สมรรถนะและสภาพแวดล้อมภายในเซลล์ตรึงสำหรับกระบวนการไนตริฟิเคชันแบบไม่สมบูรณ์
ภายใต้การเปลี่ยนแปลงของสัดส่วนเซลล์ต่อเจลและความเข้มข้นอาหาร

นางสาวภัทรพร คุนาพงษ์กิติ

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Performance and internal environment of partial nitrification-
entrapped cells under changes in cell-to-matrix ratio and substrate concentrations

Miss Pattaraporn Kunapongkiti

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Faculty of Engineering
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Thesis Title   Performance and internal environment of partial nitrification-entrapped cells under changes in cell-to-matrix ratio and substrate concentrations

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ภัทรพร คุนาพงษ์กิติ : สมรรถนะและสภาพแวดล้อมภายในเซลล์ตรึงสำหรับกระบวนการไนตริฟิเค
ชันแบบไม่สมบูรณ์ภายใต้การเปลี่ยนแปลงของสัดส่วนเซลล์ต่อเจลและความเข้มข้นอาหาร
(Performance and internal environment of partial nitrification-entrapped cells under changes
in cell-to-matrix ratio and substrate concentrations) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ตะวัน
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งานวิจัยนี้ใช้พอลีไวนิลคลอไรด์เป็นส่วนผสมในการทำเซลล์ตรึง เพื่อใช้ในการสนับสนุนการเกิด
กระบวนการไนตริฟิเคชันแบบไม่สมบูรณ์ในถังปฏิกรณ์จุลินทรีย์แบบเซลล์ตรึงโดยการใช้กลยุทธ์การจำกัด
ออกซิเจน การทดลองแบ่งออกเป็น 3 ส่วน ได้แก่ 1) ศึกษากลุ่มประชากรไนไตรต์ออกซิไดซ์ซิงแบคทีเรีย (NOB)
ในถังปฏิกรณ์ที่เดินระบบด้วยความเข้มข้นออกซิเจนละลายที่แตกต่างกัน 2) ศึกษาผลของสัดส่วนเซลล์ต่อเจล
(ร้อยละ 1 และ 4) ต่อสมรรถนะการเกิดไนตริฟิเคชันแบบไม่สมบูรณ์ของถังปฏิกรณ์ และศึกษาสภาวะภายใน
เซลล์ตรึงรวมถึงกลุ่มประชากรจุลินทรีย์และตำแหน่งของจุลินทรีย์ที่เกี่ยวข้องภายในเซลล์ตรึงในการเดินระบบ
ระยะยาว และ 3) ศึกษาผลของอัตราภาระแอมโมเนียและสารอินทรีย์ต่อประสิทธิภาพและเส้นทางการกำจัด
ไนโตรเจน รวมถึงการผลิตไนตรัสออกไซด์ในถังปฏิกรณ์จุลินทรีย์แบบเซลล์ตรึง การทดลองแรกพบว่าระดับของ
กระบวนการไนตริฟิเคชันแบบไม่สมบูรณ์เกิดขึ้นมากกว่าร้อยละ 60 ในถังปฏิกรณ์ที่เดินระบบด้วยออกซิเจน
ละลายเข้มข้น 2 และ 3 มก./ล. โดยพบไนไตรท์ออกซิไดซ์ซิงแบคทีเรียที่มีความชอบออกซิเจนแตกต่างกัน
(*Nitrobacter* and *Nitrospira* lineage I และ *Nitrospira* lineage II) อยู่ร่วมกันในเซลล์ตรึงที่ได้จากถังปฏิกรณ์
ทั้งสองถัง ผลการทดลองแสดงให้เห็นว่าไนไตรท์ออกซิไดซ์ซิงแบคทีเรียดำเนินกิจกรรมการออกซิไดซ์ไนไตรท์
อยู่บ้าง โดยไม่ส่งผลกระทบต่อระดับไนตริฟิเคชันแบบไม่สมบูรณ์โดยรวม เนื่องจากกิจกรรมการออกซิไดซ์ใน
ไตรท์ส่วนใหญ่ถูกจำกัด การทดลองที่สองเดินระบบด้วยถังปฏิกรณ์แบบเซลล์ตรึงที่มีสัดส่วนเซลล์ต่อเจลร้อยละ
1 และ 4 ผลการทดลองพบว่าการ ไล่ระดับของออกซิเจนในเม็ดเจลมีผลต่อระยะเวลาการเข้าสู่กระบวนการไนตริ
ฟิเคชันแบบไม่สมบูรณ์ แต่หลังจากที่ระบบเข้าสู่สภาวะคงตัว ประสิทธิภาพการเกิดไนตริฟิเคชันแบบไม่สมบูรณ์
ในถังปฏิกรณ์ทั้งสองถังเหมือนกัน และพบสภาวะที่ออกซิเจนจำกัด (ความเข้มข้นออกซิเจน 0.5-1.5 มก./ล.) ที่
ตำแหน่ง 10-230 ไมโครเมตรจากผิวของเซลล์ตรึง ซึ่งคาดว่าน่าจะมีส่วนสนับสนุนการยับยั้งการทำงานของไน
ไตรท์ออกซิไดซ์ซิงแบคทีเรีย การทดลองสุดท้ายพบว่ากระบวนการ ไนตริฟิเคชันแบบไม่สมบูรณ์เกิดขึ้นเฉพาะถัง
ปฏิกรณ์ที่ไม่เติมสารอินทรีย์และเดินระบบด้วยอัตราภาระแอมโมเนียมากกว่าเท่ากับ 0.3 กก./ลบ.ม./ว. ขณะที่
กระบวนการเกิดไนตริฟิเคชันแบบสมบูรณ์เกิดขึ้นในถังปฏิกรณ์ที่เดินระบบด้วยอัตราภาระแอมโมเนียน้อยกว่า
เท่ากับ 0.2 กก./ลบ.ม./ว. กระบวนการการเกิดขึ้นพร้อมกันของไนตริฟิเคชันและดีไนตริฟิเคชันเกิดขึ้นเมื่อมีการ
เติมสารอินทรีย์ลงไปในระบบ นอกจากนี้ยังพบมีการผลิตไนตรัสออกไซด์ร้อยละ 0.1-0.24 เมื่อเทียบกับอัตรา
ภาระแอมโมเนียที่เข้าสู่ระบบ

ภาควิชา วิศวกรรมสิ่งแวดล้อม	ลายมือชื่อนิสิต	
สาขาวิชา วิศวกรรมสิ่งแวดล้อม	ลายมือชื่อ อ.ที่ปรึกษาหลัก	
ปีการศึกษา 2560	ลายมือชื่อ อ.ที่ปรึกษาร่วม	

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KEYWORDS: ENTRAPPED-CELL-BASED REACTOR / PARTIAL NITRIFICATON / OXYGEN-LIMITING CONDITION / NITRITE-OXIDIZING BACTERIA / OXYGEN CONCENTRATION GRADIENT / SIMULTANEOUS NITRIFICATION AND DENITRIFICATION

PATTARAPORN KUNAPONGKITI: Performance and internal environment of partial nitrification-entrapped cells under changes in cell-to-matrix ratio and substrate concentrations. ADVISOR: ASSOC. PROF. TAWAN LIMPIYAKORN, Ph.D., CO-ADVISOR: ASST. PROF. CHAIWAT RONGSAYAMANONT, Ph.D., pp.

In this study, partial nitrification performance was investigated in entrapped cell-based reactors prepared using phosphorylated polyvinyl alcohol gel (PPVA) and operated using oxygen limiting strategy. The study was divided into three main parts: 1) to investigate the community of nitrite-oxidizing bacteria (NOB) in the reactors operated at different bulk dissolved oxygen (DO) concentrations, 2) to investigate the effect of cell-to-matrix ratio (1% and 4%) on partial nitrification performance of the entrapped-cell-based reactors and to observe internal environment, microbial community, and microbial localization within the gel matrix during long-term operation of the reactors, and 3) to study effect of ammonia and organic loading rates on performance and pathways of nitrogen removal as well as nitrous oxide (N_2O) production in the entrapped-cell-based reactors. In the first part, a comparable level of partial nitrification (> 60%) was achieved in entrapped-cell-based reactors operated at bulk DO concentration of 2 and 3 mg l^{-1}. NOB having distinct oxygen affinity, including *Nitrobacter* and *Nitrospira* lineage I and *Nitrospira* lineage II, coexisted in both reactors. The results indicated that the entrapped-cell system allow a variety of NOB to exist and perform some activity, although the majority of nitrite-oxidizing activity was inhibited in the system. In the second part, two entrapped-cell-based partial nitrification reactors were operated using gel beads containing different cell-to-matrix ratios of 1% and 4%. Results showed that oxygen concentration gradient determined the periods of which partial nitrification could be achieved. However, after partial nitrification was achieved, both reactors showed similar degree of nitrite accumulation during long-term operation. The oxygen-limiting zone (DO = 0.5-1.5 mg l^{-1}), where nitrite-oxidizing activity was suggested to be suppressed, occurred at 10-230 µm from the surface of gel matrix. In the last part, varying ammonia and organic loading rates showed that partial nitrification could only be maintained in the reactors without organic feeding and with ammonia loading rate of ≥0.3 kg m^{-3} d^{-1}. While, complete nitrification occurred in the reactors without organic feeding and with ammonia loading rate of ≤0.2 kg m^{-3} d^{-1}. Simultaneous nitrification and denitrification was found in most reactors fed with organic. Moreover, N_2O was produced between 0.1 and 0.24% of the ammonia loading rate in the studied reactors.

Department:	Environmental Engineering	Student's Signature	
Field of Study:	Environmental Engineering	Advisor's Signature	
Academic Year:	2017	Co-Advisor's Signature	

ACKNOWLEDGEMENTS

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Partial nitrification is a vital part of shortcut biological nitrogen removal process that excluded nitrite oxidation. This process reduces 25% of oxygen requirement caused by nitrite oxidation (Turk and Mavinic (1989)). Maintaining 'low dissolved oxygen (DO)', 'high pH' and 'high temperature' conditions are three main operational strategies that promote ammonia-oxidizing bacteria (AOB) activity while suppressing nitrite-oxidizing bacteria (NOB) activity which can lead to an achievement of partial nitrification. Among those 3 strategies, the 'low DO' (at which DO concentration is reduced to 0.5-3.0 mg l^{-1} and 0.4-6.0 mg l^{-1} for suspended and attached growth system) is the most commonly used strategy.

An underlying principle of partial nitrification is to promote the activity of AOB while suppressing the NOB activity. Oxygen limitation is one of the most effective strategies promoting partial nitrification. This strategy is based on differential oxygen affinity of AOB and NOB. At low oxygen environment, AOB obviously show higher competitiveness for oxygen over NOB due to its lower oxygen half-saturation coefficient (K_o) than that of NOB (Laanbroek and Gerards, 1993; Laanbroek et al., 1994; Wiesmann, 1994; Ciudad et al., 2006; Blackburne et al., 2008; Rongsayamanont et al., 2010). The K_O values of AOB are between 0.3 and 0.74 mg l^{-1} and the K_O values of NOB are between 0.4 and 1.9 mg l^{-1} (Hanaki et al., 1990; Laanbroek and Gerards, 1993; Laanbroek et al., 1994; Ciudad et al., 2006; Blackburne et al., 2008; Rongsayamanont et al., 2010). Based on the principle, partial nitrification with attached growth system can be maintained with higher oxygen concentration in bulk solution than suspended cell system because the attacthed growth system provides a barrier to interup oxygen diffusion and promote a creation of oxygen concentration gradient. A number of research in attached growth system, such as in biofilm and aerobic granule, indicated that the formation of oxygen concentration gradient with in the attached growth matrices relied on aerobic heterotroph and autotroph (AOB and NOB) activities that distributed within the matrices (Rathnayake et al., 2013; Song et al., 2013;

Rathnayake et al., 2015; Wang et al., 2017a). Entrapped-cell-bed reactor is one of attached growth system that succeeded to maintain partial nitrification in bulk DO concentrations of 2-3 mg l^{-1} (Rongsayamanont et al., 2014). Heterotrophs and autotrophs (AOB and NOB) in the gel beads normally placed themselves from the surface to gel core according to their oxygen affinity, like other immobilized cell systems. The oxygen concentration gradient results in the creation of oxygen limiting zone inside the gel beads that is a cause to promote AOB activity and suppress NOB activity. However, thus far, the oxygen concentration gradient along the depth of gel beads of entrapped-cell system has not yet been explored and should be studied.

Some studies reported that NOB can recovery their activity in low oxygen concentration environment during long-term operation of partial nitrification reactors (Huang et al., 2010b; Liu and Wang, 2013). If NOB can successfully adapt their community and activity to low DO environment, partial nitrification could be threatened seriously. Two genera of NOB, *Nitrobacter* and *Nitrospira*, are commonly found in wastewater treatment systems and the former has higher K_O than the latter (0.43-5.31 mg l^{-1} for *Nitrobacter* and 0.33-0.54 mg l^{-1} for *Nitrospira*) (Laanbroek et al., 1994; Blackburne et al., 2007; Blackburne et al., 2008; Park et al., 2017). Up to now, failure of partial nitrification at low oxygen environment in some systems has been reported but not been clearly clarified. For entrapped-cell based reactors, although partial nitrification can successfully be promoted by operating the reactors at bulk DO concentrations of 2-3 mg l^{-1}, some concentrations of nitrate were still detected (Rongsayamanont et al., 2014). This may cause by an adaptation of NOB to survive in oxygen limiting environment in the gel beads. Therefore, clarifying NOB community in the gel beads of entrapped-cell based partial nitrification reactors is of interest.

Recently, nitrogen removal has been promoted in a single reactor by the promotion of combined nitrogen transformation pathways. This includes simultaneous nitrification and denitrification (SND), partial nitification-denitrification, partial nitrification-ANaerobic AMMonia OXidation (ANAMMOX). Previous studies demonstrated that ammonia and organic loading rates as well as DO concentration impacted the performance and pathways of nitrogen removal (Bueno et al., 2017; Ma et al., 2017). This information on entrapped-cell-based reactors is limited in literatures as the studies performed previously were only focused on partial nitrification with high

ammonia loading rate and no organic (Rongsayamanont et al., 2014). Effect of threatening factors including overgrowth and oxygen-overconsumption of heterotrophic bacteria on partial nitrification is worth studying.

Moreover, the production of greenhouse gas like nitrous oxide (N_2O), which is produced during nitrification, nitrifier denitrification (Kampschreur et al., 2008a; Ahn et al., 2011; Desloover et al., 2011) and heterotrophic denitrification process (Desloover et al., 2011) is another concern. Some evidences showed that N_2O was more produced by AOB via nitrifier denitrification as compared to that of nitrification (Tallec et al., 2006; Desloover et al., 2011; Okabe et al., 2011b; Aboobakar et al., 2013). *Nitrosomonas europaea* can switch its role from nitrifying to denitrifying in oxygen-limiting environment (Kampschreur et al., 2008a; Peng et al., 2014) and N_2O is produced during that denitrification (Desloover et al., 2011; Peng et al., 2014). Therefore, N_2O is likely produced via either nitrification or denitrification in oxygen-limiting environment of entrapped cell and its production is worth being investigated.

1.2 Research hypotheses

1) NOB are present in gel matrix of entrapped-cell-based partial nitrification reactors, although partial nitrification is maintained in long run.
2) The extent of partial nitrification in entrapped-cell-based reactors is determined by oxygen concentration gradient along the depth of gel beads.
3) Volumetric ammonia and organic loading rates influence performance and pathway of nitrogen removal in entrapped-cell-based reactors.

1.3 Research objectives

1) To investigate communities of NOB in gel beads of entrapped-cell based partial nitrification reactors
2) To investigate the effect of cell-to-matrix ratio (1% and 4%) on partial nitrification performance of the entrapped-cell-based reactors and to observe internal environment, microbial community, and microbial localization within the gel matrix during long-term operation of the reactors
3) To investigate effect of volumetric ammonia and organic loading rates on performance and pathways of nitrogen removal

1.4   Aims of the study

The experimental framework is shown in Figure 1.1 and the structure of thesis is organized as the following:

1.4.1   Chapter 1

Chapter 1 presents an introduction, research hypotheses and objectives.

1.4.2   Chapter 2

Chapter 2 presents theoretical background and previous research finding as particularly related to current topics. The chapter consists of several topics including conventional and novel nitrogen removal processes in wastewater treatment system, microorganisms involved in ammonia and nitrite oxidation and strategies for achieving partial nitrification. Adaptation on low oxygen environment of autotrophic nitrifying microorganisms, partial nitrification by cell immobilization, and oxygen concentration gradient and spatial distribution of microorganisms in gel matrix were also described and discussed. Evidences of adapting to low DO environment of NOB and their implication on partial nitrification were shown on this chapter as well, and problems encountering operation of partial nitrification reactors using cell immobilization were reported.

1.4.3   Chapter 3

Chapter 3 presents partial nitrification efficiency of entrapped-cell-based reactors operated at bulk DO concentrations 2 and 3 mg l^{-1}. NOB community including *Nitrospira* and *Nitrobactor* were investigated by clone library and describe high-oxygen-affinity-NOB proportion on metabolic activity.

1.4.4   Chapter 4

Chapter 4 presents partial nitrification performance of entrapped-cell-based reactors with increasing cell-to-matrix ratios. Investigation of *in situ* oxygen concentration along the depth of gel beads on AOB and NOB activity, the relative abundance and spatial distribution of heterotrophs and autotrophic AOB were carried out.

1.4.5   Chapter 5

Chapter 5 presents partial nitrification and nitrogen removal performance of entrapped-cell-based reactors operated at various volumetric ammonia and organic

loading rates in continuous flow entrapped-cell-based reactors. Wastewater containing low, medium, and high concentrated ammonia and organic were fed into the reactors to investigate the effect of ammonia and organic loading rates on partial nitrification, nitrogen removal, oxygen concentration gradient in gel matrix and also the localization AOB and NOB within the gel matrix. N_2O production was investigated in some selected reactors.

1.4.6 Chapter 6

Chapter 6 presents research summary and the suggestion for future studies.

1.4.7 Experimental framework
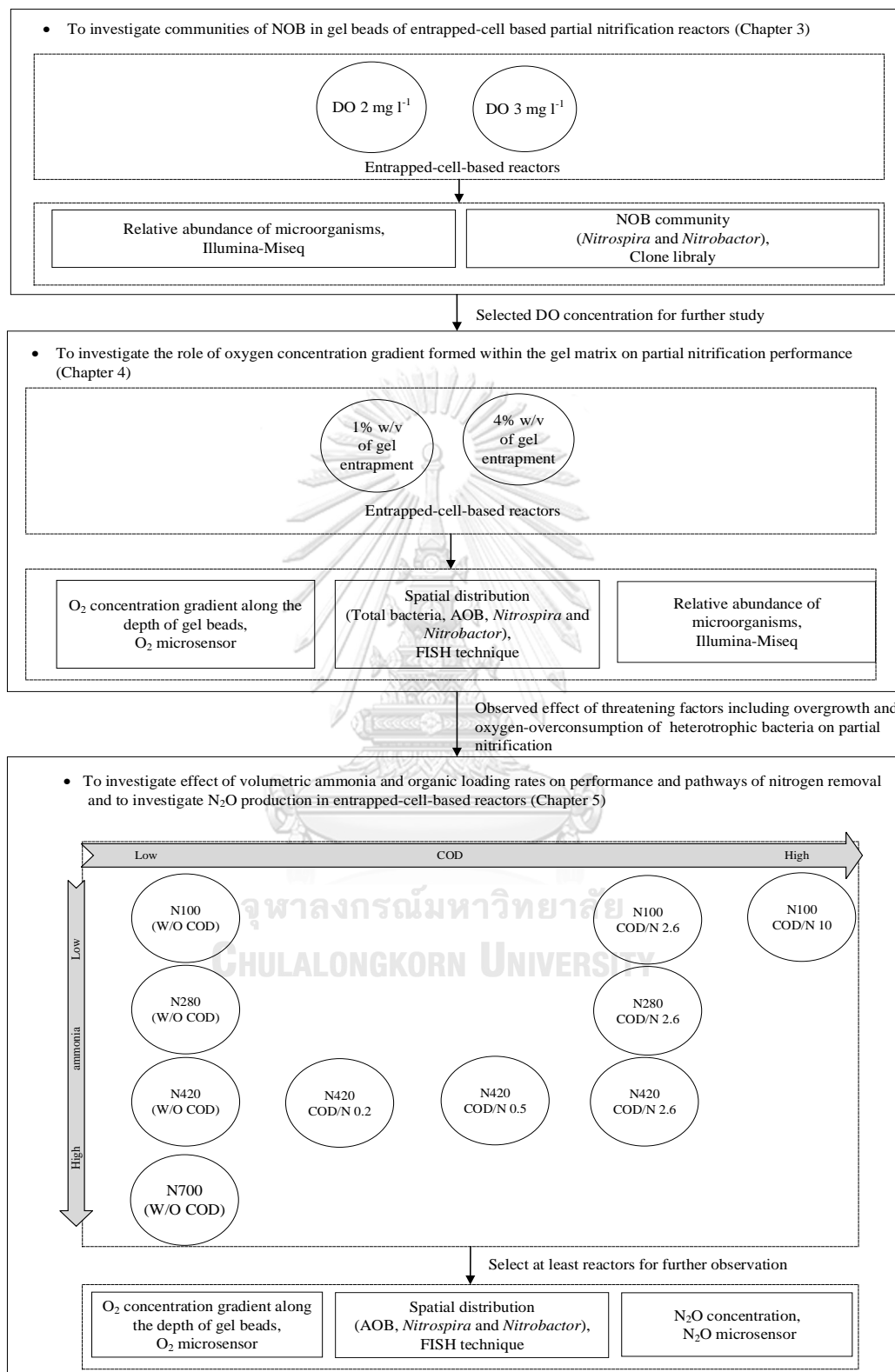
Figure 1. 1 Experimental framework

CHAPTER 2
LITERATURE REVIEW

2.1 Introduction

Conventional nitrogen removal from wastewater relies on nitrification followed by denitrification (see Figure 2.1). Nitrification is a two-step process comprising of the oxidation of ammonia (NH_4^+) to nitrite (NO_2^-) and the oxidation of nitrite to nitrate (NO_3^-). The produced nitrate from nitrification is then reduced to gaseous nitrogen (N_2) by heterotrophic denitrifying microorganisms in denitrification. Nowadays, novel nitrogen removal process becomes increasingly popular and tends to replace the conventional nitrogen removal approach in future. The novel nitrogen removal process can reduce a significant amount of oxygen (O_2) required to oxidize ammonia and organic carbon needed to reduce nitrate. The process relies on the oxidation of ammonia to nitrite without further being oxidized to nitrate which is called "partial nitrification". Then, the produced nitrite from partial nitrification is further reduced to N_2 via nitrite denitrification or anaerobic ammonium oxidation (ANAMMOX), which utilized ammonia as an electron donor during nitrite reduction.

An underlying principle of partial nitrification is to promote the activity of ammonia-oxidizing microorganisms (AOMs) while suppress the nitrite-oxidizing microorganisms (NOMs) activity to allow only ammonia to be oxidized to nitrite without nitrite being subsequently oxidized to nitrate. 'Limiting dissolved oxygen (DO)', 'promoting high pH' or/and 'maintaining high temperature' conditions are the three common operational strategies that lead to an achievement of partial nitrification (Anthonisen et al., 1976; Hellinga et al., 1998; Ciudad et al., 2006; Sinha and Annachhatre, 2007; Blackburne et al., 2008). In recent years, ultrasound treatment has been introduced for partial nitrification fulfilment (Zheng et al., 2013; Zheng et al., 2016).

Cell immobilization, including cell attachment, cell granulation and cell entrapment, has recently been employed to promote partial nitrification (Hill and Khan, 2008; Rongsayamanont et al., 2010; Rongsayamanont et al., 2014; Jeong et al., 2016). With the assistance of cell immobilization, oxygen concentration gradient can be formed along the depth of cell immobilization matrices leading to the creation of oxygen-limiting zone somewhere within the cell immobilization matrices to support partial nitrification (Rathnayake et al., 2013; Song et al., 2013). However, the application of cell immobilization for partial nitrification are still at the middle stage. Several aspects regarding microenvironments within the cell immobilization matrices and biochemical reaction pathway as well as microorganisms involved in partial nitrification have not yet been clearly clarified. This review provides current advancement of using cell immobilization to promote partial nitrification via oxygen-limiting strategy. The review is divided into 3 parts describing 1) general information required for operating partial nitrification reactors, 2) the application of cell immobilization to promote partial nitrification, and 3) problems encountering operation of partial nitrification reactors using cell immobilization.

2.2 Conventional and novel nitrogen removal processes

Nitrogen can be removed from wastewater by transforming active nitrogen species (ammonia, nitrite, and nitrate) to an inactive nitrogen (N_2) which is promptly released to atmosphere (Figure 2.1). Conventional nitrogen removal relies on nitrification-denitrification processes which are arose under aerobic and anoxic conditions, respectively. In nitrification, the oxidation ammonia to nitrite is done by AOMs at the expense of 1.5 mole O_2 per a mole ammonia oxidized. Subsequently, NOB oxidized nitrite to nitrate which consume 0.5 mole O_2 per a mole nitrite oxidized. In denitrification, heterotrophic denitrifiers reduce nitrate to N_2 at by using organic compounds as electron donors while nitrite, nitric oxide (NO) and nitrous oxide (N_2O) are produced as intermediates.

