

Mean size of iron ore particles (mm)	Total P removal rate (mg P/ L·d)	P removal rate by adsorption (mg P/ L·d)	P removal rate by precipitation (mg P/ L·d)	Fe ²⁺ production rate (mg / L·d)	P removed by precipitation/ Fe ²⁺ produced (mol/mol)
0.6	5.94 $\pm$ 0.03	3.68 $\pm$ 0.02	2.26	3.41 $\pm$ 0.02	1.20
2.4	5.99 $\pm$ 0.04	3.73 $\pm$ 0.03	2.26	10.35 $\pm$ 0.03	0.39
7.6	6.02 $\pm$ 0.05	4.08 $\pm$ 0.03	1.94	25.85 $\pm$ 0.02	0.14

Table 3.6 Molar ratio of P removed by precipitation to ferrous produced from iron ore particles of different sizes in static and rotating cultivation processes

Mean sizes of iron ore particles (mm)	P removed by precipitation/Fe ²⁺ produced (mol/mol)	
	Static reactor	Rotating reactor
0.6	0.11	1.20
2.4	0.35	0.39
7.6	1.17	0.14

The size of iron ore particles and anaerobic conditions are the major factors affecting the ferrous production and phosphate removal in BioIronTech process. The concentration of ferrous produced from iron ore reduction affects the efficiency of phosphorus removal by precipitation. The experimental results of the chemical precipitation of phosphorus by ferrous salt showed that the different molar ratios of Fe/P affected the phosphorus removal efficiency by precipitation (Fig. 3.15); as can be seen on the graph, more than 90% phosphorus could be removed only when the molar ratio Fe/P was not less than 2. A correlation, which is in fact a polynomial function between the removal efficiency with Fe/P molar ratio, is also illustrated in

Fig. 3.16. The relationship confirmed that a high ferrous production rate could cause a high phosphorus removal rate.

Fig. 3.15 Phosphorus removal efficiency with different molar ratios of Fe/P.

$$y = -13.753x^2 + 73.781x \quad (x \leq 3; R^2 = 0.9981) \quad (3-3)$$

Where x is the molar ratio of Fe to P

Fig. 3.16 The relationship between phosphorus removal efficiency by precipitation and molar ratio of Fe/P.

In spite of the similar phosphate removal efficiencies in static reactors with iron ore particle sizes of 0.6 mm, 2.4 mm, and 7.6 mm, the use of 7.6 mm size was still

preferable choice for phosphorus removal from reject water due to the lower risk of clogging in continuous process and the high removal efficiency of 90%. With the same particle size, the highest phosphorus removal rate of 25 mg P/ L·d, maximum phosphorus removal efficiency of 98%, and highest ferrous production rate of 44.5 mg/ L·d could be achieved in rotating reactor. Therefore, iron ore with mean size of 7.6mm is the most suitable for phosphorus removal from reject water using anoxic bio-reduction of iron ore.

3.3.3 TOC removal

Changes in dissolved TOC concentrations during both cultivation processes are shown in Fig. 3.17 and Fig. 3.18. After 28 days of static cultivation, TOC removal efficiencies for iron particles of 0.6 mm, 2.4 mm, 7.6 mm, 17 mm, and 35 mm were 71%, 86%, 89%, 87%, and 84%, respectively. In rotating reactor, the efficiencies for the sizes of 0.6 mm, 2.4 mm, and 7.6 mm were 31%, 41%, and 63%, respectively. The different dynamic behaviors of the TOC removal in Fig. 3.17 could be explained hypothetically by the different properties of iron ore particles surface. Iron ore particles with the sizes of 0.6 mm and 2.4 mm were selected by sieving after crushing; and iron ore particles with the sizes of 35 mm, 17.0 mm, and 7.6 mm were manually selected without crushing. Therefore, iron ore particles with the sizes of 0.6 mm and 2.4 mm had fresh and active surfaces, and iron ore particles with the sizes of 35 mm, 17.0 mm, and 7.6 mm had old surfaces. The iron ore particles with the sizes of 0.6 mm and 2.4 mm also had more surface area, they probably adsorbed TOC in the initial stage, microbial biomass probably developed the biofilm on their surface in the subsequent stage and the adsorption process was stopped, and TOC was mainly biodegraded in the final stage. Iron ore particles with the sizes of 35 mm, 17.0 mm, and 7.6 mm had inactive surfaces and less surface area, therefore, the TOC was probably removed mainly by degradation.

Fig. 3.17 Concentration of dissolved TOC in static reactors with iron ore particles of different sizes: ◆ 0.6 mm; ■ 2.4 mm; ▲ 7.6 mm; x 17 mm; □ 35 mm.

Fig. 3.18 Concentration of dissolved TOC in rotating reactors with iron ore particles of different sizes: ◆ 0.6 mm; ■ 2.4 mm; ▲ 7.6 mm.

Reject water contains a lot of branched volatile fatty acids, mainly isobutyric acid ($C_4H_8O_2$) and isocaproic acids ($C_6H_{12}O_2$) (Ivanov et al., 2005). When TOC components in the reject water are only isobutyric and isocaproic acids with the same ratio, empirical formula of VFA of reject water can be written as $C_5H_{10}O_2$; and theoretical molar ratio of oxidized TOC/reduced Fe would be 0.19:

The experimental molar ratios of TOC removed/ Fe^{2+} produced in static reactors (Table 3.7) and rotating reactors (Table 3.8) however were higher than 0.19. Therefore, the removal of organics from reject water must be mainly due to the degradation by other anaerobic bacteria and probably the adsorption by iron ore particles.

Table 3.7 TOC oxidation and Fe production in static reactors

Mean size of iron ore particles (mm)	Fe production (mg/L)	Molar of Fe production (mol)	TOC oxidation (mg/L)	Molar of TOC oxidation (mol)	Molar ratio of TOC oxidized/ Fe produced (mol/mol)
0.6	167.75	3.00	412.50	34.38	11.46
2.4	42.26	0.75	492.80	41.07	54.76
7.6	35.49	0.63	525.00	43.75	69.44
17	20.09	0.36	495.86	41.32	114.77
35	10.22	0.18	499.49	41.62	231.22

Table 3.8 TOC oxidation and Fe production in rotating reactors

Mean size of iron ore particles (mm)	Fe production (mg/L)	Molar of Fe production (mol)	TOC oxidation (mg/L)	Molar of TOC oxidation (mol)	Molar ratio of TOC oxidized/ Fe produced (mol/mol)
0.6	61.3	1.09	153	12.75	11.70
2.4	186.3	3.33	193	16.08	4.83
7.6	465.3	8.31	289	24.08	2.90

3.3.4 Significance

The experimental data showed that iron reducing bacteria used Fe(III) from solid crystalline iron ore as electron acceptor and reduced Fe(III) to Fe(II) by coupling with the oxidation of organic compounds in reject water under anaerobic conditions. The high ferrous production rate demonstrated that iron ore could replace amorphous ferric hydroxide in ferric reduction. The high phosphorus removal rate and removal efficiency proved that anaerobic bioreduction of iron ore could out-compete conventional chemical precipitation of phosphate with iron salts in the removal of phosphorus from reject water. Since the cost of Fe in iron ore is 7.2 times cheaper than that in iron salt, the cost of phosphorus removal can be reduced significantly.

Another benefit of this technology could be recovery of phosphorus as fertilizer. It is calculated that the annual supplies of phosphorus with reject water in Singapore with 4 million populations are 5,700 tons. Considering that world population, serving with centralized sewage treatment, is 1.5 billions, and the average domestic water consumption is the same as in Singapore, the annual recovery of phosphorus from reject water of MWWTPs worldwide could be approximately 2 million tons.

3.4 Conclusions

- 1) In static cultivation process, the ferrous production rate and phosphorus removal rate were associated with the size of initially loaded iron ore particles. A linear relationship between ferrous production rate and specific surface area of iron ore particles was established. The iron ore bioreduction rate negatively correlated with the initially loaded iron ore particles. The highest ferrous production rate was 40 mg/L·d in the reactor with iron ore particles of size 0.6

mm. The phosphorus removal efficiencies were about 90% for iron ore particles of 0.6 mm, 2.4 mm, and 7.6 mm. The highest phosphorus removal rate was 11 mg P/ L·d for the iron ore particles with mean size of 7.6 mm.

- 2) In rotating reactors, the rate of iron ore bioreduction was associated with the production of fine iron ore particles from initially loaded coarse iron ore. The phosphorus removal efficiency was about 97% for iron ore particle sizes of 0.6 mm, 2.4 mm, and 7.6 mm. The highest phosphorus removal rate of 25 mg P/ L·d and highest ferrous production rate of 44.5 mg/ L·d were obtained in the reactor with iron ore particles size of 7.6 mm.
- 3) The total organic carbon removal rate in both cultivation processes was not associated with the size of initially loaded iron ore particles. The instead, it was mainly due to the degradation by other anaerobic bacteria.
- 4) The removal of phosphorus from reject water using anoxic bioreduction of iron ore can outcome the conventional chemical precipitation of phosphate with iron salts because of the high removal efficiency and cheaper material used (iron ore).

Chapter 4

The removal of nitrogen and phosphorus from reject water of municipal wastewater treatment plant using ferric and nitrate bioreductions

4.1 Introduction

Reject water in MWWTPs of Singapore contributes about 4% of the total raw sewage flow and up to 50% of the nutrients and organics loads in the plant. With the population of 4 million people, the reject water in Singapore would annually supply 13,300 tons of nitrogen and 5,700 tons of phosphorus to the main streams of MWWTPs. Considering that world population, serving with centralized sewage treatment, is 1.5 billions, provided that the average domestic water consumption is the same as in Singapore, the numbers would be approximately 5 million tons of nitrogen and 2 million tons of phosphorus. Therefore, reject water should be treated for nitrogen, phosphorus, and organics removal before its recycling to the main stream (Pitman, 1999).

Various processes for the removal of nitrogen from reject water have been discussed in Chapter 2. However, all the processes cannot be applied in the removal of both nitrogen and phosphorus from reject water. Ammonium and phosphate can be simultaneously removed from reject water by struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) formation process (Battistoni et al., 1997; 2002; 2006), but the disadvantages are the slow crystallization rate of struvite as well as the requirements of big reactor and pH adjustment.

The removal of phosphorus from reject water by BioIronTech process (Ivanov et al.,

2005; 2009a; 2009b; Ivanov and Hung, 2010; Tay et al., 2008) were studied in Chapter 3. In BioIronTech process, the reduction of hematite in iron ore using acetate as electron donor and the precipitation of phosphate by ferrous ions can be described by the following equations:

These reactions can be performed in anaerobic (anoxic) biofilter filled with iron ore particles. However, there may be diverse interactions between NO_3^- , NO_2^- , NH_4^+ , Fe^{3+} , Fe^{2+} , HPO_4^{2-} , and CH_3COO^- ions, including the competitions between these ions in oxidation/reduction, chelation, or precipitation during the anaerobic treatment of reject water (Fig. 4.1).

The aim of this research was to study the interactions between transformations of NO_3^- , NO_2^- , NH_4^+ , Fe^{3+} , Fe^{2+} , HPO_4^{2-} , and CH_3COO^- ions during anaerobic treatment of reject water and to recommend the way for the removal of both nitrate and phosphate from reject water of MWWTP.

Fig. 4.1 Hypothetical interactions between NH_4^+ , NO_3^- , NO_2^- , Fe^{3+} , Fe^{2+} , HPO_4^{2-} , and organic acids during anaerobic treatment of reject water. The block arrows show (bio)chemical process, the dashed arrows show positive effects, and the dotted arrows show negative (minus) effects. Major interacting processes are as follows: (1) denitrification and ferric reduction competing for electron donors (organic acids); (2) precipitation of phosphate with the competition of phosphate, nitrate, and organic acids ions for ferrous ions.

4.2 Materials and methods

4.2.1 Iron ore, reject water, aerobic sludge, and anaerobic sludge

Iron ore was delivered from JuYuan Trading PTE LTD (PingXiang City, GuangXi,

China). According to specification, iron ore contained 60% (w/w) iron and the major mineral content was hematite (Fe_2O_3). The sizes of iron ore particles used in the experiments were between 0.5 and 1 mm, which were separated by sieving after crushing. The porosity of iron ore particles was determined in this study and the result was 46 ± 0.5 % (v/v, average value $\pm$ s.d.).

Samples of reject water, aerobic sludge (aerobic microbial biomass from activated sludge tanks), and anaerobic sludge (anaerobic microbial biomass from anaerobic digester) were collected from a MWWTP in Singapore. Anaerobic sludge was used as a source of iron-reducing bacteria (Ivanov et al, 2009a; Nielsen, 1996; Nielsen et al., 2002). Aerobic sludge (biomass from aerobic bioreactor) was used as a source for enrichment culture of facultative anaerobic denitrifying bacteria. This enrichment culture was then grown anaerobically in reject water with the addition of 0.4 g NO_3^- -N/L (model reject water after nitrification). Electron donors for bioreduction of nitrate were organic matters of reject water.

The reject water, aerobic and anaerobic sludges, and the enrichment culture of denitrifying bacteria were stored at 4 °C before the experiments. The characteristics of one sample of reject water, aerobic sludge, anaerobic sludge, and enrichment culture of denitrifying bacteria were determined in this study; the details of analytical methods and procedures used are described in section 4.2.3 and the results are given in Table 4.1. There were significant temporal variations of the concentrations of ammonium, phosphate, and total organic carbon (TOC) in reject water of MWWTP.

Table 4.1 Characteristics of one sample of reject water, aerobic sludge, anaerobic sludge, and enrichment culture of denitrifying bacteria. Average values $\pm$ standard deviation of triplicate independent measurements are shown

Parameters	Concentrations (mg/L)			
	Reject water	Suspension of aerobic sludge	Suspension of anaerobic sludge	Enrichment culture of denitrifying bacteria
Total organic carbon (TOC)	205 $\pm$ 4	n.d. *	n.d.	n.d.
Total suspended solids (TSS)	460 $\pm$ 50	7240 $\pm$ 300	9710 $\pm$ 430	n.d.
Volatile suspended solids (VSS)	430 $\pm$ 50	6570 $\pm$ 40	7430 $\pm$ 420	2800 $\pm$ 50
Total dissolved phosphate (TDP)	208 $\pm$ 2	n.d.	n.d.	n.d.
Total ferrous iron	9.1 $\pm$ 0.1	420 $\pm$ 0.1	470 $\pm$ 8	220 $\pm$ 0.1
Dissolved NH_4^+ -N	690 $\pm$ 8	n.d.	n.d.	n.d.
Dissolved NO_3^- -N	0.68 $\pm$ 0.04	n.d.	n.d.	n.d.
Dissolved NO_2^- -N	0.05 $\pm$ 0.01	n.d.	n.d.	n.d.
pH	7.69 $\pm$ 0.02	7.06 $\pm$ 0.02	6.75 $\pm$ 0.02	7.52 $\pm$ 0.02

*Note: n. d. means that the parameter was not determined.

4.2.2 Experimental set up

Three experimental and two control bioreactors were used for batch cultivation. Two electron acceptors, nitrate and ferric, were used in experiments for oxidations

of organics of reject water and additional electron donor, acetate. The objective of three experiments was to study an effect of different concentrations of acetate on nitrate and ferric bioreductions. The objective of negative control 1 was to study an effect of an absence of nitrate on bioreduction of ferric. The objective of negative control 2 was to study an effect of absence of acetate on bioreductions of both ferric and nitrate.

The mixture of 200 g of iron ore and 1.5 L of reject water was added into each of four 2 L bioreactors for the performance of control 2, and experiments 1-3 (Table 4.2). The potassium nitrate (GR grade, Merck) was added in control 2 and experiments 1-3 to make the model reject water after nitrification process containing about 0.4 g NO_3^- -N/L nitrate (Table 4.2). The sodium acetate (GR grade, Merck) was added only in the experimental bioreactors. The concentrations of acetate there after its addition were 1.07, 2.14, and 3.21 g/L (Table 4.2). These quantities of added acetate were corresponded to the molar ratios of added acetate and nitrate equal to 100%, 200%, and 300% of theoretical molar ratio according to the Equation 4-3.

The aliquots of 150 mL of anaerobic sludge, containing iron-reducing bacteria, 120 mL of aerobic sludge, and 30 mL of enrichment culture of denitrifying bacteria were added to each bioreactor as inoculum. The bioreactors were purged with nitrogen gas for 10 minutes before and after additions of the reagents and sealed up to maintain the anaerobic conditions. The bioreactors were placed on VELP® OVERHEAD MIXER vortex 6.8 (Velp Scientifica) at 30 rpm/min and 30°C for continuous mixing and incubation for 11 days. In order to study the activity of iron-reducing bacteria without addition of nitrate in control 1, the mixture of 200 g

iron ore, 150 mL anaerobic sludge, and 1.65 L reject water was added into reactor.

Table 4.2 Set up of controls and experiments

Controls and experiments	Addition			
	Inoculum	Iron ore	Nitrate	Acetate
Control 1 (no addition of nitrate and acetate)	150 mL (0.62 g VSS/L)	111 g/L	0	0
Control 2 (no addition of acetate)	300 mL (1.10 g VSS/L)	111 g/L	0.4 g N-NO ₃ ⁻ /L	0
Experiment 1 (molar ratio of added acetate and nitrate was 100% of theoretical ratio)	300 mL (1.10 g VSS/L)	111 g/L	0.4 g N-NO ₃ ⁻ /L	1.07 g/L
Experiment 2 (molar ratio of added acetate and nitrate was 200% of theoretical ratio)	300 mL (1.10 g VSS/L)	111 g/L	0.4 g N-NO ₃ ⁻ /L	2.14 g/L
Experiment 3 (molar ratio of added acetate and nitrate was 300% of theoretical ratio)	300 mL (1.10 g VSS/L)	111 g/L	0.4 g N-NO ₃ ⁻ /L	3.21 g/L

4.2.3 Analytical methods

Total suspended solids (TSS), volatile suspended solids (VSS), pH, the concentrations of nitrate, nitrite, ammonium, total ferrous ions, dissolved phosphate, and dissolved total organic carbon (TOC) were determined in the well-mixed

samples by standard methods (APHA, 2005). All procedures were similar to those in Chapter 3. The analytical method for the porosity of iron ore particles are described in Chapter 3. Additionally, the concentrations of volatile fatty acids (VFA) in the filtrated samples of reject water were analyzed using HPLC (Perkin Elmer Series 2000, Norwalk, CT, USA) equipped with a 220 mm x 4.6 mm polypore H column and UV 210 nm detector. The mobile phase was 0.005 N H₂SO₄ with a flow rate of 0.2 mL/min. All measurements were done in triplicates. The means and standard deviations have been determined for the parameters.

4.3 Results and discussion

4.3.1 Nitrate and dissolved organics removal from reject water

The volatile fatty acids (VFAs) comprised almost all dissolved total organic carbon (TOC) of reject water. The empirical formula of volatile fatty acids in reject water was CH₂O_{0.57} and average oxidation number of carbon in these organic matters was -0.86 (Table 4.3). Using empirical formula of organic matter in reject water, the balanced equations describing the reductions of ferric or nitrate by organic matter or acetate can be written as follows:

Equation 4-4 showed that theoretical mass ratio of TOC oxidized/Fe³⁺ reduced was 0.044 g/g. From this coefficient and data in Table 4.4, the quantity of organics in reject water oxidized by Fe³⁺ was from 0.5 to 6 % of total TOC removal. Thus, the major portion of TOC was oxidized by nitrate reducers. According to Equations 4-3 and 4-5, theoretical mass ratios of TOC oxidized/N-NO₃⁻ reduced were 1.07 and

0.88 g/g for oxidation of acetate and organics in reject water, respectively.

Table 4.3 The VFA content in reject water of MWWTP

VFA	Molecular formula	Empirical formula	Molar content of VFA carbon, % of total VFA carbon
Formic acid	CH ₂ O ₂	CH ₂ O ₂	8.3
Acetic acid	C ₂ H ₄ O ₂	CH ₂ O	5.9
Propionic acid	C ₃ H ₆ O ₂	CH ₂ O _{0.67}	10.4
Isobutyric acid	C ₄ H ₈ O ₂	CH ₂ O _{0.5}	5.1
Butyric acid	C ₄ H ₈ O ₂	CH ₂ O _{0.5}	2.0
Isovaleric acid	C ₅ H ₁₀ O ₂	CH ₂ O _{0.4}	17.7
Valeric acid	C ₅ H ₁₀ O ₂	CH ₂ O _{0.4}	5.9
Isocaproic acid	C ₆ H ₁₂ O ₂	CH ₂ O _{0.33}	17.1
Caproic acid	C ₆ H ₁₂ O ₂	CH ₂ O _{0.33}	12.6
Heptanoic acid	C ₇ H ₁₄ O ₂	CH ₂ O _{0.286}	14.9
All VFAs of reject water	-	CH ₂ O _{0.57}	100

In experiments 1-3, the ratios of TOC removed/N-NO₃⁻ reduced were 0.93-1.03 g/g, which were close to the stoichiometric ratio of acetate oxidation. The ratio was only 0.40 for oxidation of organics in reject water in control 2. The difference between the theoretical and experimental ratio could be hypothetically explained by the addition of aerobic and anaerobic biomass to start up the batch process (see Table 4.2). Some portion of this biomass could be hydrolyzed and used for denitrification. The utilization of hydrolyzed biomass for denitrification in reject water has been demonstrated (Gali et al., 2006a).

Table 4.4 Maximum rates of ferrous production, nitrate reduction, and TOC removal in controls and experiments for the first 2 days of cultivation. Average values $\pm$ standard deviation of triplicate independent measurements are shown

Controls and experiments	Maximum of the parameter		
	Rate of total	Rate of nitrate	Rate of TOC
	ferrous production mg Fe ²⁺ /L·d	reduction mg N-NO ₃ ⁻ /L·d	removal mg TOC/L·d
Control 1	37 $\pm$ 0.54	n.d.*	27 $\pm$ 2.28
Control 2	21 $\pm$ 0.09	103 $\pm$ 1.61	41 $\pm$ 1.04
Experiment 1	58 $\pm$ 2.66	356 $\pm$ 6.95	367 $\pm$ 12.0
Experiment 2	48 $\pm$ 0.98	396 $\pm$ 5.10	392 $\pm$ 11.4
Experiment 3	53 $\pm$ 0.46	389 $\pm$ 2.10	361 $\pm$ 13.8

*Note: n.d. means that the parameter was not determined because nitrate was not added in control 1.

Fig. 4.2 Nitrate concentration in reject water in: Δ control 2 (no acetate added); $\blacksquare$ experiment 1; $\blacktriangle$ experiment 2; $\diamond$ experiment 3, with molar ratios of acetate to nitrate, 100, 200, and 300 % of theoretical ratio by Equation 4-3, respectively.

From Equation 4-5, the reduction of 0.4 g N-NO₃⁻ /L in reject water required at least

0.35 g/L of TOC while the actual amount available was only 0.21 g/L. Therefore, an addition of other electron donor was essential to perform the complete reduction of nitrate in reject water. It was known that denitrification of the wastewater containing high nitrate concentration required an addition of external electron donors such as acetate, methanol, or ethanol (Fernández-Nava et al., 2008). This theoretical consideration was confirmed by the experimental study. The maximum nitrate removal rate for first 2 days in control 2 (no addition of acetate) was 103 mg N/L·d, while the rates were from 356 to 396 mg N/L·d in all experiments with an addition of acetate (Fig. 4. 2 and Table 4.4).

4.3.2 Fe(II) production from iron ore

The concentrations of total Fe(II) increased gradually during the 2 days of batch cultivation in all reactors (Fig. 4.3). The maximum Fe(II) production rates during this period, which could be used for the design of the continuous process, are shown in Table 4.4. After two days of the incubation, ferric reduction was stopped in control 2 (with addition of nitrate and no addition of acetate) but was continued with almost linear rate in control 1 (no additions of nitrate and acetate) and all experimental bioreactors (Fig. 4.3 and Table 4.5). In the experiments 1-3, where both acetate and nitrate were added, maxima of ferrous production rates were 2.8 times higher than in control 2 (Table 4.4). Without addition of acetate in control 2, the iron bioreduction was over competed by nitrate reduction after 2 days of cultivation.

The competition can be theoretically explained by the sequence of redox potential. Theoretically, the degradation of organic matter is performed by the sequential reduction of O_2 , NO_3^- , Mn^{4+} , Fe^{3+} , SO_4^{2-} , and CO_2 due to the decreasing redox potential of these electron acceptors. The dominance of the related groups of

microorganism will be promoted in the same sequence, i.e. aerobes will over-compete denitrifying bacteria; denitrifiers will over-compete manganese- and ferric-reducing bacteria, which is our case; ferric-reducing bacteria will over-compete sulfate-reducing bacteria, and sulfate-reducing bacteria will over-compete methanogens reducing CO_2 (Achtnich et al., 1995). A competition between nitrate reducers and ferric reducers for electron donors is also known for soil systems (Achtnich et al., 1995), anaerobic sediment (Sorensen, 1982), pure cultures (DiChristina, 1992), and iron seeps in a sulfur-rich freshwater ecosystem (Haaijer et al., 2008).

Fig. 4.3 Production of total ferrous in controls and experiments: ◆ control 1 (no nitrate and acetate added); Δ control 2 (no acetate added); ■ experiment 1; ▲ experiment 2; ◇ experiment 3, with molar ratios of acetate to nitrate, 100 , 200 and 300 % of theoretical ratio by Equation 4-3, respectively.

Even though nitrate reducers overwhelmed the competition, when sufficient electron donors (organic acids of reject water in control 2 or added acetate in experiments) were present, both ferric and nitrate were simultaneously reduced in control 2 and experiments 1-3. The rate of the ferric reduction yet was over-competed by the nitrate reduction after two days of cultivation when the

concentration of electron donors (soluble TOC of reject water) significantly decreased. Therefore, the stages of denitrification and ferric bio-reduction/phosphate precipitation must be performed sequentially to avoid the negative interactions between nitrogen and phosphorus removals during anaerobic treatment of reject water.

Additionally, the inhibition of Fe^{3+} bio-reduction by nitrate bio-reduction could be due to the chemical re-oxidation of biologically produced Fe^{2+} by NO_2^- , a product of NO_3^- bio-reduction (Cooper et al., 2003; Coby and Pacardal, 2005). However, the effect should not be significant because the nitrite concentration was less than 1 mg N- NO_2^- /L in all experiments and control 2.

Beside the reduction by iron reducing bacteria, it is hypothesized that Fe^{3+} can be biologically reduced by ammonium to form Fe^{2+} and nitrite (Clément et al., 2005). However, the oxidation of ammonium with Fe^{3+} was absent in the described research. The concentration of ammonium remained in the range of 629-655 mg N/L and the concentration of nitrite was within the range from 0.05 to 1.7 mg N/L in all experiments and controls.

Bacteria that reduce iron ore using the organics in reject water were able to degrade m-cresol, 2, 4-dichlorophenol, p-phenylphenol, diphenylamine, estrogens such as 17β -estradiol, estriol, and 17α -ethynylestradiol as well (Ivanov et al., 2005; 2010). Therefore, the anaerobic IRB could be useful also to diminish the concentrations of xenobiotics in treated wastewater.

In terms of timeline, the duration of batch cultivation was relatively long (several weeks) because of the slow specific growth rate of iron-reducing bacteria in comparison with that of heterotrophic bacteria. Specific growth rate of

iron-reducing bacteria (μ) can be determined from the condition that production of bacterial biomass (ΔX) is proportional to production of ferrous ions (ΔP):

$$\mu = \Delta \ln X / \Delta t = \Delta \ln P / \Delta t \quad (4-6)$$

where t is time of batch cultivation. Maximum of specific growth rate of iron-reducing bacteria ($\mu_{\max}$) was 0.017 h^{-1} as calculated from the data shown in Fig. 4.3. Nevertheless, this slow growth rate is not an obstacle for the practical application of iron-reducing bacteria.

For example, during the continuous cultivation of iron-reducing bacteria in a fixed biofilm submerged reactor filled with iron ore as biofilm carrier and electron acceptor, and with the rate of bacterial biomass detachment during the steady state at the level of 1% of attached to the carrier biomass, the steady state hydraulic retention time can be determined by the following equations:

$$\mu X - 0.01DX = 0 \quad (4-7)$$

where dilution rate D = flow rate/volume of liquid in the reactor, and 0.6 h of hydraulic retention time is acceptable for industrial bioprocess.

$$\text{HRT}_{\min} = 1/D_{\max} = 1/100 \mu_{\max} = 0.59 \text{ h} \quad (4-8)$$

4.3.3 Kinetics of phosphate removal from reject water

It was shown that the ferrous ions produced by iron-reducing bacteria during the reduction of ferric hydroxide or iron ore in reject water precipitated phosphate (Ivanov et al., 2005; 2009a). In this study, the production of ferrous ions during the

bio-reduction of iron ore was also accompanied by the removal of dissolved phosphate from reject water (Fig. 4.4).

Fig. 4.4 Removal of phosphate from reject water of MWWTP: ♦ control 1 (no nitrate and acetate added); Δ control 2 (no acetate added); ■ experiment 1; ▲ experiment 2; ◇ experiment 3, with molar ratios of acetate to nitrate, 100 , 200, and 300 % of theoretical ratio by Equation 4-3, respectively.

However, presence of nitrate and acetate negatively affected the precipitation of phosphate by ferrous ions. The average rates of the phosphate removal from reject water were 14.6 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$ in control 1 (no nitrate and acetate added), 11 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$ in control 2 (no acetate added), 10.5 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$ in experiment 1, 10.3 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$ in experiment 2, and 9.7 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, in experiments 3. Maximum phosphate removal rates in control 1 (without addition of nitrate and acetate), control 2 (without addition of acetate) and experiments 1-3 were 63 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, 56 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, 56 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, 42 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, and 35 mg $\text{PO}_4^{3-}/\text{L}\cdot\text{d}$, respectively. The efficiencies of phosphate removal from reject water were 88% in control 1 (no nitrate and acetate added), 73% in control 2 (no acetate added), 66%, 65% and 64% in experiments 1, 2 and 3, respectively. Lowest final concentration of phosphate after treatment was 21 mg $\text{PO}_4^{3-}/\text{L}$ in control 1.

