GENOME-BASED INSIGHTS INTO THE CHARACTERISTICS OF ANAMMOX BACTERIA FOR NITROGEN REMOVAL FROM WASTEWATER

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CERTIFICATE OF ORIGINALITY

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ABSTRACT

Anaerobic ammonium-oxidizing (anammox)-based processes for nitrogen removal have attracted increasing attention due to its advantages: less oxygen requirement, no additional carbon matters, and less sludge production. Anammox process uses ammonium as an electron donor and nitrite as an electron acceptor and converts them to dinitrogen gas (N_2). It has been successfully applied for ammonium-rich wastewater treatment, however, the application for mainstream wastewater treatment is still in progress. Till now, anammox bacteria have been analyzed from the genomic level, and an increasing number of anammox genomes was annotated such as *Ca. Brocadia fulgida, Ca. Brocadia Sinica JPN1, Ca. Kuenenia stuttgartiensis* and *Ca. Jettenia caeni* etc. The development of molecular biotechnology enables researchers to recover new whole-genome anammox species from various anammox processes and annotate key genes involving in the anammox reaction. However, it is still vital to gain a comprehensive understanding on the potential metabolic pathways in anammox bacteria based on different environmental conditions.

This doctoral study was to investigate anammox bacteria enriched in different reactors treating low strength wastewater and explore their potential metabolic mechanisms from whole-genome level. The chapter three of this dissertation rapidly starts up partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor to treat low strength ammonium wastewater without inoculating anammox sludge. Of partial interest was to investigate the unique genes in the strain of *Ca. Brocadia sp*. Subsequently, the observed gene encoding superoxide dismutase, Fe-Mn family (SOD2) in Chapter three indicates anammox bacteria has the potential for oxygen detoxification. Chapter four recovers the potential metabolic pathways for oxygen detoxification and mixotrophic metabolism in anammox bacteria and examine the related genes expression values. To test the feasibility of freshwater anammox for saline wastewater treatment, Chapter five starts up the anammox process with low-strength wastewater

containing different seawater proportions and investigates the shift of microbial community structure. In the previous chapter, anammox species affiliated with the genus of *Ca. Brocadia* was enriched in saline environment. Therefore, it is vital to identify the anammox species and explore its potential metabolic pathways. In Chapter six, a new anammox species was *de novo* assembled, and its nitrogen transformation and sulfur-dependent pathways were reconstructed. Further, the sodium pump associated with energy conservation in the dominant anammox bacteria was investigated. Finally, Chapter seven draws the conclusions and recommendations during doctoral study.

PUBLICATIONS ARISING FROM THE THESIS

To be submitted Journal Papers:

1. JI XM., Leng L., Zheng Z., Huang XW., Lee P-H. Metagenomic insights into unique genes in anammox bacteria in a partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor.
2. JI XM., Wu ZY., Lee P-H. Metagenomics and metatranscriptomics analyses reveal oxygen detoxification and mixotrophic metabolism in an enriched anammox continuous stirred-tank reactor. (Submitted to Water Research)
3. JI XM., Wang YL., Sung SH., Lee P-H. Microbial synamics during the start-up of anammox process for low-strength seawater-based wastewater treatment.

Book Chapter:

1. X Ji, YT Huang, Q Wang, GYA Tan, JG Lin, PH Lee State-of-the-Art Anaerobic Ammonium Oxidation (Anammox) Technology, Anaerobic Biotechnology: Environmental Protection and Resource Recovery, 2015, 49-71

Conference Presentations:

1. JI XM., Huang XW., Lee P-H. Microbial Community Dynamics of Partial Nitrification/Anammox in a Fluidized Bioreactor with Diluted Wastewater, 2017 International Environmental Engineering Conference & Annual Meeting of the Korean Society of Environmental Engineers (IEEC 2017), Jeju, Korea, Nov. 2017

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Chapter 1 Introduction

1.1 Background

With the increasing pressures on water shortage, energy security and climate change, various strategies from different fields are urging adaptation for these challenges. Especially, wastewater treatment is required before discharging into natural water body due to the situation of water shortage. Although the wastewater treatment technology has achieved tremendous development over the last decades around the world, in a general sense, the eutrophication is still a troublesome issue (Qin et al. 2007). To protect the natural water body from eutrophication, domestic wastewater should be treated to remove nitrogen compounds before release. However, the aeration parts in traditional wastewater treatment cost the most part energy during the whole operational period (Longo et al. 2016). Compared to conventional nitrification and denitrification process, autotrophic nitrogen removal process based on anaerobic ammonium oxidation (anammox) is more economical and environmentally friendly (Daverey et al. 2013, Van Hulle et al. 2010). Therefore, the wastewater treatment process shown in Fig. 1-1 is regarded as an effective method to meet the requirement for domestic wastewater treatment.

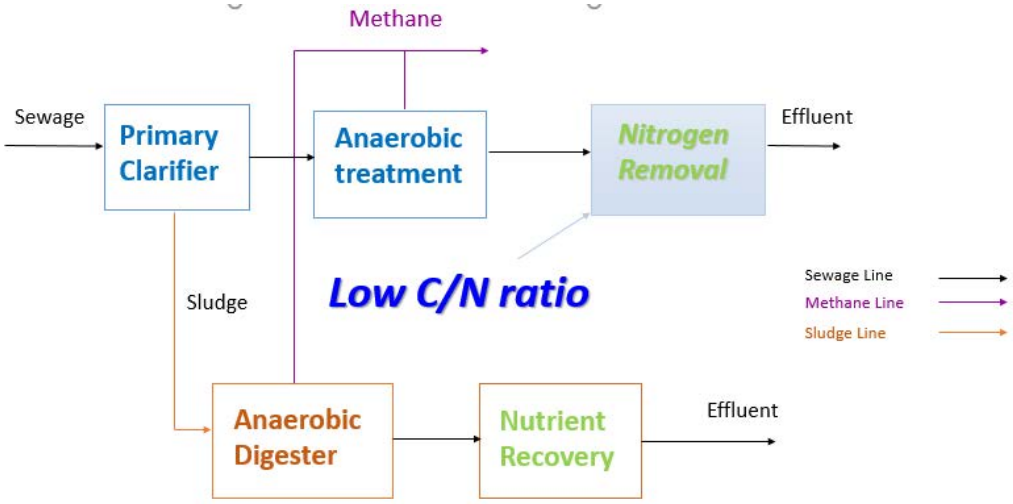

Fig. 1-1 The anaerobic domestic wastewater treatment process

To date, anammox-based processes have been successfully applied for high-strength wastewater treatment. However, the mainstream anammox wastewater treatment is still in progress due to the low nitrogen concentration and low temperature. Further, the microbial community structures in the various environment were explored using 16S rRNA sequencing. Anammox bacteria have been classified into five candidatus genera: *Brocadia, Kuenenia, Jettenia, Anammoxoglobus, Scalindua* (Ali and Okabe 2015, Strous et al. 1998, Strous et al. 1999), whereas the first four genera were detected from the freshwater environment and *Ca. Scalindua* was found in the saline ecosystem (Awata et al. 2013, Schmid et al. 2000, Strous et al. 1999). Recently, whole-genome anammox bacteria was assembled from wastewater treatment plants and bioreactors through metagenomic sequencing technology. The anammox reaction pathway was reconstructed with the annotation of key genes such as hydrazine synthase subunit (HZS) and hydrazine dehydrogenase (HDH).

Moreover, the potential microbial interaction in anammox consortia was revealed by Speth et al. (2016) and Lawson et al. (2017). This phenomenon revealed that anammox may benefit from the microbial interaction when it was implemented for low-strength wastewater treatment. Additionally, the metabolic mechanisms in anammox bacteria

are still unclear as for the unavailability of pure culture. The main motivation of this study is to develop anammox process for low strength wastewater treatment and to recover the draft genomes of dominant anammox bacteria present in the bioreactor as well as explore the potential metabolic pathways in anammox bacteria.

1.2 Research objective

The main goal of this thesis was to apply the anammox process for low-strength wastewater treatment and to investigate the potential metabolic mechanisms in anammox bacteria. In order to achieve this goal, various specific objectives are mentioned as followings:

i. Rapid start-up partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor treating low strength ammonium wastewater

This study aimed to develop a partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor for low strength ammonium wastewater treatment without inoculating anammox sludge.

ii. Oxygen detoxification and mixotrophic metabolism in anammox bacteria

This study was to explore the oxygen detoxification pathway and mixotrophic metabolism in anammox bacteria affiliated with the genus of *Ca. Brocadia*.

iii. Anammox process for low-strength seawater-based wastewater treatment

The mian goal of this experiment was to develop anammox process for low-strength seawater-based wastewater treatment and to investigate the shift of microbial community structure.

iv. Sodium pump associated with energy conservation in anammox bacteria in the saline environment

We achieved a stable anammox process for low-strength seawater-based wastewater treatment and enriched one anammox bacteria affiliated to the genus of *Ca. Brocadia*. Therefore, the objective of this experiment was to explore the specific anammox species and the intrinsic mechanisms under salinity environment, such as sodium pump, nitrogen transformation, and sulfur-dependent metabolic pathways.

1.3 Thesis structure

1.3.1 Chapter 1

This chapter states the research background and gaps during the doctoral study and the detailed research objectives and structure of this thesis.

1.3.2 Chapter 2

This chapter provides a systematic literature review of anammox mechanism and application. It presents the up-to-date information of anammox bacteria genera and their versatile metabolism pathways found by a number of advanced molecular techniques and also gives a brief summary on their potential nitrogen sources of cell synthesis through microbial thermodynamic calculations. Considering its applicability for nitrogen removal from wastewaters (both for high-strength and dilute strength), the effect of solid retention time on the nitrogen removal efficiency and inhibitors on the anammox process are discussed. Moreover, its potential involvement in syntropy for sulfate reduction and ammonium oxidation triggers scientists to wonder if it plays a significant role on the global nitrogen-sulfur cycle.

1.3.3 Chapter 3

A partial nitrification (PN)-anammox fluidized-bed bioreactor (FBR) containing granular activated carbon (GAC) particles was investigated to treat low strength ammonium wastewater with inoculating conventional activated sludge. The unique genes in the recovered anammox bacteria were investigated and their possible interaction with other microbes was also explored.

1.3.4 Chapter 4

In the previous experiment, we found that anammox bacteria was possible to detoxify superoxide and develop potential metabolic mechanisms. However, no full pathway and metatranscriptomic evidences has been reoprted to support these potential mechanisms. In this experiment, we enriched high abundance of an anammox species (~50% abundance) at room temperature without strict anaerobic condition in a lab-scale continuous stirred-tank reactor (CSTR) and explored its superoxide detoxification mechanism and mixotrophic metabolism.

1.3.5 Chapter 5

To investigate the feasibility of the anammox process for saline wastewater treatment, a continuous flow anammox reactor was started up for low-strength seawater-based wastewater treatment at room temperature without inoculating enriched anammox sludge. The reactor performance and microbial community were investigated. The anammox bacteria, ammonia oxidizing bacteria, and nitrite oxidizing bacteria were characterized.

1.3.6 Chapter 6

It was revealed that freshwater anammox bacteria affiliated with the genus of *Ca. Brocadia* was enriched for saline wastewater treatment in Chapter 5. Hence, the whole-genome of anammox species present in the CSTR was assembled. This study recovered a new anammox species with high genomic quality and provided a comprehensive understanding of its diverse metabolic activities and energy generation based on sodium pump.

1.3.7 Chapter 7

In this chapter, conclusions of previously conducted studies were present. Moreover, the limitations of the present study and recommendations for future work are proposed.

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Chapter 2 Literature review

2.1 Nitrogen cycle

Nitrogen forms the majority gas of Earth's atmosphere (78%), and thus leads to nitrogen transformation among its various chemical forms carried by biological and physical reactions. The biological nitrogen transformation pathways discovered by the end of the 19th century, consisted of assimilation, ammonification, nitrification, denitrification, and nitrogen fixation (Ahn 2006; Paredes et al. 2007; You et al. 2009). An overlooked pathway of anammox was predicted by Broda (1977). This theoretical prediction stated that the existence of Anammox should be carried by lithotrophic organisms by driving ammonium as the electron donor and nitrite or nitrate as the electron acceptor(s). Its existence was confirmed in a denitrifying bioreactor treating the Gist-Brocades fermentation effluents in the 1990s, due to the observation of ammonium concentration decrease alongside an increase in nitrogen gas concentration (Mulder et al. 1995; Kuenen and Jetten 2001). Subsequently, the mechanism of this concealed pathway has been revealed and the results showed its unconventional metabolism and attractive applications in nitrogen removal for wastewaters (Van de Graaf et al. 1996; Jetten et al. 1997; Strous et al. 1998). This knowledge completes the current understanding of the global nitrogen cycle.

2.1.1 Nitrogen Transformation

The nitrogen cycle (see Fig. 2-1) involves the following processes:

(1) Ammonification. Organic nitrogen, normally from cell lysis of deceased plants and animals, is converted to ammonium (NH_4^+) by microorganisms;

(2) Assimilation. Ammonium is utilized biologically and converted to components of organisms (organic nitrogen);

(3) Nitrification. Low valence nitrogen is converted to high valence nitrogen by ammonia oxidizing bacteria and nitrite oxidizing bacteria when electron acceptors are provided. Electron acceptors could be oxygen, or other oxidized compounds, e.g., sulfate, ferric (Fe^{3+});

(4) Assimilative reduction. Nitrate (NO_3^-) or nitrite (NO_2^-) is reduced to ammonium for cell synthesis by organisms.

(5) Denitrification. High valence nitrogen is converted to low valence nitrogen by microorganisms. Nitrate (NO_3^-) is reduced to nitrogen gas through an array of intermediate products (NO_2^-, NO, N_2O) combined with electron donors (e.g., organic matters, sulfide etc.);

(6) Nitrogen fixation. The atmospheric nitrogen is fixated to ammonium by symbiotic bacteria (mainly symbiosis with leguminous plants) or some non-symbiotic bacteria;

(7) Anammox. Autotrophic bacteria utilize ammonium as the electron donor and nitrite as the electron acceptor in a ratio of 1:1.3 during the growth phase, converting them into nitrogen gas and nitrate under anaerobic conditions in the absence organic carbons.

2.1.2 Conventional nitrogen removal

Due to the excess nitrogen discharging from waste streams directly into water bodies without advanced treatment, receiving waters are suffering from eutrophication and hypoxia. Nutrient pollution control strategy pursued by environmental authorities worldwide is calling for enforcing numeric nitrogen discharge criteria. Conventional nitrogen removal integrates nitrification (autotrophic conversion of ammonium into nitrite and further to nitrate) and denitrification (heterotrophic transfer of nitrate into nitrogen gas). Those conventional nitrogen removal processes require a large amount of oxygen, produce a substantial amount of sludge, and generate a considerable volume of N_2O (a greenhouse gas potential roughly 300 times higher than CO_2) (Peng and Zhu 2006; Sun et al. 2010). Anammox offers a sustainable alternative for the same purpose.

Its merits are: (1) a 60 % energy reduction for aeration (van Dongen et al. 2001; Paredes et al. 2007; Siegrist et al. 2008); (2) no organic donor needed for denitrification, which could otherwise be converted to energy in the form of methane (McCarty and Bae, 2011); (3) a 90% cost reduction for sludge management(Mulder 2003; De Clippeleir et al. 2011); and (4) less production of nitrous oxide (N_2O) (ICCP 2013; Kampschreur et al. 2009a). Fux and Siegrist (2004) estimated that, collectively, a 30-40% cost reduction, as well as a reduction in the environmental costs of greenhouse gas emissions, could be achieved by anammox.

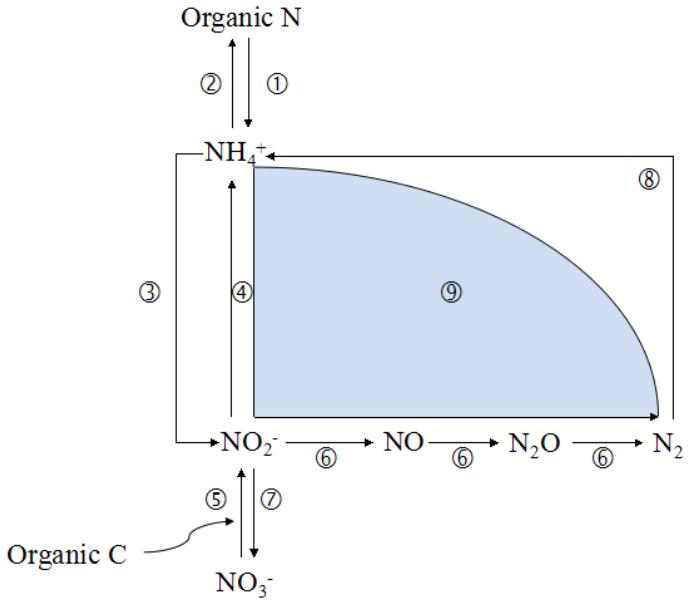

Fig. 2-1 Schematic of microbial nitrogen cycle in activated sludge floc. ① Ammonification; ②Assimilation; ③Nitrification; ④Assimilation reduction; ⑤ Denitrification; ⑥Denitrification; ⑦Nitrification; ⑧Nitrogen fixation; ⑨Anammox.

2.2 Anammox

2.2.1 Anammox genera

Anammox bacteria are chemolithoautotrophic organisms that use bicarbonate as the sole carbon source for biosynthesis of cell material and derive their energy from the

conversion of ammonium and nitrite into dinitrogen gas (van Niftrik et al. 2004). Anammox bacteria have been found not only in bioreactors, but also in natural environments, such as coastal sediments, lakes, and marine sub-oxic zones (Kuene and Jetten 2001; Schmid et al. 2007). As bacterial members of the Planctomycetes family, they are considered an ecologically and environmentally important group of microorganisms (Jetten et al. 2009).

Tab. 2-1 summarizes anammox species which have been discovered. Five known anammox genera have been described, including the genus of *Ca. Brocadia anammoxidans*, which was the first genus discovered in the denitrifying bioreactor mentioned previously (Mulder et al. 1995). Three other characterized species within the *Ca. Brocadia* genus is *Ca. Brocadia fulgida* (Kartal et al. 2008), *Ca. Brocadia sinica* (Oshiki et al. 2011), and *Ca. Brocadia caroliniensis* (Rothrock et al. 2011). *Ca. Brocadia fulgida* was an autofluorescent bacterium; *Ca. Brocadia sinica* can sustain high ammonium and nitrite load treating concentrated wastewaters; *Ca. Brocadia caroliniensis* was identified from a bioreactor treating animal waste sludge. All of these were enriched in anammox bioreactors.

Table 2-1 Anammox bacteria species

Anammox bacteria species	References
Ca. Brocadia anammoxidans	Mulder et al. 1995
Ca. Brocadia fulgida	Kartal et al. 2008
Ca. Brocadia sinica	Oshiki et al. 2011
Ca. Brocadia caroliniensis	Rothrock et al. 2011
Ca. Kuenenia stuttgartiensis	Schmid et al. 2000
Ca. Scalindua sorokinii	Kuypers et al. 2003
Ca. Scalindua richardsii	Fuchsman et al. 2012
Ca. Scalindua brodae	Schmid et al. 2003

Ca. Scalindua wagneri	Schmid et al. 2003
Ca. Scalindua arabica	Woebken et al. 2008
Ca. Scalindua pacifica	Dang et al. 2013
Ca. Scalindua profunda	van de Vossenberg et al. 2013
Ca. Scalindua sinooilfield	Li et al. 2010
Ca. Scalindua zhenghei	Hong et al. 2011
Ca. Anammoxoglobus propionicus	Kartal et al. 2007a
Ca. Jettenia asiatica	Quan et al. 2008

The only species reported within the *Candidatus Kuenenia* genus is *Ca. Kuenenia stuttgartiensis* (Schmid et al. 2000). The *Ca. Scalindua* genus consists of nine proposed species, six of which were discovered in marine environments (Kuypers et al. 2003; Woebken et al. 2008; Hong et al. 2011; Fuchsman et al. 2012; Dang et al. 2013; van de Vossenberg et al. 2013). *Ca. Scalindua sorokinii* was the first anammox species found in a natural environment (Black Sea), while *Ca. Scalindua richardsii* was also discovered from the Black Sea (Kuypers et al. 2003; Fuchsman et al. 2012). Although these two species originated from the Black Sea, they also dominated in various natural environments. A cluster associated with *Ca. Scalindua sorokinii* was detected in the lower suboxic zone where ammonium concentration was high, but nitrite concentration was low, whereas another cluster associated with *Ca. Scalindua richardsii* was found in the upper suboxic zone with a low ammonium concentration but a high nitrite concentration (Fuchsman et al. 2012). *Ca.* Scalindua brodae and *Ca. Scalindua wagneri* were both identified in wastewater treatment plants (WWTPs) (Li et al. 2010). *Ca. Scalindua arabica* originated from the Arabian Sea and the Peruvian oxygen minimum zone (Woebken et al. 2008). *Ca. Scalindua pacifica* (Dang et al. 2013) and *Ca. Scalindua profunda* with pili-like structure (van de Vossenberg et al. 2013) were retrieved from the Bohai Sea and marine sediment of a Swedish fjord, respectively. Two additional species names were tentatively proposed from molecular surveys: *Ca.*

Scalindua sinooilfield from a high temperature petroleum reservoir (Li et al. 2010) and *Ca. Scalindua zhenghei* from deep-sea subsurface sediments (Hong et al. 2011). The only known species affiliated with the *Ca. Anammoxoglobus* genus was *Ca. Anammoxoglobus propionicus*, enriched from an anammox reactor (Kartal et al. 2007a). *Ca. Jettenia asiatica* was retrieved from a granular sludge anammox reactor (Quan et al. 2008). These studies have demonstrated the ubiquity of anammox bacteria in diverse natural habitats.

2.2.2 Metabolism of anammox bacteria

Anammox's metabolism shown in Fig. 2-2 possesses a unique pathway (Strous et al. 2002). In anammox catabolism, ammonium is oxidized using nitrite as the electron acceptor for the creation of a proton motive force (PMF) over the anammoxomal membrane. With the uptake of one (+0.38V) plus three (+0.34V) low-energy electrons, nitrite is reduced to nitric oxide, which then reacts with ammonium to produce hydrazine. The conversion of hydrazine to nitrogen yields four high-energy electrons (-0.75V), which subsequently generates a positive PMF. Following which, the ATPase is energized by the PMF and further ATP is yielded in the riboplasm as demonstrated in Fig. 2-2a. To initiate high-energy electrons for carbon dioxide reduction in cell synthesis, the central catabolism is combined with nitrite reduction to nitrate through the PMF-driven reversed electron transport (RET), during which hydrazine donates high-energy electrons to ferredoxin (but not recycled). Fig. 2-2b elucidates that nitrite oxidation to nitrate yields four low-energy electrons, which must be energized by the PMF, to subsidize back into the Anammox reaction for the compensation of the four high-energy electrons flowing to ferredoxin (Kuenen 2008). Anammox's distinctive metabolism would be beneficial to biochemistry understanding and medicinal application.

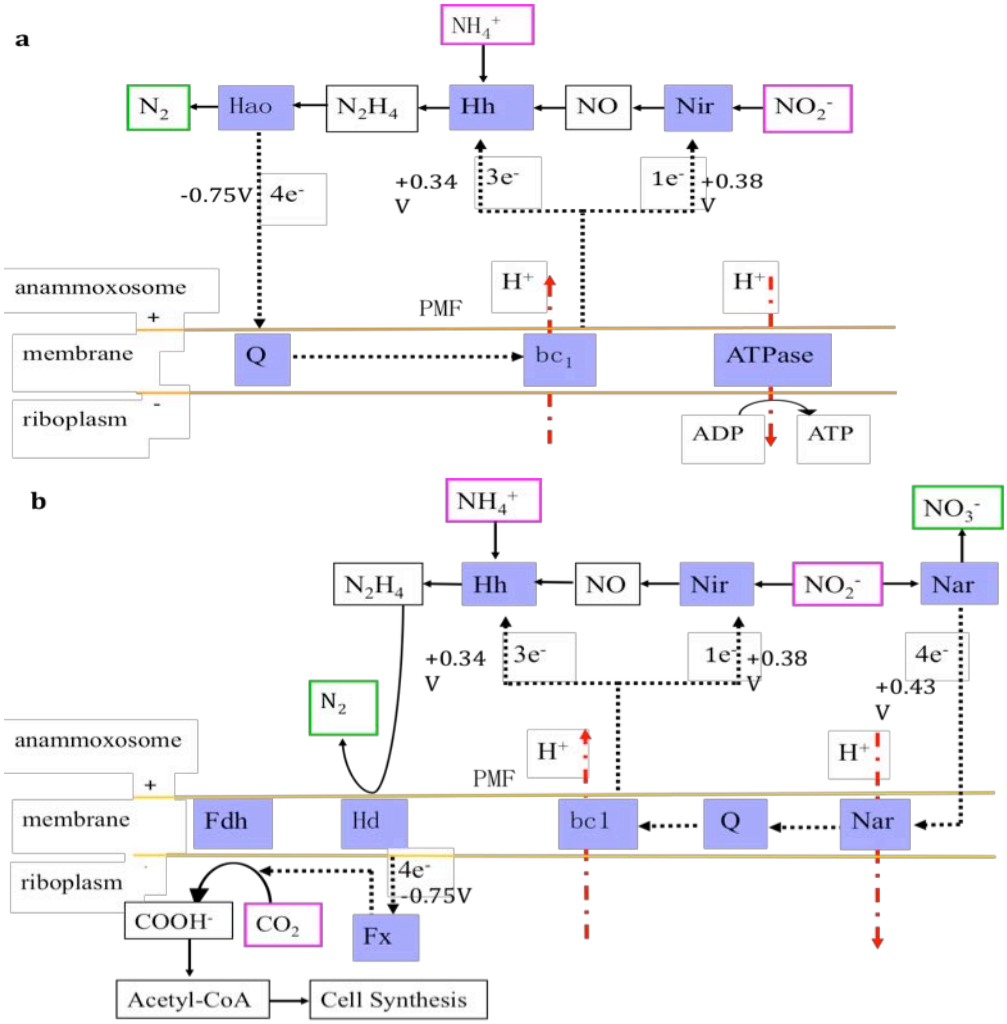

Fig. 2-2 Metabolic pathways and reversed electron transport in anammox bacteria. a): Anammox central catabolism; b): PMF-driven RET combines central catabolism with nitrate reductase to generate high potential electrons for the acetyl-CoA pathway.

2.2.3 Versatility of Anammox

Although anammox bacteria are recognized as chemolithoautotrophy, recent studies have suggested their versatility of utilizing a wide range of electron donors/acceptors for their catabolism including propionate, acetate, formate, ferric ions, manganese oxides, ammonium, and nitrite/nitrate, as summarized in Fig. 2-3 (Strous et al. 2002; Strous et al. 2006; Kartal et al. 2007b). The interesting findings at present are the ability of using propionate and acetate with the presence of ammonium and nitrate by

Candidatus Anammoxoglobus propionicus and *Candidatus Brocadia fulgida* propionicus (Kartal et al. 2007a). These two species were capable of reducing nitrite and nitrate into nitrogen while oxidizing organic into carbon dioxide for cell synthesis. The information advises that Anammox may have the potential for concurrent organic and nitrogen removal.

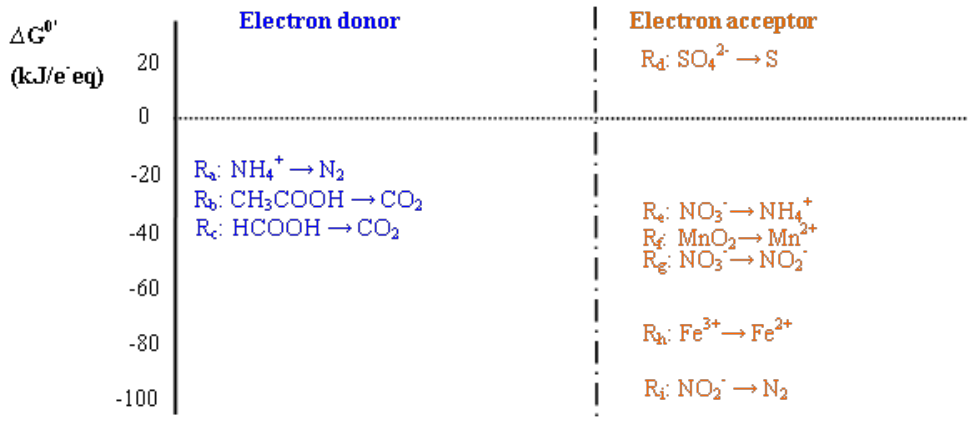

Fig. 2- 3 Half reactions and their energy yields involved in versatile Anammox process based on Gibbs standard free energy at pH 7, modified from (Gao and Tao 2011). The thermodynamic data obtained from Rittmann and McCarty (2001).

