บทบาทของกระบวนการดีเอ็นอาร์เอในระบบบำบัดไนโตรเจนทางชีวภาพ

นางสาวปกฉัตร ชูติวิศุทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรดุษฎีบัณฑิต
สาขาวิชาวิศวกรรมสิ่งแวดล้อม ภาควิชาวิศวกรรมสิ่งแวดล้อม
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

Roles of Dissimilatory Nitrate Reduction to Ammonium in Biological Nitrogen Removal System

Miss Pokchat Chutivisut

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Engineering
Department of Environmental Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2017

Thesis Title	Roles of Dissimilatory Nitrate Reduction to Ammonium in Biological Nitrogen Removal System
By	Miss Pokchat Chutivisut
Field of Study	Environmental Engineering
Thesis Advisor	Associate Professor Wiboonluk Pungrasmi, Ph.D.
Thesis Co-Advisor	Sorawit Powtongsook, Ph.D.
	Associate ProfessorFutoshi Kurisu, Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Engineering

(Associate Professor Supot Teachavorasinskun, D.Eng.)

THESIS COMMITTEE

.....Chairman

(Assistant Professor Chaiyaporn Puprasert, Ph.D.)

.....Thesis Advisor

(Associate Professor Wiboonluk Pungrasmi, Ph.D.)

.....Thesis Co-Advisor

(Sorawit Powtongsook, Ph.D.)

.....Thesis Co-Advisor

(Associate ProfessorFutoshi Kurisu, Ph.D.)

.....Examiner

(Associate Professor Tawan Limpiyakorn, Ph.D.)

.....Examiner

(Assistant Professor Benjaporn Suwannasilp, Ph.D.)

.....External Examiner

(Associate ProfessorAkihiko Terada, D.Eng.)

ปกฉัตร ชูติวิศุทธิ์ : บทบาทของกระบวนการดีเอ็นอาร์เอในระบบบำบัดไนโตรเจนทางชีวภาพ (Roles of Dissimilatory Nitrate Reduction to Ammonium in Biological Nitrogen Removal System) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.วิบูลย์ลักษณ์ พึ่งรัศมี, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.สรวิศ เผ่าทองศุข, รศ. ดร.ฟูโตชิ คูริซู, 137 หน้า.

การบำบัดไนโตรเจนโดยวิธีการทางชีวภาพเป็นกระบวนการบำบัดน้ำเสียซึ่งโดยทั่วไปจะดำเนินการด้วยระบบไนตริฟิเคชัน-ดีไนตริฟิเคชัน เพื่อเปลี่ยนของเสียไนโตรเจนให้อยู่ในรูปก๊าซที่ไม่เป็นอันตราย อย่างไรก็ตาม นอกจากทั้งสองกระบวนการนี้แล้ว ยังมีอีกกระบวนการหนึ่งที่สามารถแข่งขันกับดีไนตริฟิเคชันในการใช้ไนเตรต นั่นคือกระบวนการดีเอ็นอาร์เอซึ่งให้ผลิตภัณฑ์สุดท้ายเป็นแอมโมเนียม งานวิจัยนี้ได้ทำการศึกษากระบวนการดีเอ็นอาร์เอโดยครอบคลุมการเกิดของกระบวนการและจุลินทรีย์ที่เกี่ยวข้องกับปฏิกิริยาของดีเอ็นอาร์เอ โดยได้มีการใช้ตะกอนจุลินทรีย์จากระบบเพาะเลี้ยงสัตว์น้ำซึ่งมีการบำบัดไนโตรเจนโดยวิธีการทางชีวภาพ ผลการทดลองพบว่าตะกอนจุลินทรีย์ซึ่งนำมาจากระบบเพาะเลี้ยงสัตว์น้ำที่มีค่า C/NO_3^- ต่ำและสูง มีการแสดงปฏิกิริยาดีเอ็นอาร์เอเมื่อได้รับการกระตุ้นจากไนเตรตและไนไตรต์ นอกจากนี้ยังพบว่าการเติมซัลไฟด์เข้าไปในระบบจะกระตุ้นการสร้างแอมโมเนียมในตะกอนจุลินทรีย์จากระบบที่มี C/NO_3^- ต่ำ แต่ไม่พบว่าซัลไฟด์มีผลทางบวกต่อกระบวนการดีเอ็นอาร์เอในตะกอนจุลินทรีย์จากระบบที่มี C/NO_3^- สูง ซึ่งบ่งชี้ว่าจุลินทรีย์ดีเอ็นอาร์เอในระบบ C/NO_3^- ต่ำและสูงเป็นชนิดที่แตกต่างกัน เนื่องจากจุลินทรีย์กลุ่มนี้สามารถแบ่งออกได้เป็นกลุ่มเรสไพลาโทรีและเฟอร์เมนเททีฟ และเนื่องจากมักมีการพบกระบวนการดีเอ็นอาร์เอในระบบนิเวศน์ที่มีค่า C/NO_3^- สูง ในงานวิจัยนี้จึงได้ทำการเพาะเลี้ยงเชื้อภายใต้สภาวะ C/NO_3^- ต่ำและสูง เพื่อศึกษาประชากรจุลินทรีย์ที่เกิดขึ้นในแต่ละสภาวะ จากการจำแนกประชากรจุลินทรีย์โดยเทคนิคอิลลูมินาไมซิคพบว่า กลุ่มจุลินทรีย์ซึ่งมีความเกี่ยวข้องกับ *Sulfurospirillum* และ Lachnospiraceae เป็นประชากรหลักของระบบเลี้ยงเชื้อที่สภาวะ C/NO_3^- สูง นอกจากนี้ ในงานวิจัยนี้ยังได้ทำการพัฒนาวิธีการศึกษาจุลินทรีย์กลุ่มเฟอร์เมนเททีฟดีเอ็นอาร์เอด้วยเทคนิคเชิงโมเลกุล โดยทำการออกแบบไพรเมอร์เพื่อจับกับยีน NADH-dependent nitrite reductase หรือยีน *nirB* ซึ่งจากการทดลองใช้ไพรเมอร์ที่ทำการออกแบบพบว่า ไพรเมอร์สามารถจับ DNA และ cDNA ซึ่งมีโอกาสเป็นจุลินทรีย์เฟอร์เมนเททีฟดีเอ็นอาร์เอได้ในตัวอย่างจากระบบที่มีสภาวะ C/NO_3^- สูง แสดงให้เห็นถึงการมีอยู่และการทำงานของเชื้อชนิดนี้ในระบบ

ภาควิชา วิศวกรรมสิ่งแวดล้อม
สาขาวิชา วิศวกรรมสิ่งแวดล้อม
ปีการศึกษา 2560

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5471415221 : MAJOR ENVIRONMENTAL ENGINEERING
KEYWORDS:

POKCHAT CHUTIVISUT: Roles of Dissimilatory Nitrate Reduction to Ammonium in Biological Nitrogen Removal System. ADVISOR: ASSOC. PROF. WIBOONLUK PUNGRASMI, Ph.D., CO-ADVISOR: SORAWIT POWTONGSOOK, Ph.D., ASSOC. PROF.FUTOSHI KURISU, Ph.D., 137 pp.

Biological nitrogen removal system is a wastewater treatment process that normally utilizes nitrification-denitrification to convert nitrogen wastes to unharmful gaseous products. However, apart from these two pathways, dissimilatory nitrate reduction to ammonium (DNRA) can also compete with denitrification for nitrate and yield ammonium waste as the end product. The aim of this research is to extend knowledge on the DNRA pathway, including the study on its occurrence and microorganisms responsible for the process. To observe the presence of DNRA, microbial sludge from aquacultures which utilized biological nitrogen removal processes was applied to examine the pathway of DNRA in these systems. The results revealed that the two microbial sludge from low and high C/NO_3^- aquaculture systems performed DNRA with different inducers, *i.e.* nitrate and nitrite. The addition of sulfide was found to enhance ammonium production in the low C/NO_3^- sludge, whereas sulfide did not have positive effect on DNRA in the high C/NO_3^- sludge incubations. This suggests that different DNRA microorganisms were responsible for the process in the low and high C/NO_3^- microbial sludge, since the DNRA microorganisms can be further classified into respiratory- and fermentative-types DNRA. As the high C/NO_3^- ecosystem has been observed to sustain DNRA over denitrification, enrichment cultures maintaining under low and high C/NO_3^- ratios were operated to monitor microbial community selected for each environment. The results from Illumina MiSeq 16S rRNA gene sequencing revealed that microbial populations closely related to *Sulfurospirillum* and the family Lachnospiraceae were the dominant microorganisms in the high C/NO_3^- culture. Additionally, primers targeting the fermentative-type DNRA (F-DNRA) microorganisms were developed in this study by using NADH-dependent nitrite reductase large subunit, or *nirB* gene, as a marker for primer detection. By applying the designed primers, target DNA and cDNA sequences were detected in the high C/NO_3^- culture samples, indicating the presence and activity of potential F-DNRA microorganisms under the high C/NO_3^- ecosystem.

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

ACKNOWLEDGEMENTS

First of all, I would like to express my gratitude to my advisor and co-advisor, Assoc. Prof. Dr. Wiboonluk Pungrasmi and Dr. Sorawit Powtongsook, for their kind supports and guidance throughout the years of my Ph.D. study. The doors to their office are always opened for me. I would not have gained all the opportunities and experiences that I have without their helps.

I would also like to convey my sincere appreciation to Assoc. Prof. Dr. Futoshi Kurisu, who had been my supervisor during my research stay at the University of Tokyo, Japan, and has continued to be my Ph.D. thesis co-advisor. I would like to express my deep gratitude to his continuous supports, advice, and insightful knowledge. I do appreciate the time and efforts he has been given me all these years.

My sincere gratitude also extends to Assist. Prof. Dr. Kazuo Isobe for his kind helps and continuous advice for my experimentations, as well as those who have been supporting me throughout my Ph.D. life, though I may not be able to mention them all by name, both at the University of Tokyo and at Chulalongkorn University.

I would also like to acknowledge the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej for the financial support, as well as the Overseas Research Experience Scholarship for Graduate Student for providing me the opportunity to conduct research at the University of Tokyo.

Finally, I would like to express my deepest gratitude to my parents, who always support me in every way they possibly can. My special thanks also go to all my friends and family who have always been supporting me, both physically and spiritually.

CONTENTS

Page

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1
INTRODUCTION

1.1. Introduction to the research

Nitrogen cycle is one of the biogeochemical processes that influence all living organisms both in terrestrial and aquatic ecosystems. Nitrogen pathways govern the conversion of organic and inorganic nitrogen, *e.g.* ammonium, nitrite, nitrate, and nitrogenous gases in nature with the processes of oxidation, reduction, assimilation, and fixation. The main players taking active roles in the nitrogen cycle are microorganisms of widespread taxa. These include ammonium-oxidizing bacteria (AOB), ammonium-oxidizing archaea (AOA), nitrite-oxidizing bacteria (NOB), denitrifiers, dissimilatory nitrate reduction to ammonium (DNRA) microorganisms, anaerobic ammonium-oxidizing (anammox) bacteria, and nitrogen-fixing bacteria. Besides their roles in nature, human has applied these microorganisms in various anthropogenic activities, one of them is the application of biological wastewater treatment system. Metabolic pathways of these microorganisms have been utilized to remove nitrogen wastes in both municipal and industrial wastewater, where the process itself is satisfactory both in terms of efficiency and being economical.

Among these microbial pathways, denitrification is one of the processes that have been widely used for nitrogen removal. The process itself reduces nitrate (NO_3^-) sequentially to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and dinitrogen gas (N_2), which releases nitrogen from water to the atmosphere. Nitrate is known to be toxic to aquatic lives and can affect human health, especially in the infants (Camargo et al., 2005; Greer & Shannon, 2005). Maintaining good efficiency of denitrification in wastewater treatment system is, therefore, essential for the successful removal of nitrate. Nonetheless, denitrifiers are not the only microorganisms capable of using nitrate under anoxic condition. The other group of nitrate reducers, which is termed DNRA microorganisms, is also able to utilize nitrate and nitrite as its electron acceptors.

DNRA converts nitrate to nitrite and then nitrite to ammonium, resulting in a different end product than that of denitrification. In other environments, *e.g.* agriculture, DNRA is considered a beneficial process as it preserves nitrogen fertilizers within the soil (Silver et al., 2005). This pathway is, however, undesirable in a biological wastewater treatment system due to its production of ammonium, a waste that usually be removed by nitrification-denitrification. The occurrence of DNRA pathway would lower the efficiency of nitrate removal, increase oxygen demand for the removal of produced ammonium (by means of nitrification), as well as increase the total nitrogen in the discharged effluent. DNRA has been hypothesized and observed to occur under a condition of high electron donors to nitrate (Burgin & Hamilton, 2007; Tiedje et al., 1983), which is normally referred to as a high carbon to nitrate (C/NO_3^-) environment. Denitrification, on the other hand, seems to prefer low C/NO_3^- ecosystem. Another electron donor found to promote DNRA is sulfide, which should also support the growth of autotrophic denitrifiers. DNRA microorganisms and denitrifiers are hence competitors for both electron donors and electron acceptors where the factors governing the success pathway are still not well-studied.

Many research in the past have revealed that wastewater treatment systems could harbor DNRA microorganisms in anoxic or anaerobic processes. These include anaerobic digestion sludge (Akunna et al., 1993; Akunna et al., 1994; Kaspar & Tiedje, 1981), a pilot plant treating sulfate- and nitrate-containing wastewater (du Preez & Maree, 1994), a lab-scale denitrifying reactor (Barber & Stuckey, 2000), as well as an anaerobic digester of a marine aquaculture system (Cytryn et al., 2003). Activity of DNRA detected among these works was in a range of 18 to 70% of the nitrate added, suggesting high competitive potential of this pathway in high organic- or sulfur-loading wastewater. In addition, systems sensitive to ammonium concentration, *e.g.* aquarium or aquaculture, can be even more vulnerable to DNRA since small concentration of ammonium is already harmful to aquatic lives (Randall & Tsui, 2002). Understanding the occurrence of DNRA pathway is therefore essential for the effective control of biological nitrate removal.

Microorganisms responsible for DNRA can be further classified into two types, which are respiratory-type DNRA (R-DNRA) and fermentative-type DNRA (F-DNRA). Each of these DNRA microorganisms utilizes different metabolic pathway to produce ammonium. The two pathways, however, have received unequal attention in the research field. Numerous works have studied R-DNRA in various environments, including estuarine and marine sediments (Smith et al., 2007; Sorensen, 1978), agricultural soils (Yin et al., 2002), forest soil (Silver et al., 2001), and hot spring (Dodsworth et al., 2011). On the contrary, F-DNRA has received much less attention regarding the study on environmental samples. The works on F-DNRA microorganisms so far were conducted with well-studied pure cultures, *e.g. Escherichia coli* and *Bacillus subtilis* (Nakano et al., 1998; Wang & Gunsalus, 2000), with only a few works on isolated strains from natural environments (Bonin, 1996; Cole & Brown, 1980; Polcyn & Podeszwa, 2009). High C/NO_3^- ratio has been found to favor fermentation over oxidation process (Burgin & Hamilton, 2007), thus F-DNRA should be an important pathway that competes with heterotrophic denitrification under this condition. However, the gap in knowledge regarding the presence, activity, and identity of F-DNRA microorganisms makes it difficult to control this microbial group from occurring in denitrifying system. Furthermore, molecular technique for the specific detection of F-DNRA microorganisms is currently unavailable, while the method for identifying R-DNRA group has been adopted and widely used since 2004 (Mohan et al., 2004).

Because the roles of both R-DNRA and F-DNRA microorganisms in wastewater treatment process are presently unknown, this research therefore focused on both types of DNRA in biological nitrate removal system. The aims of this study were to examine the occurrence of DNRA under varying environmental conditions and to investigate microorganisms responsible for this activity. The expected outcomes were to fill in some part of the knowledge gap in this field of the nitrogen cycle, and to gain deeper understanding on how DNRA can be controlled in the biological nitrogen removal system.

1.2. Objectives of the research

1.2.1. Examine the presence and potential activity of DNRA in wastewater treatment systems operating with biological nitrogen removal processes

1.2.2. Study the occurrence of DNRA microorganisms in low and high C/NO_3^- ratio enrichment cultures, using activated sludge as inoculum

1.2.3. Develop a molecular method for the detection of potential F-DNRA micro-organisms in environmental samples

1.3. Scope of the research

This research could be divided into 3 main parts, which were as follows,

1.3.1. Study on the presence and activity of DNRA in wastewater treatment process: a case study in aquaculture systems

As the occurrence of DNRA in wastewater treatment process has not yet been studied systematically, the first task of this research was to observe its presence and potential activity in selected biological nitrogen removal systems. Aquacultures operating with nitrification-denitrification (low in carbon) and biofloc technology (high in carbon) were chosen as case studies for examining DNRA in the low and high carbon environments. The experiments in this part included batch incubations of the aquaculture sludge with nitrate or nitrite to observe the activity of DNRA by detecting the production of ammonium. Sulfide, as the suggested potent inducer for DNRA, was another parameter tested by adding it along with nitrate in another set of incubations. Water sampling was done in time-series and nitrogen conversion activity was observed by monitoring the change in the inorganic nitrogen (nitrate, nitrite, and ammonium) concentrations.

1.3.2. Study on the roles of DNRA microorganisms and their competition with denitrifiers under low and high C/NO_3^- enrichment cultures

Roles of DNRA microorganisms were studied in this part using enrichment culture as a means to cultivate this microbial group under their supposedly favorable condition, *i.e.* high C/NO_3^- ratio environment. Low C/NO_3^- culture maintaining under the same condition was also run in parallel in order to make a comparison between the two systems. The experiments in this part included the operation of the low and high C/NO_3^- enrichment cultures, where the change in organic carbon and inorganic nitrogen was daily monitored. After the systems reached steady-state, batch incubations of the culture sludge with stable-isotope compounds, $^{15}NO_3^-$ and $^{15}NO_2^-$, were conducted to track the formation of ammonium from nitrate/nitrite reduction. Microbial community developed under each condition was examined using Illumina MiSeq 16S rRNA sequencing method, with sludge samples collected from different time points.

1.3.3. Development of a molecular method for the detection of F-DNRA microorganisms in microbial community

As a molecular method for detecting F-DNRA microorganisms in microbial community is still unavailable, primers targeting a marker gene of this microbial group were therefore developed in an attempt to study them in environmental samples. Gene encoding NADH-dependent nitrite reductase large subunit, or *nirB*, which is an essential gene of the F-DNRA pathway, was selected as a biomarker for F-DNRA microorganisms. The primers were designed based on *nirB* nucleotide sequences of bacteria known to perform F-DNRA activity. Pure cultures of F-DNRA bacteria were used for a primer evaluation and the chosen primer pair was applied to the sludge samples taken from the low and high C/NO_3^- enrichment cultures of this study.

CHAPTER 2
LITERATURE REVIEW

2.1. Introduction

In anoxic environment, the microbial nitrogen conversion pathways that can be found include denitrification, anaerobic ammonium oxidation (anammox), and dissimilatory nitrate reduction to ammonium (DNRA). Among these processes, denitrification has long been recognized as the main pathway for nitrogen turnover in anoxic ecosystems. Recent discoveries, however, have demonstrated that anammox and DNRA can also play significant roles in certain environments (Dong et al., 2009; Dong et al., 2011; Smith et al., 2007). Favorable conditions for each of this pathway are hypothesized to depend on the type and concentration of electron donors, the amount of inorganic nitrogen compounds present, along with the redox potential in that system (Kraft et al., 2011). To date, there have been several reports on the presence and application of anammox bacteria in wastewater treatment process (Lahav et al., 2009; Tal et al., 2006; Tal et al., 2003), while DNRA microorganisms have received much less attention in this ecosystem. The reaction of DNRA and other pathways of the nitrogen cycle are as shown in Figure 2.1.

DNRA microorganisms have been suggested by Burgin and Hamilton (2007) to be classified into two groups based on their pathways, which are a fermentative-type DNRA (F-DNRA) and a respiratory-type DNRA (R-DNRA). The ability to perform DNRA is phylogenetically widespread among many bacterial taxa, and has been confirmed as one of the metabolic pathways in *Escherichia coli* (Cole, 1978); *Bacillus subtilis* (Hoffmann et al., 1998); *Enterobacter aerogenes*, *Serratia marcescens*, *Erwinia carotovora* (Bleakley & Tiedje, 1982); *Citrobacter* species (Smith, 1982); *Klebsiella* species (Dunn et al., 1979); and *Vibrio* species (Macfarlane & Herbert, 1982). However, the classification between the F-DNRA and R-DNRA microorganisms has not yet been

clearly verified. Hence, further studies are still needed in order to categorize the subgroups of DNRA microorganisms.

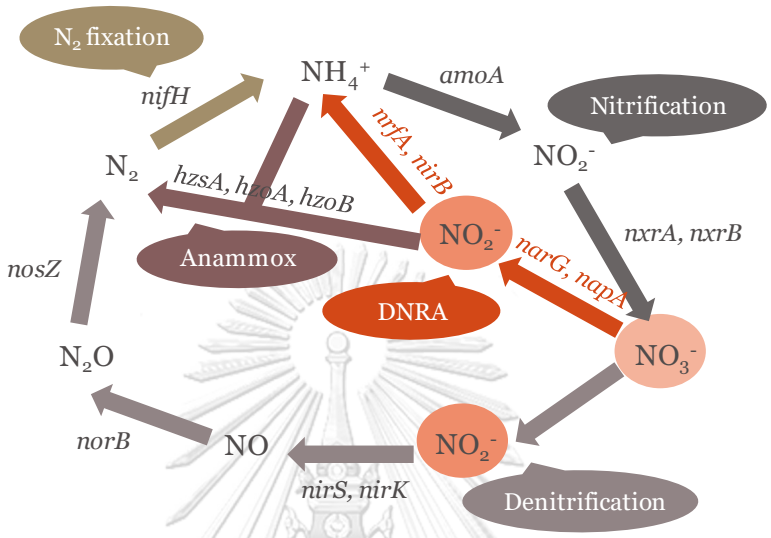


Fig. 2.1 Microbial nitrogen cycle and the marker genes involved in each pathway

One of the conditions that have been reported to favor DNRA is a high C/NO_3^- environment, where the organic carbon is rich and nitrate is limited in concentration. This condition favors fermentation over oxidative process (Polcyn & Podeszwa, 2009), and should enhance the growth and activity of F-DNRA microorganisms. Apart from organic carbon, sulfide is another parameter found to support the process of DNRA. Sulfur compounds can be involved in the nitrogen cycle when serving as an electron donor for chemolithoautotrophic denitrifiers or R-DNRA microorganisms. Moderate sulfide concentration can drive the community of chemolithoautotrophic denitrifiers under anoxic condition (Shao et al., 2011), while high sulfide is found to enhance the activity of R-DNRA microorganisms (Brunet & Garcia-Gil, 1996; Sher et al., 2008). Nevertheless, both F-DNRA and R-DNRA microorganisms have to compete with heterotrophic and autotrophic denitrifiers for the same source of electron donors and electron acceptors. The roles of both types of DNRA microorganisms in nitrate-reducing community and their ability to compete with other microbial groups are still largely

unknown. Although DNRA was found to dominate in certain environments (Dong et al., 2009), it is still not known whether F-DNRA or R-DNRA was the main player in those ecosystems. In this chapter, the overview of literatures on each type of DNRA microorganisms is given, including their competition with denitrifiers, the genes and metabolic pathways involved, and environmental parameters affecting their occurrence, which underlies the research conducted in the later chapters

2.2. Literature Review

2.2.1. Competition between denitrification and DNRA

Denitrification and DNRA are both microbial mediated processes that perform the reduction of nitrate and nitrite under anoxic condition. The difference in these two pathways is the resulting product, as denitrification generates nitrogen gas while DNRA produces ammonium. Because both of these microbial groups use the same types of electron acceptors as well as the electron donors, denitrifiers and DNRA microorganisms thus have to compete in the same environment for growth. Their competition is controlled by the ratio between available electron donors to electron acceptors, redox potential, and the kinetics and energetic values of each pathway (Kraft et al., 2011; Tiedje et al., 1983). Gibbs free energy (ΔG^0) of denitrification and DNRA when hydrogen is used as an electron donor is as shown in Table 2.1. From this table, it can be seen that the free potential energy obtained by denitrification per mole of hydrogen is higher than that obtained from DNRA. However, DNRA gains slightly higher free energy per mole of nitrate compared to denitrification. In addition, the ability to accept more number of electrons during the conversion of nitrate to ammonium seems to offer DNRA advantage over denitrification in the environment rich in electron donors but lack in electron acceptors. The predominant nitrate-reducing populations in each microbial community hence depends on the successful competition between denitrifiers and DNRA microorganisms, which in turn is controlled by the surrounding environmental parameters in that system.

Table 2.1 Energetic values gained from denitrification and DNRA processes

Process	Pathway	ΔG^0 (kcal/mole)		Electrons accepted per nitrate
		Hydrogen (H_2)	Nitrate (NO_3^-)	
Denitrification	$2NO_3^- + 5H_2 + 2H^+ \rightarrow N_2 + 6H_2O$	-53.6	-133.9	5
DNRA	$NO_3^- + 4H_2 + 2H^+ \rightarrow NH_4^+ + 3H_2O$	-35.8	-143.3	8

Modified from Tiedje et al. (1983)

2.2.2. Fermentative-type DNRA (F-DNRA) process

Fermentative-type DNRA (F-DNRA) is a pathway responsible by a group of micro-organisms that convert nitrite to ammonium during the process of fermentation. The term fermentative has been used by Burgin and Hamilton (2007) to differentiate this process from the other pathway, R-DNRA, where sulfide seems to be the main factor for its activity (Burgin & Hamilton, 2007). In the early works on DNRA, microorganisms studied were mostly enteric bacteria with fermentative pathway for growth (Tiedje et al., 1983). These enteric bacteria perform fermentation using organic compounds in the absence of inorganic electron acceptors, but they can also use oxygen, nitrate, nitrite, and other oxidizing agents for respiratory process (Mohan & Cole, 2007). In the case of nitrite, fermentative bacteria can carry out fermentation while using nitrite as the electron sink for NADH oxidation (Figure 2.2). NAD^+ is an electron carrier molecule in the cells which will be reduced to NADH after accepting electrons from organic compounds. NADH is later re-oxidized back to NAD^+ using nitrite as an electron acceptor, while nitrite is reduced to ammonium with a transfer of six electrons. It had been found that most of the DNRA microorganisms are fermentative, in contrast to denitrifiers that mostly have oxidative pathway for growth (Tiedje et al., 1983).

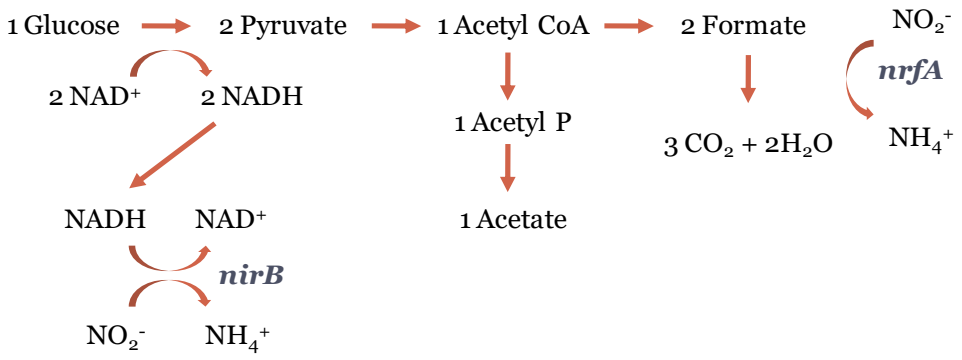


Fig. 2.2 Fermentative pathway coupling with DNRA process (Bonin, 1996)

So far, there are two identified metabolic processes for DNRA, the first is Nir pathway driven by a NADH-dependent nitrite reductase, which directly involves in fermentation. The second is Nrf pathway driven by a formate-dependent nitrite reductase, which can involve, but indirectly, to the fermentation process (Page et al., 1990). Both of these nitrite reduction pathways were identified in *E. coli* (Wang & Gunsalus, 2000), but not every DNRA microorganisms contain both of these genes in their genome. The Nrf pathway can use formate, sulfide, hydrogen, and possibly other compounds as its electron donors (Tiedje et al., 1983), while the Nir pathway depends on NADH as its sole electron source (Wang & Gunsalus, 2000). These two different and seemingly unrelated nitrite reductions both release ammonium to the environment and thus maintain nitrogen compounds within the ecosystem, whereas denitrification releases nitrogen to the atmosphere in the gaseous forms (Tiedje et al., 1983).

It should be noted that there is a difference between dissimilatory and assimilatory processes in the cell metabolism. Nitrate reduction can be catalyzed by assimilatory nitrate reduction, denitrification, and DNRA (Mohan & Cole, 2007). The assimilatory nitrate reduction is a slow pathway that reduced nitrate to ammonium for cell synthesis, which can be induced by the presence of nitrate or nitrite and is strongly repressed by the availability of ammonium (Burgin & Hamilton, 2007; Cole & Brown, 1980; Mohan & Cole, 2007; Tiedje, 1988). In this regards, nitrate and nitrite act as nitrogen sources for microbial growth, and the ammonium is produced only in an appropriate amount for cell requirement. On the other hand, both nitrate and nitrite

can also act as the electron acceptors (Stewart, 1994; Tiedje, 1988) in the process called dissimilatory nitrate/nitrite reduction. Dissimilatory nitrate reduction can occur through two microbial processes, the first is denitrification, in which nitrate is reduced to nitrite and later to nitrogenous gases (Figure 2.3a), the second is DNRA, where nitrate is reduced to nitrite and then to ammonium (Figure 2.3b) (Bonin, 1996; Tiedje, 1988). DNRA occurs rapidly with an accumulation of ammonium from cell excretion (Cole & Brown, 1980). It is induced under anoxic environment and further induced in the presence of nitrate and nitrite, while ammonium availability has no effect on it (Mohan & Cole, 2007; Page et al., 1990; Stewart, 1994; Tiedje, 1988). Furthermore, DNRA is repressed under aerobic condition whereas oxygen has little influence on nitrate assimilation (Cole & Brown, 1980; Mohan & Cole, 2007; Tiedje, 1988). Nevertheless, despite the differences in physiology of DNRA and assimilatory nitrate reduction, it has been observed that the same set of genes is utilized by both pathways for the conversion of nitrite to ammonium (Nakano et al., 1998). This complicates the differentiation between the microorganisms performing DNRA and those having this set of gene for nitrite assimilation.

The genes involved in the reduction of nitrate to nitrite in DNRA have been found to be the same as those used for denitrification, which include a membrane-bound nitrate reductase encoded by *narG* gene, and a periplasmic nitrate reductase encoded by *napA* gene (Kraft et al., 2011). In contrast, nitrite reductases in DNRA microorganisms have been found to be different from those used in denitrifiers. For denitrification, two nitrite reductases have been described, the first is a cytochrome *cd1* nitrite reductase encoded by *nirS* gene, and the second is a copper-containing nitrite reductase encoded by *nirK* gene. Both enzymes are responsible for the conversion of nitrite to nitric oxide (Figure 2.3a). For DNRA, the enzymes responsible for the reduction of nitrite to ammonium include a periplasmic nitrite reductase encoded by *nrfA* gene (Mohan et al., 2004) and a cytoplasmic nitrite reductase encoded by *nirB* gene (Figure 2.3b). These enzymes catalyze the reduction of nitrite to ammonium without generating an intermediate molecule in the process (Einsle et al., 2002). However, there has been a speculation that nitrous oxide is released as a by-

product from DNRA pathway (Kraft et al., 2011; Senga et al., 2006; Welsh et al., 2001). Therefore, DNRA may also contribute to the emission of nitrous oxide, one of the important anthropogenic greenhouse gases with high global warming potential, to the atmosphere.

Table 2.2 Nitrate and nitrite reductions occurring with different microbial pathways

Process	Gene involved*	Regulated by		Active site
		NH_4^+	O_2	
Denitrification $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$	*nirS*, *nirK*	None	Repressed	Cytoplasm/ Periplasm
Nitrate assimilation $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$	*nirB*	Repressed	None	Cytoplasm
F-DNRA $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$	*nirB*	None	Repressed	Cytoplasm
R-DNRA $NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$	*nrfA*	None	Repressed	Periplasm

*Only indicate the marker gene involved in the nitrite reduction pathway

a b

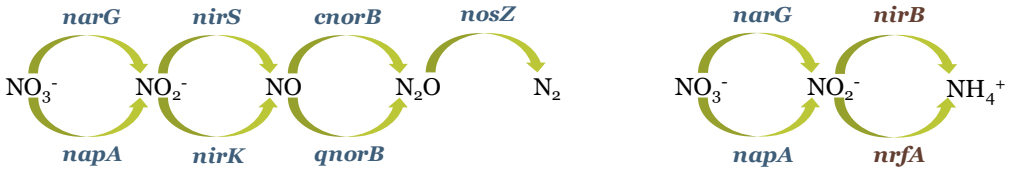


Fig. 2.3 Pathways of dissimilatory nitrate reduction with the main genes involved, (a) denitrification and (b) DNRA

2.2.3. Respiratory-type DNRA (R-DNRA) process

Respiratory-type DNRA (R-DNRA) is performing through the pathway of Nrf, which can involve sulfide, hydrogen, and formate oxidation (Tiedje, 1988). Formate-dependent nitrite reductase, the enzyme driving the Nrf pathway, occurs in the periplasmic part of the cell and is membrane-associated. It thus involves in electron transport chain and the generation of ATP by the proton motive force (Cole, 1996; Stewart, 1994; Wang & Gunsalus, 2000). Many sulfate reducers have been found to contain *nrfA* gene of the Nrf pathway. This gene thus seems to be widely distributed among many phylogenetic groups of prokaryotes and has been suggested to be more significant in the environment than the Nir pathway (Mohan et al., 2004).

Previous research has revealed that several DNRA microorganisms were closely related to sulfate reducers in the group of Deltaproteobacteria, which are mostly *Desulfovibrio* (Mohan et al., 2004; Schreier et al., 2010; Smith et al., 2007). *Desulfovibrio* spp. are the bacteria that had been intensively studied in pure cultures, and many of them, apart from reducing sulfate for energy conservation, also perform nitrate respiration with ammonium as the end product (Dalsgaard & Bak, 1994; McCready et al., 1983; Seitz & Cypionka, 1986). In the case of *D. desulfuricans*, sulfate reduction was repressed when nitrate or nitrite is present, suggesting that nitrate and nitrite are preferred over sulfate in this species (Seitz & Cypionka, 1986). However, another species of sulfate reducer, *Desulfobulbus propionicus*, can only reduce nitrite but not nitrate (Dannenberg et al., 1992). Most sulfate reducers can use various kinds of electron donors, *e.g.* sulfur compounds, hydrogen, and organic acids. Ability to oxidize sulfide means that these sulfate reducers can perform reactions on both directions of the sulfur pathways, which are sulfate reduction and sulfide oxidation. On the other hand, the involvement of the true sulfide oxidizers in DNRA had not yet been identified. Sulfide oxidizers that able to reduce nitrate were mostly found as chemolithoautotrophic denitrifiers, *e.g. Thiobacillus denitrificans*, but their roles in DNRA pathway is still obscure. So far, the sulfide oxidizer that has been found to

perform DNRA is *Wolinella succinogenes* (Liu et al., 1983), which possesses the *nrfA* gene in its genome (Greene et al., 2003).

2.2.4. E. coli as a model microorganism for DNRA

E. coli is a well-known bacterium that has been intensively studied for its ability to perform DNRA. The genome of *E. coli* contains both the genes for NADH-dependent nitrite reductase that drives the Nir pathway, and the genes for formate-dependent nitrite reductase which drives the Nrf pathway. These two sets of genes are fond to be independent from each other and are regulated by two separate operons (Page et al., 1990). NADH-dependent nitrite reductase in *E. coli* is encoded by a *nirBDC* operon consisted of *nirB*, *nirD*, and *cysG* genes (Harborne et al., 1992; Wang & Gunsalus, 2000). In other bacteria, this enzyme can either be a single polypeptide enzyme, NirB, or a two-subunit enzyme, NirB-NirD (Mohan & Cole, 2007), where NirB is the primary structural subunit and NirD is the secondary subunit of the enzyme (Harborne et al., 1992). NADH-dependent nitrite reductase is a soluble enzyme in bacterial cytoplasm that solely uses NADH as its electron donor, while reduces nitrite to ammonium in the process (Wang & Gunsalus, 2000). The Nir pathway has been hypothesized to have a role in nitrite detoxification during nitrate reduction, as the reduction of nitrate would lead to a rapid accumulation of nitrite, which is toxic to cell (Cole, 1996; Page et al., 1990). No energy conservation through oxidative phosphorylation occurs during this process of nitrite reduction, thus it is classified as a dissimilatory, not a respiratory, process (Cole & Brown, 1980; Stewart, 1994).

For the Nrf pathway, which regulated by formate-dependent nitrite reductase, energy is generated through oxidative phosphorylation during the reduction of nitrite to ammonium. This enzyme is membrane-associated and involves in the electron transport chain, which eventually produces proton motive force. Hence the process is classified as respiratory (Cole, 1996; Stewart, 1994; Wang & Gunsalus, 2000). In *E. coli*, this pathway is encoded by a *nrfABCDEFG* operon (Wang & Gunsalus, 2000) and the reaction occurs in periplasm of the cell (Cole, 1996). Since formate is used as the electron donor for this enzyme in a fermentative reaction, the pathway was named

nitrite reduction by formate or Nrf (Cole, 1996). Nevertheless, this pathway can involve a broader range of electron donors, including hydrogen (Tiedje, 1988) and sulfide (Brunet & Garcia-Gil, 1996). And because the Nir pathway does not significantly conserve the energy during the reduction of nitrite, the term dissimilatory is used to represent nitrate reduction to ammonium for both the Nir and Nrf pathways (Mohan & Cole, 2007; Stewart, 1994).