The data showed that the presence of nitrate or acetate in reject water decreased both the rate and efficiency of phosphate removal. So, an addition of acetate to increase both the nitrate removal rate and nitrate removal efficiency will negatively affect both the phosphate removal rate and efficiency. The mechanism of this inhibition could be the competition between phosphate, nitrate, and acetate anions in their interactions with ferrous cations.

4.3.4 Stoichiometry of phosphate removal from reject water

Phosphate was removed from reject water by the chemical precipitation with ferrous produced from iron ore and by adsorption on the surface of iron ore particles. The theoretical mass ratios of Fe^{2+} produced/ PO_4^{3-} precipitated for FeHPO_4 or $\text{Fe}_3(\text{PO}_4)_2$, are 0.59 and 0.88, respectively. The mass ratios of ferrous production/ phosphate precipitation in control 1 (without addition of nitrate and acetate) are shown in Table 4.5. The mass ratios of Fe^{2+} produced/ PO_4^{3-} precipitated were increased from 0.33 to 9.61 during 11 days of batch cultivation. In the first day of cultivation, the produced ferrous mainly involved in chemical precipitation of phosphate. Phosphate was also removed by the adsorption on iron ore particles because the ratio of Fe^{2+} produced/ PO_4^{3-} precipitated was less than the theoretical one. After two days of cultivation, the significant portion of the produced ferrous did not participate in the precipitation of phosphate as ferrous phosphate, but formed insoluble ferrous iron hydroxide and bound less phosphate, and phosphate was also removed due to adsorption of phosphate ions on iron ore particles. pH during nitrate reduction was increased from 7.7 to 8.5-8.7 in control 2 and experiments 1-3. This increase of pH, which stimulated the hydrolysis of Fe^{2+} ions, could be another reason of the negative effect of nitrate on phosphate precipitation with Fe^{2+} ions.

Table 4.5 Ferrous production and phosphate removal in control 1 (without addition of nitrate and acetate). Average values $\pm$ standard deviation of triplicate independent measurements are shown

Parameter	Period of cultivation, d					
	0 - 1	1 - 2	2 - 4	4 - 6	6 - 8	8 - 11
Phosphate removal	62.60 $\pm$	37.16 $\pm$	14.62 $\pm$	8.25 $\pm$	5.92 $\pm$	1.44 $\pm$
rate, mg PO ₄ ³⁻ /L·d	2.49	2.64	0.91	0.31	0.15	0.15
Ferrous production	20.59 $\pm$	36.90 $\pm$	16.56 $\pm$	26.09 $\pm$	24.22 $\pm$	13.84 $\pm$
rate, mg/L·d	0.60	0.54	0.17	0.46	0.49	0.23
Mass ratio of	0.33 $\pm$	0.99 $\pm$	1.13 $\pm$	3.16 $\pm$	4.09 $\pm$	9.61 $\pm$
Fe ²⁺ produced/ PO ₄ ³⁻ precipitated	0.02	0.07	0.07	0.13	0.13	1.0

It is known that the combination of the anoxic and oxic (aerobic) processes in MWWTP can enhance wastewater treatment efficiency (Battiston et al., 2008). Similar recommendations can be made from the experimental data shown above: the wastewater treatment in the existing MWWTP can be enhanced by the anoxic and oxic (aerobic) treatment of reject water using bioreduction and oxidation of iron compounds. The stages of denitrification and ferric bioreduction/phosphate precipitation during anaerobic treatment of reject water must be performed sequentially to avoid the negative interactions between the nitrogen and phosphorus removals. The addition of electron donor during denitrification process is essential to achieve maximum denitrification rate and efficiency. An additional stage of aerobic oxidation of ammonium and ferrous ions must be included in the process of nutrients removal from reject water in MWWTP (Fig. 4.5). Nitrate and ferric produced in aerobic oxidation stage have to be recycled to anoxic stages of the process (Fig. 4.5).

Fig. 4.5 Schematic diagram of the enhancement of municipal wastewater treatment plant with anoxic/oxic continuous treatment of reject water using bioreduction and oxidation of iron compounds. Dark block shows the schematics of the proposed innovative facility for nitrogen and phosphorus removal from reject water using iron ore (hematite, Fe_2O_3).

4.4 Conclusions

- 1) There was a competition between denitrifying and iron-reducing bacteria for electron donor during anaerobic treatment of reject water.
- 2) The presence of nitrate or acetate diminished the rate and efficiency of phosphate precipitation by ferrous ions, which were produced from bio-reduction of iron ore.
- 3) The stages of denitrification and ferric bio-reduction/phosphate precipitation must be performed sequentially to avoid the negative interactions between the nitrogen and phosphorus removals during anaerobic treatment of reject water in MWWTP.
- 4) An addition of electron donor in denitrification stage is necessary to achieve maximum denitrification rate and efficiency.

Chapter 5

The interactions between nitrate reduction, iron ore reduction and organic carbon oxidation during anaerobic treatment of reject water from MWWTPs

5.1 Introduction

The removal of ammonium from reject water can be achieved by aerobic nitrification process and anoxic denitrification process, during which organic carbon is necessary for denitrifying bacteria. The removal of phosphate from reject water can be achieved by precipitating with ferrous ions, which are reduced from $\text{Fe}(\text{OH})_3$ or iron ore by iron reducing bacteria under anaerobic conditions (Ivanov et al., 2005; 2009a; 2009b). Therefore, iron ore reduction and nitrate reduction are the key steps for the removal of nutrients from reject water, and the interactions between nitrate reduction, iron ore reduction, and organic carbon oxidation are significantly important in anaerobic treatment of reject water from MWWTPs.

Theoretically, electron acceptor with higher redox potential will be preferable and will promote sequential dominance of the related groups of microorganism. Under anaerobic environments, $\text{Fe}(\text{III})$ reduction is energetically favorable as compared with sulfate reduction and methanogenesis. However, nitrate reduction is energetically favorable as compared with $\text{Fe}(\text{III})$ reduction. The inhibition of iron reduction by nitrate is known for anaerobic sediment (Sorensen, 1982), pure culture (DiChristina, 1992), soil systems (Achnich et al., 1995), and bacteria associated with iron seeps in a sulfur-rich freshwater ecosystem (Haaijer et al., 2008). The negative impact on $\text{Fe}(\text{III})\text{EDTA}$ reduction rate in the presence of nitrate or nitrite has been reported also (Zhang et al., 2009). The mechanism of the inhibition is due

to the competition for electron donors between nitrate reducers and iron reducing bacteria. Also, nitrate tends to be more available to cells than the insoluble form of Fe(III) (Achtnich et al., 1995; DiChristina, 1992; Obuekwe et al., 1981; Sorensen, 1982). At the same time, the presence of iron oxide also partially inhibits nitrate reduction (Cooper et al., 2003). During nitrate reduction process, the biologically produced NO_2^- from NO_3^- abiotically re-oxidizes Fe^{2+} to produce Fe(III) hydroxide, which coats the cell and inhibits the transport of electron acceptors into the cell (Coby and Picardal, 2005; Cooper et al., 2003). The competitive interactions between iron reduction and nitrate reduction are shown in Fig. 5.1. However, the occurrence of such competition during anaerobic treatment of reject water is originally studied.

Fig. 5.1 The competitive interactions between nitrate reduction and iron reduction: electron donors prefer nitrate reducers thus iron reduction is inhibited; Fe(II) ions abiotically react with nitrite to produce $\text{Fe}(\text{OH})_3$ to coat the cells and inhibit the nitrate reduction.

It is known that nitrate is the theoretically favorable electron acceptor for ferrous iron oxidation (Straub et al., 2001). Anaerobic oxidation of ferrous is coupled to nitrate reduction to produce nitrogen gas and Fe(III). The process is mediated by enrichment culture or pure culture (Straub et al., 1996) or by the mesophilic denitrifying bacteria at neutral pH (Benz et al., 1998). The nitrate-dependent ferrous oxidation process is known in activated sludge anoxic (denitrification) process

(Nielsen and Nielsen, 1998 a; 1998b) and paddy soil (Ratering and Schnell, 2001). In addition to soluble Fe(II), solid Fe(II) is also used in such biological process (Weber et al., 2001). The protons produced from the Fe(II) oxidation process can neutralize the alkalinity produced from heterotrophic nitrate reduction process. However, the role of Fe(II), produced from iron ore reduction by iron reducing bacteria, in nitrate reduction during anaerobic treatment of reject water is still unknown .

It was reported that the sorption of Fe(II) produced from Fe(III) reduction on iron reducing bacteria of *Schewanella Putrefaciens* strain CN32 inhibited Fe(III) reduction (Liu et al., 2001a); and the adsorbed/precipitated Fe(II) on goethite also inhibited the extent and rate of Fe(III) oxide reduction (Roden and Zachara, 1996). Therefore, the potential interactions between nitrate reduction, iron ore reduction, ferrous oxidation, and organic carbon oxidation are complicated (Fig. 5.2). The aim of this study was to study the interactions between nitrate reduction; iron ore reduction, and organic carbon oxidation during anaerobic treatment of reject water from MWWTPs.

5.2 Materials and methods

5.2.1 Reject water, anaerobic sludge, iron ore, and iron reducing bacteria enrichment

The reject water and anaerobic sludge used in the batch experiments were collected from Jurong Wastewater Reclamation Plant (Singapore). Anaerobic sludge which contained iron reducing bacteria was used as a source of iron-reducing bacteria (Nielsen, 1996; Nielsen et al., 2002) and denitrifying bacteria. The reject water and anaerobic sludge were stored at 4 °C before being used for the experiments. Iron ore

was delivered from JuYuan Trading PTE LTD (PingXiang City, GuangXi, China). According to specification, iron ore contained 60% (w/w) iron and the major mineral content was hematite (Fe_2O_3). The sizes of iron ore particles used in the experiments were from 150 to 250 μm , which were separated by sieving after crushing. The characteristics of reject water and anaerobic sludge were determined in this study, the details of analytical methods and procedures used have been described in Chapter 3 and the results are given in Table 5.1.

Fig. 5.2 The possible interactions between organic carbon oxidation, iron ore reduction, nitrate reduction, and Fe(II) oxidation during anaerobic treatment of reject water: competition for organic carbon between iron reducing bacteria and heterotrophic denitrifying bacteria ( ); iron ore reduction process and heterotrophic nitrate reduction process ( ); Fe(II) was involved in autotrophic nitrate reduction process ( ); adsorption/precipitation of Fe(OH)₂ on iron reducing bacteria and iron ore ( ).

Table 5.1 Characteristics of reject water and anaerobic sludge. Average values $\pm$ standard deviation of triplicate independent measurements are shown

Characteristic	Concentration (mg/L)	
	Reject water	Suspension of anaerobic sludge
Total organic carbon (TOC)	543 ± 7	n.d. *
Total Fe(II)	7.1 ± 0.1	n.d.
Dissolved NO_3^- -N	0.53 ± 0	n.d.
Dissolved NO_2^- -N	0.05 ± 0.01	n.d.
Volatile suspended solids (VSS)	n.d.	7600 ± 212
pH	7.15 ± 0.01	7.17 ± 0

*Note: n.d. means the characteristic was not determined.

The medium for enrichment of iron reducing bacteria consisted of: NaHCO_3 , 2.5 g/L; NH_4Cl , 0.25 g/L; NaH_2PO_4 , 0.6 g/L; KCl , 0.1 g/L; acetate, 1.5 g/L; ferric EDTA sodium salt hydrate, 1.84 g/L; and trace element solution, 1mL/L. The trace element solution contained: EDTA-2Na, 10 g/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.12 g/L; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.12 g/L; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.03 g/L; $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 0.05 g/L; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L; $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$, 0.05 g/L; and H_3BO_3 , 0.05 g/L. 900 mL of medium was mixed with 100 mL of anaerobic sludge, purged with nitrogen gas for 10 minutes, sealed up and put on DK-OS010 Orbital Shaker (Daiki Science) (150 rpm/min) for 7 days; then 100 mL of the solution was then transferred to 900 mL fresh medium, and the same procedure was carried on to perform another 7 days of cultivation. The cultivated iron reducing bacteria enrichment solution was used in the experiment of the reactions of nitrate, nitrite, and ferrous in reject water under anaerobic conditions.

5.2.2 Experimental set up

5.2.2.1 The effects of nitrate reduction on iron ore reduction

The experiment was performed in batch culture. A mixture of 80 g of iron ore and 2L of reject water was added into each of the 2 L experimental bioreactors. An aliquot of 40 mL of anaerobic sludge containing iron reducing bacteria and denitrifying bacteria was added as inoculum to each bioreactor. All the solutions were purged with nitrogen gas for 10 minutes and sealed up to maintain the anaerobic conditions. All the reactors were put in 30 °C temperature room and continuously shaken on VELP® OVERHEAD MIXER votex 6.8 (Velp Scientifica) (15 rpm/min) for 9 days. At the 3rd day of experiment, potassium nitrate powder (GR grade, Merck) was added in 5 reactors to achieve nitrate concentrations of 0 mg N/L (biological control, reactor 1), 50 mg N/L (reactor 2), 100 mg N/L (reactor 3), 200 mg N/L (reactor 4), and 400 mg N/L (reactor 5), respectively. Abiotic control (reactor No 0) was used to evaluate the production of Fe(II) from abiotic process. For abiotic control, a mixture of 2L reject water with 40 mL anaerobic sludge was autoclaved at 121 °C for 15 minutes. The other conditions were the same as the experimental reactors. At the 6th day of experiment, 300 mL, 1300 mL, and 1300 mL of fresh reject water were replaced in the reactors 3, 4, and 5, respectively to supplement the organic carbon and partially remove previously produced Fe(II). At the 7th day of experiment, potassium nitrate powder was added in the reactors 2, 3, 4, and 5 again to achieve about 50 mg N/L, 100 mg N/L, 200 mg N/L, and 400 mg N/L NO₃⁻-N, respectively. The experimental set up is shown in Table 5.2.

Table 5.2 Set up of controls and experiments

Additions	Reactor No					
	0	1	2	3	4	5
	(abiotic control)	(biotic control)				
Reject water(L)	2	2	2	2	2	2
	(autoclaved)					
Iron ore (g)	80	80	80	80	80	80
(g iron ore/L)	(40)	(40)	(40)	(40)	(40)	(40)
Anaerobic sludge	40	40	40	40	40	40
(mL) (g VSS/L)	(autoclaved)	(0.152)	(0.152)	(0.152)	(0.152)	(0.152)
NO ₃ ⁻ -N (mg N/L)	n.a.*	n.a.	50	100	200	400
by adding KNO ₃ powder at 3 rd day						
Replacement of reject water at 6 th day (mL)	n.a.	n.a.	n.a.	300	1300	1300
NO ₃ ⁻ -N (mg N/L)	n.a.	n.a.	50	100	200	400
by adding KNO ₃ powder at 7th day						

* Note: n.a. means the addition was not performed in the reactor

5.2.2.2 The reactions of nitrite, nitrate, and ferrous in reject water under anaerobic conditions

1L reject water was filled in reactor A, and 0.98 L reject water was filled in reactors B, C, and D, respectively. All the reactors were purged with nitrogen gas for 30 minutes to ensure the anaerobic conditions. Immediately, 1.49 g FeSO₄ • 7H₂O

powder was added to reactor A (equal to 300 mg Fe/L); 1.49 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (equal to 300 mg Fe/L) powder and 20 mL iron reducing bacteria enrichment solution were added to reactor B; 1.49 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder (equal to 300 mg Fe/L), 0.228 g KNO_3 powder (GR grade, Merck) (equal to 31 mg N/L) and 20 mL iron reducing bacteria enrichment solution were added to reactor C; 1.49 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder (equal to 300 mg Fe/L), 0.1511 g NaNO_2 powder (GR grade, Merck) (equal to 30.6 mg N/L), and 20 mL iron reducing bacteria enrichment solution were added to reactor D. For control reactor E, 1L DI water was purged with nitrogen gas for 30 minutes and 1.49 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder (equal to 300 mg Fe/L) was added in immediately. All the reactors were sealed up and shaken on DK-OS010 Orbital Shaker (Daiki Science) (150 rpm/min) for 1 day. The experimental set up is shown in Table 5.3.

Table 5.3 Set up of experiments for the reactions between nitrite, nitrate, and ferrous in reject water under anaerobic conditions

Reactor	Reject water (L)	Iron reducing bacteria enrichment (mL)	KNO_3 (g)	NaNO_2 (g)	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (g)
A	1	n.a.*	n.a.	n.a.	1.4951
B	0.98	20	n.a.	n.a.	1.4939
C	0.98	20	0.228	n.a.	1.4940
D	0.98	20	n.a.	0.1151	1.4969
Control	DI water 1L	n.a.	n.a.	n.a.	1.4979

* Note: n.a. means that the addition was not performed in the reactor

5.2.2.3 MPN (Most Probable Number) analysis for iron reducing bacteria

Enumeration of iron reducing bacteria was performed using a modification of Most

Probable Number (MPN) method (Nielsen et al., 2002). The sterile medium for the enumeration comprised of NaHCO_3 , 2.5 g/L; NH_4Cl , 1.5 g/L; NaH_2PO_4 , 0.6 g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g/L; KCl , 0.1 g/L; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 g/L; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.005 g/L; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.001 g/L; ferric EDTA sodium salt hydrate, 1.84 g/L; and peptone, 1.5 g/L. The medium was adjusted to pH 7.0 and purged with nitrogen gas for 10 minutes. The MPN analysis was performed in three replicate series of ten-fold diluted samples. Sterile medium was applied with 1.5 mL Eppendorf sterile tubes and incubated in anaerobic chamber (Sheldon Manufacturing, Inc., Cornelius, OR, USA) for 10 days. The chamber was subjected to a gas mixture of, v/v: N_2 , 90%; H_2 , 2.5%; CO_2 , 2.5% for 10 days. To detect the formation of Fe(II), 0.03 mL of 0.1% Ferrozine reagent (Sorensen, 1982; Stookey, 1970) was injected into each tube with a purple color indicating a positive result. MPN values were calculated from standard MPN table (Cochran, 1950).

5.2.2.4 MPN (Most Probable Number) analysis for nitrate reducing bacteria

Enumeration of nitrate reducing bacteria was performed using a Most Probable Number method. Reject water was mixed with potassium nitrate powder (0.72 g potassium nitrate /L reject water, 100 mg NO_3^- -N/L reject water), sterilized at 121 °C for 15 minutes, adjusted to pH 7.5, and purged with nitrogen gas for 10 minutes. A triplication of ten-time diluted samples were made in sterile culture tubes (diameter 16 mm, length 150 mm) and incubated in anaerobic chamber (Sheldon Manufacturing, Inc, Cornelius, OR, USA) for 2 days. The growth of nitrate reducing bacteria was detected by the presence of large gas bubbles in the headspace of the inverted Durhan tubes (diameter 7.5 mm, length 50 mm) in the culture tubes and the reduction of nitrate concentration indicating positive result. MPN values were calculated from standard MPN table (Cochran, 1950).

5.2.3 Analytical methods

Volatile suspended solids, pH, concentrations of nitrate, nitrite, total Fe(II), and dissolved total organic carbon (TOC) were measured using the same methods in Chapter 3.

5.3 Results and discussion

5.3.1 The reactions between nitrite/nitrate and ferrous in reject water

The experimental results are shown in Table 5.4. Under anaerobic conditions, 60.5% of Fe(II) was oxidized in reject water within one day. However, Fe(II) concentration almost remained unchanged in DI water under the same conditions, which implied that Fe(II) was oxidized by some components in reject water. When the reject water contained iron reducing bacteria enrichment under the same experimental conditions, only 32.7% of Fe(II) was oxidized, which indicated the activities of iron reducing bacteria inhibited the oxidation of Fe(II). In comparison with the decrease of Fe(II) concentration, concentrations of nitrate and nitrite barely changed under the same experimental conditions. The results showed that nitrite and nitrate did not react with Fe(II) or any components in reject water. The presence of nitrite in this experiment, however, significantly enhanced the oxidation of Fe(II) with 94% of Fe(II) oxidized. The presence of iron reducing bacteria in this case did not inhibit Fe(II) oxidation. In comparison with only 32.7% Fe(II) oxidation efficiency with iron reducing bacteria in the previous case, it seemed the activities of iron reducing bacteria were inhibited by nitrite. When reject water contained nitrate and iron reducing bacteria enrichment, 48% of Fe(II) was oxidized by the components of reject water under the same experimental conditions, as compared with 60.5% Fe (II) oxidization efficiency without iron reducing bacteria and 32.7%

Fe(II) oxidization efficiency with iron reducing bacteria. It was also obvious that nitrate partially inhibited the activities of iron reducing bacteria.

Table 5.4 Change of nitrate, nitrite, and ferrous concentrations in reject water and distilled water (average $\pm$ s.d.)

		Fe(II) in reject water (A)	Fe(II) + IRB in reject water (B)	Fe(II)+ NO ₃ ⁻ + IRB in reject water (C)	Fe(II)+ NO ₂ ⁻ + IRB in reject water (D)	Fe(II) in DI water (E)
Initial	N- NO ₃ ⁻ (mg N/L)	n.d*	n.d	28.10 $\pm$ 0.32	n.d	n.d
	N- NO ₂ ⁻ (mg N/L)	n.d	n.d	n.d	30.56 $\pm$ 0.32	n.d
	Fe(II) (mg/L)	289.54 $\pm$ 0.48	286.44 $\pm$ 1.28	287.44 $\pm$ 2.28	289.44 $\pm$ 1.48	288.44 $\pm$ 0.28
Final	N- NO ₃ ⁻ (mg N/L)	n.d	n.d	26.71 $\pm$ 0.04	n.d	n.d
	N- NO ₂ ⁻ (mg N/L)	n.d	n.d	n.d	28.04 $\pm$ 0.04	n.d
	Fe(II) (mg/L)	114.26 $\pm$ 2.93	192.68 $\pm$ 1.14	149.44 $\pm$ 0.10	16.82 $\pm$ 1.98	282.21 $\pm$ 0.25

* Note: n.d. means not determined; IRB means iron reducing bacteria enrichment.

5.3.2 Iron ore reduction and ferrous production

The total Fe(II) concentrations in the reactors during the cultivation process are shown in Fig. 5.3a. The production of Fe(II) in abiotic control (reactor No 0) was

less (only 24 mg/L) during the whole process (Fig. 5.3a). Reversely, significant iron ore reduction was observed in all biological reactors from the first day. Before the addition of nitrate at the 3rd day of experiment, Fe(II) production rate was similar in all biological reactors with the average rates of 37.7 mg/L·d, 42.2 mg/L·d, 40.4 mg/L·d, 39.4 mg/L·d, and 45.0 mg/L·d in biological control (reactor 1), reactor 2, reactor 3, reactor 4, and reactor 5, respectively (Fig. 5.3a). When different quantities of NO_3^- -N were added in experimental reactors at the 3rd day of experiment to achieve 0 mg N/L (biological control reactor 1), 50 mg N/L (reactor 2), 100 mg N/L (reactor 3), 200 mg N/L (reactor 4), and 400 mg N/L NO_3^- -N (reactor 5), respectively, different iron ore reduction rates were observed. In biological control with 0 mg N/L NO_3^- -N, iron ore continued to be reduced and reached 20 mg/L·d Fe(II) production rate during day 3. In the experimental reactors with 50 mg N/L (reactor 2), 100 mg N/L (reactor 3), 200 mg N/L (reactor 4), and 400 mg N/L NO_3^- -N (reactor 5), iron ore reduction was inhibited; simultaneously, total Fe(II) concentrations were decreased 20.6 mg/L, 24.6 mg/L, 14.5 mg/L, and 129.8 mg/L in reactors 2, 3, 4, and 5 respectively (Fig. 5.3a). It was concluded that nitrate reduction inhibited iron ore reduction.

When nitrate in the reactors was completely reduced, iron reducing bacteria recovered by utilizing the remaining organic carbon in reject water. In reactors 2 and 3, the remaining TOC concentrations were 303 mg/L and 202 mg/L, respectively. Iron ore reduction consequently resumed and the total Fe(II) concentration increased accordingly during day 4 (Fig. 5.3a).

Fig. 5.3 Concentrations of total Fe(II) (a) and TOC (b) in the reactors with addition of different quantities of NO_3^- -N, mg N/L: $\blacklozenge$ abiotic control (reactor 0); $\blacksquare$ biological control (reactor 1); $\blacktriangle$ 50 mg N/L (reactor 2); $\times$ 100 mg N/L (reactor 3); $\square$ 200 mg N/L (reactor 4); $\diamond$ 400 mg N/L (reactor 5). Black arrows show addition of NO_3^- -N; dotted arrows show the replacement of reject water.

Fig. 5.4 MPN analysis for iron reducing bacteria: purple color indicated the presence of iron reducing bacteria; yellow color indicated the absence of iron reducing bacteria.

Fig. 5.5 MPN analysis for nitrate reducing bacteria: the presence of gas bubble associated with the reduction of nitrate concentration indicated the presence of nitrate reducing bacteria.

The MPN tests of iron reducing bacteria (Fig. 5.4) and nitrate reducing bacteria (Fig. 5.5) also confirmed the inhibition of iron reduction by nitrate reduction in reactors 2, 3, 4, and 5, and the recovery of iron reducing bacteria in reactors 2 and 3 after

complete nitrate reduction (Table 5.5). In reactors 4 and 5, the activities of iron reducing bacteria were inhibited by the shortage of biodegradable organic carbon, as a result, the total Fe(II) concentrations in both reactors did not increase during day 4 and day 5 (Fig. 5.3a). Therefore, iron ore reduction could resume after complete nitrate reduction provided that the organic carbon was still available. The shortage of organic carbon after complete nitrate reduction would otherwise inhibit the recovery of iron reducing bacteria.

Table 5.5 The numbers of iron reducing bacteria and denitrifying bacteria in reactors during the 1st addition of nitrate

Reactor No.	Iron reducing bacteria			Denitrifying bacteria*		
	(cells/mL)			(cells/mL)		
	Day 3	Day 4	Day 5	Day 3	Day 4	Day 5
1 Control (0 mg /L N-NO ₃ ⁻)	4.6X10 ⁶	1.3X10 ⁷	1.5X10 ⁷	7.5X10 ⁸	7.5X10 ⁸	7.5X10 ⁸
2 (50 mg/L N-NO ₃ ⁻)	1.1X10 ⁷	9.3X10 ⁶	1.5X10 ⁷	4.3X10 ⁸	1.5X10 ⁹	1.5X10 ⁹
3 (100 mg/L N-NO ₃ ⁻)	4.6X10 ⁶	2.1X10 ⁶	9.3X10 ⁶	4.3X10 ⁸	2.1X10 ⁹	2.1X10 ⁹
4 (200 mg/L N-NO ₃ ⁻)	1.1X10 ⁷	4.1X10 ⁶	4.6X10 ⁶	4.3X10 ⁸	4.2X10 ⁹	4.2 X10 ⁹
5 (400 mg/L N-NO ₃ ⁻)	2.4X10 ⁷	1.1X10 ⁷	1.2X10 ⁷	4.3X10 ⁸	0.75X10 ¹⁰	1.1X10 ¹⁰

* Note: Reactors 2, 3, and 4 achieved complete nitrate removal during day 3, and reactor 5 achieved complete nitrate removal during day 3 and day 4.

At the 6th day of experiment, in order to supplement the organic carbon for iron reducing bacteria and partially remove the produced ferrous/ferric precipitate, 300

mL, 1300 mL, and 1300 mL of fresh reject water were replaced in reactors 3, 4, and 5, respectively. Consequently, TOC concentrations in the three reactors increased to 218 mg/L, 331 mg/L, and 304 mg/L, respectively (Fig. 5.3b). As a result, iron ore reduction was significantly enhanced during day 6 with the rates of 71.9 mg/L·d, 116.5 mg/L·d, and 71.1 mg/L·d in reactors 3, 4, and 5, respectively (Fig. 5.3a). Without the replacement of fresh reject water, the Fe(II) production rates in biological control (reactor 1) and reactor 2 were only 21.9 mg/L·d and 31.7 mg/L·d during day 6 (Fig. 5.3a). Therefore, the replacement of fresh reject water was proved to significantly increase the iron ore reduction rate.

At the 7th day of experiment, potassium nitrate powder was added in reactors 2, 3, 4, and 5 again to achieve 50 mg N/L, 100 mg N/L, 200 mg N/L, and 400 mg N/L NO_3^- -N, respectively. As a result, iron ore reduction was inhibited and the total Fe(II) concentrations decreased 30.9 mg/L (reactor 2), 56.7 mg/L (reactor 3), 88.3 mg/L (reactor 4), and 24.4 mg/L (reactor 5) during day 7. The results further demonstrated the inhibition of iron ore reduction by nitrate reduction.

Similar to the first cycle of nitrate addition, nitrate reduction completed during day 7 in reactor 2 and the remaining organic carbon in reject water was 92 mg/L TOC. Iron ore reduction predictably resumed in reactor 2 and the Fe(II) concentration increased 18.9 mg/L during day 8. The complete nitrate reduction, however, was not obtained in reactors 3, 4, and 5 due to the insufficient organic carbon for denitrifying bacteria. The remaining TOC concentrations in reactors 3, 4, and 5 were only 34 mg/L, 40 mg/L, and 44 mg/L, respectively, and this organic carbon might not be biodegradable. Non-biodegradable compounds could neither be utilized by denitrifying bacteria nor iron reducing bacteria; hence, the activities of iron reducing bacteria and iron ore reduction were continuously inhibited. In reactor 3, Fe(II) concentration continued to decrease 21.9 mg/L (reduced from 98.7 mg/L to

77.0 mg/L) during day 8. In reactors 4 and 5, total Fe(II) concentrations were only 39.7 mg/L and 47.7 mg/L, which mostly remained unchanged during day 8 and day 9. In short, it was further proved that iron reducing bacteria could recover after complete nitrate reduction by utilizing the biodegradable organic carbon, and the shortage of organic carbon would inhibit the recovery.