2.2.4 Syntropic anammox bacteria

Some researchers have found that anammox may participate, in syntropy, in the coupling of the electron donor of sulfate to ammonium oxidation, which is expected to exert a significant impact on the further understanding of sulfur and nitrogen cycles and their interactions on earth. The annual N and S fluxes between the ocean and the atmosphere have been estimated to be 140 and 15-33×10^{12} g, respectively (Gruber and Galloway 2008; Malin 2006). As the oceans have been considered as a sink of ammonium and sulfate, it has been hypothesized that a significant contribution may come from the biological interaction among the N and S cycles in oceans (Cai et al.

2010; Schrum et al. 2009). It is speculated that ammonium acts as the electron donor to reduce sulfate to elemental sulfur and generates nitrogen termed “sulfate reduction and ammonium oxidation (SRAO)”.

Fdz-Polanco et al. (2001; 2001a; 2001b) have found simultaneous SRAO in a granular anaerobic fluidized-bed reactor treating vinasse from an ethanol distillery of sugar beet molasses. They proposed that this biochemical reaction could be obtained by combining reactions involving in nitrite formation and anammox reaction. This phenomenon has also been observed by other researchers in organic conditions (Reyes-Avila et al. 2004; Zhao et al. 2006). Zhang et al. (2009) reported that ammonium could be oxidized anaerobically with sulfate as the electron acceptor in the presence of digested sludge under inorganic condition. The condition of high substrate concentrations and low oxidation-reduction potential (ORP) might be favorable for such a syntrophic relationship even with its low negative Gibbs free energies gained. Liu et al. (2008) and Yang et al. (2009) have detected simultaneous ammonium and sulfate removal in an Anammox reactor, which consisted of ammonium oxidation to nitrite and sulfate deoxidization to sulfur. A new species ‘‘Anammoxoglobus sulfate” was considered to perform the critical role for SRAO reaction (Liu et al. 2008). On the other hand, the existence of SRAO in sub-seafloor has already been demonstrated through thermodynamic calculations and chemical profiles analysis of sedimentary pore water (Schrum et al. 2009). Most recently, a successful stable co-culture of Anammox bacteria and Sedimenticola sp. in a laboratory-scale system validated its achievability with the media containing sulfide, nitrate, ammonium and CO_2 (Russ et al. 2014). Although much research has been carried out, the functional microorganisms and mechanism of SRAO process, whether it in lab-scale microbial systems or natural environments, have not yet been fully identified.

2.2.5 N-source for Anammox bacteria

Microorganisms prefer ammonium as their nitrogen source since it holds the same valence as the nitrogen in protein. Most of them can also utilize nitrite, nitrate, or even nitrogen gas for nitrogen requirement during anabolism (Rittmann and McCarty 2001). It is confirmed that Anammox bacteria use ammonium as the electron donor and nitrite as the electron acceptor. Still, based on the microbial thermodynamic modeling (microbial energetics), it is proposed that Anammox bacteria may use nitrite, instead of the more general ammonium, as nitrogen source for cell synthesis was proposed, based on the microbial thermodynamic modeling (microbial energetics). The stoichiometry of Anammox reaction with the assumption of different nitrogen sources could be derived from the thermodynamic electron equivalents model (TEEM) by McCarty (1975) as shown in Tab. 2-2 (calculations in detailed not shown here). By comparing the coefficients of inorganic nitrogenous species (ammonium, nitrite, nitrate, and nitrogen gas) in anammox metabolism between the model-derived equation and the experiment experimentally-observed value provided by Strous et al. (1998), it is obvious that there is a higher agreement between the values, when nitrite is assumed as nitrogen source. Further evidence supporting this hypothesis came from numerous studies, which found that the pH of Anammox system would increase during cultivation (Strous et al. 1998; van de Graaf 1996). Anammox bacteria utilize carbon dioxide as the carbon source and protons would be produced during the conversion. The protons could be consumed and result in pH increase if nitrite is used as the nitrogen source ($4NO_2^- + H_2O + H^+ = NH_3 + 3NO_3^-$). Conversely, pH would be lower if ammonium is utilized as nitrogen source instead ($NH_4^+ + OH^- = NH_3 + H_2O$). Additionally, nitrite reductase was detected in anammox bacteria, which make the conversion of nitrite to ammonia possible (Strous et al. 2006). Accordingly, it can be deduced that nitrite not only served as the electron acceptor and reductant for carbon fixation in anammox metabolism, but also served as the nitrogen source.

Table 2-2 Stoichiometry of modeling results with NH_4^+/NO_2^- as N-source

N source	NH_4^+	NO_2^-	NO_3^-	N_2	References
NH_4^+-N	1	1.2	0.21	0.99	
NO_2^--N	1	1.27	0.26	1.00	-
Experimental	1	1.32	0.26	1.02	Strous et al. (1998)

2.2.6 Long solid retention time for less nitrate formation

According to the stoichiometry of anammox reaction, a large percentage of nitrate, over 10% of the total nitrogen, would be generated due to the use of nitrite as the reductant for cell synthesis (Strous et al. 1998). Undoubtedly, it would not allow the nitrogen removal efficiency to reach more than 90%, which is usually undesirable in anammox applications for wastewater treatment. However, a recent study exhibited that there may be exceptions with a conversion rate of only 2 to 4% to nitrate (Kwak et al. 2012). To decipher the mystery for such a low nitrate formation, Lee et al. (2013a) developed combined stoichiometric equations based on TEEM for overall Anammox metabolism. This supported that only a small fraction of ammonium would be converted into nitrate, in the range of 2% at a relatively long solid retention time (SRT), during a low growth rate. Hence, less amount of reductant, nitrite in the case of Anammox process, would be required for cell synthesis, which results in the lower formation of nitrate. This observation of a low SRT operation would enhance nitrogen removal efficiency over 90%, while generating less sludge.

2.2.7 Inhibition of anammox

Anammox has been widely studied worldwide due to its economic advantages and high nitrogen removal capacity compared to conventional nitrification-denitrification (Van Hulle et al. 2010). During the last two decades, anammox process has been developed

and applied to treat wastewater with high ammonium concentration content from the laboratory to full-scale (Hwang et al. 2005; Kimura et al. 2013; Shalini and Joseph 2012). However, it is restricted by the low growth rate and numerous inhibition factors e.g., substrate, organic matters, etc. (Jin et al. 2012). Similarly, various wastewater types and their impurities contributing to various restrictions in the application of anammox, have been investigated. Here, the inhibiting factors are summarized as follows.

2.2.7.1 Substrates

Although ammonium and nitrite are the substrates for anammox reaction, substrate inhibition should not be ignored. Results displayed that a high ammonia concentration can suppress anammox bacteria (Dapena-Mora et al. 2007; Puyol et al. 2014a). However, molecular ammonia (NH_3) caused by high ammonia and nitrite concentrations at high pH is considered as the key inhibitor rather than ammonium itself due to a relationship between ammonium ions and NH_3 (Fernandez et al. 2012; Aktan et al. 2012). The molecular NH_3 diffusing into the anammox cell, when there is a high pH gradient between extracellular and intracellular membranes, would cause cell lysis (Kadam and Boone 1996). Researchers testified that the threshold molecular ammonia concentration that greatly inhibits the anammox activity is 150 $mg \cdot l^{-1}$, but even when the ammonia and nitrite concentrations are up to 1,500 $mg \cdot l^{-1}$ and 500 $mg \cdot l^{-1}$, respectively, no serious interference to its bacterial activity was found (Aktan et al. 2012). Nevertheless, a firm conclusion of the inhibition threshold value could not be determined, due to the diverse features of various anammox species (Oshiki et al. 2011). Similar to ammonium, biotoxicity of anammox bacterial cells would incur if nitrite reaches up to a threshold concentration (Isaka et al. 2007; Bettazzi et al. 2010). However, the threshold values reported varied from 5-20 mM. Results revealed that ionized nitrite is the main inhibitor of anammox reaction at $pH > 7.1$ when nitrite exists (Puyol et al. 2014b). However, molecular nitrous acid (HNO_2), influenced by pH, is

another factor impeding anammox cells (Fernandez et al. 2012). Thus, pH value is the primary factor influencing anammox significantly. pH beyond neutral conditions is unbeneficial to anammox bacteria owing to the increase of either HNO_2 or NH_3 (Mosquera-Corral et al. 2005). Normally, a feed ratio of ammonium to nitrite ratio close to 1 or slightly lower would be advantageous to anammox reaction.

2.2.7.2 Organic matters

Due to the chemoautotrophic nature of Anammox bacteria, its slow growth rate has remained a challenge for practical application. Additionally, organic carbon comprising in wastewaters would boost the growth of heterotrophic bacteria (denitrifying bacteria) and out-compete anammox bacteria (Chamchoi et al. 2008). Even though the doubling time of heterotrophic bacteria reported are much greater than the one of autotrophic bacteria, anammox bacteria still could out-compete heterotrophic denitrifying bacteria by applying their organotrophic pathway, if the influent C/N could be lower than 2. (Strous et al. 2002; Strous et al. 2006; Kartal et al. 2007b; Guven et al. 2005). Nonetheless, some toxic organic matters e.g., methanol (Isaka et al. 2008), toluene (Martinez Hernandez et al. 2013) and antibiotic (Fernandez et al. 2009) can severely deter anammox bacteria severely. Isaka et al. (2008) exhibited that methanol is not the direct inhibitor, while formaldehyde converted by methanol is the key element due to its toxicity towards enzyme and protein.

2.2.7.3 Other factors

Other factors, e.g., salinity, heavy metals, phosphate and sulfide, influence anammox process negatively. High salinity will cause microbial death due to the rise in cellular osmotic pressure. Heavy metals are toxic to organisms through their bioaccumulation in cells. The existence of sulfate and organics could motivate sulfate to be the electron acceptor for hydrogen sulfide formation, which will be toxic to Anammox cells.

Specifically, the half maximal inhibitory concentration (IC50) of sulfide-S for anammox bacteria is 264 $mg \cdot l^{-1}$ at nitrogen concentration of 200 $mg \cdot l^{-1}$ (Jin et al. 2013). Moreover, the optimum temperature for anammox bacteria is from 30°C to 40°C (Dosta et al. 2008). Higher or lower temperatures would reduce the anammox activity. More studies are needed to understand how to enhance the anammox bacteria activity through minimizing the effect of inhibitors. Several research aspects are suggested: (1) The inhibitor species; (2) The mechanisms of the inhibitors on anammox bacteria; (3) The acclimation of anammox species to tolerate different conditions.

2.2.8 N_2O Emission

Nitrous oxide, which depletes stratospheric ozone and hastens climate change, is produced during conventional nitrification-denitrification (IPCC 2013; Kramlich and Linak 1994). In this regard, controlling its production and emissions from WWTPs garnered significant attention (Kampschreur et al. 2009b). The mechanism of N_2O formation through the conventional nitrification-denitrification lies in reducing nitrite (nitrifier denitrification) and intermediates formation during the oxidation of hydroxylamine (MH_2OH) to NO_2^- (Wunderlin et al. 2013; Scherson et al. 2014). Through this, N_2O emission can alleviate upto 14.6% of the nitrogen load (Kampschreur et al. 2009b). Conversely, due to the lack of heterotrophic denitrifiers in partial nitrification/anammox systems, N_2O emission through these systems could (Desloover et al. 2012; Scaglione et al. 2015). Kampschreur et al. (2009a) reported the N_2O emission from a single-stage nitritation-anammox process was 1.2% of the nitrogen load. Nonetheless, Scaglione et al. (2014) described that approximately 3-15% of nitrogen load was removed as N_2O in the partial nitrification reactor, indicating that a combined system integrated with anammox would be a better alternative to reduce N_2O formation. In another word, N_2O emission can be restricted through the immediate consumption of nitrite owing to higher in-situ anammox activity (Desloover et al. 2012).

Yet, N_2O has also been recognized as a rocket fuel, and thus prompts a concept of N_2O generation for energy production from wastewaters as pursued by Scherson et al. (2014). They successfully generated and collected N_2O from high ammonium content wastewaters, and used it for co-combustion with methane, achieving about 30% more energy yield in comparison with the combustion with O_2 and CH_4.

2.3 Analysis methods

2.3.1 Specific anammox activity

In order to assess the applicability of anammox processes at industrial-scale, it is necessary to estimate the maximum specific anammox activity (SAA) towards different compounds present in industrial effluents. To determinate the conditions of the maximum SAA, nitrogen gas production in batch tests are commonly used as the same concept of anaerobic digestion. Once these conditions were determined, the effects of different compounds present in wastewaters (NH_4^+, NO_2^-, NO_3^-, Na^+, Cl^-, PO_4^{3-}, SO_4^{2-}, S^{2-}, acetate, flocculants, allylthiourea, chloramphenicol) on the maximum SAA could be discovered. The total amount of N_2 gas produced was obtained from the overpressure measured in the headspace of each vial at the end of the assay based on the ideal gas law. The amount of nitrogen removed from the liquid phase was also calculated by measuring the ammonium, nitrite and nitrate concentrations at the beginning and the end of the experiment and taking into account the volume of the liquid phase (Dalsgaard and Thamdrup 2002; Dapena-Mora et al. 2007).

2.3.2 Anammox activity-isotope pairing technique

The isotope pairing technique (IPT) is a well-established ^{15}N approach for estimating denitrification of bottom-water and of NO_x^- produced via sedimentary nitrification. During the past decade, this technique has been used in numerous studies. The IPT aims

to quantify the genuine production rate of N_2 gas, i.e., ^{14}N-N production as it would occur without the addition of $^{15}NO_x^-$. This corresponds to the production rate of $^{28}N_2$ ($^{14}N^{14}N$) times 2 plus the production rate of $^{29}N_2$ ($^{14}N^{15}N$) after addition of $^{15}NO_x^-$ to the system. This production (p^{14}) is estimated from the production rates of $^{29}N_2$ ($p^{29}N_2$) and $^{30}N_2$ ($p^{30}N_2$) using the following expression of Nielsen (1992).

$$p_{14} = \frac{p^{29}N_2}{2 \cdot p^{30}N_2} \cdot \left(2 \cdot p^{30}N_2 + p^{29}N_2\right) \qquad (4.1)$$

The supposition that the genuine production rate of N_2 equals $2 \cdot 28N_2 + 29N_2$ production and that this production can be calculated from Equation 4.1 according to a number of assumptions (Risgaard-Petersen et al. 2003; Sato et al. 2012)

2.3.3 Next-generation sequencing (NGS)

The recent emergence of novel (next generation) sequencing technologies, resulting in higher sequence output and a dramatic drop in the price, defines a new era in metagenomics. NGS is a powerful technique to obtain genomic information of marine Anammox bacteria in oxygen minimum zone (OMZ) ecosystems. The coverage by solid and Ion Torrent was high enough to analyze the important *hzsA* and *hdh* core in Anammox genes. Together with 16S rRNA gene phylogenetic analysis, it was shown that the diversity of the marine Anammox in the Arabian Sea was lower than previously determined by PCR methods (Villanueva et al. 2014).

2.3.2 Fluorescence in-situ hybridization (FISH)

FISH uses fluorescent probes that can bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. Successively, fluorescence microscopy is used to identify the location of fluorescent probe bounded to the chromosomes so that the structure of anammox biofilm can be observed. FISH is

often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification (Schmid et al. 2005).

2.4 Anammox applications

2.4.1 Concentrated wastewaters

Even though anammox process was discovered in the full-scale reactor and has been successfully studied in the laboratory, the bottleneck for its full-scale applications includes the slow growth nature of anammox bacteria, substrates toxicity, accumulation of nitrite, foaming, scaling and sludge retention/setting/solids separation, and other operational problems. Generally, the process of granule or biofilm are encouraged (Fernandez et al. 2008; Figueroa et al. 2012). Besides, organic matters containing in wastewaters may lead heterotrophic bacteria out-competing with anammox bacteria as aforementioned. Furthermore, coupled with the application of anammox systems, sustainable partial nitrification is required in order to provide the electron acceptor for anammox to complete ammonium oxidation into nitrogen gas. This niche could be accomplished by a process termed "Single reactor system for High Activity Ammonium Removal Over Nitrite (Sharon)". It uses high temperature (35^{o}C) and low SRT to enrich the ammonium oxidizers and inhibit the nitrite oxidizers. This process is suitable to treat wastewaters in the meso-temperature range and with high ammonium content such as industrial reject waters (van Dongen et al. 2001). This lays the primarily groundwork for scale-up implementation of anammox.

The first full-scale anammox reaction (70 m^3) was built in Rotterdam, Netherlands (van der Star et al. 2007). However, the start-up time is up to 3.5 years as a result of lack in anammox sludge with its low growth trait. To worsen the situation, several issues occurred during the start-up period: (1) incidental nitrite toxicity; (2) biomass washout; (3) toxic to anammox biomass caused by excess methanol; (4) unexpected operational

problems (van der Star et al. 2007). Nevertheless, this study still managed to treat a flow of 750 $kg \cdot d^{-1}$ which was 50% higher than the design load. Joss et al. (2009) reported that a full-scale partial nitritation and anammox system, treating a high ammonium wastewater, was accomplished with over 90% nitrogen conversion at an ammonium oxidation rate of 0.5 $kg \cdot Nm^{-3}d^{-1}$. Beside the first discovery of anammox in the Netherlands, a full-scale landfill leachate plant operation in Keelung, Taiwan, found that 80% and 27% for total nitrogen and COD removals, respectively, were associated with anammox (Fig. 2-4). This unexpected process, termed as simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD), resulted from a low dissolved oxygen (DO) level of 0.5 $mg \cdot l^{-1}$ caused by the under-estimated aeration supply (Wang et al. 2010). Additionally, this patented process has proven to be reproducible in another landfill leachate plant in Bali, Taiwan, with treatment capacity of 800 CMD and a nitrogen loading rate of 0.6 $kg \cdot Nm^{-3}d^{-1}$ by inoculating the SNAD seed. This process also showed the practicality of treating optoelectronic wastewaters (Daverey et al. 2012, 2013). SNAD alleviates the anammox horizon by integration of denitrification for COD removal in single-stage system.

Over the past decade, many full-scale partial nitrification-anammox facilities were constructed. By 2014, more than 100 facilities were in operation. Among them, over 50% of plants are SBR, 88% of plants are single-stage systems, and 75% of plants are utilized to treat sidestream of municipal wastewaters (Lackner et al. 2014). Most of them focus on treating high ammonium concentration wastewaters, especially reject waters. Lackner et al. (2014) summarized current full-scale anammox systems. Tab. 2-3 provides a summary of the operational information for fourteen of such systems (Adapted from Ni and Zhang 2013; Lackner et al. 2014). These applications of full-scale anammox processes have shown their commercial feasibility.

Fig. 2-4 The full-scale anammox reactor in Taiwan treating landfill leachate (a) Aeration tank; (b) suspended anammox biomass; (c) attached anammox biomass (Wang et al. 2010).

Table 2-3 Scale-up anammox systems (Adapted from Ni and Zhang 2013; Lackner et al. 2014).

Location	Influent	Reactor volume·m^{-3}	Designed load·$kgNd^{-1}$	Operational year
Rptterdam, NL	Reject water	72	750	2002
Lichtenvoorde, NL	Tannery	100	150	2004
Olburgen, NL	Potato processing	600	700	2006
Mie prefecture, JP	Semiconductor	50	220	2006
Stockholm, SE	Reject water	1,400	600	2007
Niederglatt, Switzerland	Reject water	180	60	2008
Tongliao, China	Monosodium glutamate (MSG)	6,600	1,100	2009
Yichang, China	Yeast production	500	1,000	2009
NL	Reject water	425	600	2010
Tai'an, China	Corn starch and MSG	4,300	6,090	2011
Poland	Distillery	900	1,460	2011
Wuxi, China	Sweetener	1,600	2,180	2011

Shaoxing, China	Distillery	560	900	2011
Pfannenstiel, CH	Reject water	320	75	2012
Breda, NL	Reject water	1,000	990	2013
Grindsted, DK	Reject water	140	100	2013

2.4.2 Dilute wastewaters (Mainstream)

Although previous studies of anammox related systems have mostly emphasized on the treatment of high ammonium content wastewaters, the potential for extending process application to dilute wastewaters, particularly for sewage, has received increasing attention recently (Abbassi et al. 2014; Kartal et al. 2010). Anammox bacteria has been found in various environments under a wide temperature range, providing the practicability with dilute wastewaters (Rysgaard and Glud 2004; Byrne et al. 2009; Hu et al. 2011; van de Vossenberg et al. 2008). Yet, the challenges of ambient temperature and low ammonium concentration within dilute wastewaters are the limiting factors for nitritation (Dosta et al. 2008; Vazquez-Padin et al. 2011). Furthermore, dilute influents make it challenging to maintain a sustainable anammox biomass population in this process. Plus, short hydraulic retention times (HRTs) are required to meet with the conventional nitrification-denitrification. Moreover, heterotrophic bacteria competition should be a concern with organic carbon matters exiting in dilute wastewaters.

To this end, completely autotrophic nitrogen removal over nitrite (CANON), SHARON-anammox, Denitrifying ammonium oxidation (DEAMOX) have been studied with dilute wastewaters or sewage (Nozhevnikova et al. 2012; Schmidt et al. 2003). These studies showed the importance of DO control for nitrite formation so that completion nitrogen removal could be further carried out by anammox. If too little DO is delivered, incomplete ammonium oxidation would occur. However, if too much DO is supplied, nitrifiers may out-compete the Anammox organisms, resulting in excess

oxidization of ammonia to nitrate (Li et al. 2011). In both cases, total nitrogen removal efficiency would be hindered. Lee et al. (2013b) reported that a robotic DO supply strategy in a membrane biofilm reactor (MBfR) at 20°C treating synthetic diluted wastewater without organic matters. This system accumulated about 88% to 94% nitrite when feeding at a stoichiometric mole ratio of 1.5 mol O_2/ammonium fed. This research sets the foundation for sustainable nitritation by providing a benchmark oxygen control for diluted wastewaters. Based on this finding, they applied the same oxygen benchmark strategy in a single-stage nitrogen removal biofilter (NRBF). Through close control of the oxygen to ammonia ratio of 0.75 mol O_2/ammonium fed, higher than 90% nitrogen can be removed from dilute wasters at ambient temperature and with hydraulic retention times as short as one-hour HRT (Kwak et al. 2012). These studies indicate that DO control strategy for autotrophic removal of nitrogen from dilute wastewaters is a promising strategy.

Hendrickx et al. (2012) treated synthetic wastewater of 69 mg-N·l^{-1} (NH_4^++NO_2^-) at a loading rate of 0.31 g-N·(l·d)$^{-1}$ in a 4.5-l gas-lift reactor. In this study, a net anammox bacteria growth rate of 0.040 d^{-1} at 20°C and less than 0.2 mg-N·l^{-1} (NH_4^++NO_2^-) of effluent concentrations were reported. Anammox activity was maintained at a COD/N ratio up to 0.5 and ambient temperatures (18±3°C) in a lab-scale anoxic/aerobic reactor, meaning anammox bacteria successfully competed with heterotrophic bacteria (Winkler et al. 2012). Anammox granules can be formed in UASB reactor at 30°C, and even when the temperature decreased to 16°C, the sludge granules can still be maintained, which contributed to a high nitrogen removal rate (NRR) treating low strength wastewater (Ma et al. 2013). Anammox bacteria occurred in oxygen-limited autotrophic nitrification denitrification (OLAND) process and DO could greatly affect the anammox community when treating low strength nitrogen wastewater (ammonium concentrations from 66 to 29 mg-N·l^{-1}) (De Clippeleir et al. 2011). Ma et al. (2011) stated that 88.38% removal efficiency for total nitrogen was obtained in one nitritation

reactor and one anammox reactor for treating sewage containing 44.4mg·l^{-1} ammonium and 44.4 mg·l^{-1} soluble COD at HRT of 4.6 h and 27-30^{o}C. However, the temperature of wastewater is a critical problem for anammox application for mainstream treatment. Even though the optimum temperature for anammox is described from 30-40^{o}C (Dosta et al. 2008), Gilbert et al. (2014) found that anammox activity for low strength wastewater is not severe in a biofilm reactor, using 10 mm carriers, when temperature is dropped from 20^{o}C to 13^{o}C, Furthermore, Huang et al. (2013) made an important discovery that anammox bacteria can change the feeding intensity with the oxygen supply control when partial nitrification and anammox are carried out in a single-stage reactor for diluted wastewater treatment. We believe that these results will help stimulate the greater application of this energy-efficient nitrogen removal process for concentrated as well as dilute wastewaters.

2.5 Summary

Anammox has received an increased interest due to its sustainable characteristics. This chapter provides detailed information about anammox technologies including its relation to the nitrogen cycle, anammox species, molecular analysis techniques, scale-up applications, and commercialization potential. With growing population and climate change along with diminishing energy, energy-efficient anammox systems offer a promising nitrogen pollution alternative to meet lower energy consumption, no requirement of an organic donor, N_2O emission reduction, and lower sludge handling costs. Furthermore, it is conceived that anammox's involvement in syntrophy for SRAO may plays a significant role on global Nitrogen-Sulfur cycle.

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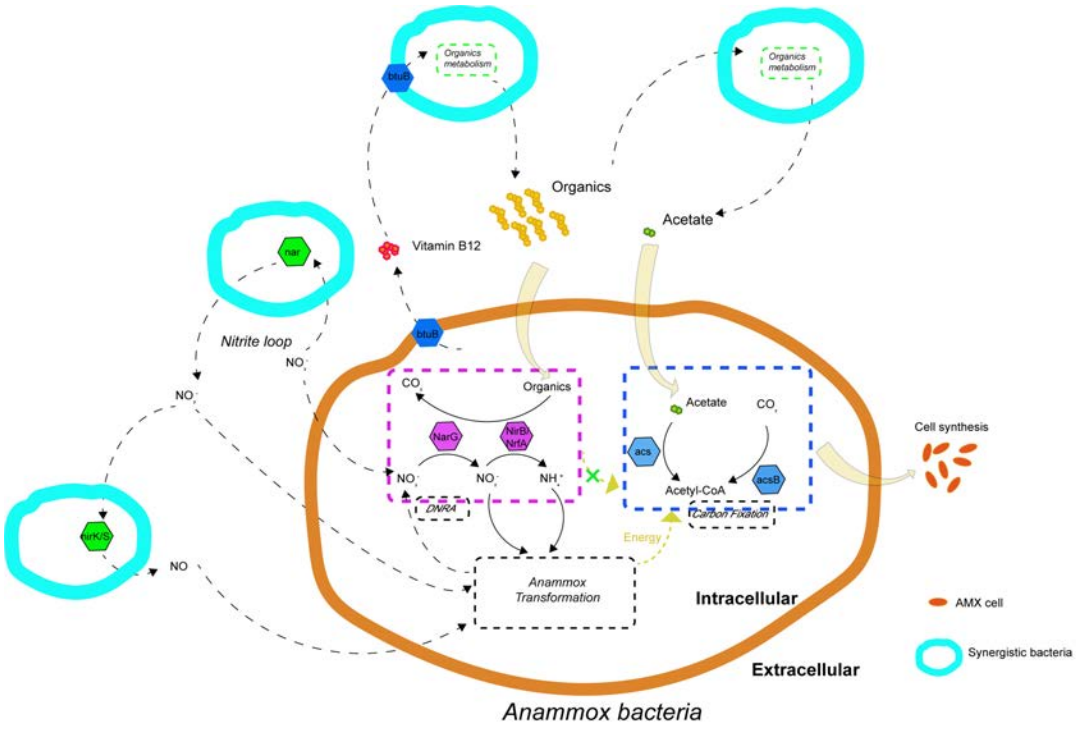
Chapter 3 Genome-based insights into anammox bacteria and microbial interaction in a partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor

GRAPHIC ABSTRACT



3.1 Introduction

Mainstream anaerobic ammonium oxidation (anammox) coupled with partial nitrification, normally regarded as single-stage PN/anammox (PN/A) process, is of great interest due to its energy-efficient nature for nitrogen removal. This process requires less oxygen consumption for nitrification, no need for organic carbon and lower sludge production, comparing to conventional nitrification-denitrification processes (De Cocker et al. 2018, Lackner et al. 2014). Despite these advantages, the slow growth rate of anammox bacteria and critical operation conditions, such as dissolved oxygen concentration, sludge retention time (SRT) and nitrogen loading rate etc., increased its difficult for the mainstream wastewater treatment application (Jin et al. 2012, Strous et al. 1999). Recently, researchers have focused on the rapid start-up of single-stage PN/A process through investigating operational conditions and reactor structures rather than the metabolisms in individual anammox bacteria and microbial interaction(Gilbert et al. 2014, Kwak et al. 2012, Laureni et al. 2016, Li et al. 2017a, Lotti et al. 2014, Persson et al. 2017a Yeshi et al. 2016). The metabolisms in anammox bacteria possibly impact the startup of anammox systems since they are related to the cell synthesis and energy harvest of anammox bacteria. Till now, it has been observed that anammox bacteria obtain energy from anammox transformation (energy for cell synthesis) and use CO_2 as carbon source for cell synthesis. Additionally, the phenomenon of microbial interaction has been demonstrated in anammox consortia, which proposed that anammox bacteria might benefit from this cooperation. Even so, further information is needed regarding the potential metabolisms in anammox bacteria and their related microbial interaction.