Novel nitrogen removal relies on partial oxidation of ammonia to nitrite which is further reduced to N_2 via heterotrophic or autotrophic denitrification. This process can lead to the reduction of the amount of oxygen required for autotrophic nitrite

oxidation and the amounts of organic carbons required for heterotrophic nitrate reduction as compared to the conventional nitrification and denitrification. If partial nitrification is followed by nitrite denitrification (Figure 2.2), 100% of influent ammonia should be oxidized to nitrite. Then, organic carbon is added in the denitrification to reduce nitrite to N_2. With this approach, 25% of oxygen supply can be saved and up to 40% of organic carbon can be reduced. In addition, the overall 20% of carbon dioxide emission can be cut as compared to the conventional nitrogen removal Van Kempen et al., 2001. If partial nitrification is connected to ANAMMOX process (Figure 2.2), around 50% of influent ammonia should be oxidized to nitrite leading to 1:1 NH_4^+:NO_2^- in the effluent of a partial nitrification reactor that enter an ANAMMOX reactor. This value is close to the stoichiometry of 1:1.32 NH_4^+:NO_2^- required for ANAMMOX microorganisms. Partial nitrification followed by ANAMMOX can save 60% of oxygen supply and no organic carbon is required as compared to the conventional nitrogen removal (Van Hulle et al., 2010).
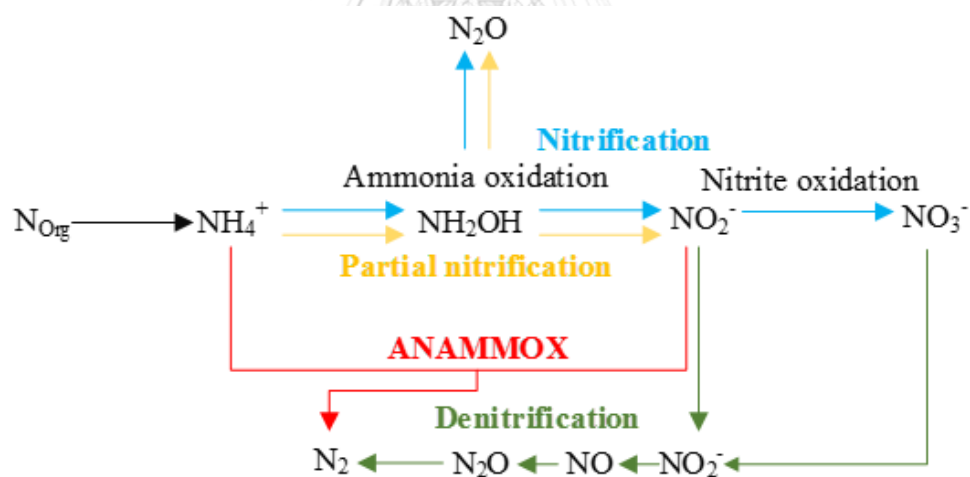
Figure 2. 1 Nitrogen cycle (modified from Thamdrup (2012))
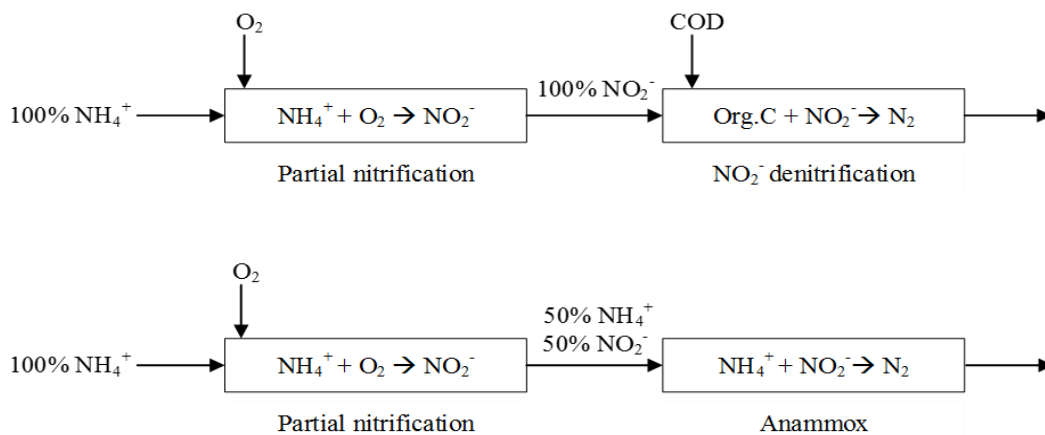
Figure 2. 2 Arrangement of reactors for novel nitrogen removal processes

2.3 Microorganisms involved ammonia and nitrite oxidation

Thus far, two groups of microorganisms, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), are known to capable of oxidizing ammonia to nitrite. AOB had long been recognized as the sole ammonia oxidizers. AOB uses ammonia monooxygenase (AMO) enzyme to oxidize ammonia to hydroxylamine (NH_2OH), then hydroxylamine oxidoreductase (HAO) enzyme oxidizes hydroxylamine to nitrite. AOB belong within two sub-phylum, *γ -Proteobacteria* and *β-Proteobacteria*. The *γ*-AOB, for examples *Nitrosococcus oceani*, is specific to marine environment only. The *β*-AOB is more general and can be found in several environments ranging from marine, estuary, and freshwater systems. *β*-AOB is classified to several clusters including *Nitrosospira* cluster, *Nitrosomonas europaea-Nitrosococcus mobilis* cluster, *Nitrosomonas communis* cluster, *Nitrosomonas oligotropha* cluster, *Nitrosomonas marina* cluster, *Nitrosomonas cryotolerans* cluster, and *Nitrosomonas* Nm143 cluster. Members of *Nitrosopira* cluster, *Nitrosomonas europaea-Nitrosococcus mobiles* cluster, and *Nitrosomonas oligotropha* cluster are generally found in wastewater treatment systems (Koops and Pommerening-Röser, 2001; Limpiyakorn et al., 2005). Recently, many studies demonstrated the potential involvement of AOA in ammonia oxidation in global nitrogen cycle (Francis et al., 2005; Könneke et al., 2005; Park et al., 2006; Roy et al., 2017; Sauder et al., 2017). AOA oxidize ammonia to nitrite directly by using AMO enzyme. AOA are classified

in a newly founded phylum, *Thaumarcheota* (Brochier-Armanet et al., 2008). AOA were found to be dominant in some wastewater treatment systems (Limpiyakorn et al., 2011; Sinthusith et al., 2015). Some studies suggested that AOA tended to be found in the low ammonia environments (Bai et al., 2012; Sauder et al., 2012). In wastewater treatment systems, AOB seemed to be the main ammonia oxidizers (Pan et al., 2018).

Nitrite-oxidizing bacteria (NOB), who oxidize nitrite to nitrate, are distributed within six genera in phylum α-*Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Nitrospinae, Nitrospirae and Chloroflexi* (Teske et al., 1994; Ehrich et al., 1995; Schloss and Handelsman, 2004; Griffin et al., 2007; Schott et al., 2010; Sorokin et al., 2012; Lücker et al., 2013). *Nitrobacter (α-Proteobacteria)*, *Nitrospira* (*Nitrospirae*) and *Nitrotoga* (*β-Proteobacteria*) are genus that highly abundant in wastewater treatment systems (Schramm et al., 1999; Daims et al., 2001; Lücker et al., 2015). *Nitrotoga* were found in low temperature (4-17°C) (Alawi et al., 2007), while *Nitrobacter* and *Nitrospira* are usually present in mesophilic condition (Lücker et al., 2015; Nowka et al., 2015). Comparing between *Nitrobacter* and *Nitrospira*, *Nitrobacter* have lower affinity to nitrite than *Nitrospira*, thus *Nitrobacter* are commonly found in high nitrite environments, while *Nitrospira* can be found in environments low in nitrite concentrations (Nowka et al., 2015).

In recent year, complete ammonia oxidizers (Comammox) have been discovered in aquaculture, full-scale wastewater treatment and drinking water treatment systems (Daims et al., 2015; Van Kessel et al., 2015; Palomo et al., 2016; Pinto et al., 2016). These microorganisms can convert ammonia to nitrate in a single microorganism by nitrite oxidoreductase (NXR) gene. Examples of Comammox are some members within *Nitrospira* lineage II (Daims et al., 2015; Van Kessel et al., 2015; Palomo et al., 2016; Pinto et al., 2016). However, thus far, their contribution to partial nitrification reactors has not been clearly described.

2.4 Strategies for achieving partial nitrification

In order to oxidize ammonia to nitrite without nitrite being oxidized to nitrate, the activity of AOB must be proceeded; in the meantime, the activity of NOB must be

suppressed. Due to the differences in physiological properties between these two groups of microorganisms, controlling some environmental parameters can lead to partial nitrification. This includes maintaining high temperature, high pH and oxygen-limiting conditions (Peng and Zhu, 2006; Sinha and Annachhatre, 2007).

2.4.1 Temperature

Partial nitrification can be achieved by operating the system at temperature higher than 35°C. At temperature of 35°C, AOB growth rate is twice NOB growth rate. SHARON® (Single reactor system for High Ammonium Removal Over Nitrite) process is one of the system configuration using temperature of 35°C as the strategy to promote partial nitrification (Hellinga et al., 1998). Moreover, temperature has an influence on free ammonia (FA) and free nitrous acid (FNA) concentrations. Increasing temperature leads to higher FA and FNA concentrations that also support partial nitrification as will be described in the following section. Recently, the first full-scale SHARON® process followed by ANAMMOX reactors has been operated at Rotterdam Dokhaven WWTP to treat rejected water from digested sludge dewatering. SHARON® process has been operated with optimum temperature at 30-40°C (Mulder et al., 2001; Van Kempen et al., 2001).

2.4.2 pH value

Both of AOB and NOB activities are inhibited by FA and FNA concentrations (Anthonisen et al., 1976). The FA and FNA rely on pH and temperature as given in equation 1 and 2 (Anthonisen et al., 1976).

$$FA = \frac{[TAN]10^{pH}}{e^{6344/(T+273)}+10^{pH}} \qquad \text{Equation 1}$$

$$FNA = \frac{[TNO_2]10^{-pH}}{e^{-2300/(T+273)}+10^{-pH}} \qquad \text{Equation 2}$$

FA and FNA are temperature and pH dependent. The higher pH, the higher FA fraction and the lesser FNA fraction are.

Influence of FA and FNA on AOB and NOB

AOB are more tolerance to FA and FNA than NOB (Anthonisen et al., 1976). Because FA and FNA concentrations are pH dependent (as shown in equation 1 and 2, respectively). Villaverde et al. (1997) indicated that nitrite higher accumulated (85%) when pH increased from 7.5 to 8.5. In addition, Sinha and Annachhatre (2007) suggested that nitrite accumulation also increased when controlled pH<7.0. From literature, increasing pH contributed high FA concentration where as high FNA concentration was influenced by low pH. In suspended-cell system, FA of 0.1-4.0 mg l^{-1} and FNA of 0.06 - 0.83 mg l^{-1} were found to inhibit nitrite oxidation Anthonisen et al., 1976; Bae et al., 2001 while ammonia oxidation is inhibited at FA of 10-150 mg l^{-1} and FNA of 0.20-2.80 mg l^{-1} (Anthonisen et al., 1976). In cell immobilization system, AOB and NOB which grew and maintained their activity in bulk FA concentrations of up to 15 mg N l^{-1} (Park et al., 2014) and 111 mg N l^{-1} (Chen et al., 2015). Higher concentrations of FA in bulk solution might be required for cell immobilization system as compared to suspended-cell system because microorganisms in cell immobilization system might be protected from the penetration of toxic substances by EPS and cell immobilization matrix.

2.4.3 DO concentration

Currently, oxyen-limiting condition is being employed to full- and pilot-scale partial nitrification reactors (Blackburne et al., 2008; Desloover et al., 2011; Rongsayamanont et al., 2014). The strategy is based on the difference in oxygen affinity between AOB and NOB. The half saturation coefficient for oxygen (K_O) of NOB is higher than that of AOB. The K_O of AOB is in a range of 0.03-0.74 mg l^{-1} and for NOB the K_O ranges from 0.4 to 1.9 mg l^{-1}. At low DO concentration, AOB growth rate become higher than NOB growth rate. For suspended-cell system, optimized DO levels for partial nitrification are in a range of 0.5-3.0 mg l^{-1} (Figure 2.3). For cell immobilization system, wider range of DO concetrations (0.4-6.0 mg l^{-1}) is allowed to promote partial nitrification (Figure 2.3). This is because biofilm can obstruct oxygen diffusion and create oxygen concentration gradient inside the biofilm. Therefore, partial nitrification can be promoted within biofilm matrix, while bulk DO concentrations can remain high level.
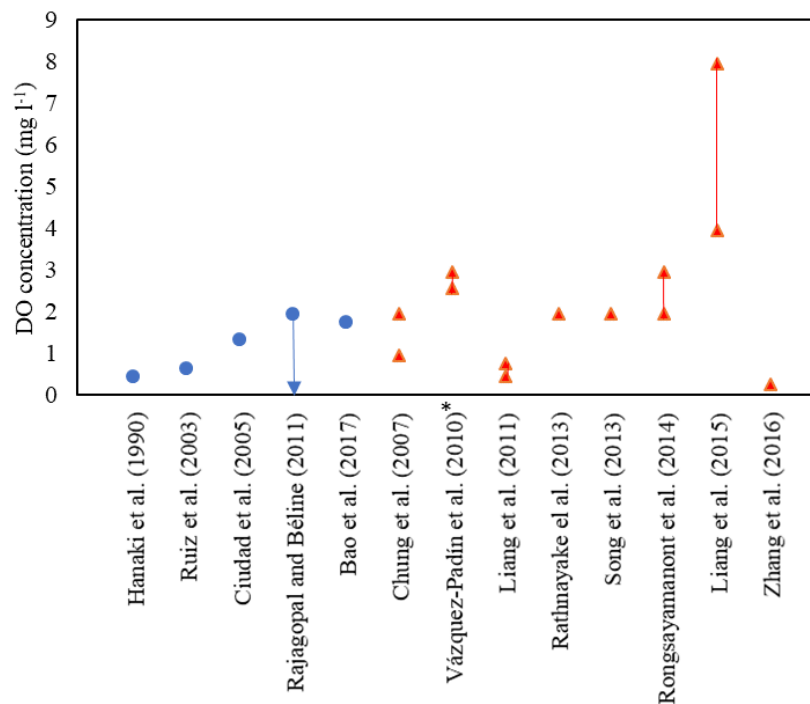
Figure 2. 3 DO concentrations used for maintaining partial nitrification in suspended-cell (circle) and cell immobilization system (triangle) systems

2.4.4 Sludge age

Sludge age can be considered as a supporting parameter for promoting partial nitrification in suspended-cell system. This parameter itself is not for stimulating the growth of AOB over NOB, but is used to exclusively promote faster-growing bacteria (AOB), while washing out slower-growing bacteria (NOB) from the system. One example is for SHARON® (The single reactor system for high-activity ammonia removal over nitrite) process (Hellinga et al., 1998). In SHARON®, temperature was controlled at $35^{o}C$. At this temperature, AOB growth rate is double of NOB growth rate. By maintaining at a certain value of sludge age that is one between minimum requirement of AOB and NOB, NOB can selectively be washed out, while AOB can still be growing in the reactor.

2.4.5 Ultrasound treatment

Low frequency and density ultrasound was applied to increase AOB and decrease NOB activities (Zheng et al., 2013; Zheng et al., 2016). Zheng et al. (2013)

indicated that the ultrasound at frequency of 40 kHz and density of 0.027 W ml^{-1} for 2 h of irradiation time improved AOB activity, while inhibiting NOB activity by creating high temperature and pH. Zheng et al. (2016) claimed that the ultrasound treatment was easier to operate and maintain partial nitrification than other methods.

2.5 Partial nitrification by cell immobilization

Principle of cell immobilization is to fix microorganisms' cells into/onto a suitable solid matrix. Benefits of cell immobilization include the enhancement of substrate loading rate, cell density, sludge separation efficiency, cell protection from environmental stress. Cell immobilization can be carried out by several approaches including cell attachment, cell granulation and cell entrapment (Wijffels, 2001). Cell attachment is the formation of biofilm onto the internal and external surface of supporting materials. Cell granulation is the self-aggregation of microorganisms into the granule-like aggregate. Cell entrapment is, for example, the encapsulation of cells into polymeric matrix.

The concept of cell immobilization can be applied to promote partial nitrification via oxygen-limiting strategy. This is because oxygen concentration gradient can be created along the depth of biofilm, granule, or entrapped-cell matrix leading to oxygen-limiting zone generated somewhere inside the biofilm, granule, and entrapped-cell matrix. With this approach, bulk DO concentration is not needed to be maintained at the oxygen-limiting level allowing more variety of other groups of microorganisms to perform activity in a reactor Rongsayamanont et al., 2014). Table 2.1 shows the application of cell immobilization including cell attachment, cell granulation and cell entrapment for partial nitrification reactors.

Regarding cell attachment system, some studies demonstrated that partial nitrification can be achieved using a few types of system configuration, for example, moving bed biofilm reactor, sequencing batch biofilm reactor, continuous-flow hybrid shortcut biological nutrient removal (Chung et al., 2007; Zhang et al., 2016; Bian et al., 2017). DO concentrations in bulk solutions were maintained in a range of 0.3-6.4 mg l^{-1}. While, temperature and pH were controlled between 6-30 ℃ and 7.3-8.0, respectively

do not support partial nitrification (Table 2.1). This implies that biofilm formation may help promote partial nitrification by creating oxygen-limiting environment inside the biofilm matrix, although oxygen concentrations in bulk solution did not meet oxygen-limiting level in some studies. Zhang et al. (2016) compared the sustainability of partial nitrification in activated sludge process and biofilm system. It is shown that partial nitrification was sustained in biofilm system as nitrite accumulation was 98.2% as compared to 90.1% in activated sludge process. Chung et al. (2007) demonstrated that partial nitrification could be maintained over 1.5 years in suspended and attached growth systems carrying polyvinyl alcohol (PVA) sponge (Chung et al., 2007). Bian et al. (2017) suggested that partial nitrification in biofilm system can be achieved by controlling the ratio of DO concentration to total ammonia nitrogen (TAN) (DO/TAN) and oxygen-limiting condition was also to be defied by DO and TAN concentration.

Aerobic granular sludge has successfully been employed to promote partial nitrification in feast-famine environment like sequencing batch airlift reactor (Wang et al.; Rathnayake et al., 2013; Song et al., 2013; Liang et al., 2017). The main limitation of this approach is the difficulty to produce granular sludge as it is time consuming and requires skillful operators. Liang et al. (2017) demonstrated that granular sludge reactor can be rapidly start-up by using seed that was mixed with nitrifying floccular sludge and 30% partial nitrifying granular sludge as compared to seed prepared from nitrifying floccular sludge alone. Rathnayake et al. (2013) and Song et al. (2013) showed that partial nitrification (1:1 effluent NO_2^-/NH_4^+) occurred in sequencing batch airlift reactors.

Entrapped-cell system has also been applied to partial nitrification reactors (Hill and Khan, 2008; Yan and Hu, 2009; Chou et al., 2012; Li et al., 2014; Rongsayamanont et al., 2014; Jeong et al., 2016). Gel matrix can be produced by a variety of polymer, such as sodium alginate, PVA, PVA/alginate, and polyethylene glycol (PEG) (Hill and Khan, 2008; Yan and Hu, 2009; Chou et al., 2012; Li et al., 2014; Rongsayamanont et al., 2014; Jeong et al., 2016). Hill and Khan (2008) demonstrated that partial nitrification can be achieved using nitrifier and denitrifier entrapped in calcium alginate gel beads. Jeong et al. (2016) showed that nitrifier

entrapped in PVA/alginate gel beads built-up nitrite in the reactor. Rongsayamanont et al. (2014) demonstrated that PVA gel matrix of entrapped cell can simply be applied to promote partial nitrification by controlling suitable DO concentrations (2 and 3 mg l^{-1}) in bulk solution. They also suggested that the preparation of suspended inoculum with having ability to partial nitrifying was not needed during the production of entrapped cell. All suspended inoculum with having non-nitrifying, nitrifying, and partial nitrifying ability entrapped in PVA gel matrix can create partial nitrification within similar periods of operation (Rongsayamanont et al., 2014).

Table 2. 1 Summary of partial nitrification reactors using cell immobilization systems (cell attachment, cell granulation and cell entrapment)

System	Reactor type	Ammonia loading rate (kg m⁻³ d⁻¹)	Influent ammonia (mg l⁻¹)	Influent COD (mg l⁻¹)	pH	Operating cycle (for SBR)	DO (mg l⁻¹)	Temperature (°C)	HRT (h)	Ammonia removal (%)	Nitrite accumulation (%)	effluent NO₂⁻: NH₄⁺	Remarks	References
Biofilm	Moving bed biofilm reactor		8.0 ± 0.5	0.67	7.3-7.4		3.8 ± 0.2	26 ± 1^{a}			$\sim100^{*c}$		Only some slected operating conditions are presented in this table	Bian et al (2017)
			13.3 ± 1.0	1.11			6.4 ± 0.5	16 ± 1^{a}			$\sim100^{*c}$			
			22.4 ± 1.0	1.87			3.8 ± 0.2	6 ± 0.5^{a}			$\sim45\text{-}90^{*c}$			
	Sequencing batch reactor of biofilm system packing		60-65		7.8	feeding 5 min; aeration 4 h; settling 0.5 h; withdraw 5 min	0.3 (an initial DO of each cycle)	$14\text{-}16^{a}$		85.7	98.2*			Zhang et al. (2016)
	Continuous-flow hybrid shortcut biological nutrient removal		1000	1600	8		2.0	30^{b}	27.1	73.9	90 ± 5*		Reactor HSBNR I	Chung et al. (2007)
				1600			1.0			75.1	≥95*		Reactor HSBNR I	
				2000			2.0			72.5	90*		Reactor HSBNR II	
				2000			1.0			77.2	≥95*		Reactor HSBNR II	

*Calculated by Nitrite accumulation $= \frac{[NO_2^-]_{eff}}{[NO_2^-]_{eff}+[NO_3^-]_{eff}}$

**Calculated by Nitrite accumulation $= \frac{[NO_2^-]_{eff}}{[NH_4^+]_{inf}+[NH_4^+]_{eff}}$

a Water or reaction temperature
b Room temperature
c Estimated from values in main text, tables or figures in the papers

Table 2. 1 Summary of partial nitrification reactors using cell immobilization systems (cell attachment, cell granulation and cell entrapment) (continued)

System	Reactor type	Ammonia loading rate (kg m⁻³ d⁻¹)	Influent ammonia (mg l⁻¹)	Influent COD (mg l⁻¹)	pH	Operating cycle (for SBR)	DO (mg l⁻¹)	Temperature (°C)	HRT (h)	Efficiency Ammonia removal (%)	Nitrite accumulation (%)	effluent NO₂⁻: NH₄⁺	Remarks	References
	Sequencing batch reator	0.99	350±21	-	7.4–7.8	feeding 3 min; aeration 232 min; settling 3 min; and withdraw 2 min	2.0±0.3		8	~52[c]		around 1		Rathnayake et al (2013)
	Sequencing batch air lift reator		200–600	200 (as TOC)	7.5–8.0	feeding 2 min; areation the remaining of 4 hr cycle time; settling 3–30 min; withdrawal 2 min	2	15–25[b]				around 1		Song et al (2013)
Aerobic granule			110–130			feeding 1 min; aeration 60–180 min; settling 10 min or 18 min; withdraw 2 min; volumetric exchange ratio of 62.5%	4.0–8.0	25[a]		80	≥95*		Seed with nitrification activated floc sludge and 30% partial nitrification granular sludge	Liang et al (2015)
	Sequencing batch reator		250–350		7.3–7.7					60	95*		Seed with nitrification activated floc sludge	
			60.0–90.0							>80	>95*		Seed with nitrification activated floc sludge and 30% partial nitrification granular sludge	
										>80	>95*		Seed with nitrification activated floc sludge	
Entrapped-cell	Continuous stirred-tank reactor	0.52	626		8.1±0.1		3	24–26[b]	28.8	46–71	14–91 **			Rongsayamanont et al (2014)
							2			50–64	65–87**			

*Calculated by Nitrite accumulation $= \dfrac{[NO_2^-]_{eff}}{[NO_2^-]_{eff}+[NO_3^-]_{eff}}$

**Calculated by Nitrite accumulation $= \dfrac{[NO_2^-]_{eff}}{[NH_4^+]_{inf}+[NH_4^+]_{eff}}$

a Water or reaction temperature

b Room temperature

c Estimated from values in main text, tables or figures in the papers

2.6   Oxygen concentration gradient and spatial distribution of microorganisms in cell immobilized matrix

Although cell immobilized has successfully been employed to promote partial nitrification, microenvironment inside cell immobilized matrix has yet been clearly observed. The information related to in situ oxygen concentration gradient inside the cell immobilized matrix of partial nitrification reactors was provided only in some studies (Table 2.2). For aerobic granules, oxygen can penetrate up to around 100-300 µ.m. from the surface of the granules (Rathnayake et al., 2013; Song et al., 2013; Rathnayake et al., 2015). while it was around 600-650 µ.m. for biofilm matrix (Wang et al., 2017a). This indicated that oxygen concentration gradient is formed along the depth of cell immobilized matrix allowing oxygen-limiting environment to be created somewhere within the matrix to support partial nitrification.

Oxygen concentration gradient can be created inside an immobilized matrix as a result of 1) air flow rate and oxygen transfer limitation at the gas-liquid interface 2) obstruction of oxygen transfer via the immobilized matrix and biofilm matrix including microbial products and inert solid etc. 3) utilization of oxygen by microorganisms distributed throughout an immobilized matrix

Aerobic microorganism is the main-playing-role microorganisms who utilize oxygen in cell immobilization system. These microorganisms influence the creation of oxygen concentration gradient in cell immobilized matrix. In the meantime, the appearance of aerobic microorganisms in immobilized cell is a result of remaining oxygen concentration distributed inside the immobilized matrix.

For cell immobilization for combined carbon oxidation and nitrification, ordinary heterotrophic organisms distributed around the surface of the matrix due to having lower oxygen affinity and higher growth rate than autotrophic nitrifier (Juhler et al., 2009). Autotrophic nitrifier usually located themselves in the deeper part of the immobilized matrix (Juhler et al., 2009; Matsumoto et al., 2010). Among the autotrophic nitrifier, although AOB have higher oxygen affinity than NOB, they are commonly shared the same position in biofilm matrix (Okabe et al., 1999; Satoh et al.,

2006). Okabe et al. (1999) indicated that AOB were found throughout of biofilm matrix where ammonia was reduced to nitrite, while NOB appeared in the inner part where nitrite was oxidized to nitrate.

In partial nitrifying immobilized matrix, Rathnayake et al. (2013) demonstrated that AOB located at the outer layer of aerobic granule collected from a labolatory-scale granular sludge reactor without receiving organic carbon. AOB was spatially distributed at 100-200 μ.m. within aerobic granule (Rathnayake et al., 2013; Song et al., 2013; Rathnayake et al., 2015). For entrapped-cell-based reactors, the oxygen concentration gradient created within the gel matrix has not yet been clearly reported although the reactors have been found to achieve partial nitrification. However, information on microbial distribution within the gel matrix has been available. Rongsayamanont et al. (2010) and Rongsayamanont et al. (2014) revealed that AOB located near the surface of gel beads (approximately 100 μ.m. from the surface) and NOB was found at the same place.

Table 2. 2 Distances of oxygen penetration from the surface of cell immobilization matrix

System	DO in bulk solution (mg l^{-1})	Distance of oxygen penetration from the surface (µ.m.)	Remarks	Reference
Aerobic granules	2	~300	Granule diameter = 2-3 mm	Rathnayake et al. (2013)
	2.5 and 7	~100	Granule diameter = 2-3 mm	Song et al. (2013)
	2 and 4	~200		Rathnayake et al. (2015)
Biofilm	0.2-0.4	~600-650		Wang et al. (2017b)

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2.7 Problems encountering operation of partial nitrification reactors using cell immobilization

2.7.1 Recovery of NOB activity after long-term operation of partial nitrification reactors

Although the efficiency of partial nitrification reactors can be decreased during long-term operation at low oxygen concentration was limited, some studies demonstrated that NOB can adapt theirs activity to survive under microaerobic condition (Lücker et al., 2010; Regmi et al., 2014). *Nitrobacter* and *Nitrospira* are NOB that commonly found in wastewater treatment systems. The oxygen affinity of these two groups of microorganisms are different with the lower affinity for *Nitrobacter* and the higher affinity for *Nitrospira* (Blackburne et al., 2007). Huang et al. (2010b) reported that *Nitrospira* was higher abundant than *Nitrobacter* at low DO concentration (<0.89 mg l^{-1}) in full-scale WWTPs. *Nitrospira* can be divided into three lineages including *Nitrospira* lineage I, II and III. The *Nitrospira* lineage I and II were usually found in nitrifying wastewater treatment systems. Park and Noguera (2008) revealed that *Nitrospira* lineage I were abundant in low DO concentration (0.12-0.24 mg l^{-1}) in contrast *Nitrospira* lineage II were highly found in high DO level (8.5 mg l^{-1}). The results indicated that some NOB groups could survive and expressed activity under oxygen-limiting condition that lead to the failure of partial nitrification under long-term operation. This characteristic of NOB is worth to further explore in cell immobilized matrix. Further works should emphasize on a change of NOB community toward the groups with extremely high oxygen affinity within cell immobilized matrix that employs oxygen-limiting strategy to promote partial nitrification.