5.3.3 Nitrate reduction and organic carbon oxidation

The concentrations of nitrate in the reactors are shown in Table 5.6. The denitrifying bacteria contained in anaerobic sludge utilized the organic carbon of reject water to perform nitrate reduction. When 50 mg N/L, 100 mg N/L, 200 mg N/L, and 400 mg N/L NO_3^- -N were added in reactors 2, 3, 4, and 5 at the 3rd day of experiment, the organic carbon in reject water was sufficient for complete reduction of nitrate in reactors 2 (50 mg N/L NO_3^- -N), 3 (100 mg N/L NO_3^- -N), and 4 (200 mg N/L NO_3^- -N) (Fig. 5.3b); thus complete nitrate reduction was achieved within one day. At the same time, the reduced Fe(II) concentration was almost similar in each reactor (Fig. 5.3a). The complete heterotrophic nitrate reduction in reactors 2, 3, and 4 made the TOC concentrations decreased 112 mg/L, 214 mg/L, and 354 mg/L, respectively (Fig. 5.3b). The ratios of TOC reduced/nitrate reduced were 2.16, 2.12, and 1.64, respectively. Since the heterotrophic nitrate reduction simultaneously produced alkalinity, pH increased from 7.99 to 8.25, 8.02 to 8.44, and 8.05 to 8.82 in reactors 2, 3, and 4, respectively.

Table 5.6 Concentration of nitrate in reject water (mg N/L). Average values $\pm$ standard deviation of triplicate independent measurements are shown

Reactor No.	Day 3	Day 4	Day 5	Day 7	Day 8	Day 9
2	52.6 $\pm$ 0.6	0.83 $\pm$ 0.01	0.7 $\pm$ 0.0	57.4 $\pm$ 0.69	0.6 $\pm$ 0.06	0.5 $\pm$ 0.04
3	102.3 $\pm$ 0.5	1.3 $\pm$ 0.04	0.7 $\pm$ 0.0	107.5 $\pm$ 0.27	20.2 $\pm$ 0.17	7.0 $\pm$ 0.04
4	217.2 $\pm$ 0.5	0.96 $\pm$ 0.03	0.5 $\pm$ 0.01	210.9 $\pm$ 0.43	49.0 $\pm$ 0.33	30.9 $\pm$ 0.36
5	398.2 $\pm$ 1.1	14.6 $\pm$ 0.23	0.6 $\pm$ 0.02	418.0 $\pm$ 1.17	291.3 $\pm$ 0.32	276.1 $\pm$ 0.4

In reactor 5, the concentration of organic carbon, on the other hand, was not sufficient for complete reduction of 400 mg N/L NO_3^- -N. During day 3, nitrate concentration decreased 384 mg/L NO_3^- -N, simultaneously, nitrite concentration increased 260 mg N/L NO_2^- -N, which demonstrated that nitrate reduction did not complete at that moment and only 124 mg N/L NO_3^- -N was completely reduced. The unchanged pH in reactor 5 during day 3 could be explained by the fact that the presence of nitrite enhanced the oxidation of 129.8 mg/L Fe(II) and the protons produced from this oxidation partially neutralized the alkalinity produced from nitrate reduction process. During day 4, the remaining 14.6 mg/L NO_3^- -N and 260 mg/L NO_2^- -N in reactor 5 were completely reduced, simultaneously, the solution pH increased from 7.99 to 8.12. The required carbon source could be hypothetically explained by the hydrolysis of anaerobic biomass; some portion of anaerobic biomass could be hydrolyzed and used for denitrification. The utilization of hydrolyzed biomass for denitrification in reject water has been reported in other study (Gali et al., 2006a).

Denitrifying bacteria utilized the organic carbon for nitrate reduction and biomass growth (Table 5.5). Iron reducing bacteria were over competed by denitrifying bacteria for organic carbon. Statistically, the increased number of denitrifying

bacteria had a reliable correlation with the reduced nitrate concentration (Fig. 5.7).

Fig. 5.6 The relationship between concentrations of denitrifying bacteria and nitrate

During the second addition of nitrate at the 7th day of experiment, the addition of 50 mg N/L NO_3^- -N in reactor 2 could be completely reduced during day 7 due to sufficient organic carbon in reject water, and the reduced Fe(II) concentration was similar to the value achieved in the first cycle. Simultaneously, the TOC concentration in reactor 2 decreased 123 mg/L and the ratio of TOC reduced/nitrate reduced was 2.17; and the solution pH increased from 8.06 to 8.26.

In comparison with the complete nitrate reduction in reactor 2, during day 7, the reduction of nitrate concentrations were 87.3 mg N/L, 161.9 mg N/L, and 126.7 mg N/L in reactor 3 (100 mg N/L NO_3^- -N), reactor 4 (200 mg N/L NO_3^- -N), and reactor 5 (400 mg N/L NO_3^- -N), respectively (Table 5.6); and 0.22 mg N/L, 1.00 mg N/L, and 0.26 mg N/L NO_2^- -N were produced. At the same time, during day 7, TOC concentrations decreased 154 mg/L, 265 mg/L, and 195 mg /L (Fig. 5.3b), and the ratios of TOC reduced/nitrate reduced were 1.76, 1.64, and 1.54, respectively; the solution pH increased also from 8.16 to 8.49, 8.06 to 8.59, and 8.42 to 8.67 in reactors 3 (100 mg N/L NO_3^- -N), 4 (200 mg N/L NO_3^- -N), and 5 (400 mg N/L NO_3^- -N), respectively.

During day 7, in comparison with almost 0 mg/L NO_2^- -N in reactor 2, the presence of nitrite in reactors 3 (100 mg N/L NO_3^- -N) and 4 (200 mg N/L NO_3^- -N) made the Fe(II) concentration decreased significantly in each reactor, and the reduced values were much higher than the values achieved during the 1st cycle. The Fe(II) concentration in reactor 5 (400 mg N/L NO_3^- -N) did not decreased significantly, which could be explained hypothetically that the remaining 47.7 mg/L Fe(II) was chelated with organic acids and protected by the acids from oxidation. Additionally, $\text{Fe}(\text{OH})_2$ probably has been adsorbed on biomass. Nielsen and Nielsen (1998b) also reported that about 56 mg/L Fe(II) could not be oxidized during the nitrate-dependent ferrous oxidation process.

As most of the biodegradable organic carbon of reject water in reactors 3, 4, and 5 was utilized by denitrifying bacteria, the remaining TOC concentrations diminished to 30-40 mg/L in day 8 (Fig. 5.3b). The nitrate reduction rate in reactors 3, 4, and 5 consequently became very slow and almost ceased in the subsequent period. During day 8 and day 9, nitrate reduction in reactor 3 only reached 13 mg N/L NO_3^- -N and remained 7 mg N/L NO_3^- -N. The same phenomena were observed in reactors 4 and 5. In reactor 4, nitrate reduction reached 18 mg N/L NO_3^- -N and remained 30.9 mg N/L NO_3^- -N. Similarly, in reactor 5, nitrate reduction reached 15 mg N/L NO_3^- -N and remained 276 mg N/L NO_3^- -N. Apparently, nitrate reduction dominated iron ore reduction; and organic carbon controlled the rate and extent of nitrate reduction.

5.3.4 The mechanisms of the inhibition of iron ore reduction by nitrate reduction

Reject water contains a lot of branched volatile fatty acids (VFA), mainly isobutyric acid ($(\text{CH}_3)_2\text{CHCOOH}$) and isocaproic acids ($(\text{CH}_3)_2\text{CH}(\text{CH}_2)_2\text{COOH}$) (Ivanov et al., 2005). When TOC components in the reject water are limited to isobutyric and

isocaproic acids with the ratio of 1:1, empirical formula of VFAs in reject water is $C_5H_{10}O_2$. Iron reducing bacteria utilized the organic carbon for iron ore reduction (Equation 5-1); and denitrifying bacteria consumed them for nitrate reduction (Equation 5-2, 5-3). As a result, denitrifying bacteria and iron reducing bacteria competed for organic carbon.

It is known that nitrate reduction is energetically favorable as compared with Fe(III) reduction and nitrate tends to be more available to cells than the insoluble form of Fe(III). Electron donors therefore prefer to transport to nitrate than iron ore. The inhibition of iron reduction by nitrate has been widely examined (Achtnich et al., 1995; DiChristina, 1992; Haaijer et al., 2008; Sorensen, 1982). When organic carbon was sufficient for complete nitrate reduction, concentration of nitrite was almost 0, nitrate reduction process was predominant and the activities of iron reducing bacteria were partially inhibited; simultaneously, Fe(II) produced from the earlier iron ore reduction was abiotically oxidized by some components in reject water, resulting in a slightly reduced Fe(II) concentration. The reduced value was affected by the characteristics of reject water and regardless of the initial nitrate concentration added. Although the chemical oxidation of solid Fe(II) by nitrite to produce N_2 or N_2O has been reported (Weber et al., 2001), our study showed that nitrite did not react with Fe(II) in reject water under anaerobic conditions. Nielsen and Nielsen (1998b) also concluded in their study that Fe(II) was not abiotically oxidized by nitrite in activated sludge anoxic process.

When organic carbon was not sufficient for complete nitrate reduction in anaerobic treatment of reject water, iron ore reduction was inhibited by nitration reduction process. A possible explanation for the inhibition was that in the competition for organic carbon between iron reducing bacteria and denitrifying bacteria, the latter is always dominant. Similar mechanism has been reported by other researchers (Achtnich et al., 1995; DiChristina, 1992; Obuekwe et al., 1981; Sorensen, 1982). The shortage of organic carbon could additionally make the nitrate reduction process stop at 1st step (Equation 5-2). Fe(II) oxidation, which can be triggered abiotically by some components in reject water, could be significantly enhanced by the presence of nitrite in the solution. This enhancement obviously inhibited the iron ore reduction process.

When nitrate was completely reduced, the inhibition of iron ore reduction was relieved; and iron reducing bacteria recovered by utilizing the remaining organic carbon and performed iron ore reduction. Sorensen (1982) also mentioned the resumed iron reduction in marine sediment after complete nitrate reduction process.

The considerably increased iron ore reduction rate by the replacement of reject water could be explained by the supplement of organic carbon for iron reducing bacteria and the partial removal of the produced Fe(II). It was known that the reactive surface-site-density of iron oxide affected the initial rate of iron ore reduction (Roden, 2003). Liu et al (2001a) emphasized that the sorption/precipitation of Fe(II) on iron reducing bacteria inhibited the activity of iron reduction bacteria. Consequently, the use of aqueous Fe(II)NTA complexant to prevent or delay Fe(II) sorption to oxide and iron reducing bacteria has been recommended (Urrutia et al., 1999). Roden and Zachara (1996) also reported the negative effects of surface-bound Fe(II) to Fe(III) oxide on crystalline iron(III) oxide reduction. The passive influence of surface-bound Fe(II) on iron reducing

bacteria surface in crystalline iron(III) oxide reduction has been suggested also (Urrutia et al., 1999). In brief, the removal of produced ferrous via aqueous-phase transport decreased the negative influence of surface bound Fe(II) on iron oxide reduction, promoted the rate and extend of microbial reduction of crystalline Fe(III) oxide and associated with bacterial growth (Roden et al., 2000; Roden and Urrutia, 1999). In our study, the replacement of fresh reject water acted as a partial removal of produced ferrous via aqueous transport, thus considerably increased iron ore reduction rate was observed and maximum iron reduction rate increased from 45 mg/L·d to 116.5 mg/L·d.

5.3.5 The contribution of ferrous oxidation for nitrate reduction

During complete nitrate reduction process, Fe(II) was oxidized by the components in reject water and such oxidation was affected by the characteristics of reject water, the Fe(II) oxidation did not make contribution for nitrate reduction. If nitrate reduction process could not complete due to the shortage of organic carbon, the activities of iron reducing bacteria were greatly inhibited; and part of nitrate reduction was stopped with nitrite as the intermediate product. Nitrite not only inhibited the activities of iron reducing bacteria (Dichristina, 1992), but also significantly enhanced the oxidation of Fe(II) and caused the largely reduced Fe(II) concentration, and during the Fe(II) oxidation process, concentrations of nitrate/nitrite did not changed. Therefore, Fe(II) abiotic oxidation process also did not made contribution during incomplete nitrate reduction process in anaerobic treatment of reject water and the nitrate-dependent ferrous oxidation process was not observed in our study.

5.4 Conclusions

- 1) Nitrate reduction inhibited iron ore reduction because denitrifying bacteria over competed iron reducing bacteria for organic carbon.
- 2) The decrease of Fe(II) concentration during nitrate reduction process was due to the reaction of Fe(II) with the components of reject water and the inhibition of the activities of iron reducing bacteria by nitrate.
- 3) If the concentration of organic carbon was not sufficient for complete nitrate reduction, nitrite produced from incomplete nitrate reduction significantly enhanced the oxidation of Fe(II). This enhancement obviously inhibited the iron ore bioreduction process.
- 4) After nitrate reduction completed, the activities of iron reducing bacteria recovered due to utilization of the remaining organic carbon. The replacement of fresh reject water effectively increased the iron ore reduction rate.
- 5) Organic carbon affected the rate and extent of nitrate reduction as well as the recovery of the activities of iron reducing bacteria after complete nitrate reduction.

Chapter 6

Interactions between ferrous oxidation and nitrate reduction during anaerobic treatment of reject water from MWWTPs

6.1 Introduction

The aerobic nitrification and anaerobic denitrification processes are the conventional processes for nitrogen removal. The addition of electron donors in denitrification stage is necessary to achieve maximum denitrification rate and efficiency for wastewater containing high nitrate concentration (Fernández-Nava et al., 2008). Organic matters of reject water are suitable electron donors for the reduction of nitrate after nitrification of reject water (Gali et al., 2006a).

Ferrous salts are used as precipitant for phosphate removal from municipal wastewater (Wang et al., 2006d). Ferrous can be produced from the biological reduction of iron ore, which is cheap source of Fe(III), by iron reducing bacteria under anaerobic conditions using organic compounds in reject water. Such cost effective and environmental friendly technology for phosphorus removal from reject water has been studied in Chapter 3. And the removal of nitrogen and phosphorus from reject water of MWWTPs using sequential ferric bio-reduction-phosphate precipitation and nitrate bio-reduction has been shown in Chapter 4 (Fig. 6.1).

It was known that when ferrous salts were used for phosphorus precipitation from municipal wastewater, ferrous could interact with nitrite or nitrate and show inhibitory effects on activated sludge (Clark et al., 2000; Philips et al., 2003). Therefore, it is important to study if ferrous produced from iron ore reduction has

negative effects on nitrate reduction, and thus determine which stage is performed first, nitrate reduction or iron reduction, to avoid the negative effects of ferrous oxidation on nitrate reduction (Fig. 6.1).

However, it is known that ferrous can also participate in nitrate reduction. The microbial nitrate-dependent oxidation of Fe(II) has been observed in paddy soil (Ratering and Schnell, 2001) and activated sludge anoxic (denitrification) process (Nielsen and Nielsen, 1998a; 1998b). Nitrate-dependent ferrous oxidation is mediated by enrichment or pure cultures of nitrate-reducing bacteria (Straub et al., 1996) or by the mesophilic denitrifying bacteria at neutral pH (Benz et al., 1998). The anaerobic oxidation of ferrous to ferric is coupled to nitrate reduction with final products as nitrogen gas (Straub et al., 1996):

The solid-phase Fe(II) compounds can also be electron donors for biological nitrate reduction (Weber et al., 2001). Hence, ferrous produced in the stage of ferric bioreduction (see the scheme proposed in Chapter 4) might be used as additional electron donor in denitrification stage (Fig. 6.1). The aim of this research was to study the effects of ferrous addition on nitrate reduction during the anaerobic treatment of the model reject water of MWWTP.

6.2 Materials and methods

6.2.1 Characteristics of reject water, anaerobic sludge, and enrichment culture of denitrifying bacteria

The reject water and anaerobic sludge were collected in a local MWWTP in

Singapore. Model reject water after nitrification process was used in experiment to study the effects of ferrous addition on nitrate reduction. Nitrate, 200 mg N/L or 300 mg N/L, was added to reject water to prepare model reject water after nitrification process. According to the Equation 6-2, the quantity of TOC needed for reduction of 200 mg N/L is 176 mg/L and for reduction of 300 mg N/L is 264 mg/L.

Fig. 6.1 Treatment of reject water to remove phosphorus and nitrogen: Proposed process is shown with black arrows; the potential enhancement of the process is shown with white arrow.

Two samples of model reject water were used: (1) reject water containing sufficient source of electron donors for complete nitrate reduction (concentration of TOC was 387 mg/L and concentration of N-NO_3^- was 200 mg N/L); (2) reject water containing insufficient source of electron donors for complete nitrate reduction (concentration of TOC was 207 mg/L and concentration of N-NO_3^- was 300 mg N/L).

Anaerobic sludge (anaerobic microbial biomass from anaerobic digester) was not only a source of iron-reducing bacteria but was also used to prepare the enrichment culture of denitrifying bacteria. The cultivation has been done anaerobically in reject water with addition of potassium nitrate (GR grade, Merck), 200 mg N/L, under shaking (shaker DK-OS010, Daiki Science) at 150 rpm/min for 10 days. 50% of the cultural medium was replaced with fresh reject water daily with nitrate addition and the medium pH was maintained between 7-8 using 5M HCl.

The reject water and anaerobic sludge were stored at 4 °C before being used for the experiments. The characteristics of reject water, anaerobic sludge, and enrichment culture of denitrifying bacteria were determined and the results are given in Table 6.1. The details of the analytical methods and procedures used were similar to that in Chapter 3

6.2.2 Experimental set up

6.2.2.1 The effects of Fe(II) addition on nitrate reduction in reject water containing organic compounds as electron donors sufficient for complete nitrate reduction without pH control

Reject water with TOC concentration of 387 mg/L was used in the experiment 1.

Enrichment culture of denitrifying bacteria, 40 mL, and reject water, 1.9 L, were placed into each of four 2 L reactors to perform control and experiments. Potassium nitrate, as source of nitrate, was added in each reactor to achieve N-NO_3^- concentration of 200 mg N/L. All the reactors were purged with nitrogen gas for 10 minutes and sealed up to maintain the anaerobic conditions. The bioreactors were placed on the overhead mixer (Rotax 6.8, VELP Scientifica, Milan, Italy) with 15 rpm/min for continuous mixing. Incubation temperature was maintained at 30 °C for 48 h. At the 6th hour of the experiment, soluble source of ferrous, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder (GR grade, Merck), was added in each reactor to achieve Fe(II) concentrations, mg/L, of 0 in control reactor, 100 in reactor 1, 200 in reactor 2, and 400 in reactor 3.

Table 6.1 Characteristics of reject water, anaerobic sludge, and enrichment culture of denitrifying bacteria

Parameter	Concentrations, mg/L		
	Reject water	Suspension of anaerobic sludge	Enrichment culture of denitrifying bacteria
Total organic carbon (TOC)	207-387	n.d.*	n.d.
Total Fe(II)	4.1-8.9	n.d.	n.d.
Dissolved NO_3^- -N	0.47-0.51	n.d.	n.d.
Dissolved NO_2^- -N	0	n.d.	n.d.
Volatile suspended solids (VSS)	n.d.	2000 ± 120	4455 ± 65
pH	7.2–7.5	7.8	7.5

*Note: n.d. means that the parameter was not determined.

6.2.2.2 The effects of Fe(II) addition on nitrate reduction in reject water containing organic compounds as electron donors sufficient for complete nitrate reduction with pH control

Model reject water with high TOC concentration of 387 mg/L and N-NO_3^- concentration of 200 mg N/L was used in experiment 2. The procedure of experiment 2 was similar to experiment 1. At the 3rd hour of the experiment, soluble source of ferrous, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder, was added in each reactor to achieve Fe(II) concentrations, mg/L, of 0 in control reactor, 100 in reactor 1, 200 in reactor 2, and 400 in reactor 3. The addition of ferrous in the model reject water caused significant pH drop. To prevent the effect of pH decrease on nitrate reduction, 5N NaOH solution was added simultaneously with ferrous sulfate to the reactors to maintain pH.

6.2.2.3 The effects of pH decrease on nitrate reduction in reject water containing sufficient organic compounds as electron donors for complete nitrate reduction

Model reject water with high TOC concentration of 387 mg/L and N-NO_3^- concentration of 200 mg N/L was used in experiment 3. The procedure of experiment 3 was similar to experiment 1, but without ferrous addition. At the 2.5th hour of the experiment, 5N HCl solution was added to the reactors to change the pH of cultural liquid. It was 7.5 in the experimental reactor 1; 7.1 in the reactor 2, and 6.6 in the reactor 3. No pH adjustment was done afterward. No HCL was added in the control reactor.

6.2.2.4 The effects of Fe(II) addition on nitrate reduction in reject water containing insufficient organic compounds as electron donors for complete nitrate reduction without pH control

Model reject water containing TOC concentration of 207 mg/L and N-NO_3^-

concentration of 300 mg N/L was used in experiment 4. Anaerobic sludge, 40 mL, was used as inoculum instead of enrichment culture of denitrifying bacteria as was used in experiment 1. Anaerobic sludge served simultaneously as the sources of denitrifying bacteria and iron reducing bacteria to convert ferric, which will be produced during ferrous oxidizing, back into ferrous. Other procedures were the same as in experiment 1. At the 9th hour of the experiment, soluble source of ferrous, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder, was added in each reactor to achieve following Fe(II) concentrations, mg/L: 0 in control reactor, 100 in reactor 1, 200 in reactor 2, and 400 in reactor 3.

6.2.2.5 Chemical interactions between nitrate, nitrite, and ferrous ions in reject water

Reject water, 1.9 L, was placed in two 2 L reactors (reactor A and reactor B). Deionized water (DI), 1.9 L, was placed the control reactor C. All reactors were purged with nitrogen gas for 20 minutes; immediately, 2.83 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder (GR grade, Merck) was added in each reactor to achieve Fe(II) concentration of 300 mg/L. Standard KNO_3 solution with concentration of 1000 mg N/L (product of Hach Company), 19 mL, was added in reactor A and in control reactor C to achieve N- NO_3^- concentration of 10 mg N/L. Standard KNO_2 solution with concentration of 2850 mg N/L, 14 ml, was added in reactor B to achieve N- NO_2^- concentration of 20 mg N/L. All reactors were sealed up and placed on the overhead mixer (Rotax 6.8, VELP Scientifica, Milan, Italy) for continuous mixing with the speed of 15 rpm/min at 30 °C for 4 hours.

6.2.3 Analytical methods

Volatile suspended solids (VSS), pH, concentrations of nitrate, nitrite, total ferrous

ions, and dissolved total organic carbon (TOC) in the well-mixed samples were determined using the same methods in Chapter 3. The length of denitrifying bacteria and sizes of ferrous hydroxide and ferric hydroxide colloidal particles were measured using the automated particle size and shape analyzer (the Sysmex FPIA-2100, Malvern Instrument).

SEM images of denitrifying bacteria, ferrous hydroxide, and denitrifying bacteria with ferrous precipitate: Distribution of denitrifying bacteria in ferrous and ferric hydroxides particles was examined with scanning electron microscope (SEM, Zeiss EV050, UK). The samples were fixed in 2% glutaraldehyde for 2 hours, washed 3 times with 0.1M sodium cacodylate buffer for 20 minutes, dehydrated in step gradients of ethanol: 50, 70, 85, and 95% ethanol for 10 minutes, kept in 100% ethanol until dried with the critical point dryer (Polavon E3100, Quorum Technologies, UK), and then sputter coated with Au-Pd with sputter coater (Emitech SC7620, Quorum Technologies, UK).

6.3 Results and discussion

6.3.1 Chemical interactions between nitrate, nitrite, and ferrous

The concentrations of nitrite and nitrate did not change in reject water with the presence of ferrous (reactor A and reactor B) after 4 hours of incubation under anaerobic conditions (Table 6.2). Under the presence of nitrate, 41% of ferrous was oxidized in reject water (reactor A); however, there was no oxidation of ferrous in DI water (reactor C). Under the presence of nitrite, 82% of ferrous was oxidized in reject water under anaerobic conditions within 4 hours of incubation (reactor B). The results show that ferrous did not interact chemically with nitrate and nitrite in reject water under anaerobic conditions. Instead, it reacted with some components

contained in reject water, which were not present in DI water, and the presence of nitrite enhanced this reaction under anaerobic conditions.

Table 6.2 Change of nitrate, nitrite, and ferrous concentrations in reject water and distilled water (average $\pm$ s.d.)

Reactor	Initial			After 4h		
	N- NO ₃ ⁻	N- NO ₂ ⁻	Fe(II)	N- NO ₃ ⁻	N- NO ₂ ⁻	Fe(II)
	(mg N/L)	(mg N/L)	(mg/L)	(mg N/L)	(mg N/L)	(mg/L)
Fe(II) + NO ₃ ⁻ in reject water (a)	9.40 $\pm$ 0.13	n.d*	284.54 $\pm$ 0.48	9.26 $\pm$ 0.05	n.d	169.21 $\pm$ 0.45
Fe(II)+ NO ₃ ⁻ in DI water (c)	10.04 $\pm$ 0.11	n.d	289.44 $\pm$ 0.28	9.88 $\pm$ 0.54	n.d	283.21 $\pm$ 0.25
Fe(II)+ NO ₂ ⁻ in reject water (b)	n.d	19.25 $\pm$ 0.13	284.44 $\pm$ 0.28	n.d	18.95 $\pm$ 0.12	49.73 $\pm$ 4.82

Note: n.d. means not determined.

6.3.2 The effects of Fe(II) on nitrate reduction in reject water containing sufficient electron donors for complete nitrate reduction without pH control

The nitrate reduction rates were similar in all reactors before the addition of ferrous in experiment 1. Average nitrate reduction rates during the first 6 hours were 14.6, 16.4, 14.9, and 16.5 mg N/L·h in control reactor, reactors 1, 2, and 3, respectively. After the addition of FeSO₄·7H₂O at the 6th hour of the experiment to achieve Fe (II) concentrations of 100, 200, and 400 mg/L in reactors 1, 2, and 3, respectively,

different nitrate reduction rates were observed during the following 3 hours. In the control reactor (0 mg/L Fe(II)), concentration of nitrate decreased quickly with an average nitrate reduction rate of 33.0 mg N/L·h. Meanwhile, in the experimental reactors with Fe(II) concentrations of 100, 200, and 400 mg/L, nitrate reduction process was inhibited and the average nitrate reduction rates were 7.9, 0.1, and 1.0 mg N/L·h, respectively (Fig. 6.2a).

Higher ferrous concentration caused the longer adaptation phase: nitrate reduction started after 0, 6, and 18 hours in experimental reactors 1, 2, and 3 after the addition of ferrous. Average nitrate reduction rate in experimental reactor 1 with 100 mg/L Fe(II) was 11.5 mg N/ L·h, which was 34% of the nitrate reduction rate in control. After the adaptation phase, the nitrate reduction in experimental reactors with Fe(II) concentrations of 200 and 400 mg/L started up again with the average nitrate reduction rates of 7.6 and 3.0 mg N/L·h, which were 23% and 9% of the rate in control, respectively. Time needed for total reduction of nitrate was 9 h in control reactor with 0 mg/L Fe(II), 24 h in reactors with Fe(II) concentrations 100 mg/L and 200 mg/L, and 48 h in reactor with 400 mg/L Fe(II) concentration.

Fig. 6.2 Concentration of nitrate (a), pH change (b), and concentration of TOC (c) in model reject water containing sufficient organic compounds as electron donors for complete nitrate reduction added with different quantities of ferrous, mg/L: 0 (♦); 100 (■); 200 (▲); 400 (x). Arrows show ferrous addition; pH was not corrected.

The addition of ferrous into the cultural liquids caused a drop of pH from 8.4 to 7.7, 7.3, and 7.0 in reactors with Fe(II) concentrations of 100, 200, and 400 mg/L, respectively (Fig. 6.2b). The soluble ferrous added immediately precipitated forming light green sediment of $\text{Fe}(\text{OH})_2$ and then it was oxidized to form brown precipitate of $\text{Fe}(\text{OH})_3$ within 3 hours in each reactor.

Since the organic compounds in reject water were utilized by denitrifying bacteria for nitrate reduction, TOC concentration was reduced to approximately 50 mg/L, corresponding to about 13% of the initial concentration (Fig. 6.2c).

6.3.3 The effects of Fe(II) on nitrate reduction in reject water containing sufficient electron donors for complete nitrate reduction with pH control

The effects of Fe(II) on nitrate reduction in reject water containing sufficient electron donors for complete nitrate reduction with pH control were studied in experiment 2. Procedures were the same as in experiment 1, except addition of sodium hydroxide altogether with soluble ferrous salt for pH maintenance. The nitrate reduction rate was the same in all reactors before the addition of ferrous, and the average nitrate reduction rates for the first 3 hours were 12.9, 13.2, 14.6, and 14.1 mg N/L·h in control reactor (0 mg/L ferrous), experimental reactors with Fe(II) concentrations of 100, 200, and 400 mg/L, respectively. The medium pH was in the range from 7.6 to 7.9 in all reactors. To prevent change of pH, 5N NaOH was added simultaneously with $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ at the 3rd hour of experiment to keep pH 7.6, 7.9, 8.0, and 8.0 in control reactor, experimental reactors with Fe(II) concentrations of 100, 200, and 400 mg/L, respectively. Different nitrate reduction rates were observed in the reactors during the next 5 hours. In the control reactor without ferrous addition, nitrate was reduced quickly with the average reduction rate of 27.1 mg N/L·h; in experimental reactors with Fe(II) concentrations of 100, 200, and 400 mg/L, the nitrate reduction however was inhibited and the average nitrate reduction rates decreased to 0.5, 1.6, and 2 mg N/L·h, respectively (Fig. 6.3). The figure clearly indicates the negative effects of ferrous ions on nitrate reduction in reject water treatment even under control pH.