In general, anammox bacteria, nitrifying bacteria and other uncultured organisms affiliated to the phyla of *Proteobacteria, Bacteroidetes, Chlorobi*, and *Chloroflexi* comprised the dominant parts of microbial community in single-stage PN/A bioreactors (Speth et al. 2016). Speth et al. (2016) and Lawson et al. (2017) revealed the possible

microbial interaction, such as nitrite loop, vitamin B12 and amino acid exchange, between heterotrophic organisms and anammox bacteria in anammox systems treating real sewage with high concentration of ammonium. Recently, anammox species, such as *Ca. Brocadia fulgida* and *Ca. Anammoxoglobus propionicus*, were reported that it could also utilize organic carbon as electron donor for nitrate reduction, termed as dissimilatory nitrate reduction to ammonium (DNRA) (Kartal et al., 2007 & 2008). Anammox bacteria possibly uses the organic carbons released by the heterotrophs for DNRA process, such as EPS or soluble microbial products (SMP) from cell decay (Winkler et al., 2012b). In turn, heterotrophs can also use the extracellular polysaccharides secreted by anammox bacteria as carbon or energy sources. Specially, the nitrogen exchange based on different forms among the microbial community could improve the reactor performance, especially in single-stage PN/A systems. Nitrate is produced not only by anammox reaction, but also by nitrite-oxidizing bacteria (NOB) since NOB generally present in single-stage PN/A systems. In the complex microbial ecosystem, nitrate can be reduced back to nitrite by some heterotrophs affiliated to the phyla *Chlorobi* and *Acidobacteria,* and the produced nitrite can act as substrates for anammox reaction. It proposed one hypothesis that if anammox bacteria could perform DNRA pathways, it not only improves the nitrogen removal performance but also enhances the energy generation by anammox bacteria for cell synthesis. Therefore, anammox bacteria can benefit from its possible metabolisms and the microbial interaction, which possibly supported its rapid accumulation.

In this study, we performed a single-stage PN/A fluidized-bed bioreactor (PN/A FBR) for low-strength synthetic wastewater treatment, which inoculated with a locally conventional activated sludge. The objective of this study was to explore the possible functional content in the enriched anammox bacteria and revealed the microbial interaction functioned on its metabolism. The changes in microbial community in the suspended sludge during the operational period was investigated using 16S rRNA

sequencing techniques. The draft genomes of the organisms in the floc and biofilm at the end of this study were separately recovered using metagenomic sequencing techniques. Possible functional genes in anammox bacteria related to heterotrophic ability and nitrogen transformation were investigated. Our results provide deeper insights into the potential functions in anammox bacteria and the beneficial microbial interaction for anammox bacteria, which will support the rapid startup of single-stage PN/A process.

3.2 Materials and Methods

3.2.1 Reactor system and operational strategy

A lab PN/A fluidized-bed bioreactor (PN/A FBR) with an effective working volume of 2.0 L (diameter: 70 mm, height: 520 mm) was set up in this study (Fig. S3-1). Granule activated carbon (GAC) particles (MRX-M, Calgon Carbon Corp., Pittsburg, PA) with 0.2 mm diameter were inserted in the reactor for biomass cultivation. The total volume of GAC placed in the reactor was 500 cm^3 and was fluidized 50% by the recirculated water through a magnetic pump. The PN/A FBR was operated continuously at room temperature with an up-flow mode by maintaining hydraulic retention time (HRT) of 4.5 hours. DO concentration was auto-controlled by a DO Proportional-Integral-Derivative (PID) controller (Burkert dissolved oxygen control system, Taiwan). The influent pH was adjusted to 8.0 and not controlled during operation. The reactor was inoculated with conventional activated sludge collected from the Shek Wu Hui Sewage Treatment Works in Hong Kong with an initially mixed liquid suspended solids (MLSS) concentration of 10,000 mg/L. The operational conditions and compositions of the synthetic wastewater were available in the supplementary information (Tab. S3-1).

3.2.2 Analytical methods

The collected influent and effluent water samples were filtrated (0.45 μm) before analysis. Concentrations of ammonium (NH_4^+-N), nitrite (NO_2^-–N) and nitrate (NO_3^-–N) were measured according to the standard methods (APHA 2005). The DO concentration and water temperature was monitored by a DO Proportional-Integral-Derivative controller. The pH was done using a pH meter (Leici Company, China).

3.2.3 Sludge sample collection and DNA extraction

For suspended sludge, five samples were collected during the operational period (Day 28, 45, 73, 106, and 220) for DNA extraction. For each sample, 200 ml of mixed liquor was collected from the mid-depth of the reactor and centrifuged to obtain the biomass. For biofilm attached on GAC, one sample was collected on day 220 for DNA extraction. Approximate 10g GAC was taken out from the reactor and washed by deionized (DI) water to remove impurities before biomass collection. Next, the biomass was washed down from GAC by ultrasound. DNA extraction was performed using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufactures' instructions. The DNA concentration of each sample was further measured using NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE). It should be pointed out that the biomass sample in biofilm was not collected before Day 220 due to the negative effect of sampling on the operation.

3.2.4 16S rRNA sequencing analysis

The overall microbial community analysis steps through high-throughput amplicon sequencing were described as by Leng et al. (2017) with some modifications. Specifically, 341F: ACTCCTACGGGAGGCAGCAG; 806R: GGACTACHVGGGTWTCTAAT were used as barcoded PCR sequencing primers.

The pair-end MiSeq reads were assembled into contigs with removing ambiguous N bases and those reads length shorter than 440 bps. The filter contigs were processed using MOTHUR (v1.36.1) to align with the V3-V4 region of the SILVA reference database. Chimera sequences identified with UCHIME were removed before operational taxonomic units (OTUs) clustering. The obtained OTUs (97 %) were used for downstream analyses.

3.2.5 Metagenome sequencing analysis

Two DNA samples (Suspended sludge on day 220; Biofilm on Day 220) were selected and sent to BGI company (Shenzhen, China) for metagenome sequencing using the Illumina HiSeq platform. The raw reads were trimmed to remove adapters and low-quality reads. The obtained clean reads were assembled using SPAdes Genome Assembler (ver. 3.5.0) by setting kmer parameters of 19,33,47,61,75 (Nurk et al. 2013). The quality of assembled metagenomes was evaluated using QUAST (Gurevich et al. 2013). Binning was performed to organize metagenome sequences into bins using MaxBin (Wu et al. 2014) and further verified with CheckM (Parks et al. 2015) to evaluate the quality and completeness of draft genomes. Prokka was used for genome annotation (Seemann 2014). BLASTP was performed to align the protein-coding sequences in anammox bins with bacterial protein database obtained from National Center for Biotechnology Information (NCBI) (Camacho et al. 2009). To identify the draft genomes recovered from the PN/A FBR, phylogenetic tree was built using PhyloPhlAn based on reference genomes available in Joint Genome Institute (JGI) and NCBI database (Segata et al. 2013). The 16S rRNA raw data has been submitted to the NCBI repository with SRP136426. Additionally, raw DNA sequences can be found on the NCBI with accession number PRJNA445626.

3.3 Results

3.3.1 Reactor performance

The operational performance of PN/A FBR over a time of 220 days is shown in Fig. 3-1. During the whole period, the influent NH_4^+-N concentration was gradually decreased from 120 to 70 mg/L with DO concentration decreasing from 1.3 to 0.7 mg/L. DO concentration was initially set at 1.3 mg/L during the first 16 days for a rapid start-up of partial nitrification. Obvious nitritation phenomenon occurred with the startup of the reactor and nitrite was over-accumulated in the effluent on day 16, which was approximate 4 times of the residual NH_4^+-N concentration. This result suggested that the oxygen was over supplied in the PN/A FBR. Next, DO concentration was reduced from 1.3 mg/L to 0.5 mg/L, which resulted the NO_2^--N/NH_4^+-N ratio lower than 1.0 from day 16 to 21. Therefore, DO concentration was further adjusted to 0.7 mg/L, consequently, a stable partial nitrification performance with a NO_2^--N/NH_4^+-N ratio of 1:1 was maintained (Fig. 3-1a). From day 23, the influent NH_4^+-N concentration was reduced from 120 to 60-70 mg/L, however, the effluent ratio of NO_2^--N/NH_4^+-N kept at 1:1. Meanwhile, an obvious total nitrogen loss phenomenon appeared from day 25 (Fig. 3-1b) and the total nitrogen removal efficiency (TNRE) reached 80% on day 106, representing a nitrogen removal rate (NRR) of 0.31 kg/(m^3•d). Later, the influent NH_4^+-N concentration was further decreased to 50 mg/L from day 115 to 220 progressively, which resulted in a drop of TNRE from 76% to 53%. An NRR represented for 0.21 kg/(m^3•d) was obtained at the end of the study. The effluent NO_3^--N concentration increased slowly and reached a concentration of 9.34 mg/L at the end of the study. Notably, part of NH_4^+-N (around 15 mg/L) remained as the residual TN in the effluent. Therefore, during the start-up of the PN/A process, it is likely that the ratio of influent NH_4^+-N to supplied DO concentration was a critical factor (Kwak et al. 2012, Lee et al. 2013). In any event, even without the optimal oxygen to ammonia ratio, over 55% total nitrogen loss with a considerable abundance of anammox species (4.70%) was achieved

within 73 days (Fig. 3-2b). Furthermore, the nitrate concentration in the effluent was relatively low (9.34 mg/L), even the relative abundance of nitrite-oxidizing bacteria (NOB) reached 29.11% and 33.73% in suspended sludge and biofilm (Fig. 3-2b), respectively. The occurrence of NOB may be caused by the long sludge retention time (SRT) due to no discharging of sludge from the reactor during whole operation (Ni et al. 2014). However, the ratio of NO_3^--N/NH_4^+-N was close to the stoichiometric value of 0.11 on the basis of single-stage PN/A process (Vlaeminck et al. 2012), indicating the successful startup of PN/A. Due to the drop of pH value from 7.5 to 5.4 in this study, oxygen-consuming bacteria such as AOB and NOB were implicitly inhibited, resulting in the residual NH_4^+-N in the effluent (Yin et al. 2016).

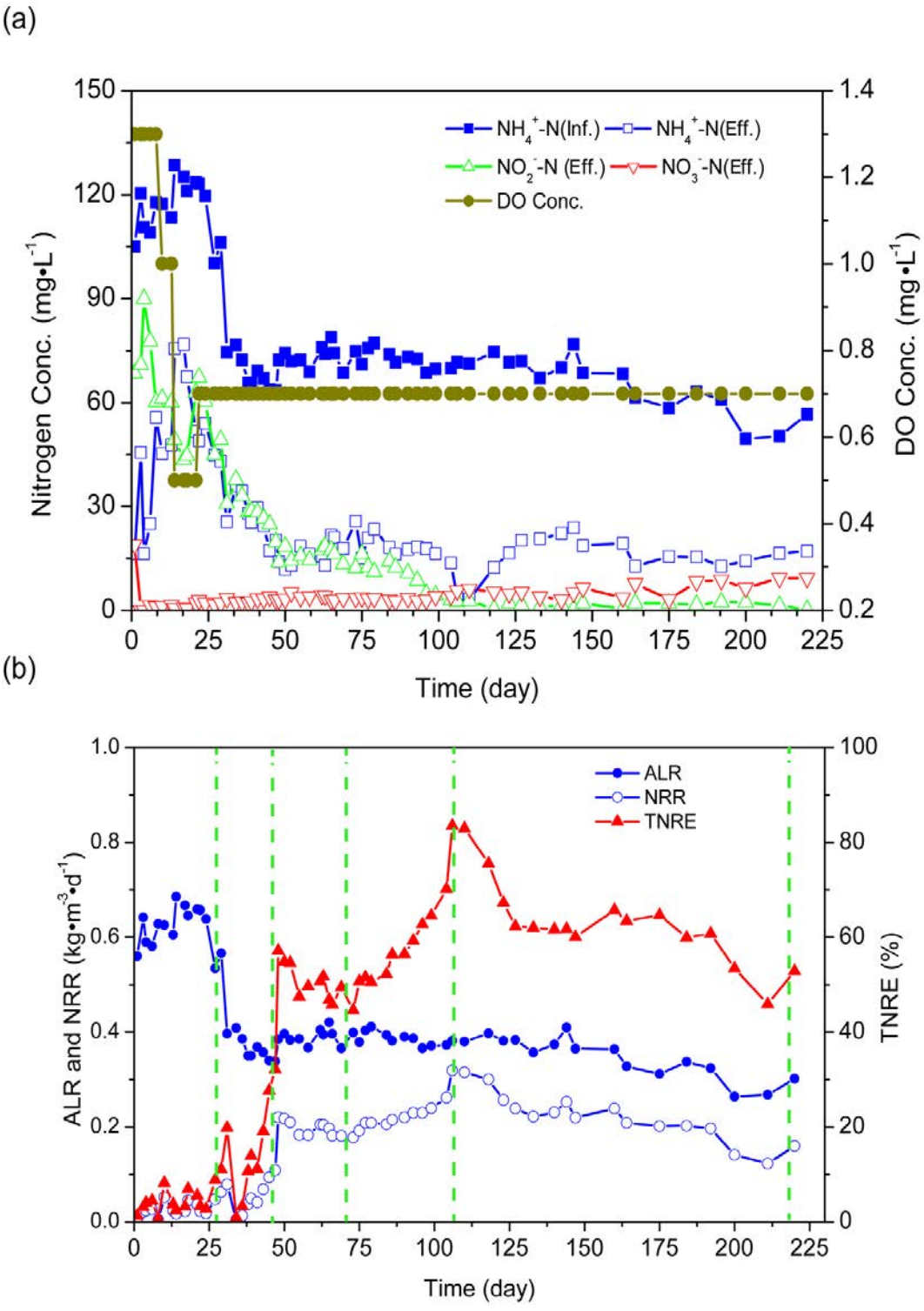


Fig. 3-1 The performance of nitrogen removal at PN/A conditions. (a): Variation of nitrogen compounds; (b): Total nitrogen removal efficiency (TNRE). ALR: Ammonium loading rate; NRR: Nitrogen removal rate. The green dash line represents the day of biomass sampling for DNA extraction.

3.3.2 Microbial community by 16S rRNA sequencing

The overall microbial communities during the operational period were obtained from 16S rRNA gene amplicon sequencing (Fig. 3-2 and Tab. S3-2). Eight phyla of bacteria with high abundance (relative abundance higher than 1% in at least one of six samples) are illustrated (Fig. 3-2a). *Proteobacteria* and *Bacteroidetes* were the most dominant phylum during the start-up of partial nitrification phase, accounting for more than 93.9% of total sequence reads in floc. However, the relative abundance of *Proteobacteria* decreased from 74.33% to 22.40% in floc with the startup of PN/A process. *Planctomycetes* became one of dominant phylum when the reactor reached steady state, accounting for 11.84% (suspended sludge on day 220) and 27.06% (biofilm on day 220). The phyla *Acidobacteria* was also enriched in floc with a relative abundance of 6.53% on day 220, while its relative abundance in the biofilm was lower than 1%. Different from the previous studies, *Chloroflexi* that was able to metabolic organic carbon derived from cell lysis was not enriched greatly in floc and biofilm (1.19% and 0.26%), respectively (Cao et al. 2016, Kindaichi et al. 2012). The phyla of *Firmicutes* and *Actinobacteria* were washed out from the reactor (Fig. 3-2a).

As expected, the co-existence of AOB and anammox bacteria was identified in floc and biofilm (Fig. 3-2b). AOB affiliated to *Nitrosomonas* with the relative abundance of 7.66% and 14.39% in floc and biofilm was detected on day 220, respectively. Anammox bacteria affiliated to *Ca. Brocadia* was enriched in the suspended sludge (11.18%) and biofilm (26.94%) on day 220, respectively, which accounted for almost all of *Planctomycetes* (Fig. 3-2b). It should be noted that a significant relative abundance (4.70%) of *Ca. Brocadia* was obtained even in the suspended sludge under a DO concentration of 0.7 mg/L on day 73. However, no anammox bacteria was detected in the inoculum activated sludge (data not provide).The enriched *Ca. Brocadia* belongs to single OUT cluster in this study, which is different from other studies (Li et al. 2017b, Park et al. 2015).

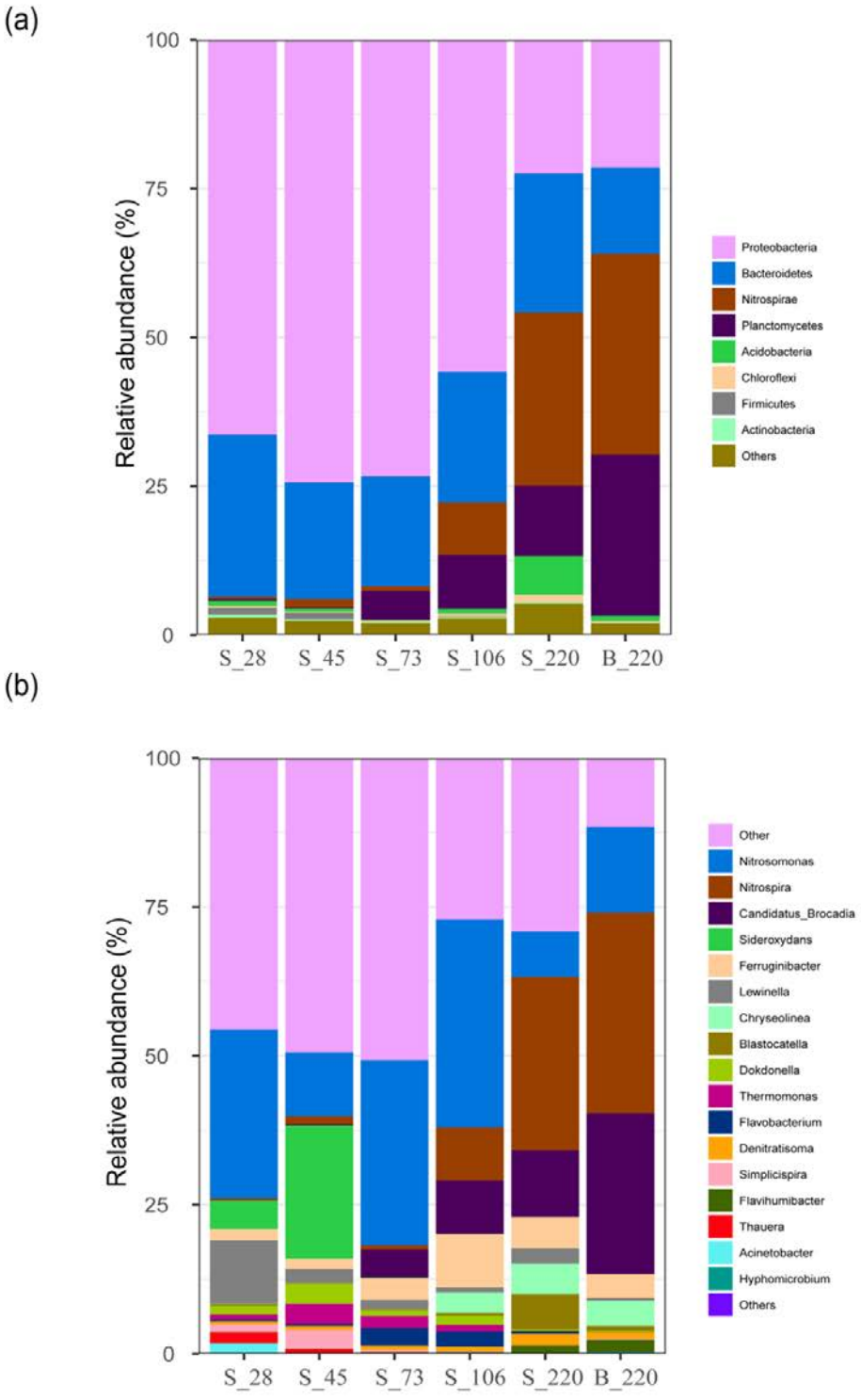


Fig. 3- 2 Taxonomic classification of microbial populations in the suspended sludge and biofilm at the phylum and genus level by 16S rRNA sequencing. S_28: suspended sludge on Day 28, S_45: suspended sludge on Day 45; S_106: suspended sludge on Day 106; S_220: suspended sludge on Day 220; B_220: biofilm on Day 220.

3.3.3 Microbial community analysis by metagenomics

Due to the coexistence of suspended sludge and biofilm in the PN/A FBR, we sequenced suspended sludge and biofilm separately and provided a comprehensive insight into the function of the recovered genomes in each phase. The microorganisms enriched in suspended sludge also played an important role in reactor performance since GAC was only filled 25% volume of the reactor. Whole-genome metagenomic analysis of two biomass samples (suspended sludge and biofilm on day 220) yielded 39,472,841 and 44,192,804 bp data after quality filtering. Assembly of the obtained clean reads from each sample generated a total of 572,897 and 370,801 contigs with N50 of 1828bp and 2653bp, respectively. Contigs were binned into population genomes and yielded 17 and 19 high-quality draft genomes (completeness > 90% and contamination < 10%), accounting for 46.09% and 46.55% of the filtered reads in floc and biofilm, respectively (Tab. 3-1 and Tab. S3-3). These genomes were affiliated to the phyla *Planctomycetes*, *Bacteroidetes, Nitrospirae*, *Acidobacteria*, and *Proteobacteria* (Tab. 3-1 and Fig. S3-2), representing a major fraction of microbial communities in the suspended sludge and biofilm. Phylogenetic tree between the recovered population genomes and reference genomes were constructed for the bacterial species identification. It illustrated that most of the recovered genomes in suspended sludge and biofilm sharing a high similarity (Fig. 3-3). Additionally, the nitrogen mark genes in recovered genomes were depicted in Fig. 3-4. Most of the recovered genomes belonged to the same phyla in anammox systems reported by Speth et al. (2016) and Lawson et al. (2017).

Table 3-1 Genome information of bins recovered from the PN/A FBR

Sample	Bin ID	Phylum	Completeness (%)	Contamination (%)	Number of contigs	Genome size (bp)	N50 (scaffolds)	GC (%)	Predicted genes
Floc	S_AMX	*Planctomycetes*	100	2.75	199	3365571	34962	42.39	3054
	S_AOB1	*Proteobacteria*	96.84	1.21	192	2778904	50637	51.39	2679
	S_AOB2	*Proteobacteria*	99.84	7.97	178	3428308	72792	48.00	3296
	S_NOB	*Nitrospirae*	95.50	3.18	135	4195058	97865	40.21	4121
	S_PRO1	*Proteobacteria*	99.88	1.75	113	4674595	99927	67.14	4009
	S_PRO2	*Proteobacteria*	95.42	0.99	322	2546225	12544	68.78	2576
	S_PRO3	*Proteobacteria*	98.62	4.88	248	2894147	31017	61.27	3007
	S_ACD1	*Acidobacteria*	94.02	1.71	104	3062164	68645	56.10	2865
	S_ACD2	*Acidobacteria*	96.58	5.21	167	4266624	164452	51.96	4033
	S_BCD1	*Bacteroidetes*	99.98	1.09	60	4052975	294533	46.18	3564
	S_BCD2	*Bacteroidetes*	94.84	1.99	259	3834309	29859	48.65	3450
	S_BCD3	*Bacteroidetes*	96.67	1.93	200	3269514	32261	38.39	2755
	S_BCD4	*Bacteroidetes*	98.27	7.59	305	4883603	43077	43.76	4620

	S_BCD5	*Bacteroidetes*	95.43	1.01	224	2820473	26963	40.23	2557
	S_BCD6	*Bacteroidetes*	99.01	5.34	219	3493162	45913	37.29	3137
	S_BCD7	*Bacteroidetes*	94.63	3.37	451	3573352	13140	50.42	3398
	S_BCD8	*Bacteroidetes*	97.85	1.25	236	3251543	26216	63.35	2900
Biofilm	B_AMX	*Planctomycetes*	100	4.94	168	3027363	34257	42.22	2802
	B_AOB	*Proteobacteria*	99.04	7.81	316	3995048	57007	48.68	3872
	B_NOB	*Nitrospirae*	88.22	1.54	88	3815059	85901	3720	3710
	B_PRO1	*Proteobacteria*	94.81	0.91	49	2605424	114497	58.90	2480
	B_PRO2	*Proteobacteria*	98.28	1.92	122	2732373	91085	61.42	2743
	B_PRO3	*Proteobacteria*	92.26	0.64	152	5548036	64180	61.31	4997
	B_PRO4	*Proteobacteria*	91.89	5.65	161	2510569	39555	58.64	2538
	B_PRO5	*Proteobacteria*	98.90	5.00	174	6348307	104045	67.68	5326
	B_PRO6	*Proteobacteria*	94.62	4.25	1462	7485915	7158	69.67	7753
	B_BCD1	*Bacteroidetes*	96.55	1.51	289	3015131	24784	40.11	2766
	B_BCD2	*Bacteroidetes*	92.35	0	29	3500712	299630	46.30	3053
	B_BCD3	*Bacteroidetes*	95.63	0.82	89	3851765	154746	43.64	3439

	B_BCD4	*Bacteroidetes*	94.53	4.41	426	3660717	15382	50.38	3459
	B_BCD5	*Bacteroidetes*	95.57	1.81	306	3603881	24217	38.38	3336
	B_BCD6	*Bacteroidetes*	97.17	1.74	279	3524898	22910	50.53	3246
	B_BCD7	*Bacteroidetes*	99.51	0.49	137	3286823	53299	37.14	2919
	B_BCD8	*Bacteroidetes*	99.19	0.54	212	3331173	31671	63.32	2939
	B_BIO1	*Candidate* Phylum	92.50	1.76	926	3955668	5731	56.30	3467
	B_BIO2	*Candidate* Phylum	91.97	1.04	578	2616139	6208	45.52	2406

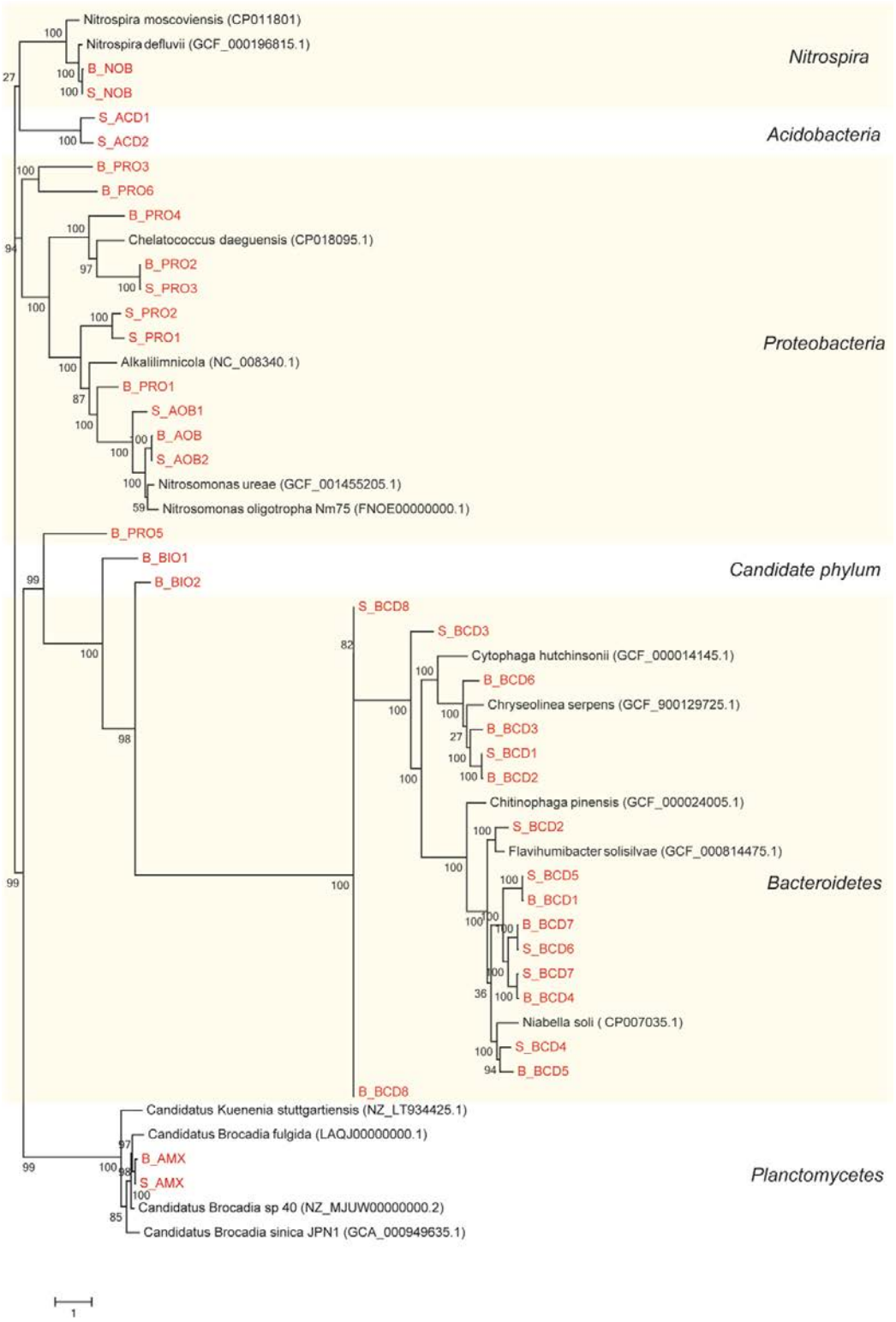


Fig. 3-3 Phylogenetic tree of the recovered genomes from the PN/A FBR. Genomes in red were obtained in this study and the reference genomes with access numbers were downloaded from the NCBI database. The tree was constructed using PhyloPhlAn based on the whole-genome sequence information.