2.2.5. Influence of C/NO_3^- ratio and nitrogen species on DNRA pathway

The ratio between carbon and nitrate (C/NO_3^-) is one of the main factors that control pathway of nitrate and nitrite reduction. F-DNRA, as a heterotrophic process, is under the influence of organic carbon available in the system. Apart from that, it also has to compete with heterotrophic denitrification for both organic carbon and nitrate/nitrite. A high C/NO_3^- ratio, the reduced condition with high electron donors and limited nitrate, is referred to as the condition favor DNRA, while denitrification is said to favor a low C/NO_3^- ratio, the condition with less electron donors and high nitrate concentration (Burgin & Hamilton, 2007; Mohan & Cole, 2007). The high electron pool may be advantageous for DNRA that transfers higher number of electrons than denitrification, and thus is a more efficient and energetically favorable pathway in rich-carbon, limited nitrate environment (Brunet & Garcia-Gil, 1996; Mohan & Cole, 2007; Tiedje, 1988). Nitrate used in DNRA is reduced into ammonium with a total transfer of eight electrons, whereas denitrification transfers only five electrons from the transformation of nitrate to dinitrogen gas (Kraft et al., 2011). Another reason for this is that the carbon-rich, electron acceptor-poor environment favors for fermentation, and hence would select for F-DNRA process rather than the oxidative denitrification (Polcyn & Podeszwa, 2009).

On the other hand, the influence of nitrate and nitrite on this competition is rarely studied in the past. Most research that examined the effect of nitrogen species on DNRA has conducted experiment on pure cultures. For example, in the study of Polcyn and Podeszwa (2009), F-DNRA pathway in *Bradyrhizobium* sp. was triggered in

the presence of nitrite. However, when nitrate was added, DNRA did not occur until nitrate was completely consumed. They hence hypothesized that fermentation occurs simultaneously with nitrite reduction to ammonium, but not with the reduction of nitrate. The activity of each nitrite reductase enzyme, the NADH- and formate-dependent nitrite reductases, had also been studied for its response to nitrate and nitrite presence. By studying *E. coli*, it was found that NADH-dependent nitrite reductase is induced by the presence of nitrate, while formate-dependent nitrite reductase is repressed by it (Page et al., 1990; Wang & Gunsalus, 2000). The explanation for this is that formate-dependent nitrite reductase is a pathway with energy conservation, and thus is repressed when nitrate, a more thermodynamically preferable electron acceptor, is present. For NADH-dependent nitrite reductase, the main purpose of this pathway is to detoxify nitrite, it hence functions along with nitrate reductase when nitrate is available (Cole, 1996). It therefore seems that the different pathways of DNRA are induced differently by nitrate and nitrite.

2.2.6. nirB as a marker gene for F-DNRA microorganisms

The gene *nrfA* of the Nrf pathway has been used as a biomarker for studying R-DNRA in the environments by several studies (Mohan et al., 2004), while the Nir pathway is so far overlooked by most of the research works. To date, the studies on F-DNRA had only been conducted with pure cultures, while the role of DNRA in microbial community was solely investigated on the R-DNRA process. As *nrfA* is a marker gene for the Nrf pathway, the gene *nirB*, which encodes the primary structural subunit of the Nir enzyme, can also be used as a biomarker for F-DNRA micro-organisms. The study on Nir pathway in *E. coli* has revealed that *nirB* is an important gene for the activity of the NADH-dependent nitrite reductase, as the mutant *E. coli* lacking *nirB* gene could not perform the reduction of nitrite to ammonium (Cole et al., 1980). Hence this gene is a suitable option for the detection and identification of F-DNRA microorganisms, when this microbial group is the target of interest in a microbial ecosystem.

2.2.7. Development of primers for the detection of nirB gene

In order to develop a primer pair for studying F-DNRA community, nucleotide sequences of the *nirB* gene from different microorganisms are required to find the conserved regions for primer annealing during the PCR reaction. Since there is a variation within the same genes in different microorganisms; the conserved region (the position in the gene that is identical across all species) is required for the detection of the gene in an environmental sample. Nevertheless, in the case of functional gene, it is often difficult to find the conserved region within the gene of interest as the gene has often evolved through time. It is therefore not practical to use a conventional primer, and so a degenerate primer has to be developed instead. Degenerate primers are a mixture of primers that have variable bases in certain positions of their nucleotide strands, which allow the primers to cover all possible sequences of the target gene in different microorganisms. Examples of degenerate primers applied for studying microorganisms in environmental samples are listed in Table 2.3

Table 2.3 Examples of degenerate primers targeting different microorganisms

Target microorganisms	Target gene	Primers	Sequence* (5´ to 3´)	References
Nitrate reducers	*narG*	narG1960f	TAY GTS GGS CAR GAR AA	Lam et al. (2009)
		narG2650r	TYT CRT ACC ABG TBG C	
	napA	v66	TAY TTY YTN HSN AAR ATH ATG TAY GG	Flanagan et al. (1999)
		v67	DAT NGG RTG CAT YTC NGC CAT RTT	
R-DNRA microorganisms	*nrfA*	nrfA F1	GCN TGY TGG WSN TGY AA	Mohan et al. (2004)
		nrfA R1	TWN GGC ATR TGR CAR TC	

*B=C+G+T, D=A+G+T, H= A+C+T, N=A+C+G+T, R=A+G, S= G+C, W=A+T, Y=C+T

These degenerate primers are designed from the conserved regions of amino acid sequences of the gene of interest. Because, though the same genes in different microorganisms have evolved through time, these genes are still coded for the same protein with the same function. Therefore, it is easier to find the identical sequences, or conserved regions, within amino acids than in the nucleotide sequences. And since each amino acid is coded by a set of three nucleotide bases in DNA called codon, and one amino acid can be coded by several of these codons (Table 2.4), the conserved amino acid regions hence can come from different nucleotide bases in the genomic DNA of the different microorganisms. This leads to the development of the degenerate primers, which have nucleotide positions containing many possible bases.

Table 2.4 Codons coded for different types of amino acids

Second base

First base	U*		C		A		G		Third base
	codon	amino acid	codon	amino acid	codon	amino acid	codon	amino acid	
U*	UUU	F	UCU	S	UAU	Y	UGU	C	U*
	UUC		UCC		UAC		UGC		C
	UUA	L	UCA		UAA	Stop	UGA	Stop	A
	UUG		UCG		UAG		UGG	W	G
C	CUU	L	CCU	P	CAU	H	CGU	R	U*
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Q	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	I	ACU	T	AAU	N	AGU	S	U*
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	K	AGA	R	A
	AUG	M	ACG		AAG		AGG		G
G	GUU	V	GCU	A	GAU	D	GGU	G	U*
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	E	GGA		A
	GUG		GCG		GAG		GGG		G

*In RNA, U is the substitute nucleotide base for T

CHAPTER 3
OCCURRENCE OF DNRA IN WASTEWATER TREATMENT PROCESS: A CASE STUDY IN AQUACULTURE SYSTEMS

3.1. Introduction

Biological nitrogen removal system depends on the presence and activity of microorganisms involving in the nitrogen cycle. This includes the pathways of nitrification, denitrification, and anaerobic ammonium oxidation (anammox), which will eventually release inorganic nitrogen waste to the atmosphere in the form of gases. Apart from these desirable processes, the production of ammonium from nitrate/nitrite by dissimilatory nitrate reduction to ammonium (DNRA) can also take place and can lead to the lower efficiency of denitrification. Key parameter known to affect the dominant microbial populations in an anoxic ecosystem is the ratio of carbon to nitrate (C/NO_3^-). Low C/NO_3^- environment has been found to sustain denitrifying community whereas DNRA activity has frequently been observed under a high C/NO_3^- condition. Wastewater containing high concentration of organic carbon compared to the level of nitrate available is thus susceptible to the occurrence of DNRA process.

The effects of ammonium formed by DNRA can be more pronounced in a system vulnerable to even a low concentration of ammonium, *e.g.* aquaculture and aquarium. This is due to the high toxicity of ammonium to aquatic animals, in which ammonium higher than 1 mg-N/L is already considered unsafe for fish and shrimp (Randall & Tsui, 2002). The activity of DNRA has previously been detected in sediment below cages of an open water fish farm, with ammonium-forming activity up to 7-fold greater than denitrification (Christensen et al., 2000), as well as in anaerobic digesters of a marine aquaculture systems in which DNRA seemed to be induced by sulfide (Cytryn et al., 2003; Sher et al., 2008). It therefore can be seen that aquaculture system can sustain DNRA and this process can compete with denitrification under certain condition. High volume of water used in aquaculture industry makes the treatment

and reuse of wastewater necessary in order to reduce water usage and environmental problems posing by the discharged effluent. Since the efficient removal of nitrogen is required for a successful water-recirculating aquaculture system, DNRA is hence needed to be controlled so that nitrate can be treated effectively through denitrification.

Besides the removal of nitrogen wastes via nitrification-denitrification, another treatment process called biofloc technology is also applied in aquaculture industry to treat the nitrogen by means of heterotrophic ammonium assimilation (Crab et al., 2012). This treatment requires an addition of organic carbon to promote the growth of heterotrophic microorganisms, which in turn would need ammonium for cell synthesis. The advantage of this process is the rapid growth of heterotrophs compared to the slower growth rate of autotrophic ammonium and nitrite oxidizers. However, the high organic carbon and the presence of nitrate due to some activity of nitrifiers can also make the biofloc system susceptible to the occurrence of DNRA.

In aquaculture system, microorganisms grow with the inorganic nitrogen and organic carbon (from animal feed and feces) form microbial sludge particles similar to those found in the activated sludge wastewater treatment process. Microorganisms responsible for the biological nitrogen removal are also residing in this sludge, making it an important source of treatment for the system. This microbial sludge from both conventional and biofloc aquacultures was analyzed in this study for the existence of DNRA pathways. The sludge was collected from aquaculture tanks and subjected to short-term batch incubations. Both of the aquaculture systems had good water quality with low concentrations of ammonium, nitrite, and nitrate. The conventional aquaculture was harboring active nitrifying-denitrifying sludge, whereas the biofloc system was daily added with starch as a carbon source to promote the growth of heterotrophic microorganisms. As both systems showed no indication of DNRA, the short-term incubations were meant to test whether the process existed by observing its activity during the addition of nitrate or nitrite. The effect of sulfide was also examined by adding sulfide along with nitrate in the other set of incubations.

Comparison between the low and high C/NO_3^- ecosystems could be made due to the different level of organic carbon available in the conventional and biofloc aquacultures. Hypothesis of this part of the research was that aquaculture system can sustain DNRA microorganisms and their activity could be observed under certain condition. The objective of the study was therefore to examine the existence of DNRA pathway by detecting its activity in the low and high C/NO_3^- aquaculture sludge with nitrate or nitrite as a sole inducer, or with sulfide as an additional stimulator.

3.2. Materials and Methods

3.2.1. Sources of aquaculture sludge

Microbial sludge from a conventional aquaculture system (having low C/NO_3^- ratio) was taken from a 4-m^3 indoor Nile tilapia culture tank with internal nitrifying-denitrifying biofilters for ammonium, nitrite, and nitrate removal. The system was observed to perform both nitrification and denitrification since the inorganic nitrogen always remained low (Table 3.1). Sludge from a biofloc system (having high C/NO_3^- ratio) was collected from an outdoor 800-L Nile tilapia biofloc tank, where starch was added daily to provide C/N ratio of 20/1. In this biofloc aquaculture, nitrogen wastes were meant to be removed by ammonium assimilation. It was found, however, that nitrate accumulated in the water (Table 3.1), hence denitrification was presumably present in the system.

Table 3.1 Water quality in the conventional (low C/NO_3^-) and biofloc (high C/NO_3^-) aquaculture systems

C/NO_3^-	NH_4^+ (mg-N/L)	NO_2^- (mg-N/L)	NO_3^- (mg-N/L)	SS* (mg/L)	DO** (mg/L)	pH	Temp. (C°)
Low	0.04±0.04	0.09±0.03	10.0±0.7	126.0±17.4	4.2±0.1	6.8±0.0	29.7±0.1
High	0.13±0.02	0.35±0.01	32.8±1.0	498.3±30.1	7.1±0.2	7.0±0.0	29.6±0.1

*Suspended solids, **Dissolved oxygen

3.2.2. Detection of DNRA pathway in the low and high C/NO_3^--N aquaculture sludge

Short-term sludge incubations were performed in batch mode in 1-L DURAN® bottles at room temperature (30°C), with approximately 10 g (dry weight) of sludge per L. In the first set of incubations, nitrate was supplied to the low and high C/NO_3^- aquaculture sludge at the concentrations of 25 and 100 mg NO_3^--N/L in the form of $NaNO_3$. Each incubation was supplemented with nutrients containing (per L): 3.8 g of Na_2HPO_4, 1.5 g of K_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 2 ml of trace element solution (van Rijn et al., 1996). Trace element solution added consisted of (per L): 5.0 g of EDTA, 2.2 g of $ZnSO_4 \cdot 7H_2O$, 0.734 g of $CaCl_2 \cdot 2H_2O$, 0.506 g of $MnCl_2 \cdot 4H_2O$, 0.499 g of $FeSO_4 \cdot 7H_2O$, 0.0195 g of $NaMoO_4 \cdot 2H_2O$, 0.157 g of $CuSO_4 \cdot 5H_2O$, and 0.161 g of $CoCl_2 \cdot 6H_2O$ (Vishniac & Santer, 1957). Organic carbon was not supplied in the incubations. The incubated sludge was mixed by using magnetic stirrer to maintain a homogenous state of the system. Water samples were taken every 30 or 60 minutes depending on the initial concentration of nitrate. For the second set of incubations, the experiment was done in the same manner with nitrite concentrations of 10, 20, and 50 mg-N/L in the form of $NaNO_2$, where only the low C/NO_3^- sludge was tested.

3.2.3. Effect of sulfide on nitrate reduction in the low and high C/NO_3^--N aquaculture sludge

Effects of sulfide on nitrate reduction was studied by adding sulfide (in the formed of $Na_2S \cdot H_2O$) at the concentrations of 50 and 100 mg-S^{2-}/L for the low C/NO_3^- aquaculture sludge, and 25 and 50 mg-S^{2-}/L for the high C/NO_3^- aquaculture sludge. The incubations were conducted in batch mode in the same manner as described for the experiments with nitrate and nitrite (*3.2.2.*). Nitrate was added in the formed of $NaNO_3$ at 100 mg-N/L for all sulfide concentrations. The medium supplemented was also the same as described in the previous experiment.

3.2.4. Analytical procedures

Nitrate was analyzed by means of ultraviolet spectrophotometric method according to the Standard Methods for Water and Wastewater Analysis (APHA, 2012). Nitrite and ammonium were both analyzed colorimetrically using sulfanilamide method (APHA, 2012) and salicylate-hypochlorite method (Bower & Holm-Hansen, 1980), respectively. Fixation of sulfide before the analysis was performed by adding 2.5 ml of the sample into 1 ml of 5% zinc acetate solution (Cytryn et al., 2003). The concentration of sulfide was measured colorimetrically by means of methylene blue method (Cline, 1969).

3.3. Results and Discussion

3.3.1. The occurrence of DNRA pathway in the low and high C/NO_3^- aquaculture sludge

The occurrence of DNRA in the low and high C/NO_3^- aquaculture sludge was observed by monitoring the production of ammonium during the course of the incubations. The systems were presumed to be anoxic due to the presence of high sludge concentration, so the trace amount of oxygen should be readily consumed during the initial phase of the experiments. Organic carbon was not supplied in the medium as the aquaculture sludge already contained carbon derived from digested animal feed and feces. The organic carbon could then be supplied intrinsically by the sludge itself. Different concentrations of nitrate/nitrite, which would result in different ratios of C/NO_3^- in the system, were applied in an attempt to find the presence of DNRA process in each incubation.

For the incubations of low C/NO_3^- sludge (from the conventional aquaculture system), nitrate addition of 25 mg-N/L demonstrated rapid reduction of nitrate where it was consumed to <2.0 mg-N/L within 120 min of the experiment (Fig. 3. 1a). This result reflects active nitrate reduction activity, which was also observed in the aquaculture system from which the sludge was taken. Nitrite was found to increase during the sharp reduction of nitrate, then decreased to <0.1 mg-N/L when nitrate reduction ceased.

Ammonium always remained below 0.2 mg-N/L throughout the incubation, therefore showed no indication of DNRA activity. The incubation of the low C/NO_3^- aquaculture sludge with 100 mg-N/L of nitrate also exhibited the same trend of nitrogen conversion, and also with no obvious sign of DNRA (ammonium always <0.2 mg-N/L) (Fig. 3.1b). The results of both incubations indicate that denitrifiers were most likely responsible for nitrate and nitrite reduction and should be the main nitrate reducers in this low C/NO_3^- aquaculture sludge.

As for the high C/NO_3^- sludge (from the biofloc system), nitrate was also reduced during the incubations but the rates observed were slower than what found for the low C/NO_3^- ones (Fig. 3.2a and b). This should be due to the lower population of denitrifiers in the biofloc system, since this process was not meant to harbor denitrification. However, it was found that nitrate reduction occurred along with the rise in ammonium concentrations. Ammonium, from as low as 0.1 mg-N/L, increased to 3.7 and 3.3 mg-N/L for the incubations with nitrate of 25 and 100 mg-N/L, respectively. Ammonium production observed most likely came from the activity of DNRA since ammonium released from organic decomposition was unlikely to occur during this short-term incubation (and the incident was not observed for the low C/NO_3^- incubations, despite the same amount of sludge used). This indicates the existence of DNRA microorganisms in the biofloc aquaculture system, which could possibly be either respiratory- or fermentative-types, or both. The possibility of being F-DNRA microorganisms, however, might be higher because most of the R-DNRA microorganisms known so far mainly use inorganic electron donors or simple organic carbon, *e.g.* formate ($CHOO^-$) (Dannenberg et al., 1992), whereas the biofloc system was provided with a complex organic carbon (*i.e.* starch). As many fermentative bacteria are known to be facultative anaerobes, these F-DNRA microorganisms should be able to grow under aerobic condition and should still be capable of fermentation and DNRA under anoxic environment.

The pathway of DNRA was previously observed in anaerobic sludge supplied with nitrate and nitrite (Akunna et al., 1993). The product of the reduction was found

to be ammonium when glucose and glycerol was utilized as the carbon source, while the supplementation with acetic acid, lactic acid, and methanol resulted in no ammonium production, and the activity occurred was essentially denitrification. This suggests that the type of electron donor is important for the selection of nitrate/ nitrite reduction pathways and that the ammonium found should be originated from nitrate/nitrite. The generation of ammonium from anaerobic sludge seemed to occur mainly during the acidogenesis (Akunna et al., 1994), which might indicate the activity of F-DNRA rather than R-DNRA. Nevertheless, as fermentation often leads to the production of acetate and formate, the two known carbon sources for R-DNRA microorganisms (Yoon et al., 2015), the co-occurrence of both types of DNRA might be common in nature. The proportion of each group under varying C/NO_3^- ratios, however, remained to be verified.

Apart from ammonium, nitrite was also found to accumulate with the highest concentrations of 5.5 and 7.6 mg-N/L for nitrate of 25 and 100 mg-N/L, respectively. This accumulation might suggest that nitrite reduction was a rate-limiting step for these microbial populations. Initial concentration of nitrate has also been indicated as a factor controlling DNRA (Akunna et al., 1994), with lower amount of nitrate leading to the higher DNRA activity. This, however, was not clearly observed in the incubations with the high C/NO_3^- sludge in this study, as nitrate of 25 and 100 mg-N/L was found to yield similar amounts of ammonium (3.7 and 3.3 mg-N/L, respectively). Concentrations of both ammonium and nitrite detected in the experiments had already exceeded the recommended value for aquaculture system. In addition, this amount of ammonium was produced during a short-term incubation, thus the accumulated ammonium could be even higher if this process was to occur in the actual system. Nevertheless, as ammonium is assimilated in the biofloc aquaculture and oxygen was supplied continuously for aquatic lives, DNRA would probably be a problem when oxygen was not dispersed thoroughly. This would then result in the occurrence of anoxic zone inside the culture system, giving an opportunity to the existing DNRA microorganisms.

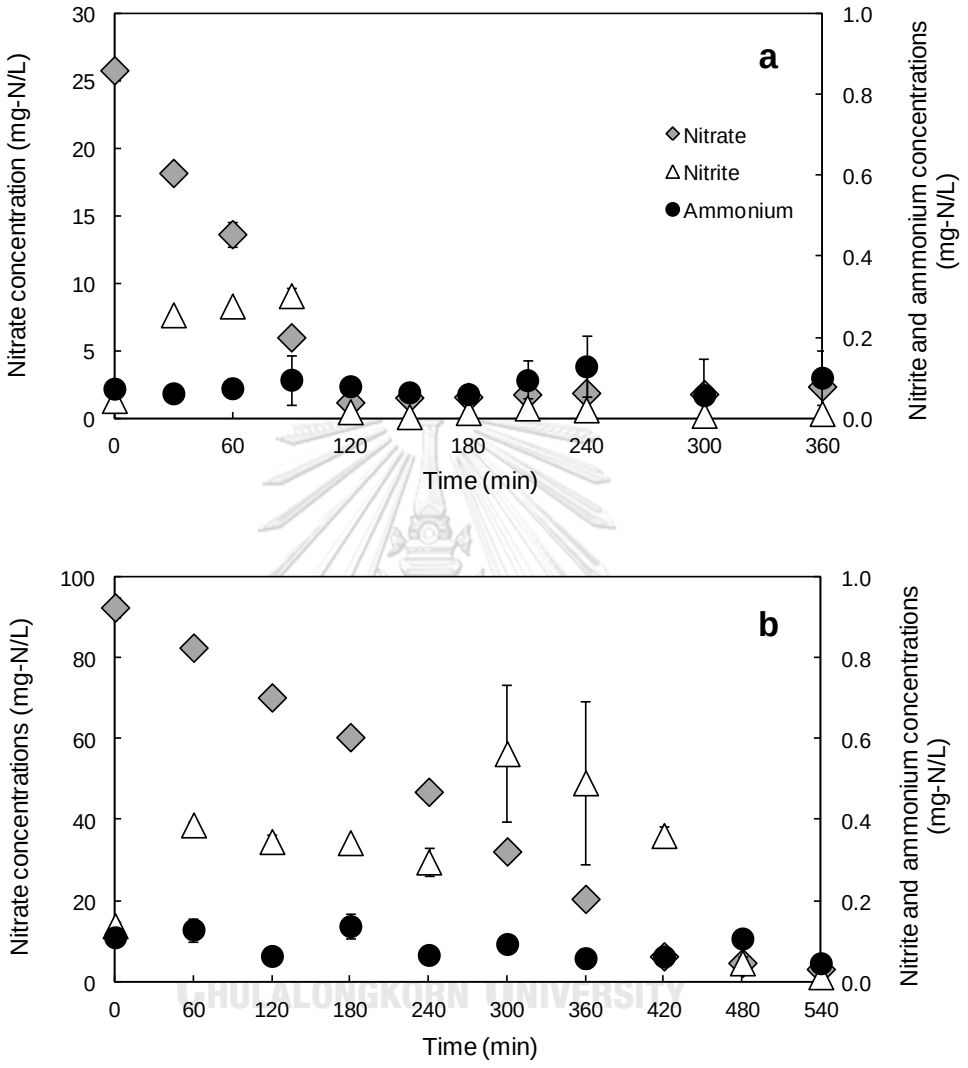


Fig. 3.1 Changes in nitrate, nitrite, and ammonium concentrations during the low C/NO_3^- sludge incubations with nitrate of 25 mg-N/L (a) and 100 mg-N/L (b)

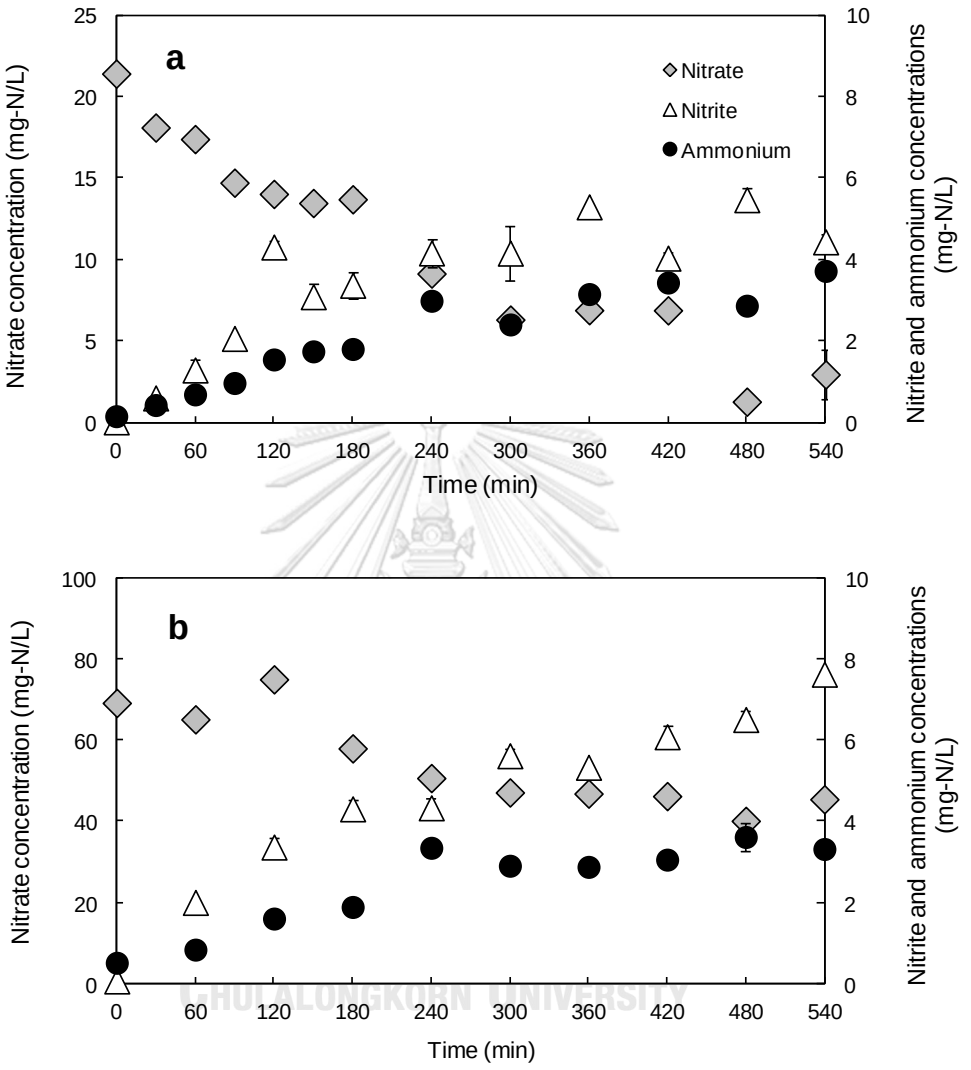


Fig. 3.2 Changes in nitrate, nitrite, and ammonium concentrations during the high C/NO_3^- sludge incubations with nitrate of 25 mg-N/L (a) and 100 mg-N/L (b)

As DNRA activity was not observed in the low C/NO_3^- sludge with the addition of nitrate, the incubations of this sludge with nitrite were tested to examine whether other nitrogen oxide could have a different effect on nitrogen reduction. Nitrite was added in a range of medium to high concentrations, *i.e.* 10, 20, and 50 mg-N/L, which were the amount previously found in aquaculture (van Rijn & Rivera, 1990). Interestingly, the addition of nitrite to the low C/NO_3^- sludge led to the increase in ammonium, which was unobservable during the incubations with nitrate (Fig. 3.3a, b, c). Nitrite supplied at 10, 20, and 50 mg-N/L resulted in ammonium production of 0.8, 3.1, and 3.2 mg-N/L, respectively, whereas ammonium never exceeded 0.2 mg-N/L with the addition of nitrate. Therefore, the results here indicate that microorganisms with DNRA function were also present in the low C/NO_3^- aquaculture sludge, though they might normally perform other metabolic pathways for growth in the actual system.

Different effect between nitrate and nitrite on DNRA was previously observed in the work of Akunna et al. (1993), where ammonium formation was also found to be higher with the presence of nitrite. Nevertheless, the influence of nitrate and nitrite demonstrated by different studies still have quite contradictory conclusions, for example, Kraft et al. (2014) reported the activity of DNRA in nitrate-supplied microcosms (where coastal sediment was used as the inoculum) while the nitrite-fed microcosms performed denitrification. van den Berg et al. (2017) conducted nitrate- and nitrite-fed high C/NO_3^- enrichment cultures and found no major difference in the two systems, as both were exhibiting DNRA and had the same dominant populations. Hence, it can be seen that the effects of the nitrogen oxide species on DNRA are currently not well-understood. The results from this current experiment, however, indicate that nitrite enhanced the activity of potential DNRA microorganisms in the low C/NO_3^- sludge, where nitrate could not. This might probably be due to the difference in nitrate/nitrite affinity between DNRA microorganisms and denitrifiers.

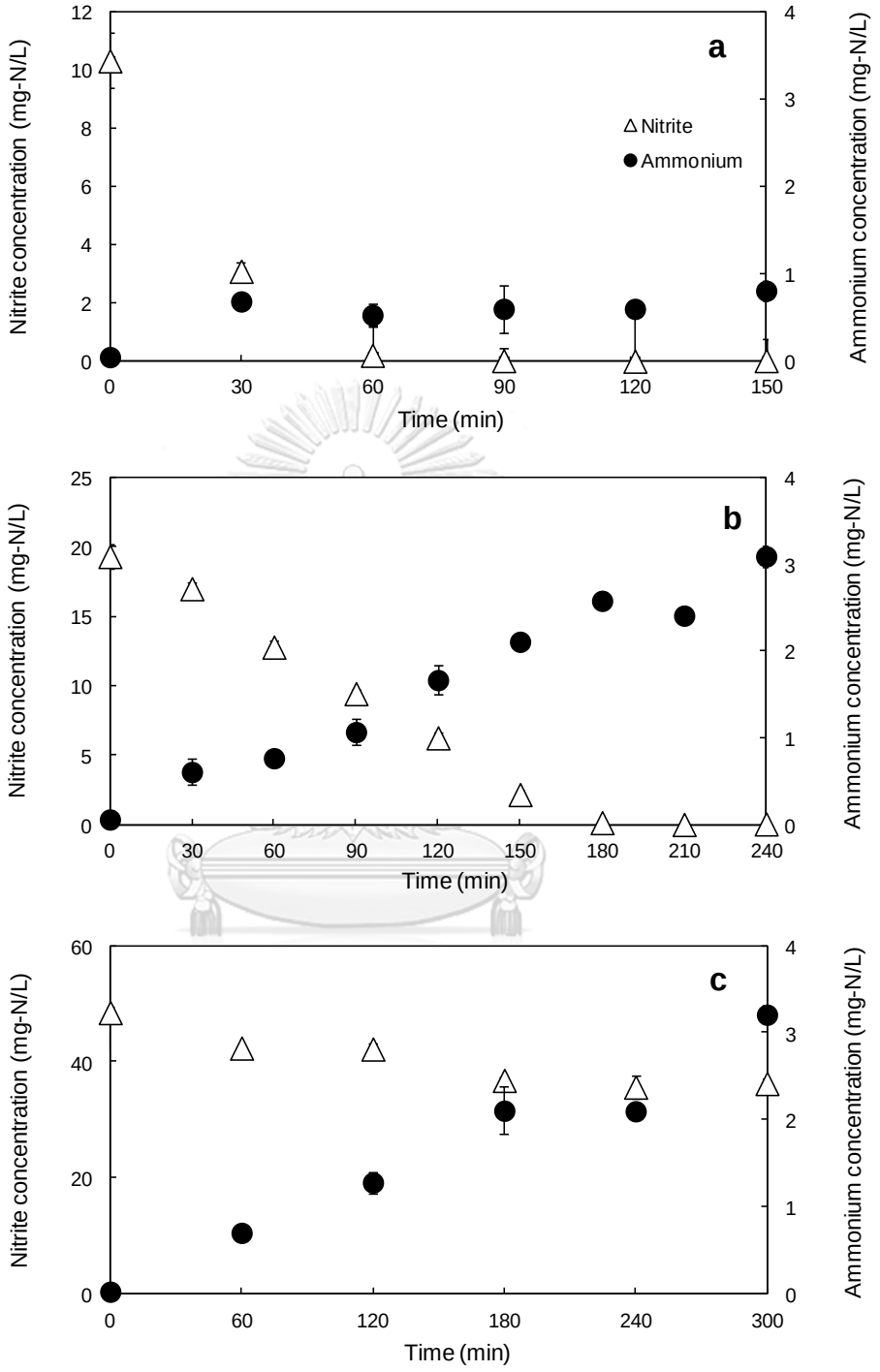


Fig. 3.3 Changes in nitrate, nitrite, and ammonium concentrations during the low C/NO_3^- sludge incubations with nitrite of 10 mg-N/L (a), 20 mg-N/L (b), and 50 mg-N/L (c)

3.3.2. Effect of sulfide addition in the low and high C/NO_3^- aquaculture sludge incubations

Another reported stimulator for DNRA process, *i.e.* sulfide, was supplied in another set of incubations to examine its effect on the low and high C/NO_3^- microbial sludge. As both sludge had performed DNRA (though with different inducers, nitrate or nitrite), sulfide addition was expected to further promote DNRA activity in both incubations. For the low C/NO_3^- sludge, nitrate and sulfide were consumed while marked increased in ammonium could be observed (Fig. 3.4), with the maximum ammonium detected of 9.5 and 12.1 mg-N/L for the sulfide added at 50 and 100 mg-S^{2-}/L, respectively (both received 100 mg-N/L of nitrate). These remarkably high ammonium concentrations for this sludge suggest the activity of DNRA when sulfide was present as its promoting factor. Nitrite was found to accumulate in the medium as high as 24.2 and 32.9 mg-N/L for the sulfide supplied at 50 and 100 mg-S^{2-}/L, respectively. This accumulation of nitrite was in accordance with those found for the high C/NO_3^- incubations with nitrate. Reduction rate of nitrite to ammonium hence might be slower than denitrifying nitrite reduction, or the presence of nitrite might lead to the formation of ammonium, as observed in the case of the low C/NO_3^- incubations with nitrite.

Considering that no ammonium production was found when nitrate alone was added to the low C/NO_3^- sludge incubations, sulfide hence seemed to provide certain advantage to DNRA microorganisms which made this pathway active during the reduction of nitrate. Denitrification was, however, still functioning judging from the portion of nitrate consumed but did not remain in the liquid phase, thus it should be released as nitrogenous gases due to the activity of denitrifiers. The occurrence of DNRA when sulfide was present suggests that the type of DNRA micro-organisms in the low C/NO_3^- sludge was, at least for a certain part, the respiratory one. This is because only R-DNRA microorganisms, which utilizing the Nrf pathway (Moreno-Vivian et al., 1999; Moreno-Vivián & Ferguson, 1998), are known to oxidize sulfide coupling to the reduction of nitrate, *e.g. Desulfovibrio desulfurican* and *Desulfobulbus propionicus*

(Dannenberg et al., 1992), whereas the Nir pathway of F-DNRA microorganisms are solely known for its organic fermentation. The presence of R-DNRA microorganisms in the low C/NO_3^- aquaculture seems quite reasonable since these microbial group can utilize sulfide, hydrogen, and simple organic carbon (Dannenberg et al., 1992) which can be present in the low C/NO_3^- system, while F-DNRA microorganisms would be expected in the high organic carbon environment (Burgin & Hamilton, 2007).

Sulfide has been reported by Brunet and Garcia-Gil (1996) to contribute as a electron donor for DNRA in a similar way as it does for denitrification. These authors also indicated that the initial concentration of sulfide was the partitioning factor for nitrate reduction, where a low amount of sulfide led to denitrification while a higher sulfide resulted in the formation of ammonium. Due to these results, the author had suggested that sulfide might inhibit nitric oxide and nitrous oxide reductases of denitrification. Sulfide has also been suggested to support DNRA by other research works (Christensen et al., 2000; Cytryn et al., 2003; Sher et al., 2008). Nevertheless, high concentration of sulfide was not always stimulated DNRA, as was found in the work of Behrendt et al. (2014) that DNRA was not promoted even when the supplied sulfide should thermodynamically favor the process over denitrification. It is possible that varying sulfide concentrations can affect each nitrate-reducing population differently, depending on the type of microorganisms present (which may have varying tolerance to sulfide), as well as other environmental parameters that may support or against DNRA/denitrification.