Fig. 6.3 Concentration of nitrate in model reject water containing sufficient organic compounds as electron donors for complete nitrate reduction added with different quantities of ferrous, mg/L: 0 (♦); 100 (■); 200 (▲); 400 (x); arrows show ferrous and NaOH addition.

6.3.4 The effects of pH change on nitrate reduction in reject water containing sufficient electron donors for complete nitrate reduction

In experiment 3, the nitrate reduction rates were similar also in all reactors before the addition of 5N HCl at the 2.5th hour of the experiment. The average nitrate reduction rates during the first 2.5 h were 34.3, 32.5, 29.2, and 32.7 mg N/L·h in control reactor, experimental reactors 1, 2, and 3, respectively. At the 2.5th hour of the experiment, 5N HCl solution was added to the reactors to achieve pH 7.5 in reactor 1, 7.1 in reactor 2, and 6.6 in reactor 3. After the pH adjustment at 2.5th h of experiment (Fig. 6.4a), in the next 1.5 hours, the nitrate reduction rates in control reactor and reactor 1 with pH value of 7.5 were 57.4 mg N/L·h and 58.2 mg N/L·h, respectively. The nitrate reduction rate was 41.9 mg N/L·h in reactor 2 with pH value of 7.1, which was 73% of the rate achieved in the control; it was 14.5 mg N/L·h in reactor 3 with pH value of 6.6, which was only 25% of the rate achieved in the control (Fig. 6.4b). The nitrate reduction in the control reactor and experimental

reactor 1 with pH value of 7.5 was completed after 4 hours; however, it took 5.5 hours in reactor 2 with pH value of 7.1 and 7 hours in reactor 3 with pH value of 6.5 to achieve complete nitrate reduction. So, the sudden drop of the pH of cultural liquid from 8.2 to 7.1 and 6.5 inhibited the nitrate reduction.

Fig. 6.4 pH value (a) and concentration of nitrate (b) in model reject water containing sufficient organic compounds as electron donors for complete nitrate reduction with different pH values by addition of 5NHCl: Control (♦); 7.5 (■); 7.1 (▲); 6.5 (x). Arrows show pH adjustment by 5N HCL addition.

6.3.5 The effects of Fe(II) on nitrate reduction in reject water containing insufficient electron donors for complete nitrate reduction

Nitrate reduction rates were similar in all reactors before the addition of ferrous, and the average nitrate reduction rates during the first 9 hours were 4.7, 4.4, 4.2, and 4.7 mg N/L·h in control reactor without Fe(II), experimental reactors with 100, 200, and 400 mg/L Fe(II), respectively. Compared with about 15 mg N/L·h nitrate reduction rate in experiment 1 and 13 mg/L·h nitrate reduction rate in experiment 2, the relatively low nitrate reduction rate in this experiment could be explained by low concentration of denitrifying bacteria in anaerobic sludge which was used as inoculum. However, denitrification rate increased rapidly after the first 9 hours and reached 18.9 mg N/L·h during the next 3 hours in control reactor with 0 mg/L Fe(II). Soluble ferrous was added in experimental reactors at the 9th hour of the experiment. The average nitrate reduction rates from 9th to 12th hours in experimental reactors with 100, 200, and 400 mg/L Fe(II) were 12.1, 13.7, and 10.5 mg N/L·h, respectively. These nitrate reduction rates were approximately 64, 72, and 56 % of the rate in the control (Fig. 6.5a). It showed that the inhibition of nitrate reduction by ferrous in the case of insufficient electron donors for nitrate reduction was not so significant in comparison with that when sufficient electron donors were supplied.

Addition of ferrous caused the decrease of pH from 7.9 in control to 7.3, 6.8, and 6.4 in reactors with 100, 200, and 400 mg/L Fe(II), respectively (Fig. 6.5b). It was shown that added ferrous was oxidized within 3 hours (from 6th hour to 9th hour) in experiment 1. However, in the present experiment, it needed much longer time (15 hours, 9th hour to 24th hour) to completely oxidize added ferrous in the reactors with 100, 200, and 400 mg/L Fe(II), respectively. The study of chemical interactions between nitrite and ferrous showed that the presence of nitrite enhanced ferrous oxidation (Table 6.2). In experiment 1, the presence of nitrite also enhanced the

ferrous oxidation during 6th and 9th hour (Table 6.3). However, in this experiment, the nitrite was absent during 9th and 12th hours (Fig. 6.5c), therefore, ferrous was not completely oxidized between 9th and 12th hour.

Table 6.3 The concentrations of Fe(II), nitrite, and nitrate in reactors with 100, 200, and 400 mg/L Fe²⁺ at 6th h and 9th h of experiment 1 (average $\pm$ s.d.)

Reactor containing Fe ²⁺ , mg/L	Concentration, mg/L at time of experiment					
	Fe ²⁺		N- NO ₂ ⁻		N- NO ₃ ⁻	
	6 th h	9 th h	6 th h	9 th h	6 th h	9 th h
100	100 $\pm$	1.9 $\pm$	38.4 $\pm$	40.0 $\pm$	101 $\pm$	77.4 $\pm$
	0.5	0.1	0.1	0.3	0.3	2.6
200	200 $\pm$	2.7 $\pm$	32.7 $\pm$	38.0 $\pm$	110.4 $\pm$	110.6 $\pm$
	2.0	0.1	0.6	0.1	0.3	0.4
400	400 $\pm$	4.8 $\pm$	41.7 $\pm$	44.3 $\pm$	99.5 $\pm$	96.4 $\pm$
	3.0	0.1	0.1	0.1	0.8	4.0

Organic carbon in reject water in the control reactor with 0 mg/L Fe(II) was mostly consumed by denitrifying bacteria within 12 hours and concentration of TOC was 76.9 mg/L at the 12th hour of the experiment. As a result, nitrate reduction ceased in the control when TOC concentration in reject water was decreased to 50 mg/L. On the other hand, the TOC concentrations in reactors with 100, 200, and 400 mg/L Fe(II) were 139, 176, and 168 mg/L, respectively, at the 12th hour of the experiment, and the nitrate reduction still proceeded slowly in these reactors due to the availability of electron donors (Fig. 6.5d). The remaining organic compounds, 60 mg/L TOC, might not be suitable for denitrifying bacteria thus could not be consumed by them for nitrate reduction. Throughout the cultivation process, nitrate

removal efficiencies were 31.5, 34.5, 49.2, and 41.8% in control reactor with 0 mg/L Fe(II), reactors with 100, 200, and 400 mg/L Fe(II), respectively. The slightly higher nitrate reduction efficiency in the reactors with 200 and 400 mg/L Fe(II) was due to the incomplete reduction of some removed nitrate. At the end of the process, 50.9 and 35.9 mg N/L of nitrite were remained in the reactors with 200 and 400 mg/L Fe(II), respectively (Fig. 6.5c).

Fig. 6.5 Concentration of nitrate (a), pH change (b), concentrations of nitrite (c), and TOC (d) in model reject water containing insufficient organic compounds as electron donors for complete nitrate reduction added with different quantities of ferrous, mg/L: 0 (♦); 100 (■); 200 (▲); 400 (x). Arrows show ferrous addition.

6.3.6 The reduction of reject water pH by ferrous addition

There are a number of researches regarding the pH change after the addition of iron salts into medium containing nitrates. Nielsen and Nielsen (1998b) in their study about the microbial nitrate-dependent oxidation of ferrous ions in activated sludge anoxic (denitrification) process stated that the change of medium pH due to the

addition of ferrous ions was not observed. Meanwhile, Philips et al. (2003) reported that the addition of ferrous to activated sludge under aeration led to a small pH drop, followed by a recovery in the remaining time of the batch period.

In the present study, the addition of ferrous under anaerobic conditions immediately caused the decrease of medium pH. Prior to the addition of ferrous, the medium pH was slightly alkaline, which was 8.2-8.4 in reactors with sufficient electron donors and 7.8-7.9 in reactors with insufficient electron donors. Ferrous added in the reject water spontaneously formed ferrous hydroxide (Equation 6-3). The produced protons during ferrous precipitation significantly decreased the medium pH and the decrease of pH value positively correlated with the amount of added ferrous (Fig. 6.6).

Fig. 6.6 The relationship between the reduction of solution pH and added ferrous concentration.

6.3.7 The mechanisms of inhibition of nitrate reduction by ferrous in reject water containing sufficient organic compounds as electron donors.

Philips et al. (2003) suggested that the negative impacts of iron salt in activate sludge process were partially due to the decrease of pH upon formation of iron hydroxide. The decrease of pH upon formation of iron hydroxides and the impairment of the floc structure could partially explain the toxicity of ferrous added in different quantities. Although the denitrifying bacteria were reported to be not very sensitive to pH in the range of 7 and 8 (Metcalf & Eddy, 2004), in our study, the significant change in medium pH, which meant a sudden alteration in microenvironment of the cells, caused the reduction of biomass activity. The results of experiment 3 confirmed that when the medium pH was suddenly dropped to 7.1 and 6.6, nitrate reduction rates decreased from 57.4 mg N/L·h in control to 41.9 mg N/L·h in reactor with medium pH of 7.1 and 14.5 mg/L·h in reactor with medium pH of 6.6. It was shown that the decrease of pH value positively correlated with ferrous concentration (Fig. 6.6), and the quantity of ferrous hydroxide formed was also positively related to ferrous concentration, therefore, the extent of inhibition was associated with ferrous concentration.

At the same time, ferrous hydroxide and ferric hydroxide particles were mixed with denitrifying bacteria cells (Fig. 6.7). The size of denitrifying sludge was 44.1 ± 2.2 μm and the sizes of colloidal particles of ferrous hydroxide and ferric hydroxide were 10.8 ± 0.1 μm and 14.7 ± 0.3 μm , respectively. Ferrous hydroxide and ferric hydroxide colloid particles occupied the space surrounding denitrifying bacteria, partially covered their surface area, and inhibited the diffusion of substrate into the cells (Fig. 6.7). It was shown that adsorption of Fe(II) on the surface of iron-reducing bacteria *Shewanella putrefaciens* caused a lag-phase in bacterial reduction of Fe(III) aqueous complex (Liu et al., 2001a). Therefore, the partial

coverage of the surface of denitrifying bacterial by iron-hydroxides particles can be reason of adaptation phase of nitrate reduction and decrease of the nitrate reduction rate. The results of experiment 2 confirmed the negative effect of ferrous on nitrate reduction in reject water under anaerobic conditions.

Fig. 6.7 SEM images of ferrous hydroxide (a) enrichment culture of denitrifying bacteria (b) and enrichment culture of denitrifying bacteria with ferrous hydroxide precipitate (c).

Another hypothetical mechanism explained the inhibition of nitrate and nitrite reduction by *Shewanella putrefaciens* in the presence of goethite was the formation of Fe(III) hydroxide (Equation 6-4) coating on the cells which physically blocked the transport of soluble electron acceptors into the cells (Coby and Picardal, 2005; Cooper et al., 2003):

In our study, such mechanism, however, was not involved in the inhibition of nitrate reduction by ferrous, as the mass ratio of Fe^{2+} oxidized/ NO_2^- reduced was 8 g Fe^{2+} oxidized/g NO_2^- reduced (Equation 6-4). However in our experiments the ferrous in each reactor was rapidly oxidized within 3 hours (from the 6th to the 9th hour of the experiment) when reject water contained sufficient organic compounds as electron donors for complete nitrate reduction (experiment 1) (Table 6.3). The nitrate reduction in reactors with 200 and 400 mg/L Fe(II) on the contrary was

almost stopped (adaptation phase) during the same period. Meanwhile, the nitrite concentration in reactors with 100, 200, and 400 mg/L Fe(II) remained unchanged or slightly increased (Table 6.3). Obviously, ferrous was not biologically oxidized by nitrite. The chemical interactions between nitrite and ferrous also showed that ferrous was not chemically oxidized by nitrite or nitrate in reject water. Therefore, the inhibition of nitrate reduction by ferrous was mainly attributed to the significant change of medium pH by ferrous addition and the space competition between denitrifying bacterial and iron-hydroxides precipitates.

6.3.8 The contribution of nitrate-dependent ferrous oxidation and iron reduction processes in nitrate reduction in reject water treatment

Fe(II) can be biologically oxidized to Fe(III) with nitrate or nitrite as electron acceptor. The nitrate-dependent ferrous oxidation process (Straub et al., 1996; Benz et al., 1998) was demonstrated in activated sludge process for nitrate removal (Nielsen and Nielsen, 1998a; 1998b). However, our study showed that when reject water contained sufficient organic compounds as electron donors for complete nitrate reduction, ferrous was not involved in nitrate-dependent ferrous oxidation process or did not enhance nitrate removal.

When reject water contained insufficient organic compounds as electron donors for complete nitrate reduction, the nitrate reduction was inhibited due to the shortage of organic compounds and nitrate was only partially reduced. The nitrate reduction efficiencies of 49.2% in experimental reactor with 200 mg/L Fe(II) and 41.8% in experimental reactor with 400 mg/L Fe(II) were higher than the efficiency of 31.5% in control reactor with 0 mg/L Fe(II). The higher nitrate reduction efficiencies in experimental reactors with 200 and 400 mg/L Fe(II) could be explained by the partial nitrate reduction process happened in the reactors. The nitrate reduction

process consists of two sequential steps with nitrite as the intermediate product:

The high nitrite concentration remained in the experimental reactors with 200 and 400 mg/L Fe(II) showed that the nitrate reduction was stopped at the first stage due to the shortage of electron donors (Fig. 6.5c). Therefore, the nitrate dependent ferrous oxidation process did not contribute to the relatively higher nitrate reduction efficiency in experimental reactors with 200 and 400 mg/L Fe(II).

The oxidation of insoluble Fe(OH)₂ with high oxidation rate under nitrate reducing conditions produced amorphous Fe(III) hydroxide (Senko et al., 2005). Amorphous Fe(III) hydroxide is an electron acceptor for iron-reducing bacteria, which were present in the anaerobic sludge (Nielsen et al., 2002), to produce Fe(II) (Ivanov et al., 2005; Stabnikov et al., 2004). However, iron reduction process was inhibited by the presence of nitrite (Fig. 6.5c) and the shortage of available organic carbon. It is agreed with the data reported previously (DiChristina, 1992; Sorensen, 1982). The inhibitory effects of nitrate NO₃⁻ and nitrite NO₂⁻ on Fe(III) reduction by iron reducing bacteria *Shewanella putrefaciens* 200 were shown (DiChristina, 1992). Additions of NO₃⁻ and NO₂⁻ to the active anaerobic slurries of marine sediment stopped the Fe³⁺ reduction, and the process resumed only after a depletion of the added compounds (Sorensen, 1982).

In the present study, ferric reduction by iron reducing bacteria to produce ferrous was neglected and iron reduction was not counted in the cycle of ferrous ions. Moreover, ferrous did not chemically react with nitrate in reject water under anaerobic conditions. Meanwhile, ferrous was chemically oxidized by some

components containing in reject water under anaerobic conditions. This further demonstrated that the oxidation of ferrous in reject water did not contribute to nitrate reduction.

6.4 Conclusions

- 1) When reject water contained amount of electron donors sufficient for complete nitrate reduction, the significant change of medium pH due to ferrous addition and the partial coverage of the surface of denitrifying bacteria by iron-hydroxides particles caused the inhibition of nitrate reduction.
- 2) When reject water contained amount of electron donor, which was not sufficient for complete nitrate reduction, addition of ferrous did not significantly inhibit nitrate reduction and nitrate reduction was limited by the shortage of electron donors.
- 3) There is no need to recycle produced Fe(II) after Fe(III) reduction back the anoxic nitrate reduction tank during removal of nitrogen and phosphorus from reject water of municipal wastewater treatment plant using sequential ferric bio-reduction-phosphate precipitation and nitrate bio-reduction. Moreover, the nitrate reduction stage should be performed prior to ferric reduction stage to avoid the negative effects of ferrous, produced in iron ore reduction, on nitrate reduction.

Chapter 7

The removal of nitrogen from reject water using iron ore as biofilm carrier

7.1 Introduction

Nitrification process, which is carried out mainly by aerobic autotrophic bacteria, consists of two steps: ammonium is oxidized to nitrite by ammonium-oxidizing bacteria (AOB), and then nitrite is oxidized to nitrate by nitrite-oxidizing bacteria (NOB). The most commonly recognized ammonium oxidizers and nitrite oxidizers are bacteria from genus *Nitrosomonas* and *Nitrobacter*, respectively. The oxidation of ammonium by AOB produces protons thereby causes acidification (Equation 7-1).

The optimum pH for *Nitrosomonas* and *Nitrobacter* is between 7.5 and 8.5 (U.S.EPA, 1975). The mechanism of pH affecting the rate of nitrification is based on the pH-dependent equilibriums of ammonia-ammonium and nitrite-nitrous acid. High pH stimulates the production of ammonia while low pH favors the formation of nitrous acid. Both free ammonia and free nitrous acid inhibit nitrifying bacteria (Anthonisem, 1974). It was reported that nitrification would cease at or below pH 6.0 (Painter and Loveless, 1983); as a result, pH control was crucial during the nitrification process. Theoretically, 7.14 mg/L alkalinity as CaCO_3 is required for 1 mg N/L NH_4^+ -N oxidized (U.S.EPA, 1975). The reject water normally contains about 1:1 molar ratio of alkalinity as CaCO_3 to ammonium. Therefore, the addition of 3.57 mg/L alkalinity as CaCO_3 is required for nitrification of 1 mg N/L NH_4^+ -N in reject water.

Since nitrifying bacteria have low growth rate (approximately 0.08/h at 30 °C) (Wiesmann, 1994), and are very sensitive to environmental factors, it is difficult to maintain a sufficient amount of them in the reactor (Moreau et al., 1994; Ochoa et al., 2002). Biofilm as a complex coherent structure of microbial cells and cellular products (van Loosdrecht et al., 1995b), is an ideal survival mode for sensitive bacteria like nitrifiers. The biofilm structure can protect them from detachment, harm, and toxicants (Bishop, 1997). In the biofilm reactor, microorganisms are immobilized/attached to static biofilm carrier (Liu and Tay, 2002) or suspended carriers (particle-support biofilm) (Nicolella et al., 2000), which can accumulate high biomass concentration in the reactor and achieve high biodegradation rate. Another benefit is the simultaneous occurrence of various biological reactions made by co-existing microbial populations in the biofilm (Bishop, 1997).

The development of biofilm is gradual process and consists of several steps (Simoes et al., 2010). The roughness of biofilm carrier surface protects the initial adhering bacteria from the detachment by shear force (van Loosdrecht et al., 1988) and enhances the development of biofilm (Heijnen et al., 1992). It is important to apply certain shear force to enhance the formation of dense, compact, and strong biofilm (Kwok et al., 1998; Liu and Tay, 2002; van Loosdrecht et al., 1995a). And a high substrate loading rate leads to a high biomass production rate and determines biofilm growth rate (van Loosdrecht et al., 1995a). Therefore, the proper balance of shear force and organic loading rate can result in smooth, stable, and strong biofilm (Kwok et al., 1998; Liu et al., 2003b; Tijhuis et al., 1995a). Moreover, the increase of N/C ratio in substrate enhances the development of nitrifying bacteria in biofilm (Ohashi et al., 1995).

The difference in growth rate between heterotrophic bacteria and nitrifying bacteria together with their competition for substrates, oxygen, and space due to limitation

of oxygen diffusion rate (Zhang et al., 1994; 1995) induce a layered structure within the biofilm (Costerton et al., 1981; Nicoletta et al., 2000; van Loosdrecht et al., 1995b). With heterotrophs occupying the outer layer and autotrophs in the inner layer, autotrophic nitrifiers are partially inhibited under high organic loading rate (Elenter et al., 2007; Nogueira et al., 2002; Okabe et al., 1996). High N/C ratio favors high nitrifying bacteria activity in the biofilm (Moreau et al., 1994; Ochoa et al., 2002), whereas high oxygen concentration reduces oxygen competition (Furumai et al., 1994). A simultaneous removal of nitrogen and organic carbon has been reported (Liu et al., 2010). However, the limited oxygen diffusion is not detrimental in all the cases. Denitrifying bacteria can be developed in the deepest layer of biofilm (Biesterfeld et al., 2003; Mehlhart, 1994; Revsbech et al., 1989), allowing simultaneous nitrification and denitrification (SND) process to take place in a single biofilm reactor (Rong et al., 2007; Walters et al., 2009).

In term of material, chalk (CaCO_3) has been used as biofilm carrier and buffering reagent for nitrification in fluidized bed reactor at low pH (4.5-5.5) (Green et al., 2001; 2002; Tarre et al., 2004). Grain-slag in biological aerated filter is another successful buffering material as it can strip CO_2 into wastewater to buffer pH value (Yu et al., 2008). Similarly, iron ore containing about 60% (w/w) of iron, can be a potential buffering material. The decrease of solution pH due to protons produced from nitrification process would possibly be buffered by ferric oxide in iron ore according to the following reaction:

So far, the development of biofilm using iron ore as carrier has not attracted much attention. In this study, the biofilm developed on iron ore surface was used to remove ammonium from reject water with and without alkalinity addition,

respectively; the cultivated biofilm denitrified nitrate/nitrite under anaerobic conditions and aerobic conditions, respectively; and the complete removal of nitrogen under alternating aerobic-anaerobic conditions and high oxygen concentration were also studied.

7.2 Materials and Methods

7.2.1 Reject water, activated sludge, iron ore, and chalk

Reject water and aerobic sludge were collected monthly from the local municipal wastewater treatment plant; they were stored at 4 °C before being used for experiments. Activated sludge was used as seed sludge for nitrifying bacteria and biofilm. The characteristics of activated sludge were determined and the results showed that the seed sludge had a mixed liquor suspended solids (MLSS) concentration of 7.24 ± 0.3 g/L, mixed liquor volatile suspended solids (MLVSS) concentration of 6.57 ± 0.04 g/L and pH of 7.06 ± 0.01 . Reject water was used as the food source for biofilm development and mature biofilm. The characteristics of the collected reject water were determined monthly and the results are summarized in Table 7.1. As this research has been carried on for about 2 years, therefore, there were significant temporal variations of the characteristics of reject water used for biofilm development and mature biofilm.

Iron ore was delivered from JuYuan Trading PTE LTD (PingXiang City, GuangXi, China). According to specification, iron ore contained 60% (w/w) iron and the major mineral content was hematite (Fe_2O_3). The sizes used in batch experiment were from 2 to 4 mm by sieving after crushing. The determined porosity was 50.5 ± 0.5 % (Ave $\pm$ s.d; v/v) and the determined density was 4045 ± 3 g/L.

Chalk, with CaCO_3 as the main content, was purchased from POPULAR book store in Singapore. It was scrapped and sieved to select the particles of the same sizes with iron ore (2-4 mm). The determined porosity was higher ($67.3 \pm 1 \%$) and the determined density was lower ($1420 \pm 5 \text{ g/L}$) as compared to iron ore.

Table 7.1 Characteristics of reject water used as influent to biofilm development and mature biofilm

Concentration (mg/L)	Reject water fed for biofilm development (Ave $\pm$ s.d.)	Reject water fed for mature biofilm
Total organic carbon (TOC)	205.5 ± 3.7	113-550
Total suspended solids (TSS)	460 ± 50	180-420
Volatile suspended solids (VSS)	430 ± 50	140-290
$\text{NH}_4^+ \text{-N}$	690 ± 8.5	278-715
$\text{NO}_3^- \text{-N}$	0.68 ± 0.04	0.20-1.05
$\text{NO}_2^- \text{-N}$	0.05 ± 0.01	0.04-0.07
Alkalinity (as CaCO_3)	2596 ± 3.9	1002-2980
pH	7.69 ± 0.02	7.15-8.29
Alkalinity (as CaCO_3)/ $\text{NH}_4^+ \text{-N}$ (molar ratio)	1.05:1	1.01-1.17
Phosphorus (mg P/L)	65.8 ± 1.2	52.6-157
Total Ferrous	9.21 ± 0.21	2.06-9.21

7.2.2 Experimental setup

7.2.2.1 The development of biofilm on iron ore surface and nitrification

Nitrification experiment was performed in bench-scale reactor and 500 mL conical

flasks (IWAKI Pyrex, Asahi Technology Glass; bottom diameter, 10.5 cm; up diameter, 3.5 cm; total height, 16.5 cm; 500 mL working volume height, 8 cm) at room temperature. The experimental configurations are listed in Table 7.2. The conical flasks were put on the DK-OS010 Orbital Shaker (Daiki Science, 150 rpm/min). Air was pumped to the conical flask with a flow rate of 0.5 L/min, distributed evenly through air stone at the bottom of the flask. The dissolved oxygen concentration obtained in the solution was about 7.2 mg/L. The reactors were run as sequencing batch mode with a solution exchange volume ratio of 50%. To help the bacteria to adapt to the high ammonium concentration, 250 mL (1 + 3) reject water was exchanged/week during the first 2 weeks of cultivation; in the following 4 cycles (3 days/cycle), 250 mL (1 + 1) reject water/cycle was exchanged. During the next 15 cycles (3 days/cycle), the working volume of 400 mL was fixed with an exchange volume ratio of 50%. 200 mL reject water was exchanged/cycle in the remaining period. The reactors were operated for the first 5 months without any addition of external alkalinity to obtain the biofilm nitrification profiles under low pH. The experimental data were collected from the 26th day of cultivation until the end of the next 38 cycles.

Table 7.2 The experimental configurations of batch experiment

Reactor number	Experiment	Control	
		1	2
Biofilm carrier	50 mL (2-4 mm) iron ore (about 100 g, void 50.5% v/v)	50 mL (2-4 mm) chalk (about 23.21 g, void 67.3% v/v)	0
Activated sludge	50 mL	50 mL	50 mL
Return liquor	1 + 3 reject water to make up to 500 mL	1 + 3 reject water to make up to 500 mL	1 + 3 reject water to make up to 500 mL

After 5 months of operation, the flask containing iron ore biofilm was stored at 4 °C fridge for 5 months, and then re-operated without shaking at room temperature and fed with reject water. In the first 5 cycles, the cycle time was 3 days and volume exchange ratio was 50%. After the 6th cycle, the cycle time was reduced to 2 days; exchange volume ratio was increased to 62.5%, which indicated 250 mL reject water was exchanged/2days. External alkalinity was added at the same time to compensate the alkalinity consumed by nitrification. After 21 cycles of operation at cycle time of 2 days, the cycle time was further reduced to 1 day; the operation was extended for another 45 cycles.

7.2.2.2 Nitrate reduction in iron ore biofilm under anaerobic and aerobic conditions

Nitrate reduction in iron ore biofilm under nitrogen gas: The experiment was performed in batch mode. In the biofilm reactor containing 500 mL reject water, potassium nitrate (GR grade, Merck) was added to maintain a nitrate concentration of 220 mg N/L. 0.25 L/min of nitrogen gas was distributed through air stone to maintain the anaerobic conditions and provide the necessary mixing. It was reported that the required COD/N ratio required for complete denitrification was from 3.5 to 15 g COD/g N (about 1.32-5.64 g TOC/g N) (Henze, 1991). Sodium acetate (NaCH₃COO • 3H₂O) (GR grade, Merck) as an external carbon source was fed to the reactor at the beginning of anaerobic stage. The theoretical mass ratio of sodium acetate/NO₃-N is 6.07 g/g NO₃-N (or 1.07g TOC/ g NO₃-N) according to the following equation:

Besides, the biomass needs carbon source for synthesis. Thus, 200% of theoretical

quantity of acetate required (12.14 g/ g NO₃-N) was added to the reactor to achieve complete nitrate reduction. TOC concentration was monitored to be above 100 mg/L by adding more sodium acetate when it was about to drop. The solution pH was maintained from 7 to 9 using 5 N HCl.

Nitrate reduction in biofilm under aerobic conditions: The operation of this experiment (liquid volume, TOC control, and pH control) was relatively the same with that under anaerobic conditions, except a higher nitrate concentration of 270 mg N/L was created at the beginning, and the pretreated reject water, contained 0 mg N/L ammonium, nitrite, and nitrate, was used in the experiment. Obviously nitrogen gas was replaced by air with a flow rate of 0.5 L/min to maintain an oxygen concentration of 7.2 mg/L. Again, sodium acetate was added at the dosage of 370% of the theoretical value to cover the organic carbon demands of both heterotrophs and denitrifiers.

7.2.2.3 The complete removal of nitrogen from reject water by iron ore biofilm

The removal of nitrogen under alternating aerobic and anaerobic conditions: 500 mL reject water was added in the biofilm reactor. Aerobic stage was maintained for 20 hours with an air flow rate of 0.5 L/min. Anaerobic stage during 4 hours was maintained by purging 0.2 L/min of nitrogen gas. 150% of the theoretical quantity of sodium acetate was added at the beginning of anaerobic stage.

Simultaneous nitrification and denitrification (SND) process under aeration:

The same volume of reject water was used. Air was distributed through air stone at the flow rate of 0.5 L/min. 250% of the theoretical quantity of sodium acetate was added at the 20th h of experiment.