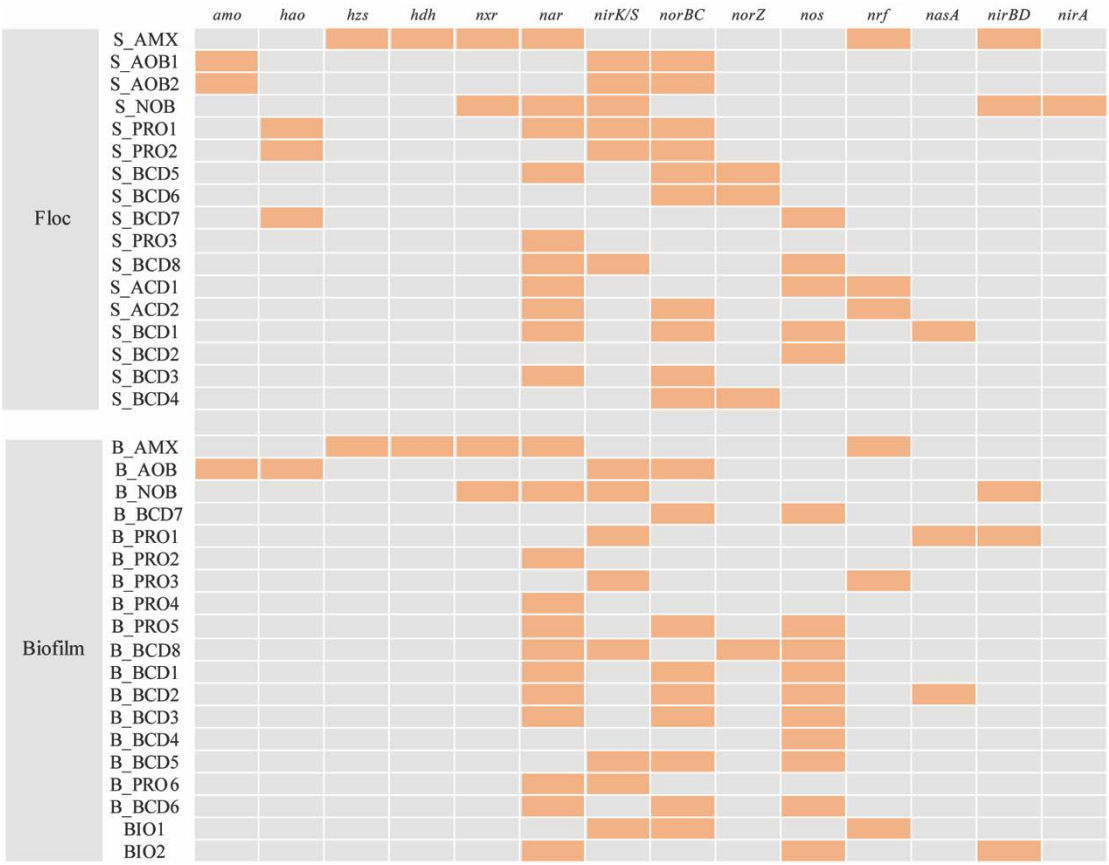


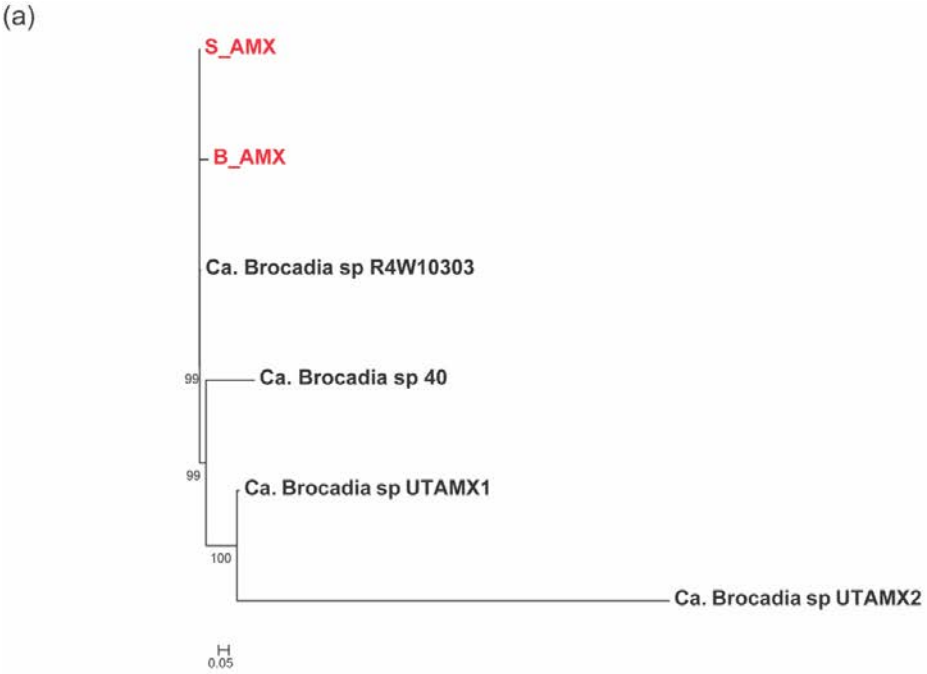
Fig. 3-4 Nitrogen cycle marker genes from the draft genomes recovered from floc sludge and biofilm. Orange indicates the presence and light grey indicates the absence.

3.3.4 Anammox metagenomic insights

High abundance of anammox species (4.70%-26.96%) affiliated to *Ca. Brocadia* was detected in the suspended sludge and biofilm on day 75, 106 and 220. The draft genomes affiliated to the strain of *Ca. Brocadia sp.* (S_AMX and B_AMX) was assembled in suspended sludge and biofilm, respectively. The protein numbers in the recovered anammox genomes were less than 3,000, which was similar to the draft genome of *Ca. Brocadia sp. 40* (Ali et al. 2016). Phylogenetic tree between the reference *Ca. Brocadia sp* genomes: (1) *Ca. Brocadia sp R4W10303* (PRJNA383754), (2) *Ca. Brocadia sp 40* (SAMN05817825), (3) *Ca. Brocadia sp UTAMX1* (SAMN06342761), (4) *Ca. Brocadia sp UTAMX2* (SAMN06342762) and the two

recovered anammox genomes was constructed using PhyloPhlAn (Fig. 3-5a). As shown in Fig. 3-5, the recovered anammox genomes were most similar to *Ca. Brocadia sp R4W10303*. Besides, these two anammox draft genomes were recovered in the same reactor and they shared a high similarity with an average nucleotide identity (ANI) of 99.69% and genome alignment fraction (AF) of 88.24%. Therefore, the recovered anammox genomes in this study were possibly belonging to the same strain of *Ca. Brocadia sp*.

(a)



(b)

	S_AMX	B_AMX	Ca. Brocadia sp R4W10303	Ca. Brocadia sp UTAMX1	Ca. Brocadia sp UTAMX2	Ca. Brocadia sp 40
S_AMX	✳	99.69 (88.24)	99.85 (94.13)	96.22 (83.26)	80.38 (53.62)	98.67 (81.94)
B_AMX	99.92 (97.36)	✳	99.86 (94.76)	96.19 (83.94)	80.07 (53.91)	98.61 (82.11)
Ca. Brocadia sp R4W10303	99.96 (97.73)	99.77 (88.84)	✳	96.23 (85.94)	80.21 (56.16)	98.74 (84.57)
Ca. Brocadia sp UTAMX1	96.45 (87.32)	96.26 (79.77)	96.43 (86.84)	✳	79.78 (55.96)	96.23 (79.53)
Ca. Brocadia sp UTAMX2	80.33 (52.98)	80.10 (48.10)	80.36 (52.96)	79.92 (51.86)	✳	80.23 (49.82)
Ca. Brocadia sp 40	98.90 (93.50)	98.77 (84.53)	98.93 (92.71)	96.16 (86.36)	80.11 (58.32)	✳

Fig. 3-5 (a) Phylogenetic tree indicating the relationships within the recovered anammox-related genomes and published strains of *Ca. Brocadia sp* using PhyloPhlAn. (b) Average nucleotide identity (ANI) and genome alignment fraction (AF) calculation based on BLAST+. The reference anammox genomes were obtained from NCBI and JGI database.

Previous studies confirmed that nitric oxide−nitrite oxidoreductase (*nir*S) was annotated in *Ca. Kuenenia* and *Ca. Scalindua* and copper-containing nitrite reductase (*nirK*) was found in *Ca. Jettenia*, while *Ca. Brocadia* might employ another unclassified nitrite reductase for nitrite reduction in anammox reaction(Hira et al. 2012, Lam et al. 2009, Li et al. 2011, Speth et al. 2015). In the S_AMX and B_AMX, genes encoding *nirS* or *nirK* were missing. Hydrazine synthase subunit (*hzs*) the key gene involving in anammox reaction, which could be used as the mark gene for anammox bacteria identification. In this study, gene encoding *hzsC* was annotated in S_AMX and B_AMX (Tab. S3-4 and S3-5), respectively. Genes encoding hydroxylamine dehydrogenase (*hao*-like), which was considered as similar function as gene hydrazine dehydrogenase (*hdh*) (Lawson et al. 2017), were also recovered in S_AMX and B_AMX (Tab. S3-4 and S3-5). These results suggest the recovered S_AMX and B_AMX belong to anammox species.

Interestingly, the major genes involving in DNRA pathway were recovered in the recovered anammox genomes. Specially, genes encoding nitrate reductase alpha subunit (*narG*) responsible for nitrite production from nitrate, nitrite reductase (NADH) large subunit (*nirB*) and cytochrome c-552 precursor (*nrfA*) acting on nitrite reduction to ammonium were observed. Much like this study, gene *narG* was also observed in *Ca. Kuenenia stuttgartiensis CH1*, *Ca. Brocadia sinica JPN1*, *Ca. Brocadia sp. 40*, and *Ca. Jettenia caeni*. Gene *narG* can be classified into four forms (Fig. 3-6d): two forms for heterotrophs, one form for archaea and one form for anammox bacteria. The phylogenetic distance illustrated that the evolution position of gene *narG* in anammox bacteria was different from other bacterial species, such as denitrifiers (Fig. 3-6d and Tab. S3-6(b)). The clade of anammox bacterial gene *nrfA* is distinct from others (Fig. 3-6b and Tab. S3-6(d)) in the phylogenetic tree. In addition to gene *nrfA*, the observed gene *nirB* suggests this anammox strain developed metabolic flexible to perform DNRA. Note that gene *nirB* was not observed in B_AMX, which might be a *de novo*

splicing gap in the draft genome. Much like gene *narG*, the evolution position of *nirB* in anammox species was distinct from that in heterotrophic bacteria and archaea (Fig. 3-6c and Tab. S3-6(c)). The evolution positions of gene *nrfA*, as well as *narG* and *nirB*, revealed that this anammox strain could perform a unique DNRA pathway, comparing to other published anammox bacteria (Fig. 3-6d and Tab. S3-6(d)). The function of genes encoding *narG* and *nrfA/nirB* could accomplish the anammox stoichiometric NH_4^+-N/NO_2^--N subtract ratio as long as ammonium or/and nitrite is insufficient. These findings can be beneficial for achieving higher TNRE as its metabolic adjustment can be tailor-made according to the nitrogen species level. Further, the functional differences of DNRA between anammox species and heterotrophic bacteria as well as archaea became the next intriguing research gap.

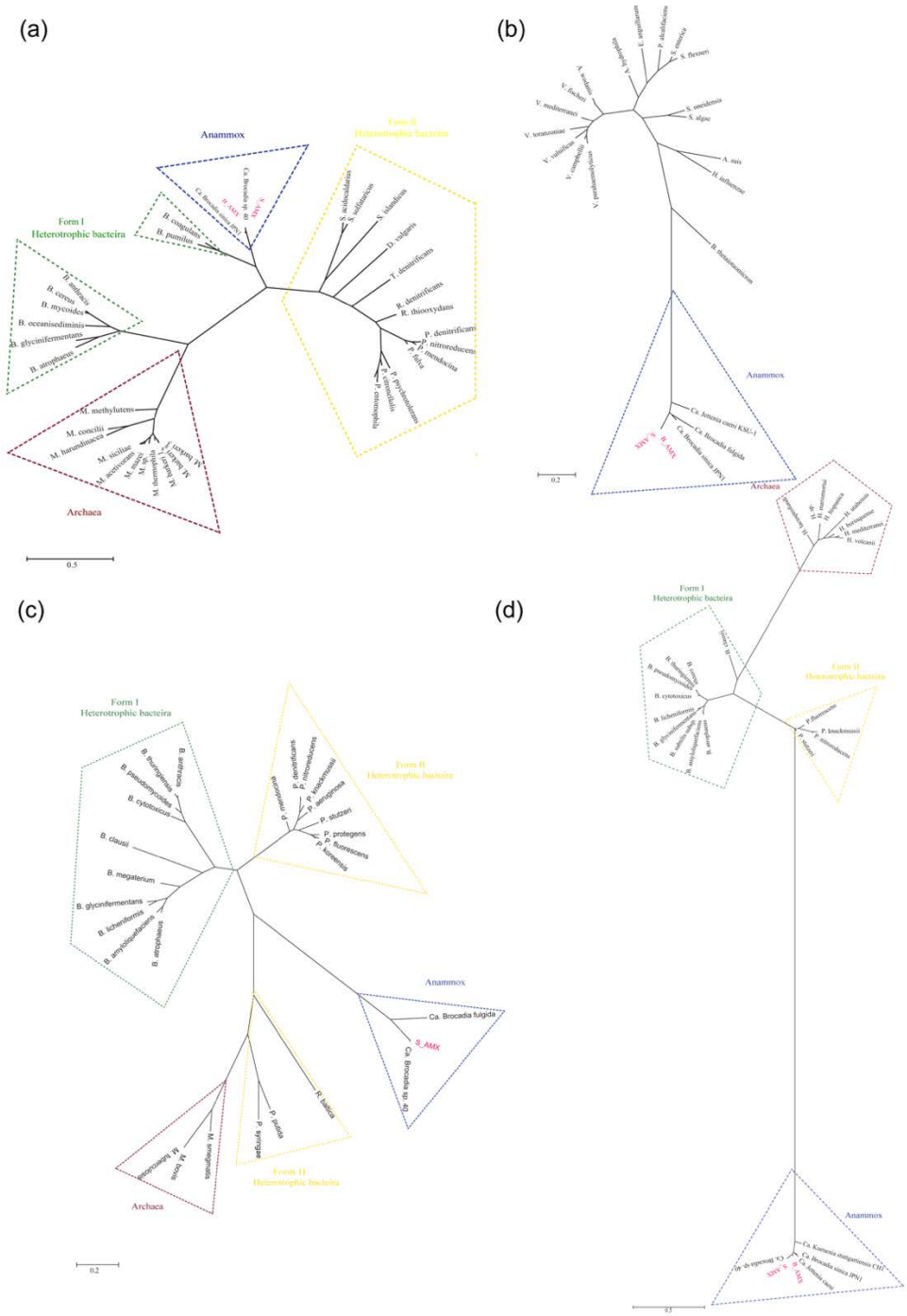


Fig. 3-6 Phylogenetic tree of (a) acetyl-CoA synthetase (*acs*), (b) nitrite reductase (cytochrome c-552) (*nrfA*), (c) nitrite reductase (NADH) large subunit (*nirB*), (d) large subunit of nitrate reductase (*narG*). The researched protein sequences are indicated in red. Aligned reference protein sequences were obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/). Protein sequences obtained from anammox genomes were imported in MEGA 7.0.21, aligned with the reference protein sequences by

ClustalW and phylogenetic trees were constructed with Maximum likelihood method, bootstrap of 1000 replications and Jones-Taylor_Thornton(JTT) model.

Anammox species are known as autotrophic bacteria that utilize CO_2 as carbon source for cell synthesis through forming Acetyl-CoA by acetyl-CoA synthase (*acsB*) in the *Wood-Ljungdahl* pathway (Strous et al. 2006). The major genes involved in the *Wood-Ljungdahl* pathway were recovered in S_AMX and B_AMX, suggesting it could perform cell synthesis similar as reported anammox bacteria. Additionally, one copy of gene encoding acetyl-CoA synthetase (*acs*) was annotated in anammox genomes. Although the reaction performed by *acs* is reversible, it also indicates that this strain of anammox bacteria has the potential ability to utilize acetate for cell synthesis. Among published anammox genomes, the genes *acs* are also observed in *Ca. Kuenenia stuttgatirnsis*, *Ca. Scalindua sp.*, and *Ca. Jettenia caeni*. However, they share a low similarity with that observed in *Ca. Brocadia sp. 40* and this study (data not shown). The phylogenetic tree revealed the evolution position of gene *acs* among archaea, heterotrophic bacteria and anammox species (Fig. 3-6a and Tab. S3-6(a)). The isoforms of gene *acs* are categorized into four groups: methanogenic archaea, anammox species, form I in heterotrophic bacteria *Bacillus* and form II in other heterotrophic bacteria in the phylogenetic tree (Fig. 3-6a), which are distinct from each group. Interestingly, the amino acid sequences of isoform *acs* genes in this study shared a high homology with that of heterotrophic bacteria *Bacillus coagulans* and *Bacillus pumilus*. This finding revealed that the overall functions of gene *acs* in S_AMX and B_AMX were possibly developed from heterotrophic bacteria affiliated to *Bacillus*.

3.3.5 Potential nitrogen transformation in PN/A FBR

As the reactor was built to start up the PN/A process for low strength wastewater treatment, the nitrogen mark genes involved in nitrification, denitrification, anammox, and respiratory ammonification were investigated in the genomes recovered from

suspended sludge and biofilm (Fig. 3-4). In the suspended sludge, the key genes responded to ammonia (*amo* and *hao*) and nitrite oxidation (*nxr*) were annotated in AOB and NOB genomes, respectively, which is similar to the results conducted by Speth et al. (2016). S_NOB, S_PRO1,3, S_AMX, S_ACD1,2, S_BCD1,3,5,8 encoded gene nitrate reductase (*nar*), possibly used for nitrate reduction to cope with the produced nitrate by NOB and AMX and maintained a low concentration of nitrate in the effluent. Genes encoding *nirK* was observed in S_AOB1,2, S_NOB, S_PRO1,2, and S_BCD8, which was regarded as detoxification from nitrite. S_ACD1 and S_BCD1 encoded the key gene for assimilatory nitrate reduction (*nasA*), possibly used for coping with the produced nitrate. It indicated that, in addition to denitrification and DNRA, flexible nitrate reduction way existed in the reactor. The key genes encoding dissimilatory nitrate reduction to ammonia (*nrf*) were observed in Acidobacteria (S_ACD1-2) and anammox bacteria (S_AMX). Proteobacteria (S_PRO1,2), Bacteroidetes (S_BCD1,3-6), Acidobacteria (S_ACD2), and S_AOB1,2 contained genes encoding nitrous oxide reductase (*norBC* or *norZ*), which potentially contributed on the nitrous oxide production in single-stage PN/A system. Finally, S_BCD1-2 and S_BCD7,8 encoded nitrous oxide reductase (*nosZ*) for nitrogen gas production via nitrous oxide reduction.

In the biofilm, as expected, genes encoding nitrate reductase (*nar*) were observed in *Bacteroidetes* (B_BCD1-3,6,8), *Proteobacteria* (B_PRO2,4-6), and anammox (B_AMX) genomes (Fig. 3-4), indicating the possibility of nitrate reduction. It should be noted that, among these organisms, only B_BCD8 and B_PRO6 encoded nitrite reductase, suggesting the possibility of microbial interaction based on nitrite cyclic feeding. In addition to B_BCD8 and B_PRO6, gene encoding *nirK* was widespread in B_PRO1,3,6, B_BCD5, B_BIO1. Additionally, B_PRO5, B_BCD1-3,5-7, and B_BIO1 encoded a *norBC* or *norZ*, possible for the nitric oxide (NO) reduction to nitrous oxide (N_2O). Similar to the results observed from suspended sludge, the gene

encoding assimilatory nitrate reduction catalytic subunit (*nasA*) was obtained in B_PRO1 and B_BCD2. The possibility for nitrous oxide reduction was more widespread compared to suspended sludge since more bacterial genomes such as Proteobacteria (B_PRO5), Bacteroidetes (B_BCD1-8), and B_BIO2 encoded nitrous oxide reductase (*nosZ*).

Interestingly, none of the recovered genomes encoded complete denitrification pathway from nitrate to dinitrogen gas. Moreover, B_BCD5 was the unique one encoded complete denitrification pathway from nitrite to dinitrogen gas. In addition, genomes encoded nitrate reduction were widespread in the suspended sludge and biofilm, suggesting partial denitrification and assimilatory nitrate reduction might facilitate a nitrite loop with anammox bacteria to cope with the nitrate production.

3.4 Discussion

3.4.1 Microbial community and nitrite loop

Metagenomic sequencing has been widely used in anammox-based processes to access the anammox bacteria and microbial interactions from the genome level. However, to our knowledge, it is the first report showing details about the anammox bacteria, microbial communities and interactions in a single-stage PN/A system treating low strength wastewater without organic carbon fed. In addition, different from the studies conducted by Steph et al. (2016) and Lawson et al. (2017), in which only granular sludge was analyzed, the microbes in both of suspended sludge and biofilm were sequenced in this study, providing a comprehensive view on the microbial community in the PN/A FBR. It should be noted that no genomes affiliated to *Chlorobi* were assembled in this study. One possible explanation for this result was that a relatively higher DO concentration (0.7 mg/L) in this study compared to previous studies (Gilbert et al. 2014, Speth et al. 2016, Yang et al. 2018). Additionally, it is vital to point out that

the PN/A FBR was fed with low ammonium concentration wastewater containing no organic matters rather than the full-scale PN/A reactor fed with real wastewater (Gonzalez-Martinez et al. 2015). Thus, it is speculated that the wastewater component is another probable reason resulted in the absence of *Chlorobi* species in the PN/A FBR.

As except, AOB, NOB and anammox bacteria was the three most dominant species in the PN/A system. It should be noted that a relatively high abundance of NOB affiliated with *Nitrospira* (S_NOB and B_NOB) was enriched in the reactor, intriguingly, without noticeable nitrate production in the effluent. During the startup period, the ratio of nitrate production to ammonium influent was 0.05, which was lower than the reported PN/A stoichiometry (0.11) (Vlaeminck et al. 2012). Thus, it indicated that excess produced nitrate in the PN/A FBR might be respired by denitrifies or anammox bacteria to convert it back to nitrite, termed as "nitrite loop" (Winkler et al. 2012a), and even further to ammonium. Though external organic matters were absent from influential medium in our study, somewhat endogenous decay (hydrolysis and acidification) from cell lysis potentially produced organic carbons, as there was no discharge of biomass from PN/A FBR. Besides, soluble microbial products and extracellular polymeric substances (EPSs) were proved to present in PN/A systems (Laspidou and Rittmann 2002), corresponding to the accumulation of *Actinobacteria* and *Acidobacteria* in the PN/A FBR. Moreover, though high relative abundance of NOB in both of suspended sludge and biofilm was obtained, excess accumulation of nitrate did not occur in the system since the relationship of microbial abundance and activity was weak (Stewart et al. 2012). Furthermore, metagenomics analysis provided the possible evidence of nitrite loop, which was discussed as below. However, more experimental evidence (metatranscriptomics and metaproteomics) need to be conducted to support this speculation.

3.4.2 Heterotrophic ability of anammox bacteria

In the present study, the major genes in DNRA pathway and gene *acs* for acetate utilization were recovered in anammox genome. These results indicate that this anammox specie not only can perform DNRA for energy harvest, but also use acetate for cell synthesis. Anammox bacteria could couple DNRA with the anammox reaction to improve the nitrogen removal performance as well as the energy generation. Genes encoding *narG*, *nrfA*, *nirB* are annotated in this anammox species, hence, two ways could be performed for this cooperation: partial DNRA and full DNRA. For partial DNRA, nitrate is only reduced to nitrite and the produced nitrite is used by anammox bacteria with external ammonium for anammox reaction. For full DNRA, nitrate is reduced to nitrite and one part of formed nitrite is further reduced to ammonium. Afterward, anammox bacteria used the produced nitrite and ammonium for anammox reaction. The observed DNRA possibly supports anammox bacteria to regulate the products from the nitrate reduction when the substrates are limited. Although organic carbon was absence in the influent, the cell decay and EPS might act as the carbon sources for DNRA. Notably, the produced nitrite from DNRA could be immediately used for anammox transformation without further reducing to ammonium due to the residual ammonium in the effluent. Though anammox bacteria do not use the extra energy when performing the DNRA pathway for autotrophic cell synthesis (Kartal et al., 2013), anammox bacteria can generate more energy from anammox transformation due to the nitrite regulation by DNRA pathway. However, the observed gene *acs* can benefit from this additional energy since it utilizes acetate for cell synthesis with energy consumption. It proposes one hypothesis that if anammox bacteria could perform DNRA pathway and heterotrophic cell synthesis, it was likely accumulated more easily than that regarded by conventional views. However, more experimental evidence (metatranscriptomics and metaproteomics) need to be conducted to support this speculation.