For the incubations of the high C/NO_3^- sludge with sulfide, it appeared that sulfide did not enhance DNRA process in this system (Fig. 3.5a, b), as ammonium produced were relatively the same as the incubations without sulfide (2.0 and 2.8 mg-N/L for the sulfide added at 25 and 50 mg-S^{2-}/L, respectively). The influence of sulfide on the high C/NO_3^- aquaculture sludge was quite surprising since this sludge had performed DNRA with nitrate as the sole inducer, so sulfide was expected to further enhance the DNRA activity. This therefore suggests that the dominant DNRA microorganisms in the low and high C/NO_3^- aquaculture systems should belong to the

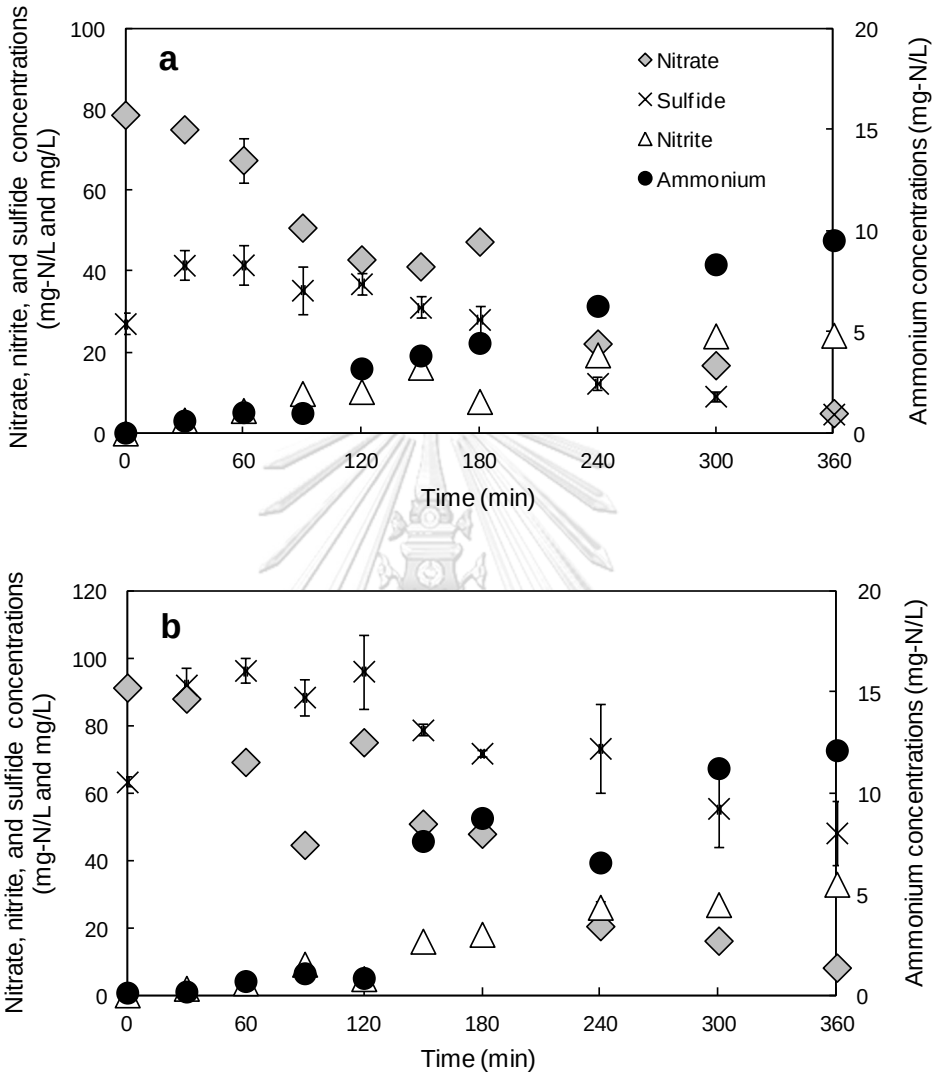


Fig. 3.4 Changes in nitrate, nitrite, ammonium, and sulfide in the low C/NO_3^- sludge incubations with 50 mg-S^{2-}/L (a) and 100 mg-S^{2-}/L (b) of sulfide and 100 mg-N/L of nitrate

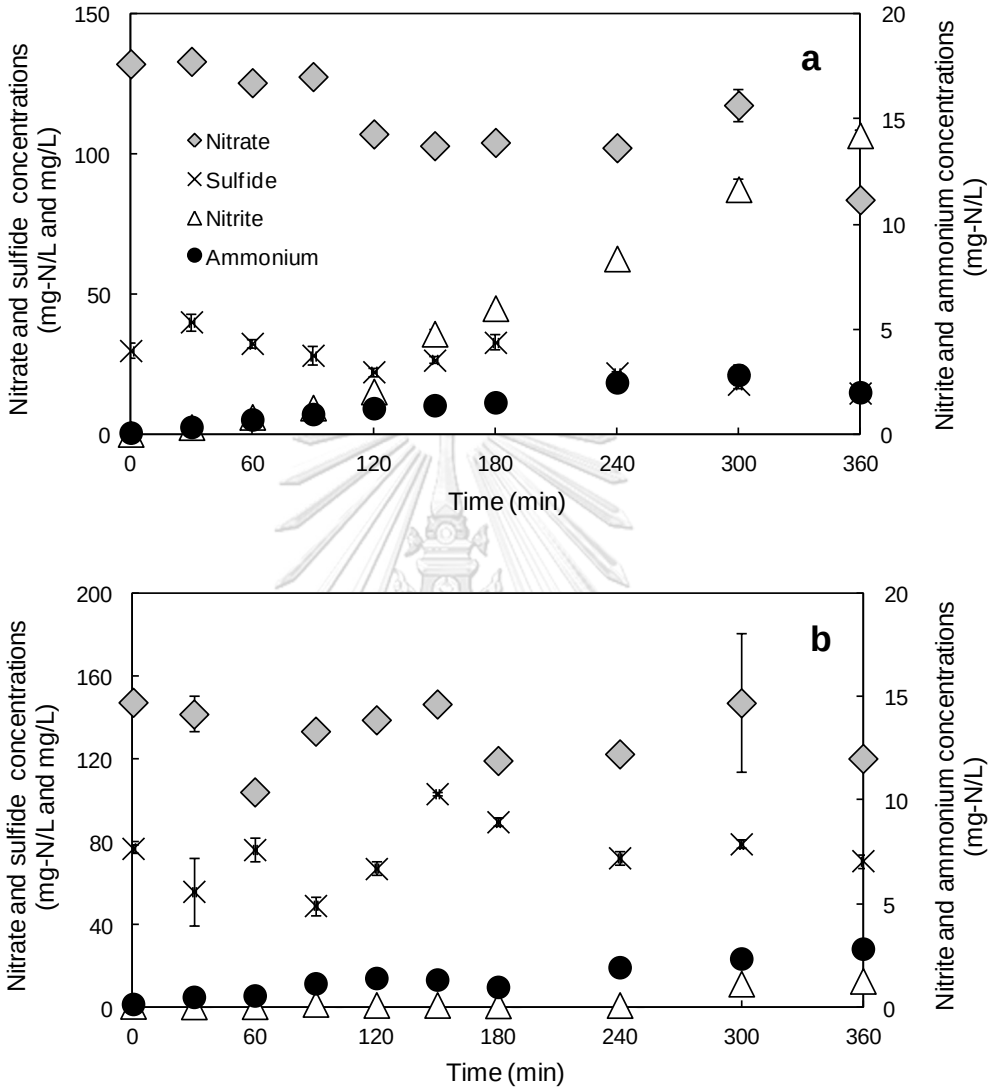


Fig. 3.5 Changes in nitrate, nitrite, ammonium, and sulfide in the high C/NO_3^- sludge incubations with 25 mg-S^{2-}/L (a) and 50 mg-S^{2-}/L (b) of sulfide and 100 mg-N/L of nitrate

different DNRA types. It is reasonable to presume that the high C/NO_3^- condition would promote the growth of microorganisms with F-DNRA ability. And since sulfide did not seem to have a positive effect on DNRA for the high C/NO_3^- sludge, the major microbial group forming ammonium from nitrate hence should be F-DNRA rather than R-DNRA microorganisms. The effect of sulfide on F-DNRA microorganisms is not known and whether both types of DNRA normally exist in the same environment is still unclear (Burgin & Hamilton, 2007). Nevertheless, the results of these incubations demonstrate that sulfide does not always enhance the pathway of DNRA, and that R-DNRA might not play significant role in certain high C/NO_3^- ecosystem.

3.3.3. Net changes in inorganic nitrogen conversions observed in the low and high C/NO_3^- sludge incubations

Net changes in nitrate, nitrite, and ammonium in all the low and high C/NO_3^- sludge incubations are illustrated in Fig. 3.6. From the bar charts, it can be seen that for all the incubations tested, most of the nitrate consumed did not remain in the medium (reported as 'Others' in the chart). Inorganic nitrogen not detected in the liquid phase was thus assumed to be converted to gases by denitrifiers. Judging from the results, denitrification was hence the major contributor for both the low and high C/NO_3^- microbial sludge, with or without sulfide. This is not surprising for the low C/NO_3^- aquaculture sludge, as denitrification was the nitrate removal process in this system. The main portion of nitrate was also found to be converted to nitrogenous gases in the high C/NO_3^- sludge from the biofloc system. However, this might or might not entirely come from denitrification. Production of nitrous oxide by ammonium-producing pure cultures has been previously demonstrated (Smith, 1983), so DNRA was also suggested to release nitrous oxide during its metabolic process (Welsh et al., 2001). Nitric oxide has also been detected during DNRA activity (Corker & Poole, 2003; Vermeiren et al., 2009). These previous studies thus indicated that DNRA micro-organisms can also be responsible for the generation of gases from the reduction of nitrate/nitrite.

Although DNRA seems to contribute a smaller part in nitrate/nitrite reduction for both the low and high C/NO_3^- aquaculture sludge, its activity was still quite surprising considering that no ammonium generation was observed in both of the aquaculture systems. The results revealed by these experiments therefore show that DNRA microorganisms might be common inhabitants in biological nitrogen removal systems and can be active under certain environmental conditions. As many known DNRA bacteria seem to have quite versatile metabolisms, *e.g. Vibrio*, *Bacillus*, and *Desulfovibrio*, these microorganisms may normally use other pathway for growth and switch to DNRA when the process becomes thermodynamically or kinetically favorable for them.

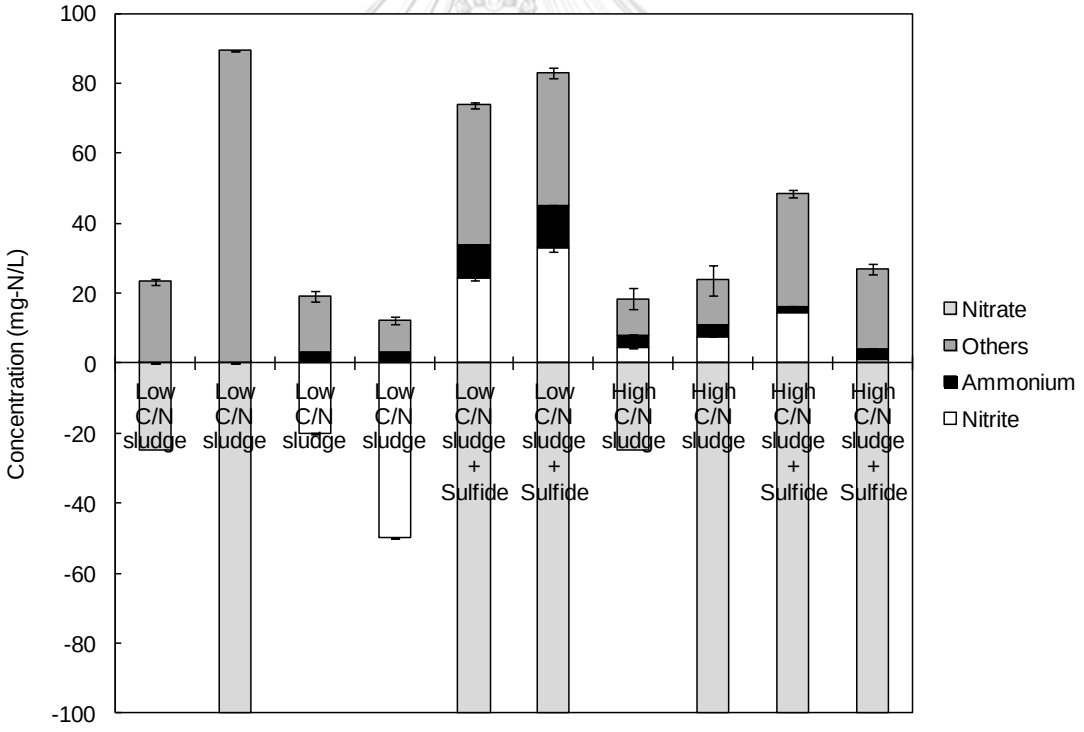


Fig. 3.6 Net changes in nitrate, nitrite, and ammonium in all the low and high C/NO_3^- sludge incubations

Although the ammonium produced in all the incubations was rather small compared to the consumed nitrate/nitrite, the concentrations detected were already harmful to aquatic animals. Since DNRA microorganisms were found to be quite common than what would be expected, the occurrence of this pathway therefore can happen if the applied treatment process is to fail under some circumstances. As DNRA can occur under very similar condition to denitrification, *i.e.* in anoxic environment with nitrate/nitrite available, understanding the mechanisms governing this process is thus necessary for the efficient control of the biological nitrate removal system.

3.4. Conclusions

The occurrence of DNRA process in the low and high C/NO_3^- aquaculture systems was investigated in this part of the research by means of anoxic batch experiments. Incubations of the microbial sludge from both aquaculture systems, in which good biological nitrogen removal was exhibited, revealed the existence of DNRA in the sludge and their activity could be induced under certain conditions. The low C/NO_3^- sludge incubations showed no indication of DNRA with either of the nitrate concentrations tested, whereas the addition of nitrite led to the production of ammonium. This suggests different inducing effects between nitrate and nitrite on the DNRA populations. Sulfide was also found as another stimulator for DNRA in the low C/NO_3^- sludge, as a significant increase in ammonium could be observed in the presence of sulfide. R-DNRA microorganisms, which are able to use sulfide as their electron donors, were thus expected to contribute to the formation of ammonium in the low C/NO_3^- aquaculture sludge.

For the high C/NO_3^- aquaculture sludge, nitrate alone could already promote the ammonium production, in which, judging from the elevated carbon environment, was likely responsible by the F-DNRA microorganisms. Stimulation by sulfide on this DNRA community was not observed under the tested conditions. This supports the speculation that F-DNRA microorganisms should be the main ammonium producers in the high C/NO_3^- sludge, since this microbial group is not known to utilize sulfide. The

occurrence of DNRA in this experiment suggests that the microorganisms capable of this pathway could present in both conventional and biofloc aquacultures with good biological nitrogen removal, and this can lead to the production of ammonium when its stimulators are available under anoxic condition.

CHAPTER 4
ROLES OF DNRA MICROORGANISMS AND THEIR COMPETITION WITH DENITRIFIERS IN LOW AND HIGH C/NO_3^- ENRICHMENT CULTURES

4.1. Introduction

Dissimilatory nitrate reduction is a part of nitrogen cycle driven by microorganisms that are capable of using nitrate as their electron acceptor. Known dissimilatory nitrate-reducing pathways include denitrification, which generates nitrogenous gases as its products, and DNRA which produces ammonium. Both of the processes occur under anoxic environments and utilize the same types of electron donors, *e.g.* organic carbon, sulfur, and iron (Brunet & Garcia-Gil, 1996; Cole & Brown, 1980; Robertson et al., 2016). Denitrifiers and DNRA microorganisms are therefore competitors for both electron donors and electron acceptors (nitrate and nitrite) as well as for the habitat in which they grow in. However, whilst much is known about those responsible for denitrification, knowledge on DNRA microorganisms is still limited to a certain extent.

Different nomenclatures have been termed for this ammonium-forming process, *e.g.* nitrate/nitrite ammonification, but mainly dissimilatory nitrate reduction to ammonium (DNRA). DNRA microorganisms have been hypothesized and observed to occur in high electron donors, limited nitrate environments which normally referred to as a high carbon to nitrate (C/NO_3^-) condition (Burgin & Hamilton, 2007; Tiedje et al., 1983).This microbial group can be further classified into respiratory-type DNRA (R-DNRA) and fermentative-type DNRA (F-DNRA) (Burgin & Hamilton, 2007; Kraft et al., 2011; Moreno-Vivián & Ferguson, 1998), where different metabolic pathways are used by each group to catalyze the formation of ammonium. Environmental studies have mostly focused on the R-DNRA microorganisms, whereas F-DNRA micro-organisms have received less attention with limited research targeted on isolated strains (Bonin, 1996; Cole & Brown, 1980; Polcyn & Podeszwa, 2009). Despite that, the active roles of DNRA

have been discovered over the past years in various natural ecosystems, including estuarine and marine sediments (Smith et al., 2007; Sorensen, 1978), the oxygen minimum zone of the ocean (Lam et al., 2009), and soils (Silver et al., 2001).

Thermodynamically, the energy gained per mole electron donor and per mole nitrate differs for denitrification and DNRA, where the former obtains a higher amount of energy per glucose molecule while the latter gains more energy per nitrate molecule (Strohm et al., 2007). This suggests how DNRA microorganisms can compete with denitrifiers in a nitrate-limiting ecosystem. The influence of C/NO_3^- ratio on the nitrate-reducing communities or on pure culture was recently reported (Kraft et al., 2014; van den Berg et al., 2016; van den Berg et al., 2017; van den Berg et al., 2015; Yoon et al., 2015), where the selective pressure of the C/NO_3^- ratio on the nitrate reduction pathways could be observed. The carbon limitation imposed by a low C/NO_3^- ratio selected for the growth of denitrifiers, whereas a nitrate limitation due to a high C/NO_3^- ratio promoted the activity of DNRA microorganisms. Other environmental parameters, *e.g.* pH, temperature (Yoon et al., 2015), fermentability of the available carbon (Burgin & Hamilton, 2007); and kinetic parameters, *e.g.* specific growth rate and substrate affinity (van den Berg et al., 2016); as well as the dilution rate applied to the culture system (Rehr & Klemme, 1989) have also been reported to influence the product of nitrate reduction. However, the extent to which each parameter controls the competitive success of denitrification and DNRA remains poorly understood, while the results from different studies have sometimes found to be inconsistent.

Apart from the mechanisms governing nitrate fate, the microbial populations developed under DNRA condition is another interesting aspect that requires further investigation. Hence, in order to observe potential DNRA microorganisms in an ecosystem that supports their growth, enrichment culture maintained at a high C/NO_3^- ratio was applied to monitor the subsequent microbial community adaptation using activated sludge as the inoculum. In direct comparison, a low C/NO_3^- enrichment culture was maintained under otherwise the same environmental conditions and using the same inoculum. The objectives of this study were 1) to investigate the presence

and activity of DNRA process under low and high C/NO_3^- ecosystems and 2) to identify the composition of the resulting microbial community using Illumina MiSeq 16S rRNA sequencing analysis. To trace the occurrence of DNRA, stable-isotope labeling compounds, $^{15}NO_3^-$ and $^{15}NO_2^-$, were utilized to track the formation of ammonium in both the low and high C/NO_3^- enrichment cultures.

4.2. Materials and Methods

4.2.1. Enrichment culture operations

Enrichment cultures were maintained under low and high C/NO_3^- ratios operated in a semi-continuous sequencing batch mode. Both of the enrichment cultures were started with an inoculum (1,393±42 mg-SS/L) from a municipal wastewater treatment plant operated with an anaerobic/oxic/anoxic/oxic (AOAO) process. The cultures were run in 1-L working volume reactors with six-blade turbine stirrers, and were maintained at 20°C in a constant temperature room. Mixing was performed at 150 rpm to keep a homogenous state inside each system. During day 1 to 15 of the operation, the reactor cycle was set to 6 h which comprised of 320 min of continuous feeding and mixing, 30 min of sludge settling, and 10 min of effluent withdrawal. The volume exchanged per cycle was set at 1/4 of the reactor working volume, resulting in a dilution rate of 0.047 h^{-1} and hydraulic retention time (HRT) of 24 h. After day 15, the cycle was changed to 12 h (680 min of continuous feeding and mixing), while the exchanged volume remained same, and so the dilution rate during this period was 0.022 h^{-1} with 48 h of HRT. The flow rates of both the influent and effluent were controlled by peristaltic pumps (Masterflex, Cole-Parmer), whereas the reactor cycle was set using timers.

In this study, glucose was selected as the carbon and energy source for microbial growth, in which its concentration was varied to achieved COD/NO_3^--N ratios of 4/1 and 8/1 for the low and high C/NO_3^- enrichment cultures, respectively (for detail, see Table 1.). Nitrate was supplied at the same concentration in both systems. The medium fed to the cultures were composed of (per L) 90 mg of $MgSO_4 \cdot 7H_2O$, 160 mg

of $MgCl_2 \cdot 6H_2O$, 42 mg of $CaCl_2 \cdot 2H_2O$, 122 mg of peptone, 20 mg of yeast extract, 50 mg of NH_4Cl, 11.33 mg of KH_2PO_4, 25.67 mg of $Na_2HPO_4 \cdot 12H_2O$, and 0.3 ml of nutrient solution (Ginige et al., 2005) for the 6-h cycle period. The concentrations of all nutrients were doubled during 12-h cycle in order to obtain the same loading as previously. The media were autoclaved and later supplemented with filter-sterilized glucose solution to meet the specified C/NO_3^- ratio for each culture. The media were then flushed with argon gas for 30 to 60 min (depending on the volume prepared) before being connected to the system.

Table 4.1 Glucose and nitrate supplied to the low and high C/NO_3^- enrichment cultures during the 6-h and 12-h cycle periods

Enrichment cultures	COD/NO_3^--N ratio	During 6-h SBR cycle			During 12-h SBR cycle		
		Glucose (mg-COD/L)	Glucose (mg/L)	Nitrate (mg-N/L)	Glucose (mg-COD/L)	Glucose (mg/L)	Nitrate (mg-N/L)
Low C/NO_3^-	4/1	400	375	100	800	750	200
High C/NO_3^-	8/1	800	750	100	1,600	1,500	200

4.2.2. Analytical methods for the chemical analysis

Water samples were collected daily from both enrichment cultures to monitor the change in inorganic nitrogen and organic carbon in the systems. The samples were first filtered through a glass microfiber filter with a 0.7-μm pore size (Whatman®, Grade GF/F) before the chemical analysis. The levels of nitrate, nitrite, and sulfate were analyzed by ion chromatography (IC) equipped with an anion column (861 Advanced Compact IC, Metrohm), whereas ammonium was measured by an IC with a cation column (761 Advanced Compact IC, Metrohm). Organic carbon was monitored in the form of dissolved organic carbon (DOC) by a total carbon analyzer (TOC-V, Shimadzu). Changes in biomass were quantified in terms of mix liquor suspended solids (MLSS) as

described by the Standard Methods for the Examination of Water and Wastewater (APHA, 2012). The pH of each withdrawn sample was measured using a pH meter.

4.2.3. Stable-isotope experiments

Stable-isotope labeling compounds, $^{15}NO_3^-$ and $^{15}NO_2^-$, were applied to verify the occurrence and extent of DNRA in the low and high C/NO_3^- enrichment cultures. This was performed by monitoring the amount of $^{15}NH_4^+$ produced from the $^{15}NO_3^-$ or $^{15}NO_2^-$ reduction. Sludge for the incubations was taken from the cultures on day 60 and day 54 for the low and high C/NO_3^- systems, respectively. The experiments were conducted in batch mode and samples were collected in time-series. The $^{15}NO_3^-$ and $^{15}NO_2^-$ were used in the form of $Na^{15}NO_3$ and $Na^{15}NO_2$ (NLM-157-PK and NLM-658-PK, respectively, both 98%+ ^{15}N atom, Cambridge Isotope Laboratories, Inc.). Before the incubations, the sludge taken from the cultures was centrifuged to remove the original medium, washed once with fresh medium (same composition as prepared for the enrichment cultures, excluding NH_4Cl), and then placed into 100-mL serum bottles along with 20 ml of the new medium. The serum bottles were closed with butyl rubber stoppers and sealed with aluminum caps before flushing with argon for 10 min. The incubations were then started by injecting either $^{15}NO_3^-$ or $^{15}NO_2^-$ into the serum bottles to a final concentration of 20 mg-N/L, along with $^{14}NH_4Cl$ to a final concentration of 100 mg-N /L, and glucose as appropriate to make the same C/NO_3^- ratio as in the cultures. All the stock solutions were flushed with argon before use. The samples were placed on a rotary shaker set at 150 rpm and the temperature was kept at 20°C. All the experiments were performed in triplicate.

The measurement of $^{15}NH_4^+$ produced from $^{15}NO_3^-$ or $^{15}NO_2^-$ reduction was conducted as previously described (Isobe et al., 2011). In brief, the filtered sample was added into a 30-mL polyethylene bottle containing 0.03 g of MgO and an acidified 1-cm GF/D glass fiber filter (Whatman®) enveloped inside PTFE tape. Each sample was added along with 2 M KCl (to supply enough salt in the solution so that the liquid would not penetrate the PTFE envelop) and the bottle was closed immediately to

prevent any ammonia gas produced from escaping. The 1-cm GF/D glass fiber filter and all the chemicals were combusted at 450°C for 4 h to remove any contaminating nitrogen before use. All the samples were then incubated at room temperature overnight. The acidified GF/D filters containing the diffused ammonium were removed from the PTFE envelop and individually placed inside a glass tube. Persulfate-oxidizing reagent (5 g $K_2S_2O_8$, 3 g boric acid, and 100 ml of 1.52% (w/v) NaOH.) was added at 2 ml along with 1 ml of distilled water to convert ammonium to nitrate by persulfate oxidation. The samples were then autoclaved at 121°C for 1 h.

The nitrate generated from the captured ammonium was further reduced to nitrous oxide by denitrifier method (Casciotti et al., 2002). *Pseudomonas chlororaphis* subsp. *aureofaciens* (ATCC 13985^T), a denitrifying bacterium incapable of nitrous oxide reduction, was used so that the nitrous oxide produced remained the final product of the reaction. This bacterium was purchased from the Japan Collection of Microorganisms (JCM) as strain JCM 20509, and was cultured in a 500 ml serum bottle containing 450 ml of tryptic soy broth, 10 mM KNO_3, 7.5 mM NH_4Cl, and 36 mM KH_2PO_4. The serum bottle containing the medium was autoclaved before the addition of *P. chlororaphis* from a stock culture. The cell culture was maintained at 30°C on a rotary shaker at 150 rpm for 6 d, then centrifuged to concentrate the cells for the denitrifier method. The concentrated *P. chlororaphis* cells were dispensed into 20-mL vials (autosampler vial, 20-CV, Chromacol, Ltd.), sealed with butyl rubber stoppers and aluminum caps, and flushed with ultrapure helium for 30 min to remove any residual oxygen and nitrous oxide. Samples were injected into the prepared vials through syringes and needles, then incubated upside-down (to prevent nitrous oxide leakage) overnight. Standard solutions were prepared from $^{15}NH_4Cl$ (99.9% ^{15}N atom, Isotec®) at a ^{15}N atom of 0.364, 1.05, 5.07, 9.28, 21.0, 50.0, and 99.9% with 2 M KCl in duplicate, and underwent the same procedure as the samples. The microbial reaction was terminated by injecting 0.3 ml of 6 M NaOH solution. The processed samples were then analyzed by gas chromatography/mass spectrometry (GC/MS) using GCMS-QP2010 Plus (Shimadzu) instrument with a CP-PoraPLOT Q-HT column (25 m × 0.32 mm, Varian). As the original media had been washed-off the sludge before the incubations,

$^{14}NO_3^-$ and $^{14}NO_2^-$ in the samples were hence considered negligible and $^{15}NO_3^-$ and $^{15}NO_2^-$ were measured by the IC method (861 Advanced Compact IC, Metrohm).

4.2.4. Microbial community analysis with Illumina MiSeq sequencing method

The microbial communities of the low and high C/NO_3^- enrichment cultures were examined using the Illumina MiSeq, 16S rRNA sequencing method. Samples analyzed included the sludge collected on days 27, 30, 38, 42, and 48 from both systems as well as the inoculum used for stating the cultures. The total microbial DNA was extracted from the samples using FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC) as described by the manufacturer's protocol. The extracted DNA was then used as template for PCR amplification with the 341F and 805R primer pair (targeting V3 and V4 region of the 16S rRNA gene). The PCR reaction mixture (25 µl) was prepared from a *TaKaRa Ex Taq*™ kit (Takara) with: 12 ng of DNA template, 0.2 µM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, and 1.25 U of *TaKaRa Ex Taq*™. The PCR thermal steps were as follows: 94°C for 3 min, 25 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. Four PCR reactions were conducted per sample to reduce biases during PCR amplification. The PCR products were then checked by 1.5% (w/v) agarose gel electrophoresis to ensure that the corrected-size products were amplified (approximately 460 bp).

Subsequently, the four replicates of each sample were combined and purified with NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL) according to the manufacturer's protocol. The concentration of PCR products was then examined with spectrophotometer (NanoDrop). The obtained 16S rRNA amplicons were prepared in a 50-µl reaction containing 5 µl of each Illumina Nextera XT index primer (Illumina) and 2X KAPA HotStart ReadyMix (Kapa Biosystems) to index each sample. The PCR thermal steps were set as follows; 94°C for 3 min; 8 to 10 cycles of 98°C for 20 s, 55°C for 30 s, 72°C for 30 s, and then a final extension at 72°C for 5 min. Subsequently, the indexed PCR amplicons were purified with Agencourt AMPure XP beads (Agencourt Bioscience), pooled and diluted to a final loading concentration of 4 pM. DNA sequencing was

conducted on the Illumina MiSeq platform with a MiSeq Reagent Kits v3 (600-cycle; Illumina) at the Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). The sequences were assembled by merging the forward and reverse reads. The OTU picking process was performed using the QIIME software (version 1.9.0), with an open-reference method. Taxonomic assignment from the OTUs to related taxa was done using UCLUST against the Greengenes database (version 13_8, with a 97% sequence identity threshold). The sequence reads in this study can be accessed at the NCBI Short Read Archive (SRA) under the accession numbers SRX3440460-SRX3440470.The OTUs with ≥1% and ≥2% maximum relative abundances were selected as representatives for class-level and genus-level analyses, respectively. Statistical analysis of the results was achieved using two-sample t-test on SPSS Statistic ver. 22 (IBM Corp.).

4.3. Results and Discussion

4.3.1. Competition between denitrification and DNRA in the low and high C/NO_3^- enrichment cultures

Cultures enriched with a nitrate-reducing community were maintained under low and high C/NO_3^- ratios in order to select for the growth of microbial populations favored under each condition. Both enrichment cultures were operated in a semi-continuously fed sequencing batch mode, which was able to maintain a stable C/NO_3^- ratio (Fig. 4.1) as well as maintained the biomass within the systems. Apart from the C/NO_3^- ratio, the applied dilution rate also affects the nitrate competition in a culture setting, where DNRA microorganisms seem to require a low dilution rate for growth (Rehr & Klemme, 1989). Therefore, the dilution rate used in this study was selected from a range that should allow DNRA to occur. All other operating parameters, besides the glucose concentration, were the same in the low and high C/NO_3^- enrichment cultures, so as to allow the C/NO_3^- ratio to be the determining factor for the successful nitrate-reducing pathway in each ecosystem.

Competition between the two nitrate reduction pathways was initially verified by observing changes in the organic carbon and inorganic nitrogen profiles of the low and high C/NO_3^- enrichment cultures (Fig. 4.2 and 4.3, respectively). The DOC measurement revealed that the supplied organic carbon was consumed in a range of 65.7 to 87.9% and 64.7 to 93.8% for the low and high C/NO_3^- systems, respectively. Increase in DOC was observed in both cultures during the transition from 6-h to 12-h SBR cycle, due to the change in the media concentration. The systems regained their steady condition after 7 to 8 days of the transition phase and remained relatively stable, though certain fluctuation could be observed in the case of high C/NO_3^- culture. The COD/NO_3^--N applied to the high C/NO_3^- system (that is, 8/1) seems to cause nitrate limitation in the culture as sulfate was found to be reduced along with nitrate (Fig. 4.4), indicating that the organic carbon added was in excess and therefore available for the reduction of sulfate. Sulfate supplied in the media was 24.6±0.66 mg-S^{2-}/L for both enrichment cultures (during 12-h cycle), which mostly remained in the liquid phase for the low C/NO_3^- system compared to the high C/NO_3^- one.

During the initial phase of the low C/NO_3^- culture operation, nitrate was detected in the system in the range of 17.7 to 52.5 mg-N/L (47.8 to 82.3% nitrate conversion) but was consumed to below 1.0 mg-N/L (>99% nitrate conversion) after day 17, which could either mean that nitrate had become limited for heterotrophic growth or that it was used by autotrophic nitrate reducers. Nitrite was only detected on day 4 (1.7 mg-N/L) and then remained below detection limit afterwards (Fig. 4.4). The level of ammonium (23.2 to 95.1 mg-N/L) was always lower than the added concentration, and so there was no obvious indication of DNRA activity or at least not at a level exceeded the process of ammonium assimilation. The major nitrate-reducing pathway under the COD/NO_3^--N ratio of 4/1 was, therefore, assumed to be denitrification since the inorganic nitrogen depleted from the culture was most likely converted to nitrogenous gases. The existence of DNRA process in this system was subsequently verified with stable-isotope tracers.

For the high C/NO_3^- enrichment culture, the COD/NO_3^--N ratio was started at 8/1 and the system was observed whether it could sustain the growth of DNRA microorganisms. After 4 days of operation, the nitrate level was depleted (<0.2 mg-N/L), which was likely to be due to the higher glucose level in the high C/NO_3^- culture than the low C/NO_3^- one, which in turn would require electron acceptors, *i.e.* nitrate and nitrite, to complete the reactions catalyzed by the microorganisms. Therefore, nitrate became a limiting substrate in this system whereas nitrite was undetectable throughout the experiment, suggesting the rapid conversion of nitrite to other end products.

The concentration of ammonium in the high C/NO_3^- enrichment culture was raised to a remarkably higher level than the supplied value, possibly accounted for 30.1 to 64.4% and 68.6 to up to 100% of the nitrate conversion during day 20 to 37 and day 38 to 54, respectively. The active formation of ammonium found under this anoxic condition most likely indicates the occurrence of DNRA. Judging from the portion of nitrate converted to ammonium during day 20 to 37, DNRA micro-organisms and denitrifiers seem to have relatively similar share in the reduction of nitrate. However, after day 37, DNRA microorganisms appear to be the principal nitrate reducers in the system, with a minor fraction of reduced nitrate being released as gases (<14.5% during the peak ammonium-forming activity). Since the COD/NO_3^--N ratio of 8/1 could already support the growth and activity of DNRA microorganisms, the ratio was not further increased from this value.