7.2.3 Analytical methods

The analytical methods for TSS, MLVSS, MLSS, alkalinity, pH, dissolved phosphorus concentration, dissolved NH_4^+ -N concentration, dissolved NO_3^- -N concentration, dissolved NO_2^- -N concentration, total ferrous concentration, dissolved TOC concentration, and the porosity were presented in Chapters 3, 4, and 5. The oxygen concentration was measured by DO meter (YSI Incorporate, Yellow Springs Ohio, USA). Sludge volume index (SVI) of granules was analyzed by standard methods (APHA, 2005). The sizes of small iron ore particles and small crushed chalk particles were measured by an automated laser particle size analyzer (Malvern Master Sizer Series 2600, Malvern Instruments LTD, Malvern, UK). The size of the granular sludge was measured by an image analysis (IA) system (Image-Pro Plus, V4.0, Media Cybernetics) with an Olympus SZX9 microscope. Morphologies of granules and biofilm were observed by an image analysis (IA) system or scanning electron microscope (SEM, Zeiss EV050, UK).

SEM images of biofilm and granules: The Morphologies of biofilm and granules were examined with scanning electron microscope (SEM, Zeiss EV050, UK). The biofilm and granules samples, collected from the reactor, were fixed in 2% glutaraldehyde for 2 hours, washed 3 times with 0.1M sodium cacodylate buffer for 20 minutes, dehydrated in step gradients of ethanol: 50, 70, 85, and 95% ethanol for 10 minutes, kept in 100% ethanol until dried with the critical point dryer (Polavon E3100, Quorum Technologies, UK), and then sputter coated with Au-Pd with sputter coater (Emitech SC7620, Quorum Technologies, UK).

Detection of nitrifying bacteria in biofilm by fluorescence *in situ* hybridization (FISH): The iron ore biofilm sample, collected from the reactor, was fixed with fresh 4% paraformaldehyde (PFA) with a 3:1 ratio for 3 hours at 4 °C. After fixing,

the specimen was rinsed with phosphate buffered saline (PBS) buffer for 2-3 times, and then stored in 1 volume ratio of 100% ethanol at -20 °C. The specimen was immobilized on a gelatin coated slide glass and air dried for over night. The dried sample was dehydrated by successive passage through 50%, 80%, and 100% ethanol for 3 minutes and then air-dried before being used for fluorescent *in situ* hybridization (FISH).

Table 7.3 16S rRNA-targeted oligonucleotide probes used in experiments

Probe	Specificity	Sequence (5'-3')	Formamide concentration in hybridization buffer (%) (v/v)	NaCL concentration in washing buffer (mM)
EUB 338	Domain Bacteria	GCTGCCTCCCGT AGGAGT	20	0.225
NSO 190	ammonia-oxidizing β- proteobacteria	CGATCCCCTGCT TTTCTCC	55	0.020
NSO 1225	ammonia-oxidizing β- proteobacteria	CGCGATTGTATT ACGTGTGA	35	0.079
NIT 3	<i>Nitrobacter</i> spp.	CCTGTGCTCCAT GCTCCG	40	0.056
NEU	halophilic and halotolerant members of the genus of <i>Nitrosomonas</i>	CCCCTCTGCTGC ACTCTA	40	0.056

The following 16S rRNA-targeted oligonucleotide probes were used (Table 7.3): (1) EUB 338 (labeled with the fluorochrome Cy5), a general probe to target all eubacteria (Okabe et al., 1999); (2) NSO190 (labeled with the fluorochrome Cy3), a genus-specific probe for the 16S rRNA region of ammonia-oxidizing β -proteobacteria (Okabe et al., 1999); (3) NIT3 (labeled with the fluorochrome Cy3), a genus-specific probe for *Nitrobacter* spp. (Okabe et al., 1999); (4) NSO1225 (labeled with fluorochrome Cy3), a genus-specific probe for ammonia-oxidizing β -proteobacteria (Mobarry et al., 1996); (5) NEU (label with fluorochrome Cy3), a genus-specific probe for halophilic and halotolerant members of the genus of *Nitrosomonas* (Okabe et al., 1999).

The hybridization buffers contained 0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), and different concentrations of formamide (Table 7.3). Hybridization was performed for 1.5 hours at 46 °C in an isotonicly equilibrated humid chamber; followed by a 20 minutes washing with 50 mL of prewarmed washing buffer containing 20 mM Tris-HCl, 0.01% SDS, and different concentrations of NaCl (Table 7.3) at 48 °C. For combination of probes with different optimal hybridization stringencies, two hybridizations were done. The first hybridization was done with the probe that required the higher formamide concentration (conditions of highest stringency), which was followed by second hybridization at a lower stringency. Finally, the images of iron biofilm were acquired by a Fluoview 300 Confocal Laser Scanning Microscope (CLSM) (Olympus, Japan).

7.3 Results and Discussion

7.3.1 The development of biofilm on the iron ore surface

Fig. 7.1a shows that the bacteria started to attach to the rough iron ore surface after

15 days of cultivation. The micrograph of matured biofilm (Fig. 7.1b) displays the mushroom-like structure of biofilm surface and the cocci shaped bacteria as well as rod-shaped bacteria dominating the outer layer of biofilms. The bacteria were embedded in the extracellular polysaccharides (EPS) and formed a smooth, compact, dense, and stable structure. It was known that EPS mediated the cohesion and adhesion of cells (Tay et al., 2001c).

Fig. 7.1 Morphology and structure of biofilm by SEM: a) bacterial cells attached to the rough iron ore surface; b) the mushroom-like and channel structures of mature biofilm.

In the control reactor 1, chalk was used as biofilm carrier. The chalk was crushed under shear force, forming fine particles and suspending in the solution. The quantity of chalk in the solution diminished with time. The fine chalk particles ($102 \pm 1.7 \mu\text{m}$) easily dispersed in reject water and caused the high solution turbidity. The dissolution made it difficult for biofilm to attach and grow on chalk surface. In control reactor 2, activated sludge was suspended in the solution and some of the sludge was attached to the flask wall.

During the cultivation, reject water was exchanged either weekly or in 3 days interval. Probably, the bacteria starved. It was known that starvation facilitated the microbial adhesion and aggregation, (Liu and Tay, 2004; Tay et al., 2001a). Therefore, the bacteria attached to the iron ore surface. Similar phenomenon was observed in control 2, where the bacteria were observed to attach to the flask wall. The roughness of biofilm carrier surface is important in protecting the initially adhering bacteria from detachment under shear force (van Loosdrecht et al., 1988; Heijnen et al., 1992). Probably, the rough iron ore particles in our study (Fig. 7.1a) helped the bacterial cells to stay long enough on the surface to develop biofilm.

The development of biofilm is the result of biomass growth and detachment. Biofilm growth is due to the diffusion of substrate and the conversion of substrate in the biofilm. High substrate loading rate leads to high biomass production rate and determines the growth rate of biofilm (van Loosdrecht et al., 1995a). The shear force, which affecting the detachment of biofilm, enhances the formation of dense, compact, and strong biofilm (Kwok et al., 1998; Liu and Tay, 2002; van Loosdrecht et al., 1995a). Therefore, the proper balance of the shear force and organic loading rate achieves smooth, stable, and strong biofilm (Liu et al., 2003b; Tijhuis et al., 1995a; Kwok et al., 1998). All the above characteristics of biofilm process were fitted to this study. With a relatively high organic carbon and nitrogen loading rate

(Table 7.1), the growth of heterotrophic and nitrifying bacteria in the biofilm was enhanced. The shear force induced by air flow and vigorous movement on shaker contributed to the smooth and dense structure of biofilms. The balance between detachment of biomass due to shear force and growth of biomass due to substrate loading successfully maintained biofilm structure.

The mass ratio of N/C in reject water was about 3.4/1 (Table 7.1). This high ratio enhanced the development of nitrifying bacteria in biofilm, which was proven in previous researches (Moreau et al., 1994; Ochoa et al., 2002; Ohashi et al., 1995). Under similar shear force, the biofilm populated with nitrifying bacteria (as in our case) has a denser structure than that with heterotrophic bacteria (Tijhuis et al., 1995b). The mushroom structure developed in the biofilm could be due to dense population of nitrifiers developed under high N/C ratio. The formation of this structure has been observed in other biofilms (Costerton et al., 1981; Wimpenny and Colasanti, 1997) and granules (Liu et al., 2004).

7.3.2 The distribution of biomass in the cultivated biofilm

The spatial distribution of ammonium oxidizing bacteria and nitrite oxidizing bacteria was analyzed by fluorescence *in situ* hybridization (FISH). The FISH images obtained using probe NEU and probe EUB 338 shows that the ammonium oxidizers were mainly found in the inner layer while heterotrophs located in the outer layer of biofilm (Fig. 7.2a and b). Similar distribution pattern was detected by probe NSO190 and probe EU338 (Fig. 7.2c), as well as probe NSO 1225 and probe EU338 (Fig. 7.2d). Fig. 7.2e depicts nitrite oxidizing bacteria were in the inner layer, surrounded by heterotrophic bacteria in the outer layer. Heterotrophs clearly existed in the whole outer layer of biofilm and additionally formed a cluster in the deepest layer (Fig. 7.2a-e). It was likely that this cluster, which was near to the substratum,

was denitrifying bacteria.

Fig. 7.2 *In situ* hybridization of iron ore aerobic biofilm viewed by Confocal Laser

Scanning Microscopy (CLSM): (a) and (b) Yellow part; ammonium oxidizing bacteria detected by both Cy3 labeled probe NEU and Cy5 labeled probe EU338. Red part: other bacteria detected by Cy5 labeled probe EU338. (c) Yellow part: Ammonium oxidizing bacteria detected by both Cy3 labeled probe NSO190 and Cy5 label probe EU338. Red part: other bacteria detected by Cy5 labeled EU338. (d) Yellow part: Ammonium oxidizing bacteria detected by both Cy3 labeled probe NSO1225 and Cy5 label probe EU338. Red part: other bacteria detected by Cy5 labeled EU338 (e) Yellow part: nitrite oxidizing bacteria detected by both Cy3 labeled probe NIT3 and Cy5 label probe EU338. Red part: other bacteria detected by Cy5 labeled EU338.

In all the FISH pictures, the red-fluorescence microorganisms (stained with Cy5 labeled probe EU338) were located around the cluster of ammonium oxidizing bacteria (stained with Cy3 labeled probe NSO 190, probe NEU, and probe NSO 1225) and nitrite oxidizing bacteria (stained with Cy3 labeled probe NIT3). The growth of nitrifying bacteria, heterotrophic denitrifying bacteria, and other heterotrophic bacteria in biofilm seemed to be simultaneous and interacted closely to each other. The organic acids and ammonium in reject water were the abundant substrates for heterotrophs and nitrifiers, respectively, while the products of ammonium oxidation (nitrite and nitrate) together with organic acids in reject water enhanced the development of denitrifying bacteria in the deepest layer of biofilm.

It was known that the distribution of bacteria in the biofilm was significantly affected by the particular microenvironment around them; bacteria developed channels in the microenvironment that improved the diffusion in biofilm (Watnick and Kolter, 2000). Specifically, bacteria are sensitive to the substrate concentration gradient, the oxygen diffusion limitation, and the different growth rates among them in the biofilm (Nicolella et al., 2000; Tjihuis et al., 1994b; 1995b; van Loosdrecht et

al., 1995b). Heterotrophic and nitrifying bacteria always compete for substrate, oxygen, and space in biofilm (Zhang et al., 1994; 1995). Being the faster growers, heterotrophic bacteria tend to dominate at the surface of biofilm to consume most of the oxygen and substrate available, the slower “eater” nitrifiers stay in the deeper layer and receive the remaining nutrients penetrating inside (Bishop, 1997; Nicolella et al., 2000; Okabe et al., 1996).

7.3.3 Biofilm nitrification without addition of external alkalinity

Nitrification was successfully achieved in iron ore biofilm reactor, activated sludge reactor (control 2), and chalk reactor (control 1). Ammonium oxidizing bacteria were cultivated in iron ore biofilm reactor (Fig. 7.3) after 26 days of cultivation, while nitrite oxidizing bacteria were developed there after 44 days of cultivation. The conversion of ammonium to nitrate was complete since nitrite concentration in the effluent was almost 0 (Fig. 7.3a). During the first nineteen cycles, the ammonium removal efficiency was about 50% (Fig. 7.3b). The alkalinity contained in reject water was sufficient to buffer the changes of pH due to 50% of nitrification. After the alkalinity was completely consumed, the pH reduced below 6.5 and nitrification thereby was inhibited. Fig. 7.3b shows that when nitrite oxidizing had not started, the effluent pH was between 6.19 and 5.64 (1st cycle to 6th cycle). When nitrite oxidation took place, the effluent pH reduced continuously, eventually dropped to the range between 5.64 and 4.67 (6th cycle to 19th cycle).

From the 20th cycle onward, when the influent contained high TOC concentration (550 mg/L), heterotrophic bacteria in the biofilms performed organic carbon oxidation, which contributed additional alkalinity, followed by increased nitrification efficiency.

Within 18 cycles (20th to 38th), nitrification efficiency increased from 60% to as high as 90%. At the same time, the effluent pH was further reduced to 4.05 (Fig. 7.3b); on the contrary to the fast removal of ammonia as nitrification process accelerated, nitrate production was insignificant (Fig. 7.3a). The phenomenon revealed that another type of bacteria consumed produced nitrate and most probably they were the denitrifiers hiding in the deepest layer of the biofilm. In that case, the consumption of organic carbon in reject water and the enhancement of solution alkalinity would have been shared with denitrifiers.

Fig. 7.3 Iron ore biofilm nitrification without addition of external alkalinity.

Fig. 7.4 Nitrification in control 2 which was activated sludge process without addition of external alkalinity.

In the activated sludge reactor (control 2), ammonium oxidation was observed after 26 days of cultivation; whereas nitrite oxidation developed only after 62 days of cultivation (Fig. 7.4a). Before the achievement of complete nitrification (1st cycle to 12th cycle), nitrification efficiency was about 50%, and effluent pH was between 6.15 and 5.72. After that (13th cycle to 19th cycle), the effluent pH further reduced to 4.88 (Fig. 7.4b).

When the influent contained high TOC concentration (550 mg/L) from 20th cycle, nitrification efficiency increased from 53% to about 78% during 20th cycle to 38th cycle due to the additional alkalinity contributed by organic carbon oxidation. The

effluent pH first reduced from 4.82 to 4.06 (20th cycle to 26th cycle), then slightly increased from 4.06 to 5.29 (26th cycle to 38th cycle) (Fig. 7.4b).

Fig. 7.5 Nitrification in control 1 using chalk as carrier without addition of external alkalinity.

In the chalk reactor (control 1), ammonium oxidation was also obtained after 26 days of cultivation. Nitrite oxidation was not fully completed during the whole process (1st cycle to 38th cycle). During the 1st cycle to 20th cycle, most of nitrification efficiencies were lower than 40%, while effluent pH was between 6.38 and 5.90 (Fig. 7.5 b). When the reject water containing high TOC was fed from 20th cycle, nitrification efficiency increased from 40% to about 70% during 20th cycle to 38th cycle, and the effluent pH was reduced from 5.90 to 5.0 (Fig. 7.5 b).

Fig. 7.6 Ammonium concentration (a) and pH (b) value in 3 reactors in the 33rd cycle.

The changes of ammonium concentration and pH in one cycle are shown in Fig. 7.6. It can be seen that ammonium in all reactors was removed significantly in the first day. At the same time, pH in the iron ore biofilm reactor increased from 7.2 to 8.46 due to organic carbon oxidation and some nitrate reduction. Activated sludge reactor also witnessed pH increment from 7.28 to 8.27 due to organic carbon

oxidation, whereas chalk reactor experienced pH reduction from 7.27 to 6.51. In the 2nd day, nitrification in iron ore reactor and activated sludge reactors continued, followed by the decrease of pH to 4.47 and 5.26, respectively. Nitrification was very slow in chalk reactor due to the 1st day low pH; hence pH was slightly decreased to 5.52. Such low pH led to the cease of nitrification in all reactors in the 3rd day. Final pH in iron ore biofilm reactor, activated sludge reactor, and chalk reactor was 4.07, 4.36, and 5.40, respectively.

Fig. 7.7 Nitrification efficiency in different reactors without addition of external alkalinity.

Nitrification efficiencies in 3 reactors are plotted in Fig. 7.7. The trends were similarly high in iron ore biofilm reactor and activated sludge process but low in chalk reactor during the first 21 cycles. When reject water containing high TOC concentration was fed from 20th cycle, nitrification efficiencies in all reactors increased. It was the highest in iron ore biofilm reactor, middle in activated sludge reactor and lowest in chalk reactor. The improved efficiency was due to the increased alkalinity, which was attributed to organic carbon oxidation by heterotrophic bacteria, and nitrate reduction in iron ore biofilm reactor further contributed additional alkalinity, therefore, the nitrification efficiency in iron ore

biofilm reactor was the highest.

It was known that the optimum pH for *Nitrosomonas* and *Nitrobacter* was between 7.5 and 8.5 (U.S.EPA, 1975). Painter and Loveless (1983) reported that nitrification process ceased at or below pH 6.0. Therefore, the removal of ammonium from reject water by nitrification should optimize pH in a suitable range ($7.5 < \text{pH} < 8.5$). As the molar ratio of alkalinity to ammonium in reject water was from 1.01 to 1.17 (Table 7.1), the alkalinity was only enough for about 50% of ammonium oxidation. When this alkalinity of reject water was completely consumed by protons produced in nitrification process, the solution pH reduced. pH of below 7 inhibited nitrification process and limited ammonium removal efficiency in all 3 reactors. When the reject water contained a lot of organic carbon, the oxidation of these substrates partially contributed to the alkalinity, raising pH, and nitrification efficiency at the same time. The limited oxygen diffusion in the deepest layer of iron ore biofilm became the favorable condition for denitrifying bacteria to develop (Fig. 7.2a-e). Similar explanation has been mentioned in previous research (Biesterfeld et al., 2003; Mehlhart, 1994; Revsbech et al., 1989). The additional alkalinity contributed by denitrifiers through nitrate reduction process boosted nitrification efficiency in iron ore biofilm reactor to the highest.

It was known that *Nitrosomonas* had higher growth rate than *Nitrobacter* at temperature higher than 20 °C (Hellings et al., 1998). Therefore, ammonium oxidizers developed first and fully grew after 26 days of cultivation. During the cultivation process, nitrite oxidizers developed faster in iron ore biofilm than in activated sludge and chalk reactor, as the rough iron ore surface was more favorable for the bacteria to attach and growth. Therefore, ammonium oxidizing bacteria and nitrite oxidizing bacteria clustered on the rough iron ore surface and were covered by the heterotrophic bacteria (Fig. 7.2a-e). On the contrary, the highly dispersed

chalk particles in reject water made it difficult for nitrifying biofilm to attach and growth. Green et al. (2001) reported that the thickness of the biofilm developed on the chalk surface was less than 30 μm during one year experimental period. In the activated sludge process, the nitrifying bacteria clustered on the glass wall instead of forming biofilm. In term of turbidity, chalk reactor was the worst due to the dispersed particles while both activated sludge and iron ore biofilm reactor were very clear.

Theoretically, iron ore can react with the protons produced from nitrification process (Equation 7-2). The result of this study, however, demonstrated that the buffer capacities of iron ore and chalk in this process were not efficient. When nitrite oxidizers have not been fully developed in iron ore biofilm and activated sludge, the effluent pH was already reduced to about 5.70. When complete nitrification was achieved in iron ore biofilm and activated sludge reactor or partially achieved in chalk reactor, the effluent pH was further reduced to 4.05, 4.06, and 5.0, respectively.

Iron ore (Fe_2O_3) or chalk (CaCO_3) did not contribute to the buffering. The buffer capacity of chalk and high nitrification rate were all achieved in the pH range of 4.5-5.5 (Green et al., 2001; 2002; Tarre et al, 2004). In this study, the effluent pH was reduced to 5.0, but nitrification almost ceased at that time. Moreover, the crashing due to shear force resulted in fine chalk particles dispersing in the solution and reduced amount of chalk, hence not suitable for biofilm to develop.

In the iron ore biofilm reactor, the high shear force also crashed small part of iron ore into fine particles with a mean size of $159 \pm 5.4 \mu\text{m}$ (Fig. 7.8). These particles had good settling ability due to the high density of the iron ore, and high specific area. As a result, fine iron particles were excellent biofilm carrier, on which stable,

compact, and strong biofilm can form. SEM pictures reveal that biofilms on small iron particles had the mushroom-like structure (Fig. 7.9a, b), which was similar to that of biofilm on static iron ore surface (Fig. 7.1b). Therefore, it is potentially advantageous to operate the biofilm reactor as the combination of static fixed biofilm reactor and biofilm airlift suspended (BAS) reactor.

Fig. 7.8 The morphology of fine iron ore particles by IA system.

Fig. 7.9 The morphology of biofilm on small iron ore particles: a) Over view of biofilm on small iron ore particles by SEM; b) mushroom-like structure of biofilm surface developed on small iron ore particles.

7.3.4 Biofilm nitrification with the addition of external alkalinity

After 5 months of storage at 4 °C, the iron ore biofilm was reactivated by feeding reject water with a cycle time of 3 days and volume exchange ratio of 62.5%. The reject water collected from Kranji wastewater treatment plant had an ammonium concentration of 666 mg N/L, TOC concentration of 150 mg/L, and alkalinity concentration of 2427 mg/L as CaCO₃. The activity of biofilm recovered within 3 cycles (Table 7.4). The result showed an inhibition over nitrite oxidizers hence nitrification was considerably stopped at nitrite formation step. The alkalinity contributed to about 50% of nitrification, which was comparable to the result before the storage. Effluent pH was about 6.0, similar to what achieved before the storage when nitrite oxidation did not fully started. The quick recovery of biofilm activity proved that iron ore biofilm had good storage ability at 4 °C.

Table 7.4 The profiles of ammonium, nitrite, nitrate and pH during 4th cycle and 5th cycle of recovery

Parameters	4 th cycle	5 th cycle
Initial NH ₄ ⁺ -N (mg N/L)	438.32	490.42
Effluent NH ₄ ⁺ -N (mg N/L)	227.74	222.27
Initial NO ₂ ⁻ -N (mg N/L)	105.93	76.82
Effluent NO ₂ ⁻ -N (mg N/L)	254.04	255.37
Initial NO ₃ ⁻ -N (mg N/L)	3.10	2.30
Effluent NO ₃ ⁻ -N (mg N/L)	6.87	9.38
Initial pH	7.63	7.93
Effluent pH	6.05	5.96
NH ₄ ⁺ -N removal efficiency (%)	48%	54.6%

After the recovery of biofilm activity, the reactor cycle time was reduced to 2 days. In the 1st cycle, nitrification efficiency was 50.8% without any addition of external alkalinity and (Fig. 7.10). The effluent pH was 5.97. At the beginning of the 2nd cycle, bicarbonate was added in the reactor to compensate the alkalinity consumed by nitrification. As a result, pH increased to 9.0 and the nitrification efficiency of 79% was mainly attributed to the air stripping of ammonium. From the 4th cycle, alkalinity was added in the 2nd day of experiment; nitrification efficiency then increased to more than 80% and reached maximum level of 91% during the period between 4th cycle and 21st cycle. The activities of nitrite oxidizers were partially inhibited hence most of the nitrite produced could not be converted further (Fig. 7.10).

Fig. 7.10 Iron ore biofilm nitrification with the addition of external alkalinity.

After the 22nd cycle, the reactor cycle time was further reduced to 24 hours. Bicarbonate was added in at the 8th hour to compensate the alkalinity consumed by nitrification. In the following 10 cycles (22nd to 32nd), nitrification efficiencies remained about 80% at ammonium loading rate of 330-400 mg N/L d. The dominance of nitrite over nitrate concentration revealed that the activities of nitrite oxidizers were seriously inhibited (Fig. 7.10).

In the next 8 cycles (33rd to 41st) nitrification process was very effective with a general efficiencies of more than 90%, and a maximum efficiency of 99.9%. The activities of nitrite oxidizers, however, were still inhibited. Ammonium loading rate was 390-486 mg N/L d and maximum nitrification capacity was 428 mg N/L d. During the 42nd cycle to 66th cycle, nitrification efficiencies were even higher (95%) with a maximum value of 99.76% at nitrogen loading rates of 380-468 mg N/L d, the maximum nitrification capacity was 464 mg N/L d, and nitrification stopped at ammonium oxidation step as usual.

In short, the experimental results proved that the addition of alkalinity was crucial to achieve high nitrification efficiency. The maximum capacity of 464 mg N/L d at a loading rate of 380-468 mg N/L d in this study was better than that achieved by Rodgers et al. (2006), which was 300 ± 100 mg N/L d at ammonium loading rate of 700 ± 20 mg N/L d in a vertically moving biofilm reactor.

Fig. 7.11 The profile of ammonium, nitrite, nitrate, and pH during the cycle with optimum pH control.

The optimum pH for *Nitrosomonas* and *Nitrobacter* is between 7.5 and 8.5 (U.S.EPA, 1975). If bicarbonate was added to the reactor in a few times, the solution pH could be maintained within this optimum range at all time and nitrification rate would be enhanced consequently. Experimental results really showed that a higher nitrification rate, which was 437 mg N/L ammonium oxidized within 18.5 h, could be achieved when pH was tightly controlled (Fig. 7.11). Nitrification was stopped at ammonium oxidation step, the reason of which will be discussed in the following section. The average and maximum nitrification rates were 23.6 mg N/L h and 33.04 mg N/L h, respectively. The maximum rate was slight lower than the result reported by Welander et al. (1997), which was 40 g (NH₄-N)/(m³ reactor h). In that study, landfill leachate was treated in a 14 h-HRT

biofilm reactor using small cubes of macroporous cellulose as suspended carrier.

It was known that free ammonia (NH_3 , FA) (Abeling and Seyfried, 1992; Surmacz-Górska et al., 1997) and free nitrous acid (HNO_2 , FNA) contributed to the transient nitrite build-up. The FA inhibition thresholds are between 10 and 150 mg N/L for *Nitrosomonas* and 0.1-1 mg N/L for *Nitrobacter* (Abeling and Seyfried, 1992; Anthonisen, 1974; Anthonisen et al., 1976; Surmacz-Górska et al., 1997). FA value can be estimated using Equation 7-4 (Anthonisen et al., 1976):

$$\text{FA (mg N/L)} = 17[\text{NH}_4\text{-N}] * 10^{\text{pH}} / 14(\text{Ka/Ke} + 10^{\text{pH}}) \quad (7-4)$$

Where $[\text{NH}_4\text{-N}]$ is the ammonium concentration, mgN/L

$$\text{Ka/Ke} = \exp [6334/(273+T)]$$

T is temperature, °C

It could be seen from the equation that FA concentration depends on ammonium concentration and pH. At 25 °C, when the influent reject water had an ammonium concentration of 400 mg N/L and pH of 7.8, the FA concentration was 16.8 mg N/L. The high FA concentration obviously caused the inhibition of nitrite oxidizers. Another explanation for the inhibition was reported by Brockmann and Morgenroth (2010). They concluded that the out-competition for oxygen by ammonium oxidizers over nitrite oxidizers caused the phenomenon; and the inhibition by free ammonia (FA) was not required to maintain partial nitrification in biofilm. However, the competition for oxygen was not serious in this study since the bulk oxygen concentration was high (7.2 mg/L). Furthermore, nitrite oxidizers and ammonium oxidizers located in the same layer that was close to the biofilm surface (Fig. 7.2 a-e).

Fig. 7.12 Nitrate reduction in iron ore biofilm under anaerobic conditions.

7.3.5 Biofilm denitrification under anaerobic conditions and aerobic conditions

When nitrogen gas was purged through the solution to create anaerobic conditions, and external carbon source was supplied, nitrate reduction started immediately (Fig. 7.12). The simultaneous reduction in nitrate and TOC concentrations showed the activity of denitrifying bacteria in the biofilm. At the 7.5th h of experiment, when the initial dosed TOC was almost consumed by denitrifying bacteria, additional TOC was added in again to achieve complete nitrate reduction. During the whole process, average and maximum nitrate reduction rates were 21.96 mg N/L h and 30.4 mg N/L h, respectively. The mass ratio of TOC utilized/nitrate reduced was 3, which was significantly higher than the theoretical value of 1.07 in Equation 7-3. The average TOC utilization rate was 67.3 mg/L h during the whole process, while maximum rate of 81.9 mg/L h was achieved in the 1st h of experiment. As the biofilm reactor was switched from aerobic stage to anaerobic stage without any lag period, some of the TOC removal during denitrification process must have been performed by heterotrophic bacteria using the left over oxygen in the solution.

Fig. 7.13 Nitrate reduction in iron ore biofilm under aerobic conditions.

Fig. 7.13 shows the immediate and continuous denitrification process under aerobic conditions. The oxygen concentration in bulk solution was 7.2 mg/L. Similar to the anaerobic operation, additional carbon source was dosed to the solution at the 7th h and 9.5th h of experiment when TOC concentration was very low. During the whole process, the average and maximum nitrate reduction rates were 25.8 mg/L h and 35.9 mg/L h, respectively. In the end, the average TOC utilization rate was 185.2 mg/L h. The mass ratio of TOC utilized/nitrate reduced was 7.2, which was much higher than the theoretical value and the value achieved under anaerobic conditions.

As discussed extensively in the previous sections, denitrifiers usually locate in the deepest layer of biofilm (Fig 7.2a-e). Biesterfeld et al. (2003), Mehlhart (1994) and Revsbech et al. (1989) also reported the development of the bacteria in the deepest layer of trickling biofilm filter. In term of activity, it was suggested that denitrifying capacity was inherent in the biofilm (Biesterfeld et al., 2003). Therefore, with the availability of organic carbon, denitrifying bacteria should be able to perform nitrate reduction under anaerobic conditions.