2.7.2 Nitrous oxide production in partial nitrification reactors operated using oxygen-limiting strategy

N_2O is a powerful greenhouse gas. Full-scale plants with partial nitrification and anammox generated N_2O between 1.2 and 12% (Kampschreur et al., 2008a; Desloover et al., 2011). Nitrification can produced N_2O via two different pathways, nitrifier nitrification and nitrifier denitrification (Kampschreur et al., 2008; Desloover et al.,

2011; Ahn et al., 2011). With nitrifier nitrification partway, N_2O is produced as a byproduct of hydroxylamine, which is an intermediate in the oxidation of ammonia to nitrite. Terada et al. (2017) showed that most N_2O, produced in batch tests (DO 0.79-4.3 mg l^{-1}) of suspended cells from partial nitrification sequencing batch reactors, was originated from AOB with NH_2OH oxidation pathway. For nitrifier denitrification pathway, some AOB, such as *Nitrosomonas europaea* can switch their activity from nitrification to nitrifier denitrification in oxygen-limiting environment (DO <1 mg l^{-1}) (Kampschreur et al., 2008a; Peng et al., 2014) and nitrous oxide is produced during the reduction of nitrite to nitrogen gas (Kampschreur et al., 2008; Desloover et al., 2011; Peng et al., 2014 Ahn et al., 2011). Peng et al. (2015) studied N_2O production under various nitrite and DO concentrations (0.35-3.5 mg l^{-1}). It was indicated that nitrifier denitrification was the dominant pathway in most cases and the contribution of nitrifier denitrification increased as DO decreased. Wang et al. (2017a) observed N_2O production by applying N_2O microelectrode to biofilm from one-stage completely autotrophic nitrogen removal process. The results indicated that nitrifier denitrification was the key pathway contributing to N_2O production, which produced N_2O >1.7 times higher than nitrifier nitrification pathway.

Because oxygen-limiting condition occurred within cell immobilized matrix, it is highly probable that high N_2O can be produced in this type of partial nitrification reactors. Reactor configuration and operational mode can influence the amounts of N_2O produced. Characteristics of immobilized matrix, such as size, cell-to-matrix ratio, type of polymeric gel, can also affect the extent of N_2O production. This requires further clarification to optimize the immobilize-cell-based partial nitrifying reactors to reduce N_2O production.

2.7.3 Influence of organic matters on partial nitrification performance using oxygen-limiting strategy

Organic matters were found to have an influence on partial nitrification performance. Organic matters stimulate growth of heterotrophic microorganisms leading to an increase in oxygen demand. Thus, oxygen available for nitrifying microorganisms can be affected. The presence of organic matters can provide both

advantage and disadvantage to partial nitrification performance. Under certain ranges of organic matters, partial nitrification performance can be enhanced but if the amount of organic matters is over a threshold level, partial nitrification may be deteriorated.

Xu et al. (2015) mentioned that organic matters can enhance partial nitrification efficiency and heterotrophic bacteria provided oxygen-limiting condition for AOB in immobilized cell system. Xu et al. (2015) demonstrated that partial nitrification was achieved with ammonia removal of 79-95 % and nitrosation of 80-99 %, when COD/N ratio was at 2.6 in a biofilm system. Partial nitrification was shorter achieved as compared to the reactor with no organic supplied. However, Rodriguez-Sanchez et al. (2016) demonstrated that organic matter decreased ammonia oxidation and relative abundance of AOB when compared to organic free reactors where high nitrite accumulation was observed.

Nitrification may fail when organic are going to too much feed. oxygen was plentifully consumed by aerobic heterotroph until inadequate oxygen in cell immobilized matrix causing to inhibit AOB activity. Therefore, optimizing organic feeding should also been focused on maintaining of partial nitrification with immobilized-cell-based reactor.

CHAPTER 3

COMMUNITIES OF NITRITE-OXIDIZING BACTERIA IN ENTRAPPED-CELL BASED PARTIAL NITRIFICATION REACTORS OPERATED WITH DIFFERENT BULK DISSOLVED OXYGEN CONCENTRATIONS

3.1 Introduction

Shortcut nitrogen removal rely on the oxidation of ammonia to nitrite, followed by the reduction of nitrite to nitrogen gas by heterotrophic microorganisms, which utilize organic matters as electron donors or by anaerobic ammonium oxidation (ANAMMOX) microorganisms, which use ammonia as an electron donor. As compared to conventional nitrogen removal, shortcut nitrogen removal can reduce amounts of oxygen required for nitrification and organic matters needed for denitrification. Partial nitrification, the oxidation of ammonia to nitrite without nitrate as the end product, is an inevitable step of shortcut nitrogen removal. The process can be promoted by providing environmental conditions that suppress the activity of nitrite-oxidizing microorganisms, while allowing only the activity of ammonia-oxidizing microorganisms to occur. Such environmental conditions include maintaining pH in a range of 7.5-8.5 (Villaverde et al., 1997), temperature >35°C (Hellinga et al., 1998), or oxygen at the oxygen-limiting levels (0.3-0.5) (Blackburne et al., 2008; Chen et al., 2016).

Entrapped-cell-based technique has recently been applied to promote partial nitrification for treating ammonia-rich wastewater (Rongsayamanont et al., 2014). The entrapped cells can be prepared by a variety of polymeric materials such as phosphorylated-polyvinyl alcohol (PPVA), sodium alginate, and PVA/alginate (Hill and Khan, 2008; Yan and Hu, 2009; Chou et al., 2012; Li et al., 2014; Rongsayamanont et al., 2014; Jeong et al., 2016). Besides retaining high cell density,

allowing more substrate loading rate, and improving cell-liquid separation, the entrapped-cell approach helps provide microenvironment inside the gel matrix that promote partial nitrification. When suitable dissolved oxygen (DO) concentrations in bulk solution are maintained, oxygen concentration gradient generating along the depth of the gel matrix creates oxygen-limiting zone somewhere in the gel matrix. At the DO range of 2-3, ammonia-oxidizing activity is promoted over nitrite-oxidizing activity, leading to the oxidation of ammonia with the remaining nitrite as the end product.

Previously, partial nitrification can be achieved using PPVA entrapped cells operating at dissolved oxygen concentrations in bulk solution of 2 and 3 mg l^{-1} (Rongsayamanont et al., 2014). Although partial nitrification can be maintained, some concentrations of nitrate (39.2-87.0 mg l^{-1} (DO 2) and 25.7-345.8 mg l^{-1} (DO 3) from 625.7 mg l^{-1} of the influent ammonia concentration) can still be detected along the operation periods (Rongsayamanont et al. (2014). It is demonstrated that the activity of nitrite-oxidizing bacteria (NOB) was not fully suppressed in the partial nitrification reactors. Previous works have shown that different groups of NOB are different in affinities to oxygen. For example, the half-satulation coefficient for oxygen (K_O) of *Nitrobacter* (0.43-5.31 mg l^{-1}) (Blackburne et al., 2008; Blackburne et al., 2007; Laanbroek et al., 1994) is higher than that of *Nitrospira* (0.33-0.54 mg l^{-1}) (Blackburne et al., 2007; Park et al., 2017). This leads to the fact that NOB can adapt themselves by changing their communities to survive under different oxygen concentration environmentsThis leads to the fact that NOB can adapt themselves by changing their communities to survive under different oxygen concentration environments. In partial nitrification reactors, NOB have been reported to shifted thieir communities to survive under hypooxia condition in the reactors, leading to the lowering of partial nitrification performance under long-term operation (Liu and Wang, 2013). With this reason, a better understanding of NOB in oxygen-limiting environments of partial nitrification reactors is required to further maintain partial nitrification in long run. In this study, communities of NOB were investigated in entrapped-cell matrices taken from partial nitrification reactors operated with two different DO concentrations in bulk solution of 2 and 3 mg l^{-1}. Apart from understanding communities of NOB, this part of the work

preliminary provided suitable bulk DO concentrations for promoting partial nitrification in further chapters.

3.2   Materials and methods

3.2.1   Preparation of entrapped cells

Sludge from a municipal wastewater treatment plant was entrapped using PPVA method as introduced by Chen and Lin (1994). The mixed liquor sludge sample was concentrated via centrifugation at a speed of 500 rpm for 15 min. Then, one liter of PVA gel solution, prepared by100 g of PVA powder per one liter of de-ionized (DI) water, was mixed with the concentrated sludge to achieve an initial cell-to-matrix ratio of 4% w/v. The formation of spherical gel matrices was conducted by dropping the mixture into boric acid solution at a flow rate of 0.83 ml min^{-1}. Then, the spherical gel matrices were immersed into 1 M of phosphate buffer solution at pH 7.0 for 2-3 h to harden the gel matrices. Normally, diameter of gel bead varied between 4-6 mm. When gel bead was in the reactors, the diameter increased to 6-8 mm.

3.2.2   Synthetic wastewater

The synthetic wastewater which contained ammonia at 700 mg N l^{-1}, was prepared by mixing $(NH_4)_2SO_4$ (3.3 g), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), $FeSO_4 \cdot 7H_2O$ (0.09 g), H_3BO_3 (30 mg), $MnCl_2 \cdot 4H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.19 g), $NiCl_2 \cdot 6H_2O$ (0.024 g), $CuCl_2 \cdot 2H_2O$ (0.02 g), $ZnSO_4 \cdot 7H_2O$ (0.144 g), $Na_2MoO_4 \cdot 2H_2O$ (0.36 g), and $NaHCO_3$ (6.89 g) in one liter of DI water. The composition was modified from Widdel and Bak (1992); Rongsayamanont et al. (2010); Rongsayamanont et al. (2014).

3.2.3   Setup and operation of entrapped-cell-based reactors

Two continuous stirred tank reactors with an effective volume of 2 l were inoculated with the prepared entrapped cells with cell-to-matrix ratio of 4% w/v. The final concentration of cells in both reactors was 2000 mg SS. DO concentrations in the reactors were maintained around 2 and 3 mg l^{-1} (reactors DO2 and DO3, respectively) using DO controllers (HI8410 DO controller, Hanna, USA). Air flow rate was

controlled at 1 l min^{-1}. pH in bulk solution was maintained at 7.8±0.2 to ensure that partial nitrification only occurred by oxygen-limited condition. The pH was controlled by pH controllers (Alpha pH 560, Thermo scientific, USA) with pH electrodes (Eutech instrument, USA) and was automatically adjusted by adding 0.2 M NaOH solution through peristaltic pumps (505U, Watson Marlow, United Kingdom). To maintain an ammonia loading rate of 500 mg N l^{-1}d^{-1}, hydraulic retention time of the reactors was controlled around 1.4 days using peristaltic pumps (505U, Watson Marlow, United Kingdom). The reactors were operated under complete-mixing condition by stirring with mechanical mixer (R20, IKA, Germany) at 250 rpm, room temperature (28-30°C) and under dark condition.

3.2.4   *Chemical analysis*

The concentration of mixed liquor suspended solid (MLSS) was determined according to the standard method described in APHA (APHA, 2012). Water samples from the reactors were filtered through 0.45 µm GF/C filter paper before ammonium, nitrite, and nitrate concentrations were analyzed. Ammonium concentration was analyzed using an ion selective electrode (NH500, WTW, Germany) in accordance with APHA (APHA, 2012). Nitrite and nitrate concentrations were analyzed by colorimetric and UV spectrophotometric methods, respectively (APHA, 2012).

3.2.5   *Relative abundance of microorganisms*

Gel beads were collected from the reactors DO2 and DO3 on day 148 and dissolved completely in DI water at 70°C for 5 min. Genomic DNA was extracted using FastDNA® SPIN Kit for Soil (Qbiogene, USA). The extracted DNA (5-10 ng µl^{-1}) from reactor DO2 was PCR-amplified for 16S rRNA gene fragment using the primers for the V3 region 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG 3′ and V4 region 5′GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′ (Klindworth et al., 2013). The condition for performing PCR amplification was 3 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and a final extension of 5 min at 72°C. The primers *515F*

(5′ GTGYCAGCMGCCGCGGTAA 3′) (Ding et al., 2014) and 806R (5′GGACTACHVGGGTWTCTAAT 3′) (Caporaso et al., 2011). The condition for PCR amplification was 3 min at 95°C followed by 23 cycles of 30 s at 95°C, 30 s at 53°C, and 30 s at 72°C and a final extension of 5 min at 72°C. For both samples, the PCR mixture was prepared by *Taq* polymerase (Thermo Scientific, USA for DO3, and New England Biolabs, USA for DO 2) was the PCR reaction was performed in a thermal cycler (Biorad Laboratories, USA). The PCR product was purified by a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified PCR product was sent to the Omics Sciences and Bioinformatics Center, Chulalongkorn University, Thailand, for Illumina library construction and data analysis. Index was attached to the purified PCR product using 2X KAPA hot-start ready mix and 5 µl of each Nextera XT index primer in 50 µl reaction. The attached index product was then purified by AMPure XP beads (Beckman Coulter, USA), pooled and diluted to 4 pM. After an Illumina MiSeq was performed, quality of the sequencing reads was checked by FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), then assembling overlapping paired end reads by PEAR (Zhang et al., 2014). FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) was used to filter assembled reads that at least 90% of bases had a quality score of less than 30 and were shorter than 400 bp. Chimeras was removed using the UCHIME method (Edgar et al., 2011) as implemented in VSEARCH 1.1.1 (Rognes et al., 2016) using –uchime_ref option against chimera-free Gold RDP database. The *pick_open_reference_otus.py* command in QIIME 1.9.0 was employed to generate operational taxonomic unit (OTU). SortMeRNA was used for the reference picking, and SUMACLUST (https://git.metabarcoding.org/obitools/sumaclust/wikis/home) was used to *de novo* cluster the subsampled failure reads. Subsequently, the OTU sequences were taxonomically assigned to the Green gene database. In addition, OTUs that have less than 0.1% reads were excluded. Raw data sequences (for DO2) were deposited in the Sequence Read Archive (SRA) under the SRA accession number SRP133361.

3.2.6   Communities of nitrite-oxidizing bacteria

The gel beads collected from both reactors on day 148 were analyzed for NOB communities. The amplification of 635-based pair fragment of *Nitrobactor* 16S rRNA gene and 350-based pair fragment of the 16S rRNA gene of *Nitrospira* were carried out with the primer sets P338f (5′ ACTCCTACGGGAGGCAGCAG 3′) and NIT3 (5′ CCTGTGCTCCATGCTCCG 3′) (Jie and Daping, 2008), and the primer sets EUB338f (5′ACTCCTACGGGAAGC3′) and Ntspa0685r (5′GGGAATTCCGCGCTCCTGGC 3′) (Regan et al., 2002), respectively.. The PCR mixture was prepared using Takara polymerase (Takara Bio Inc., Japan). The PCR reaction was conducted using a thermal cycler (Biorad Laboratories, USA) for 5 min at 95°C, followed by 30 cycles for 1.5 min at 95°C, 30 s at 65°C, and 1 min at 72°C. The PCR product was purified by gel electrophoresis method using NucleoSpin Extract II Kit (Clontech Laboratories Inc., USA). The purified PCR product was cloned using the pGEM-T Easy vector system (Promega, USA). For each library, 25 to 30 clones were randomly selected for sequencing at Macrogen Inc. in Korea.

An arrangement of operational taxonomic units (OTUs) of the analyzed sequences was determined at 99% cutoff using CD-HIT (Huang et al., 2010a). The representative sequences of *Nitrobacter* and *Nitrospira* were separately aligned and phylogenetic trees of the two microorganisms were generated using MEGA7 program (Kumar et al., 2016) and ARB program package (http://www.arb-home.de).

3.2.7   Experimental framework and reactor setup
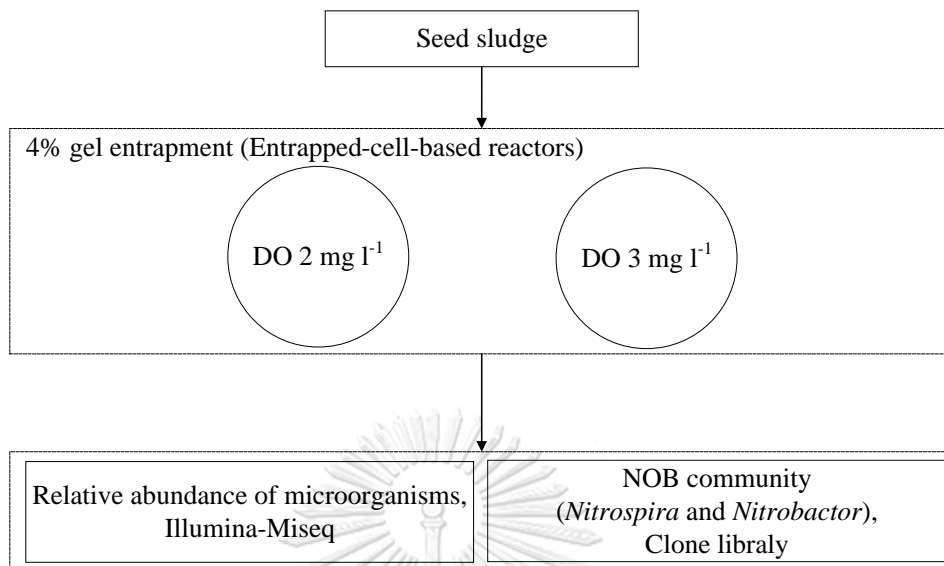
Figure 3. 1 Experimental framework
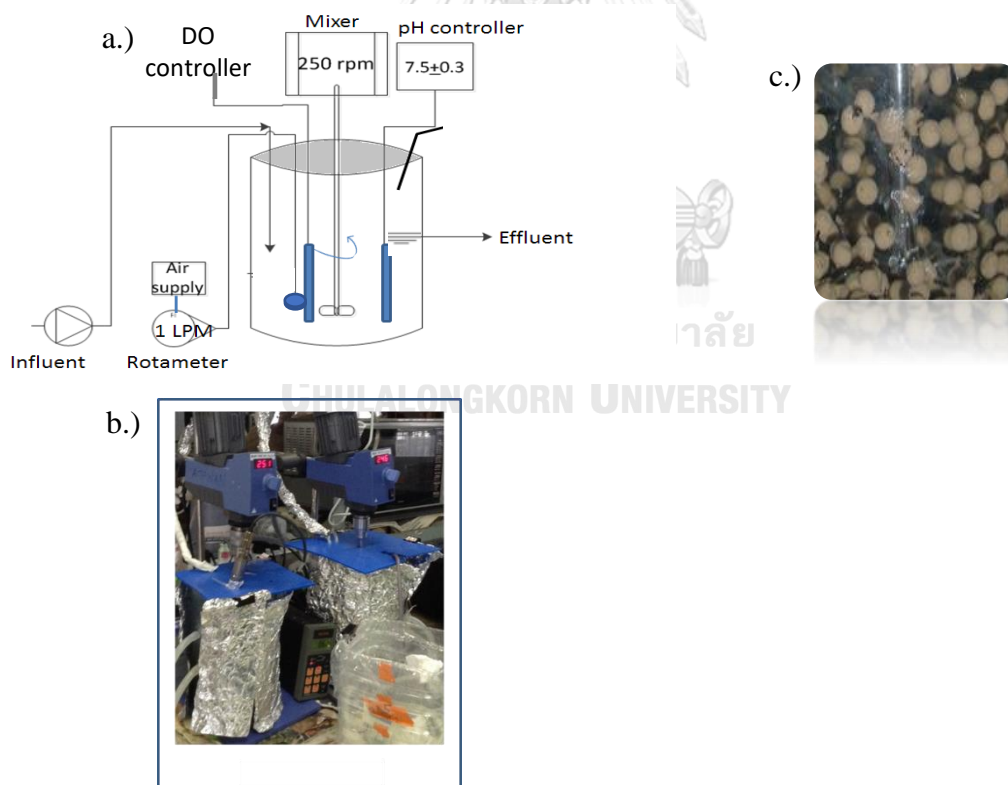
Figure 3. 2 Completely mixed reactor setup (a. and b.) and gel beads in a reactor (c.)

3.3   Results and discussion

3.3.1   Reactor performance

Figure 3.3 shows nitrogen concentrations in the entrapped-cell-based reactors during the operation periods of 165 days. At the start-up period, the ammonia removal efficiency was 49±9% and 46±13% and the nitrite accumulation was 50±19% and 59±21% for reactors DO2 and DO3, respectively. More than 60% of ammonia was removed and nitrite was accumulated after days 60 and 88 of reactors DO2 and DO3 operation, respectively. At the partial nitrification period, both reactors achieved similar degree of ammonia removal and nitrite accumulation, although both reactors were operated with different DO concentrations in bulk solutions. Ammonia removal was 68±12% and 65±12% for reactors DO2 and DO3, respectively. Nitrite accumulation of 65±9% and 66±23% can be maintained for reactors DO2 and DO3, respectively.
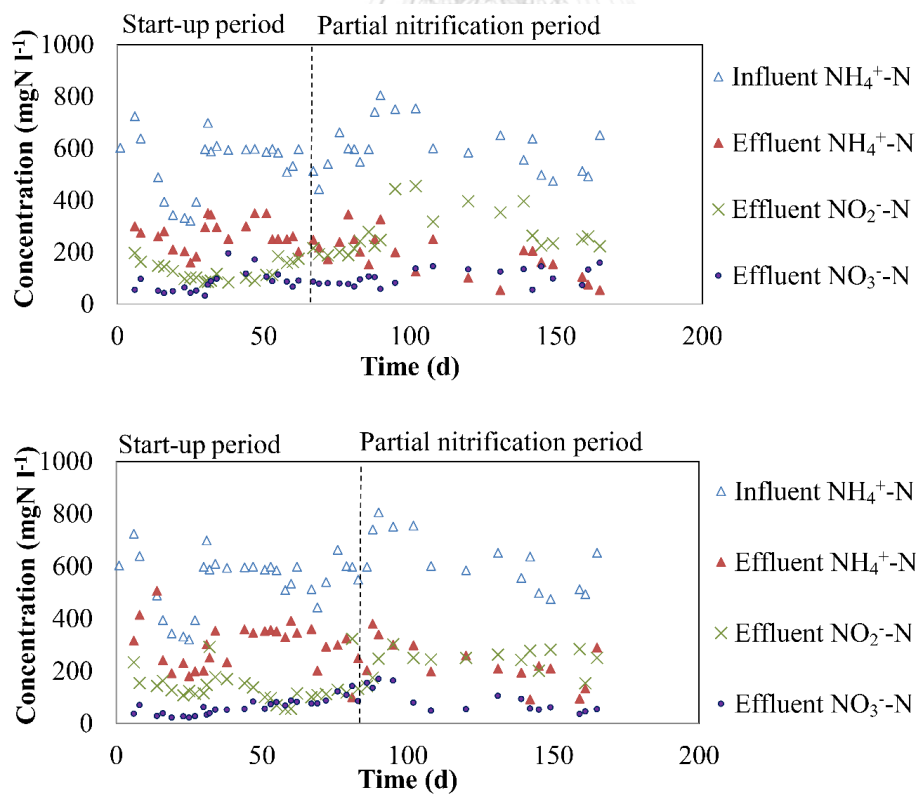
Figure 3. 3 Partial nitrification performance of reactors DO2 (up) and DO3 (down)

Both reactors were operated at temperature between 28 and 30 °C. This range of temperature lower than the range that partial nitrification can be promoted by high temperature (35 °C). Free ammonia (FA) concentrations were in a range of 3-16 mgN l^{-1} for reactor DO2 and were between 4 and 18 mg l^{-1} for reactor DO3. Vadivelu et al. (2007) reported that, in suspended cell systems, 12% of *Nitrobacter* activity decreased at FA concentration of 9 mgN l^{-1}. While, AOB in suspended cell system can be tolerant to FA levels of 10-150 mg l^{-1} (Anthonisen et al., 1976Anthonisen et al., 1976) reported that, in a suspended cell system, AOB and NOB began to be inhibited by FA levels of 10-150 mg l^{-1} and 0.1-1.0 mg l^{-1}, respectively. However, in an immobilized cell system, AOB and NOB may perform their activity under FA levels higher than those reported for a suspended cell system because the immobilized cell system may help reduce concentrations of toxicants that come in to contact to microorganisms' cells. Chen et al. (2015) suggested that NOB in aerobic granules were not affected by FA as high as 111 mg l^{-1}. Therefore, FA accumulation may not be the major factor that promoted partial nitrification in our reactor. The use of entrapped cells and maintaining DO concentrations in bulk solution of 2 and 3 mg l^{-1} were expected to be key features to promote partial nitrification in the reactor. Rongsayamanont et al. (2014) indicated that partial nitrification can be obtained using PVA entrapped-cell matrix at bulk DO concentrations of 2 and 3 mg l^{-1}. The entrapped-cell system may help limit oxygen diffusion and transfer inside the gel matrix and oxygen concentration gradient may be created along the depth of the matrix to generate oxygen-limiting zones that suited for partial nitrification. Rathnayake et al. (2013) and Song et al. (2013) demonstrated that oxygen-limiting zones were created inside aerobic granules and may support NOB inhibition.

Although partial nitrification can be achieved in both reactors, small levels of nitrate (101±3 mg l^{-1} for reactor DO2 and 79±44 mg l^{-1} for reactor DO3) were detected in both reactors. This indicated that NOB can still perform some nitrite-oxidizing activity although the operating condition limited their main performance.

3.3.2   Relative abundance of microorganisms

Figure 3.4 and 3.5 show the relative abundance of microorganisms at the phylum and family levels for reactors DO2 and DO3, respectively. Proteobacteria (83.70 % and 49.24 % for reactors DO2 and DO3) and Bacteroidetes (8.9 % and 27.27 % for reactors DO2 and DO3) were the most dominant phylum in the gel beads from both reactors.