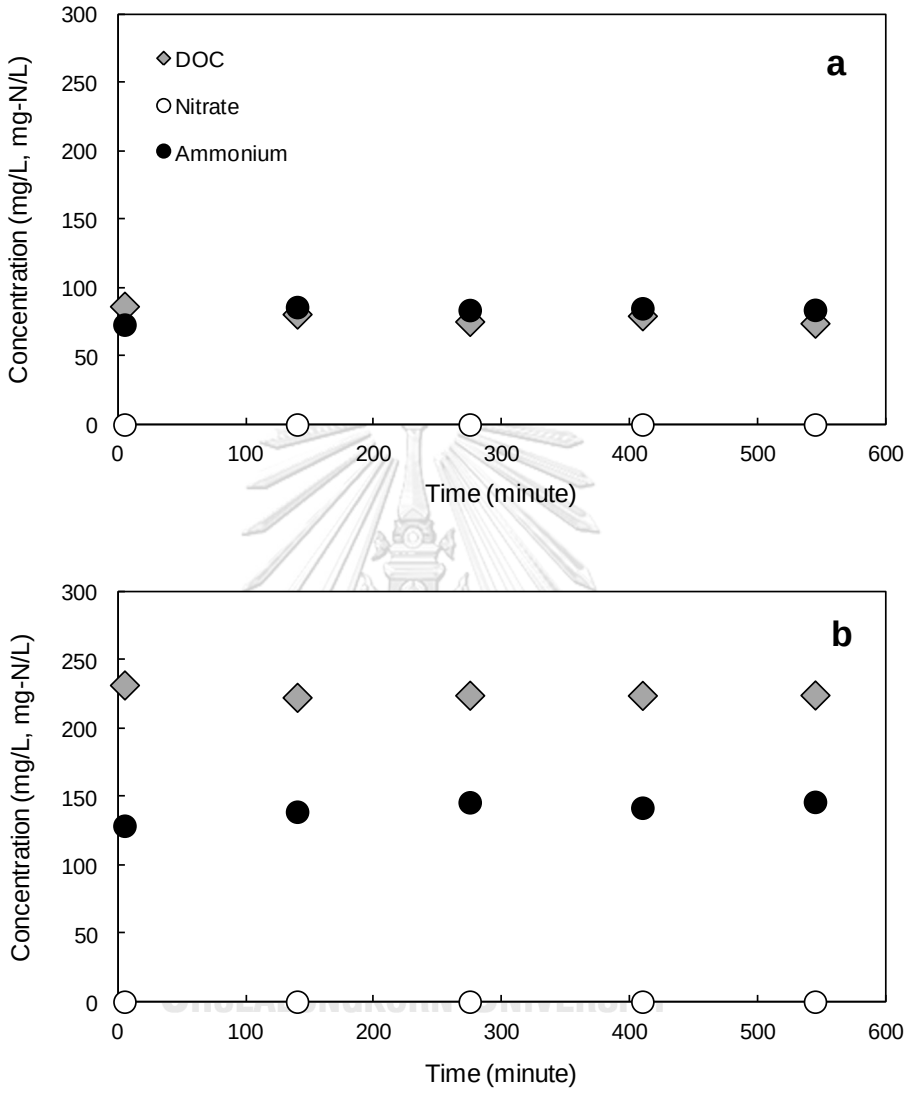


Fig. 4.1 Stable nitrate, ammonium, and DOC concentrations observed during one cycle of the low C/NO_3^- (a) and high C/NO_3^- (b) enrichment cultures (on day 20)

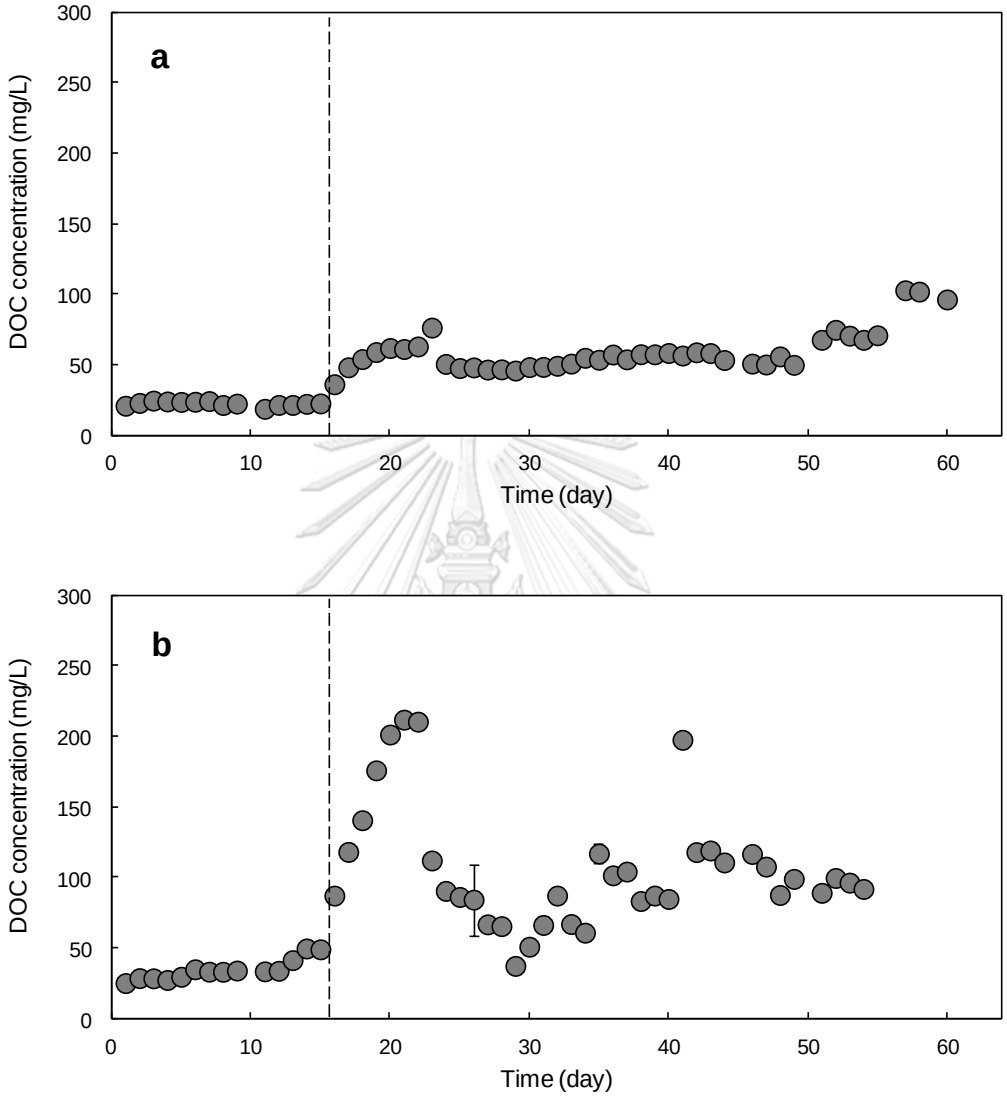


Fig. 4.2 Changes in dissolved organic carbon (DOC) concentrations in the low C/NO_3^- (a) and high C/NO_3^- (b) enrichment cultures; vertical line indicates the point of change in SBR cycle

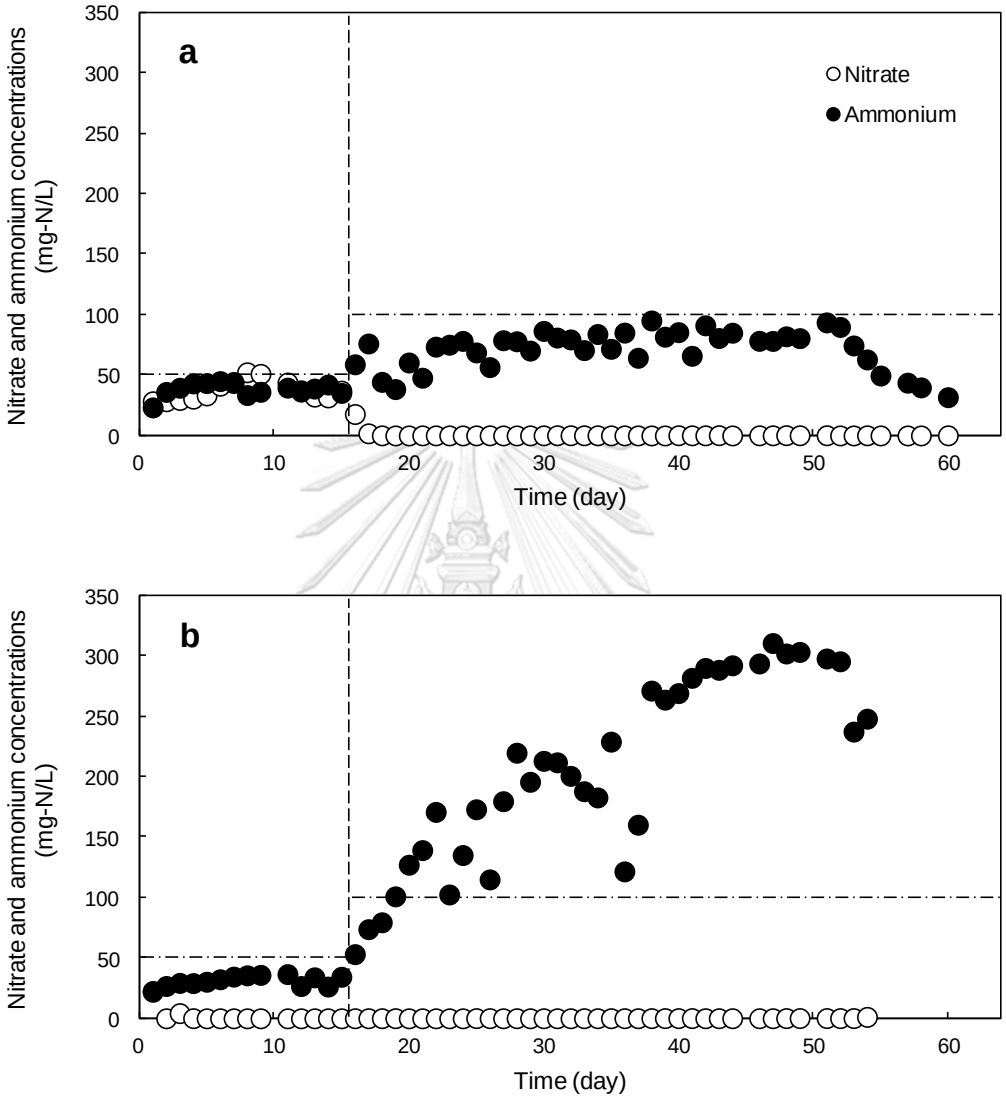


Fig. 4.3 Changes in nitrate and ammonium concentrations in the low C/NO_3^- (a) and high C/NO_3^- (b) enrichment cultures; vertical line indicates the point of change in SBR cycle, horizontal lines indicate the ammonium concentrations supplied in the media

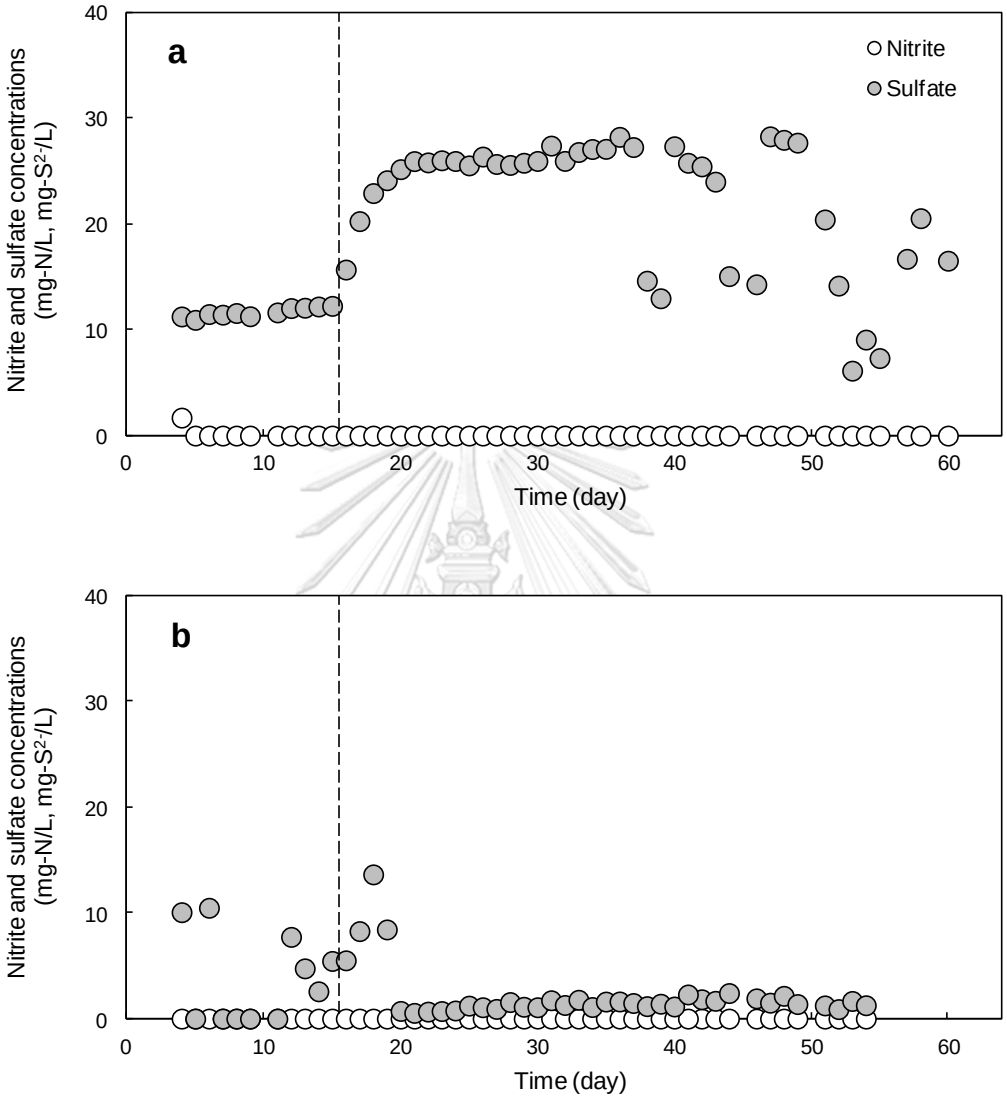


Fig. 4.4 Changes in nitrite and sulfate concentrations in the low C/NO_3^- (a) and high C/NO_3^- (b) enrichment cultures; vertical line indicates the point of change in SBR cycle

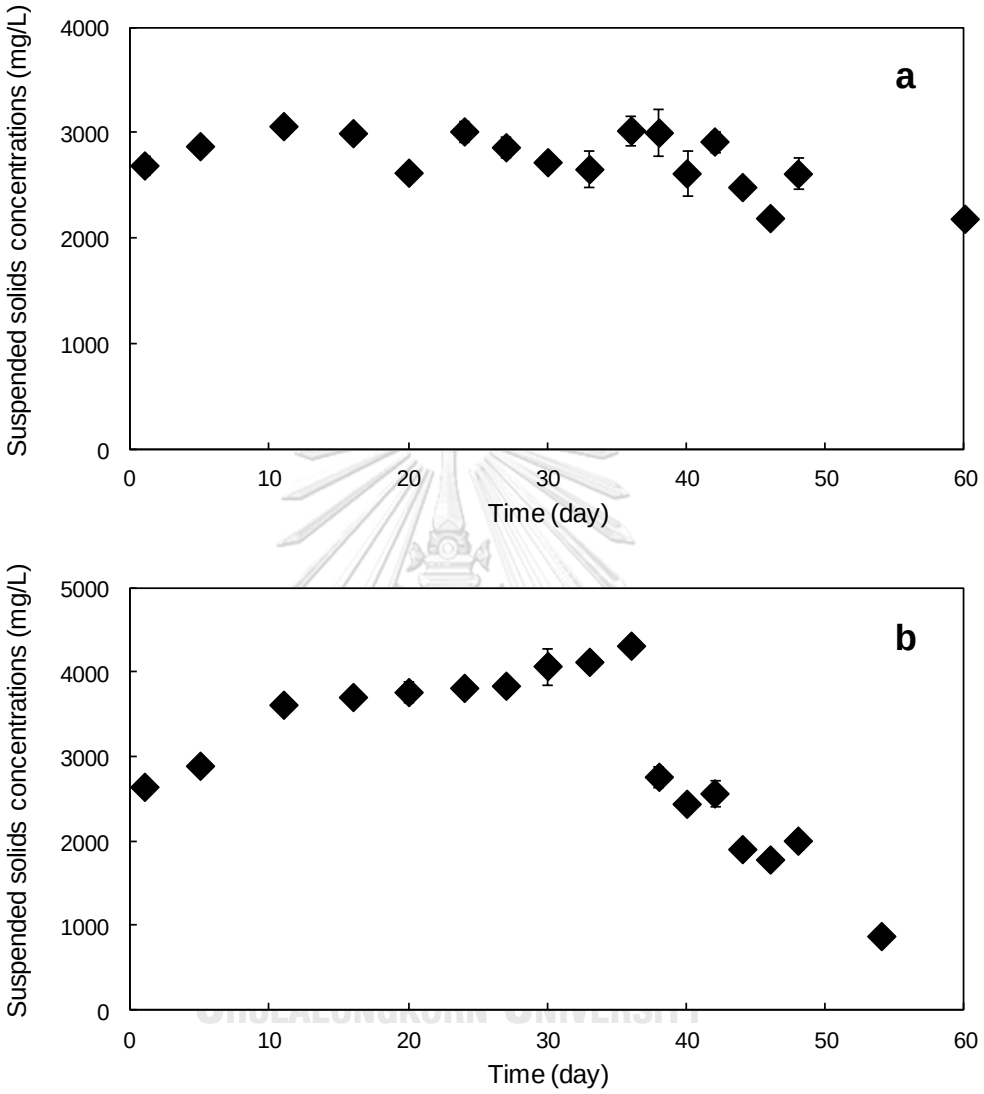


Fig. 4.5 Changes in suspended solid concentrations measured in the low C/NO_3^- (a) and high C/NO_3^- (b) enrichment cultures

With respect to the cell biomass grown in the two enrichment cultures, the biomass in the high C/NO_3^- system was detected in higher concentrations than that observed in the low C/NO_3^- one (Fig. 4.5). Since the same amount of nitrate was supplied to both cultures, the higher cell mass in the high C/NO_3^- system could be due to the higher mole of organic carbon obtained per mole of nitrate for DNRA than for denitrification (Strohm et al., 2007), thus providing more carbon and energy source for microbial cells. Additionally, it could also be caused by the growth of other microorganisms apart from nitrate reducers, because organic carbon above the stoichiometric requirement for nitrate reduction should also support other anaerobic heterotrophs, *e.g.* fermentative bacteria and sulfate reducers. Sulfide produced from sulfate reduction could sustain DNRA microorganisms by acting as an electron donor (Brunet & Garcia-Gil, 1996). Sulfide could also lower a redox potential of the system (van den Berg et al., 2016), which was hypothesized to favor DNRA as well. However, the effect of sulfide in the high C/NO_3^- culture should be small compared to that of the added glucose. This can be seen from the DNRA reaction with sulfide as the electron donor as follows,

$$4\ HS^- + NO_3^- + 6\ H^+ \rightarrow NH_4^+ + 4\ S^0 + 3\ H_2O \quad (1)$$

The DNRA reaction requires four moles of sulfide per one mole of nitrate. Therefore, if all the supplied sulfate (24.6±0.66 mg-S^{2-}/L) was reduced to sulfide, 2.69 ±0.07 mg-N/L of nitrate would be used by the sulfide-driven DNRA reaction, which is equal to 1.35% of the nitrate added. The DNRA pathway induced by the presence of sulfide hence should be negligible when considering the portion of nitrate that can be utilized by the process.

For the low C/NO_3^- system, certain amount of sulfate was utilized but most of the time was left in the culture, which was likely due to the limitation of the supplied organic carbon. Nevertheless, biomass in the high C/NO_3^- culture was found to decrease since day 38 onwards due to the occurrence of sludge with filamentous-like character, which led to the loss of cell mass from sludge bulking. These filamentous microorganisms grew during the same period as the detection of the peak in DNRA

activity. However, it is unclear whether these organisms were also responsible for the DNRA process or present due to other metabolic function. The low C/NO_3^- biomass, on the contrary, remained floc-like with good settleability, and no filamentous sludge could be observed throughout the operation.

Successful mix cultures of DNRA microorganisms have been previously demonstrated by Kraft et al. (2014) and van den Berg et al. (2015). Kraft et al. (2014) conducted anoxic incubations inoculated with coastal sediments and supplied with complex fermentable substrates. DNRA was found to prevail under nitrate-limiting condition and when a low dilution rate was applied. Likewise, van den Berg et al. (2015) performed acetate-fed enrichment cultures seeded with activated sludge with a dilution rate low enough to support both nitrate-reducing pathways, resulting in DNRA being detected at a high C/NO_3^- ratio which then caused nitrate limitation in the systems. These findings suggest the importance of the operating parameters, *i.e.* C/NO_3^- ratio and dilution rate, and the resulting environmental condition, *i.e.* nitrate limitation, on the occurrence of DNRA. The relationship between low dilution rate and the growth of DNRA microorganisms was first established by Rehr and Klemme (1989), and seems to be related to the kinetic aspect of these microorganisms. This is because dilution rate affects generation time of the microorganisms growing in the culture as well as the substrate concentration provided to the system. The low dilution rate needed for DNRA microorganisms could either mean they require a longer generation time for growth (Kraft et al., 2014) or that they need a nitrate concentration low enough to compete with denitrifiers kinetically, or both.

Although the high C/NO_3^- ratio has been proposed to be the main force selecting for DNRA, this does not always occur even when the energy gained should be thermodynamically favorable for the process (Behrendt et al., 2014). Strohm et al. (2007) has demonstrated that denitrification can obtain higher energy per mole of glucose than DNRA, whereas the opposite occurs for the energy gained per mole of nitrate, which can be illustrated in redox equations (when glucose is used as an electron donor) as follows

$$5\ C_6H_{12}O_6\ +\ 24\ NO_3^-\ +\ 24\ H^+\ \rightarrow\ 12\ N_2\ +\ 30\ CO_2\ +\ 42\ H_2O \quad (2)$$

$$C_6H_{12}O_6\ +\ 3\ NO_3^-\ +\ 6\ H^+\ \rightarrow\ 3\ NH_4^+\ +\ 6\ CO_2\ +\ 3\ H_2O \quad (3)$$

According to the first equation, $\Delta G^{\circ\prime}$ gained is -2,727 kJ per mole glucose and -568 kJ per mole nitrate for denitrification, while for DNRA the $\Delta G^{\circ\prime}$ obtained was -1,835 kJ per mole glucose and -612 per mole nitrate. It can be seen from these two equations that, when carbon becomes limited, the pathway yielding higher energy thermodynamically would be denitrification (that is, higher energy gained per mole carbon). Under nitrate limitation, on the other hand, the pathway of DNRA can obtain higher energy per nitrate and becomes more thermodynamically favorable in that system. This higher energy gained per nitrate is actually due to the fact that DNRA can receive eight electrons in the reduction of nitrate to ammonium, whereas denitrification can transfer only five when converting nitrate to dinitrogen gas.

Nevertheless, as the competition between microorganisms is also controlled by the growth kinetics of the respective competitors, kinetic parameters (*e.g.* specific growth rate and affinity for the limiting substrate) should also be considered in the context of microbial competition. Affinity for nitrate/nitrite of the two nitrate reduction pathways has been discussed previously in several works (Behrendt et al., 2014; Kraft et al., 2014; Tiedje et al., 1983; van den Berg et al., 2016; van den Berg et al., 2017; van den Berg et al., 2015), although with quite controversial conclusions. Kraft et al. (2014) speculated that denitrification and DNRA should have similar affinity for nitrate since they utilize the same nitrate reductases. These authors also suggested that the difference of the two pathways should be on the affinity for nitrite (as they use different enzymes for the nitrite reduction), where nitrite affinity for denitrification (cytochrome *cd*1 nitrite reductases) was found to be slightly higher. Tiedje et al. (1983) and Behrendt et al. (2014) made suggestions that nitrate affinity of denitrification should be higher than DNRA based on the half saturation constant (K_s) values of the known pure cultures, *e.g.* Ks of 5 to 10 μM nitrate was reported for denitrifiers and 100 to 500 μM for DNRA bacteria (Jørgensen et al., 1990). On the contrary, by observing an

enrichment culture, van den Berg et al. (2016) reported a higher nitrate affinity for DNRA microorganisms which led to their domination under nitrate limitation. Reports from these literatures hence emphasize that additional information on kinetic parameters of DNRA is still needed in order to gain more insight into the competition between the two nitrate reducers.

4.3.2. Potential activity of DNRA microorganisms in the sludge from the low and high C/NO_3^- enrichment cultures

The formation of ammonium by DNRA microorganisms was tracked using the method of ^{15}N stable-isotope tracers. Both $^{15}NO_3^-$ and $^{15}NO_2^-$ were applied to observe the stimulating effect on DNRA of each nitrogen oxide species. Samples were taken from the low and high C/NO_3^- enrichment cultures during the period of stable nitrate-reducing activity and were each incubated in batch mode. The batch incubations with low C/NO_3^- culture sludge revealed that most of the $^{15}NO_3^-$ and $^{15}NO_2^-$ were reduced to end products that did not remain in the liquid phase (Fig. 2a, b), and so they were assumed to be converted to nitrogenous gases. This was most likely from the activity of denitrifiers, which produce nitric oxide, nitrous oxide and dinitrogen gases. However, a slight increase in $^{15}NH_4^+$ could be measured with both $^{15}NO_3^-$ and $^{15}NO_2^-$ tracers, although at only small concentrations (0.19 and 0.23 mg-N/L, respectively). This minor activity indicates a small proportion of DNRA microorganisms in the low C/NO_3^- community. The $^{15}NH_4^+$ and $^{15}NO_3^-$/$^{15}NO_2^-$ assimilation in all the incubations should be suppressed due to the presence of a high level of $^{14}NH_4^+$ (100 mg-N/L). The process was, therefore, not taken into account for the balance of nitrogen conversion, and so all the $^{15}NH_4^+$ produced was assumed to be left in the media.

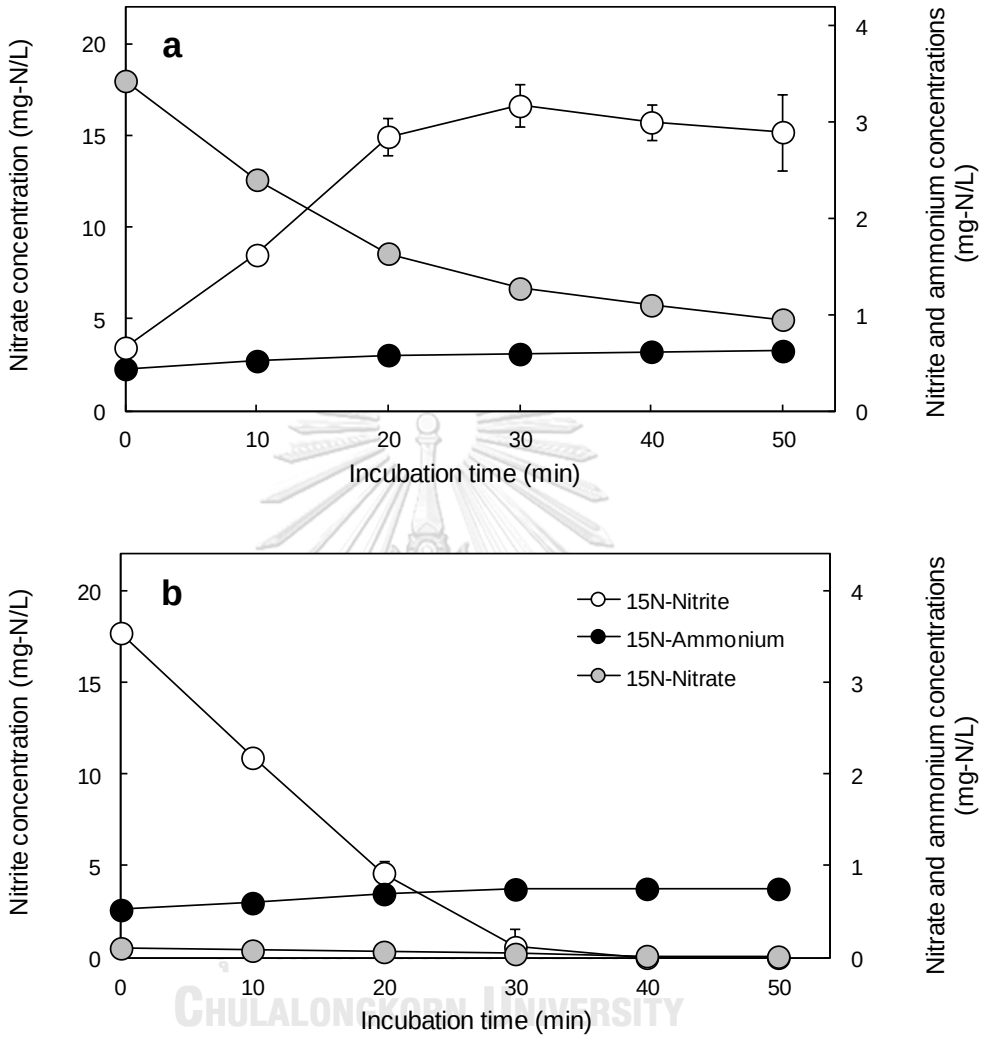


Fig. 4.6 Changes in $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ during stable-isotope tracer experiments with the low C/NO_3^- sludge incubated with $^{15}NO_3^-$ (a) and $^{15}NO_2^-$ (b)

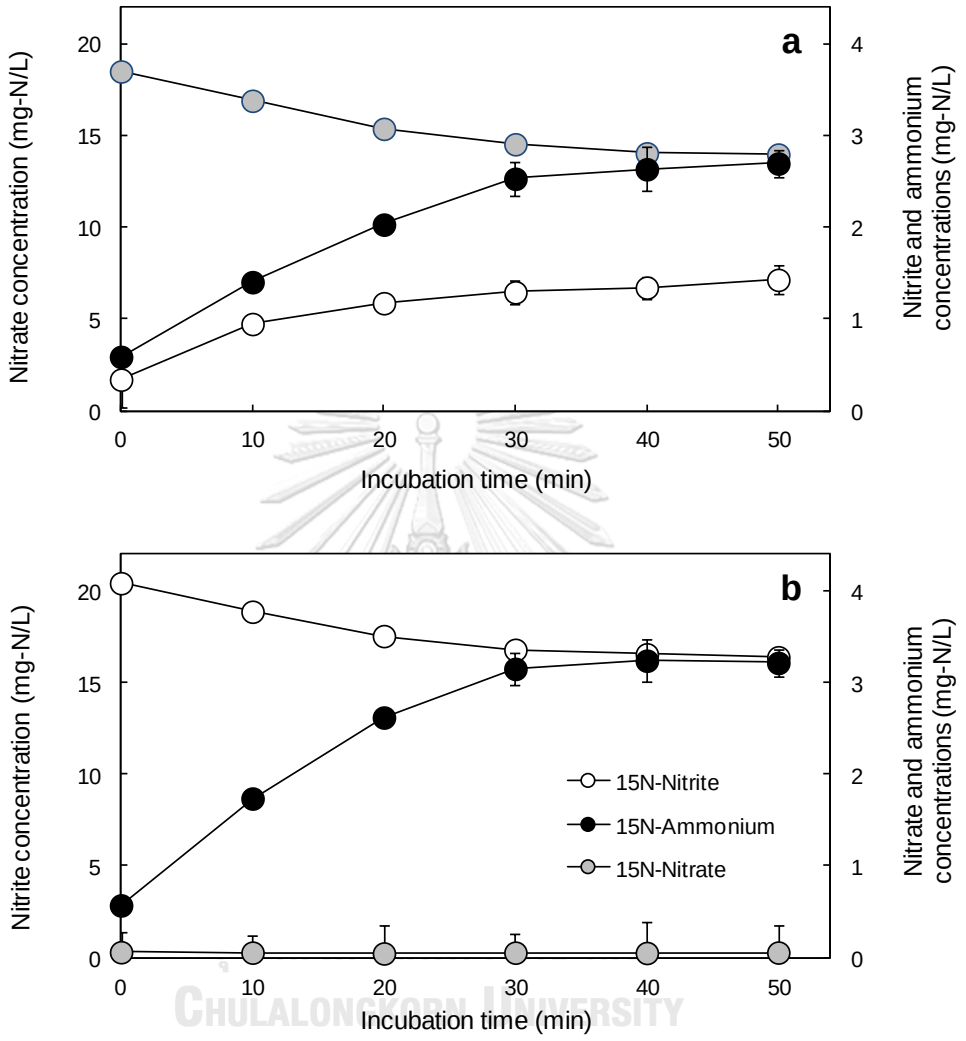


Fig. 4.7 Changes in $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ during stable-isotope tracer experiments with the high C/NO_3^- sludge incubated with $^{15}NO_3^-$ (a) and $^{15}NO_2^-$ (b)

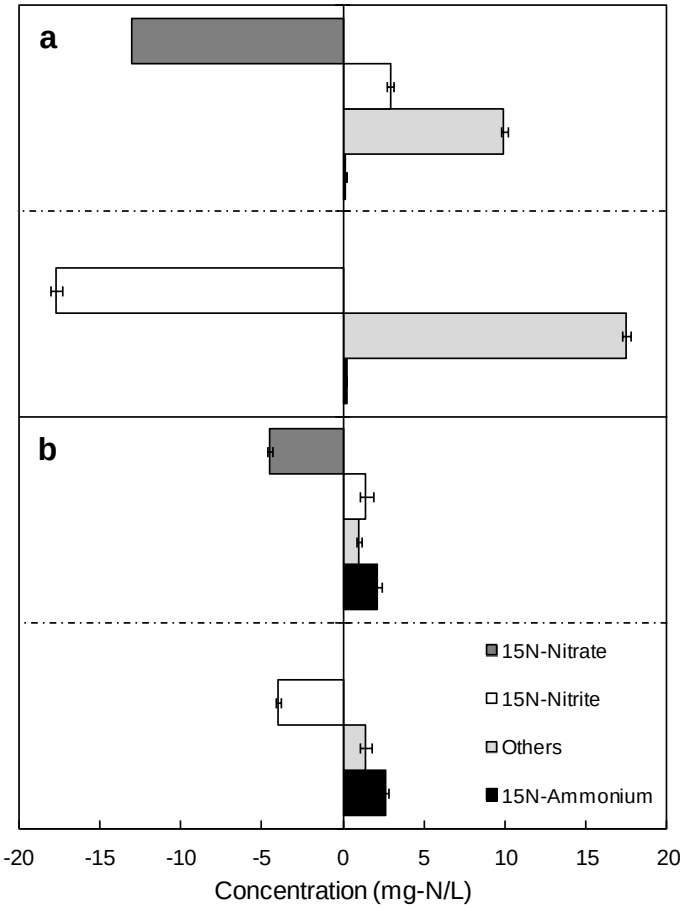


Fig. 4.8 Net change in $^{15}NO_3^-$ and $^{15}NO_2^-$ incubations with low C/NO_3^- (a) and high C/NO_3^- (b) culture sludge

For the high C/NO_3^- culture sludge, a marked increase in the level of $^{15}NH_4^+$ was observed when either $^{15}NO_3^-$ or $^{15}NO_2^-$ were added (Fig. 2c, d). DNRA activity was hence confirmed by the evidence of ammonium production from nitrate as well as nitrite. The portion of consumed $^{15}NO_3^-/^{15}NO_2^-$ not detected in the liquid phase was assumed to be converted to gaseous products. The addition of $^{15}NO_2^-$ instead of $^{15}NO_3^-$ did not significantly change the activity of DNRA microorganisms in both the low and high C/NO_3^- sludge incubations. Hence, nitrite does not seem to have a different effect on the nitrogen conversion pathway than that observed for nitrate, at least under the conditions tested in this experiment.

Net changes in the ^{15}N-nitrogen for all incubations (calculated based on the initial and final concentrations of $^{15}NO_3^-$, $^{15}NO_2^-$ and $^{15}NH_4^+$) are shown in Fig. 3. The results reveal that the main products of $^{15}NO_3^-$ and $^{15}NO_2^-$ reduction in the low C/NO_3^- sludge were in gaseous forms, and so denitrifiers were assumed to be the main contributors to the nitrogen conversion in these samples. The $^{15}NH_4^+$ produced from DNRA microorganisms inhabiting the low C/NO_3^- enrichment culture was found to be 1.4 and 1.3% of the amount of $^{15}NO_3^-$ and $^{15}NO_2^-$ consumed, respectively (Fig. 3a) Thus, DNRA microorganisms also had a small role in this denitrifiers-dominating community. In contrast, the main product in the high C/NO_3^- sludge was found to be $^{15}NH_4^+$ with both the $^{15}NO_3^-$ and $^{15}NO_2^-$ incubations (Fig. 3b). The proportion of nitrogenous gases produced in the high C/NO_3^- incubations was lower than that converted to $^{15}NH_4^+$, being 21.9 and 34.5% of the consumed $^{15}NO_3^-$ and $^{15}NO_2^-$ respectively. These gases, however, might not come exclusively from the activity of denitrification. Previous research has revealed that DNRA might also release nitric oxide and nitrous oxide from its reaction (Smith, 1983; Vermeiren et al., 2009; Welsh et al., 2001), although the exact pathway is still unclear. Since the high C/NO_3^- culture sludge was predominated with DNRA activity, the nitrogenous gases produced could, therefore, come either from DNRA or denitrification.

At the end of the incubations, the net $^{15}NH_4^+$ produced from the high C/NO_3^- sludge was at 46.6 and 65.5% of the consumed $^{15}NO_3^-$ and $^{15}NO_2^-$, respectively. However, it should be noted that the higher $^{15}NH_4^+$ formed in the presence of $^{15}NO_2^-$ does not necessary mean nitrite can induce DNRA better than nitrate, since in the $^{15}NO_3^-$ incubation a certain amount of ^{15}N still remained as $^{15}NO_2^-$ and the reaction was still ongoing. Nonetheless, the stable-isotope tracers have provided evidence that the ammonium observed in the high C/NO_3^- enrichment culture was mainly the product of DNRA. Therefore, the addition of glucose at COD/NO_3^--N ratio of 8/1 could select for DNRA activity, although denitrification might still co-exist at a certain level.

4.3.3. Microbial community of the low and high C/NO_3^- enrichment cultures

Illumina MiSeq analysis revealed that, from all the samples collected, 98.4 to 99.8% were identified as Bacteria and ≤0.01% as Archaea, while 0.2 to 1.6% of the reads could not be designated. Operational taxonomic units (OTUs) assigned from the sequence reads could be classified into 17 main classes (Fig. 4) belonging to 7 phyla. These phyla included Proteobacteria, Firmicutes, Bacteroidetes, Chloroflexi, and the candidate divisions GN02, OD1, and TM7. Classes with <2% maximum relative abundance were grouped as 'Others', this accounted for 2.0 to 13.7% of the total OTUs among 11 samples. For the high C/NO_3^- culture, the class found with the highest relative abundance was Clostridia (38.0%), followed by Epsilonproteobacteria (34.0%), Bacteroidia (20.4%), BD1-5 of the candidate GN02 division (12.9%), Erysipelotrichi (12.4%), Deltaproteobacteria (11.9%), and Gammaproteobacteria (9.4%). For the low C/NO_3^- culture, the high relative abundance classes included Saprospirae (25.4%), Betaproteobacteria (24.0%), Bacteroidia (22.9%), and Alpha-proteobacteria (13.8%). It can be seen from the class level that the majority of the OTUs is not shared between the two enrichment cultures, except for the class Bacteroidia. The distinctive community observed explains the partition in nitrate-reducing activity found in the two systems. Denitrifiers and DNRA microorganisms were hence expected to dominate the low and high C/NO_3^- enrichment cultures, respectively.

Among the major OTUs identified in the high C/NO_3^- culture, two of them were found to be related to the known DNRA bacteria. One was assigned as closely related to the genus *Sulfurospirillum* (OTU105876), while the other was related to the species *Geobacter lovleyi* (OTU586655), with maximum relative abundances of 33.8 and 8.5% respectively. The genus *Sulfurospirillum*, a member of the class Epsilonproteobacteria, has species members known to reduce nitrate and formed ammonium (Hubert & Voordouw, 2007; Luijten et al., 2003). *Sulfurospirillum* spp. were found both as sulfur reducers and sulfide oxidizers which capable of utilizing organic carbon, *e.g.* succinate and lactate, as their electron donors (Hubert & Voordouw, 2007; Stolz et al., 1999). Their versatile metabolisms should allow them to reduce nitrate and oxidize organic

carbon in the high C/NO_3^- enrichment culture, whereas the influence of sulfur compounds (either sulfate or sulfide) should be minimal compared to the available nitrate and organic carbon concentrations.

Sulfurospirillum spp. are known to use cytochrome c nitrite reductase (NrfA) to catalyze DNRA (Mohan et al., 2004; Schumacher et al., 1994), they are hence considered as respiratory-type DNRA(R-DNRA) microorganisms. Interestingly, not just one but three OTUs related to *Sulfurospirillum* were found in elevated abundances in the high C/NO_3^- community, with the other two OTUs observed at the maximum abundances of 15.4% (OTU562868) and 12.4% (OTU4350611). In the low C/NO_3^- culture, the three OTUs were present at ≤0.02% of the total community. This suggests that the ratio applied to the high C/NO_3^- system highly selected for these populations.

Another OTUs affiliated with known DNRA microorganisms were those closely related to the species *Geobacter lovleyi* (Sung et al., 2006), in which two separate OTUs were identified. These OTUs, designated as OTU586655 and OTU4453636, were found with maximum abundances of 8.5 and 2.5%, respectively. Populations related to *Geobacter lovleyi* were previously reported by van den Berg et al. (2015) to be the dominating bacteria in acetate-fed enrichment cultures performing DNRA. However, *Geobacter* spp. seem to prefer acetate and unable to use glucose (Lovley et al., 1993; Sung et al., 2006; van den Berg et al., 2015). The populations related to *Geobacter* found in the high C/NO_3^- culture thus might utilize fermented organic products from other bacteria in the community for their growth.