3.4.3 Special metabolic interaction

The interaction among the whole community of the single-stage PN/A system is a pivot in nitrogen removal. As described above, most of the retrieved genomes encode nitrate reductase (*nar*), representing for the potential on performing partial nitrification or dissimilatory nitrate reduction. Specifically, the presence of heterotrophic organisms, such as S_BCD8, B_PRO1, B_PRO3, and B_PRO6, encoded nitrite reductase (*nirK/S*) might compete the produced nitrite from AOB with anammox bacteria and NOB (Speth et al. 2016). Interestingly, the downstream pathway of NO reduction was missing in these genomes. Furthermore, the recovered anammox genomes (S_AMX and B_AMX) lack the key gene *nirK/S* of anammox pathway. One possibility was that the synergic association between *Ca. Brocadia sp* and other organisms are through nitric oxide (NO) via the anammox reaction, though it was regarded that one unknown gene instead of gene *nirK/S* in *Ca. Brocadia* reduced nitrite to NO. Besides, several organisms, S_BCD1,3-4,6, S_ACD2, B_PRO5, and B_BCD1-3,6-7, also lack a nitrite reductase, however, they encode nitric oxide reductase, indicating that they may also consume the product (NO) in competition with anammox bacteria.

We annotated the genes (*nar*, *nirB/nrfA* and *ac*s) in S_AMX and B_AMX, suggesting the DNRA or cell synthesis via organic utilization in anammox bacteria. Anammox bacteria might respire nitrate using organic matter as the electron donor, making cyclic nitrite or ammonium feeding. These recovered genes in anammox genomes proved the possibility of microbial interaction between anammox bacteria and heterotrophic organisms, such as B_BCD1-6,8, B_PRO6, S_BCD1-4, S_ACD1-2, and S_BCD8, which consistency with the nitrite loop as described above. This agrees with the recent observation by Gori et al. (2011) and Jenni et al. (2014). In addition, the recovered *acs* gene shows its possibility of acetate utilization by the strain of *Ca. Brocadia sp*, however, acetate was absence in the influent. Therefore, nitrite loop and

heterotrophic cell synthesis in anammox consortia were two considerable microbial interaction ways in the PN/A FBR.

Metabolic exchange in the microbial community, such as amino acids and vitamins exchange, is vital to shaping the microbial ecosystems (Mee et al. 2014). In this study, we observed several genomes affiliated with heterotrophic organisms, indicating that they could synthesize essential organic matters for cell synthesis in the PN/A system. Gene encoded TonBdependent vitamin B12 transporter (*btuB*) was annotated in most of retrieved genomes (except S_BCD2-3,8, B_BCD6,8, B_PRO4, B_BIO1-2). In addition, autotrophic organisms (AOB, NOB, AMX, etc.) possess the B12 vitamin synthesis pathways, probably for other microbes that do not have, as the lack of organic matters in the influent of the PN/A FBR, Furthermore, considering the widespread of gene *btuB* in the recovered genomes, vitamin B12 might be a key substrate for metabolic interactions in the PN/A FBR. This suggests the possibility of vitamins B12 translocation in the microbial communities of the anammox-based processes (Ludington et al. 2017), as was also proposed by Lawson et al. (2017).

3.5 Conclusion

In this study, a lab-scale PN/A FBR was rapidly started up to treat low-strength ammonium wastewater without anammox sludge inoculum at room temperature. The major conclusions and finds can be drawn as follow:

1) A high nitrogen removal rate (>0.21 kg/(m^3•d)$^{-1}$) was obtained along with limited nitrate production (<9.34 mg/L). One anammox species affiliate to the strain of *Ca. Brocadia sp* was enriched in the PN/A FBR under a DO concentration of 0.7 mg/L.

2) The evolution positions of genes encoding *nar, nirB/nrfA* and *acs* in S_AMX and B_AMX reveal that anammox bacteria possibly perform unique DNRA pathway and heterotrophic cell synthesis.
3) Genes encoding nitrate or nitrite reductase, *nar,* and *nirB/nrfA*, were widespread in retrieved genomes, hinting at the possibility of enhancing the reactor performance by nitrite loop.
4) The discovery of the *btuB* gene for vitamin B12 exchange in the anammox bacteria may shape such microbial interaction community for nitrogen transformation with dilute wastewater.

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Supporting material for chapter 3

Table S3-1. The composition in synthetic wastewater

Composition	Concentration (mg/L)
NH_4Cl	Depend
$KHCO_3$	1000
KH_2PO_4	25
$CaCl_2$	230
$MgSO_4$	200
$FeSO_4$	6.25
Trace elements*	1 mL/L

*Trace elements:

Composition	Concentration (g/L)
$MnCl_2 \cdot 4H_2O$	0.99
$NaMoO_4 \cdot 2H_2O$	0.22
$CuSO_4 \cdot 5\ H_2O$	0.25
$CoCl_2 \cdot 6\ H_2O$	0.24
$ZnSO_4 \cdot 7\ H_2O$	0.43
$NiCl_2 \cdot 6\ H_2O$	0.19
$NaSeO_4 \cdot 10\ H_2O$	0.21
H_3BO_4	0.014
EDTA	15

Table S3-2. The diversity of microbial community based on 16S rRNA sequencing in the PN/A FBR

group	invsimpson	shannon	chao	ace	coverage(%)
S_28	25.7813	4.799138	3185.521	4168.758	0.96981
S_45	18.30384	4.560254	2911.468	3804.462	0.972988
S_73	6.058734	2.992336	1969.83	3757.032	0.983856
S_106	7.982893	3.28287	2413.497	4465.411	0.979939
S_220	9.735649	3.968959	4604.604	7328.194	0.965033
B_220	5.193747	2.746354	2337.998	3743.231	0.980736

Table S3-3. Metagenomic filtered reads mapping on the obtained high-quality genomes

Sample	Bin ID	No. of reads	Mapped reads (%)	Coverage (RPKM)
Floc	Total filtered reads	39,472,841	46.09	-
	S_AMX	1,519,490	3.85	91.12
	S_AOB1	887,360	2.25	60.33
	S_AOB2	674,170	1.71	32.73
	S_NOB	7,564,299	19.16	358.64
	S_PRO1	3,620,668	9.17	158.32
	S_PRO2	1,334,363	3.38	96.37
	S_PRO3	133,412	0.34	5.68
	S_ACD1	338,310	0.86	21.85
	S_ACD2	245,537	0.62	11.40
	S_BCD1	292,285	0.74	11.52
	S_BCD2	182,862	0.46	9.80
	S_BCD3	107,619	0.27	6.36
	S_BCD4	166,135	0.42	5.65
	S_BCD5	362,099	0.92	26.11
	S_BCD6	276,634	0.70	16.06
	S_BCD7	265,392	0.67	14.66
	S_BCD8	223,125	0.57	13.40
Biofilm	Total filtered reads	44,192,804	46.55	-
	B_AMX	3,820,444	8.64	257.15
	B_AOB	1,330,334	3.01	52.68
	B_NOB	11,530,718	26.09	641.18

	B_PRO1	367,246	0.83	25.70
	B_PRO2	162,080	0.37	9.31
	B_PRO3	227,630	0.52	9.02
	B_PRO4	116,451	0.26	6.11
	B_PRO5	560,671	1.27	18.99
	B_PRO6	312,383	0.71	8.76
	B_BCD1	292,080	0.66	21.00
	B_BCD2	266,906	0.60	16.57
	B_BCD3	242,350	0.55	13.24
	B_BCD4	169,152	0.38	9.51
	B_BCD5	160,110	0.36	8.76
	B_BCD6	120,316	0.27	7.32
	B_BCD7	506,144	1.15	34.59
	B_BCD8	253,108	0.57	16.17
	B_BIO1	86,638	0.20	4.38
	B_BIO2	46,980	0.11	3.66

Table S3-4 Coding DNA sequences (CDS) predicted to be involved in the metabolism of main pathways in S_AMX genomes

Predicted function	Gene name	e-value	Accession no.	Closely related protein
Anammox reaction				
hydrazine synthase subunit	*hzsC*	2.91e-5	SOH06074.1	hydrazine synthase subunit C [*Candidatus Kuenenia stuttgartiensis*]
hydrazine synthase subunit	*hzsC*	6.10e-5	SOH06074.1	hydrazine synthase subunit C [*Candidatus Kuenenia stuttgartiensis*]
hydrazine synthase subunit	*hzsC*	3.57e-5	SOH06074.1	hydrazine synthase subunit C [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05157.1	hypothetical protein KSMBR1_2670 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	3.20e-80	SOH04660.1	hypothetical protein KSMBR1_2163 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH04263.1	hypothetical protein KSMBR1_1764 [*Candidatus Kuenenia stuttgartiensis*]

hydroxylamine dehydrogenase	*hao*	5.31e-84	SOH05896.1	hypothetical protein KSMBR1_3422 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05211.1	hypothetical protein KSMBR1_2724 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH04660.1	hypothetical protein KSMBR1_2163 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH04263.1	hypothetical protein KSMBR1_1764 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05518.1	hypothetical protein KSMBR1_3040 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH06265.1	hypothetical protein KSMBR1_3792 [*Candidatus Kuenenia stuttgartiensis*]
Carbon fixaztion pathway				
acetyl-CoA synthetase	*acs*	0	SOH05368.1	AMP-forming acetyl-CoA synthetase [*Candidatus Kuenenia stuttgartiensis*]
DNRA metabolism pathway				

nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG*	0	WP_070067086.1	respiratory nitrate reductase subunit beta [*Candidatus Brocadia sp. 40*]
nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG*	0	WP_070066136.1	nitrate oxidoreductase subunit alpha [*Candidatus Brocadia sp. 40*]
nitrite reductase (cytochrome c-552)	*nrfA*	0	GAN35163.1	formate-dependent nitrite reductase periplasmic cytochrome c552 subunit [*Candidatus Brocadia sinica JPN1*]
Detoxification of superoxide				
superoxide dismutase, Fe-Mn family	SOD2	2.84e-115	WP_070067090.1	superoxide dismutase [*Candidatus Brocadia sp. 40*]

Table S3-5 Coding DNA sequences (CDS) predicted to be involved in the metabolism of main pathways in B_AMX genomes

Predicted function	Gene name	e-value	Accession no.	Closely related protein
Anammox reaction				
hydrazine synthase subunit	*hzsC*	2.91e-05	SOH06074.1	hydrazine synthase subunit C [*Candidatus Kuenenia stuttgartiensis*]
hydrazine synthase subunit	*hzsC*	6.10e-5	SOH06074.1	hydrazine synthase subunit C [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	5.31e-84	SOH05896.1	hypothetical protein KSMBR1_3422 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05518.1	hypothetical protein KSMBR1_3040 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH06265.1	hypothetical protein KSMBR1_3792 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH04660.1	hypothetical protein KSMBR1_2163 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05211.1	hypothetical protein KSMBR1_2724 [*Candidatus Kuenenia stuttgartiensis*]

hydroxylamine dehydrogenase	*hao*	0	SOH04263.1	hypothetical protein KSMBR1_1764 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	3.20e-80	SOH04660.1	hypothetical protein KSMBR1_2163 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH04263.1	hypothetical protein KSMBR1_1764 [*Candidatus Kuenenia stuttgartiensis*]
hydroxylamine dehydrogenase	*hao*	0	SOH05157.1	hypothetical protein KSMBR1_2670 [*Candidatus Kuenenia stuttgartiensis*]
Carbon fixaztion pathway				
acetyl-CoA synthetase	*acs*	0	SOH05368.1	AMP-forming acetyl-CoA synthetase [*Candidatus Kuenenia stuttgartiensis*]
DNRA metabolism pathway				
nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG*	0	WP_070067086.1	respiratory nitrate reductase subunit beta [*Candidatus Brocadia sp. 40*]
nitrate reductase / nitrite	*narG*	0	KKO18624.1	nitrate oxidoreductase subunit alpha [*Candidatus Brocadia fulgida*]

oxidoreductase, alpha subunit				
nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG*	1.47e-49	WP_070066136.1	nitrate oxidoreductase subunit alpha [*Candidatus Brocadia sp. 40*]
nitrite reductase (cytochrome c-552)	*nrfA*	1.33e-112	WP_070067376.1	nitrite reductase [*Candidatus Brocadia sp. 40*]
Detoxification of superoxide				
superoxide dismutase, Fe-Mn family	SOD2	6.33e-130	WP_070067090.1	superoxide dismutase [*Candidatus Brocadia sp. 40*]

Table S3-6 (a) List of gene acs in reference genomes for evolution analysis

Specie	Genomic Sequence (NCBI)	Abbreviation
Bacillus atrophaeus subsp. Globigii	WP_003325073.1	B. atrophaeus
Bacillus oceanisediminis 2691	WP_019383059.1	B. oceanisediminis
Bacillus pumilus	WP_058015048.1	B. pumilus
Bacillus cereus ATCC 14579	NP_834351.1	B. cereus
Bacillus anthracis str. Ames	NP_847096.1	B. anthracis
Bacillus coagulans DSM 1 = ATCC 7050	WP_029141442.1	B. coagulans
Bacillus mycoides	WP_003191809.1	B. mycoides
Bacillus glycinifermentans	WP_046130829.1	B. glycinifermentans
Pseudomonas fulva 12-X	WP_013791735.1	P. fulva
Rhodanobacter denitrificans	WP_015446345.1	R. denitrificans
Pseudomonas entomophila L48	WP_011535759.1	P. entomophila
Pseudomonas psychrotolerans	WP_058772626.1	P. psychrotolerans
Pseudomonas fluorescens F113	WP_014340246.1	P. fluorescens
Pseudomonas nitroreducens NBRC 12694	WP_065327875.1	P. nitroreducens
Pseudomonas citronellolis	WP_064584366.1	P. citronellolis
Pseudomonas mendocina ymp	WP_004374086.1	P. mendocina
Rhodanobacter thiooxydans	WP_008438848.1	R. thiooxydans
Desulfovibrio vulgaris str. Hildenborough	YP_009969.1	D. vulgaris
Methanosaeta harundinacea 6Ac	WP_014586840.1	M. harundinacea
Methanosaeta concilii GP6	WP_013718636.1	M. concilii

Methanosarcina barkeri str. Wiesmoor	WP_011307146.1	M. barkeri 3
Methanosarcina barkeri str. Fusaro	WP_011307146.1	M. barkeri 1
Methanosarcina barkeri CM1	WP_048116506.1	M. barkeri 2
Methanosarcina acetivorans C2A	WP_048065557.1	M. acetivorans
Methanococcoides methylutens MM1	WP_048205265.1	M. methylutens
Methanosarcina mazei LYC	WP_048038839.1	M. mazei
Methanosarcina siciliae T4/M	WP_048171355.1	M.siciliae
Methanosarcina sp. MTP4	WP_048178813.1	M. sp.
Methanosarcina thermophila TM-1	WP_048166722.1	M. thermophila
Sulfolobus acidocaldarius	WP_011277224.1	S. acidocaldarius
Sulfolobus islandicus REY15A	WP_014514142.1	S. islandicus
Sulfolobus solfataricus P2	WP_009989839.1	S. solfataricus
Rhodanobacter thiooxydans	WP_008438848.1	R. thiooxydans
Thiobacillus denitrificans ATCC 25259	WP_011311016.1	T. denitrificans
Paracoccus denitrificans DSM 415	SFQ95566.1	P. denitrificans
Candidatus Brocadia sinica JPN1	GAN33747.1	Ca. Brocadia sinica JPN1
Candidatus Scalindua sp. husup-a2	WP_096894216.1	Ca. Scalindua sp.
Candidatus Jettenia caeni	WP_007220468.1	Ca. Jettenia caeni
	Gene ID (JGI)	
Candidatus Kuenenia stuttgartiensis	642556154	Ca. Kuenenia stuttgartiensis

Table S3-6 (b) List of gene narG in reference genomes for evolution analysis

Specie	Genomic Sequence(NCBI)	Abbreviation
Pseudomonas stutzeri	WP_013981797.1	P. stutzeri
Pseudomonas fluorescens F113	WP_014339178.1	P. fluorescens
Pseudomonas knackmussii B13	WP_043249492.1	P. knackmussii
Pseudomonas nitroreducens NBRC 12694	WP_084359857.1	P. nitroreducens
Bacillus subtilis subsp. subtilis str. 168	NP_391609.2	B. subtilis
Bacillus thuringiensis] serovar konkukian	YP_036283.1	B. thuringiensis
Bacillus licheniformis DSM 13	WP_011198014.1	B. licheniformis
Bacillus cytotoxicus NVH 391-98	WP_012095856.1	B. cytotoxicus
Bacillus atrophaeus subsp. Globigii	WP_003326509.1	B. atrophaeus
Bacillus cereus ATCC 14579	NP_831884.1	B. cereus
Bacillus glycinifermentans	WP_046131387.1	B. glycinifermentans
Bacillus clausii KSM-K16	WP_011245570.1	B. clausii
Bacillus amyloliquefaciens DSM 7	WP_013353945.1	B. amyloliquefaciens
Bacillus pseudomycoides DSM 12442	WP_033799165.1	B. pseudomycoides
Haloferax mediterranei ATCC 33500	WP_004056332.1	H. mediterranei
Halorhabdus utahensis DSM 12940	WP_012795090.1	H. utahensis
Halogeometricum borinquense DSM 11551	WP_006054602.1	H. borinquense
Halorubrum lacusprofundi ATCC 49239	WP_015910069.1	H. lacusprofundi
Haloarcula marismortui ATCC 43049	WP_011223493.1	H. marismortui
Haloarcula hispanica N601	WP_014040599.1	H. hispanica
Haloferax volcanii DS2	WP_004041371.1	H. volcanii
Haloarcula sp. CBA1115	WP_050038198.1	H. sp.
Candidatus Jettenia caeni	GAB63886.1	Ca. Jettenia caeni
	Gene ID (JGI)	

Candidatus Kuenenia stuttgartiensis CH1	2553461265	Ca. Kuenenia stuttgartiensis CH1
Candidatus Brocadia sp. 40	2708412991	Ca. Brocadia sp. 40

Table S3-6 (c) List of gene nirB in reference genomes for evolution analysis

Specie	Genomic Sequence (NCBI)	Abbreviation
Pseudomonas aeruginosa PAO1	NP_250472.1	P. aeruginosa
Pseudomonas putida KT2440	NP_743862.1	P. putida
Pseudomonas syringae] pv. tomato str. DC3000	NP_793045.1	P. syringae
Pseudomonas denitrificans ATCC 13867	WP_015476869.1	P. denitrificans
Pseudomonas protegens CHA0	WP_015634754.1	P. protegens
Pseudomonas stutzeri	WP_025242187.1	P. stutzeri
Pseudomonas fluorescens F113	WP_014337337.1	P. fluorescens
Pseudomonas nitroreducens NBRC 12694	WP_084359108.1	P. nitroreducens
Pseudomonas koreensis	WP_041479128.1	P. koreensis
Pseudomonas knackmussii B13	WP_043251195.1	P. knackmussii
Pseudomonas mendocina ymp	WP_012018512.1	P.mendocina
Rhodopirellula baltica SH 1	NP_864646.1	R. baltica
Bacillus thuringiensis serovar konkukian str. 97-27	YP_036299.1	B. thuringiensis
Bacillus clausii KSM-K16	WP_011246465.1	B. clausii
Bacillus cytotoxicus NVH 391-98	WP_011983336.1	B. cytotoxicus
Bacillus licheniformis DSM 13 = ATCC 14580	WP_003178981.1	B. licheniformis
Bacillus atrophaeus subsp. globigii	WP_003328057.1	B. atrophaeus
Bacillus amyloliquefaciens DSM 7	WP_013350961.1	B. amyloliquefaciens
Bacillus pseudomycoides DSM 12442	WP_018781999.1	B. pseudomycoides
Bacillus megaterium NBRC 15308 = ATCC 14581	WP_034654272.1	B. megaterium

Bacillus glycinifermentans	WP_046131614.1	B. glycinifermentans
Bacillus atrophaeus	WP_088116465.1	B. atrophaeus
Mycobacterium bovis AF2122/97	YP_009357588.1	M. bovis
Mycobacterium tuberculosis H37Rv	NP_214766.1	M. tuberculosis
Mycobacterium smegmatis str. MC2 155	YP_884840.1	M. smegmatis str.

Table S3-6 (d) List of gene nrfA in reference genomes for evolution analysis

Specie	Genomic Sequence (NCBI)	Abbreviation
Shewanella oneidensis MR-1	NP_719510.1	S. oneidensis
Salmonella enterica subsp. enterica serovar	NP_463142.1	S. enterica
Actinobacillus suis ATCC 33415	WP_014992038.1	A. suis
Providencia alcalifaciens Dmel2	WP_006661113.1	P. alcalifaciens
Vibrio mediterranei NBRC 15635	WP_006074229.1	V. mediterranei
Haemophilus influenzae Rd KW20	NP_439227.3	H. influenzae
Vibrio fischeri ES114	YP_204937.1	V. fischeri
Vibrio parahaemolyticus RIMD 2210633	NP_798308.1	V. parahaemolyticus
Vibrio vulnificus	WP_039447673.1	V. vulnificus
Vibrio toranzoniae	WP_060467485.1	V. toranzoniae
Edwardsiella anguillarum ET080813	WP_034165380.1	E. anguillarum
Aeromonas hydrophila subsp. hydrophila	YP_856978.1	A. hydrophila
Shigella flexneri 2a str. 301	NP_709846.2	S. flexneri
Bacteroides thetaiotaomicron VPI-5482	NP_810330.1	B. thetaiotaomicron
Shewanella algae	WP_044733844.1	S. algae
Aliivibrio wodanis	WP_045102327.1	A. wodanis
Vibrio campbellii ATCC BAA-1116	WP_041853314.1	V. campbellii
	Gene ID (JGI)	
Candidatus Brocadia fulgida	2673238468	Ca. Brocadia fulgida
Candidatus Jettenia caeni KSU-1	2532548089	Ca. Jettenia caeni KSU-1
Candidatus Brocadia sinica JPN1	2621315867	Ca. Brocadia sinica JPN1

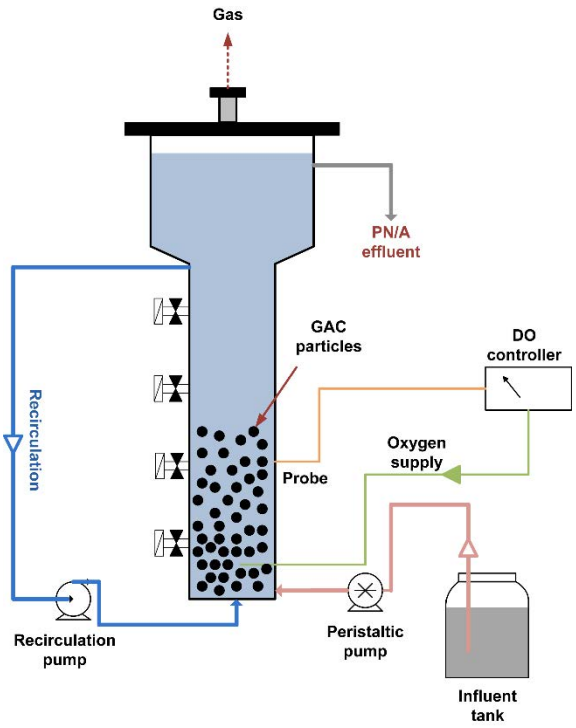
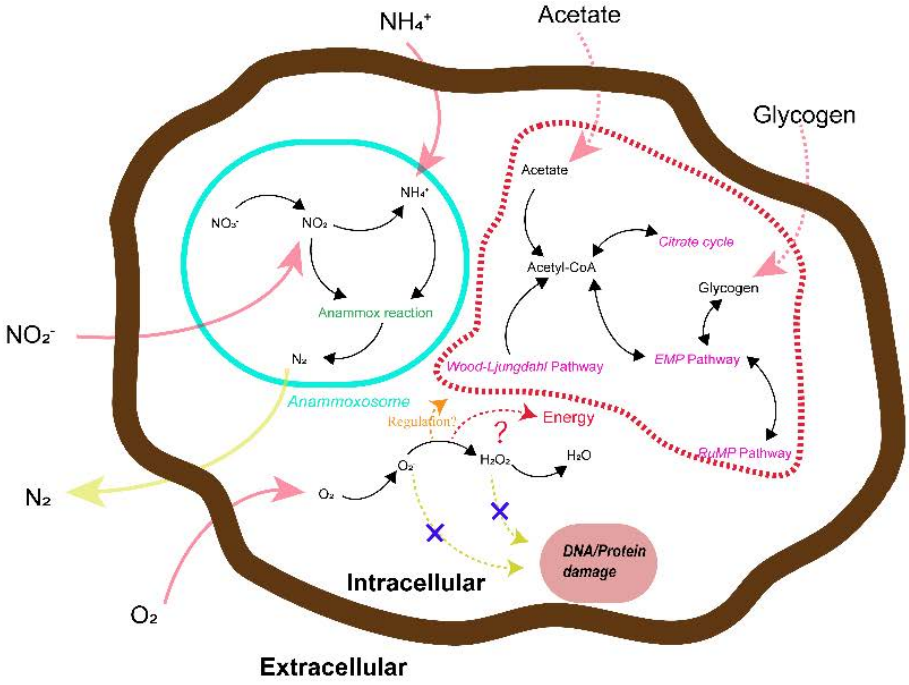


Fig. S3-1 Schematic diagram of the partial nitrification/anammox fluidized-bed bioreactor (PN/A FBR)

Fig. S3-2 CheckM plot for the quality assessment of MaxBin results describing bin completeness, contamination, and heterogeneity.

Chapter 4 Metagenomics and metatranscriptomics analyses reveal oxygen detoxification and mixotrophic metabolism in an enriched anammox continuous stirred-tank reactor

GRAPHIC ABSTRACT



4.1 Introduction

Anaerobic ammonium oxidation (Anammox)-based processes have been widely applied to wastewater treatment due to the advantages of energy and co-substrates saving compared to conventional nitrogen removal processes (Kartal et al., 2011). Especially, the research on mainstream anammox process has gained lots of interests, expanding the applicability of anammox process (Laureni et al., 2016; Lotti et al., 2015). In practice, suitable environmental conditions were controlled for anammox enrichment, such as temperature, pH, dissolved oxygen, substrates, etc. (Thuan et al., 2004), either through the combined process with or without nitritation (single-stage or two-stage) in various types of reactors (Chamchoi and Nitisoravut, 2007; Lopez et al., 2008). Some reactor configurations intend to form granular or biofilm, protecting anammox bacteria from the poisoning caused by oxygen diffusion (Abma et al., 2010; Rosenwinkel & Cornelius, 2005), however, these processes are difficult to maintain strict anaerobic environment. Specially for the single-stage partial nitrification/anammox process, low dissolved oxygen (DO) concentration was maintained in the reactor for partial nitrification (Lackner et al., 2014). For example, despite the formation of granular or biofilm, suspended anammox cell identified as *Ca. Brocadia sp 40* survived in partial nitrification/anammox (PN/A) system under DO concentration of 0.1-0.16 mg/L (Yeshi et al., 2016). Additionally, even though the oxygen reversely inhibit their activity, anammox bacteria could survive under oxygen exposure (Liu et al., 2008)-(Kalvelage et al., 2011). Anammox species are currently regarded as anaerobic bacteria (Chamchoi & Nitisoravut, 2007), and anaerobic bacteria would be poisoned by superoxide ($O_2^{\cdot}$) or hydrogen peroxide (H_2O_2) in the case of aerobic conditions due to the lack/none of catalase or superoxide dismutase (SOD) (McCord et al., 1971). With the presence of dissolved oxygen, molecular oxygen transported from the environment is converted into superoxide ($O_2^{\cdot}$) in the cell, termed reactive oxygen species (ROS), which are toxic to anaerobic bacteria (Bryukhanov et al., 2015). Interestingly, we found the presence of gene encoding SOD Fe-Mn family (SOD2) associated with the conversion of $O_2^{\cdot}$ to H_2O_2 in *Ca. Brocadia sp 40* (SAMN05817825), *Ca. Brocadia sinica JPN1*

(SAMD00036619) and *Ca. Brocadia fulgida* (SAMN03103735), indicating the potential capacity of oxygen detoxification by anammox bacteria. Hence, it is vital to comprehensively understand the oxygen detoxification mechanism in anammox bacteria.