Comamonadaceae was relatively highest abundant in family level (35.70%) in reactor DO2 while *Cytophagaceae* showed the highest abundant (23.04%) in reactor DO3. Some of both family members have been reported to be capable of denitrification (Khan et al., 2002; Tomasek et al., 2017; Alrashed et al., 2018; Chen et al., 2018). During the operations, both reactors showed some nitrogen loss of around 6±6% for reactor DO2 and 11±17% for reactor DO3. The relative abundance of *Nitrosomonadaceae* was 16.01% and 18.21% for reactors DO2 and DO3, respectively. The results indicated that AOB were found in relatively high abundant in both reactors. Regarding to NOB, *Bradyrhizobiaceae* were accounted as 10.40 % and 4.44% of the analyzed sequences of reactors DO2 and DO3, respectively. Because *Nitrobacter* are only a part of this family member, it cannot be implied directly that the relative abundance of *Bradyrhizobiaceae* found related to *Nitrobacter*. *Nitrospira* have been reported to be within the family *Nitrospiraceae*. Using Illumina Miseq technique, the family *Nitrospiraceae*, was not detected in both reactors.
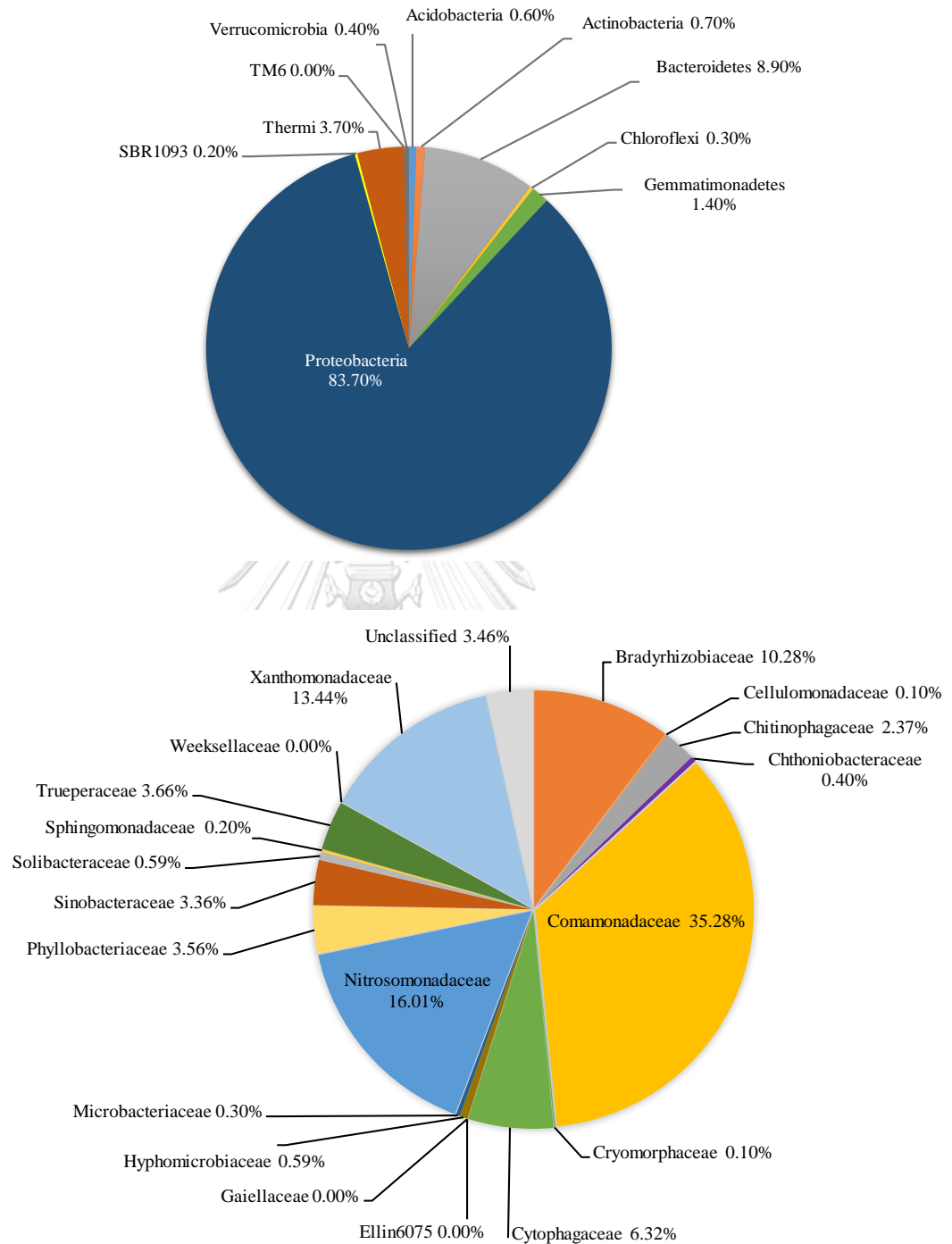
Figure 3. 4 Relative abundance of microorganisms at the phylum level (up) and family level (down) in gel matrix of reactor DO2
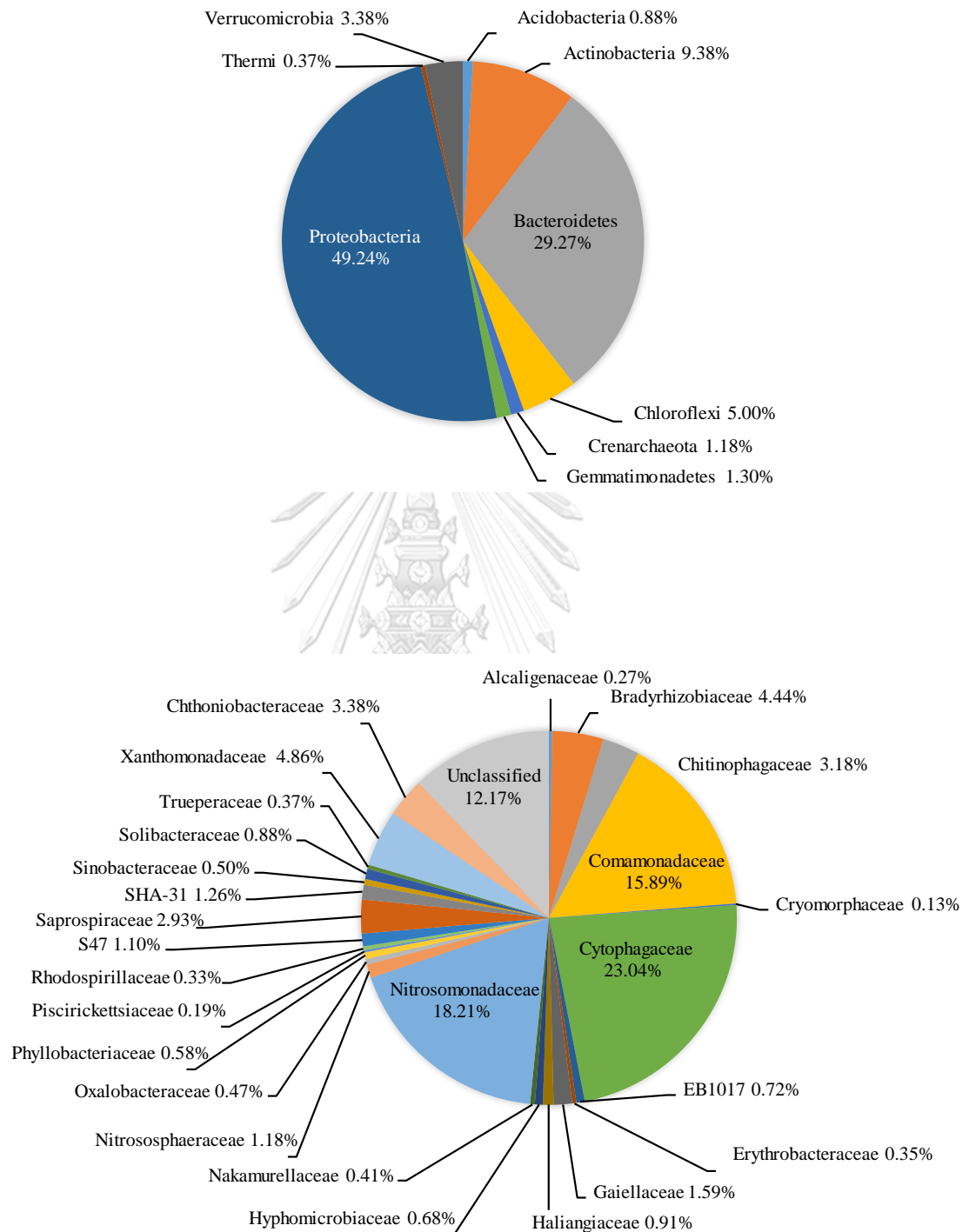
Figure 3. 5 Relative abundance of microorganisms at the phylum level (up) and family level (down) in gel matrix of reactor DO3

3.3.3   Communities of nitrite-oxidizing bacteria
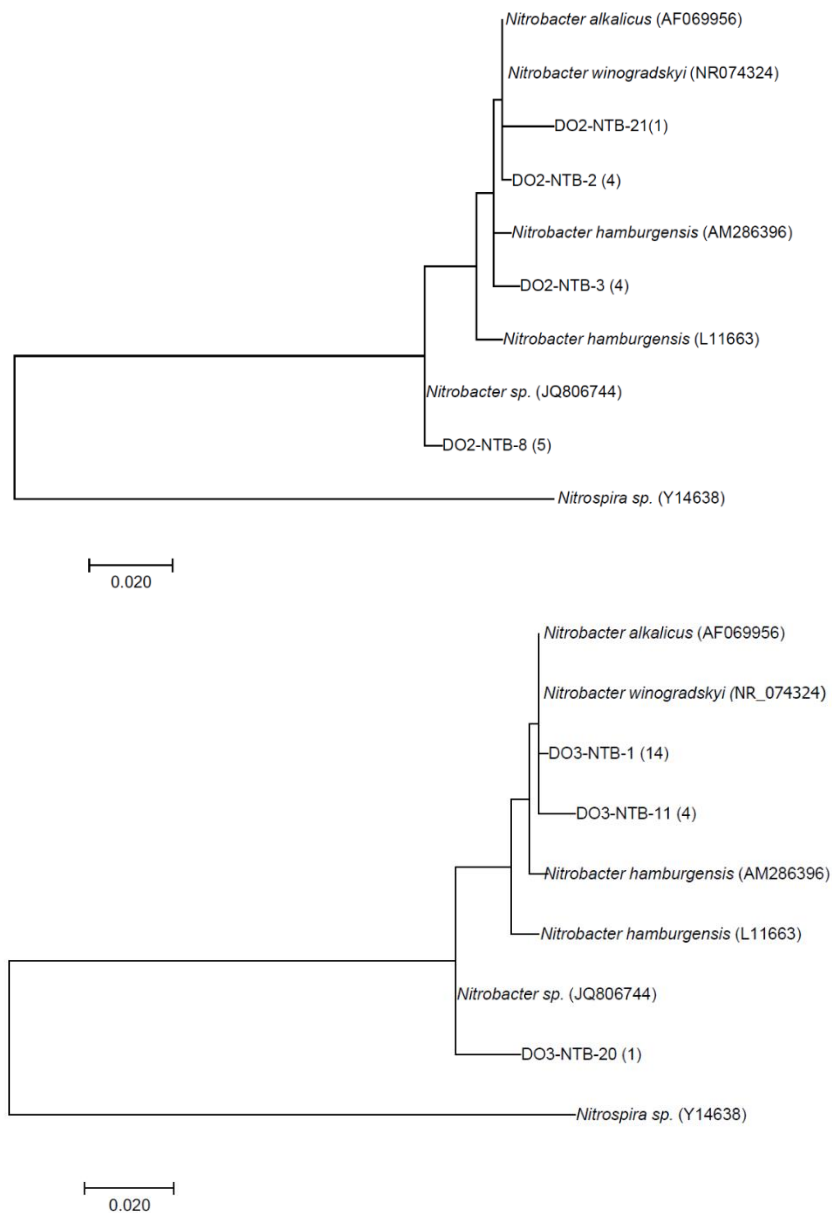
Figure 3. 6 Phylogenetic trees calculated based on 492 bp of the 16S rRNA gene sequences of *Nitrobacter* using the maximum likelihood method for reactors DO2 (up) and DO3 (down). The numbers in parentheses of the analyzed sequences represent numbers of sequences in the same OTUs.
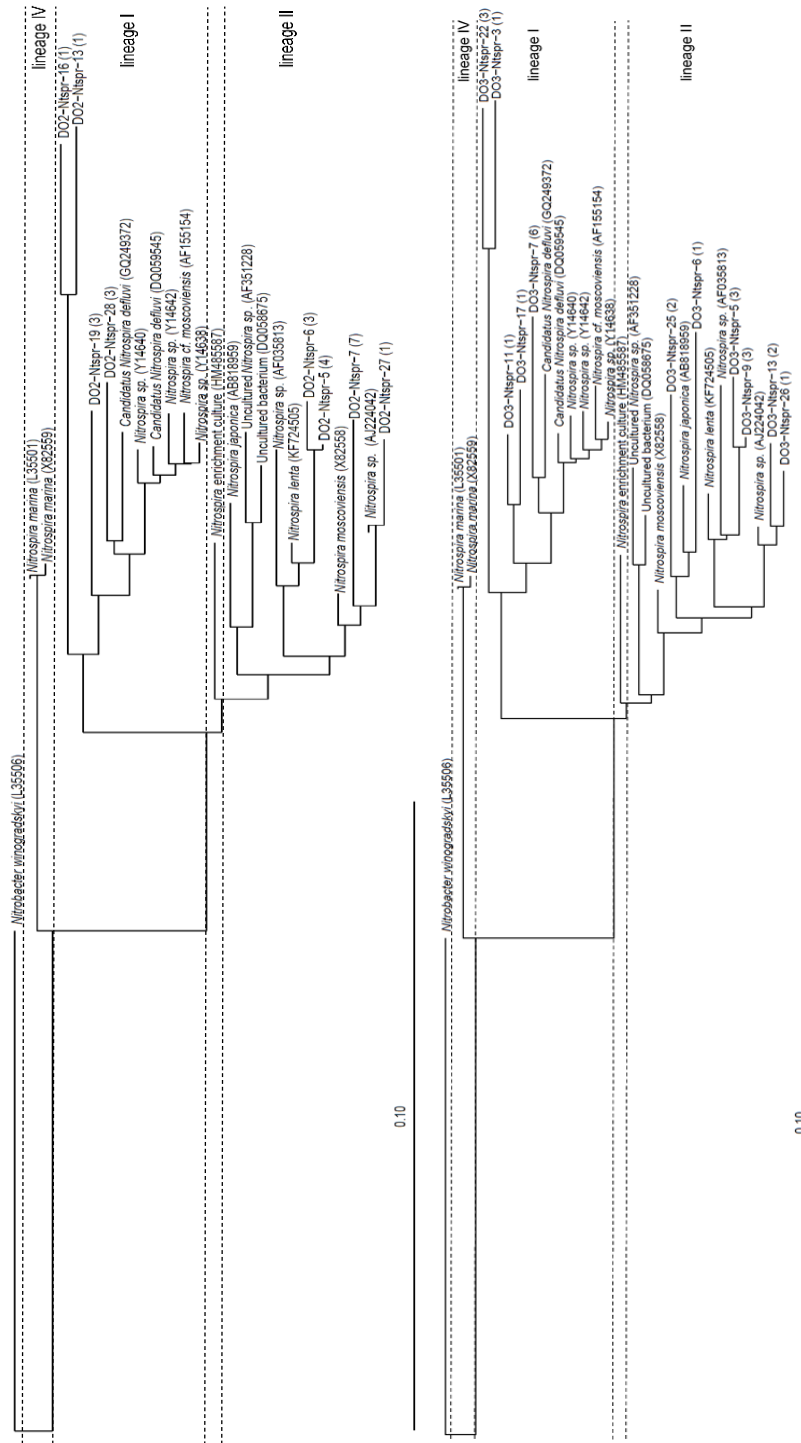
Figure 3. 7 Phylogenetic trees calculated based on the 16S rRNA gene sequences of *Nitrospira* from reactors DO2 (up) and DO3 (down). The trees were constructed by adding approximately 236-bp sequences using the parsimony method into the tree prior constructed with> 1400-bp sequences of reference *Nitrospira* using the neighbor joining method. The numbers in parentheses of the analyzed sequences represent numbers of sequences in the same OTUs.

During the reactor operation, small amounts of nitrate were detected indicating certain level of nitrite-oxidizing activity in the reactors. Because Illumina Miseq did not provide direct link to NOB communities in the reactors, clone libraries were constructed from the PCR-amplified products obtained using the specific primers targeting 16S rRNA gene fragments of *Nitrobacter* and *Nitrospira*.

Out of the 30 clones analyzed for reactors DO2 and DO3, 14 and 19 clones were identified as *Nitrobacter*, while the others were found to be non *Nitrobacter*, as analyzed by Blast (https://blast.ncbi.nlm.nih.gov/). Figure 3.4 shows the phylogenetic trees of *Nitrobacter* 16S rRNA gene sequences. For *Nitrospira*, 23 and 24 out of the 25 clones analyzed for reactors DO2 and DO3, respectively, were identified *Nitrospira*. The phylogenetic trees of *Nitrospira* 16S rRNA gene sequences are shown in Figure 3.6. The analyzed *Nitrospira* sequences fell within *Nitrospira* lineage I and II. Figures 3.6 and 3.7 indicated that the communities *Nitrobacter and Nitrospira* were similar in both reactors. This indicated that maintaining bulk DO concentrations at 2 and 3 mg l^{-1} did not affect the communities of *Nitrobacter and Nitrospira*.

Nitrobacter and *Nitrospira* are NOB that were found commonly in wastewater treatment systems (Daims et al., 2001; Whang et al., 2009). However, these two groups of NOB are different in substrate affinities. *Nitrobacter* have lower nitrite and oxygen affinities than *Nitrospira* (Schramm et al., 1999; Nowka et al., 2015). The half-satulation coefficient for nitrite (K_S) of *Nitrobacter* pure cultures was 0.69-7.6 mg-N l^{-1}, while *Nitrospira* pure cultures showed lower values of 0.13-0.38 mg-N l^{-1} (Nowka et al., 2015). The half-satulation coefficient for oxygen (K_O) in mixed cultures of *Nitrobacter* was 0.43-5.31 mg l^{-1} (Blackburne et al., 2008; Blackburne et al., 2007; Laanbroek et al., 1994). These values for *Nitrospira* was 0.33-0.54 mg l^{-1} (Blackburne et al., 2007; Park et al., 2017). The difference of K_S and K_O between *Nitrobacter* and *Nitrospira* allows them to be dominant in distinct environmental conditions. *Nitrobacter* can be found to be the dominant NOB in environments high in nitrite and oxygen, while *Nitrospira* can be dominant in lower nitrite and oxygen environments (Schramm et al., 1999; Schramm et al., 2000; Huang et al., 2010b).

Nitrospira can be divided into a few lineages, for examples lineages I, II, III, IV, V, and VI. *Nitrospira* lineage I and II were found in wastewater treatment bioreactors (Daims et al., 2001). Some studies suggested that *Nitrospira* lineage I and II may be different in oxygen affinity (Park and Noguera (2008)). Park and Noguera (2008) operated two chemostat reactors with low and high DO concentrations (0.12-0.24 and 8.5 mg l^{-1}, respectively) using seed sludge from the same source. *Nitrospira* lineage I were found in the low-DO reactor, while both *Nitrospira* lineage I and II were found in the high low-DO reactor. The results suggested that *Nitrospira* lineage I may have higher oxygen affinity than *Nitrospira* lineage II (Park and Noguera (2008)).

In the current study, *Nitrobacter* and *Nitrospira* lineage I and II were found to coexist in the gel beads although each group of NOB was reported to be distinct in substrate affinity. Using entrapped-cell system allows substrate concentration gradients to be formed along the depth of gel beads. Higher substrate concentration can be found at the gel bead surface and the concentrations decline along the distance toward the center of the beads. This provided environments of various substrate concentrations in the gel matrix allowing microorganisms with different substrate affinity to exist in the system. In addition to the substrate concentration gradient formed within the gel matrix, entrapped-cell matrix does not allow cells to be washed out from the reactors although some of those cells were slow in grow rate and activity. Therefore, in case of NOB, although the entrapped-cell matrix helped inhibit the majority of NOB activity, the entrapped-cell matrix provided, in the same time, some space for NOB to be survived and perform some activity.

It is noted that, in this current study, quantitative analysis of each group of NOB has not been performed. Therefore, the extent of the appearance of each group of NOB cannot be included in the discussion. In addition, some undiscovered NOB may coexist in the gel beads of the reactor and was not accounted for consideration. Also, some known NOB may be able to perform special function in nitrogen transformation in the gel beads. For example, recently, some members of *Nitrospira* have been reported to directly oxidize ammonia to nitrate as called "COMplete AMMonia OXidiser" (Comammox") (Daims et al., 2015; Van Kessel et al., 2015). These microorganisms

have been found in various sources, such as, hot water, recirculation aquaculture system, full-scale wastewater treatment and drinking water treatment systems (Daims et al., 2015; Van Kessel et al., 2015; Palomo et al., 2016; Pinto et al., 2016). All the points mentioned can be challenged regarding their contribution to nitrite oxidation in entrapped-cell based partial nitrification reactors.

3.4 Conclusions

Maintaining bulk DO concentrations of 2 and 3 mg l^{-1} did not provide any differences of partial nitrification performance. Partial nitrification can be achieved within 88 days of operation of both reactors. Therefore, operating at bulk DO concentration of 2 mg l^{-1} was selected for further parts of experiments. The operation of the reactor with bulk DO concentrations of 2 mg l^{-1} was extended to day 502 and used in the next experiment (Chapter 4). NOB communities were not different in the gel matrices from both reactors. *Nitrobacter* and *Nitrospira* lineage I and *Nitrospira* lineage II coexisted, although they have different substrate affinity. This indicated that entrapped-cell system allow NOB to be survived and perform some activity, although the majority of NOB activity was inhibited by the entrapped cells.

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CHAPTER 4

IN SITU OXYGEN CONCENTRATION GRADIENT IN PHOSPHORYLATED POLYVINYL ALCOHOL GEL MATRICES WITH DIFFERENT CELL-TO-MATRIX RATIOS IN ENTRAPPED-CELL-BASED PARTIAL NITRIFICATION REACTORS

4.1   Introduction

Shortcut nitrogen removal provides benefits in term of cost effectiveness and environmental impact for treating nitrogen-containing wastewater. The process helps reduce energy consumption, organic carbon requirement, and greenhouse gas emission as compared to conventional nitrogen removal scheme. The first step in shortcut nitrogen removal is partial nitrification, which relies on the oxidation of ammonia to nitrite without further oxidation to nitrate. This allows shortcut nitrogen removal to reduce oxygen requirement in nitrite oxidation by 25% as compared to complete nitrification Turk and Mavinic, 1989. The key factor in promoting partial nitrification is to concurrently promote ammonia-oxidizing bacteria (AOB) activity and suppress nitrite-oxidizing bacteria (NOB) activity to avoid nitrite oxidation into nitrate.

Partial nitrification can be maintained by controlling operational conditions, such as low oxygen, high pH, or high temperature environments. Among these 3 strategies, maintaining an oxygen-limited condition is the most attractive because it requires no additional energy or chemicals and is applicable for a broader range of wastewater types. Under an oxygen-limited condition, AOB has a higher degree of competitiveness for oxygen than NOB, resulting in the inhibition of NOB activity while AOB activity can still be maintained (Laanbroek and Gerards, 1993; Laanbroek et al., 1994; Wiesmann, 1994; Ciudad et al., 2006; Rongsayamanont et al., 2010; Zeng et al., 2013)

Partial nitrification with oxygen-limiting strategy can be carried out in either suspended- or attached-growth systems. For suspended-growth system, partial nitrification can occur when bulk dissolved oxygen (DO) concentrations are in the range of 0.5-1.5 mg l^{-1} (Fudala-Ksiazek et al., 2014). However, sludge bulking may be a problem when operating within this low oxygen concentration range (Ge et al., 2015) The bulk DO concentration range can be higher (1.0-5.0 mg l^{-1}) when an attached-growth system is employed (Kim et al., 2003; Fux et al., 2004; Ciudad et al., 2005; Rajagopal and Béline, 2011; Rongsayamanont et al., 2014; Liu et al., 2017), and this provides higher flexibility for long-term operations.

An entrapped-cell-based reactor, a type of an attached-growth system, provides quick achievement of partial nitrification when operated under oxygen-limited conditions, with the added benefits of high sludge retention time, easy sludge separation, and high chemical tolerance. Partial nitrification by cell entrapment in a phosphorylated polyvinyl alcohol (PPVA) gel matrix had previously been achieved at bulk DO concentrations of 2 and 3 mg l^{-1} (Rongsayamanont et al., 2014). Although partial nitrification with the aid of the PPVA gel matrix had been demonstrated, some essential features within the gel matrix, such as its 'oxygen concentration gradient', had never been explored. This work, therefore, aims to 1) investigate the effect of cell-to-matrix ratio on startup and long-term-operation performance of entrapped-cell-based partial nitrification reactors and 2) clarify microenvironment as well as microbial community and localization within the gel matrix during long-term.

4.2 Material and methods

4.2.1 Preparation of entrapped cells

Entrapped cells were prepared using the PPVA method as outlined by Chen and Lin (1994). Mixed liquor was taken from an aeration tank of an activated sludge system in a municipal wastewater treatment plant and was concentrated through centrifugation at 5000 rpm for 15 min. Aliquots of concentrated sludge containing 10 and 40 g of suspended solid (SS) were mixed with 1 l polyvinyl alcohol (PVA) gel solution (10 g PVA per liter of de-ionized (DI) water) to achieve initial 1% and 4% w/v cell-to-matrix ratios. These mixtures were then dropped into boric acid solution at a flow rate of 0.83

ml min^{-1} to form the spherical gel matrices which were then immersed in 1 M phosphate buffer pH 7.0 for 2-3 h to harden the gel matrices. Normally, diameter of gel bead varied between 4-6 mm. When gel bead was in the reactors, the diameter increased to 6-8 mm.

4.2.2 Synthetic wastewater

Synthetic wastewater was prepared with $(NH_4)_2SO_4$ (3.3 g), Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO_4 \bullet 7H_2O$ (0.05 g), $CaCl_2 \bullet 2H_2O$ (0.01 g), $FeSO_4 \bullet 7H_2O$ (0.09 g), H_3BO_3 (30 mg), $MnCl_2 \bullet 4H_2O$ (0.1 g), $CoCl_2 \bullet 6H_2O$ (0.19 g), $NiCl_2 \bullet 6H_2O$ (0.024 g), $CuCl_2 \bullet 2H_2O$ (0.02 g), $ZnSO_4 \bullet 7H_2O$ (0.144 g), $Na_2MoO_4 \bullet 2H_2O$ (0.36 g), and $NaHCO_3$ (6.89 g) in one liter of DI water (modified from Widdel and Bak, 1992; Rongsayamanont et al., 2010) . The resultant ammonia concentration in the synthetic wastewater was equivalent to 700 mg N l^{-1}.

4.2.3 Setup and operation of entrapped-cell-based reactors

Two continuous stirred tank reactors with an effective volume of 2 l were inoculated with the prepared entrapped cells with cell-to-matrix ratios of 1% and 4% w/v. The final concentration of cells in both reactors was equivalent to 2000 mg SS. DO concentration in the reactors was maintained at 2 mg l^{-1} (Rongsayamanont et al., 2014) using DO controllers (HI8410 DO controller, Hanna, USA). Air flow rate was controlled at 1 l min^{-1} by an air valve, air pump, and diffuser. pH in the bulk solution was controlled at 7.8 ± 0.2 to ensure that partial nitrification occurred in oxygen-limited condition only. pH was controlled by pH controllers (Alpha pH 560, Thermo scientific, USA) with pH electrodes (Eutech instrument, USA) and was adjusted by injections of 0.2 M NaOH solution with peristaltic pumps (505U, Watson Marlow, United Kingdom). Hydraulic retention time was controlled at around 1.4 days by peristaltic pump (505U, Watson Marlow, United Kingdom) to maintain an ammonia loading rate of 500 mg N l^{-1}d^{-1}. Complete-mixing condition was sustained in the reactors by stirring with mechanical mixer (R20, IKA, Germany) at 250 rpm. Reactors were operated at room temperature (28-30°C) under dark condition.