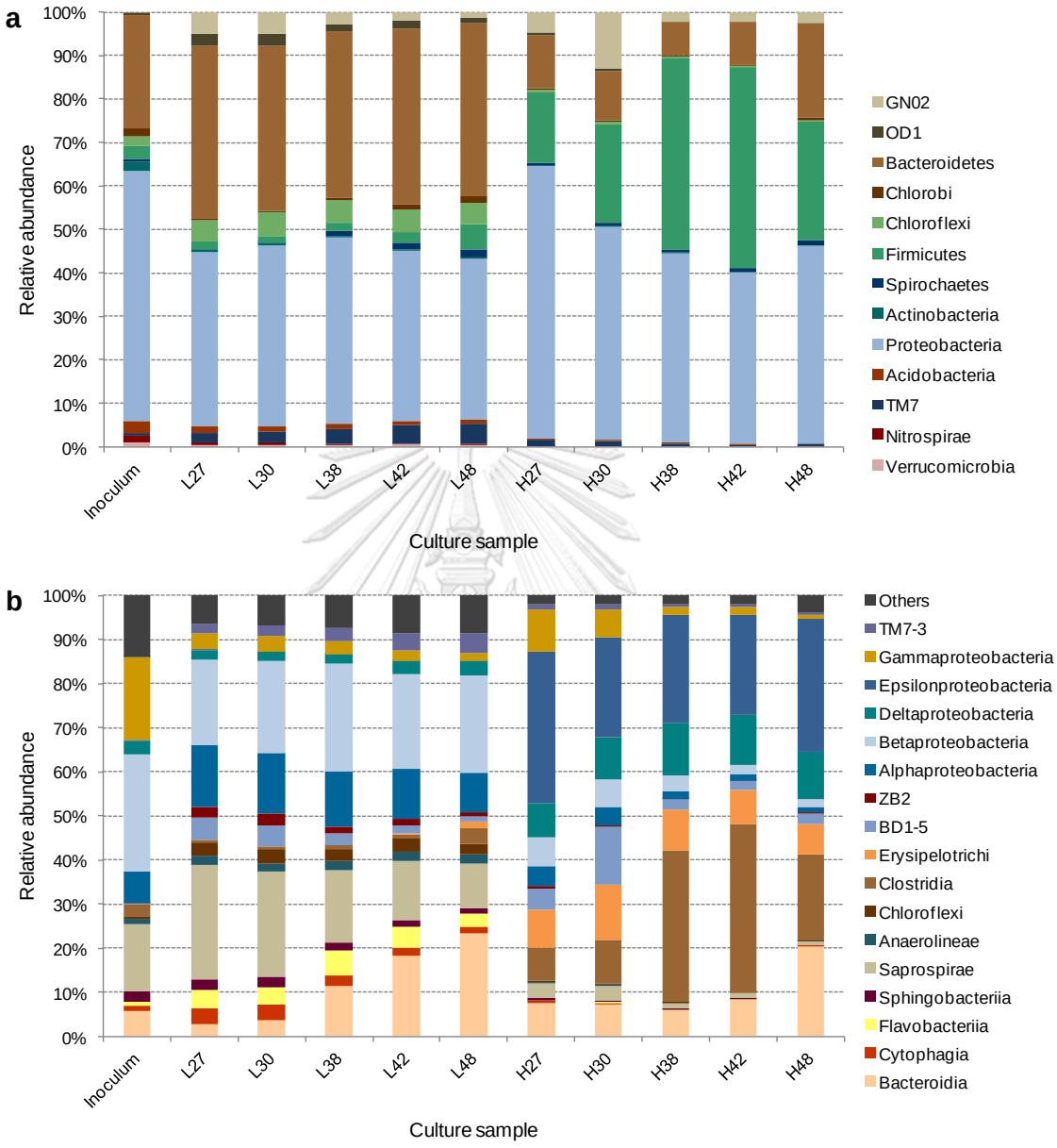


Fig. 4.9 Relative abundances of the dominant phyla (a) and classes (b) in the inoculum and the low C/NO_3^- (L27, L30, L38, L42, and L48) and high C/NO_3^- (H27, H30, H38, H42, and H48) enrichment cultures

Besides those related to known DNRA microorganisms, the involvement in nitrate reduction for other OTUs was not as apparent. Many OTUs identified in the high C/NO_3^- culture were related to the bacteria with fermentative ability, these include the populations affiliated with the family Lachnospiraceae (OTU567875), members of the class BD1-5 (OTU65013), a genus *Tolumonas* (OTU4439030), and a genus *Paludibacter* (OTU72348). However, the presence of fermentative bacteria in this system is not surprising, as organic carbon level in the high C/NO_3^- culture was supplied in excess while nitrate was limited, hence it should allow fermentation to occur along with nitrate reduction.

The OTU assigned to the family Lachnospiraceae (in the class Clostridia, Firmicutes phylum) was one of the most abundant taxa in the high C/NO_3^- community, with a 33.2% maximum abundance detected. Several members of Lachnospiraceae were classified as fermentative bacteria (Cotta & Forster, 2006), and were mainly found as a part of gut microbiota in mammals (Cotta & Forster, 2006; Meehan & Beiko, 2014). It is interesting that microorganisms related to *Lachnospira*, members of the Lachnospiraceae family, were also detected in anoxic microcosms performing DNRA in the work of Kraft et al. (2014). Their metatranscriptomic results revealed that these bacteria might have partial ammonifying ability as part of their metabolisms, though their full capacity on this function is still not known. The high proportion of this OTU discovered in this work suggests their essential role in this DNRA community. Nonetheless, whether this population was performing exclusively organic fermentation, or conducting fermentative-type DNRA (F-DNRA) under a high C/NO_3^- environment, still remains to be determined.

Three OTUs affiliated with the class BD1-5 (in the candidate GN02 division) were identified in the high C/NO_3^- community, with maximum relative abundances of 9.7% (OTU65013), 2.1% (OTU643385), and 0.3% (OTU71839). BD1-5 were mostly observed in anaerobic ecosystems, *e.g.* sediment aquifer (Miller et al., 2013), deep-sea sediment (Li et al., 1999), and submarine tufa columns (Vester et al., 2014). The functional capabilities of BD1-5 are largely unknown due to the absence of a representative

culture (Hanke et al., 2014; Wrighton et al., 2012). Previous metagenomic study revealed fermentative capacity in certain BD1-5 (Wrighton et al., 2012), while they seem to lack enzymes known to involve dissimilatory nitrate/nitrite reduction (Hanke et al., 2014). Base on the current knowledge, the OTUs related to BD1-5 in this study were most likely fermentative bacteria acted as contributors supplying fermented products to the rest of the populations. Apart from the high C/NO_3^- community, the OTU71839 of BD1-5 was also detected in the low C/NO_3^- culture, at a 4.6% maximum relative abundance, whereas the other two OTUs were almost exclusively found in the high C/NO_3^- system. The role in the organic carbon conversion of those related to BD1-5 was thus also expected under the low C/NO_3^- environment.

The OTU closely related to the genus *Tolumonas* (OTU4439030) was found with a maximum abundance of 4.9% in the high C/NO_3^- community. *Tolumonas*, members of the family Aeromonadaceae in the class Gammaproteobacteria, are also identified as fermentative bacteria and, at least from the known species, seem unable to conduct nitrate reduction (Caldwell et al., 2011; Fischer-Romero et al., 1996). Fermentation products of *Tolumonas* from glucose include ethanol, acetate, formate, and lactate (Fischer-Romero et al., 1996; Caldwell et al., 2011). The OTU affiliated with *Tolumonas* thus might produce fermented organic carbons that should support the growth of DNRA microorganisms, *i.e.* those related to *Sulfurospirillum* and *Geobacter lovleyi*, which might prefer these organic substrates to glucose.

Another OTUs found in similar proportion to the one assigned as *Tolumonas* were those closely related to the genus *Paludibacter*, in which three OTUs were detected in the high C/NO_3^- culture with maximum abundances of 4.1% (OTU72348), 2.1% (OTU4322518), and 0.6% (OTU356639). Known species of *Paludibacter* were unable to use nitrate as electron acceptor, but capable of fermenting glucose to acetate and propionate (Qiu et al., 2014; Ueki et al., 2006). However, *nrfA* gene belonging to *Paludibacter* sp. was identified from a previous metagenomic study (Kantor et al., 2017). Therefore, it is possible that the populations affiliated with *Paludibacter* in the high C/NO_3^- system were performing R-DNRA. Nonetheless, these

OTUs could also conduct the role of providing fermented carbon substrates to the bacteria involved in nitrate reduction, as speculated for those related to BD1-5 and *Tolumonas*.

Among the top abundant OTUs found in the high C/NO_3^- culture, little is known about the functions of an OTU assigned to a genus PSB-M-3 (OTU355578). The proposed genus PSB-M-3, belonging to the family Erysipelotrichaceae, the Firmicutes phylum, shared the high C/NO_3^- community with a maximum abundance of 12.2%. PSB-M-3 was previously detected in microbial community fed with high amounts of acetate and lactate (Smith et al., 2016), suggesting its preference for elevated carbon environment. Additionally, as several members of the family Erysipelotrichaceae were known to ferment organic carbons (Fujimoto et al., 2004), fermentation ability of PSB-M-3 might be possible.

For the low C/NO_3^- culture, the OTU detected with the highest abundance was assigned as members of the family Comamonadaceae in the class Beta-proteobacteria (OTU143252), with a maximum abundance of 8.4%. Several members of this family were recognized as denitrifying bacteria, *e.g. Comamonas* and *Acidovorax* (Willems, 2014). Other OTUs affiliated with known denitrifiers include those related to *Rhodobacter* (OTU321409), *Dechloromonas* (OTU536847), and *Flavobacterium* (OTU188193) (Betlach & Tiedje, 1981; Byrne & Nicholas, 1987; Coates et al., 2001). OTUs related to members of the family Chitinophagaceae (OTU181810 and OTU4083690) and the family Oxalobacteraceae (OTU782472) were also identified in which some species members of these two families were known to reduce nitrate or perform complete denitrification, *e.g.* certain species of *Chitinophaga*, *Terrimonas* (Kämpfer et al., 2011), and *Noviherbaspirillum* (Ishii et al., 2017). The finding of these major OTUs in the low C/NO_3^- culture was in accordance with the denitrifying activity found in this system, whereas microorganisms with potential DNRA was not detected among the dominant populations.

Table 4.2 Microbial community richness (Chao1 index) and biodiversity (Shannon index) of the samples taken from inoculum and the low C/NO_3^- enrichment culture

Index	Inoculum	L27	L30	L38	L42	L48
Chao1	3688.6	4288.4	4720.5	4772.0	4978.1	5024.6
Shannon	8.10	7.84	7.78	7.86	7.98	7.90

Table 4.3. Microbial community richness (Chao1 index) and biodiversity (Shannon index) of the samples taken from the high C/NO_3^- enrichment culture

Index	H27	H30	H38	H42	H48
Chao1	3872.5	3533.4	2855.1	2401.4	3157.5
Shannon	5.30	5.48	4.57	4.48	5.70

Between the two enrichment cultures, there were only a few dominant taxa shared among them. These few OTUs include those related to BD1-5 and *Paludibacter*, which seem chiefly involved in organic carbon conversion. Micro-organisms with dual nitrate-reducing functions, as has been described for *Shewanella loihica* and *nirK*-containing denitrifiers (Helen et al., 2016; Yoon et al., 2015) was not detected, or at least were not clearly distinguishable. Observing a heatmap showing relative abundances of the major OTUs from all samples (Fig. 5), one would see that the dominating OTUs in the high C/NO_3^- culture rarely occurred in the low C/NO_3^- system, and vice versa. This emphasizes the role of those major taxa in the high C/NO_3^- ecosystem in partitioning nitrate reduction to DNRA and formed ammonium-producing community. It is also interesting to see that the high C/NO_3^- condition enriched for microbial composition markedly different from that of the inoculum, while smaller changes was observed for the low C/NO_3^- community. This was, however, not surprising since the inoculum was obtained from a wastewater treatment system operating with denitrification, whereas the high C/NO_3^- ratio selected for those with different functional abilities.

Microbial richness, as estimated by the Chao1 index, and microbial diversity, as calculated by the Shannon index (Table 1), revealed a significant difference ($P<0.05$) between the low and high C/NO_3^- microbial populations. A lower richness and biodiversity were found in the high C/NO_3^- samples, while the low C/NO_3^- populations were more diverse and had a higher community richness. Therefore, it would seem that the higher C/NO_3^- ratio allowed certain bacteria to thrive with elevated proportions whereas the lower ratio of C/NO_3^- nurtured diverse bacteria that, among the dominating ones, had relatively fair shares of microbial composition.

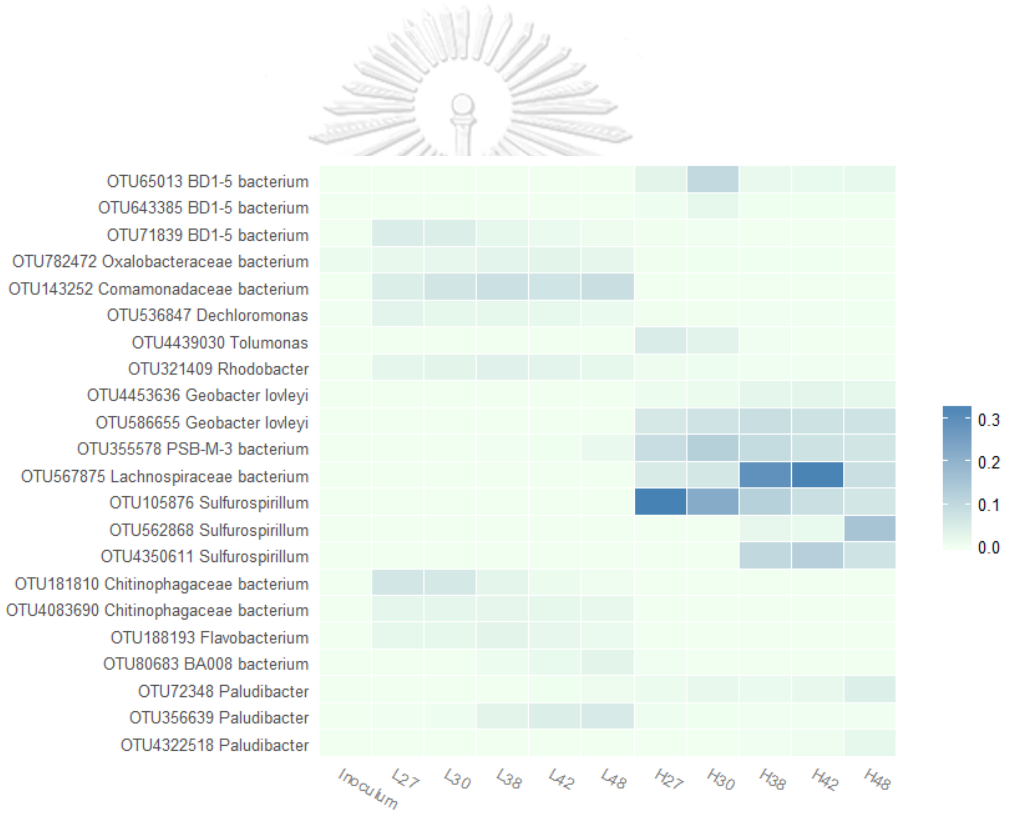


Fig. 4.10 Heatmap showing relative abundance of the predominant OTUs in inoculum and the low C/NO_3^- (L27, L30, L38, L42, and L48) and high C/NO_3^- (H27, H30, H38, H42, and H48) enrichment cultures (the scale is in a range 0.0 to 1.0)

4.4. Conclusions

In the high C/NO_3^- enrichment culture, DNRA could be observed by the marked increase in the ammonium concentration. Tracing nitrogen conversions with $^{15}NO_3^-$ and $^{15}NO_2^-$ revealed that DNRA microorganisms were the major contributors in nitrate/nitrite reduction under a high COD/NO_3^--N ratio of 8/1. With a low COD/NO_3^--N ratio of 4/1, denitrifiers obtained major role as the main nitrate/nitrite reducers. Interestingly, the condition provided by the high C/NO_3^- ratio enriched for distinct microbial populations markedly different from the inoculum, which composing of OTUs closely related to the known dissimilatory ammonifiers, *i.e. Sulfurospirillum* and *Geobacter lovleyi*, as well as fermentative bacteria and those that might be capable of both functions. These bacteria were hardly present in the low C/NO_3^- culture, which harboring community of denitrifiers. The ratios applied to each enrichment culture hence provided the environment that partitioned the pathways of nitrate reduction to either nitrogenous gases or ammonium as the end product, which resulted in distinctive microbial compositions found in each system.

CHAPTER 5
PRIMER DESIGN FOR THE DETECTION OF FERMENTATIVE-TYPE DNRA MICROORGANISMS

5.1. Introduction

Microorganisms capable of DNRA have been suggested by Burgin & Harmilton (2007) to be divided into two groups based on their metabolic and physiological functions: which are respiratory-type DNRA (R-DNRA) and fermentative-type DNRA (F-DNRA). As the name implied, R-DNRA microorganisms are those using nitrate/nitrite for respiration, where the energy is conserved during the process through oxidative phosphorylation (Moreno-Vivian & Ferguson, 1998; Mohan & Cole, 2007). This pathway is catalyzed by periplasmic cytochrome c nitrite reductase, or Nrf, which encoded by a *nrf* operon, and the gene *nrfA* has been utilized as a marker for studying R-DNRA microorganisms since the design of their primers in the year 2004 (Mohan et al., 2004). Therefore, in most of the research targeting on DNRA micro-organisms in environmental samples, the respiratory-type were what usually being referred to (though mostly called in general as DNRA microorganisms). R-DNRA has also been termed as a sulfide-induced process (Burgin & Harmilton, 2007), since it seems to use sulfide as an electron donor (Brunet & Garcia-Gil, 1996). Other electron donors for the Nrf pathway of R-DNRA include hydrogen (Dannenberg et al., 1992) and formate (Mohan & Cole, 2007), which is a simple organic carbon. It is not clearly known whether R-DNRA microorganisms can utilize more complex carbon substrate. This microbial group therefore is anticipated to mainly involve with a sulfide-rich environment or perhaps organic-rich system with formate as an end product from microbial fermentation.

Compared to the R-DNRA, not much is known about the microorganisms capable of the F-DNRA pathway. Most studies in the past were focusing on bacterial pure cultures, in which one of them was the most studied bacterium *Escherichia coli* (Bleakley & Tiedje, 1982). Apart from *E. coli*, many enterobacteria (of the class

Gammaproteobacteria) have also been recognized to perform DNRA along with fermentation, including certain species of *Klebsiella*, *Serratia*, *Enterobacter*, and *Citrobacter* (Bleakley & Tiedje, 1982; Dunn et al., 1979; Smith, 1982). Other Gammaproteobacteria reported to perform this function are *Vibrio* (Prakash & Sadana, 1973) and *Proteus* (Behrendt et al., 2015). Isolated strains of the class Alpha-proteobacteria were also reported in the work of Polcyn & Podeszwa (2009) to exhibit F-DNRA ability where ethanol was fermented to acetate. The strains isolated were composed of the genera *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium*, all of which are associated with the rhizosphere ecosystem.

The other bacterial taxon found to contain F-DNRA microorganisms is the phylum Firmicutes. These include the genera *Staphylococcus*, *Paenibacillus*, and *Bacillus* (Bleakley & Tiedje, 1982; Mohan & Cole, 2007; Schirawski & Unden, 1995). The genus *Arthrobacter* of the phylum Actinobacteria was another bacteria reported to perform F-DNRA (Eschbach et al., 2003). All of the bacteria described as F-DNRA microorganisms here use the enzyme NADH-dependent nitrite reductase, or Nir, to catalyze the reduction of nitrite to ammonium. It is called fermentative-type DNRA because the Nir enzyme utilizes NADH (a carrier molecule transferring electrons during fermentation) as its electron donor and uses nitrite as its electron acceptor. As Nir is recognized as a cytoplasmic enzyme, it hence does not seem to be involved with the electron transport chain of the membrane and its major mechanism has been hypothesized to be nitrite detoxification. Additional amount of ATP is, however, produced from F-DNRA through substrate-level phosphorylation, which makes this pathway more preferable than the process of fermentation alone (Cole & Brown, 1980). It therefore can be seen that the physiological functions of the R-DNRA and F-DNRA are very different from each other. The environmental factors affecting their occurrence thus may also differ depending on the regulation of each DNRA pathway by the microbial cells.

It can be seen from the known F-DNRA microorganisms that the ability to perform this function is phylogenetically widespread among many bacterial taxa. And though these bacteria have been previously studied in detail, their actual roles in natural and engineered ecosystems are mostly unknown. The lack of study on F-DNRA microorganisms seems partly be due to the absence of a molecular method for detecting them in environmental samples. Primers specific for *nirB* (a gene encoding for the Nir enzyme) of the genus *Proteus* has previously been developed in the work of Behrendt et al (2015), but primers covering the majority of F-DNRA micro-organisms have not yet been designed. Therefore, in this study, primer pairs for the detection of microorganisms capable of F-DNRA were developed using *nirB* gene as the target. The objective of this part of the research was to design the *nirB* primers and apply them to environmental samples, so as to verify whether F-DNRA micro-organisms were present and functionally active in those selected ecosystems.

It should be noted, however, that there is one complication associated with the use of *nirB* as a marker gene for F-DNRA microorganisms. Even though *nirB* has been studied by several research works for its dissimilatory function, it has also been observed to catalyze nitrite assimilation by generating ammonium for cell synthesis (Nakano et al. 1998). Regulators used by the cells to control dissimilatory and assimilatory functions are, however, different and respond differently to the presence of oxygen and ammonium. For DNRA (which is a dissimilatory pathway), oxygen will suppress the process while the presence of ammonium has no effect on it, whereas nitrate assimilation is repressed by ammonium and unaffected by oxygen (Mohan & Cole, 2007). The proportion of the microorganisms containing *nirB* for DNRA and those having this gene for solely assimilatory function in the environment is still not known. What we know so far is that *E. coli* can conduct both R-DNRA and F-DNRA pathways, but cannot perform nitrate assimilation (Wang & Gunsalus, 2000). On the other hand, NADH-dependent nitrite reductase in *B. subtilis* was found to catalyze both DNRA and nitrate assimilation. Despite this complication, nitrite reduction to ammonium is still the partitioning pathway that distinguishes the process of DNRA from denitrification.

Therefore, the *nirB* gene encoding for NADH-dependent nitrite reductase is still the best option for the detection of F-DNRA microorganisms in environmental samples.

5.2. Materials and Methods

5.2.1. Retrieval, screening, and analysis of amino acid and nucleotide sequences

Sequences of amino acid and nucleotide used for the analysis were retrieved from four online databases, including Genbank, European Nucleotide Archive (ENA), Universal Protein Resource (Uniprot), and the Integrated Microbial Genomes and Microbiome Samples (IMG/M). For the analysis of NirB amino acid, a total of 5,650 sequences were collected. An online program CD-HITS (Huang et al., 2010) was applied to reduce sequence redundancy and to obtain a reasonable number of sequences for a phylogenetic tree construction. The sequence clustering was done using a threshold of ≥75% similarity, where a representative sequence was selected by the program for further analysis. After clustering, 676 NirB amino acid sequences were obtained for the construction of phylogenetic tree. The sequences were aligned with Clustal W algorithm in MEGA program ver. 6, and the tree was constructed with the Maximum Likelihood algorithm using the same program.

Protein domains (conserved sections of the protein sequence) within NADH-dependent nitrite reductase large subunit (NirB) were identified via Pfam, the protein families database (Bateman et al., 2004), using the NirB of *E. coli* as a representative sequence. Protein motifs (patterns in protein which usually have significant function) within each domain were later examined with the aligned amino acid sequences. As motifs are usually quite conserved within the same enzyme across different species, nucleotide positions corresponding to the selected motifs were therefore selected as candidates for the primers designing. Conserved regions within the aligned nucleotide sequences were also observed directly for potential primer binding positions outside the protein domains. All the alignments of the sequences were done using Clustal W algorithm in the MEGA program.

5.2.2. Primers design for targeting nirB genes

Due to the large number of *nirB* sequences available in the online databases and unclear classification between the genes encoding for nitrate assimilation and DNRA, therefore, only *nirB* nucleotide sequences from microorganisms with evidence for F-DNRA ability were used for the designing of the primers. From reviewing the literatures on F-DNRA, 25 bacterial species from 4 taxa were found to perform this process. The list of these selected bacteria is as shown in Table 5.1.

Although smaller number of sequences was applied for the primer designing, the alignment of *nirB* nucleotide sequences still showed high variation of bases used across the selected bacteria. The bacteria were then grouped together according to their class/phylum and the primers were designed specifically for each taxon. The primers were initially designed as degenerate primers (that is, a mixture of primers containing varying nucleotide bases at certain positions) in order to cover all the possible targets. However, due to the problems of non-specific binding of these designed primers (see Results and Discussion for detail), primers were eventually designed as 'consensus-degenerate' primers. The method for designing this type of primer was developed by Rose et al. (1998), in which the primer was designed as consensus towards the 5' end position and as degenerate towards the 3' end positions. For the consensus region, each nucleotide base was chosen from those most frequently used among the aligned sequences, whereas for degenerate region a mixture of different bases was used to cover all the possible targets. These primers were developed separately for each clade of the *nirB* sequences (the clades were assigned based on the phylogenetic tree construction), designated *nirB*-I (for clade I) and *nirB*-II (for clade II) (see Results and Discussion for detail). Several consensus-degenerate primers were designed for each clade and later evaluated with selected pure cultures from both clades I and II to examine their coverage and specificity.

Table 5.1 Bacterial species with evidence for F-DNRA ability

Bacterial species	Classes/Phyla	References
Escherichia coli str. K-12	Gammaproteobacteria	Bleakley and Tiedje, 1982
Citrobacter freundii	Gammaproteobacteria	Smith, 1982
Salmonella typhimurium	Gammaproteobacteria	Tiedje, 1988.
Klebsiella pneumoniae	Gammaproteobacteria	Satoh et al., 1983
Enterobacter aerogenes	Gammaproteobacteria	Bleakley and Tiedje, 1982
Serratia marcescens	Gammaproteobacteria	Bleakley and Tiedje, 1982
Pectobacterium carotovorum	Gammaproteobacteria	Bleakley and Tiedje, 1982
Vibrio fischeri	Gammaproteobacteria	Prakash and Sadana, 1973
Vibrio parahaemolyticus	Gammaproteobacteria	MacFarlane and Herbert, 1982
Lelliottia amnigena	Gammaproteobacteria	Fazzolari et al., 1990
Proteus vulgaris	Gammaproteobacteria	Behrendt et al, 2015
Proteus mirabilis	Gammaproteobacteria	Behrendt et al, 2015
Bradyrhizobium sp.	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Mesorhizobium amorphae	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Mesorhizobium loti	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Rhizobium etli	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Sinorhizobium meliloti	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Rhizobium leguminosarum	Alphaproteobacteria	Polcyn and Podeszwa, 2009
Staphylococcus carnosus	Firmicutes	Mohan and Cole, 2007
Bacillus subtilis	Firmicutes	Bleakley and Tiedje, 1982
Bacillus mycoides	Firmicutes	Lipman, 1909
Bacillus vireti	Firmicutes	Mania et al., 2014
Bacillus licheniformis	Firmicutes	Sun et al., 2016
Paenibacillus macerans	Firmicutes	Schirawski and Unden, 1995
Arthrobacter globiformis	Actinobacteria	Eschbach et al., 2003

5.2.3. Primer evaluation with in silico PCR

The *nirB* primers designed were initially evaluated with FastPCR program (Kalendar et al., 2014) using *in silico* PCR option. The *nirB* gene sequences were used as templates and the evaluation was done by observing the specificity of the primers to the target and possible mismatches within those sequences. Primer characteristics, including melting temperature (T_m) and %GC content, were also calculated with the FastPCR. Selection of the primers from *in silico* PCR evaluation was based on their coverage as well as the absence or low level of mismatches to the tested templates.

5.2.4. Primer evaluation with pure cultures

To evaluate the designed primers, 22 bacterial pure cultures known to contain *nirB* gene were used for testing the PCR amplification of each primer. All of the strains were purchased from the Japan Collection of Microorganisms (JCM), except for *E. coli* K-12, which was derived from a culture stock in the laboratory. These bacteria included *L. amnigena* (JCM 1237), *C. koseri* (JCM 1659), *Yersinia aldovae* (JCM 5892), *Aeromonas salmonicida* (JCM 7874), *S. marcescens* (JCM 11315), *Aliivibrio fischeri* (JCM 18803), *Pseudomonas putida* (JCM 20111), *P. carotovorum* (JCM 20699), *Halomonas elongate* (JCM 21044), *B. japonicum* (JCM 10344), *R. leguminosarum* (JCM 20680), *Ensifer meliloti* (JCM 20682), *M. loti* (JCM 21556), *M. amorphae* (JCM 21563), *R. etli*, (JCM 21823), *P. macerans* (JCM 2500), *S. piscifermentans* (JCM 6057), *S. carnosus* (JCM 6069), *B. mycoides* (JCM 9801), *B. subtilis* (JCM 20036), and *B. vireti* (JCM 21711). Each bacterium was cultured using medium and condition as recommended by the JCM catalogue. After culturing overnight, DNA of each bacterium was extracted from the harvested cells using heat extraction method (boiled at 95°C in a heating block for 15 min) with the addition of InstaGene™ Matrix (BIO-RAD) and Triton™ X-100 to assist the DNA extraction. The obtained DNA was further used as the templates for the primer analysis.

Several PCR conditions were adjusted for the amplification of each primer, these included the change in annealing temperature, denaturing/annealing/extension time, as well as the use of touchdown PCR method. PCR reagents were also varied, ranging from the concentrations of primer and $MgCl_2$, and the use of PCR additives (bovine serum albumin and DMSO). The results of the primer amplification were observed by gel electrophoresis using 1.5% agarose gel.

5.2.5. Detection of the presence and expression of nirB-containing microorganisms in environmental samples

The selected primer pairs from the evaluation step were applied to samples taken from the low and high C/NO_3^- enrichment cultures of this study (see Chapter 4 for detail), which exhibited active nitrate-reducing activity. Sludge samples for DNA analysis were collected on days 27, 30, 38, 42, and 48 from both cultures, as well as from the inoculum used for starting the systems. These samples were examined for the presence of *nirB* gene with the selected primer pairs *nirB*-I and *nirB*-II. Apart from the DNA, RNA samples were also collected to observe the expression of *nirB* gene during the period of elevated ammonium accumulation in the high C/NO_3^- culture, which were on the days 38, 40, 42, 44, 46, and 48. RNA samples from the low C/NO_3^- system were also obtained on the same sampling days for a comparative analysis. Additionally, batch incubations with nitrate and nitrite were also conducted with both low and high C/NO_3^- culture sludge to induce DNRA activity, and thus enhance the expression of the *nirB* gene. These incubations were prepared by adding 20 mg-N/L of nitrate/nitrite to the sludge taken from the enrichment cultures on days 60 and 54 for the low and high C/NO_3^- systems, respectively. The obtained sludge was harvested by centrifugation and washed once with a new medium (the same constituents as supplied to each culture, excluding NH_4Cl), then placed into 100-ml serum bottles along with 20 ml of the new medium. The serum bottles were closed with butyl rubber stoppers and sealed with aluminum caps before flushing with Ar gas for 10 min. Nitrate and nitrite (in the forms of $NaNO_3$ and $NaNO_2$, respectively) was injected into each

bottle, along with 100 mg-N/L of NH_4Cl (to repress nitrate/nitrite assimilation) and sterile glucose solution to make the same C/NO_3^- ratios as in the enrichment cultures. All of the experiments were performed in triplicate and the stock solutions were flushed with Ar gas for 10 min before use. Sludge samples for RNA analysis were collected at one time point during the period of exponential nitrate- or nitrite-reducing activity.

The obtained RNA samples were first preserved with RNA*later*™ Stabilization Solution (Invitrogen™) and kept at -80°C before extraction. RNA extraction was done using RNeasy Mini Kit (QIAGEN) with an additional bead beating step with FastPrep® Lysis Beads and Matrix Tubes (Lysing Matrix E, MP Biomedicals, LLC) to enhance cell rupture. Other procedures were conducted according to the manufacturer's protocol with a step of DNA elimination using RNase-free DNase digestion set (QIAGEN). The extracted RNA was subsequently converted to complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's instruction. DNA was extracted using FastDNA™ SPIN Kit for Soil (MP Biomedicals, LLC). Both the DNA and cDNA were then analyzed with the selected primer pairs and the presence of the expected PCR product was examined with gel electrophoresis (using 1.5% agarose gel).

5.3. Results and Discussion

5.3.1. Primer design and evaluation

From screening the online databases for amino acid and nucleotide sequences (Genbank, ENA, Uniprot, and IMG/M), a large quantity of *nirB* sequences were observed in various bacterial taxa including Proteobacteria (Gamma-, Alpha-, Beta-, Delta-, and Epsilon-), Firmicutes, Actinobacteria, and Bacteroidetes. So far, there is no report on *nirB*-containing archaea. However, the widespread occurrence of *nirB* gene across many bacterial phyla makes it difficult to develop a molecular method to cover the detection of all *nirB*-containing bacteria. Problems on primer coverage have previously

been observed with the functional genes of denitrifiers, *nirS* and *nirK* (Helen et al., 2016; Wei et al., 2015), as well as with the functional gene of R-DNRA microorganisms, *nrfA* (Welsh et al., 2014). This is because both denitrifiers and DNRA microorganisms are highly phylogenetically diverse compared to other nitrogen-converting microorganisms, *e.g.* ammonium-oxidizing bacteria/archaea (AOB/ AOA), nitrite-oxidizing bacteria (NOB), and anammox bacteria, which therefore complicates the detection and identification of denitrifiers and DNRA microorganisms in environmental samples.

The first attempt to design primer pairs targeting the *nirB* gene was done by screening the online databases for the selection of *nirB* sequences as templates for the primer design. However, as too large quantity of the sequences were deposited and the function of *nirB* gene in different bacteria is still in question, primers were eventually designed using bacteria that have previously been reported to perform F-DNRA. From reviewing the literature, 25 bacterial species were identified (Table 5.1). This included bacteria from Gammaproteobacteria, Alphaproteobacteria, Firmicutes, and Actinobacteria. After retrieving the representative *nirB* genes from the databases, the sequences were aligned with Clustal W algorithms and the results were obtained as follows,

```
Bacillus_mycoides           AAATCATCCCGCTTCGTTAATGAAAGAATGAATGGCAATATTCAGCATGATGGTACATTT
Staphylococcus_carnosus     AAAGCATCACGTTTTGCAAATGAACGATACCATGCCAATATTCAAAATGATGGAACATTC
Bradyrhizobium_sp.          CAGCAGTCGCGCTTCATCAACGAGCGCGTCCACGCCAATATCCAGAAGGACGGCACCTAT
Mesorhizobium_amorphae      TACCAGTCGCGCTTCATCAACGAGCGCGTCCATGCCAACATCCAGAAGGACGGCACCTAT
Mesorhizobium_loti          TACCAGTCACGCTTCATCAATGAGCGGGTGCATGCCAACATCCAGAAGGACGGCACCTAT
Sinorhizobium_meliloti      TACCAGTCGCGCTTCATCAACGAGCGTGTCCACGCCAATATCCAGAAGGACGGCACCTAT
Rhizobium_etli              TACCAGTCGCGTTTCATCAATGAGCGCGTCCACGCCAACATCCAGAAGGACGGCACCTAC
Rhizobium_leguminosarum     TACCAGTCGCGTTTCATCAATGAACGCGTGCATGCCAACATCCAGAAGGACGGCACCTAC
Paenibacillus_macerans      AAAGAATCCCGGTTCGTCAACGAACGCTACCACGCGAATATTCAAAAGGACGGAACTTTT
Bacillus_vireti             CGTGATTCCCGCCTCGTCAATGAAAAAATGCACGCCAATATTCAGAAGGACGGAACCTAC
Bacillus_licheniformis      AGAGAATCCCGCTTCGTCAATGAACGGATGCACGCCAACATTCAAAAAGACGGCACATAT
Bacillus_subtilis           AGAACTTCCCGATTTGTGAACGAACGGATGCACGCCAATATTCAAAAAGACGGAACATAC
Arthrobacter_globiformis    GGCACCCTGCAGGACACCAACGACCGTGCGCTGGCCAACATGCAGAAGGACGGCACGTAT
Vibrio_fischeri             GTTAAACTGCATGATACTAACGATAACTTCTTAGGCAACATGCAAAAAGATGGTACTTAT
Vibrio_parahaemolyticus     GTTAAGCTGCACGACACGAACGATAACTTCCTAGGTAACATCCAAAAAGACGGTACTTAC
Proteus_mirabilis           GTTGCGCTACAAGATACGAATGATAACTTCCTCGCCAATCTGCAAAAAGATGGCACTTAC
Proteus_vulgaris            GTCTCTCTGCAAGATACTAATGATAACTTTCTCGCCAATATGCAAAAAGATGGGACTTAT
Pectobacterium_carotovorum  ACAGCGCTACAGGACTCCAACGATAACTTCCTCGGCAACATCCAGAAAGACGGCACGTAC
Serratia_marcescens         ACGCCGCTGCAGGATACCAACGACAACTTCCTCGGCAATATCCAGAAAGACGGCACCTAC
Enterobacter_aerogenes      ACGCCGCTGCAGGACACCAACGATAACTTCCTCGCCAACATCCAGAAAGATGGTACCTAC
Klebsiella_pneumoniae       ACGCCGCTGCAGGACACCAACGACAACTTCCTTGCCAACATTCAGAAAGATGGCACCTAC
Escherichia_coli            ACTCCGCTGCAGGATTCTAACGACAACTTCCTCGCTAACATCCAGAAAGACGGCACCTAC
Lelliottia_amnigena         ACTCCGCTTCAGGACACCAACGATAACTTCCTGGCCAATATTCAGAAAGACGGGACTTAC
Citrobacter_freundii        ACGCCGCTGCAAGACACCAACGACAACTTCCTGGCGAATATCCAGAAAGACGGCACCTAC
Salmonella_enterica         ACGCCGCTACAGGATACTAACGATAACTTCCTAGCGAATATCCAGAAAGACGGTACTTAC
                                     *        ** **          *  **  * **  * ** ** ** *
```

```
Bacillus_mycoides              TTAGAAATGGTAGATACACCTCACAAAATGAAAATGGGTGTAACTGGTTGTCCACGTAAC
Staphylococcus_carnosus        TTTGAATATATCGATACGCCGCATAAATTTAAAATGGGTGTTTCTGGTTGTCCGCGTAGC
Bradyrhizobium_sp.             ATGTGGGGCTCCTGGACGCCGGCCAAGGTCAAGCTCGGCGTCTCCGGCTGCCCGCGCAAC
Mesorhizobium_amorphae         ATGTGGGGCTCGTGGACACCGGCCAAGGTCAAGATGGCGGTGTCGGGTTGCCCGCGAAAC
Mesorhizobium_loti             ATGTGGGGCTCCTGGACGCCCGCCAAGGTCAAGATGGCGGTGTCGGGCTGCCCCAGAAAC
Sinorhizobium_meliloti         ATGTGGGGCTCCTGGACGCCGGCCAAGCTGAAGATGGCCGTCTCCGGCTGCCCGCGCAAT
Rhizobium_etli                 ATGTGGGGCTCATGGACGCCGGCCAAATTGAAGATGGCCGTGTCCGGCTGCCCGCGCAAT
Rhizobium_leguminosarum        ATGTGGGGCTCCTGGACGCCGGCCAAGCTGAAGATGGCCGTCTCCGGCTGCCCGCGCAAC
Paenibacillus_macerans         TTCGAACGCTTGAATACGCCGGGCAAGGTGAAGCTGGCCGTATCCGGATGCCCCCGCAAC
Bacillus_vireti                TTTGAACGTCTGGATACCCCGCATAAAGTAAAAATGGGCGTATCAGCGTGCCCACGTAAC
Bacillus_licheniformis         TTTGAAGGGCTCTACACGCCTCACAAAATTAAAATGGCGGTTTCCGCTTGTCCGAGAAAC
Bacillus_subtilis              TTCGAAGGGCTCAATACACCTCATAAAGTCAAAATGGCCGTGTCTGCCTGCCCGCGGAAC
Arthrobacter_globiformis       TACCGCGGCCTCCGCAGCCCGCACAAGCTCAAGATGGGCGTCTCCGGCTGCGCCCGCGAG
Vibrio_fischeri                TACAAAGGTATTCGTACACCTCATAAAATGAAGTTCGGTGTATCTGGTTGTACTCGTGAA
Vibrio_parahaemolyticus        TACAAAGGCATCCGTACGCCTCACAAGATGAAGTTCGGTGTTTCTGGCTGTACTCGTGAG
Proteus_mirabilis              TATAAAGGGATCCGCACGCCGCATAAAATGAAATTTGGGGTATCTGGTTGTACCCGTGAA
Proteus_vulgaris               TATAAAGGCATTAGAACACCTCATAAGATGAAATTTGGTGTGTCAGGTTGTACTCGTGAA
Pectobacterium_carotovorum     TACAAAGGCATCCGCACGCCGCACAAAATGAAATTTGGCGTCTCCGGCTGTACGCGGGAA
Serratia_marcescens            TACAAAGGCATCCGCACCCCGCACAAAATGAAGTTCGGCGTCTCGGGCTGCACCCGTGAA
Enterobacter_aerogenes         TATAAAGGTATCCGTACCCCGCACAAAATGAAGTTCGGCGTCTCCGGTTGTACCCGTGAA
Klebsiella_pneumoniae          TACAAAGGCATCCGCACCCCGCACAAAATGAAGTTCGGCGTCTCCGGCTGCACCCGCGAA
Escherichia_coli               TACAAAGGCATCCGTACGCCGCACAAAATGAAGTTCGGTGTCTCCGGCTGTACCCGTGAA
Lelliottia_amnigena            TACAAAGGTATCCGTACGCCGCACAAAATGAAGTTCGGCGTCTCAGGGTGTACCCGTGAA
Citrobacter_freundii           TACAAAGGCATTCGTACGCCGCATAAGATGAAGTTCGGTGTCTCTGGCTGTACCCGTGAA
Salmonella_enterica            TACAAAGGCATTCGTACCCCGCACAAAATGAAATTCGGCGTCTCCGGCTGTACCCGTGAA
                                     *  **    **  * **  * *  **  * *  **  *  *
```

These alignments show the two regions of highly conserved positions within the gene *nirB* (* indicates the position where all the sequences are using the same nucleotide base). Although these two regions are already highly conserved compared to the other sections of the gene, they still contain too many variable base positions. Since normally a primer should be in a range of 17- to 24-base long, to design a primer from these regions, mix bases thus have to be added to the varied nucleotide positions which then would result in a degenerate primer. However, high degeneracy (number of primers in the mixture) of the resulting primers makes them inapplicable for using with the real sample. Therefore, new strategy had to be employed in order to obtain degenerate primers with reasonable degeneracy so that it would be usable in the real application.