Extra electron is generally required by relevant enzymes in the process of oxygen detoxification to reduce superoxide (McCord et al., 1971). Apart from ammonia as the electron donor by anammox bacteria, organic carbon sources can serve as the one. Though anammox bacteria are initially considered as autotrophs and utilize inorganic carbon through Wood-Ljungdahl pathway for cell synthesis (Kartal et al., 2008; Kartal et al., 2012), genome-based information combined with reactor performance revealed that they were susceptible to be affected by the addition of organic carbon (Leal et al., 2016; Li et al., 2015; Shu et al., 2016). For example, metabolomic study revealed that acetate addition triggered the conversion of Adenosine triphosphate (ATP) to Adenosine monophosphate (AMP) in *Candidatus Brocadia* (Feng et al., 2018). Likewise, the phenomenon of organics utilization was observed in other anammox cultures (Gori et al., 2011; Kartal et al., 2007; Park et al., 2017). Furthermore, nitrate reduction using organic carbon as electron donor in anammox bacteria, termed dissimilatory nitrate reduction to ammonium (DNRA), was also reported (Winkler et al., 2012b)-(Winkler et al., 2012a). In addition to organic utilization, organic extracellular polymeric substances (EPS) secretion by anammox bacteria for the unfavorable environment adaption were revealed (Tang et al., 2011). Based on these results, one hypothesis is proposed that the organic carbon metabolism in anammox bacteria may be partly associated with the oxygen detoxification. Accordingly, the exploration of organic carbon metabolism pathway could provide a comprehensive understanding on the possibility of oxygen detoxification in anammox bacteria.

Here, we set up a continuous stirred-tank reactor (CSTR) to obtain high abundance of anammox bacteria without strict anaerobic condition through inoculating the dried

power sludge from a full-scale simultaneous partial nitrification, anammox and denitrification (SNAD). Of particular interest was to elucidate the capabilities of oxygen detoxification and mixotrophic metabolism (nitrogen transformation and carbon metabolism). To this end, metagenomic and metatranscriptomic analyses were performed to reconstruct the metabolic pathways and profiling the gene expression from the recovered anammox genome. These findings reveal the potential metabolic mechanism in anammox bacteria and widen the application of anammox-based processes.

4.2. Material and methods

4.2.1 Reactor set-up, inoculum and operation strategy

A lab-scale anammox Continuously Stirred Tank Reactor (CSTR) with an effective volume of 1.0 L was set up and operated with continuous mode (Fig. 4-1) at room temperature. Magnetic stirring with 50 rpm was used for mixing in the reactor. Biomass was intercepted through the inclined plate installed in the sedimentation area. The reactor was inoculated with dried powdered sludge taken from a SNAD reactor treating landfill leather in Taiwan (Taiwan, China). The dried powder sludge was activated in the laboratory before inoculum.

The reactor was fed with synthesis wastewater contained NH_4Cl, $NaNO_2$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and 1 mL/L of trace element solution as described by Van de Graaf et al. (1996). The influent concentration of ammonium and nitrite were configured as needed. The influent pH was adjusted to 8.0, and it was not controlled in the reactor. Three operational stages with different hydraulic retention times (HRT), representing 12 hours, 9.6 hours, and 7.6 hours, were conducted in this study, respectively.

4.2.2 Analytical methods

The collected influent and effluent water samples were filtered (0.45μm) before measurement. The water quality parameters of NO_3^--N, NO_2^--N, NH_4^+-N were measured according to standard methods (APHA 2002) and the pH was done using a pH meter (Leici Company, China).

4.2.3 Sludge sampling, DNA extraction, and 16S rRNA sequencing

Biomass samples were obtained from the reactor by centrifugation (5 min, 10000 *g*, 4 °C) on Day 55, 105, 144 and 234, were named as AX6, AX7, AX8 and AX11 (Tab. 4-1). DNA extraction was performed using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instruction. The quality and concentrations of extracted DNA samples were measured using NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE).

DNA extracts were amplified by polymerase chain reaction (PCR) in the V3-V4 region of the 16S rRNA gene using primers 341F: ACTCCTACGGGAGGCAGCAG and 806R: GGACTACHVGGGTWTCTAAT, and were sequenced using the Illumina MiSeq platform (BGI, Shenzhen). PCR conditions were adopted based on Kapoor et al. (2015). The raw reads were processed using MOTHUR software to remove chimeric and low-quality sequences. The obtained high-quality sequences with an average length of 440 bp were clustered into operational taxonomic units (OTUs) with 97% similarity cutoff and assigned to the SILVA reference database.

4.2.4 Whole-genome metagenomics analysis

Whole-genome metagenome sequencing was performed to recover genomes of dominant anammox bacteria enriched in CSTR and to obtain a deep understanding of the potential functional genes contained in anammox species. Genomic DNA sample

(50 μg) collected at the end of this study was sequenced by BGI company (Shenzhen, China) using Illumina HiSeq platform. The raw reads obtained were filtered to remove adapters, low-quality data and N bases through trimming progress and ensured that the length of the clean read was over 150 bases. The clean reads were assembled into contigs using SPAdes Genome Assembler (ver. 3.10.1) by setting kmer parameters of 19,33,47,61,75 (Nurk et al., 2013). QUAST was used to access the quality of assembled contigs (Gurevich et al., 2013). The obtained contigs were binned into draft genomes using MaxBin (Wu et al., 2014). One anammox genome with completeness of 99.5% was recovered from CSTR. The quality of the anammox genome was evaluated using CheckM (Parks et al., 2015). The functional genes in anammox genome were annotated using Prokka (Seemann, 2014), which packages used were described as Park et al. (2017). JSpeciesWS was used to calculate the pairwise average nucleotide identity (gANI) and alignment frequency (AF) between recovered draft anammox genome and published anammox genomes based on BLASTP+ (Richter et al., 2015).

The phylogenetic tree was constructed using PhyloPhlAn to identify the relationship between the draft anammox genome enriched in CSTR and reference anammox genomes. Six published draft anammox genomes from JGI and NCBI database were used as templates for phylogenetic tree construction; (1) *Ca. Brocadia fulgida* (SAMN03103735), (2) *Ca. Brocadia sinica JPN1* (SAMD00036619), (3) *Ca. Brocadia sp R4W10303* (PRJNA383754), (4) *Ca. Brocadia sp 40* (SAMN05817825), (5) *Ca. Brocadia sp UTAMX1* (SAMN06342761), (6) *Ca. Brocadia sp UTAMX2* (SAMN06342762).

4.2.5 Metatranscriptomics library construction and analysis

Metatranscriptomics analysis was performed to access the expressed functional genes in recovered anammox genome. Sludge sample for metatranscriptomics analysis was collected at the same time as metagenomic sampling. About 5 mL mixed liquor was collected from the reactor and added 10 mL buffer to protect the RNA sample. The

mixed sludge sample was sent to BGI company (Shenzhen, China) for RNA extraction and library construction, and was sequenced using Illumina HiSeq4000 platform. Metatranscriptome sequence reads with an average length of 100 bp were generated, which were filtered to remove adaptor sequences, contamination and low-quality reads from raw reads. All clean reads obtained from sequencing company were trimmed using Trimmomatic to filter the reads again. *De novo* assembly of reads was performed using Trinity v2.5.1 (Grabherr et al., 2011). Transcriptomic reads were mapped to the recovered draft anammox genome from metagenomics analysis using BBMap with a minimum alignment identity of 95% to investigate the expression value of functional genes (Konstantinidis & Tiedje, 2005). The number of raw reads mapped for each potential functional gene was normalized to reads per kilobase per million reads mapped (RPKM) (Mortazavi et al., 2008). The raw reads of 16S rRNA were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database with BioProject PRJNA475660. Additionally, raw DNA and RNA sequences can be found on the NCBI with accession number SRP150295.

4.3. Results and discussion

4.3.1 Reactor performance

The CSTR was operated continuously at room temperature for 300 days to cultivate anammox bacteria and the overall nitrogen removal performance was shown in Fig. 4-1. The operational period was divided into three stages based on the hydraulic retention time (HRT). At stage I (days 1-75), the reactor was feed with an average concentration of 25.55 mg/L of NH_4^+-N and 31.88 mg/L NO_2^--N (Fig. 4-1A), corresponding to a nitrogen loading rate (NLR) of 0.119 kg-N/(m^3·d) (Fig. 4-1B). The total nitrogen removal efficiency (TNRE) increased gradually and reached around 60% on day 55. At stage II (days 76-147), HRT was reduced from 12 hr to 9.6 hr, resulting in an increase of NLR from 0.192 to 0.337 kg-N/(m^3·d). It should be noted that the nitrogen removal performance deteriorated suddenly with an increase of NLR from 0.189 to 0.288 kg-

N/(m^3·d) on day 117, representing for a decrease of TNRE from 66.24% to 32.04%. The TNRE reverted to 54.11% slowly even NLR further increased to 0.338 kg-N/(m^3·d). At stage III (days 148-294), NLR increased from 0.408 to 1.277 kg-N/(m^3·d) gradually as the shorten of HRT from 9.6 to 7.6 hr and the increase of substrates (NH_4^+-N and NO_2^--N) concentration. The nitrogen removal performance increased constantly, with a TNRE of 80% at the end of this study. Simultaneously, the reactor reached a NRR of 1.032 kg-N/(m^3·d), which show similar performance reported in previous anammox studies (Bagchi et al., 2010; Cao et al., 2016; Guo et al., 2016).

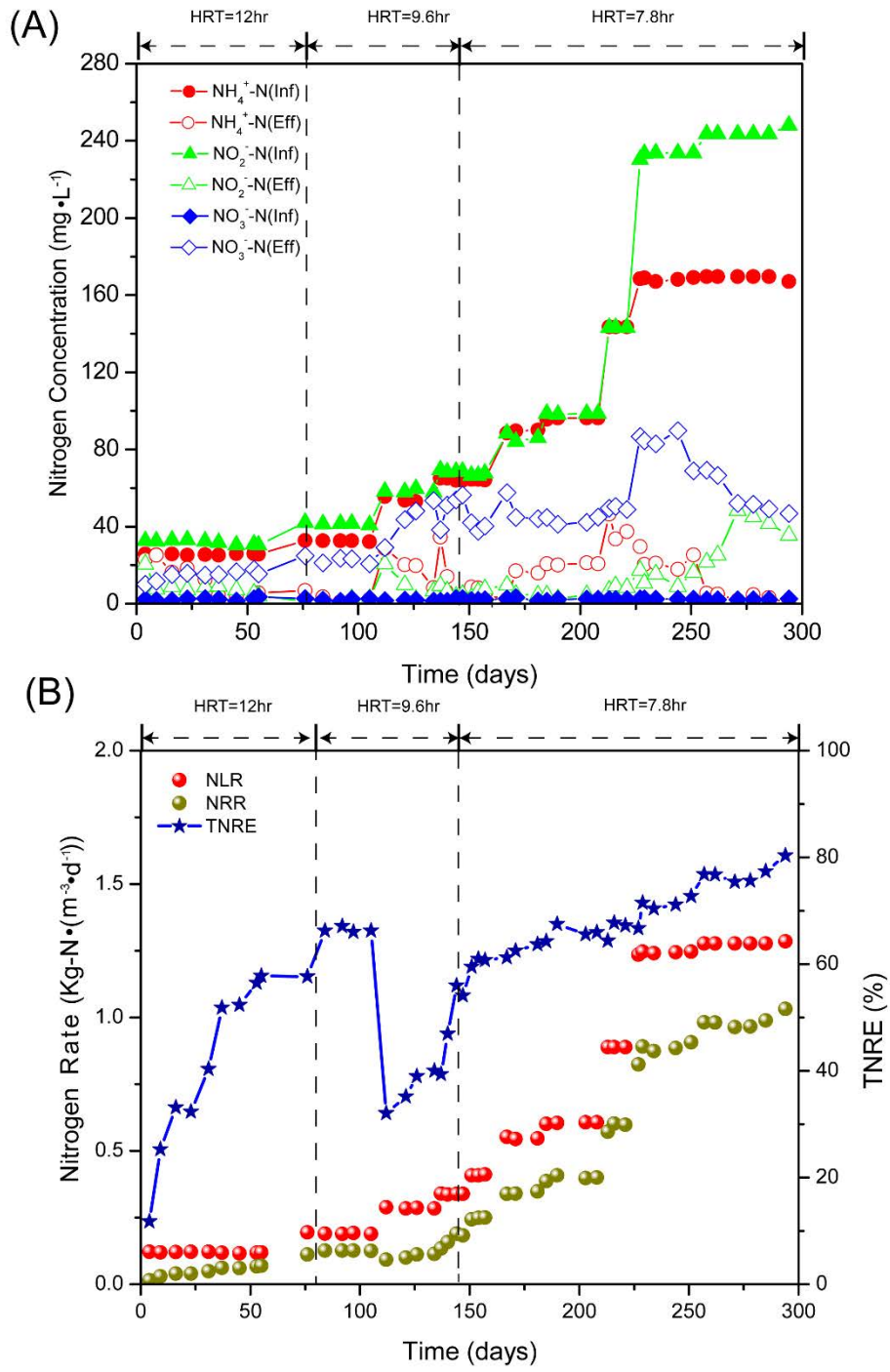


Fig. 4-1 The overall performance of nitrogen removal in CSTR. (A): Variation of nitrogen compounds; (B): Total nitrogen removal efficiency (TNRE). NLR: Nitrogen loading rate; NRR: Nitrogen removal rate.

4.3.2 The overall shifts of microbial community

The microbial community at different operating time in CSTR was conducted using 16S rRNA amplicon sequencing. which generated more than 76550 reads for each sample. The diversification of microbial community revealed the apparent reduction of community diversity during the enrichment period (Tab. S4-2). Taxonomic results showed that the dominant bacteria mainly affiliated with the phyla of *Planctomycetes, Proteobacteria, Bacteroidetes, Acidobacteria* , similar to the dominant phylum in other reported anammox reactors (Cao et al., 2016; Guo et al., 2016). As except, *Planctomycetes* became the most dominant phyla, reaching a relative abundance of 50.93% at the end of this study (Fig. S4-2A). *Candidatus Brocadia* was the most dominant genus with a relative abundance of 50.81% in CSTR, representing for approximate 99% of *Planctomycetes* (Fig. S4-2A and S4-2B). The genera *Sideroxydans* was also enriched synchronously with anammox bacteria and responsible for a relative abundance of 12.41%. Furthermore, over 99% sequences of *Candidatus Brocadia* belong to one OTUs cluster, indicating almost only one anammox bacterium was enriched in this study.

4.3.3 Whole-genome metagenomic analysis

As high abundance (50.81%) of *Ca. Brocadia* was enriched, whole-genome metagenomic analyses were performed to recover the draft genome of dominant anammox species and reconstruct its potential metabolic pathways. This resulted in the recovery of one high-quality draft anammox genome (CSTR001). Results from Maxbin revealed that CSTR001 contained 1 rRNA, 37 tRNA, and 185 contigs with an N50 of 35660 bp. The completeness and contamination of recovered CSTR001 was 99.94% and 1.65%, respectively (Fig. S4-3 and Tab. S4-3). The average GC content of CSTR001 was 42.3%, similar to other recovered anammox genomes (around 40%) (Ali et al., 2016; Oshiki et al., 2016a; Oshiki et al., 2015). The genome size was about 3.1Mb with a protein number of 2829, closed to *Ca. Brocadia sp 40* (Tab. S3) (Ali et al., 2016).

4.3.4 Phylogenetic relationship of recovered anammox genome

The 16S rRNA sequence in CSTR001 genome was extracted using CheckM with command of SSU and employed to access the phylogenetic relationship between recovered CSTR001 genome and other published anammox species (Fig. 4-2A). The neighbor-joining phylogenetic tree shown a similarity of 99% between CSTR001 and *Ca. Brocadia sp*. Moreover, taxonomic analysis and phylogenetic tree by 16S rRNA sequence revealed that CSTR001 was classified into the genus of *Ca*. Brocadia. Therefore, whole-genome phylogenetic tree was constructed between CSTR001 genome and reference anammox genomes belonging to *Ca. Brocadia* genus (Fig. 4-2B). This analysis verified the results conducted above and further confirmed that CSTR001 was one strain of *Ca. Brocadia sp*. Furthermore, average nucleotide identity (ANIb) and nucleotides alignment (AF) were used to compare the relatedness between CSTR001 and above six published *Ca. Brocadia* genomes (Fig. 4-2C). The results revealed that CSTR001 was most similar to *Ca. Brocadia R4W10303* among these reference anammox genomes. Correspondingly, the observed values of ANIb and AF were higher than 96% and 84%, respectively (Fig. 2C). Bring the results from taxonomic classification, phylogenetic analysis based on 16S rRNA sequence, whole-genome, and ANIb value together, it indicated that the recovered CSTR001 genome belonged to the strain of *Ca. Brocadia sp.*

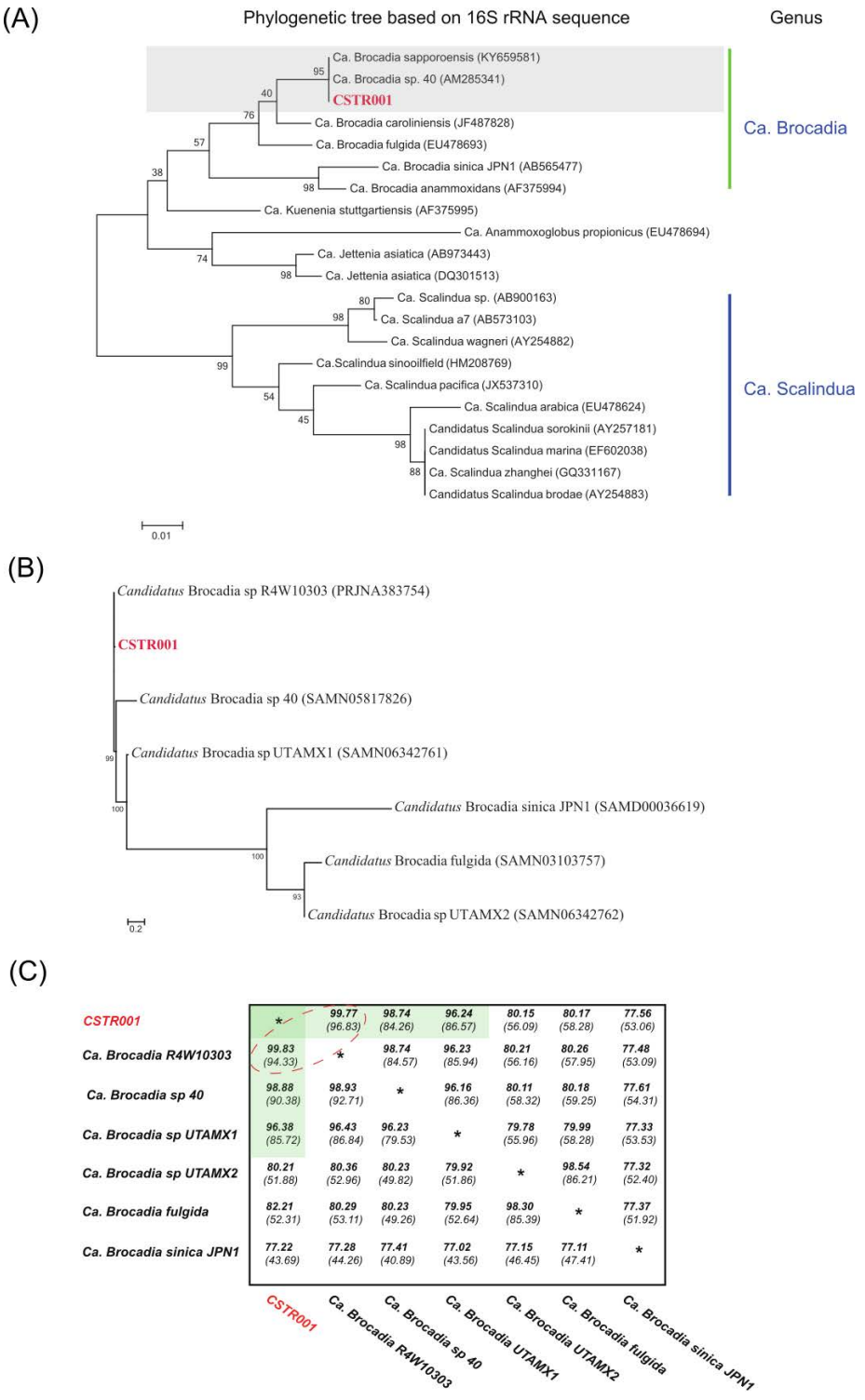


Fig. 4-2 (A) Phylogenetic tree of the anammox sequence based on 16S rRNA gene sequences. The tree was constructed by MEGA 7 with the neighbor-joining method, bootstrap of 1000 replications and Maximum Composite Likelihood model. Scale bar represents 1% estimated sequence divergence. (B) Phylogenetic relationships of the recovered anammox genome and published *Ca. Brocadia sp* genomes using

PhyloPhlAn. (C) Average nucleotide identity (ANI) and aligned nucleotides (%) between public databases of *Ca. Brocadia* and CSTR001 recovered in this study using BLAST. The reference anammox genomes were obtained from Joint Genome Institute (JGI) and NCBI database.

4.3.5 Metabolism pathways in anammox genome

4.3.5.1 Nitrogen metabolic pathway

The nitrogen metabolic pathway was recovered and annotated in CSTR001 (Fig. 4-3A). To date, genes relevant to ammonium, nitrite, and nitrate transporters have been reported in reference anammox genomes. The observed CSTR001 contains two copies of nitrate/nitrite transporter (NRT), one copy of ammonium transporter (*amt*) and formate/nitrite transporter (f*ocA*) (Tab. S4-4A). The presence of NRT in CSTR001 indicated its potential capability on nitrate utilization. Ammonium and nitrite (NH_4^+-N and NO_2^--N) are the two main substrates for anammox bacteria. Nitrite was reduced to nitric oxide (NO) by nitric oxidenitrite oxidoreductase (*nirS*) or copper-based nirK-type nitrite reductase. However, anammox species of *Ca. Brocadia sinica* and *Ca. Brocadia fulgida* may employ an unidentified gene for nitrite reduction due to the absence of *nirK/S* genes (Gori et al., 2011; Oshiki et al., 2015; Oshiki et al., 2016a). Notably, *Ca. Brocadia sinica* may reduce nitrite to hydroxylamine rather than nitric oxide as intermediate (Oshiki et al., 2016a). In this study, none of *nirS* or *nirK* genes were recovered in CSTR001 genome, suggesting CSTR001 might utilize unidentified genes to generate hydroxylamine or NO from nitrite reduction. Hydrazine synthase subunit (HZS) has been recognized as the specific gene for anammox bacteria, which was known as three types: *hzsA, hzsB*, and *hzsC* (Harhangi et al., 2012; Oshiki et al., 2016a; Oshiki et al., 2016b; Strous et al., 2006). Therefore, hzs encoding genes were used to identify the anammox organisms. In this study, two copies of the *hzs* genes (*hzsA* and *hzsC*) from the CSTR001 aligned with high nucleotide identities (95% and 92%) to *Ca. Brocadia fulgida* (SAMN03103735) using Local BLASTP (Tab. S4-4A) (Gori et al.,

2011). Metatranscriptomic results showed that gene *hzs*, especially *hzsA* cluster, was one of most highly expressed genes, revealing its activity in nitrogen transformation (Fig. 4-3B). Six copies of Hydroxylamine oxidoreductase-like (hao-like) genes, converting hydrazine and ammonium into nitrogen gas, were observed in CSTR001 (Tab. S4-4A). All of these hao-like genes were highly expressed in CSTR001 (Fig. 4-3B), suggesting it developed the metabolic flexibility to generate nitrogen gas.

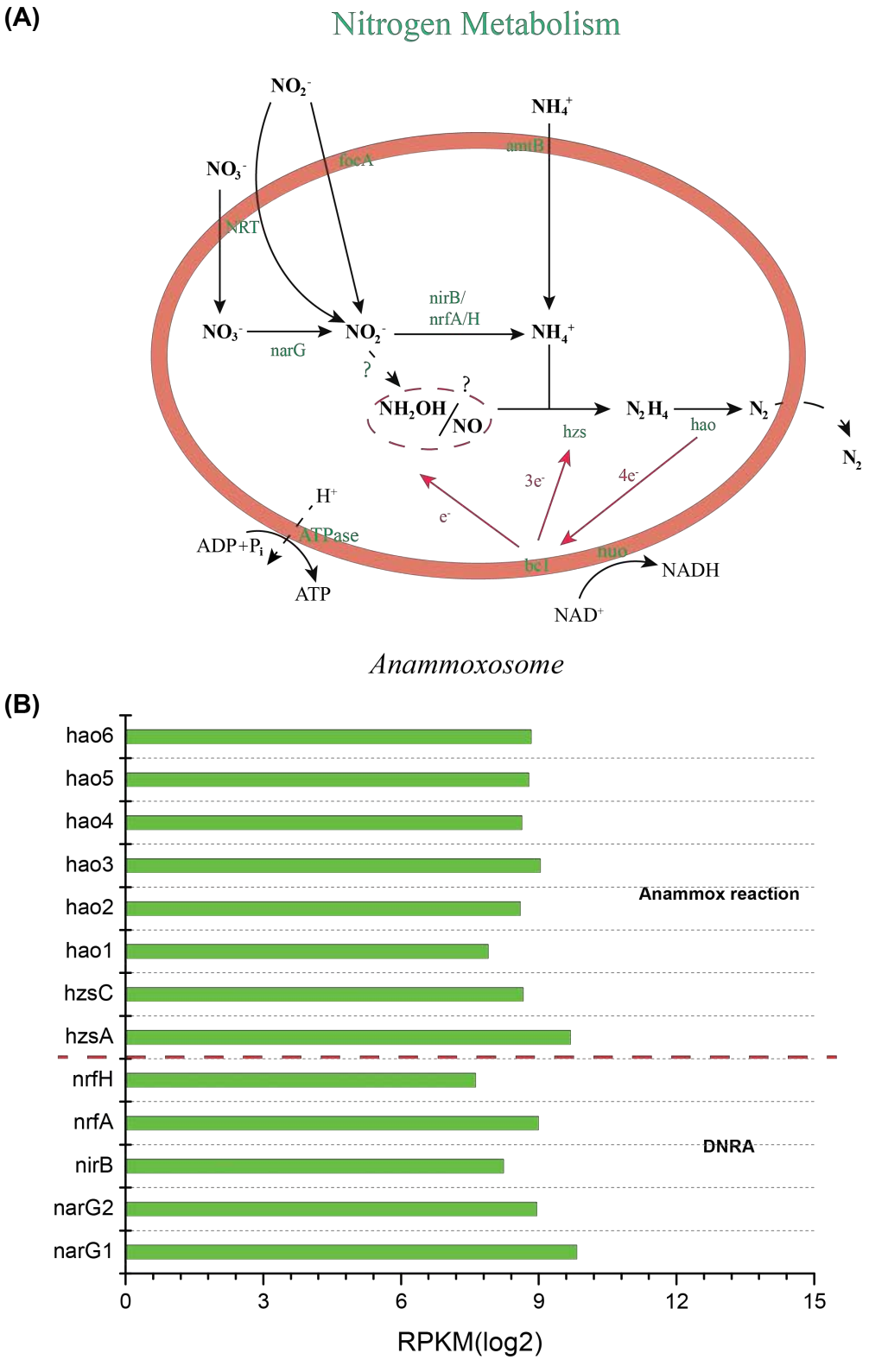


Fig. 4-3 (A) Overall of nitrogen metabolism of CSTR001. (B) The expression value of genes (log2 RPKM) related to nitrogen metabolism. The dotted black line indicates the missing of the required genes and the dotted red circle means the unidentified pathway.