4.2.4   Chemical analysis

The concentration of mixed liquor suspended solid (MLSS) was determined gravimetrically according to the standard method described in (APHA, 2012). Before ammonia, nitrite, and nitrate concentrations were analyzed, samples were filtered through 0.45 µm GF/C filter paper. Ammonia concentration was analyzed using an ion selective electrode (NH500, WTW, Germany) in accordance (APHA, 2012). Nitrite and nitrate concentrations were analyzed by colorimetric and UV spectrophotometric methods, respectively (APHA, 2012).

4.2.5   In situ oxygen concentrations in gel matrix

In situ oxygen concentrations in the gel matrix were analyzed by an oxygen microsensor with a tip diameter of 8-12 µm (OX-10, Unisense, Denmark) held on a micromanipulator (MM 33, Märzhäuser wetzlar, Germany) and motorized stage (Ex 30F20, Jenny Science, Switzerland). The microsensor was connected to a microsensor monometer (Unisense, Denmark). With the help of the micromanipulator and motorized stage, the microsensor was placed into the gel matrix to take measurements at every 10 µm from the surface of the gel matrix under stereomicroscope. Oxygen uptake rate (OUR) was determined for each oxygen gradient profile using Fick's second law of diffusion with an oxygen diffusion coefficient for PVA of 5×10^{-7} cm^2 s^{-1} Tamai and Tanaka, 1998.
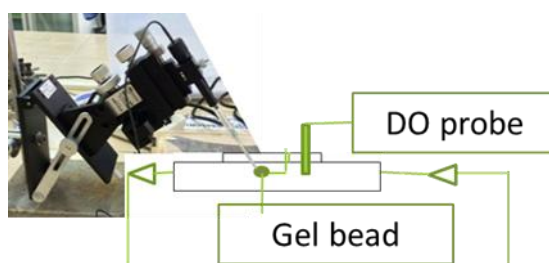
Figure 4. 1 Oxygen microprobe set up

4.2.6   Scanning electron microscope (SEM)

Samples of gel matrices were collected from the reactors for SEM analysis. Samples were first rinsed with phosphate buffer solution, then fixed by 0.1 M phosphate buffered saline (PBS) in 50% ethanol solution, and finally soaked in liquid nitrogen and a series of ethanol solutions. The samples were cut, placed on a stub, and coated under vacuum with gold. The samples were observed under SEM (JEOL, JSM-5410LV, Tokyo, Japan).

4.2.7   Microbial communities

Gel beads were collected from the 1% and 4% cell-to-matrix ratio reactors, dissolved completely in DI water at 70°C for 5 min, then genomic DNA was extracted from gel matrices using FastDNA® SPIN Kit for Soil (Qbiogene, USA). The extracted DNA (5-10 ng µl^{-1}) was Polymerase Chain Reaction (PCR)-amplified for 16S rRNA gene fragment using the primers for the V3 region 5′ TCGTC GGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 3′ and V4 region 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTAC HVGGGTATCTAATCC 3′ (Klindworth et al., 2013). The PCR mixture was prepared using *Taq* polymerase (New England Biolabs, USA) and performed in a thermal cycler (Biorad Laboratories, USA). The PCR amplification was carried out for 3 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C and a final extension of 5 min at 72°C. Each sample was PCR amplified in triplicate, then pooled and purified using a NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). Illumina library preparation and bioinformatics analysis were proceeded through the Omics Sciences and Bioinformatics Center, Chulalongkorn University, Thailand. Briefly, index was added to the purified PCR product using 2X KAPA hot-start ready mix and 5 µl of each Nextera XT index primer in 50 µl reaction. The condition was carried out for 3 min at 94°C, followed by 8 cycles of 20 s at 98°C, 30 s at 55°C, 30 s at 72°C, and 5 min at 72°C. The indexed amplicon was purified by AMPure XP beads (Beckman Coulter, USA), then pooled and diluted to 4 pM. Cluster generation and 300-bp paired-end read sequencing were carried out by an Illumina MiSeq. FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was

employed for checking the quality of sequencing reads and PEAR (Zhang et al., 2014). was used for assembling overlapping paired end reads. Assembled reads that at least 90% of bases had a quality score of less than 30 and were shorter than 400 bp were filtered out using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit). Chimeras removal was conducted using the UCHIME method (Edgar et al., 2011) as implemented in VSEARCH 1.1.1 (Rognes et al., 2016) using –uchime_ref option against chimera-free Gold RDP database. The *pick_open_reference_otus.py* command in QIIME 1.9.0 was implemented for operational taxonomic unit (OTU) picking, specifying that SortMeRNA was used for the reference picking. Taxonomy was assigned against the SILVA database.

The subsampled failure reads were clustered de novo with SUMACLUST (https://git.metabarcoding.org/obitools/sumaclust/wikis/home). Then, OTUs that have less than 0.1% reads were excluded. Raw data sequences were deposited in the Sequence Read Archive (SRA) under the SRA accession number SRP133361.

4.2.8 *Spatial distribution of total bacteria, AOB, and NOB in gel matrix*

Spatial distribution of heterotrophs, AOB, and NOB in gel matrix was investigated using fluorescence *in situ* hybridization (FISH) technique. Gel matrix was first suspended in an ice-cold PBS solution. Then, the gel matrix was transferred and fixed in a paraformaldehyde solution (4% in PBS at pH 7.4) at 4°C for 16 h. The fixed sample was embedded in a Tissue-Tek OCT compound (Sakura Finetek, USA Inc.) and cut into 10 μm sections at -20°C using a cryomicrotome (Leica CM 1950) and then immobilized onto a poly-L-lysine coating slide. The slide was air-dried and dehydrated by 50%, 80% and 98% v/v ethanol (3 min each), respectively.

In this study, commercially synthesized oligonucleotide probes which were fluorescently labeled at the 5' end by Alexaflour 488 or CY3 (ThermoHybaid, Ulm, Germany) were used. Oligonucleotide probes were hybridized with the target 16S rRNA genes at 46°C for 2 h in a buffer solution (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), formamide) containing 5 ng of probe μl^{-1}. The sections of gel matrix were immersed into a pre-heated washing buffer (20 mM Tris-HCl, 0.01%

SDS, NaCl) at 48°C for 15 min and then subsequently rinsed with DI water, dried, and mounted with an anti-fading solution (SlowFade, Antifade kit, Molecular Probes, Eugene, OR, USA). For simultaneous hybridization, two hybridizations were performed successively with the probe having higher stringency was performed first (Wagner et al., 1996). Confocal laser scanning microscopy (CLSM) (FluoView FV10i, Olympus, Japan) was used to observe the hybridized samples, with the corresponding FISH-CLSM images processed by FluoView FV10i-Viewer version 4.2 (Olympus, Japan). The Alexaflour 488–labeled probe was visualized by excitation between 460 and 495 nm and the collection of fluorescence emission at 510 nm. The Cy3-labelled probe was excited between 530 and 550 nm and its fluorescence emission was collected at 575 nm. Table 4.1 shows the oligonucleotide probes and their corresponding hybridization conditions used in this study.

Table 4. 1 Oligonucleotide probes for analyzing spatial distribution of microorganisms in gel matrix

Probe	Sequence (5' to 3')	Label	Target organisms	Formamide (%)
EUB 338	GCTGCCTCCCGTAGGAGT	CY3	Most bacteria	15
Nso190	CGATCCCCTGCTTTTCTCC	AF	Many but not all ammonia oxidizing *β-Proteobacteria*	55
Ntspa 662	GGAATTCCGCGCTCCTCT	Cy3	*Nitrospira* genus	35
Nit3	CCTGTGCTCCATGCTCCG	Cy3	*Nitrobacter spp.*	40

4.2.9   Experimental framework

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┌─────────────────────┐
│     Seed sludge     │
└─────────────────────┘
           │
           ▼
┌─────────────────────────────────────────────────────────────────────┐
│ Entrapped-cell-based reactors                                        │
│                                                                       │
│         ╱‾‾‾‾‾‾╲                      ╱‾‾‾‾‾‾╲                         │
│        │ 1% w/v │                    │ 4% w/v │                        │
│        │ of gel │                    │ of gel │                        │
│        │entrapment│                  │entrapment│                      │
│         ╲_____╱                      ╲_____╱                         │
└─────────────────────────────────────────────────────────────────────┘
           │
           ▼
┌─────────────────────────────────────────────────────────────────────┐
│ ┌──────────────────┐ ┌──────────────────┐ ┌──────────────────┐       │
│ │ O₂ concentration │ │ Spatial distribution│ │                  │     │
│ │gradient along the│ │(Total bacteria, AOB,│ │Relative abundance of│  │
│ │  depth of gel    │ │  Nitrospira and   │ │  microorganisms, │     │
│ │    beads,        │ │   Nitrobacter),   │ │  Illumina-Miseq  │       │
│ │  O₂ microsensor  │ │  FISH technique   │ │                  │       │
│ └──────────────────┘ └──────────────────┘ └──────────────────┘       │
└─────────────────────────────────────────────────────────────────────┘
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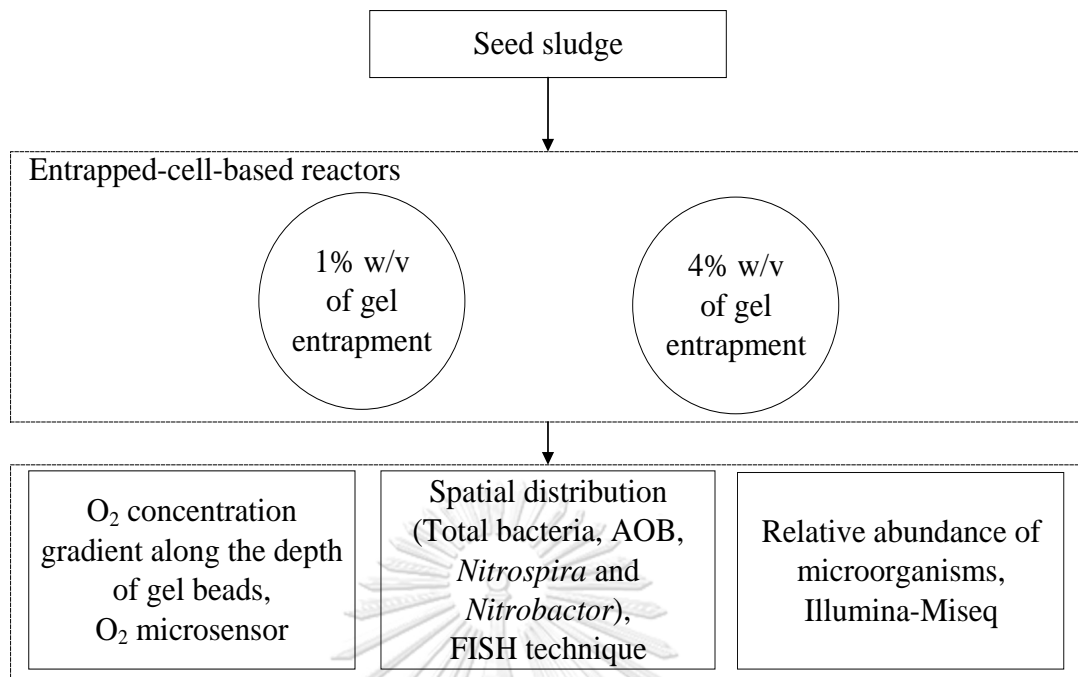
Figure 4. 2 Experimental framework

4.3   Results and discussion

4.3.1   Reactor performance

The entrapped-cell-based reactors containing gel matrices of 1% and 4% cell-to-matrix ratios were monitored for 362 and 502 days, respectively (Figure 4.3). A slightly higher ammonia removal efficiency was observed for the reactor with 1% cell-to-matrix ratio ($62.4\pm7.3\%$) as compared to the reactor with 4% cell-to-matrix ratio ($54.4\pm13.3\%$). The reactor with 4% cell-to-matrix ratio required less time (60 days) to achieve partial nitrification as defined by >50% nitrite accumulation (nitrite accumulated per ammonia removed) than the reactor with 1% cell-to-matrix ratio (184 days). This indicated that the entrapped cell with higher cell-to-matrix ratio can accelerate partial nitrification, while ammonia oxidation was not much reduced (Figure 4.3).

Although the initial cell-to-matrix ratio determined the success of partial nitrification at the early state of operation, this parameter did not affect degree of partial nitrification during long-term operation. Nitrite accumulation in the reactor with 1% cell-to-matrix ratio increased from around 15 – 35% before day 184 (Figure 4.3) to $58.7\pm18.2\%$ after day 184, which was comparable to the reactor with 4% cell-to-matrix ratio ($61.1\pm16.8\%$). Reason behind the increase in nitrite accumulation of the reactor with 1% cell-to-matrix ratio is described in the following section. From a practical point of view, cell entrapment with 4% cell-to-matrix ratio is more preferable to the 1% cell-to-matrix ratio as it can shorten the startup period of the reactors.
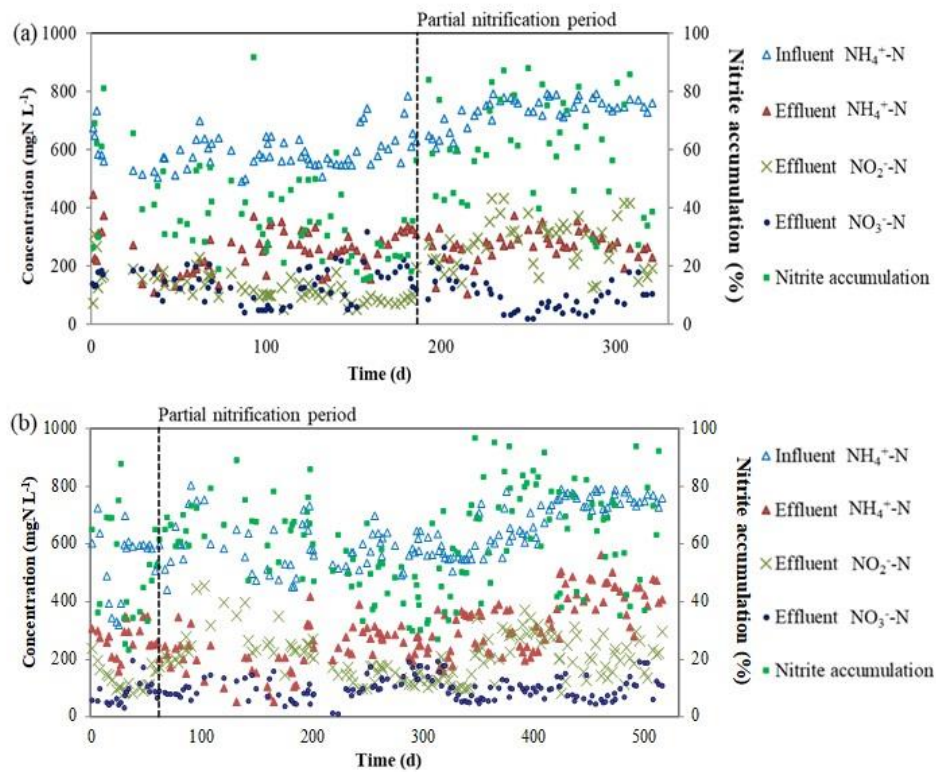
Figure 4. 3 Performance of entrapped-cells-based reactors containing gel matrices of (a) 1% and (b) 4% cell-to-matrix ratios.

As shown in Table 4.2, the ammonia removal efficiencies obtained in this study's reactors are comparable to those reported for other attached-growth reactors. Those attached-growth reactors gained benefits from temperature of $\geq 30^{\circ}$C or bulk DO concentrations of < 2 mg l^{-1} to promote partial nitrification (Okabe et al., 2011a; Park et al., 2014; Ali et al., 2016). Entrapped-cell-based reactors were superior to other attached-growth systems in achieving stable nitrite accumulation at room temperature and bulk DO concentration than 2 mg l^{-1}. Fux et al. (2004) suggested the difficulties in maintaining long-term partial nitrification in attached-growth systems due to the nature of the attached-growth systems that do not allow NOB cells to be washed out from the reactors, though NOB inhibiting environment was provided. Yang et al. (2013) reported a gradually switching from partial nitrification to full nitrification in a pulse-feed anoxic/oxic granular sludge reactor after 111 days of operation. Recovering the activity of the retained NOB cells in some periods of oxygen fluctuation above the oxygen-limiting concentration for NOB activity was suggested to be the main cause of this

failure. In the current work, stable partial nitrification can be maintained over $1 - 1.5$ years of operation using the entrapped-cell-based reactors.

Table 4. 2 Long-term operational performance of partial nitrification reactors with attached-growth systems

Reactor[a]	DO (mg l⁻¹)	Temp (°C)	Influent NH₃ (mg N l⁻¹)	ALR[c] (kg N m⁻³ d⁻¹)	NH₃ removal (%)	NO₂⁻ accumulation (%)	Operation (d)	References
AGS	-	30	450	1.8	99	99	224	Liu et al. (2008)
AGS	2.7	18-24	400	1.6	50	99	880	Vázquez-Padín et al. (2010)
AGS	5.6-6.7	28	100	0.3	99	80	250[f]	Yang et al. (2013)
CSGS	0.3-1.7	33-35	350	4.07	99	85	253	Ali et al. (2016)
CFBR	0-0.8	35	270	1.6	50	99	400	Okabe et al. (2011)
CFBR	2.5	30	150	0.3	40	99	200	Park et al. (2014)
EBR	2–2.5	r.t.[b]	700	0.5	62.4[d] 54.4[e]	58.7[d] 61.1[e]	362[d] 502[e]	This study This study

[a] AGS = Aerobic granular sludge reactor; CSGS = Combined suspended and granular sludge reactor; CFBR = Continuous flow biofilm reactor; EBR = Entrapped-cell-based reactor; [b] r.t. = room temperature, [c] ALR = ammonia loading rate; [d] EBR with 1% cell-to-matrix ratio; [e] EBR with 4% cell-to-matrix ratio; [f] unintentionally being converted to full nitrification after the first 111 days of operation.

4.3.2 *Oxygen concentration gradient, oxygen uptake rate, and gel matrix structure*

Gel matrices were collected from the reactors with 1% and 4% cell-to-matrix ratios to determine the oxygen concentration gradient and OUR along the depth of gel matrices after partial nitrification was achieved. Figure 4.4 shows the profiles of oxygen concentrations inside the gel matrices collected from the reactor with 1% cell-to-matrix ratio on day 289 and 294, and the reactor with 4% cell-to-matrix ratio on day 479 and 488. The DO concentrations in bulk solution of both reactors were controlled between 2.0-2.5 mg l⁻¹ during the examination of oxygen concentration gradient.

The gel matrix with 1% cell-to-matrix ratio exhibited different patterns of oxygen concentration gradient profiles on day 289 and 294 (Figure 4.4a). Oxygen concentrations started from 1.9±0.2 mg l⁻¹ at the surface of the gel matrices. However, oxygen available zones (>0 mg l⁻¹) arose down to approximately 120 and 320 µm from

the surface for the first and second patterns, respectively. The oxygen concentrations reached the levels of 0.5-1.5 mg l^{-1}, the so called 'oxygen-limiting zone', at around 10-50 µm and 40-230 µm from the surface for the first and second patterns, respectively. The gel matrix with 4% cell-to-matrix ratio showed oxygen concentrations of 1.88±0.07 mg l^{-1} at the surface (Figure 4.4d), and penetrated down to 167.5±48.9 µm from the surface while the oxygen-limiting zone occurred at around 20-110 µm from the surface. Positive values of OUR (shown in Figure 4.4b, c, and e) indicated that the formation of oxygen concentration gradients within the gel matrix is contributed by not only oxygen diffusion, but also utilization of oxygen through microbial respiration.

Oxygen-limiting condition and high free ammonia (FA) concentrations were considered as two main factors promoting partial nitrification in the reactors. The analysis of *in situ* oxygen concentrations in gel matrices showed that oxygen limiting zones were created inside the gel matrices collected from both reactors. Numerous research have reported that the oxygen concentration range of 0.5-1.5 mg l^{-1} strongly limits NOB activity while allowing AOB activity (Ruiz et al., 2003; Ma et al., 2009; Fudala-Ksiazek et al., 2014; Yao et al., 2017). FA concentrations in bulk solution were in the ranges of 3-13 and 3-27 mg N l^{-1} in the reactors with 1% and 4% cell-to-matrix ratios, respectively. Previous studies have reported that for suspended-growth system, AOB activity can be suppressed by bulk FA concentrations of 10-150 mg N l^{-1} (Anthonisen et al., 1976; Yao et al., 2017), while NOB activity can be inhibited by lesser bulk FA concentrations of 0.1-4.0 mg N l^{-1} (Anthonisen et al., 1976; Bae et al., 2001). In attached-growth system, both microorganisms' activities were more resistant to bulk FA concentrations than suspended-growth system because of a reduction in inhibitory effects by cell entrapment. AOB and NOB which grow in biofilm systems can tolerate bulk FA concentrations than suspended-growth system because of a reduction in inhibitory effects by cell attachment and entrapment. AOB and NOB which grew in biofilm systems can tolerate bulk FA concentrations of up to 15 mg N l^{-1} (Park et al., 2014) and 111 mg N l^{-1} (Chen et al., 2015). Therefore, partial nitrification was achieved in both reactors as a result of oxygen-limiting conditions created inside the gel matrices rather than the inhibition by the presence of bulk FA.

It is likely that it was harder for oxygen to migrate into the inner parts of the gel matrices with 4% cell-to-matrix ratio as compared to the gel matrices with 1% cell-to-matrix ratio (Figure 4.4a, c). SEM images (Figure 4.5) showed that the gel matrix with 4% cell-to-matrix ratio (collected on day 515) was filled by a denser layer of microorganisms and debris around the surface (between the red lines in Figure 3), while the gel matrix with 1% cell-to-matrix ratio (collected on day 321) showed a looser layer of microorganisms and debris and contained more holes and spaces around the surface. The difference in gel matrix structure may have caused the distinct patterns of oxygen concentration gradients created inside the gel matrices. This may explain why partial nitrification was achieved faster in the reactor with 4% cell-to-matrix ratio (60 days) than the reactor with 1% cell-to-matrix ratio (184 days) (Figure 1). The faster partial nitrification achieved in the reactor with 4% cell-to-matrix ratio is most likely caused by less oxygen being available inside the gel matrix due to the greater density of cells which led to higher microbial respiration and lesser surface area for oxygen diffusion.
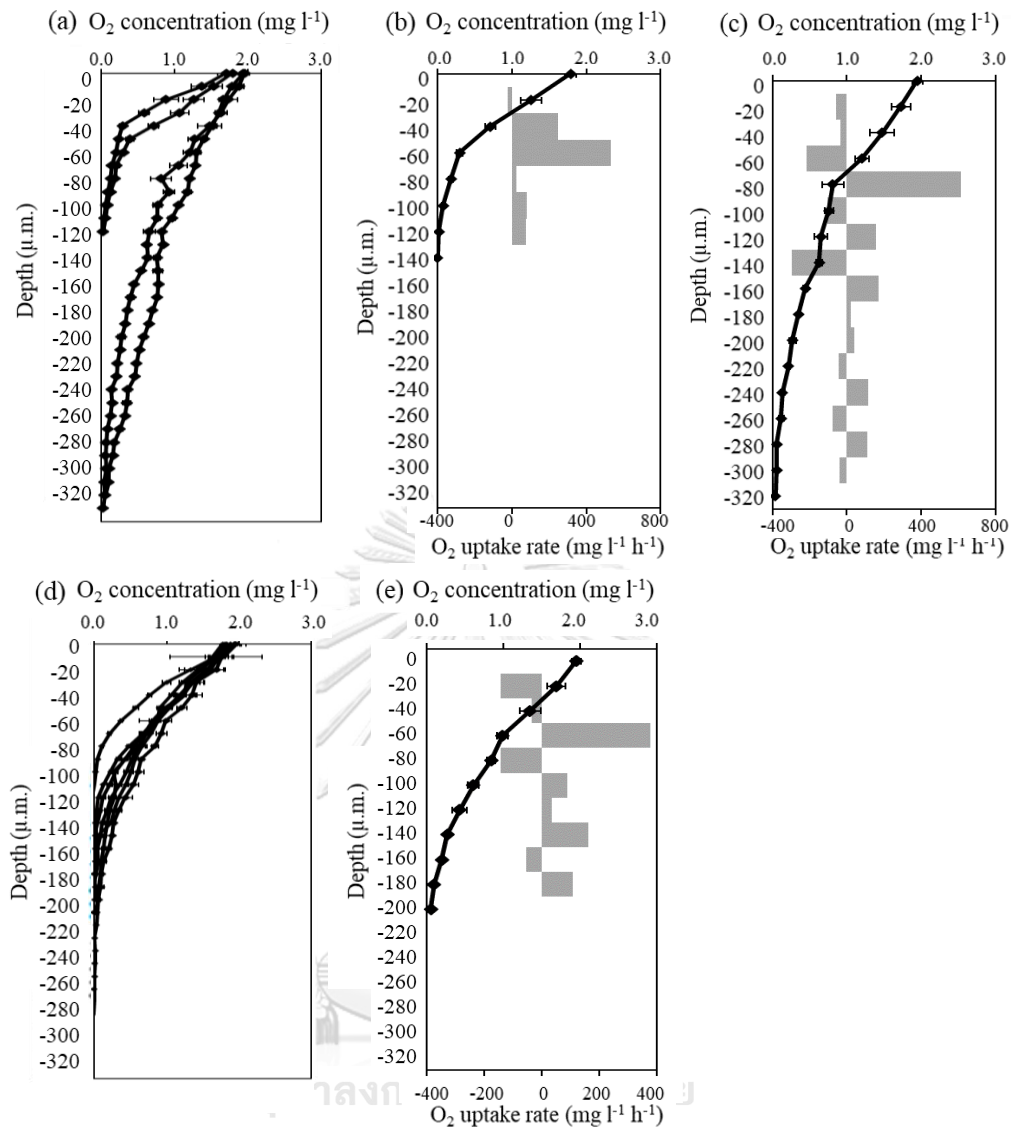
Figure 4. 4 Oxygen concentration gradients (line) and OURs (bar) along the depth of gel matrix. (a) oxygen concentration gradients and (b, c) selected OUR in gel matrix of 1% cell-to-matrix ratio collected on day 289 and 294. (d) oxygen concentration gradients and (e) selected OUR in gel matrix of 4% cell-to-matrix ratio collected on day 479 and 488.
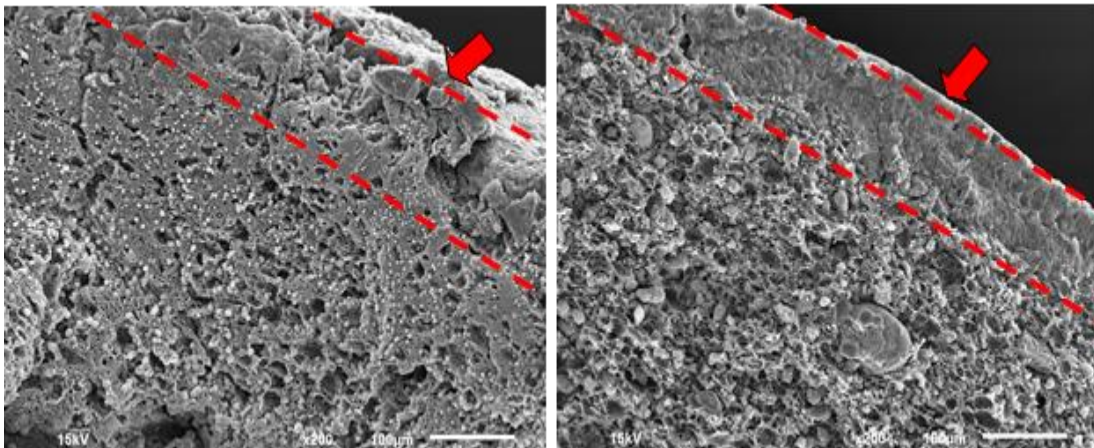
Figure 4. 5 SEM images showing the structure of gel matrices of (a) 1% cell-to-matrix ratio collected from the reactor on day 321, and (b) 4% cell-to-matrix ratio collected from the reactor on day 515 (×200)

4.3.3 Microbial communities

Analyzes were performed on the microbial communities within the gel matrices collected from the 1% cell-to-matrix ratio reactor on days 80 (N1) and 360 (N3), and the 4% cell-to-matrix ratio reactor on days 148 (N2) and 515 (N4) (13 days after the end of the monitoring). The relative abundance of microorganisms in the gel matrices at the phylum and family levels is shown in Figure 4.6. Because of complete cell retention, microbial communities were found to have slightly changed between the two sampling occasions of both reactors. At the phylum level, *Proteobacteria* was found to be the dominant group in all samples, accounting for 78.0% - 83.7%.