As the bacteria known to perform F-DNRA are belonging to four different taxa, the primers were then designed based on the bacteria of each taxon due to the fact that more conserved regions can be found within closely related microorganisms. Nonetheless, as only one bacterium in the phylum Actinobacteria has been identified as F-DNRA microorganisms (*i.e. A. globiformis*), this bacterium hence has to be excluded from the current primer designing process. Eventually, the primers were designed to

separately target the *nirB*-containing bacteria in the class Gamma-proteobacteria, Alphaproteobacteria, and in the phylum Firmicutes.

Several degenerate forward and reverse primers have been designed for the detection of each group of *nirB*-containing bacteria (Table 5.2). However, several of these primers gave numerous non-specific products after PCR amplification, whereas some were more specific but could not cover all of the tested targets (pure cultures known to contain *nirB* gene). Examples of these fail results of the primers designed for Gammaproteobacteria are as shown in Fig. 5.1. Likewise, most of the primer pairs for Alphaproteobacteria also resulted in a high number of non-specific bands (Fig. 5.2), even though the primers were already designed from the closely related *nirB* sequences. The results of the Alphaproteobacterial primers were quite surprising considering that the primers have far lower degeneracy than those of the Gamma-proteobacteria. By inspecting the sequences using *in silico* analysis in the FastPCR program, it seemed that *nirB* genes of the Alphaproteobacteria have some repeated positions within their sequences. These repeats were not exactly matched but the similarity might be high enough for the primers to bind non-specifically. Increasing the annealing temperature to avoid these non-specific bindings resulted in the lost of the target bands, as shown in Fig. 5.3.

The attempt to design the primers for Firmicutes revealed that, although coming from different phyla, *nirB* sequences of Firmicutes and Alphaproteobacteria share similarity to a certain level. These similarities might not be so high but they were enough to hinder the design of the *nirB* primers specifically for each taxon. In addition, the best results from using the Gammaproteobacterial primers still could not amplify all the tested strains (Fig 5.4), which might be due to the coverage of the primers themselves. The best results from the Alphaproteobacterial primers still show clear non-specific bands along with the targets, and the increase in annealing temperature also made the target band disappear, which are as shown in Fig. 5.5.

In order to improve the primers designing, NirB amino acid sequences were analyzed to make an appropriate clustering of the NirB sequences. Amino acid was used instead of nucleotide because it better reflects the evolutionary function of the sequences. So it might be possible to observe the evolutionary divergence between the NirB with dissimilatory and assimilatory functions. Amino acid sequences of all available NirB from the selected online databases were therefore collected and clustered to reduce the number of the processed sequences. A total of 676 NirB amino acid sequences were then used for the phylogenetic tree construction, and the result is as shown in Fig. 5.6.

From the constructed phylogenetic tree, it can clearly be seen that NirB can be categorized into two clades, where one of them (designated in this work as clade I) contains mainly Gammaproteobacteria, Actinobacteria, and Bacteroidetes, while the other (designated as clade II) contains mainly Firmicutes, Alphaproteobacteria, and Betaproteobacteria. However, the crossover of the NirB sequences between the two clades can also be observed, *e.g.* some NirB belonging to Gammaproteobacteria were classified as clade II. The same phenomena can be seen for Alphaproteobacteria, Betaproteobacteria, and Actinobacteria. Additionally, as NirB of the known F-DNRA bacteria are located in both clades I and II, the constructed tree hence does not seem to differentiate the NirB functioning as DNRA from those functioning as nitrate assimilation. And since the classification of the bacterial taxa was based on 16S rRNA gene, the clustering of a functional gene, which might not be evolutionary related to the 16S rRNA gene, thus can yield different grouping results from their original taxa. The evolutionary divergence of a functional gene seems to be quite common, as has been evidenced in the case of *nirS* (Wei et al. 2015), *nirK* (Helen et al., 2016), and *nrfA* (Welsh et al., 2014). Therefore, it would seem improbable to use only one pair of primers to detect all targets within a complex microbial community. The idea of designing primers for a specific clade or cluster of the studied functional gene has previously been employed to overcome this problem, as has already been done in the case of denitrifiers (Wei et al. 2015).

Table 5.2 Examples of primers designed specifically for *nirB* Gammaproteobacteria and Alphaproteobacteria

Primer	Nucleotide sequence (5´ - 3´)	Length (bp)	Degeneracy
Gammaproteobacteria			
Forward primers:			
G-nirB1471F	TAY TCN CGY CAR GAR CTG TWC CA	23	128
G-nirB1471Fm	TAY TCN CGY CAR GAR CTG T	19	64
G-nirB1477F	CGY CAR GAR CTG TWC CA	17	16
G-nirB1555F	TAY GGY TGY GAR GTN TGT AA	20	64
G-nirB2005F	CGY ACB CCK CAY AAR ATG AA	20	48
G-nirB2122F	GGY AAY GGC GGB ATG AA	17	12
Reverse primers:			
G-nirB1928R	CC NAC RCA SGT YTT HGC CAT	20	96
G-nirB1925R	AC RCA SGT YTT HGC CAT	17	24
G-nirB2012R	GG VGT RCG RAT RCC TTT	17	24
G-nirB2015R	TG MGG VGT RCG RAT RCC TTT	20	48
G-nirB2144R	CG NGG YTT CAT VCC GCC	17	24
G-nirB2150R	GC RTG RCG NGG YTT CAT	17	16
G-nirB2225R	CG RAT RTA GAA CAT CAT RAA GCG	23	8
G-nirB2231R	GC VGT RCG RAT RTA GAA CAT CAT	23	24
Alphaproteobacteria			
Forward primers:			
A-nirB1797F	G GTS AAG GTS ACS GGC G	17	8
A-nirB1750F	GAR YTK CGC GCC ATC GCC G	20	8
A-nirB1720F	CGS ATG TGG GGC GGC GT	17	2
A-nirB1681F	GCC AAY ATC CAG AAG GAC G	19	2
A-nirB1687F	ATC CAG AAG GAC GGC ACC TA	20	0
A-nirB1376F	CS TCS TGC GGY TCC TG	16	8
A-nirB1389F	C TGC ACC GGS CTS GTC G	17	4
A-nirB1273F	ATY TGY GGC TGC AAC GG	17	4
A-nirB1285F	AAC GGC GTN TGC AAG GG	17	4
A-nirB1282F	TGC AAC GGC GTN TGC AA	17	4
Reverse primers:			
A-nirB2141R	GC DCC KGC RAA ATG GAT	17	12
A-nirB2024R	GT CCA SGA GCC CCA CAT GAA	20	2
A-nirB1976R	GA ATC CTG VGT GCC GAA	17	3
A-nirB1706R	TA GGT GCC GTC CTT CTG GAT	20	0
A-nirB1709R	GA RTA GGT GCC GTC CTT	17	2
A-nirB1738R	T SAC GCC GCC CCA CAT	16	2
A-nirB1403R	AC SAG SCC GGT GCA GGA	17	4

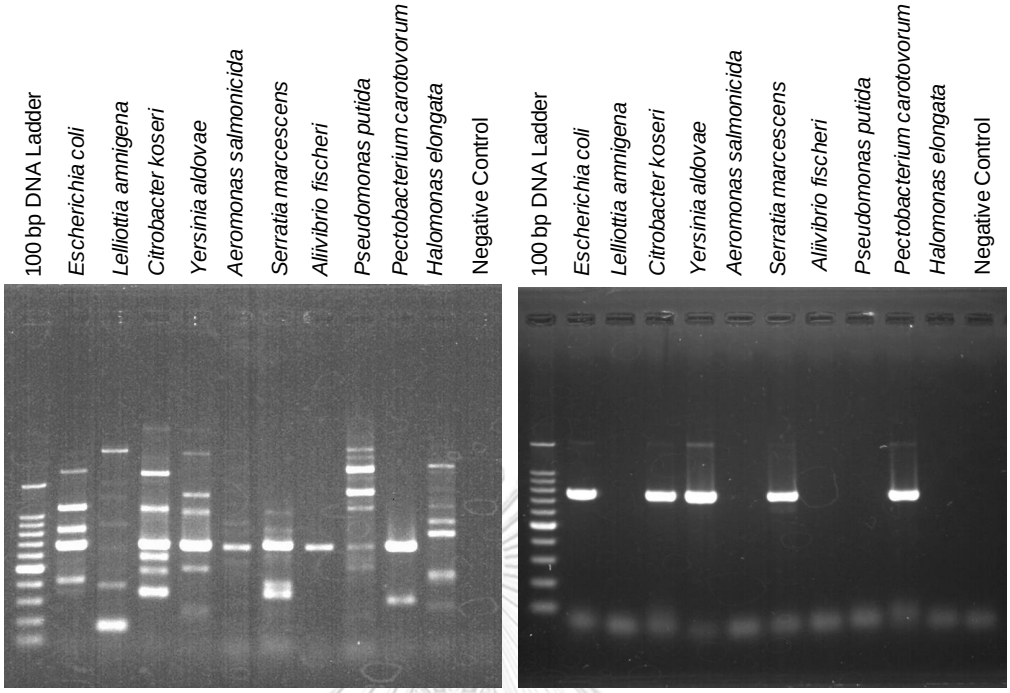


Fig. 5.1 Examples of *nirB* Gammaproteobacterial primer evaluation: 1471F + 2144R (left) and 1471F + 2231R (right) primer pairs

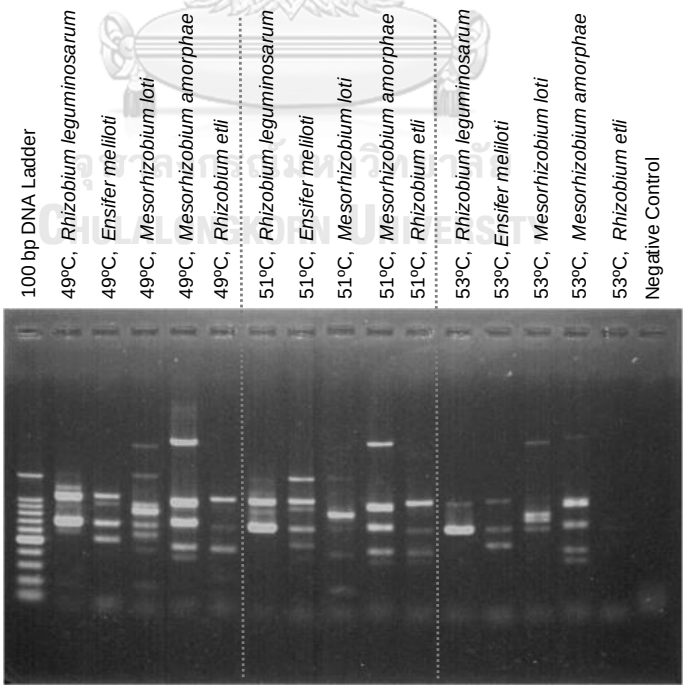


Fig. 5.2 Examples of *nirB* Alphaproteobacterial primer evaluation: 1273F + 1976R primer pair testing with different annealing temperatures

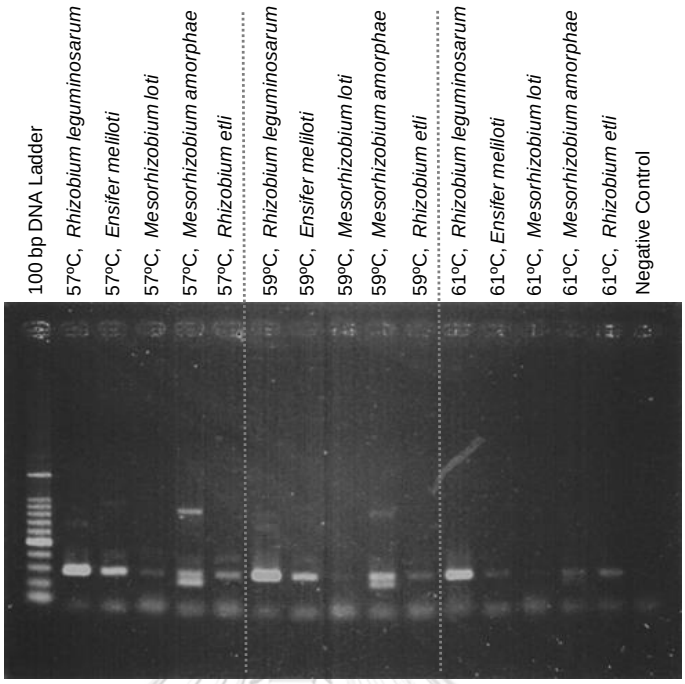


Fig. 5.3 Examples of *nirB* Alphaproteobacterial primer evaluation: 1681F + 1976R primer pair testing with different annealing temperatures

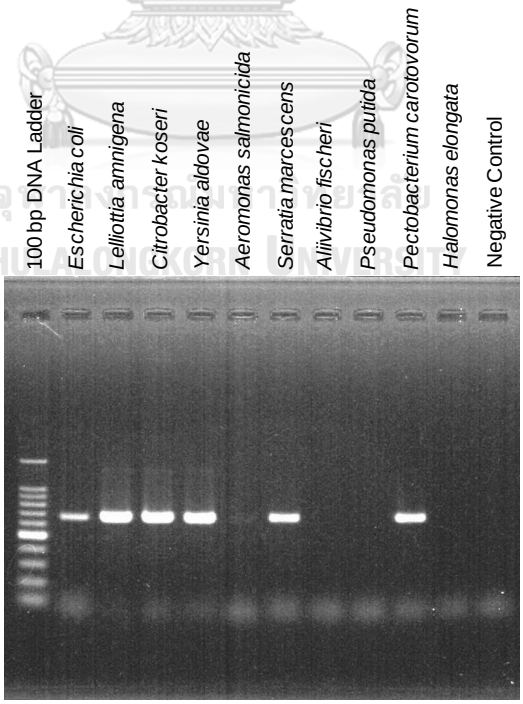


Fig. 5.4 Examples of *nirB* Gammaproteobacterial primer evaluation: 1555F + 2231R primer pair

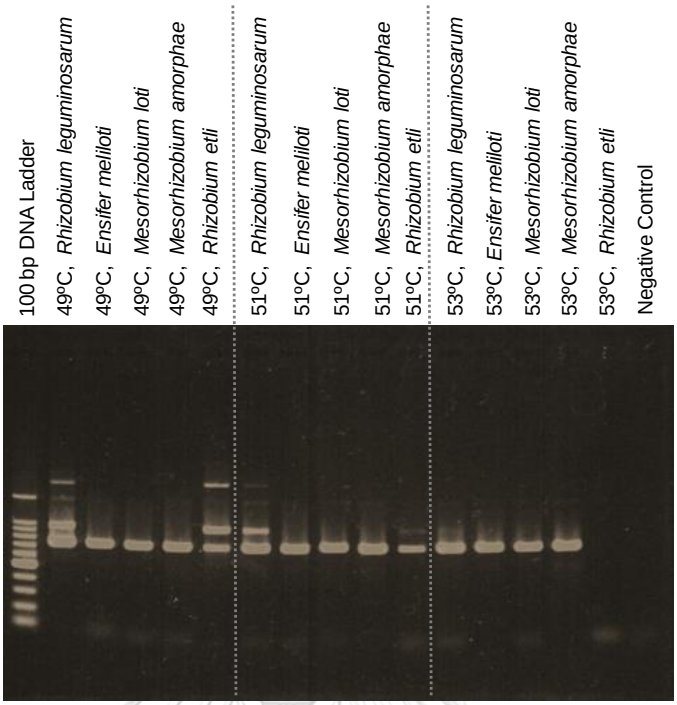


Fig. 5.5 Examples of *nirB* Alphaproteobacterial primer evaluation: 1282F+2024R primer pair testing with different annealing temperatures

As the divergence of NirB into two clades has now been identified in this study, primers targeting each clade were therefore developed for the detection of *nirB*-containing bacteria. However, since the constructed phylogenetic tree cannot differentiate between DNRA and nitrate assimilation pathways, primers were still designed based on the known F-DNRA microorganisms. The NirB sequences were further evaluated to identify the protein domains (which should contain conserved regions) using the Pfam database. The search result revealed that NirB is composed of four domains including pyridine nucleotide-disulphide oxidoreductase, BFD-like [2Fe-2S] binding domain, nitrite/sulfite reductase ferredoxin-like half domain, and nitrite and sulphite reductase 4Fe-4S domain.

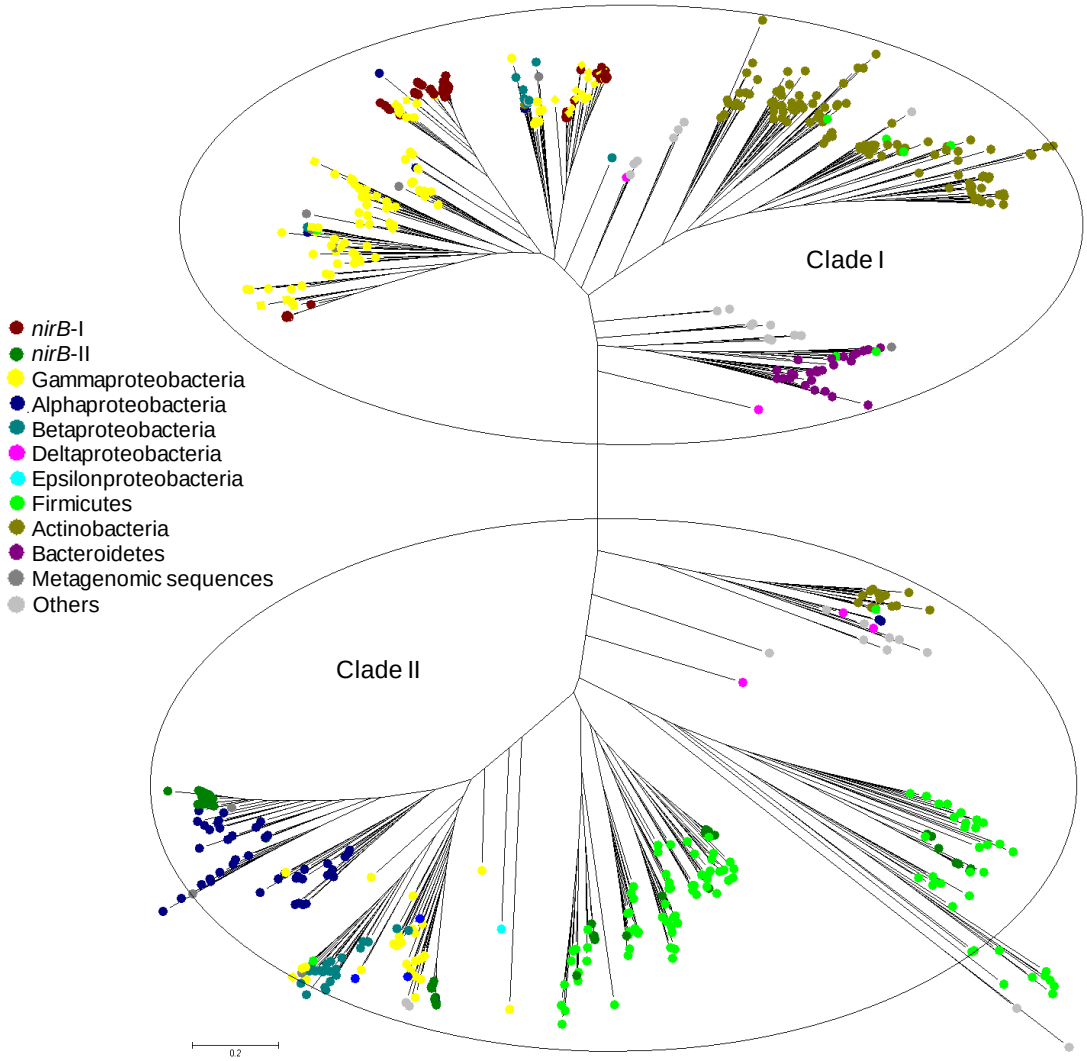


Fig. 5.6 Phylogenetic tree of the clustered NirB amino acid sequences

The search for proteins that share similarity to the NirB enzyme was also conducted using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). This was done to avoid designing primers on the region that shares high similarity with other enzymes, which can lead to a non-specific binding of the primers to a non-target gene. BLAST analysis also revealed four putative conserved domains within the NirB protein, where the first domain was found to share similarity with an enzyme nitric oxide reductase, which also depends on the NADH molecule. Positions for the primer

design were hence selected within the other three domains of NirB to avoid the binding of the primers to non-target sequences.

For primers targeting the *nirB* genes in clade I, motif within the second (BFD-like [2Fe-2S] binding) domain was selected as the position for the primer design. This region contains two highly conserved amino acids cysteine (C) in the motif CX_2C (where X can be any amino acids), which act as binding sites for an enzyme co-factor (Andrews, 1998). According to Rose et al (1998), four highly conserved amino acids are required for designing the degenerate positions on the 3' end of the primers. The consensus positions on the 5' end can be less conserved since the amplification will start from the 3' end side, while the 5' end will be acting like a clamp stabilizing the primer into position. The consensus-degenerate primer is therefore designed to be longer than the conventional primer to allow more base positions to bind to the targets (since mismatches would be unavoidable in the case of consensus positions). In the case of NirB in clade I, a conserved region (V/I)CKP within the second domain was identified and selected as the degenerate positions for the forward primer. The forth (nitrite and sulphite reductase 4Fe-4S) domain, which also contains two highly conserved cysteine CX_3C (Crane et al., 1995), was selected as the region for designing the reverse primer, where a motif CTRE was used as the degenerate positions.

For the NirB in clade II, the motif CX_2C in the second domain was also used for designing the forward primer, where a motif CRPA was selected as the degenerate positions. Region for the reverse primer was also from the conserved cysteine CX_3C in the forth protein domain, where a motif CPRN was used for the degenerate positions. The alignments of the NirB amino acid sequences showing the selected positions for the primer design are as shown in Fig. 5.7.

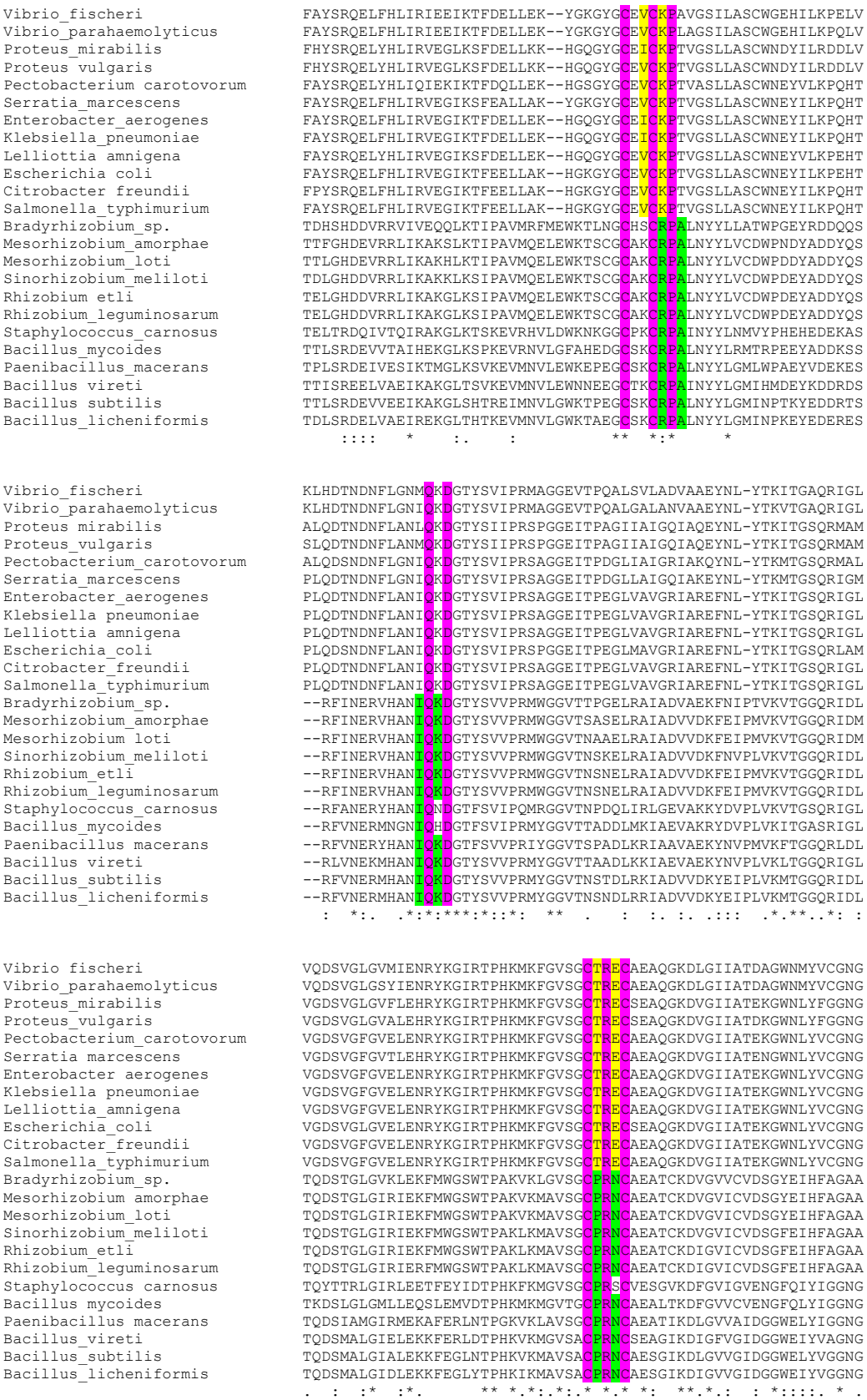


Fig. 5.7 NirB sequence alignment showing the positions selected for the primer design

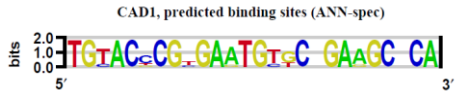
GGCTACGGCTGTGAArtntgyaarcc

Primer: *nirB*-I-1552F

Motif: (V/I)CKP

T_m: 62.1℃

GC content: 53.8%



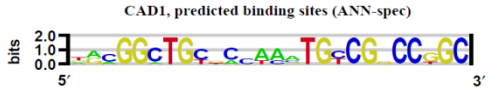
tgyacncgngarTGTGCGGAAGCTCA
TGAGCTTCCGCACAytcncgngtrca

Primer: *nirB*-I-2066R

Motif: CTRE

Tm: 66.1℃

GC content: 57.7%



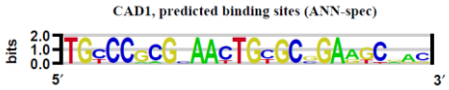
TACGGCTGCGCCAAAtgycgnccngc

Primer: *nirB*-II-1555F

Motif: CRPA

T_m: 70.3℃

GC content: 67.3%



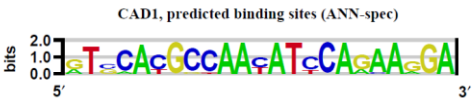
tgyccnmgnaaCTGCGCGGAAGCGAC
GTCGCTTCCGCGCAGttnckнggrca

Primer: *nirB*-II-2063R

Motif: CPRN

T_m: 68.9℃

GC content: 65.4%



GTCCACGCCAACathcarmanga

Primer: *nirB*-II-1651F

Motif: IQKD

T_m: 65.3℃

GC content: 57.1%

Fig. 5.8 The consensus-degenerate primers designed for *nirB* gene of clades I and II, the lower-case letters indicate degenerate positions while the upper-case letters indicate the consensus positions

All *nirB* nucleotide sequences of the known F-DNRA microorganisms (except for *A. globiformis*, in which, although belonging to clade I, still too diverge from other known *nirB*-I sequences to be included in the primer design) were retrieved from the online databases for designing the consensus-degenerate primers. After retrieving, the sequences were clustered using ≥97% similarity to reduce the sequence number and redundancy. Finally, a total of 60 and 62 sequences were used for designing the primers for clades I and II, respectively. The high number of sequences was due to the availability of more than one *nirB* sequence per bacterial species. In addition, these sequences, although belonging to bacteria of the same species, can have similarity of <97% and thus more than one sequence was required for designing a high coverage primer.

The evaluation of *nirB*-I primers, 1552F and 2066R, revealed that the primers could amplify PCR products of the expected sized (512 bp) from all the pure culture strains tested (Fig. 5.9). Nevertheless, since the results from conventional PCR still could not yield clear band for some strain, touchdown PCR was then employed to boost the amount of the PCR products. The PCR thermal steps were as follows, 94°C for 5 min; 20 cycles of 94°C for 30 s, 67°C for 30 s (-0.5°C per cycle), 72°C for 1 min; 20 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min; and a final extension at 72°C for 10 min. It was found, however, that non-specific bands was increased along with the targets. Evaluation of the *nirB*-I primers with clade II pure cultures was also performed to test the specificity of the *nirB*-I primers to their intended targets. It was found that these primers could not amplify the *nirB* of clade II (Fig. 5.10), which confirmed their specificity to the *nirB* of clade I. However, some non-specific bands could still be observed from the amplification. Therefore, the PCR condition will still need to be adjusted for each sample where it may contain different possible non-specific targets to these primers.

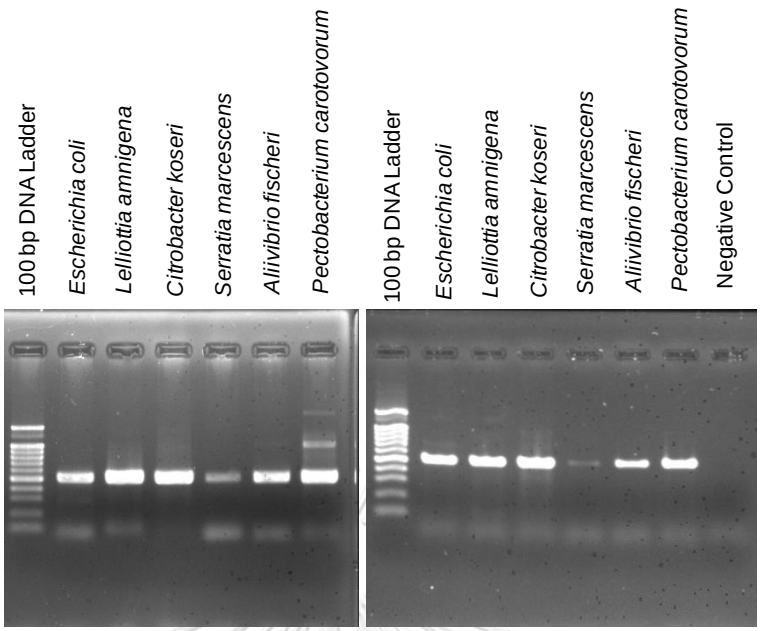


Fig. 5.9 Primer evaluation with pure cultures from clade I, using *nirB*-I primer pair 1552F + 2066R, amplified with touchdown (left) and conventional (right) PCR methods

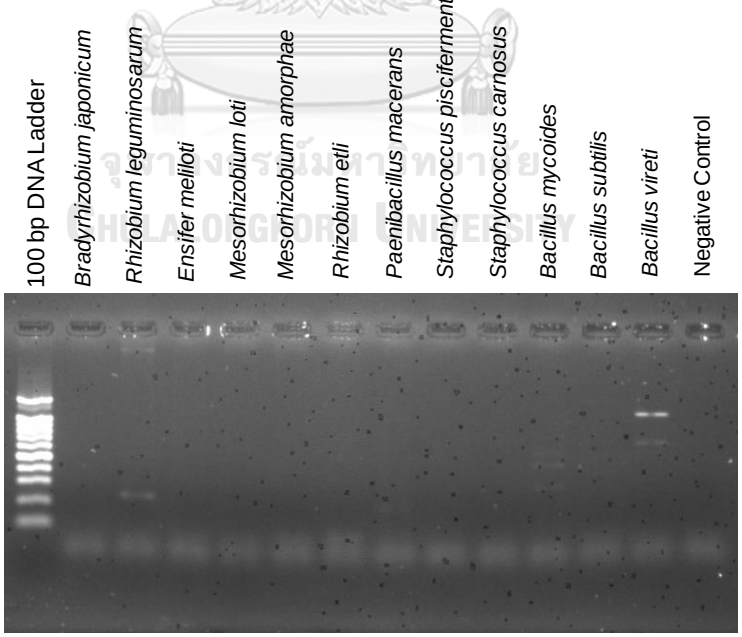


Fig. 5.10 Primer evaluation with pure cultures from clade II, using *nirB*-I primer pair 1552F + 2066R

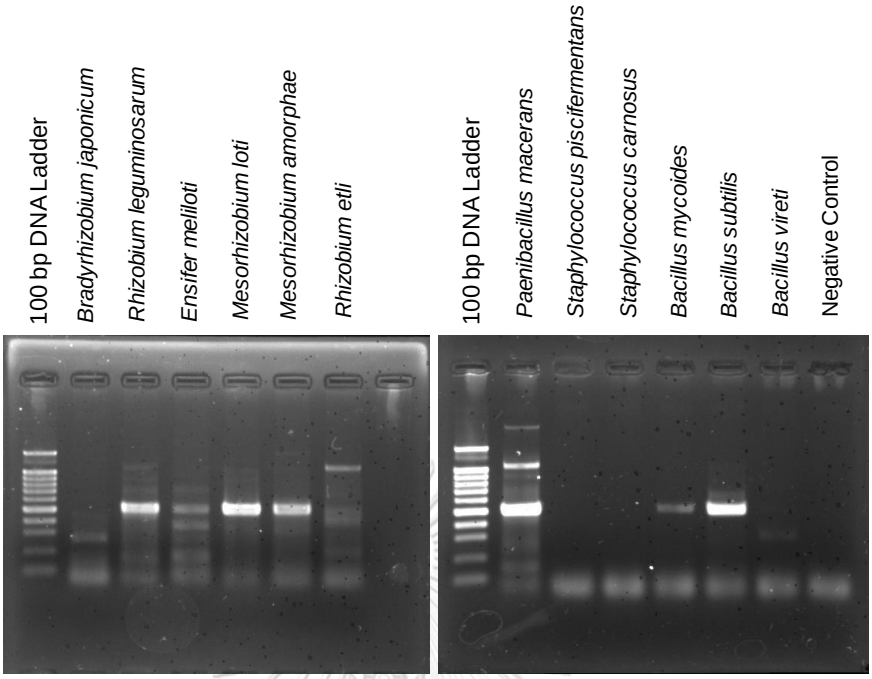


Fig. 5.11 Primer evaluation with pure cultures from clade II, using *nirB*-II primer pair 1555F and 2063R

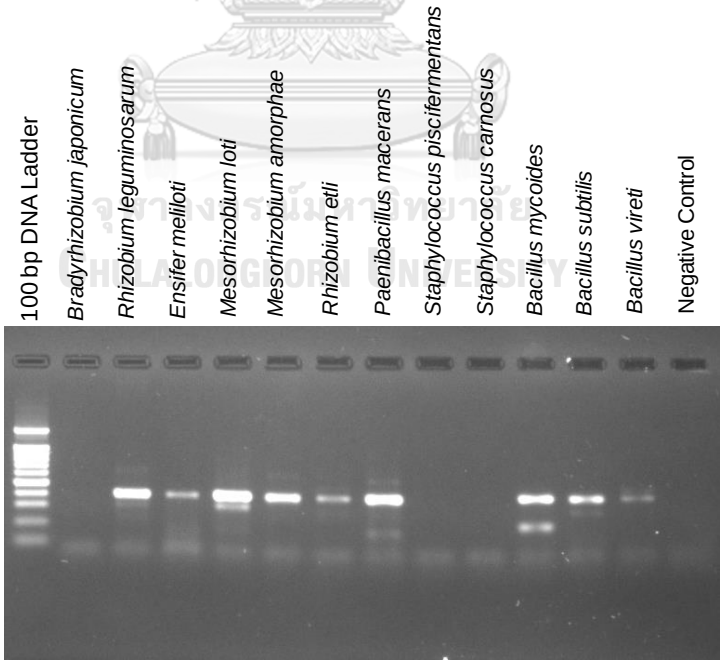


Fig. 5.12 Primer evaluation with pure cultures from clade II, using *nirB*-II primer pair 1651F and 2063R, amplified with conventional PCR method

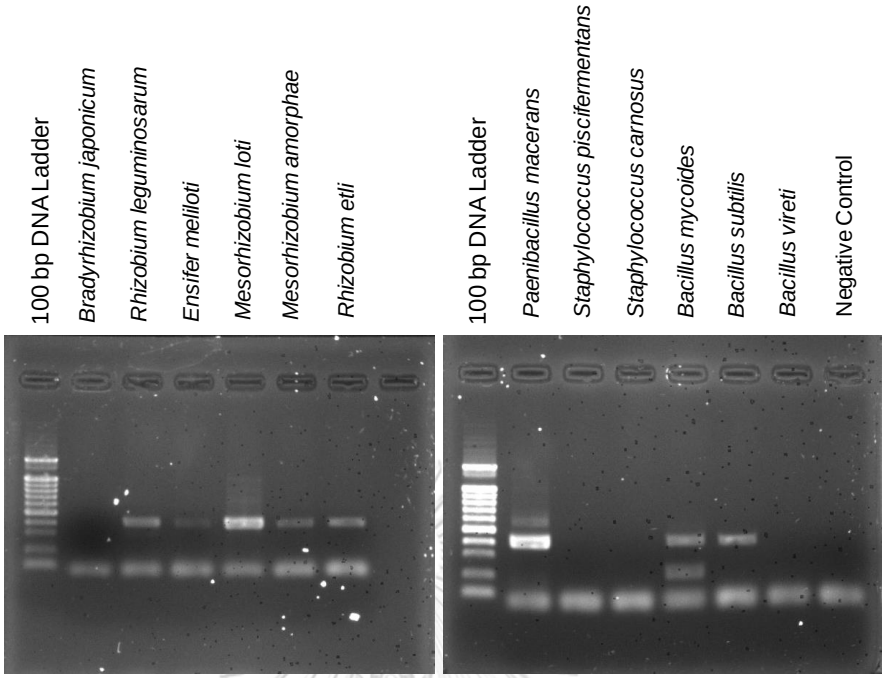


Fig. 5.13 Primer evaluation with pure cultures from clade II, using *nirB*-II primer pair 1651F and 2063R, amplified with touchdown PCR method

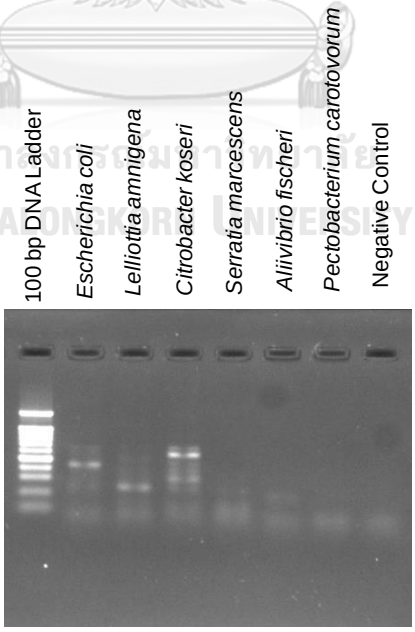


Fig. 5.14 Primer evaluation with pure cultures from clade I, using *nirB*-II primer pair 1651F and 2063R

For the *nirB*-II primers, the combination of 1555F and 2063R pair could not yield satisfactory results for any of the PCR conditions tested (an example is shown in Fig. 5.11). Although designing from highly conserved positions, the PCR results still contained several non-specific bands for some strains and no band at all for others. To overcome this problem, another forward primer, 1651F, was designed and tested for its efficiency. This primer was selected from a motif IQKD in the third protein domain of NirB. Although with unknown function, this motif was chosen based on its conservation throughout the aligned sequences. The results of the 1651F evaluation showed that this primer, combining with 2063R, could amplify 9 out of 12 strains of the clade II pure cultures (Fig. 5.12), which was the best result obtained so far for the *nirB*-II primer set. It is possible that the primers designed might not fully cover the *nirB* sequences of *Bradyrhizobium* and *Staphylococcus*, as these two genera were less closely related to the rest of the Alphaproteobacteria and Firmicutes that used for the primer designed, respectively. Therefore, the consensus positions of the primers would contain higher number of mismatches to the *nirB* genes of these two bacterial genera.