Even when oxygen is present in bulk solution, denitrification could be achieved

(Biesterfeld et al., 2003). High TOC and oxygen concentrations in solution naturally induce concentration gradient in the biofilm (Nicolella et al., 2000). Furthermore, organic oxidation rate in biofilm is directly proportional to COD loading rate, and high organic loading rate in return reduces the transfer of oxygen into the biofilm layer (Bovendeur et al., 1990).

In this study, the high activity of heterotrophic bacteria located in the outer layer of biofilm consumed most of the organic carbon and oxygen. Denitrifying bacteria, protected from oxygen, consumed the little amount of TOC that could reach the deepest layer of biofilm. The mass ratio of TOC consumed/nitrate reduced therefore was increased significantly. High denitrification rate of 3.09-5.55 g N/m²d with no initial lag period under the oxygen concentration of 2-4 mg/L was also achieved before by Biesterfeld et al. (2003).

7.3.6 The removal of ammonium and organic carbon from reject water by iron ore biofilm under alternating aerobic and anaerobic conditions

The complete removal of ammonium from reject water was achieved by iron ore biofilm under alternating aerobic and anaerobic conditions (Fig. 7.14). In the aerobic stage, due to the inhibition over nitrite oxidizers, ammonium concentration decreased, nitrite concentration increased while nitrate content stayed unchanged. In the initial stage of experiment, organic carbon and ammonium in reject water were removed simultaneously. TOC concentration decreased from 98.4 mg/L to 75.5 mg/L. In the following period, organic carbon was not further degraded. The remaining TOC might not be degradable for aerobic heterotrophic bacteria. The average and maximum ammonium removal rates were 19.8 mg N/L h and 27.06 mg N/L h, respectively.

In the 20th h of experiment, the iron ore biofilm reactor was converted to anaerobic stage by purging with nitrogen gas through air stone at a flow rate of 0.2 L/min. As soon as external organic carbon was added, nitrite/nitrate reduction started without any lag period. The average and maximum nitrite reduction rates were 158.3 mg N/L h and 229.4 mg N/L h, respectively; while average and maximum TOC utilization rates were 295 mg/L h and 359 mg/L h, respectively.

Fig. 7.14 The profiles of ammonium, nitrite, nitrate, and TOC during one cycle under alternating aerobic and anaerobic conditions.

Heterotrophs and nitrifiers grew simultaneously in biofilm. Fig 7.2 shows nitrifying bacteria were covered by a thin layer of heterotrophic bacteria. The high concentration of dissolved oxygen and low concentration of organic carbon in solution were favorable for simultaneous nitrification and organic carbon oxidation. It was reported that nitrification in biofilm would be inhibited by high organic loading rate (Elenter et al., 2007; Nogueira et al., 2002; Okabe et al., 1996), which in return reduced the transfer of oxygen into the biofilm layer. The reduction of nitrification rate by simultaneous COD oxidation can be quantified as $-0.015\text{g/m}^2\text{d}$ $\text{NH}_4\text{-N}$ nitrified per $\text{g/m}^2\text{d}$ COD removed (Bovendeur et al., 1990).

However, Liu et al. (2010) could obtain simultaneous nitrification and carbon oxidation at a COD/N ratio of 5. Their nitrification was only seriously inhibited at the COD/N ratio of 6. In this study, reject water contained high concentration of ammonium and relatively low concentration of organic carbon, therefore, nitrification was not negatively affected by organic carbon oxidation.

The organic carbon undoubtedly enhanced the growth of heterotrophic bacteria in the outer layer of biofilm. The SEM image in this case depicts biofilm was a very compact and dense structure. The surface was dominated by rod bacteria tightly linking cell to cell (Fig. 7.15). The description was identical to the morphology of acetate-fed granules in the study of Tay et al. (2002b).

Fig. 7.15 The SEM morphology of the iron ore biofilm for simultaneous nitrogen and carbon removal under alternating aerobic and anaerobic conditions.

7.3.7 The removal of ammonium and organic carbon from reject water by iron ore biofilm under aerobic conditions

Similar phenomena with previous experiments were observed in the simultaneous

removal of organic carbon and nitrogen from reject water under aerobic conditions by iron ore biofilm (Fig. 7.16). In the initial stage of experiment, ammonium and organic carbon were oxidized simultaneously and TOC concentration reduced from 91.6 to 68.8 mg/L. The remaining TOC could not be degraded by aerobic heterotrophic bacteria in biofilm. Most of ammonium oxidized to nitrite instead of nitrate as the activities of nitrite oxidizers were inhibited.

When acetate was added in as external carbon source at the 20th h of experiment, simultaneous nitrification and nitrite reduction started immediately. The average and maximum ammonium removal rates for the first 20 hours were 17.56 mg N/Lh and 20.29 mg N/Lh, respectively. Average ammonium removal rate was reduced to 10.18 mg N/L h due to high TOC loading rate during the SND process. Average and maximum nitrite reduction rates were 97.8 mg N/L h and 119.8 mg N/L h, respectively; while average and maximum TOC consumption rates were 277 mg/L h and 331 mg/L h, respectively.

Fig. 7.16 The profiles of ammonium, nitrite, TOC, and nitrate during one cycle under aerobic conditions.

As discussed in the section 7.3.5 on denitrification in iron ore biofilm, the high

organic carbon loading rate induced high heterotrophic oxidation in the outer layer hence prevented oxygen from diffusing to deeper layer of biofilm and reduced nitrification rate. Similar results were shown in previous studies (Elenter et al., 2007; Nogueira et al., 2002; Okabe et al., 1996). The consumption of oxygen in outer layer favored simultaneous nitrification and nitrate reduction (SND).

The simultaneous nitrification and nitrate reduction process has attracted attention over the years. It was reported that SND in trickling biofilm filter contributed 80% nitrogen removal (Mehlhart, 1994); and trickling biofilm reactor could perform SND process at an oxygen concentration of 2-4 mg/L (Biesterfeld et al., 2003). Rong et al. (2007) used fixed biofilm hybrid system to treat coke plant wastewater by SND at the suitable DO concentration. Both COD and TN removal efficiency were higher than 94% with the supply of sufficient electron donors for denitrification. In this study, SND could be achieved with an oxygen concentration of 8 mg/L and addition of sufficient organic carbon. The competition between heterotrophs and denitrifiers for organic carbon made it more cost-saving to add the carbon source at the later stage of experiment to save the cost of carbon source. Similar to Tay et al. (2002b) study, the high concentration of acetate added in reactor contributed to the compact and dense structure of biofilm (Fig. 7.17).

7.3.8 The development of aerobic granules from biomass detached from the iron ore biofilm

The biomass detached from iron ore biofilm was accumulated in the medium, and grew in iron ore biofilm reactor at cycle time of 24 h and 62.5% volume exchange ratio. After 20 days, biomass became compact aerobic granules (Fig. 7.18a-c). The SEM pictures reveal the same structure of microbial aggregate as before: compact, strong, dense, and were dominated by tightly linked rod-shaped bacterial cells

embedded in the EPS. The mean size of the granules was 1.45 ± 0.16 mm. The settling velocity was 55.7 ± 5 m/h, and SVI_{3min} value was 16.6 ± 0.54 mL/g.

Fig. 7.17 The SEM morphology of iron ore biofilm for simultaneous removal of organic carbon and nitrogen from reject water under aerobic conditions.

Fig. 7.18 Morphology and structure of microbial granules cultivated from the detached biomass of iron ore biofilm: a) morphology of granules by image analysis (IA); b) over view of microbial granules by SEM; c) compact and dense structure of granules surface.

It is known that aerobic granules can be considered as a special case of biofilm (Liu and Tay, 2002). Therefore, the operating strategy for biofilm development should be also suitable for the development of granules. During the development of biofilm, the high shear force applied on the biofilm surface enhanced the retention of bacteria with strong adhesion and aggregation. Together with the suitable substrates in reject water, the biomass detached from biofilm was easily aggregated into

granules under the operating conditions of iron ore biofilm reactor. Identically, DiIaconi et al (2005) obtained smooth granules from the biomass detached from biofilm under high shear force condition.

7.4 Conclusions

- 1) The biofilm was successfully developed on the surface of suspended fine iron ore particles and static iron ore particles. The rough iron ore surface, moderate organic substrate loading rate, short period of starvation, and high shear force contributed to this development.
- 2) The FISH results pictured the distribution of bacteria in biofilm, in which heterotrophic bacteria occupied the outer layer, ammonium oxidizers and nitrite oxidizers located in the inner layer, and denitrifying bacteria grew in the deepest layer of biofilm.
- 3) Without any addition of alkalinity, iron ore biofilm could achieve 50% nitrification efficiency for reject water containing low TOC, and 90% for reject water containing high TOC. The effluent pH was reduced to 4.05. Iron ore biofilm reactor achieved either comparable or better nitrification efficiency than activated sludge and chalk reactor. Iron ore could not be used to buffer the solution in nitrification process.
- 4) With the addition of alkalinity, iron ore biofilm reactor achieved average and maximum nitrification efficiencies of above 95% and 99.76%, respectively. The nitrogen loading rates was 380-468 mg N/L d, and maximum nitrification capacity was 464 mg N/L d. The average and maximum nitrification rates of 23.6 mg N/L h and 33.04 mg N/L h were achieved with optimum pH control.
- 5) Under anaerobic conditions, iron ore biofilm achieved the average and maximum nitrate reduction rates of 21.96 and 30.4 mg N/L h, respectively. The results were 25.8 and 35.9 mg/L h, respectively for aerobic conditions. TOC

consumption, however, was significantly higher under aerobic conditions.

- 6) Iron ore biofilm reactor achieved simultaneous removal of organic carbon and nitrogen from reject water under alternating aerobic and anaerobic conditions. The average and maximum ammonium removal rates were 19.8 mg N/L h and 27.06 mg N/L h, respectively. The average nitrite reduction rate and maximum nitrite reduction rate were 158.3 mg N/L h and 229.4 mg N/L h, respectively.
- 7) Iron ore biofilm reactor achieved simultaneous removal of organic carbon and nitrogen from reject water under aerobic conditions. The average and maximum ammonium removal rates were 17.56 mg N/L h and 20.29 mg N/L h. The simultaneous nitrification and nitrite reduction (SND) process was achieved at DO concentration of 8 mg/L. The average ammonium removal rate reduced to 10.18 mg N/L h during SND process due to high TOC loading rate. The average and maximum nitrite reduction rates were 97.8 mg N/L h and 119.8 mg N/L h, respectively.
- 8) The biomass detached from iron ore biofilm could be easily developed to smooth, compact, and dense aerobic granules.

Chapter 8

The removal of nitrogen and organic carbon from reject water using microbial granules

8.1 Introduction

Aerobic granulation is a phenomenon of cell-to-cell self-immobilization instead of attaching to solid surface, to form a compact and dense structure (Liu, 2008). Normally, aerobic granulation can be regarded as special case of biofilm (Liu and Tay, 2002).

Compared to conventional activated sludge, the aerobic granules have unique advantages: better settling ability, better mass transfer efficiency and bioactivity (Su and Yu, 2005a); regular, compact, smooth round shape, and strong microbial structure (Ivanov et al., 2006a); can achieve high biomass concentration and high biodegradation rate in the reactor, withstand high organic loading rate (OLR) and shock loading; tolerant to the toxic chemicals and heavy metals in wastewater (Liu and Tay, 2004). Moreover, aerobic granules have good storage stability without the supplies of substrate and oxygen at room temperature and 4 °C; they can remain the structure integrity and metabolic potential; the microbial activity can be recovered back within short period with the supplies of substrate and oxygen (Pijuan et al., 2009; Wang et al., 2008; Zhu and Wilderer, 2003).

Aerobic granules have been cultivated using various substrates including synthetic wastewater, toxic wastewater, real municipal wastewater, and industrial wastewater. Specifically, the contents of these wastewater are: sucrose (Zheng et al., 2006), ethanol (Beun et al., 1999), phenol (Jiang et al., 2006), tert-butyl alcohol (Tay et al.,

2005a; Zhuang et al., 2005), 2, 4-dichlorophenol (Wang et al., 2007a), particulate organic matter-rich wastewater (Schwarzenbeck et al., 2004), domestic sewage (de Kreuk and van Loosdrecht, 2006; Wang et al., 2009), wastewater containing p-nitrophenol (Yi et al., 2006), abattoir wastewater (Cassidy and Belia, 2005), soybean-processing wastewater (Su and Yu, 2005a; 2005b), paper making wastewater (Wang et al., 2006b), brewery wastewater (Wang et al., 2007b), and industrial wastewater (Arrojo et al., 2004; Inizan et al., 2005; Schwarzenbeck et al., 2005). To extend the applicability of aerobic granule, this study focused on the development of aerobic granules in reject water.

Aerobic granulation is a gradual process from seed sludge to dense aggregates, granular sludge, and finally compact mature granule (Tay et al., 2001a). Some important factors recommended to successfully cultivate aerobic granules from activated sludge are: high organic loading rate (Liu et al., 2003a; Moy et al., 2002); high hydrodynamic shear force (Chen et al., 2008; Tay et al., 2001b); relative short settling time (Adav et al., 2009; Beau et al., 1999; Morgenroth et al., 1997; Qin et al., 2004a; 2004b); optimized hydraulic retention time (HRT) (Beau et al., 1999; Tay et al., 2002a; 2005a); Feast-Famine regime or short time starvation (Liu and Tay, 2008); high exchange ratio (Wang et al., 2006c); short discharge time (Wang, 2007), and addition of Fe to assist the formation of granule core (Tsuneda et al., 2004). All of the above factors could also affect the cultivation of granules in reject water.

Normally, the development of aerobic granules containing nitrifying bacteria takes longer time than that of aerobic granules containing heterotrophic bacteria (Tsuneda et al., 2003). So does the cultivation of aerobic granules in reactor fed with toxic wastewater (Tay et al., 2005a; Yi et al., 2006). The aerobic granulation process can be divided into 4 phases: acclimating, shaping, developing, and matured (Liu and

Tay, 2002; Su and Yu, 2005a). Cell surface hydrophobicity promotes strong cell-cell interactions and the formation of a denser structure (Liu et al., 2003c).

It was reported that extracellular polysaccharides (EPS) mediated the cohesion and adhesion of cells and enhanced the maintenance of integrated granular structure (Tay et al. 2001c). The selection pressure theory proposed by Liu et al. (2005) indicated that the minimum settling velocity, which was a function of the settling time, exchange ratio, and discharge time in SBRs, should be controlled at a level higher than the settling velocity of suspended sludge to achieve successful aerobic granulation. The mechanisms of the addition of Fe in feed solution to assist the formation of nitrifying granular core (Tsuneda et al., 2004) and the positive charged particles formed the core of phosphorus accumulation granules (Wu et al., 2010) have also been recommended. It was reported that aerobic granules could be cultivated in wide range of substrate N/COD ratios and the increased N/COD ratio enhanced the activity of nitrifying bacteria (Yang et al., 2003).

However, high content of free ammonia inhibites the formation of aerobic granules (Yang et al., 2004). In terms of reactor design, column SBR with relatively high height to diameter ratio ($H/D = 4-24$) is preferable in most studies (Kong et al., 2009; Qin and Liu, 2004a; 2004b). In this specific case, beaker-scale reactor with low H/D was used to cultivate aerobic granules fed with reject water.

The characteristics of aerobic granules in terms of settling velocity (Qin et al., 2004a, Wang et al., 2007b), size (Cassidy and Belia, 2005; Jang et al., 2003), microbial activity (Ivanov and Tay, 2006; Qin et al., 2004a; Tay et al., 2001b), and storage stability (Pijuan et al., 2009; Tay et al., 2002b; Wang et al., 2008; Zhu and Wilderer, 2003) have been widely studied. All of the above characteristics however have not been examined in the aerobic granules cultivated in reject water.

In general, the substrates can penetrate into aerobic granules deeper than oxygen (Li and Liu, 2005; Li et al., 2008). The dissolved oxygen concentration (DO) affects the distribution of microbial population in aerobic granules, the conversion rate of different components, and overall nutrient removal efficiency (de Kreuk et al., 2007). DO is absent inside the deepest layer of granule when the granule size is larger than 0.5 mm (Jang et al., 2003; Li and Liu, 2005; Su and Yu, 2005b). The growth of heterotrophic and autotrophic bacteria in granule is simultaneous. They compete for oxygen, which may only penetrate into the outer layer of granule. Nitrifying bacteria, as a result, mainly locate in the outer layer of the granule (Ivanov, 2006; Ivanov and Tay, 2006; Jang et al., 2003; Ni et al., 2008; Tay et al., 2002c). In case of high DO, the penetration is deeper thus autotrophic bacteria can accumulate in the inner zone of granule as well (de Kreuk et al., 2007; Xavier et al., 2007). The mushroom-like structure is developed in granules when the substrate contains high N/COD ratio (Liu et al., 2004). Channels and pores contained in the granules penetrate into the depth of 900 μm below the granule surface (Tay et al., 2002c; 2003). The anaerobic bacteria locate at depth of 800 to 900 μm from the granule surface (Ivanov and Tay, 2006; Tay et al., 2002d). Thus, it was expected that aerobic granules cultivated in reject water would achieve simultaneous removal of organic carbon and nitrogen under suitable operating conditions.

Aerobic granules have been widely used for the removal of nitrogen from wastewater (Belmonte et al., 2009; Liu et al., 2008; Shi et al., 2010; Tsuneda et al., 2003); simultaneous removal of organic carbon and nitrogen (Arrojo et al., 2004; Liu et al., 2007b; Wang et al., 2007b); removal of nitrogen and organic carbon under alternative aerobic and anaerobic conditions (Qin and Liu, 2006; Yang et al., 2003; Jang et al., 2003); simultaneous nitrification, denitrification (SND) and COD removal (Beun et al., 2001; Wan and Sperandio, 2009); P removal (Wu et al., 2010); simultaneous removal of phosphorus, nitrogen, and COD (Cassidy and Belia, 2005;

De Kreuk et al., 2005; Wang et al., 2009). It was concluded that optimum DO concentration for simultaneous nitrification and denitrification process (SND) was at 3.2 mg/L (Beun et al., 2001; 2002).

The positive effects of ferrous on anaerobic granules in upflow anaerobic sludge blanket (UASB) reactor are known (Vlyssides et al., 2009). Having mentioned the inhibitory effects of ferrous on nitrate reduction during anaerobic treatment of reject water in previous Chapter 6, it is important to further examine its effect on nitrification by aerobic granules in reject water.

This study was aiming to achieve the simultaneous removal of nitrogen and organic carbon from reject water. The objective of this chapter was to study the cultivation of aerobic granules in reject water, the characteristics of the granules, the effects of ferrous on nitrification by aerobic granules in reject water, and the removal of organic carbon and nitrogen from reject water under different operating conditions.

8.2 Materials and methods

8.2.1 Reject water and aerobic sludge

Reject water and aerobic sludge were collected from local municipal wastewater treatment plants and stored at 4 °C before being used for experiments. Activated sludge was used as seed sludge, the characteristics of activated sludge were determined in this study and the results showed that the sludge had a mixed liquor volatile suspended solids (MLVSS) of 6.60 ± 0.31 g/L, sludge volume index (SVI)₃₀ of 127 ± 2.8 mL/g, and mean size of 87.83 ± 0.62 µm. Reject water, collected from Jurong Water Reclamation Plant (Singapore), was used for the cultivation of aerobic granules from activated sludge. The compositions of this reject water were

measured and the results were as follows (Ave $\pm$ s.d.): soluble total organic carbon (TOC), 543 ± 7 mg/L; total Fe(II), 7.1 ± 0.1 mg/L; dissolved NO_3^- -N, 0.53 ± 0 mg N/L; dissolved NO_2^- -N, 0.05 ± 0.01 mg N/L; dissolved NH_4^+ -N, 277.8 ± 0.56 mg N/L; the mass ratio of N/COD was about 19/100; pH, 7.15 ± 0.01 ; and alkalinity as CaCO_3 , 1050 ± 20 mg/L. For the feeding of the matured granules, reject water was collected monthly from Kranji Water Reclamation Plant (Singapore), and the compositions of reject water were measured immediately after the collection. As this research has been carried on for about 2 years, therefore, there were significant temporal variations of the characteristics of reject water. The results were as follows: pH, 7.85-8.29; dissolved NH_4^+ -N, 645-715 mg N/L; P, 52.6-156.7 mg P/L; dissolved NO_3^- -N, 0.20-1.05 mg N/L; dissolved NO_2^- -N, 0.05-0.07 mg N/L; total Fe(II), 0.68-2.06; TOC, 112.5-223.4 mg/L; Total Suspended Solids (TSS), 240-267 mg/L; Volatile Suspended Solids (VSS), 207-210 mg/L; and alkalinity as CaCO_3 , 2427-2980 mg/L.

8.2.2 Experimental set up

8.2.2.1 Cultivation of aerobic granules in beaker under aerobic conditions

A 5L plastic beaker (16.5 cm diameter, 26 cm height, and 18 cm working volume height, $H/D = 1.1$) was filled in with 2 L reject water and inoculated with 2 L fresh activated sludge to provide an initial biomass concentration of 3300 mg/L. The reactor was maintained at 25 °C. Because of the high ammonium concentration in reject water, it required 16-36 hours aerobic detention time for nitrification (Water Environment Federation, 2006). Therefore, the reactor was operated sequentially in 24 h cycles with 1 min of filling, 23h 38 min of aeration, 20 min of settling, and 1 min of effluent withdrawal. Air stone was used to distribute air evenly within the beaker during aeration. The airflow rate was 3.5 L/min, maintaining a dissolved

oxygen concentration of 6.2-7 mg/L. After the sludge settled, 2L effluent was poured out and replaced with 2L new reject water. The sludge attached to the beaker wall was scraped by 3 mL plastic dropper to detach from the beaker wall and sink down at the bottom of beaker at the end of each cycle. As the reject water contained about 1:1 molar ratio of alkalinity as $\text{CaCO}_3/\text{NH}_4^+\text{-N}$, no additional bicarbonate was required in the first 2 weeks of cultivation. In the third week, additional bicarbonate was added step-up daily to support the growth of nitrifying bacteria. After 2 weeks, an optimum bicarbonate/ammonium nitrogen mass ratio of 7.2 mg/mg N was achieved. The whole cultivation lasted 40 days.

8.2.2.2 Cultivation of the granules under alternating aerobic anaerobic conditions

1L of matured granules achieved in aerobic cultivation stage was transferred to a 2L column SBR (10 cm diameter and 27 cm working volume height) with a tapered based. 1L reject water was added to achieve an initial MLVSS concentration of 1.5 g/L. The reactor was operated sequentially in 24 h cycles with 2 min of filling, 20 h 28 min of aerobic reaction, 3 h of anaerobic reaction, 25 min of aeration to strip the nitrogen gas and remove the remained organic carbon or ammonium, 3 min of sludge settling and 2 min of effluent discharge. In order to maintain additional alkalinity for nitrification, the solution pH was monitored all time.

In aerobic reaction stage, when pH was lower than 7.3, anaerobic reaction was performed for 0.5-0.75 h to achieve 50-80 mg N/L nitrite/nitrate reduction to provide the necessary alkalinity for nitrification. In the final 3 h of anaerobic reaction stage, when pH was higher than 9.0, 5 M HCL solution was added to maintain the solution pH less than 8.5. Effluent was discharged through middle port at a volumetric exchange ratio of 50%. The reactor was refilled at the beginning of

each cycle. In aerobic stage, air was supplied evenly throughout the reactor with an air stone. Air flowrate was 2.0 L/min, maintaining a dissolved oxygen concentration of 6.0–7.0 mg/L.

In anaerobic stage, pure nitrogen gas was purged through air stone at a flow rate of 0.5 L/min to maintain anaerobic conditions as well as to mix well the solution. It was reported that the COD/N ratio required for complete denitrification ranged from 3.5-15 g COD/g N (about 1.32-5.64 g TOC/g N) (Henze, 1991). Sodium acetate ($\text{NaCH}_3\text{COO} \cdot 3\text{H}_2\text{O}$) (GR grade, Merck) was used as external carbon source at the beginning of anaerobic stage. According to the following equations:

the theoretical mass ratios of sodium acetate/ $\text{NO}_3\text{-N}$ and sodium acetate/ $\text{NO}_2\text{-N}$ are 6.07 g/g $\text{NO}_3\text{-N}$ (or 1.07 g TOC/ g $\text{NO}_3\text{-N}$) and 3.65 g/g $\text{NO}_2\text{-N}$ (or 0.644 g TOC/ $\text{NO}_2\text{-N}$), respectively. To achieve complete NO_x reduction as well as to supply carbon source for cell synthesis, the dosage of sodium acetate added in this study was 200% theoretical required value (12.14 g/ g $\text{NO}_3\text{-N}$ or 7.3 g/g $\text{NO}_2\text{-N}$).

8.2.2.3 The nitrification and denitrification characteristics of the aerobic granules cultivated under aerobic conditions

Nitrification batch experiment: The batch experiment was tested for nitrification ability of the aerobic cultivated granules. A mixture of 500 mL reject water and 500 mL DI was filled in 1L serum bottle, aerated at a flow rate of 1 L/min to maintain oxygen concentration of 6.2 mg/L. Bicarbonate was added in when pH dropped below 7.2. MLVSS concentration of the solution was 2.3 g/L.

Denitrification experiment: Denitrification ability of the aerobic cultivated granules was also tested using batch experiment. The cultivated aerobic granules were activated for 2 days at the beginning. Aerobic granules were mixed with 1L reject water in 1L serum bottle to achieve a MLVSS concentration of 0.5 g/L. Potassium nitrate (GR grade, Merck) was added to achieve a nitrate concentration of 250 mg N/L. Sodium acetate (GR grade, Merck) was added as carbon source based on the 200% theoretical value (12.14 g/ g $\text{NO}_3\text{-N}$). Short stirring bar (2.5 cm) was used to avoid colliding with the granules. The solution was purged with nitrogen gas for 10 minutes and sealed to maintain anaerobic conditions; then the bottle was placed on on Magnetic Stirrer REXIM RS-6A (Asone) at 100 rpm/min and room temperature for continuous mixing for 48 hours. When the activation process completed and the granules settled, 500 mL effluent was replaced with fresh reject water. The same amount of potassium nitrate and sodium acetate were added again, same procedure was performed and the experiment lasted for 10 h. In the control, no granule was added.

Nitrite reduction batch experiment: The granules from denitrification experiment were used for nitrite reduction batch test. 1L reject water was filled in 1L serum bottle; sodium nitrite (GR grade, Merck) was added to achieve 300 mg N/L nitrite, sodium acetate was added at a dosage of 7.3 g/g $\text{NO}_2\text{-N}$. The solution was purged with nitrogen gas for 10 minutes and sealed to maintain anaerobic conditions; then the bottle was placed on on Magnetic Stirrer REXIM RS-6A (Asone) at 100 rpm/min and room temperature for continuous mixing for 5.5 h.

The effect of ferrous on the nitrification by aerobic granulation: The batch experiment was to study the effect of ferrous on nitrification by granules. 30 mL of granular biomass was poured into 1L of reject water in each of the experimental and control reactors to achieve MLVSS concentration of 600 mg/L. In experimental

reactors 1 and 2, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ powder was added to achieve 200 mg Fe^{2+} /L and 400 mg Fe^{2+} /L, respectively. Air was distributed at a flow rate of 1 L/min to maintain the oxygen concentration of 6 mg/L. The solution pH was monitored at all time. When pH dropped below 7.3, sodium bicarbonate (GR grade, Merck) was added in to maintain the optimum pH.

The storage stability of aerobic granules cultivated under aerobic conditions:

The cultivated granules were kept at 4 °C in the mixture of 500mL reject water and 500 mL DI water in a closed 1L serum for 11 months. After that, the granules were re-activated in reject water. The reactivation was done in a 24 h cycles with 50% volume exchange ratio, 3 min of settling time, 2 min of filling, and 2 min of effluent discharge. Aeration rate of 1L/min was applied. 3.6 mg/mg N bicarbonate was added as alkalinity everyday at 12th hour of experiment.

8.2.2.4 The organic carbon removal, denitrification, and nitrogen removal by the granules cultivated under alternating aerobic and anaerobic (O/A) conditions

The removal of TOC by granules cultivated under alternating aerobic and anaerobic (O/A) conditions: 2L treated reject water, in which nitrogen and biodegradable TOC had been completely removed, was fill in experimental reactor. Aerobic granules were added to achieve MLVSS concentration of 4.37 g/L. Sodium acetate was used as sole organic carbon source to obtain initial TOC concentration of 677 mg/L. Air was supplied at a flow rate of 1 L/min. pH was regulated to be below 9.0 using 5N HCl.

Denitrification under nitrogen gas and air aeration: Similar treated reject water, in which nitrogen has been completely removed, was filled in experimental and

control reactors (10 cm diameter and 27 cm working volume height). The granules cultivated under alternating aerobic and anaerobic (O/A) conditions were added in each reactor to achieve an MLVSS concentration of about 4.4 g/L. Potassium nitrate was added in both reactors to achieved nitrate concentration of 250 mg N/L. In the control, pure nitrogen gas was distributed through air stone at the bottom of the reactor. Nitrogen flow rate was set at 0.5 L/min to maintain the anaerobic conditions and provide the necessary mixing. In the experimental reactor, 3.5 L/min air flow rate was maintained to achieve an oxygen concentration of 8-9 mg/L. Sodium acetate was added in each reactor at the beginning of the experiment at a dosage of 300% theoretical value (18 g $\text{NaCH}_3\text{COO} \cdot 3\text{H}_2\text{O}/\text{g NO}_3\text{-N}$). pH of both reactors was monitored all the time, and 5M HCL was used to adjusted pH when the solution pH was higher than 9.0. The TOC concentration in reactor was monitored all the time, and when the TOC concentration was lower than 100 mg/L, the additional sodium acetate was added in to ensure the sufficient organic carbon for the complete nitrate reduction.