Among the nitrifying bacterial population, there was a relatively high abundance of *Nitrosomonadaceae* (16.4-20.7%) in the gel matrices of both cell-to-matrix ratios. The relative abundance of *Nitrosomonadaceae* was a little higher in the gel matrix of 1% cell-to-matrix ratio (20.3% and 20.7% for N1 and N3, respectively) than the gel matrix of 4% cell-to-matrix ratio (16.4% and 16.5% for N2 and N4, respectively). The family *Bradyrhizobiaceae,* to which the genus *Nitrobacter* belongs, accounted for around 10% across all samples. The presence of these microorganisms in the gel matrices may have been responsible for some nitrite-oxidizing activity

(72.1 ± 40.7 and 71.4 ± 27.8 mg N $l^{-1}d^{-1}$ for the gel matrix of 1% and 4% cell-to-matrix ratio, respectively) as indicated by the presence of small nitrate concentrations in both reactors throughout the operational periods.

Among heterotrophic microbial populations, the most abundant families across all samples were *Comamonadaceae* and *Xanthomonadaceae*, accounting for 21.3-35.7% and 13.5-20.8%, respectively. The families *Comamonadaceae* and *Xanthomonadaceae* are associated with denitrifying bacterial assemblage. The *Comamonadaceae* was found in a hypoxia habitat where the oxygen concentration was relatively low (<1 mg l^{-1}) (Sadaie et al., 2007). The *Xanthomonadaceae* are known as key microorganisms for the production of extracellular polymeric substances (EPS) in bacterial aggregate (Weissbrodt et al., 2014). Previous research reported that EPS strongly determines the microenvironment and affected oxygen transfer within high-density microbial aggregates like biofilm and granular sludge (Fan et al., 2017). Although the amount of EPS generated within the gel matrix has not been determined, it is possible that EPS may occur as one of the main components of substances in the gel matrix, and the EPS may contribute to the obstruction of oxygen penetration through the gel matrix.
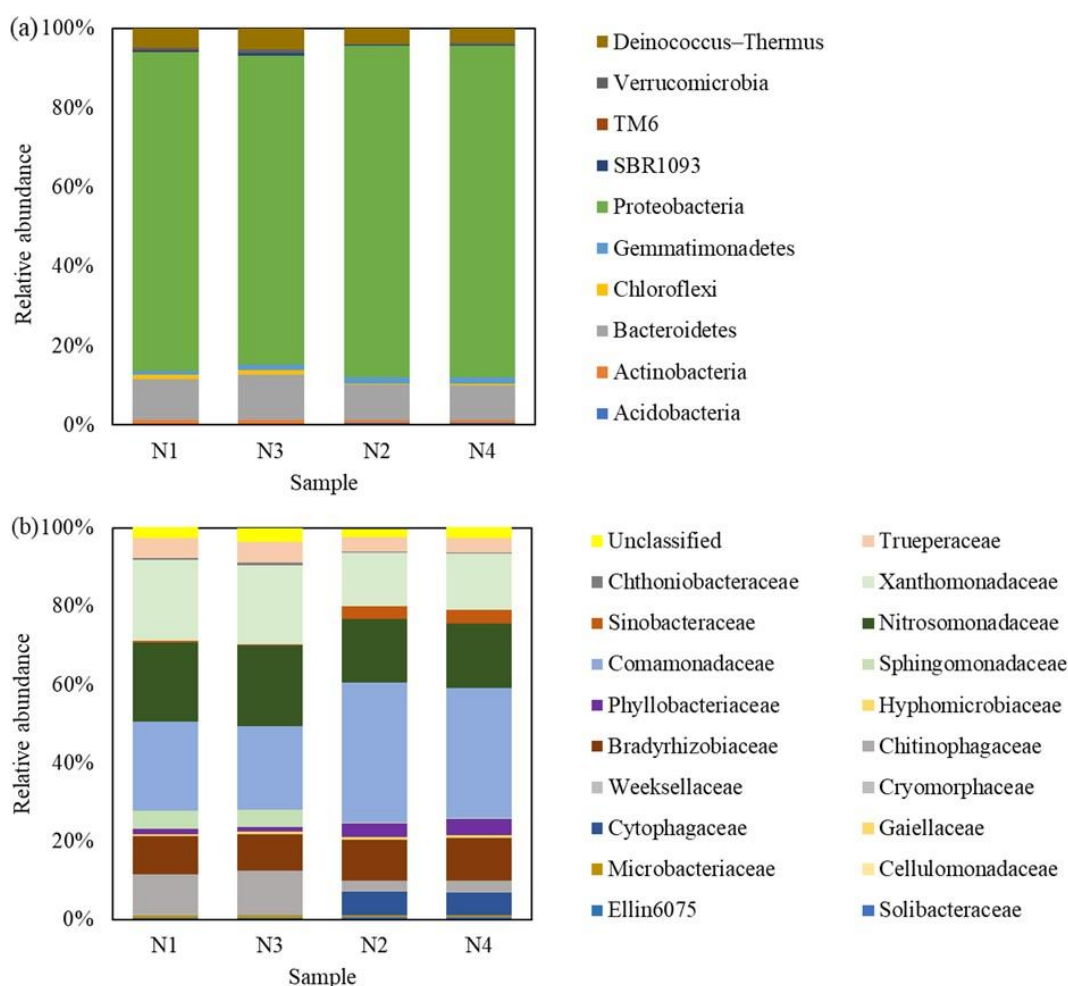
Figure 4. 6 Relative abundance of microorganisms at the (a) phylum level and (b) family level in gel matrices of 1% cell-to-matrix ratio collected on day 80 (N1) and 386 (N3), and 4% cell-to-matrix ratio collected on day 148 (N2) and 515 (N4).

4.3.4 *Spatial distribution of total bacteria, AOB, and NOB in gel matrix*

Spatial distribution of total bacteria, AOB, and NOB in gel matrix was observed using FISH-CLSM. Observations were performed on gel matrices of 1% cell-to-matrix ratio collected on day 149 and 321, when $18.9\pm4.1\%$ and $35.8\pm2.5\%$ of partial nitrification was achieved (Figure 4.7). The observed gel matrices of 4% cell-to-matrix ratio were collected on day 261, when $48.1\pm8.3\%$ of partial nitrification was reached (Figure 4.8). For both types of gel matrices, high signals of AOB aggregates were found within the first 50 µm from the surface of the gel matrix, where oxygen concentrations were between 0.5 and 1.5 mg l^{-1} which is known to be able to promote the growth of

AOB but suppress the growth of NOB (Fudala-Ksiazek et al., 2014). NOB has a lower degree of competitiveness for oxygen at oxygen-limiting conditions than AOB as indicated by the higher affinity to oxygen of AOB than NOB. The half saturation constant for oxygen of AOB and NOB are 0.03-0.74 mg l^{-1}, and 0.4-1.9 mg l^{-1} respectively (Hanaki et al., 1990; Laanbroek and Gerards, 1993; Laanbroek et al., 1994; Wiesmann, 1994; Ciudad et al., 2006; Blackburne et al., 2008; Rongsayamanont et al., 2010; Regmi et al., 2014).

No NOB cells were detected in both types of gel matrices. Nevertheless, some nitrite-oxidizing activity was still found in both reactors as indicated by nitrate concentrations in the effluent of the reactors (Figure 4.2). In addition, Illumina MiSeq analysis indicated that the family *Bradyrhizobiaceae*, with which *Nitrobacter sp* is associated, was present in both reactors. Signals of NOB rRNA in the samples were undetectable by FISH-CLSM analysis, implying some portion of NOB cells in the samples may have been inactive due to them being suppressed by the oxygen-limiting microenvironment in the gel matrix.

FISH-CLSM images of the gel matrix of 1% cell-to-matrix ratio during the startup period (day 149) (Figure 4.7a and b) demonstrated that AOB formed loose aggregates and distributed throughout the oxygen-available zone (approximately 120 and 320 µm from the surface as shown in Figure 4.4a). The AOB aggregates became denser and located mostly around the surface of the gel matrix on day 321. These results demonstrate that the AOB reorganized their aggregates after partial nitrification was reached on day 184. This finding indicates that during the startup period, oxygen is likely to penetrate into deeper parts of the gel matrix. However, after a certain period of operation, the oxygen concentration gradient leads to the formation of a layer-like structure for AOB aggregates around the surface of gel matrix, resulting in more resistance to oxygen penetration into the inner parts of the gel matrix. This layer-like structure of AOB aggregates likely plays a key role in reducing the oxygen available for the growth of NOB whose colonies are typically found adjacent to those of AOB (Rongsayamanont et al., 2010). The layer-like structure of AOB aggregates was also observed in the gel matrices of 4% cell-to-matrix ratio collected on day 261 after partial

nitrification was reached (Figure 4.8). The results demonstrated that cell entrapment with polymeric gel initially created oxygen gradient environment that forced nitrifying bacteria to rearrange their aggregates in the gel matrix leading to the enhancement of partial nitrification.
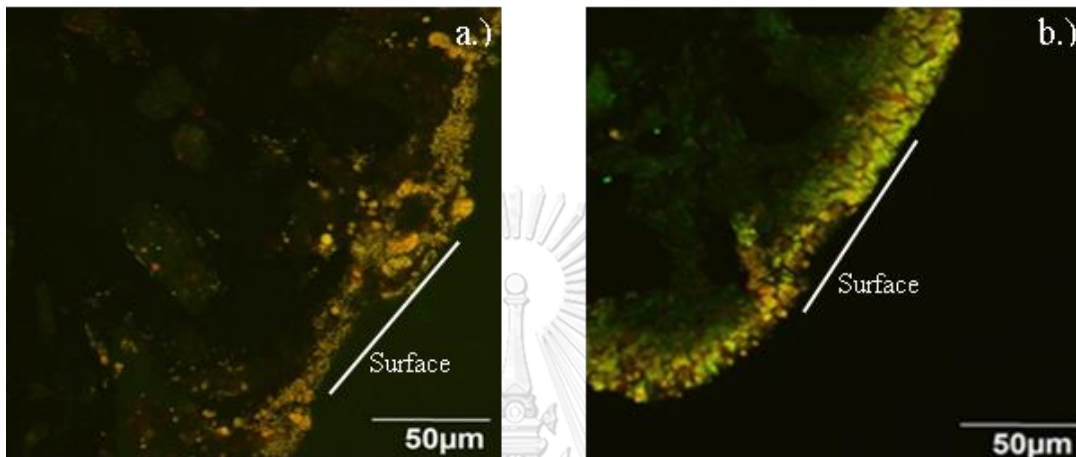
Figure 4. 7 FISH images showing total bacteria and AOB in gel matrix of 1% cell-to-matrix ratio collected on (a) day 149 and (b) day 321. Cy3 (red) was used to label EUB 338 probe targeting most bacteria and Alexa Flour 488 (green) labeled NSO 190 probe targeting AOB. The target cells complimentary to both probes were shown in orange-yellow. Bright colors were auto-fluorescence
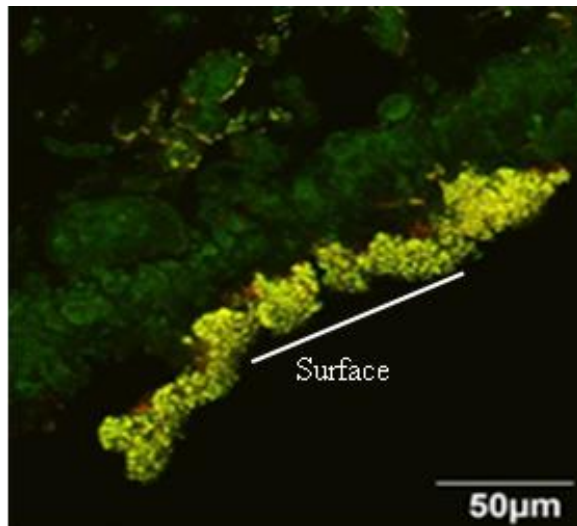
Figure 4. 8 A FISH image showing total bacteria and AOB in the gel matrix of 4% cell-to-matrix ratio collected on day 261. Cy3 (red) was used to label EUB 338 probe targeting most bacteria and Alexa Flour 488 (green) labeled NSO 190 probe targeting AOB. The target cells complimentary to both probes were shown in orange-yellow. Bright colors were auto-fluorescence

4.4   Conclusions

The entrapped cell with higher cell-to-matrix ratio speeded up the startup period of partial nitrification. However, after partial nitrification was achieved, high and comparable degree of partial nitrification can be long-term maintained, regardless of the initial cell-to-matrix ratio. The oxygen-limiting zone (DO = 0.5-1.5 mg l^{-1}) was found at 10-230 µm from the surface of gel matrices indicating that microenvironment driving partial nitrification arose inside the gel matrix. Layer-like structure of AOB was found in this zone and was suggested to obstruct oxygen penetrated into the inner part of gel matrices that provided suitable microenvironment for partial nitrification. These findings confirmed an appropriateness of an entrapped-cell system in generating and prolonging operation of partial nitrification.

CHAPTER 5

EFFECT OF AMMONIA AND ORGANIC LOADING RATE ON PARTIAL NITRIFICATION PERFORMANCE OF ENTRAPPED-CELL-BASED-REACTOR

5.1 Introduction

Conventional biological nitrogen removal is driven by two sequential steps comprising of nitrification and denitrification. The first step, ammonia (NH_3) is oxidized to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) then nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB) in aerobic condition. The second step, nitrate (NO_3^-) is reduced to gaseous nitrogen by denitrifying bacteria in oxygen-free environment. New nitrogen removal process scheme as shortcut biological nitrogen removal (SBNR) via nitrite route has been becoming more popular particularly for treating nitrogen rich wastewater because of its much lower cost for supplying oxygen (O_2) and external organic source than conventional biological nitrogen removal (BNR) via nitrate pathway. However, in order to shift the BNR toward nitrite pathway, ammonia need to be partially nitrified up to nitrite which is called partial nitrification under aerobic condition. After that, nitrite is promptly reduced further into gaseous nitrogen under anoxic environment.

An underlying principle of partial nitrification is to promote the activity of AOB while suppress the NOB activity. Oxygen limitation is one of the most effective strategies promoting partial nitrification. Promoting partial nitrification using oxygen limitation is based on differential oxygen affinity of AOB and NOB. At low oxygen environment, AOB obviously show higher competitiveness for oxygen over NOB due to its lower oxygen half-saturation coefficient (K_O) than that of NOB (Ciudad et al., 2006; Blackburne et al., 2008; Rongsayamanont et al., 2010). Cell entrapment with phosphorylated-polyvinyl alcohol (PPVA) has been recently used to easily promote

partial nitrification via creating an oxygen-limited zone within the gel matrix of entrapped cell (Rongsayamanont et al., 2014). However, partial nitrification on entrapped-cell-based reactor was studied only at high ammonia loading rate (0.5 kg m^{-3} l^{-1}) (Rongsayamanont et al., 2014). While lower ammonia loading rate (<0.5 kg m^{-3} l^{-1}) challenge on maintaining partial nitrification.

Because oxygen is always shared among key functional groups of microorganism in entrapped-cell-based partial nitrification reactor including AOB, NOB and heterotrophic bacteria. Undoubtedly, loading more organic into reactor can surely lead to encourage oxygen competition among these key microbes. Depending on the extent of organic loading rate, partial nitrification may be promoted as more NOB is likely suppressed. However, partial nitrification can be drastically reduced also as too high organic is loaded into reactor resulting of inhibiting ammonia oxidation at very oxygen limiting condition. Although partial nitrification by entrapped cell is likely very sensitive to organic substance in wastewater, robustness of entrapped-cell-based partial nitrification reactor has never been tested in organic containing wastewater.

In addition, several benefits can be gained from applying cell entrapment for SBNR but the generation and emission of an intermediate substance like nitrous oxide (N$_2$O) in gel matrix can be another main concern for wide application of entrapped-cell-based reactor (Rathnayake et al., 2013). N$_2$O is generally produced under oxygen-limiting condition in nitrite-concentrated solution via both nitrifier and heterotrophic denitrification pathways. Therefore, it is worth investigating if N$_2$O is generated from microaerobic condition in entrapped-cell-based partial nitrification reactor fed with organic containing wastewater.

For all the above reasons, this study was hypothesized that ammonia and organic loading rate strongly affect partial nitrification and the production of N$_2$O. In order to prove the hypothesis, continuous flow PPVA-entrapped-cell-based reactors fed with low, medium, and high-strength ammonia nitrogen wastewater having various ratio of chemical oxygen demand (COD)/N was used to determine its effect on partial nitrification and nitrogen removal and also the abundance, localization, and *in situ* oxygen utilization of heterotrophs and AOB within the gel matrix.

5.2 Material and methods

5.2.1 Preparation of entrapped cells

Chen and Lin (1994) has proposed the PPVA method for preparing entrapped cells. The mixed liquor was sampled from the aeration tank of an activated sludge system in a municipal wastewater treatment plant and then centrifuged using speed at 500 rpm for 15 min. One liter of polyvinyl alcohol (PVA) gel solution prepared by10 g PVA per liter of de-ionized (DI) water was mixed with aliquots of concentrated sludge which containing 40 g of suspended solid (SS) to reach initial 4% w/v cell-to-matrix ratios. The spherical gel matrices formation was performed by dropping these mixtures into boric acid solution using a flow rate of 0.83 ml min^{-1}. Then, the spherical gel matrices were immersed into 1 M of phosphate buffer at pH 7.0 for 2-3 h to generate the gel matrices. Normally, diameter of gel bead varied between 4-6 mm. When gel bead was in the reactors, the diameter increased to 6-8 mm.

5.2.2 Synthetic wastewater

The preparation method for preparing synthetic wastewater was further developed from Widdel and Bak (1992); Rongsayamanont et al. (2010); Rongsayamanont et al. (2014). The synthetic wastewater containing ammonia concentrations which are 100, 280, 420 and 700 mg N l^{-1}. Wastewater was prepared by using the mixture of $(NH_4)_2SO_4$, Na_2HPO_4 (4.05 g), K_2HPO_4 (2.1 g), $MgSO4 \cdot 7H_2O$ (0.05 g), $CaCl_2 \cdot 2H_2O$ (0.01 g), $FeSO4 \cdot 7H2O$ (0.09 g), H_3BO_3 (30 mg), $MnCl_2 \cdot 4H2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.19 g), $NiCl_2 \cdot 6H_2O$ (0.024 g), $CuCl_2 \cdot 2H_2O$ (0.02 g), $ZnSO_4 \cdot 7H_2O$ (0.144 g), $Na_2MoO_4 \cdot 2H_2O$ (0.36 g), and $NaHCO_3$ (6.89 g) with one liter of DI water. The chemical oxygen demand (COD) was prepared by adding glucose stock solution into synthetic wastewater.

5.2.3 Setup and operation of entrapped-cell-based reactors

The partial nitrification in continuous flow reactors (parent reactors) is simulated by using the activated sludge being entrapped into polyvinyl alcohol gel, or called PPVA entrapped cell. Two parent reactors (P1 and P2) were continuous stirred

tanks inoculated using prepared entrapped cells with cell-to-matrix ratio of 4% w/v. The effective volume of parent reactors were 4 and 8 L for reactor P1 and P2, respectively.

The final concentration of cells in both reactors was 2,000 mg of SS. DO concentration in the reactors was maintained at 2 mg l^{-1} (Rongsayamanont et al., 2014) using DO controllers (HI8410 DO controller, Hanna, USA). Air flow rate was controlled at 1 l min^{-1}. pH in the bulk solution was maintained at 7.8±0.2 to ensure oxygen-limiting condition for partial nitrification. The pH was controlled by pH controllers (Alpha pH 560, Thermo scientific, USA) with pH electrodes (Eutech instrument, USA) and was automatically adjusted by adding 0.2 M NaOH solution through peristaltic pumps (505U, Watson Marlow, United Kingdom). Hydraulic retention time of the reactors was controlled around 1.4 days using peristaltic pump (505U, Watson Marlow, United Kingdom) to maintain an ammonia loading rate at 500 mg N l-1d-1. The reactors were operated under complete-mixing condition by stirring with mechanical mixer (R20, IKA, Germany) at 250 rpm under room temperature (28-30°C). At least 50-60 % of nitrite accumulation and also 50% of ammonia conversions were achieved steady-state for parent reactors (reactor P1 and P2).

5.2.4 *Operation of entrapped-cell-based reactors with various ammonia and organic loading rates*

After reactors P1 and P2 achieved partial nitrification, which defined as the ammonia removal and nitrite accumulation of >50% at the steady-state operation, gel beads were collected and used as seeds for nine daughter reactors (N100, N100 COD/N 2.6, N100 COD/N 10, N280, N280 COD/N 2.6, N420, N420 COD/N 0.2, N420 COD/N 0.5, and N420 COD/N 2.6). The reactors N100, N280 and N420 received only ammonia at the concentrations of 100, 280, and 420 mg l^{-1}, respectively. Influent of reactor N100 COD/N 2.6 and N100 COD/N 10 contained ammonia at the concentration of 100 mg l^{-1}, and had COD/N ratio of 2.6/1 and 10/1, respectively. Influent of reactors N280 COD/N 2.6 had the ammonia concentration of 280 mg l^{-1}, and COD/N of 2.6/1. Influent of reactors N420 COD/N 0.2, N420 COD/N 0.5, and N420 COD/N 2.6 had the ammonia concentration of 420 mg l^{-1}, and COD/N ratios of 0.2/1, 0.5/1 and 2.6/1,

respectively. All daughter reactors were operated as same as manner for the parent reactors, except that their effective volume was 1 l.

5.2.5 Chemical analysis

The concentration of mixed liquor suspended solid (MLSS) was determined in accordance with the standard method described in APHA (APHA, 2012). Samples were filtered through 0.45 µm GF/C filter paper before ammonia, nitrite, nitrate, and COD concentrations were analyzed. Ammonia concentration was analyzed using an ion selective electrode (NH500, WTW, Germany) in accordance with APHA (APHA, 2012). Nitrite and nitrate concentrations were analyzed by colorimetric and UV spectrophotometric methods, respectively (APHA, 2012). COD was analyzed by closed reflux method.

5.2.6 In situ oxygen concentrations in gel matrix

In situ oxygen concentrations in the gel matrix were analyzed using an oxygen microsensor with a tip diameter of 8-12 µm (OX-10, Unisense, Denmark) held on a micromanipulator (MM 33, Märzhäuser wetzlar, Germany) and motorized stage (Ex 30F20, Jenny Science, Switzerland). The microsensor was connected to a microsensor monometer (Unisense, Denmark). The micromanipulator and motorized stage using to move the microsensor was placed into the gel matrix to take measurements at every 10 µm from the surface of the gel matrix under stereomicroscope.

5.2.7 Spatial distribution of total bacteria, AOB, and NOB in gel matrix

To investigate microbial localization within gel matrix, gel beads samples were collected from entrapped-cell-based reactors. Fluorescence *in situ* hybridization (FISH) technique was selected for determining spatial distribution of heterotrophs, AOB, and NOB in gel matrix. Gel matrix was first suspended in an ice-cold PBS solution. Then, the gel matrix was transferred and then fixed in a paraformaldehyde solution (4% in PBS at pH 7.4) at $4°C$ for 16 h. The fixed sample was embedded in a Tissue-Tek OCT compound (Sakura Finetek, USA Inc.) and cut into 10 µm sections at $-20°C$ using a

cryomicrotome (Leica CM 1950) and then immobilized onto a poly-L-lysine coating slide. The slide was air-dried and dehydrated by 50%, 80% and 98% v/v ethanol (3 min each), respectively.

In this study, commercially synthesized oligonucleotide probes which were fluorescently labeled at the 5' end by Alexaflour 488 or CY3 (ThermoHybaid, Ulm, Germany) were used. Oligonucleotide probes were hybridized with the target 16S rRNA genes at 46°C for 2 h in a buffer solution (0.9 M NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), formamide) containing 5 ng of probe μl^{-1}. The sections of gel matrix were immersed into a pre-heated washing buffer (20 mM Tris-HCl, 0.01% SDS, NaCl) at 48°C for 15 min and then subsequently rinsed with DI water, dried, and mounted with an anti-fading solution (SlowFade, Antifade kit, Molecular Probes, Eugene, OR, USA). For simultaneous hybridization, two hybridizations were performed successively with the probe having higher stringency was performed first (Wagner et al., 1996). Confocal laser scanning microscopy (CLSM) (FluoView FV10i, Olympus, Japan) was used to observe the hybridized samples, with the corresponding FISH-CLSM images processed by FluoView FV10i-Viewer version 4.2 (Olympus, Japan). The Alexaflour 488–labeled probe was visualized by excitation between 460 and 495 nm and the collection of fluorescence emission at 510 nm. The Cy3-labelled probe was excited between 530 and 550 nm and its fluorescence emission was collected at 575 nm. Table 1 shows the oligonucleotide probes and their corresponding hybridization conditions used in this study.