Despite the lower primer coverage than originally expected, 1651F and 2063R were still applied for the detection of clade II F-DNRA microorganisms in this study. Touchdown PCR was first employed in an attempt to reduce non-specific bands during PCR amplification. The results were, however, still not satisfactory since band intensity of many tested strains was still low and the target band of *B. vireti* was absent in any of the touchdown PCR conditions tested (example is as shown in Fig. 5.13). Conventional PCR was hence applied with these primers and the evaluation was continue to the amplification with the clade I strains. The PCR thermal steps were as follows, 94°C for 5 min; 30 cycles of 94°C for 1 min, 63°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. Unfortunately, the *nirB*-II primers seem to be able to amplify the target gene in *E. coli*, while gave non-specific bands to some others (Fig. 5.14). Nevertheless, since this primer pair was still the best one obtained so far in the experiment, it was further applied with samples that might contain F-DNRA microorganisms within their communities.

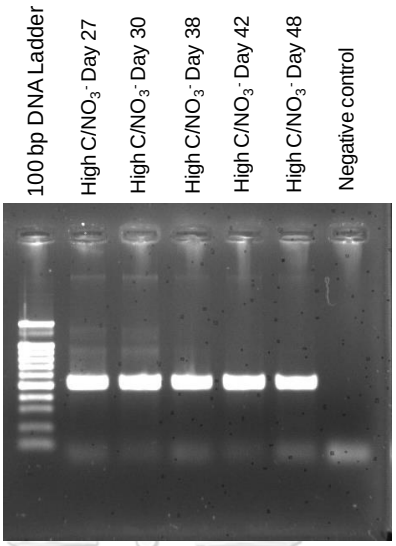


Fig. 5.15 Primer amplification with the high C/NO_3^- enrichment culture DNA samples collected from different days, using *nirB*-I primer pair 1552F + 2066R

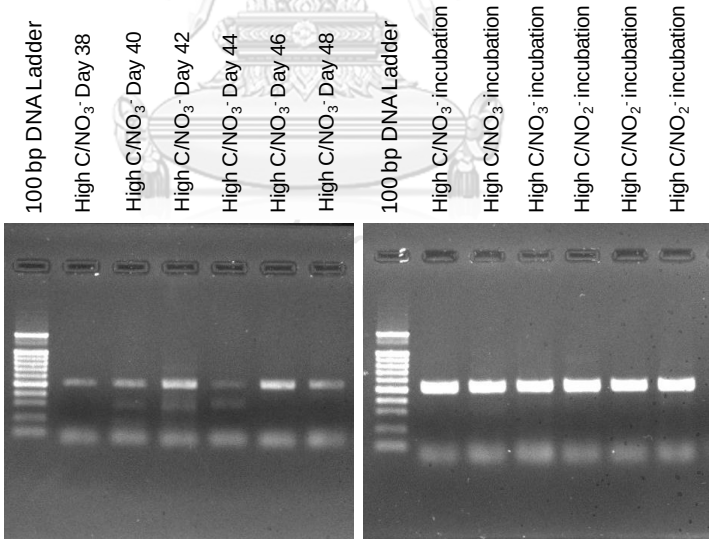


Fig. 5.16 Primer amplification with the high C/NO_3^- enrichment culture cDNA samples collected from different days (left) and from the nitrate/nitrite incubation samples (right), using *nirB*-I primer pair 1552F + 2066R

5.3.2. Detection of the presence and expression of potential F-DNRA microorganisms in environmental samples

For the use of the designed primers with environmental samples, sludge from the low and high C/NO_3^- enrichment cultures of this study (see Chapter 4 for detail), was collected and subjected to PCR amplification with the designed primers from both clades I and II. The cultures were exhibiting active nitrate-reducing activity, with the high C/NO_3^- system performing DNRA process. Therefore, the F-DNRA community might be present in the high C/NO_3^- culture, whereas the low C/NO_3^- samples were also analyzed in comparison and to observe whether *nirB*-containing microorganisms are commonly present in a nitrate-reducing community. Both DNA and RNA samples collected from the enrichment cultures were used in the analysis. Additionally, RNA samples were also collected from incubations of the culture sludge with nitrate and nitrite which were meant to induce the expression of *nirB* in the sludge samples.

The results of the *nirB* gene detection in the high C/NO_3^- culture samples with the *nirB*-I primers 1552F and 2066R revealed that PCR products of the expected size (515 bp) could be observed in all the analyzed DNA samples (days 27, 30, 38, 42, and 48) (Fig. 5.15). The size of the PCR products was in accordance with the results obtained from the pure cultures (bacteria known to contain *nirB* gene in clade I). These PCR products were hence most likely the *nirB* genes of F-DNRA micro-organisms in clade I, which contains members of the class Gammaproteobacteria. The results from the PCR amplification with cDNA (converted from the collected RNA) also revealed the expression of presumably *nirB* gene of clade I in all the RNA samples collected. These samples were taken from the period of peak ammonium formation in the high C/NO_3^- system (days 38, 40, 42, 44, 46, and 48), which suggests that certain part of the detected activity was coming from F-DNRA microorganisms. The cDNA results from the batch experiments also showed clear PCR product bands of the size expected to be the amplified *nirB* genes for both the nitrate and nitrite incubations. This therefore provides an additional evidence for the activity of F-DNRA microorganisms in the high C/NO_3^-

enrichment culture, as nitrate/nitrite assimilation should be suppressed under the presence of high ammonium concentration.

For the inoculum and the low C/NO_3^- sludge samples, PCR products of the expected size were also observed with the *nirB*-I primers 1552F and 2066R (Fig. 5.17). This indicates that potential F-DNRA microorganisms can be present not only in a high C/NO_3^- environment but also in a low C/NO_3^- one. Results from the cDNA amplification, however, revealed no detection of the putative *nirB* expression in the low C/NO_3^- enrichment culture (Fig. 5.18). This then suggests that, although the *nirB*-containing microorganisms were present in the system, there seems to be no gene expression involving F-DNRA activity. Interestingly, expression of the putative *nirB* gene was detected in the batch incubations with both nitrate and nitrite (Fig. 5.18). It hence seems that microorganisms with the *nirB* gene can exist under a low C/NO_3^- condition and are able to perform F-DNRA under certain stimulating conditions. It also seems that nitrate might induce the F-DNRA process better than nitrite. The detection of DNRA activity in batch incubations was in accordance with the results from the stable-isotope experiment with the low C/NO_3^- culture sludge, in that minor DNRA activity was observed in the low C/NO_3^- culture sludge (see section *4.3.2* in Chapter 4 for detail). Certain part of this detected DNRA process thus seems to come from the activity of F-DNRA microorganisms.

For the *nirB*-II primers 1651F and 2063R, PCR amplification also showed bands of the expected size from all the low and high C/NO_3^- culture samples collected, although still with the presence of non-specific bands (Fig. 5.19 and 5.21). The results thus indicate the presence of F-DNRA microorganisms in clade II in both the low and high C/NO_3^- cultures. Fainted bands could also be observed for the PCR amplification with the cDNA samples from the high C/NO_3^- system and from the batch incubations (Fig. 5.20), while no bands of cDNA could be detected in the low C/NO_3^- samples (Fig. 5.22). Judging from the PCR band intensity, it seems that F-DNRA microorganisms in clade II were present in lower level than those detected for clade I. Nonetheless, the lower band intensity could also be a result of a lower coverage of the *nirB*-II primers

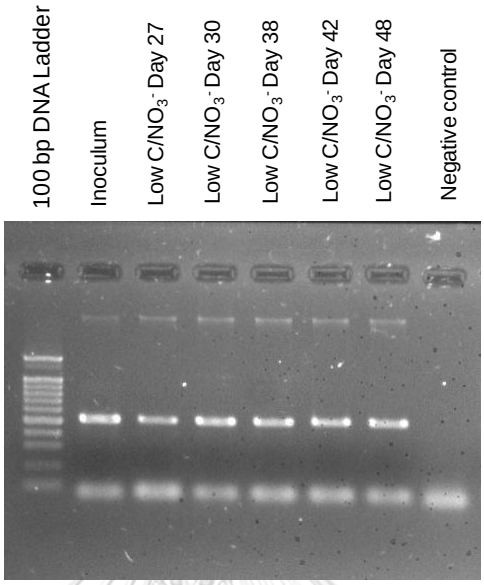


Fig. 5.17 Primer amplification with inoculum and the low C/NO_3^- enrichment culture DNA samples collected from different days, using *nirB*-I primer pair 1552F + 2066R

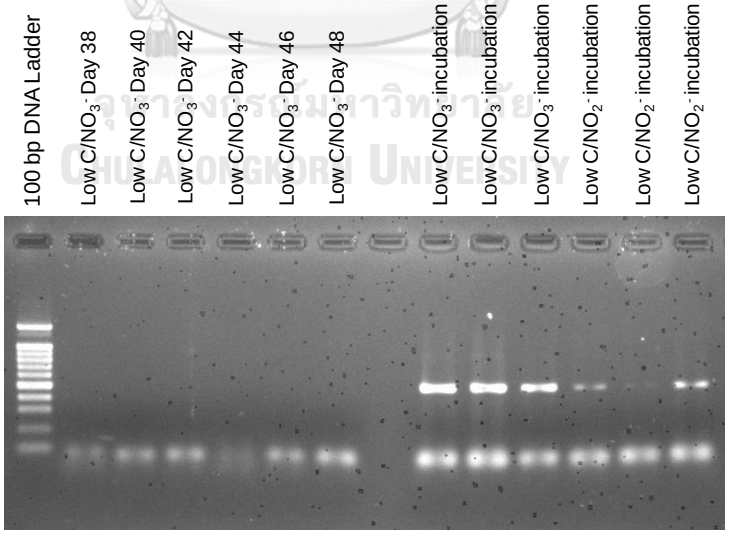


Fig. 5.18 Primer amplification with the low C/NO_3^- enrichment culture cDNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-I primer pair 1552F + 2066R

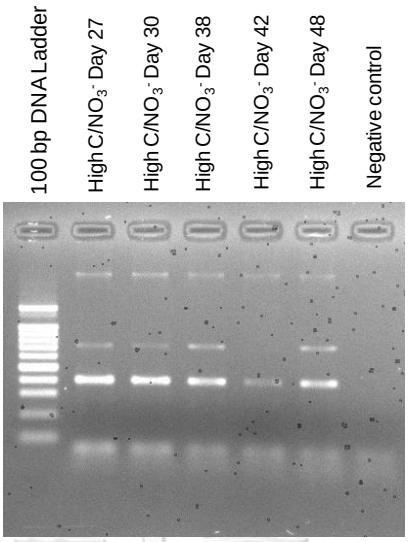


Fig. 5.19 Primer amplification with the high C/NO_3^- enrichment culture DNA samples collected from different days, using *nirB*-II primer pairs 1651F and 2063R

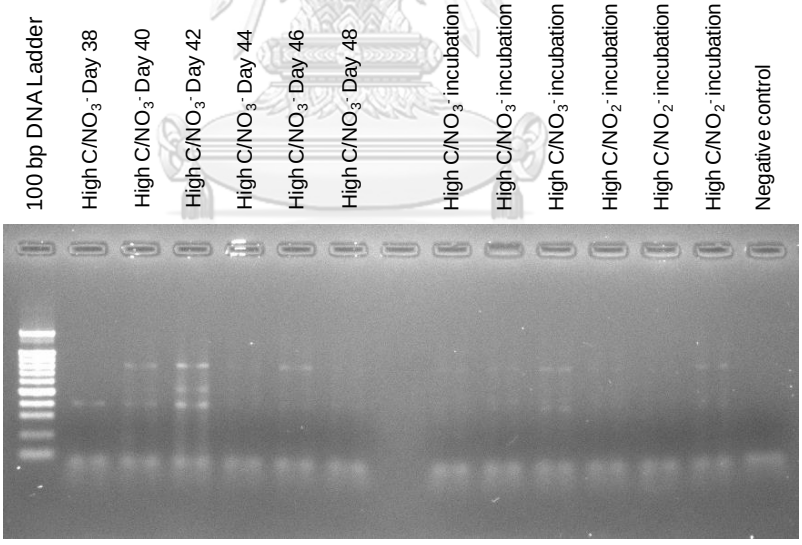


Fig. 5.20 Primer amplification with the high C/NO_3^- enrichment culture cDNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-II primer pairs 1651F and 2063R

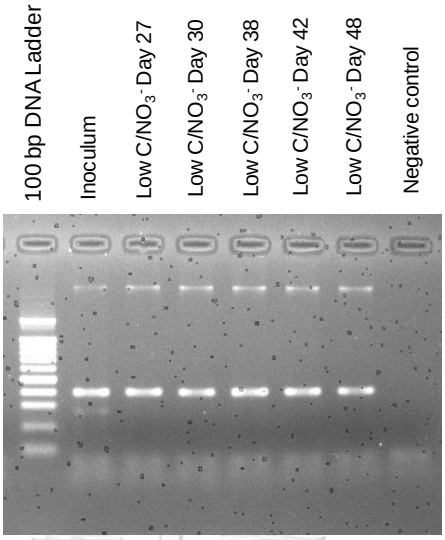


Fig. 5.21 Primer amplification with the low C/NO_3^- enrichment culture DNA samples collected from different days, using *nirB*-II primer pairs 1651F and 2063R

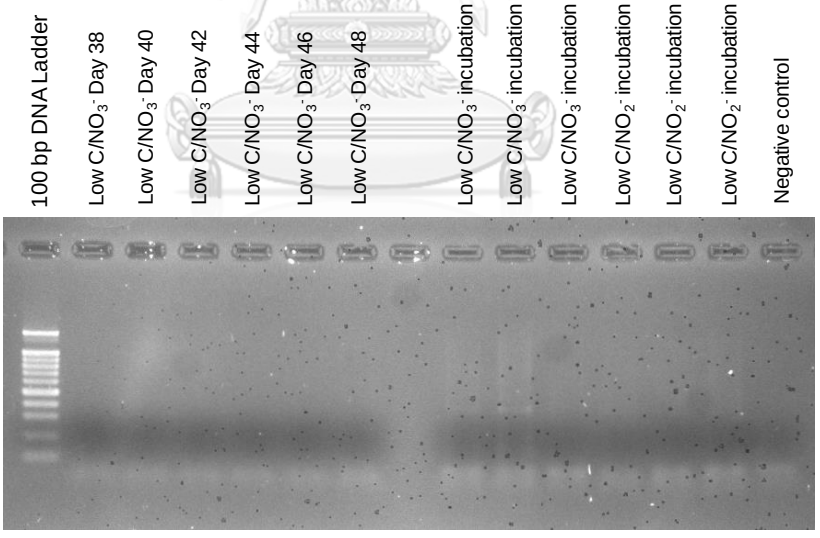


Fig. 5.22 Primer amplification with the low C/NO_3^- enrichment culture cDNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-II primer pairs 1651F and 2063R

Table 5.3 Percentage of *nirB* primer coverage with the *nirB* sequences other than those used for the primer design

Taxa	Percent coverage		Total number of clustered sequences used
	nirB-I	*nirB*-II	
Actinobacteria	-	-	91
Alphaproteobacteria	2.0	76.8	254
Bacillales	-	39.6	48
Bacteria	8.0	13.8	87
Bacteroidetes	1.1	-	176
Betaproteobacteria	-	23.0	61
Deltaproteobacteria	-	25.0	16
Enterobacterales	57.4	-	68
Epsilonproteobacteria	-	25.0	4
Firmicutes	-	5.3	57
Gammaproteobacteria	43.6	16.0	94
Proteobacteria	-	46.7	15
Rhizobiales	-	78.0	205
Vibrio sp.	55.3	-	38

to the presenting clade II F-DNRA microorganisms in the samples. This issue hence still remains to be verified in the future study.

The results of the *nirB*-I and *nirB*-II primers tested with the *in silico* FastPCR program using *nirB* sequences other than those applied for the primer design are as shown in Table 5.3. The sequences were clustered using ≥97% similarity (with the online program CD-HITS) and a representative *nirB* was selected for the *in silico* evaluation. The analysis revealed that the *nirB*-II primers have higher coverage to the other *nirB* sequences than those detected by the *nirB*-I primers. On the other hand, the *nirB*-I primers are more specific to *nirB* sequences of Gammaproteobacteria (including Enterobacterales and *Vibrio* sp.). Nevertheless, these obtained results are solely based on available *nirB* sequences in the online databases. The coverage of the

nirB primers to *nirB*-containing microorganisms in environmental samples is thus still a subject for further investigations, where collaborative works are needed to fulfill the gap in knowledge regarding the F-DNRA microorganisms.

5.4. Conclusions

In an attempt to study the presence and activity of F-DNRA microorganisms in environmental samples, primers targeting their marker gene, *nirB*, were developed and evaluated to test the primers' coverage and specificity. However, due to the high variation within the *nirB* sequences, the task has proven to be challenging and several trials have been made to improve the designed *nirB* primers. Eventually, a combination between consensus and degenerate primers was applied to obtain primers with less degree of degeneracy and still contain high coverage to the targets. The primers were designed specifically for each *nirB* clade as the *nirB* genes were found to be too diverged to be covered by a single pair of primers. PCR amplification with DNA samples from the low and high C/NO_3^- enrichment cultures revealed potential candidates for F-DNRA microorganisms in clade I. Furthermore, detection of the *nirB* gene expression by amplifying the cDNA samples showed activity of potential F-DNRA microorganisms in the high C/NO_3^- culture as well as in the high C/NO_3^- sludge incubations with both nitrate and nitrite. It hence seems that the F-DNRA microorganisms belonging to clade I were taking active part in the ammonium formation in the high C/NO_3^- enrichment culture. Nonetheless, additional information on the PCR products detected from both clades is still needed in order to confirm the identity of the amplified sequences and to determine the diversity of these potential F-DNRA microorganisms.

CHAPTER 6
CONCLUSIONS AND SUGGESTIONS

6.1. Conclusions of the research

By using biological nitrogen removal processes in the low and high C/NO_3^- aquaculture systems for studying the presence of DNRA, it was found that DNRA activity could be observed in both the low and high C/NO_3^- aquaculture sludge incubations. The ammonium-forming activity in the low C/NO_3^- sludge most likely came from the R-DNRA microorganisms, whereas the ammonium production in the high C/NO_3^- sludge was presumably responsible by the F-DNRA microorganisms.

Partitioning in the nitrate reduction pathways could be observed when different C/NO_3^- ratios were applied to enrichment cultures inoculated with activated sludge. The lower C/NO_3^- ratio led to the presence of denitrification, whereas the higher C/NO_3^- ratio resulted in the activity of DNRA. The occurrence of DNRA was proved by the method of stable-isotope tracers using $^{15}NO_3^-$ and $^{15}NO_2^-$, where $^{15}NH_4^+$ could be detected as the major product of both the $^{15}NO_3^-$ and $^{15}NO_2^-$ incubations.

Microbial community analysis with Illumina MiSeq 16S rRNA method revealed distinctive populations in the DNRA-dominating enrichment culture, where marked difference in microbial compositions could be observed compared to the denitrifying culture and inoculum. Dominant microbial populations were found to be related to *Sulfurospirillum* and the members of Lachnospiraceae family. The high abundances of these two bacterial taxa suggest their major role in the DNRA process.

Primers designed for the detection of *nirB*, the marker gene for F-DNRA micro-organisms, revealed the presence of potential candidates for this microbial group in both the low and high C/NO_3^- enrichment cultures. The expression of putative *nirB* genes in clade I was also detected in the high C/NO_3^- system as well as in the high C/NO_3^- sludge incubations with nitrate and nitrite. It therefore seems that F-DNRA

microorganisms in clade I were taking active role in the nitrogen conversions in the high C/NO_3^- enrichment culture, whereas the clade II type seems to be present in lower level and seemingly taking less active part in this ecosystem.

6.2. Suggestions

Since the study on DNRA is still limited to a certain extent, many aspects of the process thus still need further investigation in order to understand the competition between DNRA and denitrification. Among the controlling factors of the process, kinetic values of DNRA are hardly known, with only limited research examining on this topic. The author therefore encourages the study on kinetic parameters of both F-DNRA and R-DNRA pathways, so as to further extend insight into the mechanisms governing this process of nitrate reduction.

The detection of potential F-DNRA microorganisms using PCR amplification with the newly designed *nirB* primers has proven to be a challenging task due to the diverged nature of the target gene. And since the primers were designed based on only the known F-DNRA microorganisms, the coverage of the primers when applying to an environmental sample is therefore still in question. Nevertheless, the designed primers would allow the detection of possibly unknown F-DNRA microorganisms in the environment. Further detection and identification of this microbial group hence will allow adjustment to be made to the primers in order to expand their coverage to more F-DNRA microorganisms in the future.

REFERENCES

Akunna, J.C., Bizeau, C., Moletta, R. 1993. Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. Water Research 27, 1303-1312.

Akunna, J.C., Bizeau, C., Moletta, R. 1994. Nitrate reduction by anaerobic sludge using glucose at various nitrate concentrations: ammonification, denitrification and methanogenic activities. Environmental Technology 15, 41-49.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. 1990. Basic local alignment search tool. Journal of Molecular Biology 215, 403-410.

Andrews, S.C. 1998. Iron storage in bacteria, pp. 281-351.

APHA. 2012. *Standard Methods for the Examination of Water and Wastewater*. *20th ed*, Washington DC, USA.

Barber, W.P., Stuckey, D.C. 2000. Nitrogen removal in a modified anaerobic baffled reactor (ABR): 1, denitrification. Water Research 34, 2413-2422.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L.L., Studholme, D.J., Yeats, C., Eddy, S.R. 2004. The Pfam protein families database. Nucleic Acids Research 32, D138-D141.

Behrendt, A., Tarre, S., Beliavski, M., Green, M., Klatt, J., de Beer, D., Stief, P. 2014. Effect of high electron donor supply on dissimilatory nitrate reduction pathways in a bioreactor for nitrate removal. Bioresource Technology 171, 291-7.

Behrendt, U., Augustin, J., Spröer, C., Gelbrecht, J., Schumann, P., Ulrich, A. 2015. Taxonomic characterisation of *Proteus terrae* sp. nov., a N_2O-producing, nitrate-ammonifying soil bacterium. Antonie van Leeuwenhoek 108, 1457-1468.

Betlach, M.R., Tiedje, J.M. 1981. Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. Appl Environ Microbiol 42, 1074-84.

Bleakley, B.H., Tiedje, J.M. 1982. Nitrous oxide production by organisms other than nitrifiers or denitrifiers. Applied and Environmental Microbiology 44, 1342-8.

Bonin, P. 1996. Anaerobic nitrate reduction to ammonium in two strains isolated from coastal marine sediment: a dissimilatory pathway. FEMS Microbiology Ecology 19, 27-38.

Bower, C.E., Holm-Hansen, T. 1980. A Salicylate–hypochlorite method for determining ammonia in seawater. Canadian Journal of Fisheries and Aquatic Sciences 37, 794-798.

Brunet, R.C., Garcia-Gil, L.J. 1996. Sulfide-induced dissimilatory nitrate reduction to ammonia in anaerobic freshwater sediments. FEMS Microbiology Ecology 21, 131-138.

Burgin, A.J., Hamilton, S.K. 2007. Have we overemphasized the role of denitrification in aquatic ecosystems? A review of nitrate removal pathways. Frontiers in Ecology and the Environment 5, 89-96.

Byrne, M.D., Nicholas, D.J.D. 1987. A membrane-bound dissimilatory nitrate reductase from *Rhodobacter sphaeroides* f.sp. denitrificans. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 915, 120-124.

Caldwell, M.E., Allen, T.D., Lawson, P.A., Tanner, R.S. 2011. *Tolumonas osonensis* sp. nov., isolated from anoxic freshwater sediment, and emended description of the genus *Tolumonas*. International Journal of Systematic and Evolutionary Microbiology 61, 2659-63.

Camargo, J.A., Alonso, A., Salamanca, A. 2005. Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58, 1255-1267.

Casciotti, K.L., Sigman, D.M., Hastings, M.G., Böhlke, J.K., Hilkert, A. 2002. Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. Analytical Chemistry 74, 4905-4912.

Christensen, P.B., Rysgaard, S.Â.r., Sloth, N.P., Dalsgaard, T., Schwarter, S. 2000. Sediment mineralization, nutrient fluxes, denitrification and dissimilatory nitrate reduction to ammonium in an estuarine fjord with sea cage trout farms. Aquatic Microbial Ecology 21, 73-84.

Cline, J.D. 1969. Spectrophotometric determination of hydrogen sulfide in natural water. Limnology and Oceanography 14, 454-458.

Coates, J.D., Chakraborty, R., Lack, J.G., O'Connor, S.M., Cole, K.A., Bender, K.S., Achenbach, L.A. 2001. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature 411, 1039-1043.

Cole, J. 1996. Nitrate reduction to ammonia by enteric bacteria: redundancy, or a strategy for survival during oxygen starvation? FEMS Microbiology Letters 136, 1-11.

Cole, J.A. 1978. The rapid accumulation of large quantities of ammonia during nitrite reduction by *Escherichia coli*. FEMS Microbiology Letters 4, 327-329.

Cole, J.A., Brown, C.M. 1980. Nitrite reduction to ammonia by fermentative bacteria: a short circuit in the biological nitrogen cycle. FEMS Microbiology Letters 7, 65-72.

Cole, J.A., Newman, B.M., White, P. 1980. Biochemical and genetic characterization of *nirB* mutants of *Escherichia coli* K12 pleiotropically defective in nitrite and sulphite reduction. Microbiology 120, 475-483.

Corker, H., Poole, R.K. 2003. Nitric oxide formation by *Escherichia coli*. Dependence on nitrite reductase, the NO-sensing regulator Fnr, and flavohemoglobin Hmp. The Journal of Biological Chemistry 278, 31584-92.

Cotta, M., Forster, R. 2006. The family Lachnospiraceae, including the genera *Butyrivibrio*, *Lachnospira* and *Roseburia*. in: *The Prokaryotes*, pp. 1002-1021.

Crab, R., Defoirdt, T., Bossier, P., Verstraete, W. 2012. Biofloc technology in aquaculture: beneficial effects and future challenges. Aquaculture 356-357, 351-356.

Crane, B.R., Siegel, L.M., Getzoff, E.D. 1995. Sulfite reductase structure at 1.6 AA: evolution and catalysis for reduction of inorganic anions. Science 270, 59-67.

Cytryn, E., Gelfand, I., Barak, Y., Van Rijn, J., Minz, D. 2003. Diversity of microbial communities correlated to physiochemical parameters in a digestion basin of a zero-discharge mariculture system. Environmental Microbiology 5, 55-63.

Dalsgaard, T., Bak, F. 1994. Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil: sulfide inhibition, kinetics, and regulation. Applied and Environmental Microbiology 60, 291-7.

Dannenberg, S., Kroder, M., Dilling, W., Cypionka, H. 1992. Oxidation of H_2, organic compounds and inorganic sulfur compounds coupled to reduction of O_2 or nitrate by sulfate-reducing bacteria. Archives of Microbiology 158, 93-99.

Dodsworth, J.A., Hungate, B.A., Hedlund, B.P. 2011. Ammonia oxidation, denitrification and dissimilatory nitrate reduction to ammonium in two US Great Basin hot springs with abundant ammonia-oxidizing archaea. Environmental Microbiology 13, 2371-2386.

Dong, L.F., Smith, C.J., Papaspyrou, S., Stott, A., Osborn, A.M., Nedwell, D.B. 2009. Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne estuary, United Kingdom). Applied and Environmental Microbiology 75, 3171-9.

Dong, L.F., Sobey, M.N., Smith, C.J., Rusmana, I., Phillips, W., Stott, A., Osborn, A.M., Nedwell, D.B. 2011. Dissimilatory reduction of nitrate to ammonium, not denitrification or anammox, dominates benthic nitrate reduction in tropical estuaries. Limnology and Oceanography 56, 279-291.

du Preez, L.A., Maree, J.P. 1994. Pilot-scale biological sulphate and nitrate removal utilizing producer gas as energy source. Water Science and Technology 30, 275-285.

Dunn, G.M., Herbert, R.A., Brown, C.M. 1979. Influence of oxygen tension on nitrate reduction by a *Klebsiella* sp. growing in chemostat culture. Microbiology 112, 379-383.

Einsle, O., Messerschmidt, A., Huber, R., Kroneck, P.M.H., Neese, F. 2002. Mechanism of the six-electron reduction of nitrite to ammonia by cytochrome c nitrite reductase. Journal of the American Chemical Society 124, 11737-11745.

Eschbach, M., Möbitz, H., Rompf, A., Jahn, D. 2003. Members of the genus *Arthrobacter* grow anaerobically using nitrate ammonification and

fermentative processes: anaerobic adaptation of aerobic bacteria abundant in soil. FEMS Microbiology Letters 223, 227-230.

Fischer-Romero, C., Tindall, B.J., Juttner, F. 1996. *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. International Journal of Systematic and Evolutionary Microbiology 46, 183-188.

Flanagan, D.A., Gregory, L.G., Carter, J.P., Karakas-Sen, A., Richardson, D.J., Spiro, S. 1999. Detection of genes for periplasmic nitrate reductase in nitrate respiring bacteria and in community DNA. FEMS Microbiology Letters 177, 263-270.

Fujimoto, S., Mizuno, R., Saito, Y., Nakamura, S. 2004. Clinical application of wave intensity for the treatment of essential hypertension. Heart Vessels 19, 19-22.

Ginige, M.P., Keller, J., Blackall, L.L. 2005. Investigation of an acetate-fed denitrifying microbial community by stable isotope probing, full-cycle rRNA analysis, and fluorescent in situ hybridization-microautoradiography. Applied and Environmental Microbiology 71, 8683-91.

Greene, E.A., Hubert, C., Nemati, M., Jenneman, G.E., Voordouw, G. 2003. Nitrite reductase activity of sulphate-reducing bacteria prevents their inhibition by nitrate-reducing, sulphide-oxidizing bacteria. Environmental Microbiology 5, 607-617.

Greer, F.R., Shannon, M. 2005. Infant methemoglobinemia: the role of dietary nitrate in food and water. Pediatrics 116, 784-786.

Hanke, A., Hamann, E., Sharma, R., Geelhoed, J.S., Hargesheimer, T., Kraft, B., Meyer, V., Lenk, S., Osmers, H., Wu, R., Makinwa, K., Hettich, R.L., Banfield, J.F., Tegetmeyer, H.E., Strous, M. 2014. Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between Alpha- and Gammaproteobacteria in a tidal sediment microbial community naturally selected in a laboratory chemostat. Frontiers in Microbiology 5, 231.

Harborne, N.R., Griffiths, L., Busby, S.J.W., Cole, J.A. 1992. Transcriptional control, translation and function of the products of the five open reading frames of the *Escherichia coli* nir operon. Molecular Microbiology 6, 2805-2813.

Helen, D., Kim, H., Tytgat, B., Anne, W. 2016. Highly diverse *nirK* genes comprise two major clades that harbour ammonium-producing denitrifiers. BMC Genomics 17, 155.

Hoffmann, T., Frankenberg, N., Marino, M., Jahn, D. 1998. Ammonification in *Bacillus subtilis* utilizing dissimilatory nitrite reductase is dependent on resDE. Journal of Bacteriology 180, 186-9.

Huang, Y., Niu, B., Gao, Y., Fu, L., Li, W. 2010. CD-HIT Suite: a web server for clustering and comparing biological sequences. Bioinformatics 26, 680-682.

Hubert, C., Voordouw, G. 2007. Oil field souring control by nitrate-reducing *Sulfurospirillum* spp. that outcompete sulfate-reducing bacteria for organic electron donors. Applied and Environmental Microbiology 73, 2644-52.

Ishii, S., Ashida, N., Ohno, H., Segawa, T., Yabe, S., Otsuka, S., Yokota, A., Senoo, K. 2017. *Noviherbaspirillum denitrificans* sp. nov., a denitrifying bacterium isolated from rice paddy soil and *Noviherbaspirillum autotrophicum* sp. nov., a denitrifying, facultatively autotrophic bacterium isolated from rice paddy soil and proposal to reclassify *Herbaspirillum massiliense* as *Noviherbaspirillum massiliense* comb. nov. International Journal of Systematic and Evolutionary Microbiology 67, 1841-1848.

Isobe, K., Suwa, Y., Ikutani, J., Kuroiwa, M., Makita, T., Takebayashi, Y., Yoh, M., Otsuka, S., Senoo, K., Ohmori, M., Koba, K. 2011. Analytical techniques for quantifying $^{15}N/^{14}N$ of nitrate, nitrite, total dissolved nitrogen and ammonium in environmental samples using a gas chromatograph equipped with a quadrupole mass spectrometer. Microbes and Environments 26, 46-53.

Jørgensen, B.B., Zawacki, L.X., Jannasch, H.W. 1990. Thermophilic bacterial sulfate reduction in deep-sea sediments at the Guaymas Basin hydrothermal vent site (Gulf of California). Deep Sea Research Part A. Oceanographic Research Papers 37, 695-710.

Kalendar, R., Lee, D., Schulman, A.H. 2014. FastPCR software for PCR, in silico PCR, and oligonucleotide assembly and analysis. in: *DNA Cloning and Assembly Methods*, (Eds.) S. Valla, R. Lale, Humana Press. Totowa, NJ, pp. 271-302.

Kämpfer, P., Lodders, N., Falsen, E. 2011. *Hydrotalea flava*gen. nov., sp. nov., a new member of the phylum Bacteroidetes and allocation of the genera *Chitinophaga, Sediminibacterium, Lacibacter, Flavihumibacter, Flavisolibacter, Niabella, Niastella, Segetibacter, Parasegetibacter, Terrimonas, Ferruginibacter, Filimonas and Hydrotalea* to the family Chitinophagaceae fam. nov. International Journal of Systematic and Evolutionary Microbiology 61, 518-523.