The simultaneous nitrification and denitrification (SND) under aeration: 2L of reject water was filled in the reactor (10 cm diameter and 27 cm working volume height). Granules were added to achieve MLVSS concentration of 5.85 g/L. Air flow rate was set at 3.5 L/min. Different quantities of sodium acetate were added at 14th hour, 16th hour, and 20th hour of experiment at a dosage of 250%, 450%, and 450% of theoretical value, respectively.

8.2.3 Analytical methods

The analytical methods for TSS, MLVSS, alkalinity, pH, dissolved phosphorus concentration, dissolved $\text{NH}_4^+\text{-N}$ concentration, dissolved $\text{NO}_3^-\text{-N}$ concentration, dissolved $\text{NO}_2^-\text{-N}$ concentration, total ferrous concentration, dissolved TOC

concentration have been presented in Chapter 3. Sludge volume index (SVI) was analyzed by standard methods (APHA, 2005). The oxygen concentration was measured by DO meter (YSI Incorporate, Yellow Springs Ohio, USA). The sizes of activated sludge, ferrous hydroxide, and ferric hydroxide colloidal particles were measured by automated laser particle size analyzer (Malvern MasterSizer Series 2600, Malvern Instruments LTD, Malvern, UK). Granular sludge size was obtained by an image analysis (IA) system (Image-Pro Plus, V4.0, Media Cybernetics) with an Olympus SZX9 microscope. The morphologies of granules and activated sludge were observed by an image analysis (IA) system or scanning electron microscope (SEM, Zeiss EV050, UK). The preparation for SEM samples has been presented in Chapter 7.

Specific oxygen utilization rate: The specific oxygen utilization rate by heterotrophic bacteria $(SOUR)_H$, ammonium oxidizers $(SOUR)_{NH_4}$, and nitrite oxidizers $(SOUR)_{NO_2}$ were measured using standard methods (APHA, 2005). A certain amount of granules were carefully washed with tap water, and put in a clean BOD bottle. When the bottle was fully filled with the pre-aerated nutrient and substrate solution, an oxygen-sensing probe with stirring mechanism was immediately inserted into it. The decrease in DO was recorded at each 30 seconds interval. Specific oxygen utilization rate was calculated according to the DO concentration recorded over time. 200 mg/L TOC as CH_3COONa , 50 mg N/L as NH_4Cl and 50 mg N/L as $NaNO_2$ were used as substrates for the determination of $(SOUR)_H$, $(SOUR)_{NH_4}$, and $(SOUR)_{NO_2}$, respective. The biomass concentration for each test was kept at about 500 mg MLVSS/L.

Detection of nitrifying bacteria in microbial granules by fluorescence *in situ* hybridization: The granules collected from the reactor were prepared for FISH following the procedure reported in Chapter 7. The following 16S rRNA-targeted

oligonucleotide probes were used (Table 8.1): (1) EUB 338 (labeled with the fluorochrome Cy5), a general probe for targeting all eubacteria (Okabe et al., 1999); (2) NSO190 (labeled with the fluorochrome Cy3), a genus-specific probe for the 16S rRNA region of ammonia-oxidizing β - proteobacteria (Okabe et al., 1999); (3) NIT3 (labeled with the fluorochrome Cy3), a genus-specific probe for *Nitrobacter* spp.(Okabe et al., 1999); (4) NSO1225 (labeled with fluorochrome Cy3), a genus-specific probe for ammonia-oxidizing β - proteobacteria (Mobarry et al., 1996).

Table 8.1 16S rRNA-targeted oligonucleotide probes used in experiments

Probe	Specificity	Sequence (5'-3')	Formamide concentration in hybridization buffer (%) (v/v)	NaCl concentration in washing buffer (mM)
EUB 338	Domain Bacteria	GCTGCCTCCC GTAGGAGT	20	0.225
NSO 190	ammonia-oxidizing β - proteobacteria	CGATCCCCTG CTTTTCTCC	55	0.020
NSO 1225	ammonia-oxidizing β - proteobacteria	CGCGATTGTAT TACGTGTGA	35	0.079
NIT 3	<i>Nitrobacter</i> spp.	CCTGTGCTCC ATGCTCCG	40	0.056

8.3 Results and discussion

8.3.1 Formation of the granules cultivated in beaker under aerobic conditions

The seed sludge had a mean floc size of $87.83 \pm 0.62 \mu\text{m}$ and sludge volume index (SVI_{30}) of $127 \pm 2.8 \text{ mL/g}$. The high sludge volume index indicated poor settleability of seed sludge. The morphology by IA (Fig. 8.1 a) and the micrograph by SEM (Fig. 8.1 c) shows that seed sludge was typical flocculent-activated sludge with loose, irregular, and fluffy structure. After 35 days of cultivation in the beaker under aerobic aeration, microbial granules with mean size of $0.62 \pm 0.12 \text{ mm}$ were formed. The granules had sludge volume index ($\text{SVI}_{3\text{min}}$) of $47 \pm 0.1 \text{ mL/g}$ and settling velocity of 45 m/h . It was reported that the typical settling velocity of conventional activated sludge was less than 5 m/h (Giokas et al., 2003). Obviously, the granules with higher settling ability and lower sludge volume index were significantly more advantageous than seed sludge. The morphology of cultivated granules by IA showed that the granules had spherical or elliptical outer shape with compact structure (Fig. 8.1 b). The macrostructure of granules taken by SEM demonstrated the mushroom-like structure in the cultivated granules (Fig. 8.1 d.). It also revealed that the outer layer of granule mainly consisted of cocci-shaped and rod-shaped bacteria (Fig. 8.1 e).

8.3.2 Mechanisms of aerobic granulation in beaker fed with reject water

Aerobic granulation is the result of microbial self-immobilization process and the net result of interactions between the bacterial growth and detachment (Liu et al., 2003b); it is a gradual process and normally takes 1 to 2 weeks or even more depended on the process control (Liu and Tay, 2004). In this study, it took 35 days to cultivate the granules containing nitrifying bacteria in the beaker fed with reject water. The longer cultivation time was due to the slow growth rate of nitrifying bacteria and the toxicity of reject water. Similar results have been reported previously (Tsuneda et al., 2003; Yi et al., 2006).

Fig. 8.1 Morphologies and structures of seed sludge and microbial granules cultivated in beaker under aerobic conditions: a) morphology of seed sludge by image analysis (IA); b) morphology of cultivated microbial granules under aeration in beaker by IA; c) fluffy and loose structure of seed sludge by SEM; d) over view of microbial granules by SEM; e) mushroom-like structure at surface of granules.

Aerobic granules are normally cultivated in column SBR. They can be developed in SBR with various height/diameter (H/D) ratios. The ratio is flexible and does not affect granule formation (Kong et al. 2009; Pan, 2003). In this study, a simple and

easy-to-operate 5L plastic beaker with H/D ratio of 1.1 was used as a SBR reactor for the cultivation.

High hydrodynamic shear force created by aeration is important for the formation of aerobic granules (Tay et al., 2001b). The moderate shear force in beaker cultivation was favorable for granulation. The selection pressure theory proposed by Liu et al. (2005) suggested that settling time, volume exchange ratio, and discharge time were three major selection pressures in the cultivation of granules. Wang et al. (2006c) demonstrated that the high volume exchange ratio (60% and 80%) facilitated rapid and successful aerobic granulation. On the contrary, Yang et al. (2004) reported that free ammonia concentration higher than 23.5 mg N/L inhibited the aerobic granulation. In this study, a high volume exchange ratio of 50% was used to dilute the high ammonium concentration in reject water. A short discharge time during the cultivation was also favorable for granulation.

It was noticed that short settling time helped to wash out free living and floc forming microorganisms and select good settleability bacteria for granule formation (Beau et al., 1999; Morgenroth et al., 1997). Qin et al. (2004b) reported that aerobic granules could only be successfully cultivated and became dominant in reactor operated at settling time of 5 min, while, the mixture of granules and suspended sludge was found at settling time of 20, 15, and 10 min. In this study, the settling time was 20 minute, which seemed to play an insignificant role in the cultivation.

A suitable hydraulic retention time or SBR cycle time is important for the formation of granules. Short hydraulic retention time (HRT) represents high frequency of solids discharge, suppresses the growth of suspended solids, and enhances the granules development. For slow growing nitrifying bacteria, however, too short HRT can cause completely wash out of sludge since sludge loss cannot be

compensated by the normal bacteria growth. Tay et al. (2002a) successfully cultivated nitrifying granules at SBR cycle time of 6 to 12 h. At higher cycle time (24 h), the cultivation failed due to the weak hydraulic selection pressure. Lower cycle time (3 h) on the other hand failed because all sludge was washed out. In this study, granulation was achieved even when the cycle time was maintained at 24 h. Successful cultivation with such high cycle time has also been reported in the case of tert-butyl as substrate by Tay et al. (2005a).

Aerobic aeration consisted of degradation phase and aerobic starvation phase (Liu and Tay, 2004). Aerobic starvation facilitates the microbial adhesion and aggregation, which are strategies of bacteria against starvation (Liu and Tay, 2004; Tay et al., 2001a). In this study, the continuous adhesion of bacteria on the beaker wall was observed during the initial cultivation stage, the starvation should be responsible for such phenomenon. The attached bacteria were retrieved back the reactor at the end of each cycle. Such bacteria acted as the core of the granule, around which more bacteria aggregated. It was known that extracellular polysaccharides (EPS) mediated the cohesion and adhesion of cells, and played crucial role in the maintenance of integrated granular structure (Tay et al., 2001c). Therefore, the initial formed aggregate could be glued by EPS. Subsequently, the granules development was accompanied with the interactions between the bacterial growth, attachment, and detachment; the moderate hydrodynamic shear force stabilized and enhanced the development of compact structure of granules.

The reject water contained 7 mg/L total Fe^{2+} and high concentrations of PO_4^{3-} , and NH_4^+ . The formation of granule core by positive charged particles to stimulate the granulation was reported (Wu et al., 2010). Tsuneda et al. (2004) added that trace Fe (Fe_2O_3 or $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was accumulated in the core of nitrifying granules and could promote the core formation. In this study, the roles of Fe and positive charged

particles in the granulation were not clear.

In short, the moderate shear force, high exchange ratio, short discharge time, reasonable cycle time, and starvation in the beaker cultivation system exerted good selection pressures for the biomass and achieved aerobic granules after 35 days of cultivation.

8.3.3 Characteristics and structure of aerobic cultivated granules

The low SVI_{3min} of 47 ± 0.1 mL/g for the cultivate granules was comparable to the reported values of 49 mL/g by Qin (2005) and 40 mL/g by Liu et al. (2008). A mean size of 0.62 mm for aerobic granules was achieved in this study. The value was larger than that of nitrifying granules cultivated using inorganic carbon (0.24 mm) (Liu et al., 2008), similar to granules cultivated under N/COD mass ratio of 20/100 (0.5 mm) (Yang et al., 2003), and smaller than the mean size of 0.85 mm reported by Shi et al. (2009). The high N/COD ratio in the feed favored the growth of slow growing nitrifying bacteria. Hence the mean size of the cultivated granules was reasonably small. The result agreed with the conclusions from other papers that increased substrate N/COD ratio decreased the mean sizes of mature aerobic granules (Liu et al., 2004; Yang et al., 2005).

The reject water contained volatile fatty acids and high concentration of ammonium. It was known that organic carbon source affected the granules microstructure and species diversity. Specifically, acetate-fed granules have compact bacterial structure and are dominated by rod bacteria (Tay et al., 2002b). Additionally, high N/COD in the substrate favors the formation of more compact and denser structure as well as improves the stability of aerobic granules (Liu et al., 2004; Yang et al., 2005). The granules formed in this work showed relevant characteristics namely compact

structure and rod-shaped bacteria dominated in the outer layer. It was reported that the mushroom-like structure was developed in the granules cultivated with the substrate contained high N/COD weight ratios of 20/100 and 30/100 (Liu et al., 2004); and the same structure was found in biofilm (Costerton et al., 1981). Similarly, the pattern was developed in the granules cultivated in this work. This structure enhanced the movement of slow growing nitrifying bacteria to nutrients (Liu and Tay, 2004; Prescott et al., 1999).

Fig. 8.2 The nitrification by the aerobic cultivated granules: ■ nitrate concentration; ▲ ammonium concentration; ♦ TOC concentration; x nitrite concentration.

8.3.4 The activities of nitrifying bacteria and heterotrophic bacteria in aerobic cultivated granules

Bacterial degradation activity was observed in the granules. Organic carbon was mostly removed (Fig. 8.2). The remaining TOC was either non-biodegradable or could not be utilized by aerobic heterotrophic bacteria. In terms of ammonium removal, nitrification batch experiment showed that nitrifying bacteria achieved

average ammonium removal rate of 9.1 mg N/g VSS h, and maximum ammonium removal rate of 11.41 mg N/g VSS h. The cell yield was 0.11 g VSS/g NH₄-N oxidized. However, most of the ammonium was only partially oxidized to nitrite since final nitrate concentration was only 5.9 mg N/L.

It was likely that the activity of nitrite oxidizer in the cultivated granules was inhibited. The activities of ammonium oxidizers, nitrite oxidizers, and heterotrophic bacteria were quantified by their specific oxygen utilization rates (SOUR). The specific oxygen utilization rates for ammonium oxidizer (SOUR)_{NH₄}, nitrite oxidizer (SOUR)_{NO₂}, and heterotrophic bacteria (SOUR)_H were 43.76 mg O₂/g VSS h, 3.06 mg O₂/g VSS h, and 76.57 mg O₂/g VSS h, respectively. It further showed that the good microbial activities of ammonium oxidizing bacteria and heterotrophic bacteria, and the inhibited activity of nitrite oxidizing bacteria.

The nitrifying bacteria cell yield of 0.11 g VSS/g NH₄-N in this work was lower than the theoretical value of 0.16 g VSS/g NH₄-N according the following equation (Metcalf & Eddy, 2004):

However, it was reported that the experimental values were from 0.04 to 0.13 for *Nitrosomonas* and from 0.02 to 0.07 for *Nitrobacter* (Bitton, 1999). The high ammonium oxidation rate and high (SOUR)_{NH₄} value demonstrated that the activity of ammonium oxidizing bacteria in the cultivated granules was very high. Yang et al. (2003) reported the SOUR values for the ammonium oxidizers, nitrite oxidizers, and heterotrophic bacteria in the granules cultivated under N/COD mass ratio of 20/100 were 32 mg O₂/g VSS h, 29 mg O₂/g VSS h, and 120 mg O₂/g VSS h,

respectively. The (SOUR)_{NH4} value in this work was higher but the (SOUR)_H and (SOUR)_{NO2} values were lower than those in Yang et al. study (2003).

Nitrification consists of two sequential steps: first, the ammonium is oxidized to nitrite by *Nitrosomonas*, and then nitrite is converted to nitrate by *Nitrobacter*. It was found out in previous researches that free ammonia (NH₃, FA) (Abeling and Seyfried, 1992, Surmacz-Górska et al., 1997) and free nitrous acid (HNO₂, FNA) contributed to the transient nitrite build-up. The FA inhibition thresholds are between 10 and 150 mg N/L for *Nitrosomonas*, and 0.1- 1 mg N/L for *Nitrobacter* (Abeling and Seyfried, 1992; Anthonisen, 1974; Anthonisen et al., 1976; Surmacz-Górska et al., 1997). The FA concentration can be estimated using the following equation (Anthonisen et al., 1976):

$$\text{FA (mg N/L)} = 17[\text{NH}_4\text{-N}] * 10^{\text{pH}} / 14(\text{Ka/Ke} + 10^{\text{pH}}) \quad (8-4)$$

where [NH₄-N] is the ammonium concentration, mg N/L; Ka/Ke = exp [6334/(273+T)]; T is temperature, °C

The equation illustrates that the FA concentration depends on ammonium concentration and pH. pH of reject water was about 8 after aeration. During the cultivation, ammonium concentrations in the feed were about 280 mg N/L (before the start of nitrification) and 140 mg N/L (after the start of nitrification). The corresponding FA concentrations were 18.28 mg N/L and 9.14 mg N/L, respectively. As a result, the activity of *Nitrobacter* was inhibited.

Even when ammonium and FA concentrations were reduced, pH always reached 6.5 at the end of each cycle. FNA concentration consequently increased with nitrite accumulation and pH decrease in return. Anthonisen et al. (1976) reported that the

FNA inhibition threshold for *Nitrobacter* was as low as 0.2 mg N/L HNO₂. Therefore, it was likely that the activity of *Nitrobacter* was inhibited due to the existence of FA and FNA during the cultivation. The activity of nitrite oxidizing bacteria in the cultivated granules was very low. The low (SOUR)_{NO₂} and high nitrite concentration during nitrification process further proved such mechanism.

8.3.5 The activities of denitrifying bacteria in aerobic cultivated granules

Denitrification batch experiment showed that denitrifying bacteria contained in the cultivated granules could be easily activated within two days. The activity of denitrifying bacteria was relatively high with average and maximum nitrate reduction rates in experimental reactor of 43.4 and 71.2 mg N/g VSS h, respectively. The average TOC utilization rate in experimental reactor was 84.8 mg/g VSS h (Fig. 8.3). The ratio of average TOC utilized rate/average nitrate reduction rate was 1.95, which was significantly higher than the theoretical required value (1.07 g TOC/g NO₃-N according to Equation 8-1).

In the control, the concentrations of nitrate and TOC stayed unchanged. Hence it can be concluded that the nitrate reduction was performed by denitrifying bacteria in the granule. Nitrite concentration in both experimental and control reactor were very low (Fig. 8.3). The cell yield (Y) of the denitrifying bacteria was 0.58 g VSS/g NO₃-N reduced, similar to the theoretical value of 0.525 according to the Equation 8-5 (Metcalf & Eddy, 1991; Water Environment Federation, 2006):

The value falls into the range of cell yield for denitrifying bacteria which are from

0.4 to 0.9 (Metcalf & Eddy, 1991).

Fig. 8.3 The nitrate reduction in experimental and control reactors: ■ Nitrate concentration in experimental reactor; □ Nitrate concentration in control; ◆ TOC concentration in experimental reactor; ◇ TOC concentration in control; ▲ nitrite concentration in experimental reactor; △ nitrite concentration in control.

The results of nitrite reduction batch experiment showed that the average and maximum nitrite reduction rates were 72.4 and 95.5 mg N/g VSS h, respectively (Fig. 8.4). The average nitrite reduction rate was 1.67 times of the average nitrate reduction rate. Previous researches also emphasized nitrite reduction rate was greater than nitrate reduction rate from 1.5 to 2 times (Turk and Mavinic, 1989; Abeling and Seyfried, 1992).

The cell yield achieved was 0.44 g VSS/g $\text{NO}_2\text{-N}$, lower than what achieved in nitrate reduction process (0.58 g VSS/g $\text{NO}_3\text{-N}$), but similar to the theoretical value of 0.452 g VSS/g $\text{NO}_2\text{-N}$ according to the following equation (U.S.EPA, 1975; Water Environment Federation, 2006):

Fig. 8.4 The nitrite reduction by aerobic cultivated granules: ■ nitrite concentration; ◆ TOC concentration.

The average TOC utilization rate was 47.7 mg/g VSS h (Fig. 8.4). The ratio of average TOC utilization rate/nitrite reduction was 0.66, which was quite close to the theoretical value (0.64 g TOC/g $\text{NO}_2\text{-N}$) and significantly lower than what reported in the nitrate reduction process (1.95 g TOC/g $\text{NO}_3\text{-N}$).

It was known that nitrite reduction required approximately 40% less carbon source, and could obtain higher reduction rate together with lower biomass production, (Turk and Mavinic, 1989; Abeling and Seyfried, 1992). Therefore, it is more cost effective to remove ammonium from reject water by partial nitrification and denitrification. The emission of CO_2 to the atmosphere can also be reduced (Schmidt et al., 2003).

De Kreuk et al. (2007) reported that the DO had substantial effects on the microbial population distribution in aerobic granules. Substrate can penetrate into the whole granules, but the dissolved oxygen will face diffusion limitation with granules larger than 0.5 mm (Li and Liu, 2005; Li et al., 2008; Su and Yu, 2005b). Chiu et al. (2007) concluded that most of oxygen was consumed in the surface layer with a thickness of 125-375 μm . The presence of anaerobic bacteria *Bacteroids* spp. in the deep layer of granules was found by Tay, et al. (2002d). The coexistence of heterotrophic, nitrifying, and denitrifying bacteria in the granules was also demonstrated (Qin, 2005; Qin and Liu, 2006; Yang et al., 2003). In this study, the mean size of the cultivated granules was 0.62 mm. Substrate obviously could penetrate into the whole granule, while oxygen was unlikely to penetrate into the deep anaerobic zone.

8.3.6 The distribution of biomass in the aerobic cultivated granules

The spatial distribution of ammonium oxidizing bacteria and nitrite oxidizing bacteria was analyzed by the fluorescence in situ hybridization (FISH). The FISH image obtained using probe NSO190 and probe EUB 338 shows that the ammonium oxidizing bacteria were mainly found at the outer layer of granule (Fig. 8.5 a). The FISH image obtained using probe NSO1225 and probe EU338 further confirms that ammonium oxidizing bacteria mainly located from the granules surface to the depth of 250 μm (Fig. 8.5 c, d) while heterotrophic bacteria was distributed over the whole granule. At the same time, a cluster of heterotrophic bacteria at the center of granules (Fig. 8.5 c, d) was observed. It was likely that this cluster was denitrifying bacteria. Fig. 8.5c shows a channel in the granule.

Fig. 8.5 Fluorescence *in situ* hybridization of aerobic cultivated granule viewed by Confocal Laser Scanning Microscopy and the distribution of AOB in the granule cultivated under aerobic conditions: (a) Yellow part; ammonium oxidizing bacteria detected by both Cy3 labeled probe NSO 190 and Cy5 labeled probe EU338. Red part: other bacteria detected by Cy5 labeled probe EU338. (b) Green part: nitrite oxidizing bacteria detected by Cy3 labeled probe NIT3. (c) Yellow part: Ammonium oxidizing bacteria detected by both Cy3 labeled probe NSO1225 and Cy5 label probe EU338. Red part: other bacteria detected by Cy5 labeled EU338. (d) Distribution of AOB detected by Cy3 labeled probe NSO1225 and all the bacteria detected by Cy5 labeled probe EU338 in the granule.

The FISH image obtained using Cy3 labeled probe NIT3 reveals that nitrite oxidizing bacteria located close to the surface or in the middle of granules. Nevertheless, the total number of nitrite oxidizing bacteria was significantly less than ammonium oxidizing bacteria (Fig. 8.5 b). The minority of nitrite oxidizing bacteria in the granules was due to the inhibitory effect of FA and FNA concentrations on the nitrite oxidizing bacteria during aerobic cultivation process. As a result, the nitrification process was stopped at the ammonium oxidation step and $(\text{SOUR})_{\text{NO}_2}$ of the granule was very low.

Fig 8.5 a and c also demonstrates that red-stained microorganisms (stained with Cy5 labeled probe EU338) located around the cluster of ammonium oxidizing bacteria (stained with Cy3 labeled probe NSO 190 or probe NSO 1225). It seemed that the growth of ammonium oxidizing bacteria and other bacteria including nitrite oxidizing bacteria, denitrifying bacteria, and heterotrophic bacteria in granule were simultaneous and the different bacteria interacted closely to each other. The organic acids contained in reject water provided the necessary substrates for the cultivation of heterotrophic bacteria, while nitrite/nitrate, the products of ammonium oxidation, together with the organic acids in reject water enhanced the development of denitrifying bacteria in the deep layer of cultivated granules.

It was known that the growth of heterotrophic and autotrophic bacteria in granule was simultaneous (Liu and Tay, 2004). Organic substrate can completely penetrate into the whole granule, whereas oxygen can only penetrate into the outer layer (Li and Liu, 2005; Li et al., 2008; Su and Yu, 2005b). The distribution explains the fact that autotrophic bacteria normally grow on the outer layer of granule. As the dissolved oxygen concentration in the solution was high, the competition between the heterotrophic and nitrifying bacteria for oxygen was reduced; and nitrite oxidizing bacteria could also be developed in the middle layer of granules (Fig. 8.5b). Similar conclusions have been achieved in previous researches. De Kreuk et al. (2007) and Xavier et al. (2007) observed that ammonium-oxidizing *Nitrosomonas* spp. mainly located at a depth of 70 to 100 μm from the granule surface. Tay et al. (2002c) estimated that channels and pores contained in the granules penetrate into the depth of 900 μm below the granule surface. Liu et al. (2007a) mentioned that the main active part of nitrifying granule was at the upper 150 μm layer.

Fig. 8.6 The distribution of bacteria in the granule cultivated in beaker under aerobic conditions.

In this experiment, the coexistence of heterotrophic, nitrifying, and denitrifying bacteria was found in the cultivated granules occurred (Fig. 8.6). Autotrophic ammonium oxidizing bacteria presented in the outer layer of granules, nitrite oxidizers were in the outer and middle layer, anoxic denitrifying bacteria were in the center, and aerobic heterotrophic bacteria located in the middle and outer layer of granules. The heterotrophic and autotrophic biomasses interacted in the aerobic granules. Autotrophic bacteria performed nitrification, heterotrophic denitrifying bacteria were responsible for nitrate reduction and anaerobic organic carbon oxidation, and aerobic heterotrophic biomass performed aerobic organic carbon oxidation. Similar results were reported by Ni et al. (2008).

However, it should be pointed out that nitrogen gas, the product of nitrate/nitrite reduction, produced within the granules negatively affected the granules stability and could even destroy it (Fig. 8.7a). The flocs produced from the collapse of granules were floating on the surface of solution and trapped nitrogen gas inside it (Fig. 8.7b). The mechanism of granule collapse thus was due to the production of nitrogen gas within the granule. The negative effect of biogas, produced from the anaerobic biomass, on the granule long-term stability has been suggested by other researchers (Liu and Tay, 2004). The mean size of the floating floc was $253.9 \pm 1.1 \mu\text{m}$, the floating floc could settle down very fast after the stripping of the nitrogen gas. In order to obtain granules with strong structure for nitrate reduction process,

further cultivation under alternating aerobic and anaerobic conditions (O/A) was conducted.

Fig. 8.7 The partially collapsed granules by nitrogen gas after nitrate reduction (a) and the floating flocs resulted from the destroyed granule (b).

Fig. 8.8 Nitrification by aerobic granulation under different ferrous concentrations:

◆ Control; ■ 200 mg/L Fe; ▲ 400 mg/L Fe.

Fig. 8.9 Specific oxygen utilization rates of ammonium oxidizers and nitrite oxidizers in the granules in different reactors: ■ ammonium oxidizers; □ nitrite oxidizers.

8.3.7 The effects of ferrous on nitrification by granules

The batch experimental results showed a slight deterioration in nitrification process conducted by aerobic granules when ferrous was added (Fig. 8.8). The average ammonium removal rates were 17.3 mg N/g VSS h, 14.7 mg N/g VSS h, and 14.7

mg N/g VSS h in the control, the reactor contained 200 mg/L Fe, and the reactor contained 400 mg/L Fe, respectively. In all 3 reactors, nitrification process stopped at ammonium oxidation step since the activities of nitrite oxidizers in the granules were inhibited. The high specific oxygen utilization rates of ammonium oxidizers $(SOUR)_{NH_4}$ and very low rate for nitrite oxidizers $(SOUR)_{NO_2}$ in each reactor are demonstrated in Fig. 8.9. The $(SOUR)_{NH_4}$ value in the control was higher than that of the 2 experimental reactors, which further revealed the inhibition of ferrous on nitrification by aerobic granules.

Reject water contained about 1:1 molar ratio of alkalinity as $CaCO_3/NH_4-N$. Hence when ferrous ions were added, ferrous ions was quickly hydrolyzed and oxidized under aeration. The produced protons from ferrous hydrolysis reacted with bicarbonate in reject water and made pH reduced from 7.76 to 7.35 and 7.28 in the experimental reactors containing 200 mg/L Fe and 400 mg/L Fe, respectively. Due to the partial consumption of alkalinity by the hydrolysis of ferrous, addition of more bicarbonate was necessary during nitrification process to maintain the solution pH. 2.0 g, 2.8 g, and 2.8 g sodium bicarbonate were added in control, reactors with 200 mg/L Fe, and 400 mg/L Fe, respectively.

It was indicated in Chapter 6 that the space competition between denitrifying bacterial and iron-hydroxide precipitates was responsible for the inhibition of ferrous on nitrate reduction. In this chapter, a similar mechanism was observed, the local adsorption of ferric/ferrous hydroxide on the granule surface partially inhibited the diffusion of substrate into the granules (Fig. 8.10c-f), thus prevented ammonium oxidation. The inhibition of ferrous on nitrification, however, was not significant. The mean size of granules was $620 \pm 120 \mu m$; whereas the mean sizes of ferrous hydroxide and ferric hydroxide particles were $10.8 \pm 0.1 \mu m$ and $14.7 \pm 0.3 \mu m$, respectively. The difference in size caused the different settling velocities

and induced the friction between the particles. It was this friction together with the moderate shear force produced by aeration that a portion of ferric precipitate adsorbed on granule surface was detached (Fig. 8.10 f and g) and the inhibition was eventually lessened.

Fig. 8.10 Morphologies and structures of granule and ferric hydroxide by image technology (IA) and SEM: a) morphology of ferric hydroxide by IA; b) structure of ferric hydroxide by SEM; c) morphology of granule in 200 mg/L Fe reactor by IA; d) structure of granule surface in 200 mg/L Fe reactor by SEM; e) morphology of granule in 400 mg/L Fe reactor by IA; f) structure of granule surface in 400 mg/L Fe reactor by SEM (with ferric hydroxide precipitate adsorption); g) structure of granule surface in 400 mg/L Fe reactor by SEM (without ferric hydroxide precipitate adsorption).