Table 5. 1 Oligonucleotide probes for analyzing spatial distribution of microorganisms in gel matrix

Probe	Sequence (5' to 3')	Label	Target organisms	Formamide (%)
Nso190	CGATCCCCTGCTTTTCTCC	AF	Many but not all ammonia oxidizing *β-Proteobacteria*	55
Ntspa 662	GGAATTCCGCGCTCCTCT	Cy3	*Nitrospira* genus	35
Nit3	CCTGTGCTCCATGCTCCG	Cy3	*Nitrobacter spp.*	40

5.2.8 Nitrous oxide production form entrapped-cell reactor

After reaching steady state condition, dissolved nitrous oxide (N_2O) concentration in bulk liquid was analyzed in three reactors including reactor P1, N420 COD/N 0.2 and N420 COD/N 0.5. N_2O concentration in liquid phase was investigated by N_2O microsensor with Clark-type microsensor which a tip diameter 25 µm. (Unisense, Denmark). The N_2O concentration was analyzed on days 238 and 239 for reactor P2 and days 45 and 46 for reactor N420 COD/N 0.2 and reactor N420 COD/N 0.5 and for each time point the concentration was measured in triplicate.
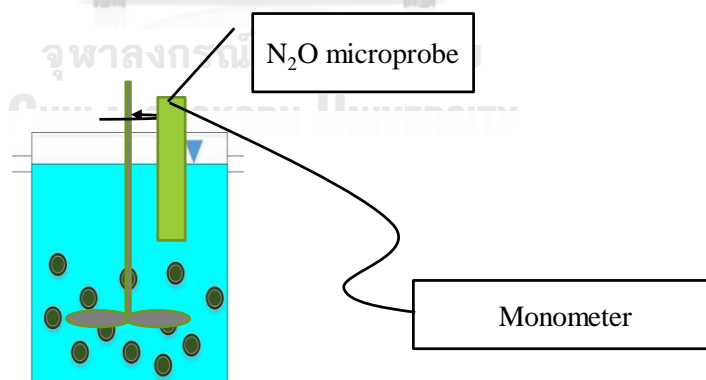

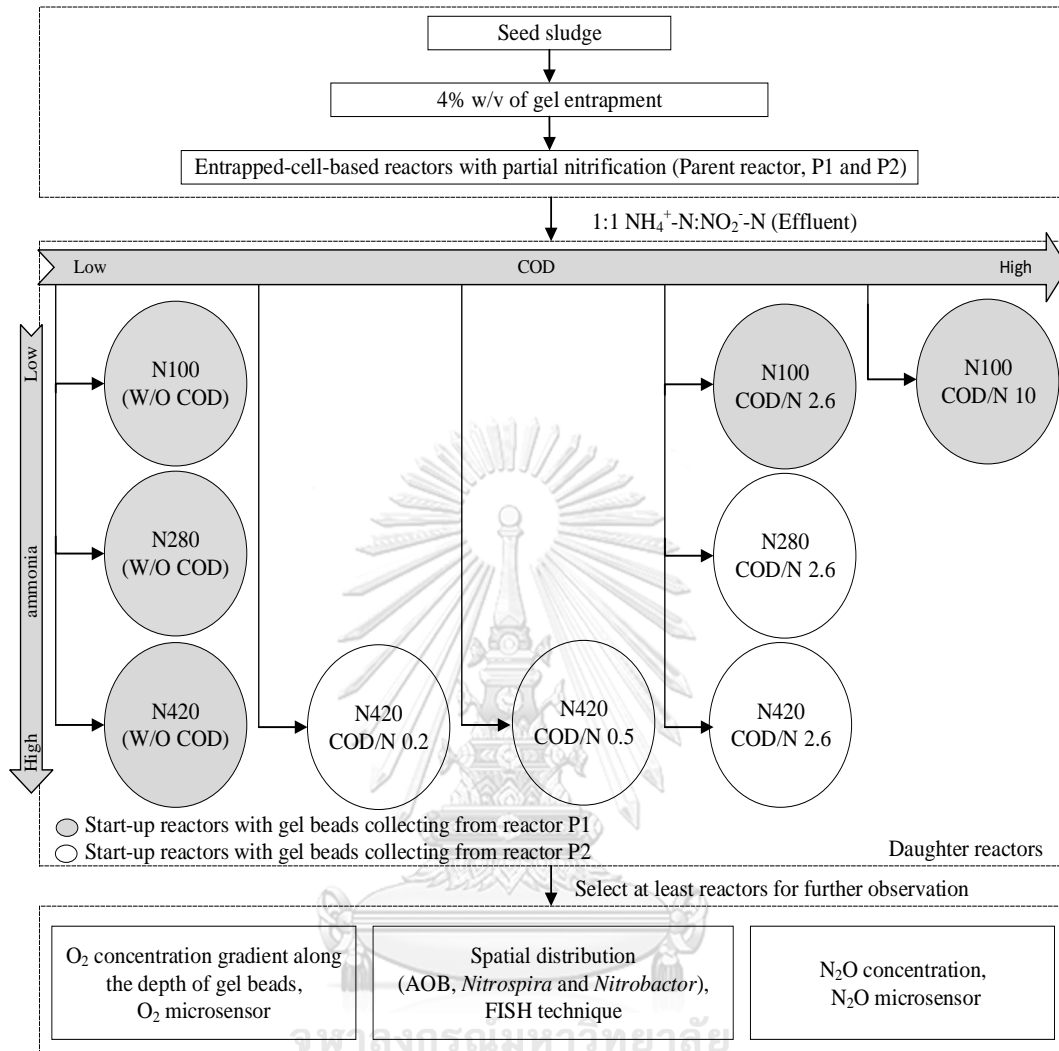**Figure 5. 1** Nitrous oxide measurement

5.2.9   Experimental framework

Figure 5. 2 Experimental framework

5.3   Results and discussion

5.3.1   Performance of parent reactors

Two parent reactors (P1 and P2) were fed with synthetic wastewater containing an ammonium concentration of 700 mg l^{-1} resulting in ammonia loading rate (ALR) of 0.5 kg m^{-3} d^{-1}. Reactor P1 was operated for 123 days and partial nitrification was achieved after day 34, while reactor P2 was operated for 196 days and partial nitrification were achieved and maintained since day 60. Figure 5.3 shows ammonium, nitrite, and nitrate concentrations in the reactors along the operation periods of 123, and 196 days. For reactor P1, nitrite accumulation increased from 52.8±19.9% to 61.0±18.3% and ammonia removal was relatively stable along the operation period (from 56.7±29.1% to 58.7±14.6%). For reactor P2, nitrite accumulation increased from 54.6±31.4% to 73.3±21.8%, while ammonia removal increased from 43.8±19.9% to 61.2±10.4%. After partial nitrification was reached, free ammonia concentration (FA) in both reactors were in a range of 4.9-20.9 mg l^{-1}, which did not affect AOB and NOB activities. Previously, Rongsayamanont et al. (2010) indicated that gel entrapment matrix can help protect AOB and NOB activities from FA up to 120 mg l^{-1}.

Although entrapped-cell-based reactor can help promote partial nitrification at high ammonia loading, its application at various ammonia and organic loading has not been well clarified. Therefore, entrapped cells from both reactors were harvested, after partial nitrification was reached (ammonia removal >50% and nitrite accumulation >50%), and used as inoculum for daughter reactors operation with different ammonia and organic loading. The gel beads were collected from reactor P1 on day 38 for start-up of reactor N100, day 58 for start-up of reactors N100 COD/N2.6 and N100 COD/N 10, and day 123 for start-up of reactors N280 and N420. While, the gel beads were collected from reactor P2 on day 83 for start-up of reactors N280 COD/N 2.6 and N420 COD/N 2.6, and day 192 for start-up of reactors N420 COD/N 0.2 and N420 COD/N 0.5.
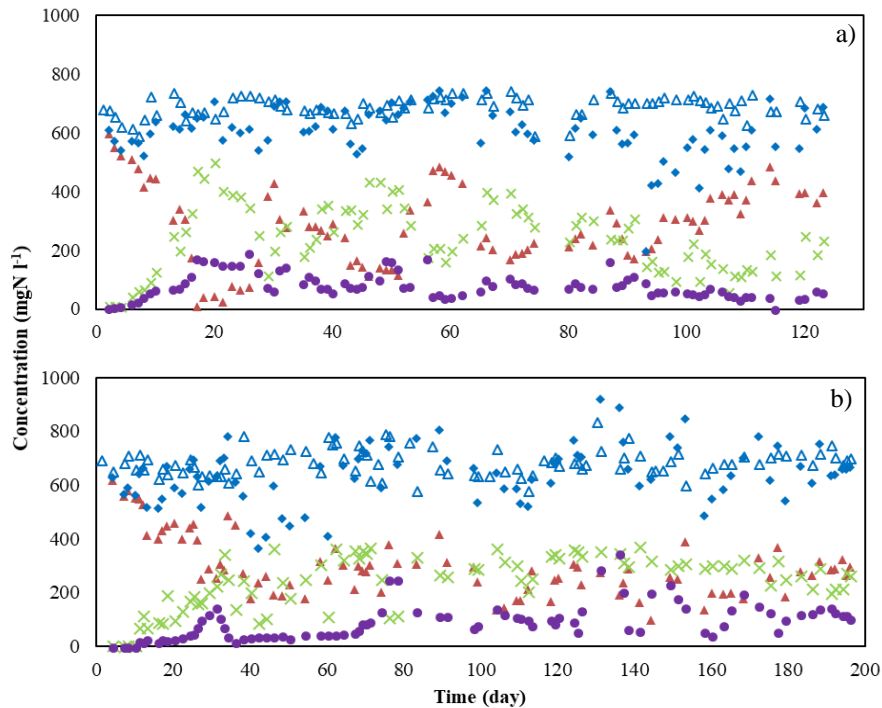
Figure 5. 3 Performance of parent reactors 1(a) and 2 (b): influent ammonia (△), effluent ammonia (▲), effluent nitrite (×), and effluent nitrate (●), total nitrogen in the effluent (◆)

5.3.2 *Effect of ammonia load on partial nitrification performance*

Gel beads from mother reactors (P1 and P2), where partial nitrification was successfully promoted, were used as seeds for operating reactors N100, N280, and N420. The reactors were fed with the influent containing no organic and varying ammonium concentrations of 100, 280, and 420 mg l^{-1}. Results from mother reactor P2 were used and analysed as a reactor N700, which received the influent without organic and ammonium concentrations of 700 mg l^{-1}. Therefore, the four reactors were different in ammonia loading rate of 0.07, 0.2, 0.3, and 0.5 kg m^{-3} d^{-1}, respectively. Figure 5.4 shows ammonium, nitrite, and nitrate concentrations in the reactors along the operation periods of 20, 87, 87, and 196 days. The summary of reactor performance at the steady state conditions was presented in Table 5.2.

At the steady state conditions, the ammonia removal efficiency was 94.4±1.8, 90.2±10.8, 66.0±7.3, and 62.9±9.3% for reactors N100, N280, N420, and N700,

respectively (Table 5.2). Partial nitrification was achieved only in reactors N420 and P2 (N700) with nitrite accumulation of 55.4±15.4 and 68.1±17.0% (Table 5.2 and Figure 5.4c and d). While, reactors N100 and N280 failed to maintain partial nitrification after certain periods of operation (nitrite accumulation of 17.7±7.2 and 21.7±8.9 %, Table 5.2 and Figure 5.4). For reactors N100 and N280, partial nitrification was able to be maintained only before days 13 and 45 (Figure 5.4). After these periods, partial nitrification turned to be complete nitrification with nitrate accumulation of 58.0±2.0 and 64.7±14.4% (Table 5.2 and Figure 5.4). The results indicated that ammonia loading rate determined partial nitrification performance. Only the reactors with higher ammonia loading rate ($\geq$0.3 kg m^{-3} d^{-1}), partial nitrification was able to be maintained.

FA concentrations along the operation period were 0.2-0.6, 3.4-11.2, 3.8-18.2, and 4.2-36.5 mg l^{-1} in reactors N100, N280, N420, and N700, respectively. These ranges of FA concentrations did not inhibit AOB and NOB activities in entrapped-cell systems. Rongsayamanont et al. (2010) demonstrated that gel entrapment matrix help protect the activities of AOB and NOB from FA concentrations up to 120 mg l^{-1}.

Figure 5.5 shows oxygen concentration gradients along the depth of gel matrices from reactors N280, N420, and N700. It is found that the oxygen concentration gradients in the gel beads from reactors N420 and N700, where partial nitrification was able to be maintained, were steeper than that in the gel beads from reactor N280, where complete nitrification was achieved. Due to the higher ammonia loading rate, the activity of AOB was higher in reactors N420 and N700 than in reactors N100 and N280. This may result in higher oxygen consumption in reactors N420 and N700 than in reactors N100 and N280 leading to steeper oxygen concentration gradient in the gel beads from reactors N420 and N700 than in reactor N280. Therefore, oxygen-limiting zone, that support partial nitrification, was more generated in the gel beads of reactors N420 and N700 than in the gel beads of reactor N280. In the gel beads from reactors N420 and N700, aerobic zone (oxygen concentration >1.5 mg l^{-1}) was created starting from the gel bead surface to the depth of approximately 40 μ.m. and oxygen-limiting zone (oxygen concentration 0.5-1.5 mg l^{-1}) was found at the depth of 40-100 μ.m. from

the gel beads surface. More oxygen was penetrated into the gel beads from reactor N280 as aerobic zone was found to located within the first 70 μ.m. from the gel bead surface and oxygen-limiting zone was at 70-170 μ.m. FISH images (Figure 5.6) revealed that AOB aggregates were denser around the edge of the gel beads from reactors N420 and N700. While in the gel beads from reactors N100 and N280, AOB aggregates were found in the deeper parts of the gel beads.

Although partial nitrification was achieved in reactors N420 and N700, some nitrate was still detected in those reactors (59.2±1.3, and 124.7±62.2 mg l^{-1}, respectively). This indicated some nitrite-oxidizing activity in the reactors though the majority of the activity was inhibited. However, NOB including *Nitrobacter* and *Nitrospira* were not detected in the gel beads of both reactors by FISH (Figure 5.6). This is because of much higher activity of AOB than NOB in the reactors resulting in more numbers of AOB than NOB in the gel beads.

For reactors N100 and N280, where complete nitrification was achieved, signals of *Nitrobacter* and *Nitrospira* were detected, but the signals were not as strong as expected from the activity (Figure 5.6a, b, e, and f). This may be due to the effect of original seed from mother reactor P2, that was operated under partial nitrification and contained extremely higher AOB than NOB.

Table 5. 3 Summary of reactor performance after the steady state conditions

Reactor	Influent ammonium concentration (mg l⁻¹)	Ammonia loading rate (kg m⁻³ d⁻¹)	Influent COD concentration (mg l⁻¹)	Influent COD:N ratio	Day to reach steady state	NH$_4^+$ removal (%)	NO$_2^-$ accumulation (%)	NO$_3^-$ accumulation (%)	SND (%)	COD removal (%)	Nitrogen loss (%)	Partial nitrification	Complete nitrification	SND	No ammonia oxidation
N100	100	0.07	-	-	13-20	94.4±1.8	17.7±7.2	58.0±2.0	24.3±8.2	-	23.0±7.8		/		
			260	2.6	21-50	86.8±7.8	9.4±9.8	34.0±8.6	56.6±10.0	92.5±5.0	49.0±8.6			/	
			1000	10	35-50	85.0±4.9	3.6±2.0	23.3±9.1	73.1±9.1	95.7±3.0	62.2±8.9			/	
N280	280	0.2	-	-	72-84	90.2±10.8	21.7±8.9	64.7±14.4	13.6±18.0	-	13.2±16.4		/		
			728	2.6	30-77	96.2±3.7	1.7±2.2	69.5±11.1	28.7±11.2	92.2±4.3	27.7±10.8			/	
N420	420	0.3	-	-	45-87	66.0±7.3	55.4±15.4	31.6±7.9	13.0±17.9	-	9.1±12.0	/			
			84	0.2	24-50	91.4±3.8	9.0±3.7	21.6±8.2	69.4±8.4	100±0.0	63.7±9.4			/	
			210	0.5	45-50	66.2±9.1	2.0±0.4	0.9±1.3	97.1±1.7	91.0±1.8	64.8±9.1			/	
			1092	2.6	22-77	32.1±14.0	3.0±3.5	33.5±43.4	63.5±45.8	87.7±6.5	22.6±13.4				/
P1	700	0.5	-	-	35-123	58.7±14.6	61.2±18.3	18.1±7.3	20.9±21.4	-	12.1±4.3	/			
P2	700		-	-	60-196	62.9±9.3	68.1±17.0	27.9±13.2	4.0±19.3	-	3.6±12.3	/			
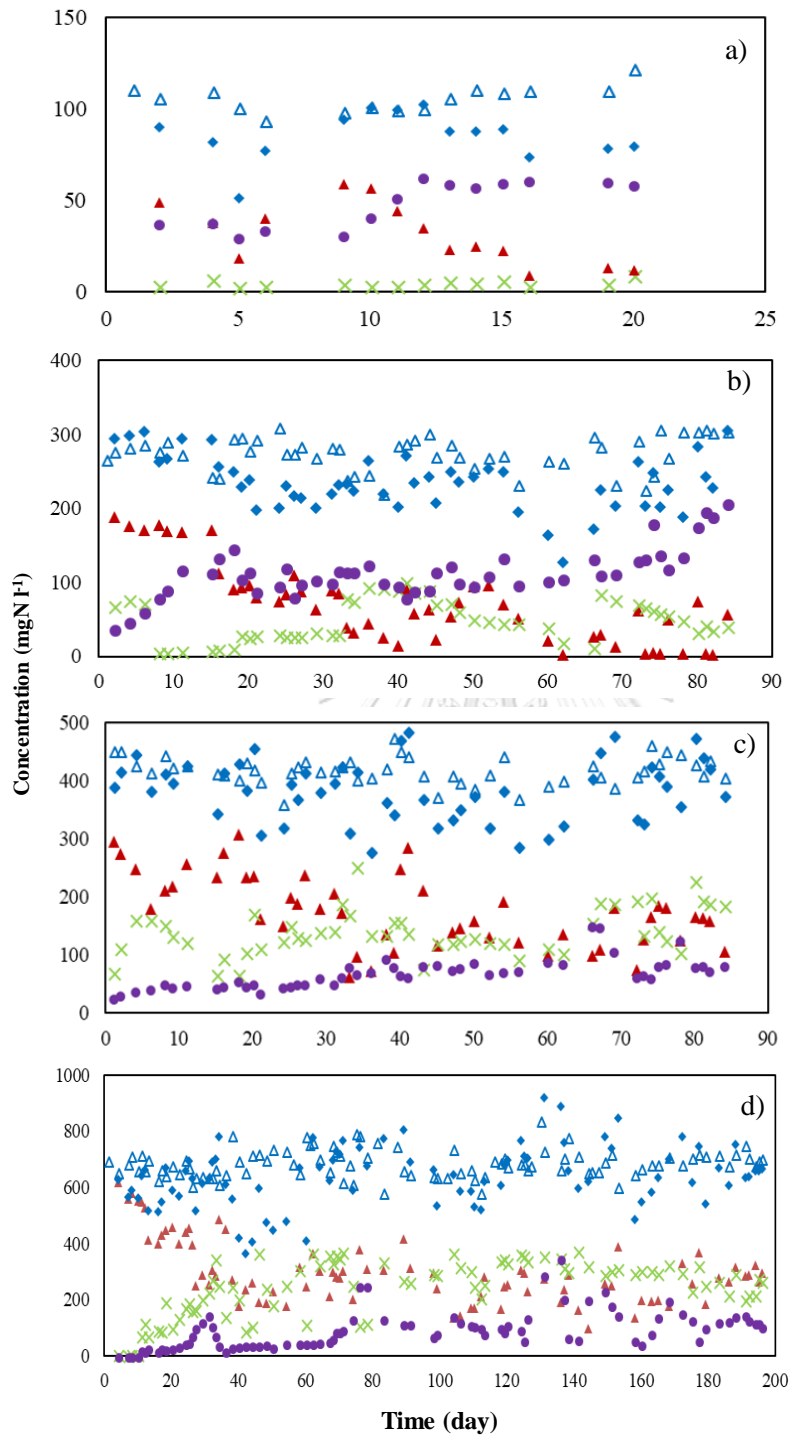
Figure 5. 4 Performance of reactor N100 1(a), N280 (b), N420 (c), and P2 (N700) (d): influent ammonia (△), effluent ammonia (▲), effluent nitrite (×), and effluent nitrate (●), total nitrogen in the effluent (◆)
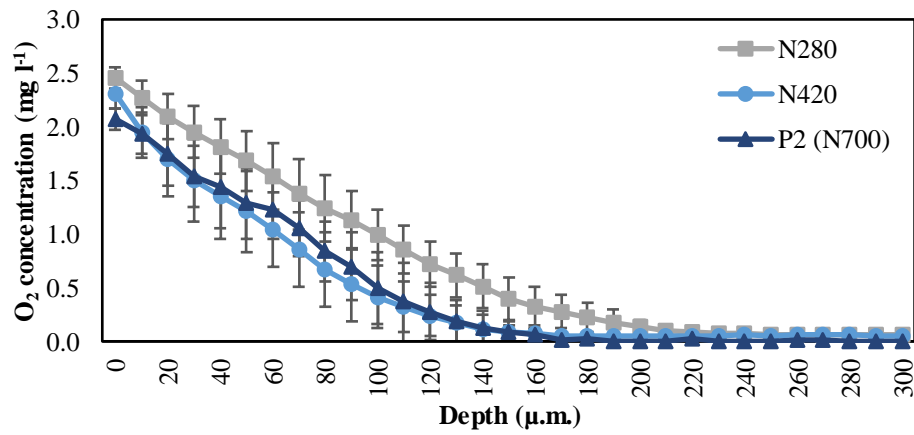
Figure 5. 5 Oxygen concentration gradients along the depth of gel matrices of reactors N280 (square), N420 (circle), and P2 (N700) (triangle). Gel beads were collected on days 192 from reactor P2 and 84 from reactor N420.
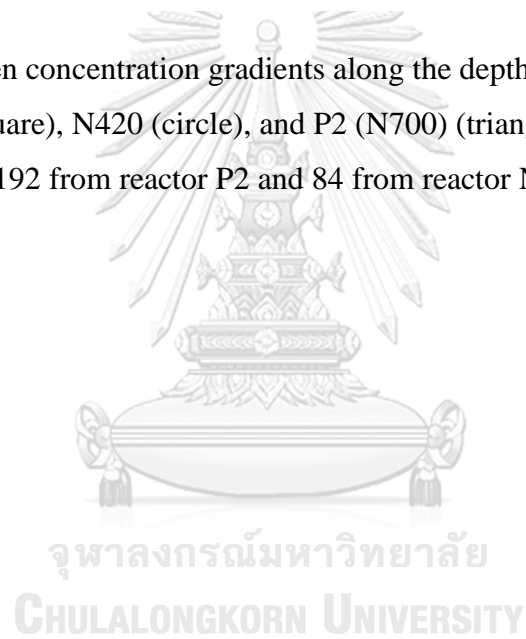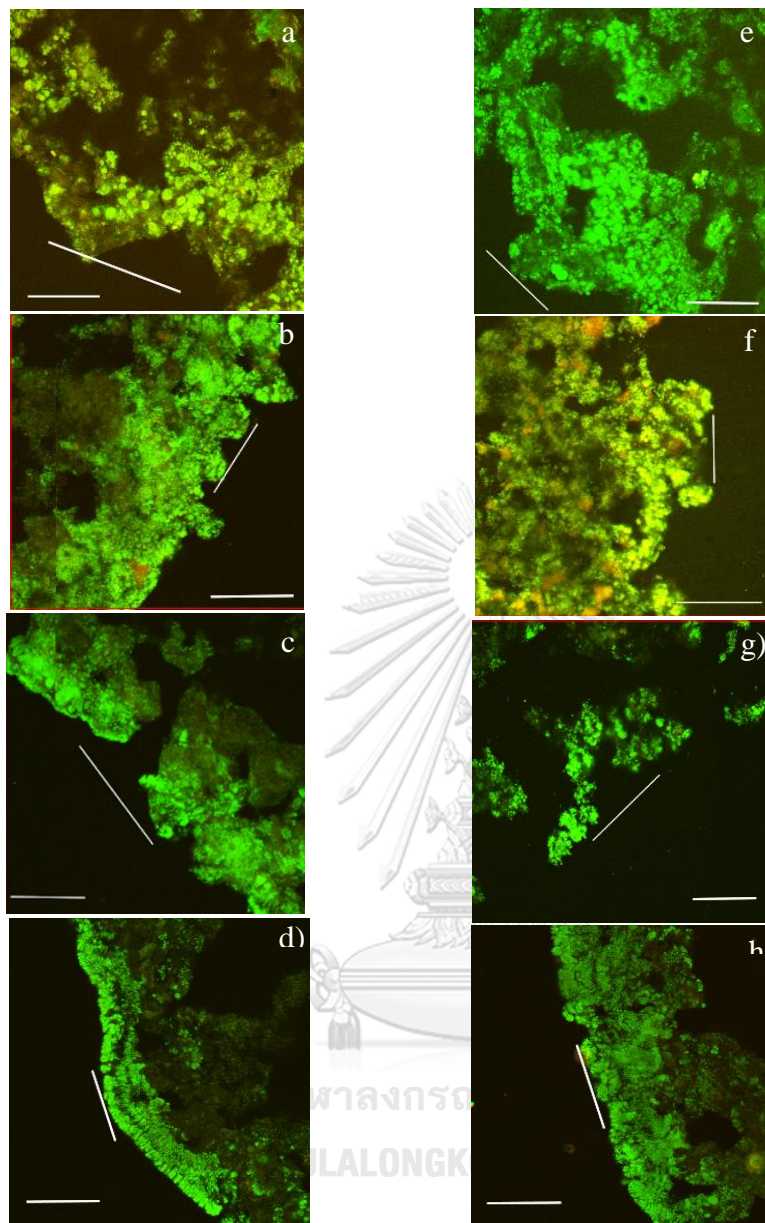
Figure 5. 6 FISH images showing AOB and *Nitrobacter* (a, b, c, and d), and AOB and *Nitrospira* (e, f, g, and h) in the gel matrices of reactors N100 (a and e), N280 (b and e), N420 (c and g), and P2 (N700) (d and h). The scale bars represent 50 µ.m.. Gel beads were collected on days 20, 84, and 192, respectively. Cy3 (red) was used to label NIT3 and Nspr662 probes targeting *Nitrobacter* (a, b, and c) and *Nitrospira* (d, e, and f), respectively and Alexa Flour 488 (green) labeled NSO 190 probe targeting AOB. The target cells complimentary to both probes were shown in orange-yellow. Bright colour was auto-fluorescence.