4.3.5.2 Oxygen detoxification

In this study, media without deoxidization and reactor operation without strict anaerobic environment likely created micro-oxygen regions in the main reaction zone of CSTR. Here, we found that anammox bacteria possessed a proposed fundamental mechanism for oxygen detoxification described in Fig. 4-4A. Genes encoding superoxide dismutase Fe-Mn family (SOD2) and superoxide reductase (dfx) were observed in the CSTR001, which prevent the extensive damage via the conversion of $O_2^{\cdot}$ to hydrogen peroxide (H_2O_2). The downstream process for H_2O_2 detoxification was likely performed by genes encoding cytochrome c peroxidase (ccp), menaquinol-cytochrome c reductase iron-sulfur subunit (MQCRA), and NADH-quinone oxidoreductase (nuo). In this study, these genes, except dfx gene, were highly expressed in CSTR001 suggesting the anammox species CSTR001 abundant in the CSTR developed an intrinsic mechanism for oxygen detoxification (Fig. 4-4B and Tab. S4-4B). Interestingly, the SOD2, rather than the dfx gene, was highly expressed in CSTR001 under this experimental condition (Fig. 4-4B). The evolutionary pressure induces organisms develop intrinsic mechanism with SOD for superoxide detoxification, for instance, the SOD2 gene observed in this study has been found even in methanogens (Miller, 2012). To date, three distinct families of superoxide dismutase have been reported, i.e. Copper/zinc superoxide dismutase (Cu/ZnSOD), Iron/manganese superoxide dismutase, (Fe/MnSOD) and Nickel superoxide dismutase (NiSOD) (Miller, 2012). The FeSOD is the most primitive version, however, more modern organisms develop a new version of SOD that require Mn for activity. Hence, the employment of inorganic chemical makeup results the driven evolution of SOD in organisms. Though FeSOD and MnSOD appear from a common ancestor, they evolved into different clusters (Wolfe-Simon et al., 2005). In anaerobic bacteria, both of FeSOD and MnSOD are found, whereas FeSOD is not found in aerobic bacteria (Amo et al., 2003; Dos Santos et al., 2000). Some organisms possess Fe/MnSODs that trend to emphasize different metal iron for superoxide metabolism based on different conditions. Specifically, Mn is more emphasized under aerobic condition, whereas Fe under

anaerobic condition (Amo et al., 2003). In this study, it is important to explore which metal iron is emphasized in the metabolic mechanism of SOD2 since the anammox bacteria survives under anoxic condition.

Generally, the major genes that play key role in H_2O_2 detoxification are catalase (CAT), ascorbate peroxidase (APX) or glutathione peroxidase (GPX), however, they are absent in CSTR001. Instead, we observed one gene encoding ccp, which used H_2O_2 as the electron acceptor for electron capture, probably from the periplasmic quinone pool under anoxic conditions (Kaya et al., 2017). Different from CAT converting H_2O_2 to O_2, the product by Ccp is H_2O, which is same as the product by APX and GPX. Nonetheless, the mechanism for APX is different from Ccp, which utilizes ascorbic acid as a substrate for the reduction of H_2O_2 in the ascorbate-glutathione cycle. Ascorbate is oxidized to mono dehydroascorbate with the conversion of H_2O_2 to H_2O, and the produced mono dehydroascorbate is further dissociate back to ascorbate and dehydroascorbate (Gapper & Dolan, 2006). Similar to APX, GPX aslo uses reduced glutathione regeneration system as electronic donor for H_2O_2 detoxification. The previous study also confirmed that Ccp could degrade micromolar H_2O_2 in E. coli at substantial rates under anoxic conditions (Khademian & Imlay, 2017). Moreover, gene ccp involved in peroxide metabolism was observed in both prokaryotes and eukaryotes, e.g., *Saccharomyces cerevisiae* (budding yeast) (Kaya et al., 2017). The observation of highly expressed ccp gene (RPKM 9.60) in CSTR001 suggested the oxygen detoxification was performed by SOD2 and Ccp. Moreover, research found that the function of Ccp in bacteria might not only to detoxify H_2O_2 for periplasm protection but also to facilitate electron transport for energy harvesting through carbon metabolism regulation (Kaya et al., 2017).

We also recovered one gene encoding ubiquinol-cytochrome c reductase cytochrome b subunit (CYTB) with high gene expression value (RPKM 8.84). CYTB is involved in the peroxide metabolism under aerobic conditions, where ubiquinone (Q) acts as a role

to reduce the oxidation of APX in H_2O_2 metabolism pathway. However, previous studies found that the oxidation of Ccp was reduced by menaquinone (MQ) rather than ubiquinone (Q) (Kaya et al., 2017; Khademian & Imlay, 2017). In this study, highly expressed MQCRA gene (RPKM 9.54) was also identified in CSTR001, indicating oxygen detoxification pathway was possibily performed by SOD2, Ccp, and MQCRA. However, it is still unclear the affordable DO range for oxygen detoxification in anammox bacteria. Further work needs to be conducted for the function of anammox bacterial Ccp and MQCRA.

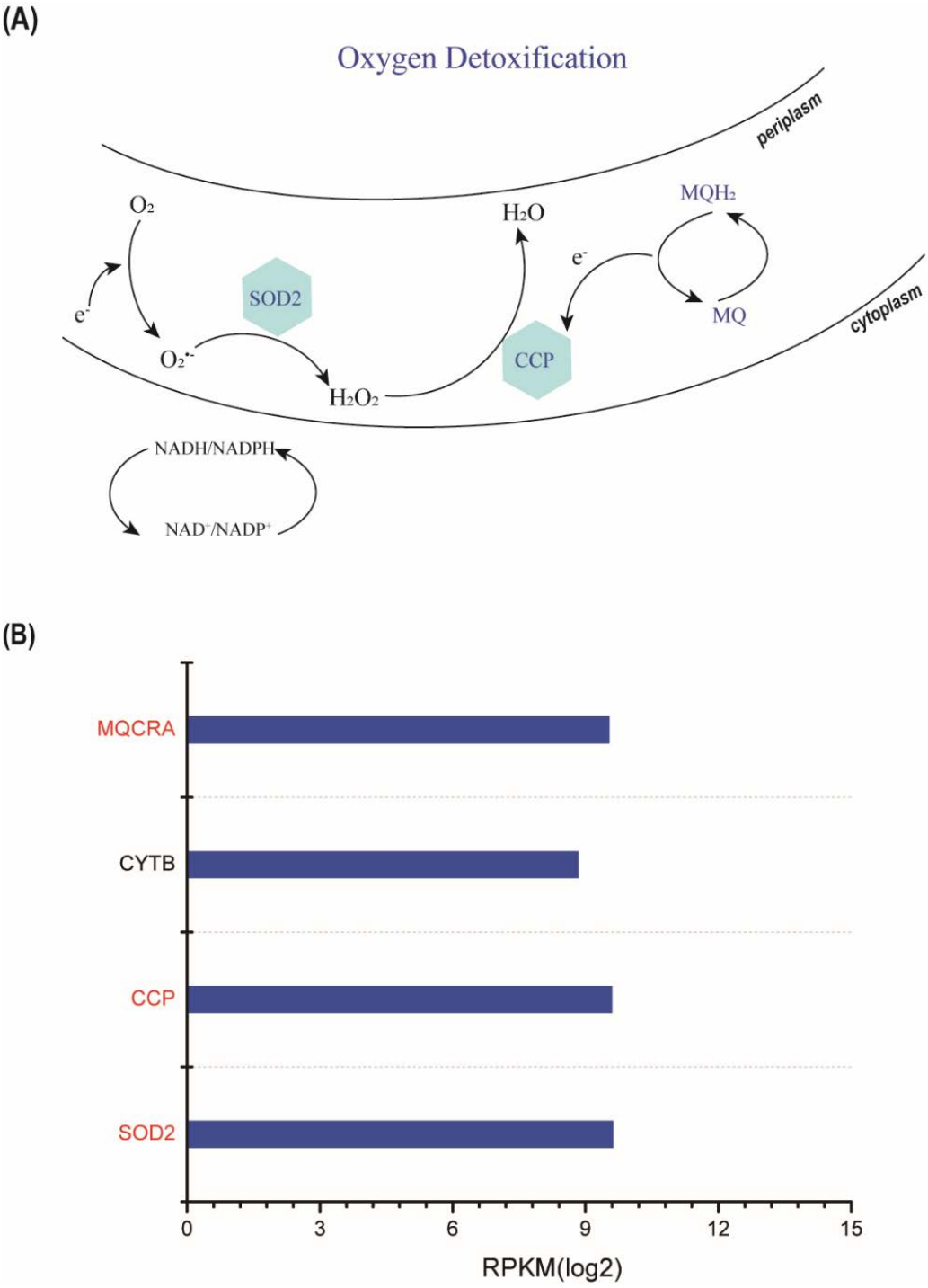


Fig. 4-4 (A) Overall of oxygen detoxification of CSTR001. (B) The expression value of genes (log2 RPKM) related to oxygen detoxification.

4.3.5.3 Oxygen Detoxification Associated with Carbon Metabolism

In oxygen detoxification, the expressed ccp possibly plays a role in the respiratory growth based on the nonfermentable carbon source utilization. This represents an amazing beneficial use of H_2O_2 in the metabolism of anammox bacteria. In this study, the presence of dissolved oxygen in the influent possibly resulted the production of exogenous and endogenous H_2O_2, which would activate anammox bacterial Ccp for oxygen detoxification. In turn, the expressed ccp may control the alternative carbon metabolism in anammox bacteria for the bioenergetics conversion or cell synthesis. The use of relevant carbon sources, such as glucose, acetate, formate etc., by bacteria for fermentation or anaerobic respiration may be directly controlled by the H_2O_2 concentration and Ccp based on oxygen availability (Kaya et al., 2017). Since the potential ability on the utilization of organic carbon sources is revealed in anammox bacteria, it is proposed that the Ccp in anammox bacteria may regulate its carbon metabolism.

In the present of study, genes encoding carbon metabolic pathways for Wood-Ljungdahl, Citrate cycle (TAC cycle), Embden-Meyerhof-Parnas (EMP), and Ribulose monophosphate (RuMP) were observed in CSTR001 (Fig. 4-5A and Tab. S4-4C). Genes encoding Wood-Ljungdahl pathway were expressed in CSTR001, suggesting this anammox lineage could fix inorganic carbon for cell synthesis as other reported anammox bacteria (Kartal et al., 2008; Park et al., 2017; Strous et al., 2006). We also observed gene encoding AMP-forming acetyl-CoA synthetase (*acs*) in CSTR001 that possibly utilized acetate to form acetyl-CoA for cell synthesis, which was also found in *Ca. Kuenenia stuttgartiensis* (Strous et al., 2006). Additionally, the expressed gene *acs* suggested the existence of this possibility (Fig. 4-5B), which coincided with the metabolomics result reported by Feng et al. (2018). In this study, even if no acetate was added in the influent, anammox bacteria could use organic matters produced by the endogenous degradation or soluble microbial products (SMP) from other microbial species. Perhaps with dilute nitrogen influent, anammox species without organic

metabolism cannot outcompete as was observed in this study. However, since the reaction is also reversible, with ATP synthesis resulting in acetate formation, it is still needed to explore whether the expressed *acs* gene involved in acetate consumption, acetate formation, or both.

In addition to the Wood-Ljungdahl pathway, the recovered EMP pathway, TCA cycle, and RuMP pathway provided a comprehensive insight into the carbon metabolism in anammox bacteria. Key genes encoding these three pathways were highly expressed (RPKM >7.29) in CSTR001, except gene rpiB that converting Ribulose-5P to Ribose-5P in RuMP pathway. Additionally, genes encoding citrate synthase (gltA) associated with the synthesis of citrate and fructose-bisphosphate aldolase, class I (Stewart et al.) functioned on fructose-1,6P_2 formation were missing from the CSTR001 genome. Perhaps, it may be a result of assembled gaps in CSTR001 genome, and thus further study is necessary. Interestingly, the expressed gene encoding glucokinase (glk) involved in EMP pathway revealed another possibility of cell synthesis through glycogen utilization in CSTR001. Further, the recovered amino acid, C4-dicarboxylate, and vitamin B12 transporters were highly expressed in CSTR001 (Tab. S4-4D), revealing its potential on organics utilization and extrusion. Therefore, the possibility of metabolic control by Ccp in anammox bacteria is partly supported by the observed expressed relevant carbon metabolism pathways including the expressed glucokinase (glk) for glucose utilization (Fig. 4-5). These results also propose a hypothesis whether or not the presence of trace oxygen can promote the growth of anammox bacteria. Meanwhile, we observed the expressed carbon metabolism pathways in anammox bacteria. Collectively, it is interesting to understand whether detoxification of oxygen, or even energy harvesting with H_2O_2, is associated with the carbon sources utilization. At least, the observed carbon metabolism pathways with expressed key genes can provide the fundamental feasibility to explore the organics utilization by anammox bacteria when they perform oxygen detoxification under anoxic conditions.

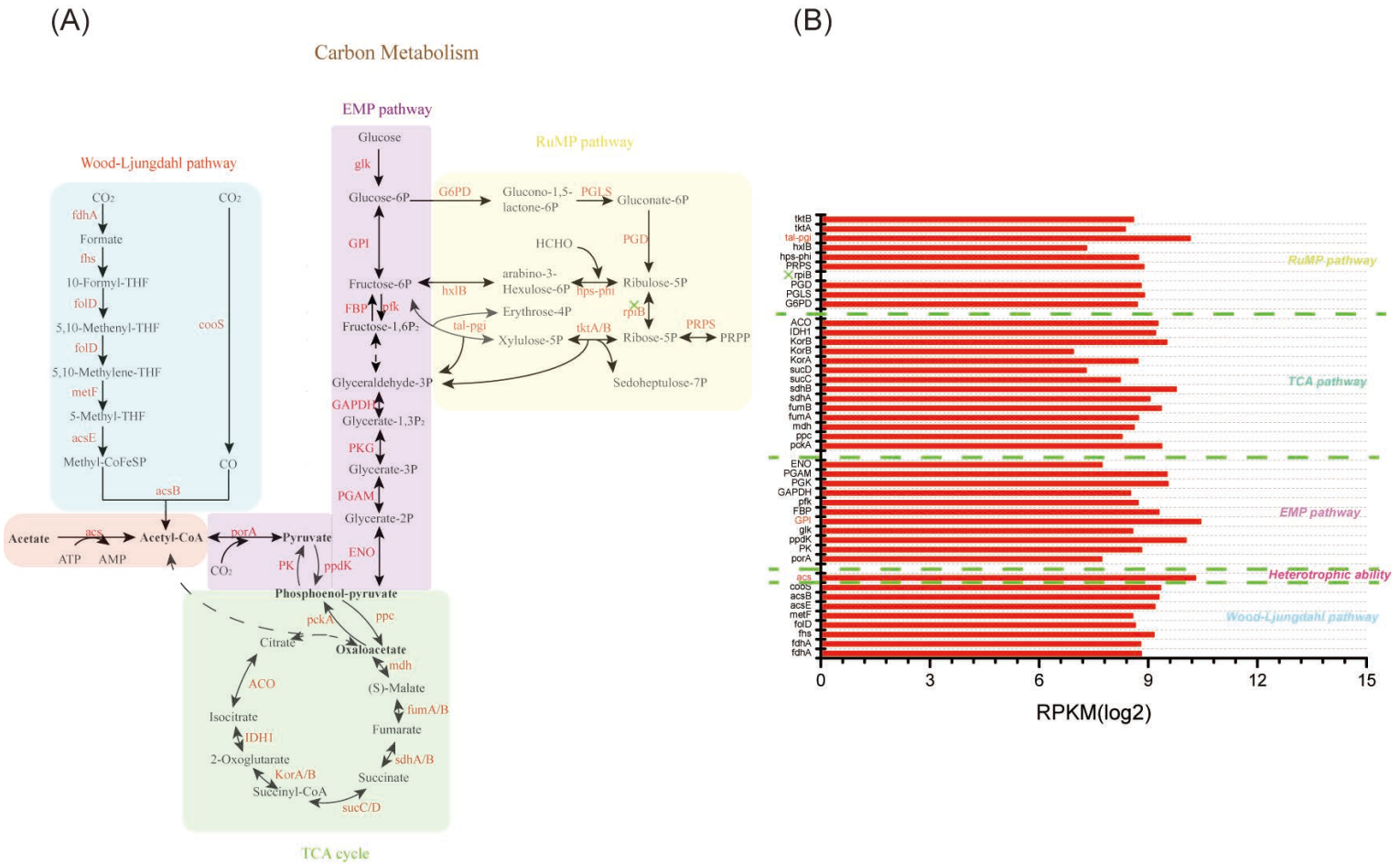


Fig. 4-5 (A) Overall of carbon metabolism of CSTR001. (B) The expression value of genes (log2 RPKM) related to carbon metabolism. Abbreviations not indicated in the text. The dotted line indicates the missing of the required genes; The green cross means the gene is missing.

4.4 Significance of This Study

The results of this study demonstrate diverse metabolisms associated with the oxygen detoxification and heterotrophic growth ability in CSTR001 affiliated to the strain of *Ca. Brocadia sp*. These findings are beneficial for mainstream anammox applications. Either through energy-efficient aeration or anaerobic treatment for the first stage carbon removal from sewage, a low-C/N-ratio treated sewage is suitable for the following-up nitritation/anammox process in the second stage (McCarty, 2018). This study is the first to elucidate the oxygen detoxification pathway in anammox bacteria based on metagenomics and metatranscriptomics. Importantly, the identified oxygen detoxification mechanism suggests that the short-foot-print nitritation-anammox process in one reactor is feasible. The oxygen detoxification pathway also indicates the possibility of energy generation or cell synthesis based on the organic carbon metabolism regulation by anammox bacteria. The remaining Biochemical Oxygen Demand (BOD), e.g., SMP, short-chain volatile fatty acids (VFAs), can serve as the organic sources for anammox bacteria. Additionally, the numerous types of organic carbon transporters, such as amino acids and vitamin B12 (Tab. S4-4D) in anammox bacteria revealed not only its aggregation capacity of microbial community or synergetic association with others (Lawson et al., 2017) but also the possibility of organic carbon utilization. As the sewage influent and operational conditions are generally unstable, the versatility of both inorganic and organic carbon for energy harvesting or cell synthesis in CSTR001 based on the regulation of carbon metabolism has promise for the system's adaptability and stability, and shortening the start-up period of anammox process. It also raised a question, if SOD2 and Ccp work, in what DO range anammox bacteria can perform oxygen detoxification for energy harvesting or cell synthesis based on organic carbons and out-compete with nitrite oxidizer, which should be explored in further studies. This study raises a new horizon both for our understanding of anammox metabolism and its mainstream application.

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Supporting material for charper 4

Table S4-1. Sludge samples for 16S rRNA sequencing, metagenomics, and metatranscriptomic analysis

Sample ID	Time (d)	16S rRNA sequencing	Metagenomics	Metatranscriptomics
AX6	55	✓	-	-
AX7	105	✓	-	-
AX8	144	✓	-	-
AX11	234	✓	✓	✓

Table S4-2. Diversification of 16S rRNA sequencing results

Sample ID	OTUs	Invsimpson	Shannon	Chao 1	Ace	Coverage	Simpson index
AX6	3149	23.078704	4.344884	11028.47	25088.22	0.972505	0.007925
AX7	3121	16.848169	4.112391	11583.74	23004.25	0.970216	0.005398
AX8	3726	10.196098	3.790793	12330.31	25777.54	0.968202	0.003105
AX11	1760	3.576716	2.441092	6657.699	13018.61	0.983104	0.002041

Table S4-3. The sequences quality of draft anammox genome

Sample	CSTR001
Completeness	99.94%
Predicted Protein	2826
Annotated (Kegg)	45.9%
GC std	0.029468119
Genome size	3161494
Longest contig	132065
Contigs	185
Contamination	1.648%
GC	42.3%
N50	35660
Ambiguous bases	0
Mean contig length	17089.1568

Table S4-4(A) Lists the expressed anammox metabolism genes in the CSTR001 genome

Gene	Length	Definition	Abbreviation	RPKM (log2)
Bin2.001_00027	891	formate/nitrite transporter	*focA*	8.995046
Bin2.001_00724	1215	nitrate/nitrite transporter	*NRT*	8.547587
Bin2.001_02173	1215	nitrate/nitrite transporter	*NRT*	8.225659
Bin2.001_00498	1383	ammonium transporter	*amt*	8.623777
Bin2.001_01110	2823	nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG1*	9.816741
Bin2.001_02719	2574	nitrate reductase / nitrite oxidoreductase, alpha subunit	*narG2*	8.949958
Bin2.001_01132	1218	nitrite reductase (NADH) large subunit	*nirB*	8.222101
Bin2.001_00136	1440	nitrite reductase (cytochrome c-552)	*nrfA*	8.980546
Bin2.001_00137	465	cytochrome c nitrite reductase small subunit	*nrfH*	7.611313
Bin2.001_02771	2433	Dipeptidyl-peptidase 5	*hzsA*	9.68331
Bin2.001_02810	1131	6-phosphogluconolactonase	*hzsC*	8.650944
Bin2.001_00988	354	NADH-quinone oxidoreductase subunit A	*nuoA*	9.004794
Bin2.001_00990	507	NADH-quinone oxidoreductase subunit C	*nuoC*	7.486558
Bin2.001_00991	1194	NADH-quinone oxidoreductase subunit 4	*nuoD*	8.835775
Bin2.001_00993	1848	NADP-reducing hydrogenase subunit HndC	*nuoF*	8.790575
Bin2.001_00996	1041	NADH-quinone	*nuoH*	8.770573

		oxidoreductase subunit H		
Bin2.001_01000	1929	NADH-quinone oxidoreductase subunit L	*nuoL*	8.728687
Bin2.001_01001	1563	NADH-quinone oxidoreductase subunit M	*nuoM*	9.184225
Bin2.001_01002	1464	NADH-quinone oxidoreductase subunit N	*nuoN*	7.541662
Bin2.001_02482	498	NADP-reducing hydrogenase subunit HndA	*nuoE*	9.09736
Bin2.001_02483	1680	NADP-reducing hydrogenase subunit HndC	*nuoF*	8.565509
Bin2.001_00052	1536	Hydroxylamine oxidoreductase	*hao1*	7.887437
Bin2.001_01491	1650	Hydroxylamine oxidoreductase	*hao2*	8.591504
Bin2.001_01613	1746	Hydroxylamine oxidoreductase	*hao3*	9.02449
Bin2.001_01757	1611	Hydroxylamine oxidoreductase	*hao4*	8.626013
Bin2.001_02305	1452	Hydroxylamine oxidoreductase	*hao5*	8.775929
Bin2.001_02708	1608	Hydroxylamine oxidoreductase	*hao6*	8.821348

Table S4-4(B) Lists the expressed ROS detoxification genes in the CSTR001 genome

Gene	Length	Definition	Abbreviation	RPKM (log2)
Bin2.001_01272	690	Superoxide dismutase, Fe-Mn family	SOD2	9.626909
Bin2.001_01130	1053	Cytochrome c551 peroxidase	CCP	9.602035
Bin2.001_00268	1788	Menaquinol-cytochrome c reductase cytochrome b subunit	CYTB	8.838193
Bin2.001_00269	489	Cytochrome b6-f complex iron-sulfur subunit	MQCRA	9.538709

Table S4-4(C) Lists the expressed carbon metabolism genes in the CSTR001 genome

Gene	Length	Definition	Abbreviation	RPKM (log2)
Wood-Ljungdahl pathway				
Bin2.001_00994	2640	formate dehydrogenase (NADP+) alpha subunit	*fdhA*	8.806517
Bin2.001_02480	2688	formate dehydrogenase (NADP+) alpha subunit	*fdhA*	8.780522
Bin2.001_00587	1764	Formate--tetrahydrofolate ligase	*fhs*	9.147196
Bin2.001_01174	912	methylenetetrahydrofolate dehydrogenase (NADP+) / methenyltetrahydrofolate cyclohydrolase	*folD*	8.639509
Bin2.001_02096	960	methylenetetrahydrofolate reductase (NADPH)	*metF*	8.565509
Bin2.001_00601	786	5-methyltetrahydrofolate:corrinoid/iron-sulfur protein co-methyltransferase	*acsE*	9.175942
Bin2.001_00595	2184	Carbon monoxide dehydrogenase/acetyl-CoA synthase subunit alpha	*acsB*	9.286533
Bin2.001_00594	1968	anaerobic carbon-monoxide dehydrogenase catalytic subunit	*cooS*	9.33724
Bin2.001_02005	1740	Acetyl-coenzyme A synthetase	*acs*	10.29249
EMP pathway				

Bin2.001_01122	1293	Pyruvate synthase subunit PorA	*porA*	7.720855
Bin2.001_00180	1419	Pyruvate kinase	PK	8.809095
Bin2.001_02212	2772	pyruvate, orthophosphate dikinase	*ppdK*	10.03569
Bin2.001_00582	960	Glucokinase	*glk*	8.565509
Bin2.001_02678	1638	Glucose-6-phosphate isomerase	GPI	10.43854
Bin2.001_01666	1020	Fructose-1,6-bisphosphatase class 1	FBP	9.285401
Bin2.001_01133	1083	ATP-dependent phosphofructokinase / diphosphate-dependent phosphofructokinase	*pfk*	8.71351
Bin2.001_02484	1002	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	8.503732
Bin2.001_02485	1227	Phosphoglycerate kinase	PGK	9.533408
Bin2.001_00080	750	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	PGAM	9.506615
Bin2.001_02201	1287	Enolase	ENO	7.727565
TCA cycle				
Bin2.001_00474	1656	Phosphoenolpyruvate carboxykinase [ATP]	*pckA*	9.363875
Bin2.001_01701	1473	Phosphoenolpyruvate carboxylase	*ppc*	8.269786
Bin2.001_01422	936	Malate dehydrogenase	*mdh*	8.602034
Bin2.001_00342	858	fumarate hydratase subunit alpha	*fumA*	8.727566

Bin2.001_00343	558	fumarate hydratase subunit beta	*fumB*	9.348278
Bin2.001_00066	1725	succinate dehydrogenase / fumarate reductase, flavoprotein subunit	*sdhA*	9.041947
Bin2.001_00287	735	succinate dehydrogenase / fumarate reductase, iron-sulfur subunit	*sdhB*	9.758154
Bin2.001_02012	912	succinyl-CoA synthetase alpha subunit	*sucC*	8.224472
Bin2.001_02013	1161	succinyl-CoA synthetase beta subunit	*sucD*	7.291247
Bin2.001_00563	1089	2-oxoglutarate oxidoreductase subunit KorA	*korA*	8.705539
Bin2.001_00562	741	2-oxoglutarate oxidoreductase subunit KorB	*korB*	6.939069
Bin2.001_01210	753	2-oxoglutarate oxidoreductase subunit KorB	*korB*	9.500856
Bin2.001_00265	1242	Isocitrate dehydrogenase [NADP]	IDH1	9.19395
Bin2.001_01909	1932	aconitate hydratase	ACO	9.25696
RuMP pathway				
Bin2.001_02249	1539	Glucose-6-phosphate 1-dehydrogenase 2	G6PD	8.691977
Bin2.001_02250	768	6-phosphogluconolactonase	PGLS	8.887437

Bin2.001_02558	1020	6-phosphogluconate dehydrogenase, NAD(+)-dependent, decarboxylating	PGD	8.799974
Bin2.001_01180	453	ribose 5-phosphate isomerase B	*rpiB*	/
Bin2.001_01508	972	Ribose-phosphate pyrophosphokinase	PRPS	8.869515
Bin2.001_00966	1290	3-hexulose-6-phosphate synthase / 6-phospho-3-hexuloisomerase	*hps-phi*	8.724207
Bin2.001_00805	579	6-phospho-3-hexuloisomerase	*hxlB*	7.29498
Bin2.001_02504	3291	transaldolase / glucose-6-phosphate isomerase	*tal-pgi*	10.14564
Bin2.001_00821	828	Transketolase A	*tktA*	8.363875
Bin2.001_00822	945	Transketolase B	*tktB*	8.588229