Kantor, R.S., Huddy, R.J., Iyer, R., Thomas, B.C., Brown, C.T., Anantharaman, K., Tringe, S., Hettich, R.L., Harrison, S.T., Banfield, J.F. 2017. Genome-resolved meta-omics ties microbial dynamics to process performance in biotechnology for thiocyanate degradation. Environmental Science & Technology 51, 2944-2953.

Kaspar, H.F., Tiedje, J.M. 1981. Dissimilatory reduction of nitrate and nitrite in the bovine rumen: nitrous oxide production and effect of acetylene. Applied and Environmental Microbiology 41, 705-9.

Kraft, B., Strous, M., Tegetmeyer, H.E. 2011. Microbial nitrate respiration--genes, enzymes and environmental distribution. Journal of Biotechnology 155, 104-17.

Kraft, B., Tegetmeyer, H.E., Sharma, R., Klotz, M.G., Ferdelman, T.G., Hettich, R.L., Geelhoed, J.S., Strous, M. 2014. The environmental controls that govern the end product of bacterial nitrate respiration. Science 345, 676-679.

Lahav, O., Massada, I.B., Yackoubov, D., Zelikson, R., Mozes, N., Tal, Y., Tarre, S. 2009. Quantification of anammox activity in a denitrification reactor for a recirculating aquaculture system. Aquaculture 288, 76-82.

Lam, P., Lavik, G., Jensen, M.M., van de Vossenberg, J., Schmid, M., Woebken, D., Gutiérrez, D., Amann, R., Jetten, M.S.M., Kuypers, M.M.M. 2009. Revising the nitrogen cycle in the Peruvian oxygen minimum zone. Proceedings of the National Academy of Sciences 106, 4752-4757.

Li, L., Kato, C., Horikoshi, K. 1999. Bacterial diversity in deep-sea sediments from different depths. Biodiversity & Conservation 8, 659-677.

Liu, M.-C., Liu, M.-Y., Payne, W.J., Peck, H.D., LeGall, J. 1983. *Wolinella succinogenes* nitrite reductase: purification and properties. FEMS Microbiology Letters 19, 201-206.

Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A., Goodwin, S. 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Archives of Microbiology 159, 336-344.

Luijten, M.L., de Weert, J., Smidt, H., Boschker, H.T., de Vos, W.M., Schraa, G., Stams, A.J. 2003. Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. International Journal of Systematic and Evolutionary Microbiology 53, 787-93.

Macfarlane, G.T., Herbert, R.A. 1982. Nitrate dissimilation by *Vibrio* spp. isolated from estuarine sediments. Microbiology 128, 2463-2468.

McCready, R.G.L., Gould, W.D., Cook, F.D. 1983. Respiratory nitrate reduction by *Desulfovibrio* sp. Archives of Microbiology 135, 182-185.

Meehan, C.J., Beiko, R.G. 2014. A phylogenomic view of ecological specialization in the Lachnospiraceae, a family of digestive tract-associated bacteria. Genome Biology and Evolution 6, 703-13.

Miller, C.S., Handley, K.M., Wrighton, K.C., Frischkorn, K.R., Thomas, B.C., Banfield, J.F. 2013. Short-read assembly of full-length 16S amplicons reveals bacterial diversity in subsurface sediments. PLoS One 8, e56018.

Mohan, S.B., Cole, J. 2007. *Biology of the nitrogen cycle*, Amsterdam : Elsevier.

Mohan, S.B., Schmid, M., Jetten, M., Cole, J. 2004. Detection and widespread distribution of the *nrfA* gene encoding nitrite reduction to ammonia, a short circuit in the biological nitrogen cycle that competes with denitrification. FEMS Microbiology Ecology 49, 433-43.

Moreno-Vivian, C., Cabello, P., Martinez-Luque, M., Blasco, R., Castillo, F. 1999. Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. Journal of Bacteriology 181, 6573-84.

Moreno-Vivián, C., Ferguson, S.J. 1998. Definition and distinction between assimilatory, dissimilatory and respiratory pathways. Molecular Microbiology 29, 664-666.

Nakano, M.M., Hoffmann, T., Zhu, Y., Jahn, D. 1998. Nitrogen and oxygen regulation of *Bacillus subtilis nasDEF* encoding NADH-dependent nitrite reductase by TnrA and ResDE. Journal of Bacteriology 180, 5344-50.

Page, L., Griffiths, L., Cole, J.A. 1990. Different physiological roles of two independent pathways for nitrite reduction to ammonia by enteric bacteria. Archives of Microbiology 154, 349-354.

Polcyn, W., Podeszwa, J. 2009. Coordinate induction of dissimilatory ammonification and fermentative pathways in rhizobia. Antonie van Leeuwenhoek 96, 79-87.

Prakash, O., Sadana, J.C. 1973. Metabolism of nitrate in *Achromobacter fischeri*. Canadian Journal of Microbiology 19, 15-25.

Qiu, Y.L., Kuang, X.Z., Shi, X.S., Yuan, X.Z., Guo, R.B. 2014. *Paludibacter jiangxiensis* sp. nov., a strictly anaerobic, propionate-producing bacterium isolated from rice paddy field. Archives of Microbiology 196, 149-55.

Randall, D.J., Tsui, T.K.N. 2002. Ammonia toxicity in fish. Marine Pollution Bulletin 45, 17-23.

Rehr, B., Klemme, J.-H. 1989. Competition for nitrate between denitrifying *Pseudomonas stutzeri* and nitrate ammonifying enterobacteria. FEMS Microbiology Letters 62, 51-57.

Robertson, E.K., Roberts, K.L., Burdorf, L.D.W., Cook, P., Thamdrup, B. 2016. Dissimilatory nitrate reduction to ammonium coupled to Fe(II) oxidation in sediments of a periodically hypoxic estuary. Limnology and Oceanography 61, 365-381.

Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M., Henikoff, S. 1998. Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. Nucleic Acids Research 26, 1628-1635.

Schreier, H.J., Mirzoyan, N., Saito, K. 2010. Microbial diversity of biological filters in recirculating aquaculture systems. Current Opinion in Biotechnology 21, 318-325.

Schumacher, W., Hole, U., Kroneck, M.H. 1994. Ammonia-forming cytochrome c nitrite reductase from *Sulfurospirillum deleyianum* Is a tetraheme protein: new aspects of the molecular composition and spectroscopic properties. Biochemical and Biophysical Research Communications 205, 911-916.

Seitz, H.-J., Cypionka, H. 1986. Chemolithotrophic growth of *Desulfovibrio desulfuricans* with hydrogen coupled to ammonification of nitrate or nitrite. Archives of Microbiology 146, 63-67.

Senga, Y., Mochida, K., Fukumori, R., Okamoto, N., Seike, Y. 2006. N_2O accumulation in estuarine and coastal sediments: The influence of H_2S on dissimilatory nitrate reduction. Estuarine, Coastal and Shelf Science 67, 231-238.

Shao, M.-F., Zhang, T., Fang, H.H.-P., Li, X. 2011. The effect of nitrate concentration on sulfide-driven autotrophic denitrification in marine sediment. Chemosphere 83, 1-6.

Sher, Y., Schneider, K., Schwermer, C.U., van Rijn, J. 2008. Sulfide-induced nitrate reduction in the sludge of an anaerobic digester of a zero-discharge recirculating mariculture system. Water Research 42, 4386-92.

Silver, W.L., Herman, D.J., Firestone, M.K. 2001. Dissimilatory nitrate reduction to ammonium in upland tropical forest soils. Ecology 82, 2410-2416.

Silver, W.L., Thompson, A.W., Reich, A., Ewel, J.J., Firestone, M.K. 2005. Nitrogen cycling in tropical plantation forests: potential controls on nitrogen retention. Ecological Applications 15, 1604-1614.

Smith, C.J., Nedwell, D.B., Dong, L.F., Osborn, A.M. 2007. Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. Applied and Environmental Microbiology 73, 3612-22.

Smith, M.S. 1982. Dissimilatory reduction of NO_2^- to NH_4^+ and N_2O by a soil *Citrobacter* sp. Applied and Environmental Microbiology 43, 854-60.

Smith, M.S. 1983. Nitrous oxide production by *Escherichia coli* is correlated with nitrate reductase activity. Applied and Environmental Microbiology 45, 1545-7.

Smith, S.L., Rizoulis, A., West, J.M., Lloyd, J.R. 2016. The microbial ecology of a hyper-alkaline spring, and impacts of an alkali-tolerant community during sandstone batch and column experiments representative of a geological disposal facility for intermediate-level radioactive waste. Geomicrobiology Journal 33, 455-467.

Sorensen, J. 1978. Capacity for denitrification and reduction of nitrate to ammonia in a coastal marine sediment. Applied and Environmental Microbiology 35, 301-5.

Stewart, V. 1994. Regulation of nitrate and nitrite reductase synthesis in enterobacteria. Antonie van Leeuwenhoek 66, 37-45.

Stolz, J.F., Ellis, D.J., Blum, J.S., Ahmann, D., Lovley, D.R., Oremland, R.S. 1999. *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the ε-Proteobacteria. lnternational Journal of Systematic Bacteriology 49, 1177-1180.

Strohm, T.O., Griffin, B., Zumft, W.G., Schink, B. 2007. Growth yields in bacterial denitrification and nitrate ammonification. Applied and Environmental Microbiology 73, 1420-4.

Sung, Y., Fletcher, K.E., Ritalahti, K.M., Apkarian, R.P., Ramos-Hernandez, N., Sanford, R.A., Mesbah, N.M., Loffler, F.E. 2006. *Geobacter lovleyi* sp. nov. strain SZ, a novel metal-reducing and tetrachloroethene-dechlorinating bacterium. Applied and Environmental Microbiology 72, 2775-82.

Tal, Y., Watts, J.E., Schreier, H.J. 2006. Anaerobic ammonium-oxidizing (anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system. Applied and Environmetal Microbiology 72, 2896-904.

Tal, Y., Watts, J.E.M., Schreier, S.B., Sowers, K.R., Schreier, H.J. 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in a closed recirculated mariculture system. Aquaculture 215, 187-202.

Tiedje, J.M. 1988. *Biology of anaerobic microorganisms*. John Wiley and Sons, New York, NY.

Tiedje, J.M., Sexstone, A.J., Myrold, D.D., Robinson, J.A. 1983. Denitrification: ecological niches, competition and survival. Antonie van Leeuwenhoek 48, 569-583.

Ueki, A., Akasaka, H., Suzuki, D., Ueki, K. 2006. *Paludibacter propionicigenes* gen. nov., sp. nov., a novel strictly anaerobic, Gram-negative, propionate-producing bacterium isolated from plant residue in irrigated rice-field soil in Japan. International Journal of Systematic and Evolutionary Microbiology 56, 39-44.

van den Berg, E.M., Boleij, M., Kuenen, J.G., Kleerebezem, R., van Loosdrecht, M.C. 2016. DNRA and denitrification coexist over a broad range of acetate/N-NO_3^- ratios, in a chemostat enrichment culture. Frontiers in Microbiology 7, 1842.

van den Berg, E.M., Rombouts, J.L., Kuenen, J.G., Kleerebezem, R., van Loosdrecht, M.C.M. 2017. Role of nitrite in the competition between denitrification and DNRA in a chemostat enrichment culture. AMB Express 7, 91.

van den Berg, E.M., van Dongen, U., Abbas, B., van Loosdrecht, M.C. 2015. Enrichment of DNRA bacteria in a continuous culture. ISME 9, 2153-61.

van Rijn, J., Rivera, G. 1990. Aerobic and anaerobic biofiltration in an aquaculture unit—nitrite accumulation as a result of nitrification and denitrification. Aquacultural Engineering 9, 217-234.

Vermeiren, J., Van de Wiele, T., Verstraete, W., Boeckx, P., Boon, N. 2009. Nitric oxide production by the human intestinal microbiota by dissimilatory nitrate reduction to ammonium. Journal of Biomedicine and Biotechnology 2009, 284718.

Vester, J.K., Glaring, M.A., Stougaard, P. 2014. Discovery of novel enzymes with industrial potential from a cold and alkaline environment by a combination of functional metagenomics and culturing. Microbial Cell Factories 13, 72.

Vishniac, W., Santer, M. 1957. The thiobacilli. Bacteriol Rev 21, 195-213.

Wang, H., Gunsalus, R.P. 2000. The *nrfA* and *nirB* nitrite reductase operons in *Escherichia coli* are expressed differently in response to nitrate than to nitrite. Journal of Bacteriology 182, 5813-22.

Wei, W., Isobe, K., Nishizawa, T., Zhu, L., Shiratori, Y., Ohte, N., Koba, K., Otsuka, S., Senoo, K. 2015. Higher diversity and abundance of denitrifying microorganisms in environments than considered previously. ISME 9, 1954-65.

Welsh, A., Chee-Sanford, J.C., Connor, L.M., Loffler, F.E., Sanford, R.A. 2014. Refined NrfA phylogeny improves PCR-based *nrfA* gene detection. Applied and Environmental Microbiology 80, 2110-9.

Welsh, D., Castadelli, G., Bartoli, M., Poli, D., Careri, M., de Wit, R., Viaroli, P. 2001. Denitrification in an intertidal seagrass meadow, a comparison of ^{15} N-isotope and acetylene-block techniques: dissimilatory nitrate reduction to ammonia as a source of N^2O? Marine Biology 139, 1029-1036.

Willems, A. 2014. The Family Comamonadaceae. in: *The Prokaryotes*, pp. 777-851.

Wrighton, K.C., Thomas, B.C., Sharon, I., Miller, C.S., Castelle, C.J., VerBerkmoes, N.C., Wilkins, M.J., Hettich, R.L., Lipton, M.S., Williams, K.H., Long, P.E., Banfield, J.F. 2012. Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. Science 337, 1661.

Yin, S.X., Chen, D., Chen, L.M., Edis, R. 2002. Dissimilatory nitrate reduction to ammonium and responsible microorganisms in two Chinese and Australian paddy soils. Soil Biology and Biochemistry 34, 1131-1137.

Yoon, S., Cruz-Garcia, C., Sanford, R., Ritalahti, K.M., Loffler, F.E. 2015. Denitrification versus respiratory ammonification: environmental controls of two competing dissimilatory NO_3^-/NO_2^- reduction pathways in *Shewanella loihica* strain PV-4. ISME 9, 1093-104.

APPENDIX

Table 1. Nitrate, nitrite, and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with nitrate of 25 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	25.87	0.65	0.049	0.009	0.066	0.011
30	18.46	0.31	0.256	0.005	0.062	0.006
60	13.94	0.92	0.278	0.001	0.074	0.023
90	6.31	0.38	0.303	0.017	0.095	0.061
120	1.20	0.19	0.016	0.006	0.079	0.007
150	1.54	0.25	0.004	0.004	0.064	0.003
180	1.60	0.28	0.014	0.006	0.060	0.008
210	1.80	0.21	0.026	0.010	0.094	0.047
240	1.99	0.03	0.021	0.125	0.128	0.075
300	1.96	0.04	0.007	0.138	0.057	0.007
360	2.37	0.02	0.012	0.003	0.100	0.067

Table 2. Nitrate, nitrite, and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	92.66	2.46	0.138	0.010	0.109	0.009
60	82.97	1.90	0.387	0.007	0.128	0.028
120	70.60	0.58	0.346	0.018	0.063	0.001
180	60.77	1.46	0.343	0.009	0.137	0.032
240	47.21	0.46	0.296	0.034	0.066	0.004
300	32.73	0.66	0.564	0.169	0.093	0.009
360	20.93	0.14	0.491	0.201	0.058	0.023
420	6.58	0.07	0.361	0.023	0.062	0.002
480	4.66	0.05	0.046	0.017	0.106	0.004
540	3.10	0.02	0.012	0.002	0.045	0.041

Table 3. Nitrite and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with nitrite of 10 mg-N/L

Time (min)	Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	10.32	0.25	0.046	0.046
30	3.09	0.10	0.684	0.684
60	0.20	0.04	0.528	0.528
90	0.02	0.02	0.599	0.599
120	0.00	0.00	0.599	0.599
150	0.02	0.03	0.806	0.806

Table 4. Nitrite and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with nitrite of 20 mg-N/L

Time (min)	Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	19.31	0.90	0.060	0.049
30	16.99	0.47	0.607	0.147
60	12.79	0.46	0.766	0.015
90	9.43	0.33	1.067	0.148
120	6.25	0.35	1.665	0.171
150	2.17	0.14	2.107	0.061
180	0.15	0.01	2.579	0.027
210	0.02	0.00	2.408	0.090
240	0.06	0.00	3.090	0.118

Table 5. Nitrite and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with nitrite of 50 mg-N/L

Time (min)	Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	48.47	0.95	0.02	0.023
60	42.36	0.33	0.70	0.082
120	42.19	1.00	1.28	0.125
180	36.78	0.44	2.10	0.272
240	35.62	2.00	2.10	0.044
300	36.24	0.72	3.21	0.107
360	32.88	2.14		

Table 6. Nitrate, nitrite, and ammonium concentrations measured during the high C/NO_3^- aquaculture sludge incubation with nitrate of 25 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	21.44	0.10	0.015	0.003	0.154	0.016
30	18.72	0.24	0.603	0.031	0.428	0.006
60	18.69	0.47	1.289	0.242	0.689	0.035
90	16.79	0.11	2.065	0.033	0.970	0.026
120	18.34	0.10	4.310	0.154	1.547	0.142
150	16.57	0.06	3.090	0.296	1.747	0.095
180	17.08	0.37	3.371	0.316	1.805	0.049
240	13.31	0.13	4.165	0.336	2.989	0.032
300	10.48	0.22	4.161	0.672	2.407	0.091
360	12.19	0.07	5.297	0.053	3.155	0.086
420	10.92	0.10	4.038	0.154	3.437	0.115
480	6.75	0.26	5.473	0.286	2.869	0.192
540	7.38	1.63	4.437	0.187	3.723	0.036

Table 7. Nitrate, nitrite, and ammonium concentrations measured during the high C/NO_3^- aquaculture sludge incubation with nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	69.32	0.58	0.058	0.013	0.507	0.084
60	67.21	1.75	1.999	0.017	0.832	0.004
120	78.46	0.20	3.366	0.204	1.599	0.057
180	62.34	1.14	4.310	0.202	1.886	0.024
240	55.03	0.60	4.336	0.236	3.347	0.064
300	52.77	1.05	5.622	0.165	2.904	0.078
360	52.20	0.20	5.337	0.040	2.876	0.069
420	52.33	0.77	6.092	0.256	3.055	0.179
480	46.64	1.71	6.517	0.228	3.615	0.331
540	53.10	0.79	7.637	0.079	3.318	0.021

Table 8. Nitrate, nitrite, and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with sulfide of 50 mg-S^{2-}/L and nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium		Sulfide	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-S^{2-}/L)	SD
0	78.77	0.45	0.05	0.00	0.03	0.01	27.10	2.58
30	78.36	0.31	3.24	0.01	0.62	0.04	41.51	3.69
60	73.14	5.50	5.62	0.08	1.04	0.01	41.63	4.97
90	60.64	1.00	9.82	0.67	1.00	0.04	35.34	6.06
120	53.26	0.61	10.31	0.27	3.20	0.26	36.94	2.79
150	57.53	0.60	16.30	0.29	3.84	0.43	31.12	2.62
180	55.22	0.23	7.85	0.23	4.46	0.41	28.12	3.45
240	41.52	1.43	19.36	0.21	6.30	0.07	12.31	1.75
300	40.86	1.09	24.05	0.35	8.35	0.47	9.14	1.26
360	29.17	0.91	24.21	0.49	9.54	0.21	4.72	0.26

Table 9. Nitrate, nitrite, and ammonium concentrations measured during the low C/NO_3^- aquaculture sludge incubation with sulfide of 100 mg-S^{2-}/L and nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium		Sulfide	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-S^{2-}/L)	SD
0	91.27	0.45	0.05	0.00	0.12	0.05	63.23	1.43
30	89.96	2.11	2.01	0.29	0.17	0.03	91.99	5.33
60	72.79	0.31	3.64	0.41	0.69	0.13	96.30	3.51
90	53.71	0.46	9.16	0.10	1.08	0.07	88.38	5.43
120	79.87	1.17	4.87	0.35	0.85	0.23	96.07	10.96
150	66.82	1.82	15.97	0.25	7.62	0.11	78.66	1.75
180	65.86	1.61	18.02	0.17	8.76	0.23	71.73	1.16
240	46.49	0.17	26.04	1.81	6.55	0.09	73.19	12.96
300	42.97	0.87	26.84	0.41	11.22	0.18	55.37	11.40
360	41.01	0.98	32.88	0.94	12.11	0.10	48.09	9.33

Table 10. Nitrate, nitrite, and ammonium concentrations measured during the high C/NO_3^- aquaculture sludge incubation with sulfide of 25 mg-S^{2-}/L and nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium		Sulfide	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-S^{2-}/L)	SD
0	132.4	2.1	0.03	0.01	0.091	0.052	29.99	2.76
30	133.6	0.9	0.36	0.01	0.365	0.027	40.23	3.09
60	126.4	0.4	0.84	0.04	0.719	0.032	32.55	1.67
90	129.1	0.7	1.28	0.10	0.977	0.046	28.30	3.08
120	109.4	0.2	2.05	0.04	1.256	0.047	22.41	1.49
150	107.9	0.6	4.80	0.25	1.397	0.090	26.55	1.20
180	110.3	1.4	6.02	0.11	1.534	0.033	32.92	2.69
240	110.8	2.0	8.38	0.06	2.485	0.207	22.00	0.69
300	125.2	1.8	11.68	0.52	2.841	0.466	17.99	1.59
360	97.5	2.2	14.24	0.27	2.015	0.167	14.83	0.31

Table 11. Nitrate, nitrite, and ammonium concentrations measured during the high C/NO_3^- aquaculture sludge incubation with sulfide of 50 mg-S^{2-}/L and nitrate of 100 mg-N/L

Time (min)	Nitrate		Nitrite		Ammonium		Sulfide	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-S^{2-}/L)	SD
0	147.2	1.0	0.074	0.012	0.167	0.075	76.7	2.1
30	141.5	8.5	0.049	0.026	0.509	0.028	56.0	16.5
60	104.0	2.9	0.059	0.023	0.576	0.042	76.1	5.4
90	133.4	1.3	0.170	0.074	1.161	0.066	49.1	4.5
120	138.8	3.2	0.121	0.028	1.418	0.034	67.0	3.2
150	146.4	0.7	0.120	0.004	1.343	0.135	103.1	0.8
180	119.2	1.6	0.101	0.054	0.990	0.066	89.5	1.9
240	122.4	1.4	0.115	0.017	1.939	0.360	72.1	3.1
300	147.6	33.6	1.146	0.023	1.785	0.999	78.9	2.1
360	121.3	3.4	1.281	0.024	2.833	0.200	70.8	3.2

Table 12. Dissolved organic carbon (DOC) concentrations measured during the enrichment culture experiment

Low C/NO_3^- enrichment culture			High C/NO_3^- enrichment culture		
Day	Conc. (mg/L)	SD	Day	Conc. (mg/L)	SD
1	21.15	0.65	1	25.17	0.30
2	23.14	0.52	2	28.68	0.31
3	24.78	0.40	3	28.54	0.96
4	24.19	0.42	4	27.37	0.64
5	23.85	0.75	5	29.65	0.62
6	23.97	0.76	6	34.95	0.62
7	24.47	0.12	7	33.25	1.49
8	21.67	0.49	8	33.18	0.17
9	22.65	0.52	9	34.06	1.08
11	18.96	0.69	11	33.49	0.99
12	21.74	0.64	12	33.88	0.70
13	21.72	0.25	13	41.45	0.20
14	22.50	0.26	14	49.70	0.58
15	22.80	0.28	15	49.10	0.93
16	36.39	0.63	16	87.18	1.55
17	48.40	1.73	17	118.16	2.90
18	54.23	1.67	18	140.61	0.54
19	59.20	0.69	19	175.97	1.21
20	62.05	0.23	20	201.35	0.87
21	61.37	0.92	21	211.90	0.75
22	63.18	0.64	22	210.48	1.22
23	76.53	3.30	23	112.08	3.28
24	50.79	2.13	24	90.53	5.43
25	47.86	1.28	25	86.23	4.74
26	48.32	1.09	26	84.31	25.20
27	46.78	0.72	27	66.81	3.07
28	46.92	1.06	28	65.54	4.94
29	46.11	1.34	29	37.35	3.39
30	48.55	1.19	30	50.96	3.00
31	48.69	1.50	31	66.37	5.53
32	49.48	2.00	32	87.19	5.22

33	51.02	0.49	33	67.02	2.43
34	55.13	0.81	34	60.90	3.06
35	53.74	1.40	35	116.98	6.56
36	57.46	1.61	36	101.55	3.20
37	54.05	0.54	37	104.21	3.65
38	57.67	0.92	38	83.34	2.93
39	57.60	0.79	39	87.21	2.23
40	58.54	0.90	40	84.81	4.29
41	56.75	1.33	41	197.61	0.02
42	59.01	0.94	42	118.01	1.50
43	58.49	0.82	43	119.18	0.39
44	53.57	0.55	44	110.69	2.85
46	51.02	1.13	46	116.70	2.22
47	50.38	2.56	47	107.73	1.96
48	56.12	1.70	48	87.62	3.60
49	50.13	0.91	49	99.03	0.79
51	67.89	0.35	51	89.15	1.90
52	74.95	1.26	52	99.68	2.12
53	70.82	1.84	53	96.38	1.39
54	67.87	1.03	54	91.89	0.54
55	71.10	0.75			
57	103.00	1.69			
58	102.06	0.71			
60	96.48	0.07			

Table 13. Nitrate, nitrite, and ammonium concentrations measured during the enrichment culture experiment

Day	Low C/NO_3^- enrichment culture			High C/NO_3^- enrichment culture		
	Nitrate. (mg-N/L)	Nitrite (mg-N/L)	Ammonium (mg-N/L)	Nitrate. (mg-N/L)	Nitrite (mg-N/L)	Ammonium (mg-N/L)
1	28.266	0	23.182	22.610	0	21.95
2	28.260	0	35.996	0.011	0	26.73
3	29.359	0	39.449	4.273	0	29.27
4	30.405	1.70	43.053	0.119	0	29.11
5	33.072	0	43.316	0.000	0	30.24
6	41.143	0	44.933	0.000	0	32.28
7	43.370	0	44.222	0.000	0	34.46
8	52.236	0	33.403	0.003	0	35.55
9	50.943	0	36.089	0.000	0	35.92
11	43.783	0	39.546	0.000	0	36.55
12	37.350	0	36.274	0.000	0	26.69
13	32.103	0	38.774	0.000	0	33.89
14	31.438	0	42.098	0.000	0	26.24
15	37.066	0	35.182	0.000	0	34.33
16	17.720	0	58.859	0.060	0	53.07
17	1.747	0	76.064	0.079	0	73.71
18	0.059	0	44.314	0.090	0	79.42
19	0.000	0	38.357	0.099	0	100.86
20	0.000	0	60.335	0.000	0	127.04
21	0.057	0	47.750	0.000	0	139.12
22	0.056	0	73.444	0.000	0	170.73
23	0.042	0	75.050	0.000	0	102.43
24	0.000	0	78.210	0.000	0	135.04
25	0.054	0	68.662	0.141	0	172.91
26	0.099	0	56.535	0.125	0	114.89
27	0.000	0	78.761	0.031	0	179.58
28	0.000	0	77.901	0.081	0	219.69
29	0.062	0	70.192	0.033	0	195.62
30	0.073	0	86.464	0.043	0	212.95
31	0.166	0	80.959	0.048	0	211.72

32	0.159	0	79.426	0.044	0	200.54
33	0.119	0	70.572	0.087	0	187.82
34	0.128	0	83.894	0.075	0	182.70
35	0.060	0	71.659	0.086	0	228.85
36	0.068	0	85.051	0.100	0	121.53
37	0.028	0	64.234	0.069	0	160.13
38	0.000	0	95.108	0.139	0	271.07
39	0.000	0	81.681	0.166	0	263.60
40	0.166	0	85.432	0.150	0	269.00
41	0.099	0	65.884	0.164	0	281.64
42	0.093	0	91.080	0.065	0	289.79
43	0.212	0	80.605	0.081	0	288.26
44	0.000	0	84.972	0.071	0	291.99
46	0.094	0	78.265	0.079	0	293.51
47	0.107	0	78.203	0.073	0	310.41
48	0.216	0	81.993	0.164	0	301.78
49	0.156	0	80.482	0.195	0	303.07
51	0.172	0	93.499	0.190	0	297.64
52	0.000	0	89.771	0.159	0	295.31
53	0.000	0	74.426	0.183	0	237.16
54	0.116	0	62.877	0.139	0	247.83
55	0.000	0	49.541			
57	0.000	0	43.655			
58	0.000	0	39.742			
60	0.113	0	31.661			

Table 14. $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ concentrations measured during the $^{15}NO_3^-$ incubations with low C/NO_3- enrichment culture sludge

Time (min)	$^{15}NO_3^-$		$^{15}NO_2^-$		$^{15}NH_4^+$	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	17.982	0.169	0.658	0.007	0.437	0.012
10	12.600	0.113	1.623	0.078	0.520	0.019
20	8.576	0.096	2.852	0.189	0.581	0.022
30	6.674	0.166	3.173	0.222	0.592	0.007
40	5.760	0.328	3.002	0.182	0.616	0.017
50	4.978	0.283	2.901	0.398	0.624	0.015

Table 15. $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ concentrations measured during the $^{15}NO_2^-$ incubations with low C/NO_3- enrichment culture sludge

Time (min)	$^{15}NO_3^-$		$^{15}NO_2^-$		$^{15}NH_4^+$	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	0.103	0.021	17.691	0.234	0.520	0.036
10	0.077	0.008	10.894	0.033	0.595	0.007
20	0.063	0.007	4.610	0.664	0.694	0.017
30	0.041	0.004	0.573	0.993	0.745	0.028
40	0.018	0.012	0.000	0.000	0.751	0.018
50	0.011	0.015	0.000	0.000	0.750	0.012

Table 16. $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ concentrations measured during the $^{15}NO_3^-$ incubations with high C/NO_3- enrichment culture sludge

Time (min)	$^{15}NO_3^-$		$^{15}NO_2^-$		$^{15}NH_4^+$	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	18.501	0.224	0.341	0.302	0.590	0.078
10	16.904	0.190	0.948	0.014	1.404	0.049
20	15.375	0.299	1.175	0.066	2.031	0.097
30	14.534	0.208	1.294	0.135	2.531	0.178
40	14.041	0.332	1.344	0.121	2.634	0.237
50	13.975	0.303	1.429	0.157	2.697	0.151

Table 17. $^{15}NO_3^-$, $^{15}NO_2^-$, and $^{15}NH_4^+$ concentrations measured during the $^{15}NO_2^-$ incubations with high C/NO_3- enrichment culture sludge

Time (min)	$^{15}NO_3^-$		$^{15}NO_2^-$		$^{15}NH_4^+$	
	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD	Conc. (mg-N/L)	SD
0	0.060	0.005	20.408	0.583	0.570	0.032
10	0.056	0.005	18.853	0.460	1.735	0.088
20	0.047	0.011	17.527	0.420	2.618	0.145
30	0.055	0.006	16.779	0.400	3.148	0.274
40	0.052	0.002	16.535	0.341	3.237	0.275
50	0.057	0.007	16.374	0.263	3.213	0.266

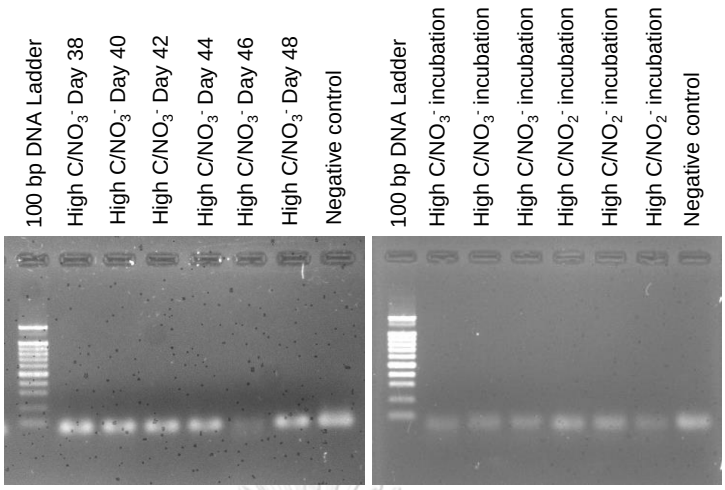


Fig. 1 Primer amplification with the high C/NO_3^- enrichment culture RNA samples collected from different days (left) and from the nitrate/nitrite incubation samples (right), using *nirB*-I primer pair 1552F + 2066R, the absence of band indicates no DNA contamination during the extraction of RNA

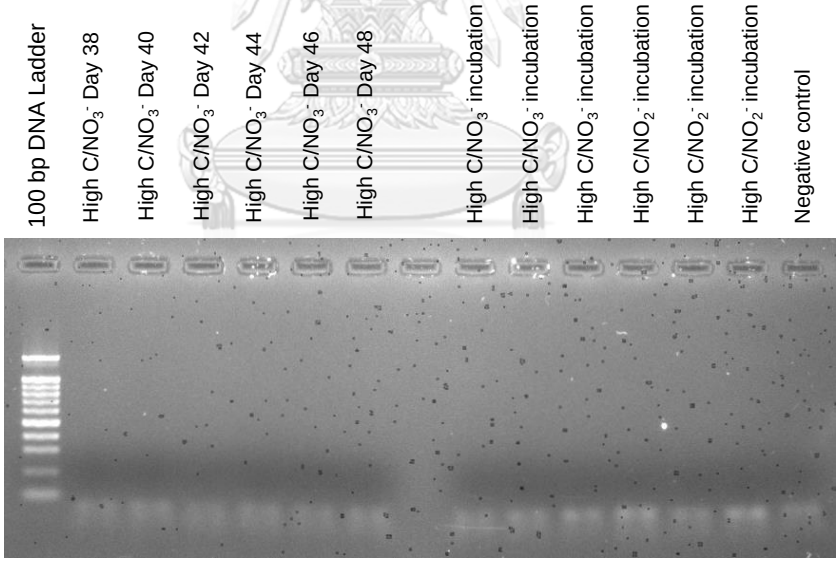


Fig. 2 Primer amplification with the high C/NO_3^- enrichment culture RNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-II primer pairs 1651F and 2063R, the absence of band indicates no DNA contamination during the extraction of RNA

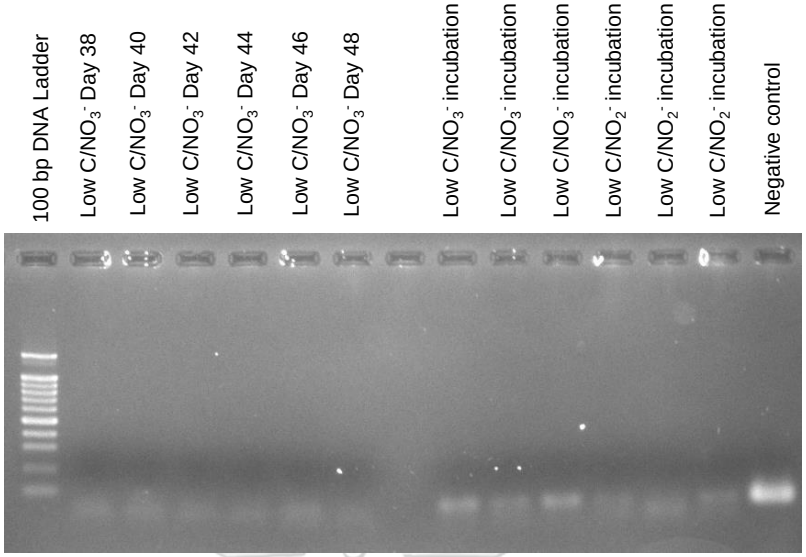


Fig. 3 Primer amplification with the low C/NO_3^- enrichment culture RNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-I primer pair 1552F + 2066R, the absence of band indicates no DNA contamination during the extraction of RNA

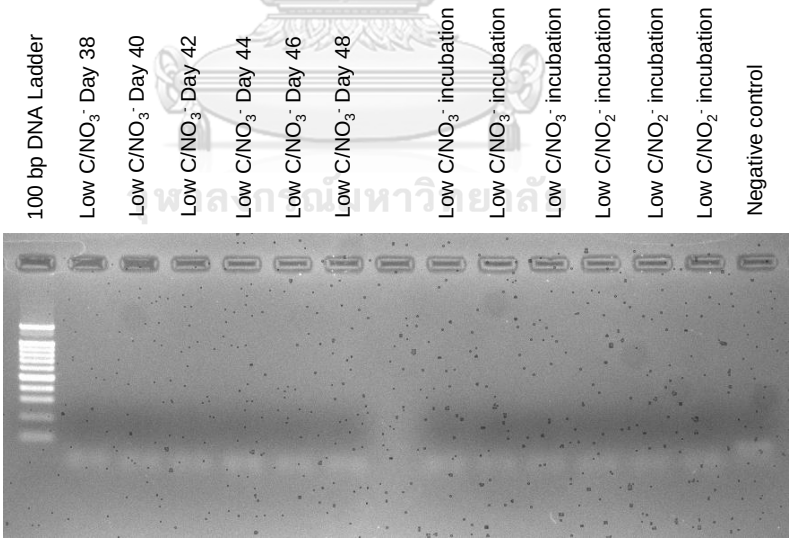


Fig. 4 Primer amplification with the low C/NO_3^- enrichment culture RNA samples collected from different days and from the nitrate/nitrite incubation samples, using *nirB*-II primer pairs 1651F and 2063R, the absence of band indicates no DNA contamination during the extraction of RNA

VITA

Pokchat Chutivisut was born on April 2, 1984 in Bangkok, Thailand. She began her academic life at Rajini Bon School and continued to be a student there for 12 years. She continued her higher study at Sirindhorn International Institute of Technology (SIIT), Thammasat University, and chose to be in the major of Environmental Technology. After granted a Bachelor Degree, she went on to study in the field of Environmental Engineering at Chulalongkorn University. Her Master thesis involved biological nitrogen removal in aquaculture system, and she stayed on the topic of nitrogen cycle ever since.