8.3.8 The storage ability of aerobic cultivated granules

After 11 months of storage at 4 °C, the granules changed from yellowish brown to dark black. In term of microbial activity, specific oxygen utilization rates (SOUR) of ammonium oxidizing bacteria, nitrite oxidizing bacteria, and heterotrophic bacteria were reduced about 90% (Fig. 8.11). After 20 days (20 cycles) of reactivation in aerated reject water at dissolved oxygen concentration of 6.2 mg/L, the microbial activity of granules fully recovered (Fig. 8.11).

Fig. 8.11 The microbial activity of granules after 11 months of storage and 20 days of reactivation: ■ ammonium oxidizers; □ nitrite oxidizers; ■ heterotrophic bacteria.

The study of Tay et al. (2002b) about storage ability of granules showed similar result i.e. acetate-fed granules lost about 90% metabolic activity after 4 months of storage at 4 °C. Wang et al. (2008) concerned about the recovery of bacteria in the granules, after storing at 4 °C for 7 months, the activities of nitrifiers and heterotrophs fully recovered within 11 days and 16 days, the recovering periods were comparative to the result of this study. As a conclusion, granules cultivated in beaker under aerobic conditions had excellent storage and recovering capabilities.

8.3.9 Formation of the granules cultivated under alternating aerobic and anaerobic (O/A) conditions

The granules formed in the beaker under aerobic conditions showed weak structure during nitrate reduction process. They were further cultivated in 2 L-reactor under alternating aerobic and anaerobic conditions for an improvement. After 3 weeks of cultivation, the granules became smoother, stronger, and more compact (Fig. 8.12 a, b). Fig. 8.12a illustrates that the cultivated granules had more regular shape, smooth

surface, and increased mean size. Fig. 8.12b emphasizes the strong and more compact structure. Fig. 8.12c demonstrates the dense layer of biomass and the dominance of rod shape bacteria on the granule surface. The cells were tightly combined with each other and embedded in the extracellular polysaccharides (EPS) produced by the bacteria. The characteristics of strengthened granules were as follow: mean size, 1.11 ± 0.16 mm; SVI_{3min} , 21.4 ± 0.8 mL/g; $(SOUR)_{NH_4}$, 32.5 mg O_2 /gVSSh; $(SOUR)_{NO_2}$, 2.36 mg O_2 /gVSSh; and $(SOUR)_H$, 116.0 mg O_2 /gVSSh.

Fig. 8.12 Morphology of the granules by IA and SEM: a) morphology of granules by IA; b) overview of granules by SEM; c) structure of granules surface by SEM.

Compared to the granules cultivated under aerobic conditions, the granules formed under alternating aerobic and anaerobic conditions had increased mean size. The measured mean size of 1.11 ± 0.16 mm was similar to those in previous studies, which were 1-1.3 mm by Jang et al., (2003) and about 0.83-1.72 mm by Qin and Liu (2006). The supply of external carbon source enhanced the growth of denitrifiers, and the fast growing of these bacteria contributed to the increase of granule size. The compact structure and the large size of the granule enhanced settling ability of granule; hence the low SVI_{3min} of 21.4 ± 0.8 mL/g was achieved. This value was only less than half of what achieved under aerobic conditions. Qin and Liu (2006) obtained a similar result in their study. Due to the more populated denitrifiers, the fraction of nitrifiers over denitrifiers and other heterotrophic bacteria reduced. As a result, $(SOUR)_{NH_4}$ reduced.

8.3.10 The removal of nitrogen and organic carbon from reject water by O/A cultivated granules under alternating aerobic and anaerobic conditions

Fig. 8.13 shows the concentration profiles of TOC, nitrate, nitrite, and ammonium during aerobic-anaerobic cycle operated at nitrogen loading rate of 370 mg N/L d. The organic carbon contained in reject water was quickly removed at the first 2 hours, the remaining 70 mg/L TOC could not be utilized by aerobic heterotrophic bacteria in the granules. As ammonium concentration decreased continuously, nitrite instead of nitrate concentration increased. The profile demonstrated an inhibition over nitrite oxidizers.

At the 13th h of experiment, pH dropped to 7.07. In order to maintain the solution pH, sodium acetate was added in to supply carbon source for denitrifying bacteria. Nitrite reduction started under anaerobic stage created by purging with nitrogen gas at the rate of 0.5 L/min. From 13th h to 13.75th h of experiment, denitrifying bacteria removed 80 mg N/L of nitrite and the solution pH was increased to 9.1. Subsequently, aerobic nitrification was performed from 13.75th h to 17th h of experiment, while the solution pH reduced to 7.3. Subsequently, sodium acetate was added and nitrite reduction started under nitrogen purging. During the next half hour, 56 mg N/L nitrite was removed by denitrifiers and the solution pH was increased to 9.3. Aerobic nitrification process took over and finally stopped at 20.5th h of experiment. Ammonium was completely oxidized at 20th h of experiment. The remaining 196 mg N/L nitrite and 1.2 mg N/L nitrate from ammonium oxidation process were completely removed by denitrifying bacteria within the next 2 hours.

Since nitrogen gas produced from nitrite/nitrate reduction was embedded in the granules, some granules were floating on the surface of the solution. The final aeration step could strip all of this gas and made the granules settled down very fast. The nitrate/nitrite reduction process did not deform the granules. It should be pointed out that the first 2 steps of anaerobic nitrite reductions were used to compensate the alkalinity consumed by nitrification process; therefore, no addition

of alkalinity was necessary during aerobic nitrification process. However, in the last anaerobic nitrite reduction stage, the solution pH was increased significantly by alkalinity produced from nitrite/nitrate reduction process according to Equation 8-1 and 8-2. 5M HCl was dropped to the solution as an adjustment.

Fig. 8.13 The concentrations of TOC, nitrite, nitrate, and ammonium during an aerobic-anaerobic cycle: ■ ammonium; ▲ nitrite; ◆ TOC; × nitrate.

During the whole aerobic cycle, the average and maximum ammonium removal rates were 6.3 mg N/g VSS h and 9.62 mg N/g VSS h, respectively. For anaerobic stage, the average and maximum nitrite reduction rates were 32.7 mg N/g VSS h and 56.7 mg N/g VSS h, respectively. The average and maximum external TOC utilization rates were 39.1 mg/g VSS h and 77.5 mg/g VSS h, respectively. And mass ratio of TOC utilized/nitrite reduced was 1.38 g TOC/g NO₂-N reduced, (about 2.1 times of the theoretical value of 0.64 g TOC/g NO₂-N). The relatively high consumption of organic carbon for nitrite reduction could be due to bacterial synthesis and oxidation by heterotrophs at the beginning when there was still some

dissolved oxygen in the solution. The required COD/N ratio for complete denitrification ranges from 3.5 to 15 g COD/g N (1.35-5.76 g TOC/g N) (Henze, 1991). Therefore, the removal of nitrogen and organic carbon from reject water by aerobic granules cultivated under alternating aerobic anaerobic (O/A) conditions is a promising alternative.

Fig. 8.14 The TOC removal by aerobic granules cultivated under alternating aerobic anaerobic conditions: ◆ TOC; ■ pH.

8.3.11 The activities of heterotrophic bacteria and denitrifying bacteria in the O/A cultivated granules

1) The TOC removal capability of the O/A cultivated granules: The fast TOC removal demonstrated high microbial ability of heterotrophic biomass. Fig. 8.14 shows an average TOC removal rate of 22.76 mg/g VSS h. The remaining TOC could not be degraded by aerobic biomass. The maximum TOC removal rate was 49.41 mg/gVSS h together with high (SOUR)_H value of 116.0 mg O₂/gVSS h confirmed again the rapid organic carbon biodegradation rate of heterotrophic biomass in the granules. Therefore, the aerobic granules can be used to treat high strength wastewater. A slight drawback of the process was the significant increase in

pH, which should be adjusted by acid solution (Fig. 8.14).

Table 8.2 The comparison of nitrate reduction under aerobic and anaerobic conditions

Parameter	Nitrate reduction under 0.5 L/min N ₂	Nitrate reduction under 3.5L/min air aeration
DO concentration (mg/L)	0	8-9
MLVSS (mg/L)	4840	4383
Average nitrate reduction rate (mg N/g VSS h)	5.98	8.17
Maximum nitrate reduction rate (mg N/g VSSh)	9.32	14.20
Average TOC utilization rate (mg/g VSS h)	14.48	55.14
Maximum TOC utilization rate (mg/g VSS h)	46.74	81.55
TOC usage/NO ₃ -N reduced (g/g)	2.42	6.74

2) Nitrate reduction by O/A cultivated granules under nitrogen gas and air aeration: Table 8.2 shows the results of this process and Fig. 8.15 illustrates the trends. It can be seen firstly that the complete nitrate reduction was achieved under both conditions, and secondly the high oxygen in bulk solution under air aeration did not negatively affect the nitrate reduction by aerobic granule. Average nitrate reduction rate under aerobic aeration was higher than that under nitrogen gas (Table 8.2). Similarly, TOC utilization rate under aerobic aeration was also higher than that under nitrogen gas (Table 8.2). Due to the high TOC consumption under aerobic condition, the solution pH increased significantly; therefore, it needed to add more

5M HCl solution to maintain the solution pH.

Fig. 8.15 Nitrate reduction and TOC utilization under nitrogen gas and air aeration: ◆ nitrate concentration under nitrogen gas; ▲ TOC concentration under nitrogen gas; ■ nitrate concentration under air aeration; × TOC concentration under air aeration.

As discussed previously, the coexistence of heterotrophic bacteria, nitrifying bacteria, and denitrifying bacteria in granule enabled the anaerobic nitrate reduction. Substrate can penetrate into the granule core, but dissolved oxygen will face diffusion limitation in granules larger than 0.5 mm (Li and Liu, 2005; Li et al., 2008; Su and Yu, 2005b). The mean size of cultivated granules under alternating aerobic anaerobic conditions was 1.11 mm, which was double the size of the ones with limited oxygen diffusion.

Aerobic heterotrophic bacteria located in the outer and middle layer of granules. Even when a lot of organic carbon was added in the solution, these bacteria would oxidize them very fast hence no more oxygen would be left for denitrifying bacteria

in the deeper layer. Organic carbon on the other hand penetrated throughout the whole granule, acting as the food source for both heterotrophic bacteria and denitrifying bacteria. TOC consumption rate in aerobic reactor unsurprisingly was significant higher than that in anaerobic reactor.

Compared to nitrate reduction in the control (anaerobic conditions), the relatively higher average nitrate reduction rate in experimental reactor (aerobic conditions) could be due to the strong shear force created by 3.5 L/min aeration. The hypothesis was proven by other researchers. Tay et al. (2001b) reported that respiration activity of bacteria could be stimulated by the increase of shear force; Liu and Tay (2004) similarly concluded that the increase of shear force increased the oxygen transfer rate at the granules-liquid interface. In the study, the high shear force created by the strong aeration stimulated the substrate transfer rate into the granule; as a result, the higher nitrate reduction rate was achieved.

8.3.12 The simultaneous nitrification and denitrification (SND) for nitrogen removal from reject water by O/A cultivated granules

The results of simultaneous nitrification and denitrification cycle are demonstrated in Fig. 8.16. From beginning to 14th h of experiment, due to the shortage of organic carbon, only ammonium oxidation was performed in the reactor. The increase in nitrite instead of nitrate concentration showed an inhibition over nitrite oxidizers. After that, sodium acetate was added as the carbon source for denitrifying bacteria. Within 45 minutes (14th-14.75th h), 94 mg N/L nitrite, 2.47 mg N/L nitrate, and 10.3 mg N/L ammonium were removed; in return, 308 mg/L TOC was consumed.

Fig. 8.16 The concentration profiles of ammonium, nitrite, nitrate, and TOC during the simultaneous nitrification and denitrification cycle: ◆ TOC concentration; ▲ nitrite concentration; ■ ammonium concentration; × nitrate concentration.

From 14.75th to 16th h, due to the shortage of biodegradable TOC, ammonium oxidation continued followed by an increase in nitrite concentration. To achieve complete nitrogen removal, the operation was repeated with the addition of sodium acetate. Subsequently, 96 mg N/L nitrite, 1 mg N/L nitrate, and 7 mg N/L ammonium were removed, and 294.5 mg/L TOC was consumed within 0.75h (16th -16.75th h).

As expected, from 16.75th h to 20th h, nitrite concentration increased again. Lastly, sodium acetate was added to create favorable conditions for simultaneous ammonium oxidation and nitrite reduction, and to completely remove nitrogen in the solution. During the whole process, the average ammonium removal rate and the maximum ammonium removal rate were 3.10 mg N/g VSS h and 5.22 mg N/g VSS h, respectively; the maximum nitrite removal rate was 30.3 mg N/g VSS h.

After about 10 cycles of simultaneous nitrification and denitrification, the mean size of the granules increased to 1.54 ± 0.20 mm, which was similar to the result of 1.7 mm in the study of Cassidy and Belia (2005). SVI_{3min} further reduced to 15.7 ± 1.2 mL/g, which was better than the result reported by Cassidy and Belia (2005). The SVI_{30} of granule in their work was 22 mL/g. In term of morphology, the granule became more compact and had smoother surface (Fig. 8.17 a; b). Fig. 8.17c shows a very dense layer of biomass on granule surface, which was dominated by rod shape bacteria.

The cells tightly adhered to each other and the “consortium” was embedded in the extracellular polysaccharides (EPS). Such characteristics were demonstrated in the work of Tay et al. (2002b), in which the acetate-fed granules showed very compact bacterial structure and were mainly occupied by tightly-linked-rod bacteria. The specific oxygen utilization rates of ammonium oxidizing bacteria ($(SOUR)_{NH_4}$), nitrite oxidizing bacteria ($(SOUR)_{NO_2}$), and heterotrophic bacteria ($(SOUR)_H$) were: 21.49 mg O_2 /gVSSh, 1.36 mg O_2 /gVSSh, and 139 mg O_2 /gVSSh, respectively.

Fig. 8.17 Morphology and structure of the granules under simultaneous nitrification and denitrification by IA and SEM: a) morphology of granules by IA; b) overview of granules by SEM; c) structure of granules surface by SEM.

As a recommendation for successful nitrite reduction, a sufficient amount of external organic carbon should be added in; as a result, the ratio of N/COD was reduced in the substrate. It was proven before that the activity of nitrifying bacteria would decrease and the activity of heterotrophic bacteria would increase with such ratio decrease (Yang et al., 2003; 2005). Consequently, $(\text{SOUR})_{\text{NH}_4}$ value of the SND granules was lower than that of alternating aerobic nitrification and anaerobic denitrification granules. The $(\text{SOUR})_{\text{H}}$ value was at the same time increased.

Most of the denitrifying bacteria were facultative, but it was known that oxygen negatively affected the denitrification efficiency. The denitrification efficiency by granules could be inhibited at 0.8 mg/L DO concentration (Yang et al., 2003). However, significant denitrification in aerobic granules occurred at DO concentration of 1.8 mg/L was reported by Wan and Sperandio (2009). Though low DO concentration favors the development of denitrifiers in anaerobic zone, it negatively affects nitrifiers as heterotrophs over compete them for oxygen.

Therefore, during the simultaneous nitrification and denitrification, DO concentration should be optimized for maximum nitrification and denitrification efficiencies. In previous works of other researchers, an optimum DO concentration of 3.2 mg/L was recommended for SND by aerobic granules (Beun et al., 2001; 2002; Cassidy and Belia, 2005) and low DO concentration of 1.8 mg/L was reported by De Kreuk et al. (2005).

Mosquera-Corral et al. (2005) however mentioned that the granules stability deteriorated at low oxygen concentration. Specifically, the granules were disintegrated and biomass was washed out at DO concentration of 3.2 mg/L. With those detailed information, it is still incompatible to compare the results of this study to others since their ammonium concentration was quite low (7-40 mg N/L $\text{NH}_4\text{-N}$); and only part of NO_x was removed in aerobic SND process due to the quick consumption of organic carbon by heterotrophic bacteria in aerobic stage (Beun et al., 2001; Cassidy and Belia, 2005; De Kreuk et al., 2005; Mosquera-Corral et al., 2005). Furthermore, in the work of Cassidy and Belia (2005), the remained NO_x was removed in the 2 h anaerobic filling phase.

In this work, SND process was achieved at high DO concentration of 7-8 mg/L with additional organic carbon. It seemed that high DO concentration did not affect SND

process negatively. The rapid oxygen consumption by heterotrophic bacteria in outer layer protected denitrifying bacteria in inner layer from oxygen and made the SND process occurred at high DO concentration. However, it should be noted that the high microbial activity of heterotrophic bacteria in outer layer of granule consumed most of the external organic carbon, total organic carbon required for SND in this case was relative higher than that in nitrogen removal by aerobic granule under alternating aerobic and anaerobic conditions.

Moreover, due to the quick consumption of organic carbon by aerobic heterotrophic during SND process, denitrification might cease and NO_x could be accumulated as indicated in previous works (Beun et al., 2001; Cassidy and Belia, 2005; Mosquera-Corral et al., 2005).

As a recommendation, 3 separate additions of organic carbon in the late stage of each cycle should be conducted to optimize its consumption and SND efficiency. Additionally, the high TOC biodegradation rate as well as nitrite reduction rate made the solution pH increase greatly, a careful control of solution pH, thus was important. Beside nitrogen and organic carbon, other researchers also made efforts to study phosphorus removal by aerobic granules (Cassidy and Belia, 2005; De Kreuk et al., 2005). The aerobic granules in this study however were unable to consume the phosphorus contained in reject water.

8.4 Conclusions

- 1) Aerobic granules were cultivated in reject water under aeration. The granules had good settleability, microbial activities, and storage ability.
- 2) An average ammonium removal rate of 9.1 mg N/g VSS h was achieved by partial nitrification by granules. The granules achieved an average nitrate

- reduction and average nitrite reduction rates of 43.4 and 72.4 mg N/g VSS h, respectively.
- 3) Heterotrophic bacteria, nitrifying bacteria, and denitrifying bacteria co-existed in the granules. FISH results showed that ammonium oxidizing bacteria located in the outer layer of granules. Nitrite oxidizers were minority and located together with heterotrophic bacteria in the outer and middle layer. The deeper layer was occupied by denitrifiers.
 - 4) The granules cultivated under alternating aerobic and anaerobic (O/A) conditions successfully achieved simultaneous nitrogen and organic carbon removal at nitrogen loading rate of 370 mg N/L d. Average ammonium removal rate was 6.3 mg N/g VSS h and average nitrite reduction rate was 32.7 mg N/g VSS h. Batch experiment also showed that the average TOC removal rate of 22.76 mg/g VSS h was achieved by the granule. Under high DO concentration of 8-9 mg/L average nitrate reduction was 8.17 mg N/g VSS h. The structure of granules was not negatively affected by nitrate/nitrite reduction and the proper solution pH control was important.
 - 5) Under high DO concentration of 7-8 mg/L, the granules were also able to perform simultaneous nitrification and denitrification (SND), during which nitrogen was completely removed. By adding organic carbon multiple times at the late stage of each cycle, the maximum ammonium removal rate of 5.22 mg N/g VSS h and maximum nitrite removal rate of 30.3 mg N/g VSS h were achieved with a nitrogen loading rate of 390 mg N/L d. The control of pH was necessary.

Chapter 9

Conclusions and recommendations

9.1 Conclusions

The study explored the removal of phosphorus from reject water of MWWTP by precipitation with ferrous which was produced from iron ore reduction by iron reducing bacteria. The removal of phosphorus and nitrogen by sequential ammonium nitrification and nitrate reduction combined with iron ore reduction-phosphorus precipitation was demonstrated. The study also provided insight into the interactions between iron and nitrate bioreductions, and the interactions between nitrate reduction and ferrous oxidation during anaerobic treatment of reject water. Finally, the aerobic biofilm using iron ore as carrier in reject water, and aerobic granules cultivated in reject water were used for removal of nitrogen from reject water. The conclusions drawn from this study are summarized as follows:

1. The removal of phosphorus from reject water could be performed using anoxic bioreduction of iron ore in either static reactor or rotating reactor. In static cultivation process, the ferrous production rate and phosphorus removal rate were associated with the size of initially loaded iron ore particles. The iron ore bioreduction rate was negatively correlated to the initially loaded iron ore particles. The highest ferrous production rate was 40 mg/L·d in the reactor with iron ore particles of size 0.6 mm. The phosphorus removal efficiencies were about 90% for iron ore particles of 0.6 mm, 2.4 mm, and 7.6 mm. The highest phosphorus removal rate was 11 mg P/ L·d for the iron ore particles with mean size of 7.6 mm. In rotating reactors, the rate of iron ore bioreduction was associated with the production of fine iron ore particles from initially loaded coarse iron ore. The phosphorus removal efficiencies were about 97% for iron ore particle sizes of 0.6

mm, 2.4 mm, and 7.6 mm. The highest phosphorus removal rate of 25 mg P/ L·d and highest ferrous production rate of 44.5 mg/ L·d were obtained in the reactor with iron ore particles of size 7.6 mm. The total organic carbon removal rate in both cultivation processes was not associated with the size of initially loaded iron ore particles and was mainly due to the degradation of other anaerobic bacteria. The removal of phosphorus from reject water using anoxic bioreduction of iron ore can outcome the conventional chemical precipitation of phosphate with iron salts because of the high removal efficiency and cheaper reagent (iron ore) used.

2. The removal of nitrogen and phosphorus from reject water of municipal wastewater treatment plant could be performed using sequential biooxidation of NH_4^+ and bioreduction of NO_3^- with precipitation of phosphate by Fe^{2+} ions produced due to bioreduction of Fe^{3+} in iron ore. However, it showed that there was a competition between denitrifying and iron-reducing bacteria for electron donors during anaerobic treatment of reject water. Addition of acetate as electron donor increased both bioreduction rates of Fe^{3+} and NO_3^- , but the presence of nitrate or acetate diminished the rate and efficiency of phosphate precipitation by ferrous ions. The stages of denitrification and ferric bioreduction/phosphate precipitation must be performed sequentially to avoid the negative interactions between the nitrogen and phosphorus removals during anaerobic treatment of reject water in MWWTP and the addition of electron donor in denitrification stage is necessary to achieve maximum denitrification rate and efficiency.

3. There were interactions between nitrate bioreduction, iron ore bioreduction, and organic carbon biooxidation in anaerobic treatment of reject water from MWWTPs. Nitrate bioreduction inhibited iron ore bioreduction because denitrifying bacteria over competed iron reducing bacteria for organic carbon source. At the same time, Fe(II) concentration was reduced during nitrate reduction process due to the reaction of Fe(II) with the components of reject water and the inhibition of the

activities of iron reducing bacteria by nitrate. If organic electron donor was not sufficient for complete nitrate reduction, nitrite produced from incomplete nitrate reduction significantly enhanced the oxidation of Fe(II) and this enhancement obviously inhibited the iron ore reduction process. However, iron reducing bacteria could be recovered after complete nitrate reduction by utilizing the remaining organic carbon and iron ore reduction resumed, and the replacement of fresh reject water containing organic carbon effectively increased the iron ore reduction rate. Organic electron donors affected the rate and extent of nitrate reduction as well as the recovery of iron reducing bacteria after complete nitrate reduction.

4. There were complicated interactions between ferrous oxidation and nitrate bioreduction during anaerobic treatment of reject water from MWWTPs. When reject water contained sufficient amount of electron donors for complete nitrate reduction, the significant change of medium pH by ferrous addition and the partial coverage of the surface of denitrifying bacteria by iron-hydroxides particles caused the inhibition of nitrate reduction by ferrous. On the contrary, when reject water contained not sufficient amount of electron donors for complete nitrate reduction, addition of ferrous did not significantly inhibit nitrate reduction, nitrate reduction was limited by the shortage of electron donors, and nitrate-dependent ferrous oxidation was not involved in nitrate reduction process in reject water treatment under anaerobic conditions. Therefore, the nitrate reduction stage should be performed prior to ferric reduction stage to avoid the negative effects of ferrous, which was produced in iron ore reduction, on nitrate reduction; and it was no need to recycle produced Fe(II) after iron reduction back the anoxic nitrate reduction tank.

5. The removal of nitrogen from reject water could be performed using iron ore as biofilm carrier. Biofilm was developed on the rough iron ore surface in reject water

under the moderate organic loading rate, short period of starvation, and high shear force. FISH results showed that heterotrophic bacteria occupied the outer layer of biofilm, while ammonium oxidizers and nitrite oxidizers located in the inner layer, and denitrifying bacteria grew in the deepest layer of biofilm. The nitrifying bacteria showed high activity and could achieve 50% nitrification efficiency for reject water containing low TOC, and 90% for reject water containing high TOC without any addition of alkalinity. However, iron ore could not be used to buffer the solution in nitrification process and the effluent pH was reduced to 4.05. The addition of alkalinity made iron ore biofilm reactor achieve average and maximum partial nitrification efficiencies of above 95% and 99.76 %, respectively, at nitrogen loading rate of 380-468 mg N/L d, and maximum nitrification capacity was 464 mg N/L d. The average and maximum nitrification rates of 23.6 mg N/L h and 33.04 mg N/L h were achieved with optimum pH control.

The denitrifying bacteria in deepest layer of biofilm achieved average and maximum nitrate reduction rates of 21.96 and 30.4 mg N/L h under anaerobic conditions, respectively. And the results were 25.8 and 35.9 mg N/L h, respectively, for aerobic conditions with significantly higher TOC consumption. Iron ore biofilm reactor achieved simultaneous removal of organic carbon and nitrogen from reject water under alternating aerobic and anaerobic conditions. The average and maximum ammonium removal rates were 19.8 mg N/L h and 27.06 mg N/L h, respectively. The average nitrite reduction rate and maximum nitrite reduction rate were 158.3 mg N/L h and 229.4 mg N/L h, respectively.

Iron ore biofilm reactor achieved simultaneous removal of organic carbon and nitrogen from reject water under aerobic conditions. The average and maximum ammonium removal rates were 17.56 mg N/L h and 20.29 mg N/L h. The simultaneous nitrification and nitrite reduction (SND) process was achieved at DO

concentration of 8 mg/L. The average ammonium reduction rate of 10.18 mg N/L h was low due to high TOC loading rate during SND process. The average and maximum nitrite reduction rates were 97.8 mg N/L h and 119.8 mg N/L h, respectively. Moreover, the biomass detached from iron ore biofilm could be easily developed into smooth, compact, and dense aerobic granules.

6. The removal of nitrogen and organic carbon from reject water could be performed using aerobic granulation of microbial biomass. Aerobic granules were cultivated in reject water under aerobic aeration. The granules had good settleability, microbial ability, and storage ability. Nitrification by the aerobic cultivated granules achieved the average ammonium removal rate of 9.1 mg N/g VSS h. FISH results showed that heterotrophic bacteria, nitrifying bacteria, and denitrifying bacteria co-existed in the granules; ammonium oxidizers located in the outer layer of granules; nitrite oxidizers were minority and located together with heterotrophic bacteria in the outer and middle layer. The deeper layer was occupied by denitrifiers. The activities of nitrite oxidizers were inhibited due to high concentrations of FA and FNA and nitrification mainly achieved by partial nitrification.

The denitrifying bacteria in the cultivated granule achieved average nitrate reduction and average nitrite reduction rates of 43.4 mg N/g VSS h and 72.4 mg N/g VSS h, respectively, but the granules structure was negatively affected by the nitrate/nitrite reduction process. The nitrification process by aerobic granule was negatively affected by ferrous ions.

The granules were successfully cultivated under alternating aerobic and anaerobic (A/O) conditions. The simultaneous removal of nitrogen and organic carbon under alternating aerobic and anaerobic conditions was achieved at nitrogen loading rate of 370 mg N/L d. Average ammonium removal rate was 6.3 mg N/g VSS h and

average nitrite reduction rate was 32.7 mg N/g VSS h. Batch experiment showed that the average TOC removal rate of 22.76 mg/g VSS h was achieved by the granules. Average nitrate reduction rate of 8.17 mg N/g VSS h was achieved under high DO concentration of 8-9 mg/L; the structure of granules was not affected by nitrate/nitrite reduction.

Moreover, the simultaneous nitrification and denitrification (SND) process for complete nitrogen removal was achieved by the granules cultivated under alternating aerobic and anaerobic conditions at high DO concentration of 7-8 mg/L. By adding multiple times of organic carbon at the late stage of each cycle, the maximum ammonium removal rate of 5.22 mg N/g VSS h and maximum nitrite removal rate of 30.3 mg N/g VSS h were achieved with a nitrogen loading rate of 390 mg N/L d. The structure of granules was not affected by nitrate reduction and the proper solution pH control during nitrate/nitrite reduction process was important.

9.2 Recommendations

The ferrous produced from iron ore reduction faced the competition between the hydrolysis and the precipitation with phosphorus. The interaction between ferrous hydrolysis and precipitation with phosphorus could be suggested to study in depth in future. Also, the microbiology of IRB involved in iron ore reduction could be suggested to study in future.

The nitrate-dependent ferrous oxidation has been reported for nitrate reduction, however, in the study, such mechanism was not involved in nitrate reduction process. The proper selection of specific organisms for nitrate dependent ferrous oxidation and the application of such organism for nitrate reduction in anaerobic

treatment of reject water could be suggested to study in future.

The aerobic biofilm and aerobic granules developed in reject water could achieve simultaneous organic removal and complete nitrogen removal under alternating aerobic and anaerobic conditions and under aerobic aeration. The distribution of heterotrophic bacteria, nitrifying bacteria, and denitrifying bacteria in biofilm/granules under different operating conditions should be qualified and quantified in future study. And the modeling of the distribution of substrate and oxygen in the biofilm/granules with depth under different operating conditions is also suggested to study in future.

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