5.3.3 Effect of organic feeding on nitrogen removal

Similar to previous part, gel beads from mother reactors (P1 and P2) were used as seeds for operating reactors receiving ammonia together with organic at various loads. Two more reactors were operated with an influent ammonium concentration of 100 mg l^{-1}, but with COD/N ratios of 2.6 and 10. One more reactor with an influent ammonium concentration of 280 mg l^{-1} was operated with a COD/N ratio of 2.6. Three more reactors were operated with COD/N ratios of 0.2, 0.5, and 2.6 and an influent ammonium concentration of 420 mg l^{-1}

Reactors N100, N100 COD/N 2.6, and N100 COD/N 10 were operated for 20, 50, and 50 days, respectively. Reactors N100 COD/N 2.6 and reactor N100 COD/N 10 were fed with the synthetic wastewater containing COD concentrations of 260 and 1000 mg l^{-1}. Figure 5.7 shows performance of reactors N100, N100 COD/N 2.6, and N100 COD/N 10. For reactor N100, partial nitrification turned to complete nitrification after day 13. Comparing both periods, ammonia removal efficiency was stable ranging between 91-97%, while nitrite accumulation decreased from 44.1±14.9% to 17.7±7.2% and nitrate accumulation increased from 41.2±12.3 to 58.0±2.0%. For reactors N100 COD/N 2.6, SND occurred and became steady after day 21. At the steady state condition, ammonia removal was 86.8±7.8%. Nitrite and nitrate accumulation was 9.4±9.8% and 33.6±9.0%, respectively. SND was 56.6±10.0% and COD removal efficiency was 92.5±5.0%. Reactor N100 COD/N 10 reached the steady state condition of SND after day 35. Ammonia removal efficiency was 85.0±4.9%. Nitrite and nitrate accumulation was 3.6±2.0% and 23.3±9.1%, respectively, while SND was 73.1±9.1% and COD removal efficiency was 95.7±3.0%.
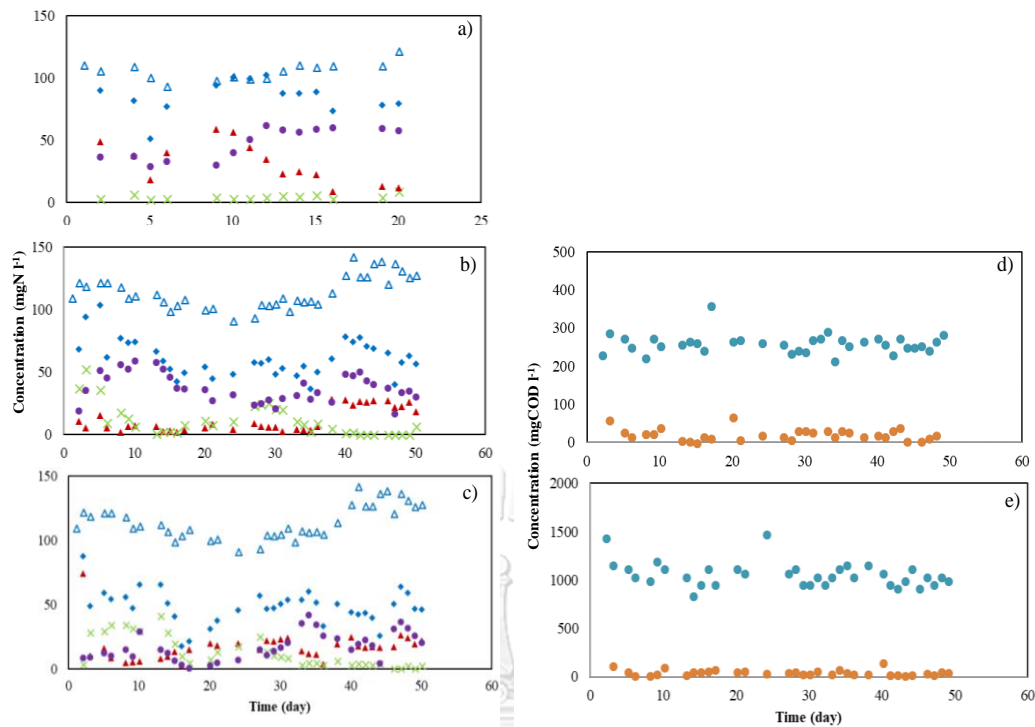
Figure 5. 7 Performance of reactors N100 (a), N100 COD/N 2.6 (b and d), and N100 COD/N 10 (c and e): influent ammonia (△), effluent ammonia (▲), effluent nitrite (×), and effluent nitrate (●), total nitrogen in the effluent (◆), influent COD (●), and effluent COD (●)

Reactors N280 and N280 COD/N 2.6 were operated for 84 and 103 days, respectively. COD concentration of 728 mg l⁻¹ was provided in the influent of reactor N280 COD/N 2.6. Performance of reactors N280 and N280 COD/N 2.6 was shown in Figure 5.8. For reactors N280, complete nitrification occurred and reached the steady state condition after day 72. At the steady state condition, ammonia removal efficiency was 90.2±10.8%. Nitrite and nitrate accumulation was 21.7±8.9% and 64.7±14.4%, respectively. Complete nitrification also arose in reactor N280 COD/N 2.6. At the steady state condition after day 30, ammonia removal efficiency was 96.2±3.7%. Nitrite and nitrate accumulation was 1.7±2.2% and 69.5±11.1%, while SND was 28.7±11.2% and COD removal efficiency was 92.2±4.3%.
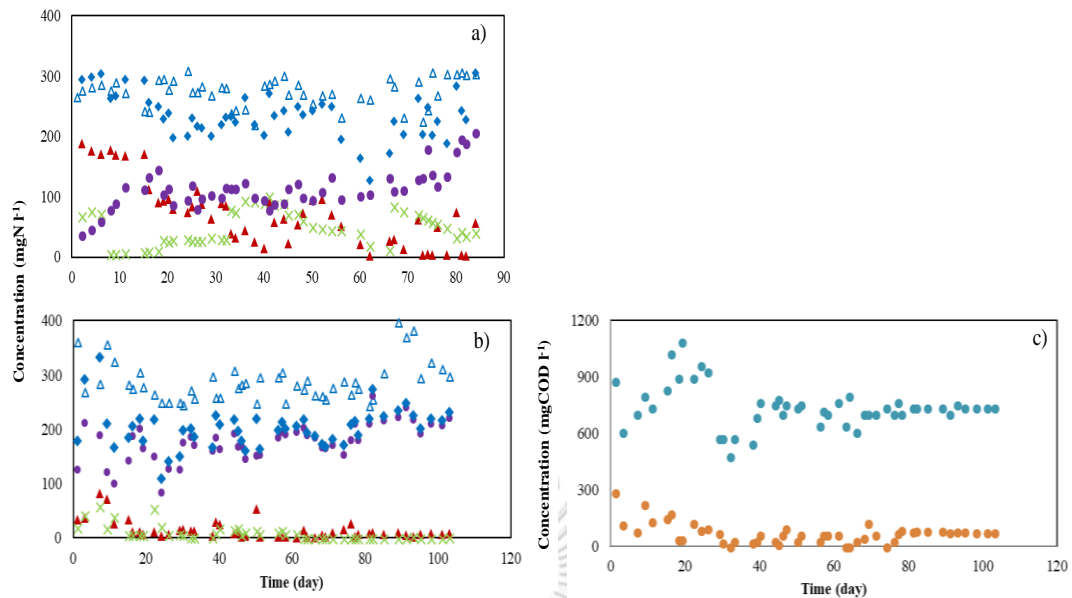
Figure 5. 8 Performance of reactors N280 (a) and N280 COD/N 2.6 (b and c): influent ammonia (△), effluent ammonia (▲), effluent nitrite (×), and effluent nitrate (●), total nitrogen in the effluent (◆), influent COD (●), and effluent COD (●)

Reactors N420, N420 COD/N 0.2, N420 COD/N 0.5, and N420 COD/N 2.6 were operated for 87, 50, 50, and 77 days, respectively. COD concentrations of 84, 210, and 1092 mg l^{-1} were provided in the influent of reactors N420 COD/N 0.2, N420 COD/N 0.5, and N420 COD/N 2.6 to achieve the desired COD/N ratios. Figure 5.9 shows performance of the reactors. For reactor N420, partial nitrification had been successfully maintained. At the steady state condition after day 45, ammonia removal efficiency was 66.0±7.3%. Nitrite and nitrate accumulation was 55.4±15.4%. and 31.6±7.9%, respectively. SND occurred in reactors N420 COD/N 0.2 and N420 COD/N 0.5. The steady state conditions of both reactors were reached after days 24 and 45. Ammonia removal efficiency in both reactors were 91.4±3.8% and 66.2±9.1%. Nitrite accumulation was 9.0±3.7% and 2.0±0.4%. Nitrate accumulation was 21.6±8.2% and 0.9±1.3%. SND reached 69.4±8.4 and 97.1±1.7% for both reactors. COD removal efficiency was 100 and 91.0±1.8%. Reactor N420 COD/N 2.6 was different from all other reactors as it failed to remove ammonia at 32.1±14.0% only.
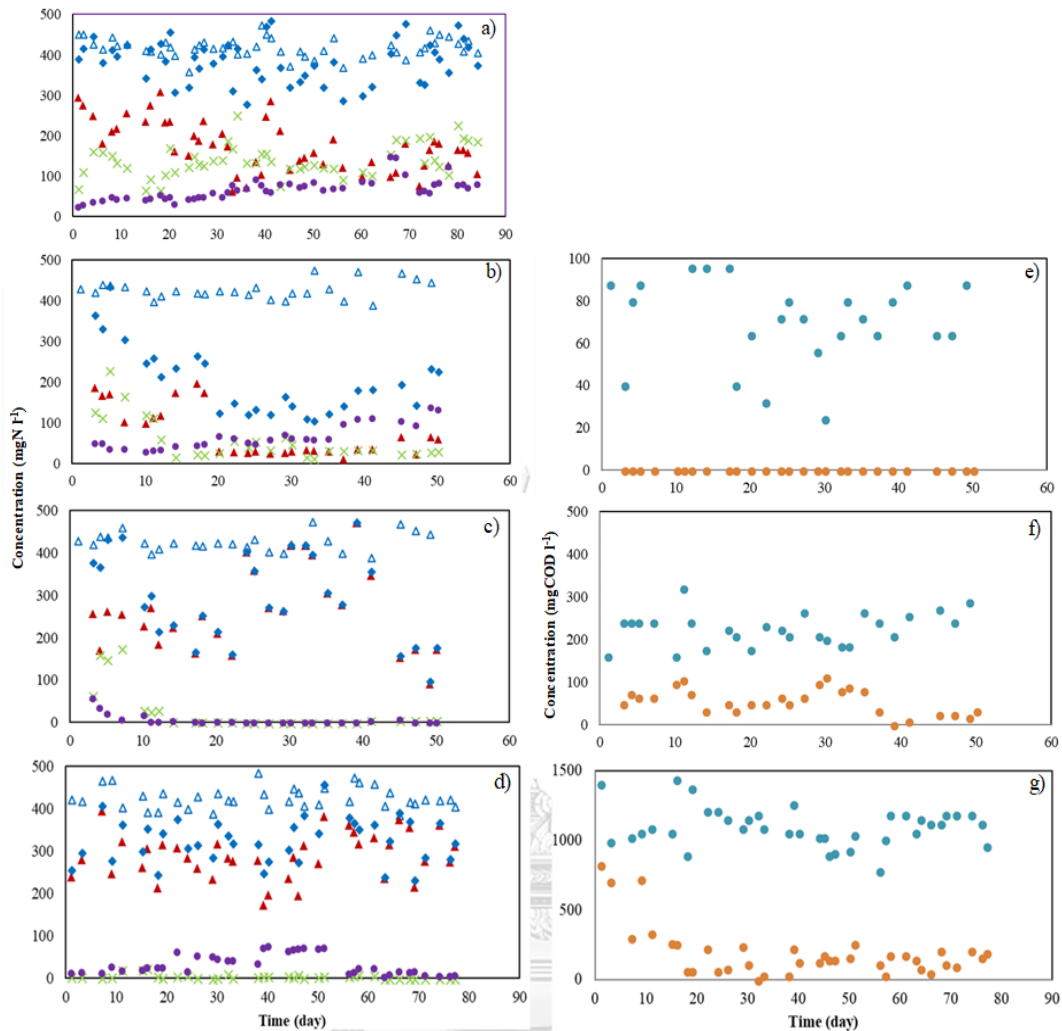
Figure 5. 9 Performance of reactors N420 (a), N420 COD/N 0.2 (b and e), N420 COD/N 0.5 (c and f), and N420 COD/N 2.6 (d and g): influent ammonia ($\triangle$), effluent ammonia ($\blacktriangle$), effluent nitrite ($\times$), and effluent nitrate ($\bullet$), total nitrogen in the effluent ($\blacklozenge$), influent COD ($\bullet$), and effluent COD ($\bullet$)

Figure 5.10 shows percentages of nitrite accumulation, nitrate accumulation, and SND in entrapped-cell based reactors. The values implied nitrogen transformation pathway indicating types of reactors achieved under each operating condition (partial nitrification, complete nitrification, or SND reactors). Partial nitrification was able to be achieved in the reactors with high ammonia loading rate and without organic feeding (reactors N420 and N700). Complete nitrification occurred in the reactors receiving low ammonia loading rate without organic feeding (reactor N100, N280). The reasons for why partial nitrification was achieved only in high ammonia loading reactors and

complete nitrification was reached only in low ammonia loading reactors has been discussed in the earlier section. Most of the reactors fed with organic showed high SND percentages (reactors N100 COD/N 2.6, N100 COD/N 10, N420 COD/N 0.2, and N420 COD/N 0.5). However, it should be noted that one exception is the reactor N280 COD/N 2.6, where complete nitrification arose. Apart from an electron donor being supplied as glucose for denitrification, operating the reactors at low DO concentration in bulk concentration of 2 mg l^{-1} and the use of entrapped cells help create anoxic condition in the gel matrix that support denitrification. Bueno et al. (2017) showed that SND was successfully achieved in activated sludge reactors operated at DO concentration of 0.5±0.2 mg l^{-1}, but not in the reactors operated at DO concentration of 2.5±0.2 mg l^{-1}. Ma et al. (2017) indicated that 81.23% of SND and 76.91% of ammonia removal efficiency was achieved when treating coal gasification wastewater using biofilm reactors operated at DO concentrations of 0.35 mg l^{-1}.

Stoichiometry indicated that organic of 1.72 g COD/g N is required to reduce nitrite to nitrogen gas, while 2.86 g COD/g N is needed for nitrate reduction to nitrogen gas (Van Hulle et al. 2010). Considering nitrite or nitrate that occurred in the reactors without organic feeding (N100, N280, N420), the organic feeding was not adequate in reactors N420 COD/N 0.2 and N420 COD/N 0.5 for denitrification.

Denitrification can be performed via two different pathways, nitrite denitrification or nitrate denitrification. It is expected that SND in reactors N100 COD/N 2.6 and N100 COD/N 10 was driven mainly through nitrification-denitrification pathway. This is because reactors N100 COD/N 2.6 and N100 COD/N 10 were operated at low ammonia loading which tend to achieve complete nitrification. On the other hand, SND in reactors N420 COD/N 0.2 and N420 COD/N 0.5 was expected to be driven mainly via partial nitrification-denitrification pathway because partial nitrification was achieved at high ammonia loading rate in reactor N420.

For reactor N420 COD/N 2.6, where high COD was loaded into reactor, only small ammonia was oxidized (32.1±14.1%). In this reactor, large amount of COD was loaded into the reactor. This results in the promotion of heterotrophic microorganisms that oxidize organic matters by utilizing oxygen with an electron acceptor. Therefore,

oxygen is not available for AOB to oxidize ammonia leading to the lowering of ammonia removal efficiency.
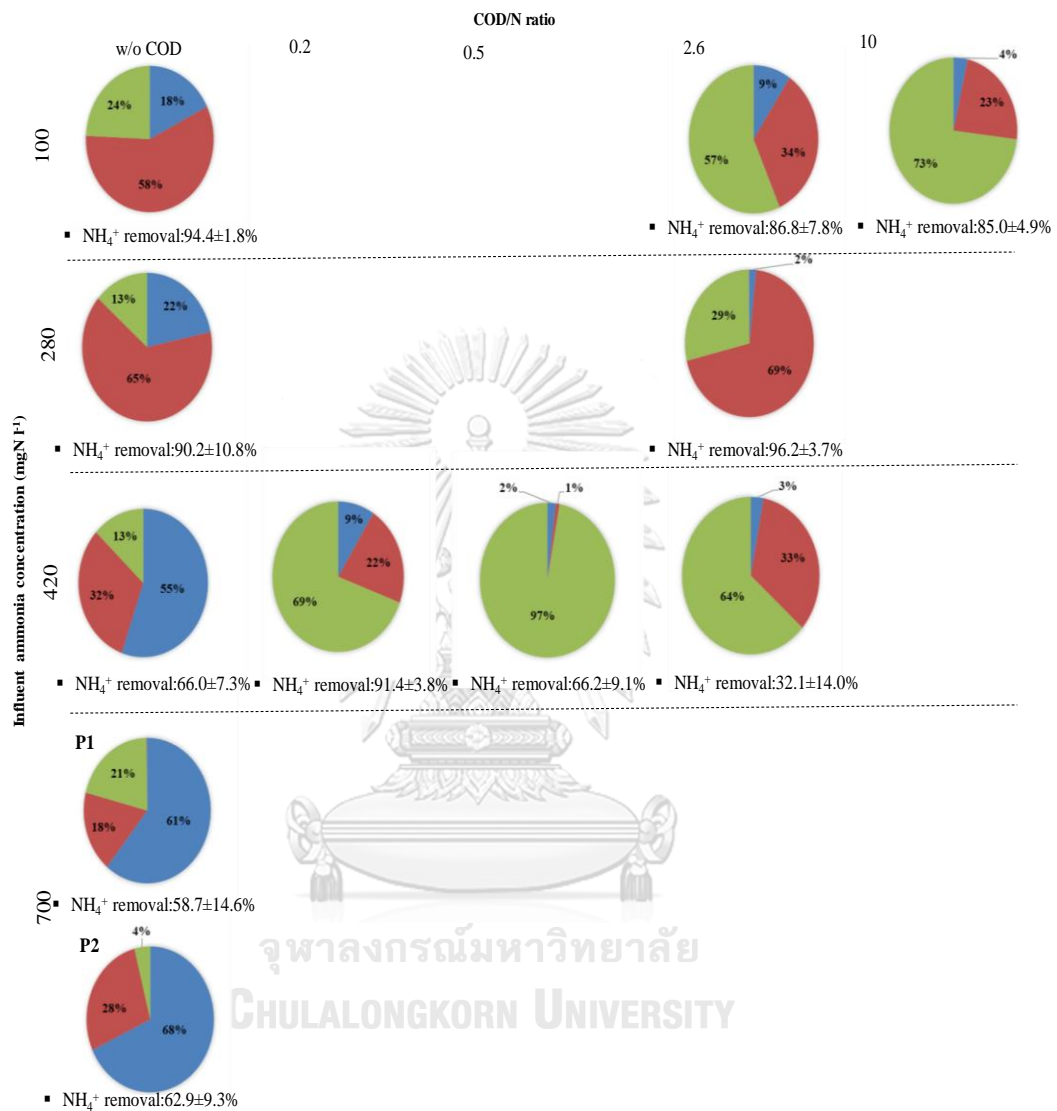
Figure 5. 10 Comparison of nitrite accumulation (blue), nitrate accumulation (red), and SND (green) in entrapped-cell based reactors operated with different ammonia loading rate and COD/N ratios

   Gel beads were collected from reactors N280 and N280 COD/N 2.6 to investigate the effect of organic on oxygen concentration gradient. Figure 5.11 shows oxygen concentration gradients along the depth of gel matrix collected from both reactors. Steeper oxygen concentration gradient presented in the gel beads from the

reactor with organic (N280 COD/N 2.6) than in organic-free reactor (N280). It is noted that small SND (28.7±11.2%) occurred in this reactor. This indicated that most of organic was removed (COD removal >90%) by heterotrophic microorganisms, resulting in the utilization of oxygen around the surface of the gel beads.
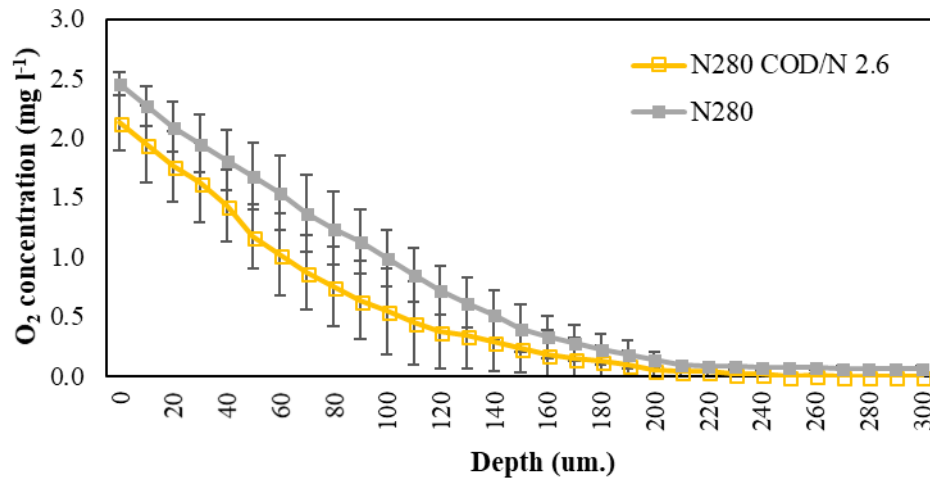
Figure 5. 11 Oxygen concentration gradients along the depth of gel matrices of N280 (square) and N280 COD/N 2.6 (empty square). Gel beads were collected on day 84 (N280) and 77 (N280 COD/N 2.6).

5.3.4 Production of nitrous oxide in the reactors

Dissolved N_2O production was investigated in reactor N420 COD/N 0.2, N420 COD/N 0.5, and P2 to determine effect of organic loading rate on N_2O production. Dissolved N_2O was measured on day 42 and 44 in reactor N420 COD/N 0.2 and N420 COD/N 0.5, and on day 235 and 237 in reactor P2. In this study, dissolved N_2O was produced at similar range of previous study. The amount of detected N_2O was corresponding with ammonia loading rate (0.24±0.14%, 0.1±0.07%, and 0.1±0.06% of ammonia loads in reactor N420 COD/N 0.2, N420 COD/N 0.5, and P2, respectively). However, N_2O generation pathway was not investigated in this experiment. Agree with some evidence indicated that N_2O was typically found in partial nitrification reactors operated using oxygen-limiting strategy (Kampschreur et al., 2008b; Ahn et al., 2011; Desloover et al., 2011; Rathnayake et al., 2013; Peng et al., 2015; Peng et al., 2016). Full-scale plants activated sludge system with partial nitrification which was operated

with DO 0.75 ± 0.05 mg l^{-1} and nitrogen was oxidized to nitrite around 45-47% and 13-15% to nitrate, detected N_2O in liquid phase was between 0.16 and 0.21% of ammonia load (Desloover et al., 2011). A lab-scale reactor partial nitrifying granular sludge reactor operated with DO 2 ± 0.2 mg l^{-1} also generated N_2O at $0.8\pm0.4\%$ of ammonia loading rate (Rathnayake et al., 2013).

5.4 Conclusions

Partial nitrification was maintained only in the reactors without organic feeding and at ammonia loading rate of ≥0.3 kg m^{-3} d^{-1}. Oxygen-limiting zone in the gel matrix was larger in the gel beads of the reactors fed with higher ammonia loading rate, where partial nitrification was able to be maintained, than the reactor with lower ammonia loading rate, where complete nitrification was achieved. Increasing organic loading rate enhanced nitrogen removal via simultaneous nitrification and denitrification. Moreover, N_2O was produced in a range 0.1 to 0.24% of ammonia loading rate in the reactors fed with ammonia of 420 and 700 mg l^{-1}.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1   Conclusions

This study investigated partial nitrification performance of entrapped cell-based reactors prepared using PPVA gel beads and operated using oxygen limiting strategy. The aims of this study were 1) to investigate the community of NOB in the reactors operated at different bulk DO concentrations, 2) to determine the role of oxygen concentration gradient formed within the gel matrix on partial nitrification, and 3) to study effect of ammonia and organic loading rates on performance and pathways of nitrogen removal as well as N_2O production in the entrapped-cell-based reactors. Three main experiments were designed to fulfill the objectives and the findings of the work can be summarized as the following.

Similar degree of partial nitrification can be achieved in entrapped-cell based reactors operated at bulk DO concentrations of 2 and 3 mg l^{-1} (**Chapter 3**). NOB communities were found not to be different in the gel matrices of both reactors. *Nitrobacter* and *Nitrospira* lineage I and *Nitrospira* lineage II coexisted in both reactors though the substrate affinity is different among them. This indicated that NOB can be survived and perform some activity in the entrapped cells, though the majority of NOB activity was inhibited by the entrapped cell system.

Two entrapped-cell-based reactors were operated with the PPVA gel matrices containing two different initial cell-to-matrix ratios at 1 and 4% (**Chapter 4**). The results showed that the reactor with 4% cell-to-matrix ratio required a shorter time to achieve partial nitrification than the reactor with cell-to-matrix ratio of 1%. However, the degree of nitrite accumulation was similar during long-run operation for both reactors. Oxygen-limiting zone (DO = 0.5-1.5 mg l^{-1}) which is preferable conditions for partial nitrification were generated at 10-230 µm from the surface of gel matrices of

both reactors. There were changes in the distribution patterns of AOB aggregates in the gel matrix during early and late stages of operation of the1% cell-to-matrix ratio contained reactor. This may be caused from the microorganisms that involved in partial nitrification can adapt themselves to appropriate ecological niches.

Nitrogen removal performance and pathways of entrapped-cell-based reactors receiving various ammonia and organic loading rates were investigated **(chapter 5)**. Without organic loading, partial nitrification can be achieved only in the reactors with higher ammonia loading rate of ≥ 0.3 kg m^{-3} d^{-1}. On the other hands, complete nitrification was found in the reactors operated at lower ammonia loading rate of ≤ 0.2 kg m^{-3} d^{-1}. The results indicated the range of ammonia loading that should be maintained to achieve partial nitrification. When organic was fed, SND occurred in most reactors with organic feeding. N$_2$O was produced in a rage 0.1 to 0.24% of ammonia loading rate in some selected reactors for study.

6.2   Recommendations

1)   Partial nitrification can be maintained in long run operation with this approach. However, NOB cells can hardly be avoided completely from the entrapped-cell based partial nitrification reactors. This may cause by the fact that NOB can shift their community composition from NOB with low oxygen affinity to NOB with high oxygen affinity under oxygen-limiting condition. To reduce threaten of high adaptive capacity of NOB to a strictly oxygen limiting condition, the switching of NOB community as influenced by the concentration of dissolved oxygen needs to be further clarified.

2)   SND occurred in most reactors with organic feeding. However, it is unclear how SND can be proceeded under this type of entrapped-cell based reactors: through partial nitrification or complete nitrification. This point should be clarified in further study.

3)   N$_2$O in liquid phase was produced in entrapped-cell-based reactors with similar degree to other studies. The emission of N$_2$O from the reactors to atmosphere needs to be concerned and required further studies to verify and reduce.

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Journal publications (not directly related to dissertation)

Nguyen T.N., Limpiyakorn T., Kunapongkiti P., Thuptimdang P., and Siripattanakul-Ratpukdi S. (2017) Influence of silver nanoparticles and liberated silver ions on nitrifying sludge: ammonia oxidation inhibitory kinetics and mechanism. Environmental Science and Pollution Reseach. Published online: 21st February 2017.

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