Table S4-4(D) Lists the expressed transporter genes in the CSTR001 genome

Gene	Length	Definition	RPKM (log2)
Bin2.001_00027	891	formate/nitrite transporter	8.995046
Bin2.001_00175	1389	ammonium transporter protein	8.617531
Bin2.001_00347	978	Cadmium, cobalt and zinc/H(+)-K(+) antiporter	9.708634
Bin2.001_00359	1365	magnesium transporter protein	8.379642
Bin2.001_00498	1383	ammonium transporter protein	8.623777
Bin2.001_00535	1290	Putative outer membrane protein	9.139244
Bin2.001_00700	744	zinc and cadmium transporter	7.93324
Bin2.001_00741	1173	arsenite transporter	8.59834
Bin2.001_00742	2118	vitamin B12 transporter	8.745841
Bin2.001_00955	1074	putative transporter protein	8.725549
Bin2.001_01069	873	High-affinity branched-chain amino acid transport system	9.509916
Bin2.001_01070	876	High-affinity branched-chain amino acid transport system permease protein LivH	9.01954
Bin2.001_01400	618	biopolymer transport protein ExbB	8.200936
Bin2.001_01792	1221	putative transporter protein	9.025907
Bin2.001_01820	576	biopolymer transport protein ExbB	7.302474
Bin2.001_01821	2280	Vitamin B12 transporter BtuB	8.902544
Bin2.001_01851	1512	basic amino acid/polyamine antiporter, APA family	9.717512
Bin2.001_01986	1095	putative C4-dicarboxylate transporter	8.960647
Bin2.001_01982	1362	potassium transporter protein	9.382816
Bin2.001_01983	1485	potassium transporter protein	9.106077
Bin2.001_02115	1284	Serine/threonine exchanger SteT	9.605401
Bin2.001_02213	1275	putative transporter protein	8.156118

Bin2.001_02628	639	cobalt/nickel transport system permease protein	7.152727
Bin2.001_02534	702	putative Mg^{2+} transporter-C (MgtC) family protein	9.017072

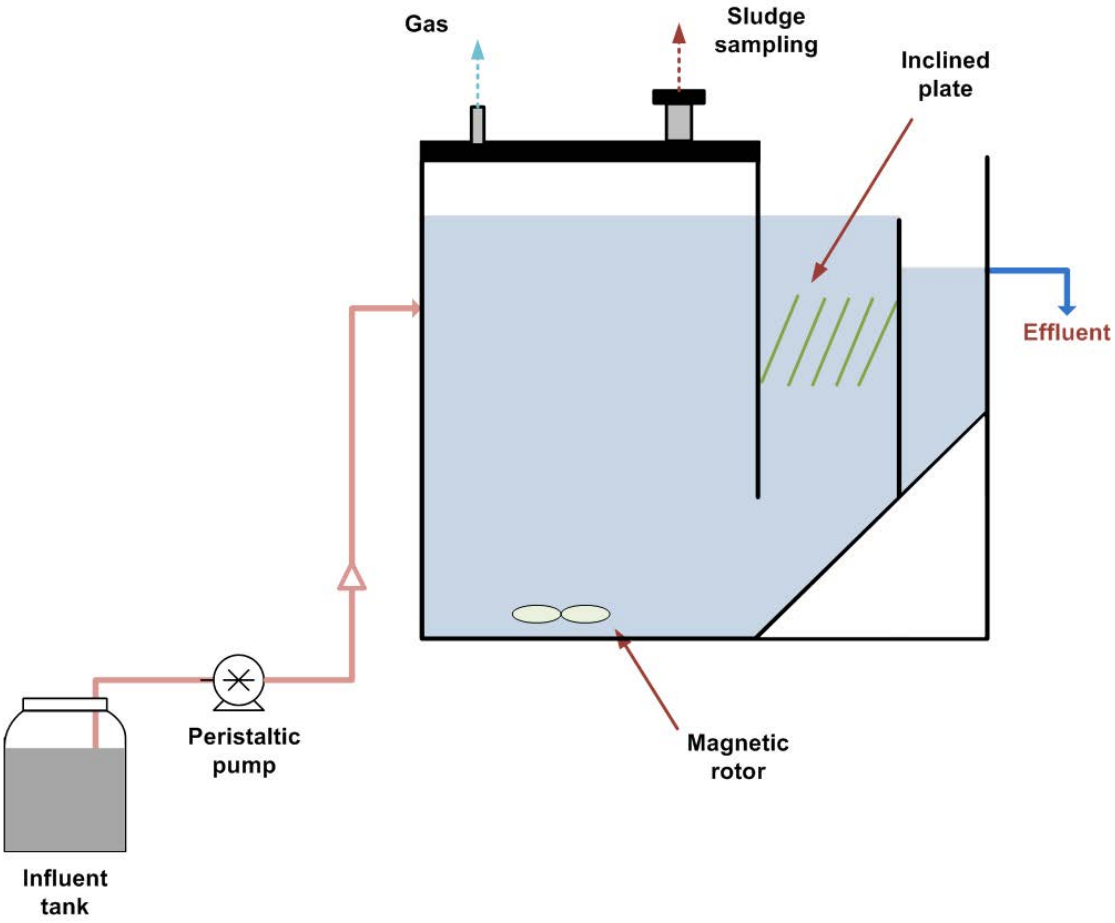


Fig. S4-1. Schematic diagram of enriched anammox reactor

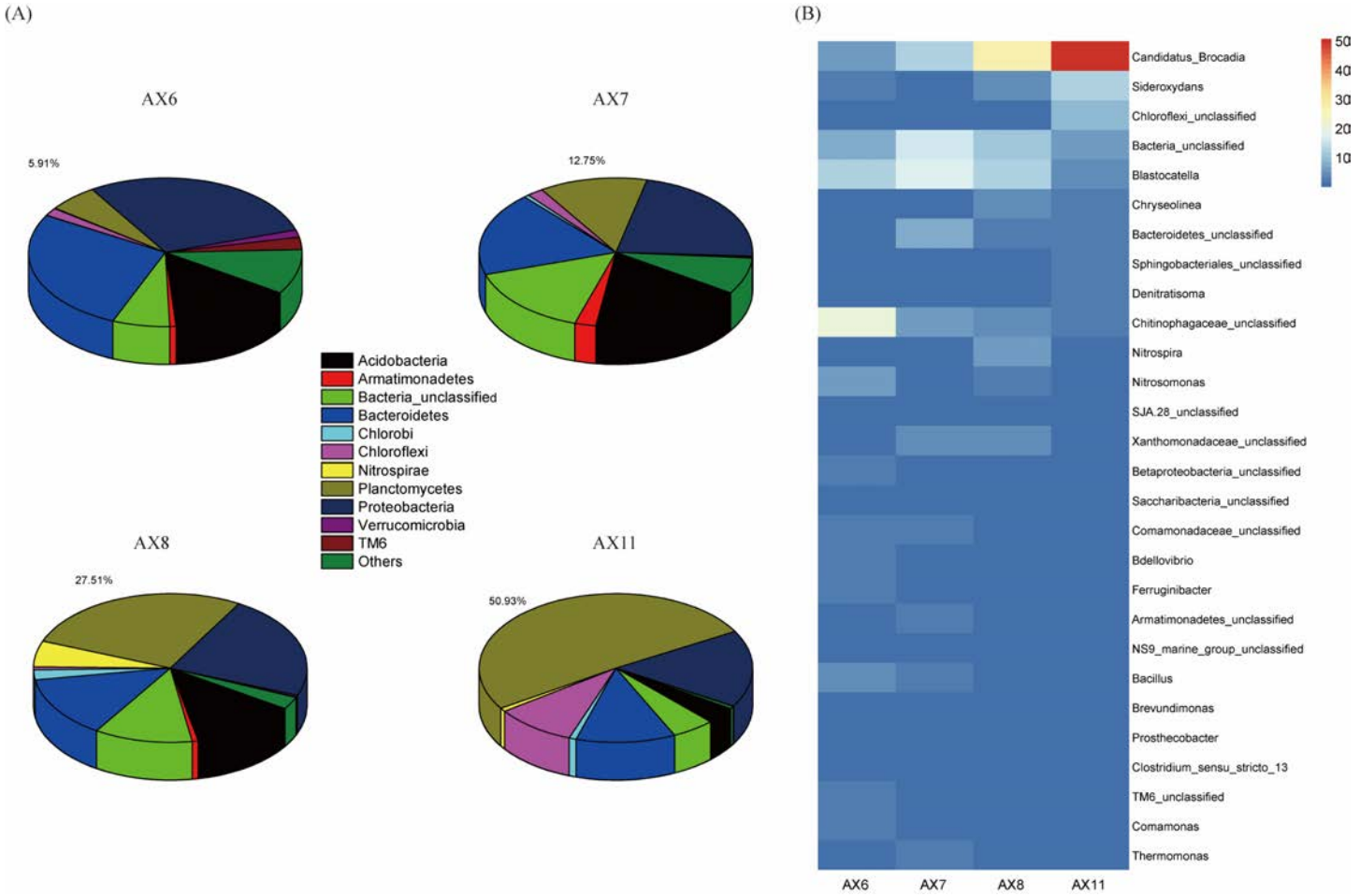


Fig. S4-2. Taxonomic classification of microbial populations at phylum level (A) and heatmap showing of the genus level (B) by 16S rRNA sequences (Phyla and genera with more than 1% relative abundance). Heatmap scale represents the relative abundance of dominant genera.

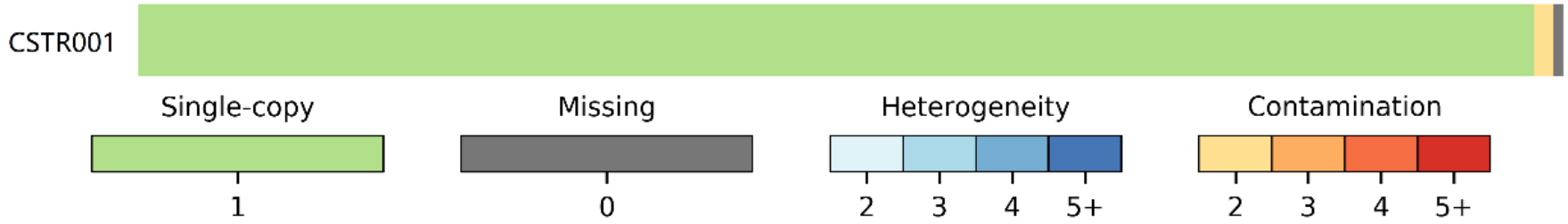
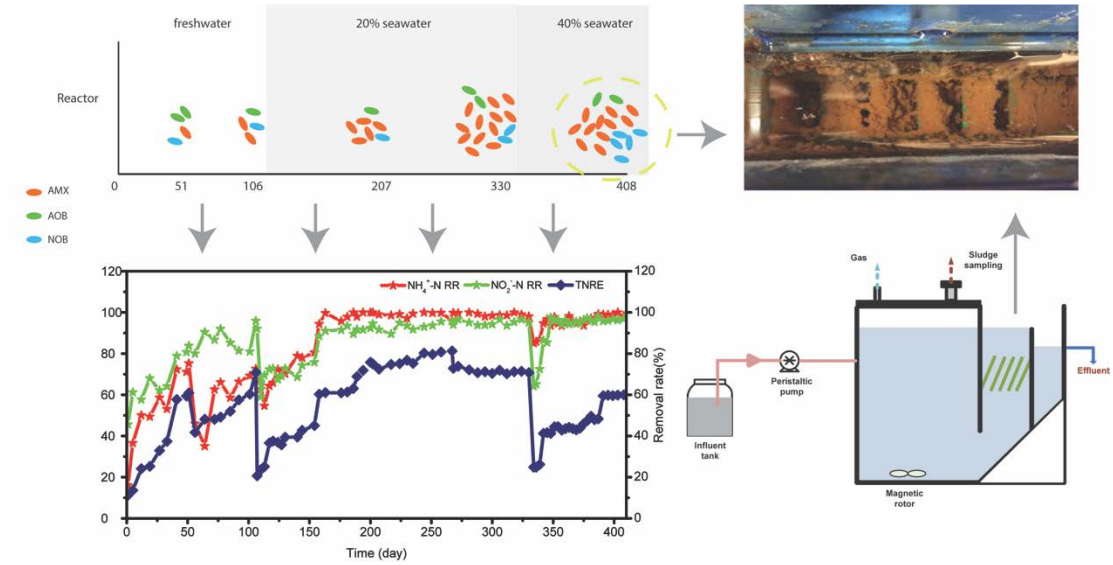


Fig. S4-3. CheckM plot for the quality assessment of draft anammox genome describing bin completeness, contamination, and heterogeneity.

Chapter 5 Microbial community of anammox process response to the introduction of real seawater for low-strength wastewater treatment

GRAPHIC ABSTRACT



5.1 Introduction

With the development of coastal cities, the production of saline wastewater is increased. One of such causes is seawater intrusion into coastal freshwater aquifers. The over-extraction of groundwater resources is one of the important reasons (Li et al., 2017). Meanwhile, seawater was directly used in coastal cities due to the shortage of freshwater. For example, seawater was used to flush toilets in Hong Kong (Leung et al., 2012). Both of these two contributors will result in an introduction of seawater in mainstream wastewater (Bear et al., 1999, Li et al., 2017), increasing the difficulty for biological wastewater treatment. Recently, anaerobic ammonium-oxidizing (anammox)-based nitrogen removal processes has attracted increasing attention due to its advantages: less oxygen requirement, no additional carbon matters and less sludge production (Du et al., 2015, Lackner et al., 2014, Ma et al., 2017, Siegrist et al., 2008). Anammox process was successfully applied for high-strength wastewater treatment (Lackner et al., 2014, Ma et al., 2016), and in recent, an increasing of studies supported its feasibility for mainstream wastewater treatment (Agrawal et al., 2018, Laureni et al., 2016). The introduction of seawater results an additional challenge for the mainstream anammox processes by driving the microbial community.

The evolution of microbial community structure could provide a comprehensive understanding on the nitrogen removal performance of anammox-based processes, and further to optimize the whole system. (Vlaeminck et al., 2012). Recently, the

microbial interaction in the anammox-based systems was investigated by Speth et al. (2016) and Lawson et al. (2017), indicating the importance of microbial community structure for reactor performance. Most of the studies explored the salinity effect on anammox bacteria and the microbial community through adding NaCl (Bassin et al., 2012, Li et al., 2018, Kartal et al., 2006, Kuypers et al., 2003, Xing et al., 2015), and limited reports available describe the application of anammox process for mainstream wastewater treatment with real seawater. Previous studies reported the dominant anammox species shift from *Ca. Brocadia* to *Ca. Kuenenia* with the increasing of salinity concentration, since *Ca. Kuenenia* could endure a higher salinity than *Ca. Brocadia* (Kartal et al., 2006, Zhang et al., 2010). Apart from anammox species, the microbial community structure was significantly affected by the wastewater constituent (Gonzalez-Martinez et al., 2015, Liu et al., 2018). Obviously, the seawater composition is much more complex than the synthetic wastewater with only NaCl. In addition to the NaCl, the presence of sulfate and organic matters would also shape the microbial community during the startup of anammox processes. For example, heterotrophic denitrifiers could benefit from the additional carbon source and might enhance the nitrogen removal efficiency through denitrification (Lotti et al., 2015). Sulfate might be metabolized by anammox bacteria or denitrifiers as electron acceptors, in turn to affect the microbial community (Wu et al., 2016, Yang et al., 2009). Additionally, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) widely present in anammox-based systems (Pereira et al., 2017). The introduce of seawater also possibly affect the distribution of AOB and NOB, further

to compete with anammox bacteria for nitrogen sources. However, limited reports describe the microbial community of anammox process with real seawater, especially for the evolution of microbial community during the start-up of anammox process for low-strength seawater-based wastewater treatment.

Therefore, this study aimed to characterize the evolution of microbial community during the startup of anammox process for low-strength wastewater treatment with different proportion of seawater. A continuous flow anammox reactor was set up at room temperature without pre-enriching high abundance of anammox bacteria and the reactor performance was investigated at different seawater proportion. The whole microbial community structure under different seawater proportion was investigated, with special attention on the anammox, AOB and NOB species.

5.2 Material and methods

5.2.1 Reactor set-up, inoculum and operation strategy

A lab-scale anammox continuous stirred-tank reactor (CSTR) with an effective volume of 1.0 L was set up and operated at room temperature (17-21℃). Magnetic stirring with 50 rpm was used for mixing in the reactor. Biomass was intercepted through the inclined plate installed in the sedimentation area. The reactor was inoculated with dried powdered sludge taken from a simultaneous partial nitrification, anammox and denitrification (SNAD) reactor treating landfilled leather in Taiwan (Taiwan,

China). The dried powdered sludge was activated in the lab before inoculum. The inoculated sludge had no anammox activity and DNA cannot be extracted.

The reactor was fed with synthetic saline wastewater (without pre-removing dissolved oxygen) containing NH_4Cl, $NaNO_2$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and 1 mL/L of trace element solution as described by Van de Graaf et al. (1996), and different proportions of seawater. Seawater was collected from the Hong Kong Victoria Harbour without any pre-treatment process, which the salinity was 3.1-3.3% at the point of collection. The influent concentration of ammonium and nitrite were configured as needed. The influent pH was adjusted to 8.0 without further adjustment. Three operational stages with different seawater proportions were conducted in this study as shown in Tab. 5-1.

Table 5-1 The different operational stages throughout this study

Stage	Time (day)	Influent NH_4^+-N concentration (mg/L)	Influent NO_2^--N concentration (mg/L)	Hydraulic retention time (h)	Nitrogen loading rate (kg-N/(m^3•d))
1	1-51	32.20 ± 1.04	41.78 ± 1.01	12	0.151
	52-106	32.47 ± 0.90	39.57 ± 0.94	9.6	0.184
	107-120	32.05 ± 0.94	39.16 ± 0.89	9.6	0.181
2	121-267	32.31 ± 1.62	36.98 ± 1.82	7.8	0.218
	268-330	30.25 ± 0.38	31.05 ± 0.48	6	0.249
3	331-387	30.29 ± 0.54	30.82 ± 0.42	6	0.248
	388-408	30.15 ± 0.26	30.85 ± 0.23	3.6	0.413

5.2.2 Analytical methods

The collected influent and effluent water samples were filtered (0.45μm) before analyzing. The influent and effluent water quality parameters of NO_3^--N, NO_2^--N, NH_4^+-N were measured according to standard methods (APHA, 2005) and pH was done using a pH meter (Leici, China).

5.2.3 Sludge sampling, DNA extraction, and 16S rRNA sequencing

Biomass samples were obtained from the reactors by centrifugation (5 min, 10000 g, 4 °C) on day 51, 106, 207, 330 and 408, were named as AS1, AS2, AS3, AS4, and AS5.

DNA extraction was performed using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instruction, and the quality and concentrations were measured using NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE).

DNA extracts were amplified by polymerase chain reaction (PCR) in the V3-V4 region of the 16S rRNA gene using primers 341F: ACTCCTACGGGAGGCAGCAG and 806R: GGACTACHVGGGTWTCTAAT, and were sequenced using the Illumina MiSeq platform (BGI, Shenzhen). The raw reads were processed using MOTHUR software to remove chimeric and low-quality sequences (Schloss et al., 2009). The obtained high-quality sequences with an average length of 430 bp were clustered into operational taxonomic units (OTUs) with 97% similarity cutoff and assigned to the SILVA reference database. Raw 16S rRNA data obtained in this study had been deposited into NCBI Sequence Read Archive database with the accession number of (PRJNA491507).

5.3 Results and discussion

5.3.1 Reactor performance

The overall nitrogen removal performance of CSTR was shown in Fig. 5-1. The reactor was fed with 31 mg/L of NH_4^+-N and 41 mg/L NO_2^--N at HRT of 12 hr for anammox bacteria accumulation, representing the nitrogen loading rate (NLR) of 0.151 kg-

N/(m^3•d). During this period, the total nitrogen removal effective (TNRE) increased with the operational time and reached around 60% on Day 55, accounting for a nitrogen removal rate (NRR) of 0.092 kg-N/(m^3•d). The NH_4^+-N and NO_2^--N removal rate of 75.29% and 80.03% was achieved on Day 55, meanwhile, the NO_3^--N concentration in the effluent decreased from 28.60 mg/L to 14.72 mg/L along with the operation. On Day 56, HRT was reduced to 9.6 hr with an increase of NLR from 0.151 kg-N/(m^3•d) to 0.186 kg-N/(m^3•d), resulting a sudden drop of TNRE from 60.86% to 41.71%. It should be noted that the NH_4^+-N removal rate also decreased from 75.29% to 46%, accounting for the main reduced part of TNRE, as the NO_2^--N removal rate and the NO_3^--N concentration kept stable. The TNRE recovered to 70.85% from Day 56 to 106, accounting for 0.130 kg-N/(m^3•d) of NLR. During this period (days 56-106), the NH_4^+-N and NO_2^--N removal rate increased from 46.00% to 72.60% and 80.03% to 95.96%, respectively, and the NO_3^--N concentration in the effluent decreased from 17.24 mg/L to 10.81 mg/L. During this period, ammonium and nitrite were removed with an average NO_2^--N/NH_4^+-N ratio of 1.55, similar to anammox reaction ratio, indicating anammox reaction was the main factor for nitrogen removal (Fig. 5-1). It is different from other anammox systems inoculated with aerobic activated sludge, nitrification sludge or anaerobic sludge, where ammonium concentration increased in the effluent due to the decay of heterotrophic bacteria (Trigo et al., 2006, Wang et al., 2009).

In the following stage (days 106 to 330), the influent wastewater contained 20% of seawater, resulting in a salinity concentration of 0.7% in influent. As a result, the TNRE

dropped from 70.85% to 20.69% sharply, similar to the result obtained by Xing et al., (2017), however, there was almost no negative effect on the NH_4^+-N and NO_2^--N removal rate. Interestingly, the increased nitrate concentration in the effluent was the main reason for the decrease of TNRE. On Day 118, HRT was further reduced to 7.8 hr, resulting in an increase of NLR from 0.180 kg-N/(m^3•d) to 0.214 kg-N/(m^3•d). Even though, the reactor performance was not affected and over 80% of TNRE was achieved on Day 254, representing for NRR of around 0.180 kg-N/(m^3•d). It showed that both of NH_4^+-N and NO_2^--N was almost removed and NO_3^--N was the main nitrogen substrate in the effluent (10.60 mg/L). Since the CSTR reached steady state, the HRT was shortened to 6 hr. Though it resulted TNRE decreasing from 81.27% to 70.74%, little impaction on the NRR was observed.

In the third stage (days 331 to 404), the seawater proportion was adjusted to 40%, resulting in a 1.5% of salinity concentration in influent. Similar to the former stage, the TNRE dropped from 70.74% to 24.86% suddenly at the point of the increase of seawater proportion. Both of the NH_4^+-N and NO_2^--N removal rates decreased, which resulted the decrease of NRR from 0.178 kg-N/(m^3•d) to 0.062 kg-N/(m^3•d). Moreover, the addition of seawater proportion yielded more negative impact on the NO_2^--N removal rate (from 95.04% to 64.12%) compared to that on the NH_4^+-N removal rate (from 98.08% to 85.05%). However, the NO_3^--N concentration in the effluent increased from 16.25 mg/L to 31.24 mg/L. The reactor reached at steady state on Day 384 with a TNRE of 48.36%, accounting for 0.122 kg-N/(m^3•d) of NRR. It was noteworthy that

both of the NH_4^+-N and NO_2^--N removal rate reached more than 98% and nitrate was the most dominant residual nitrogen compounds in the effluent, which concentration reached 23.09 mg/L. Shorter HRT (3.6 hr) was applied when the reactor reached steady state. Interestingly, the TNRE was improved to 60.03% at the end of the study. The reactor achieved 0.245 kg-N/(m^3•d) of NRR with 23.09 mg/L of NO_3^--N concentration in the effluent.

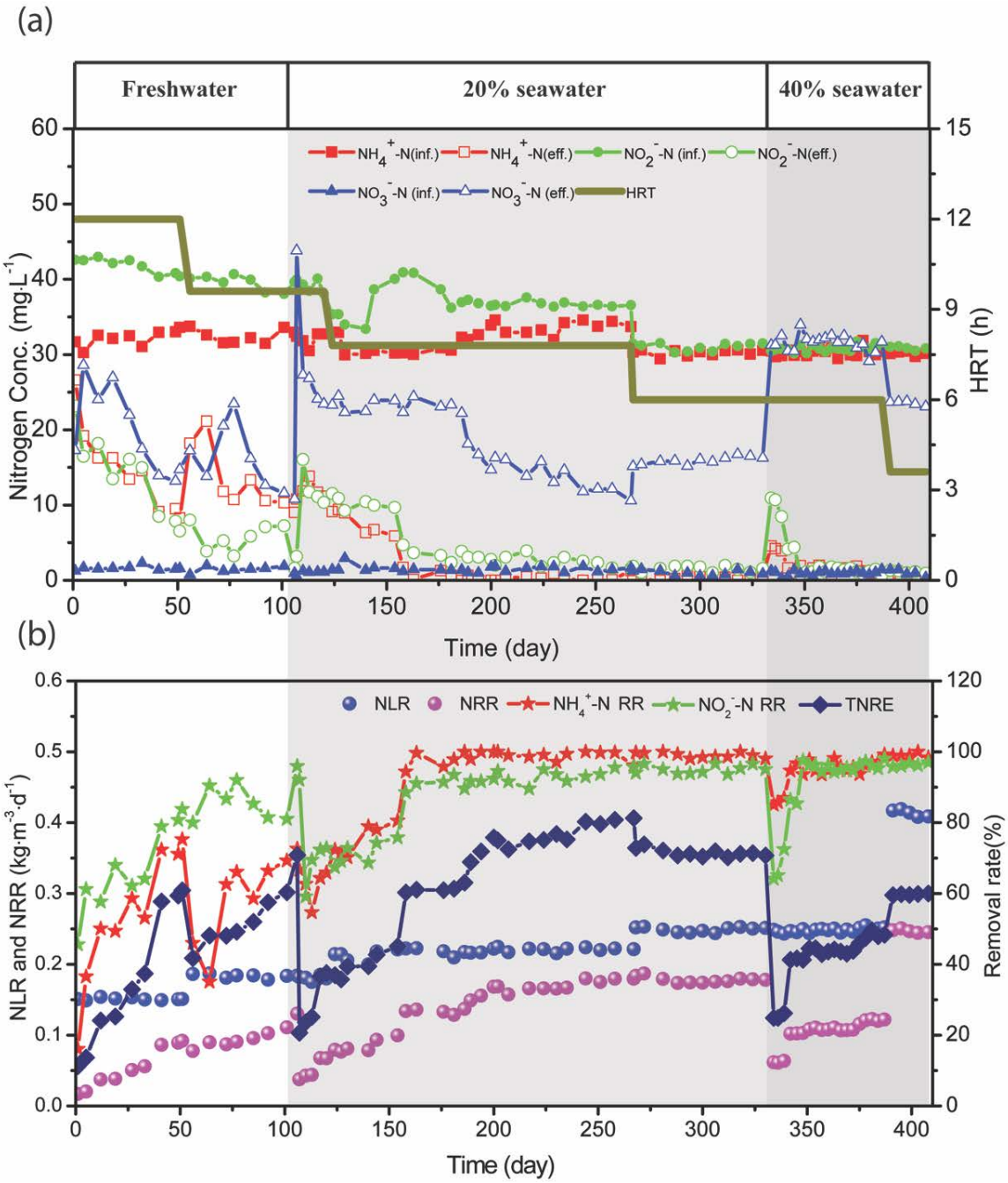


Fig. 5-1 The overall performance of nitrogen removal in CSTR during the entire operation. (a): Variation of nitrogen compounds; (b): Variation of nitrogen removal efficiency. TNRE: Total Nitrogen removal efficiency; NLR: Nitrogen loading rate; NRR: Nitrogen removal rate.

5.3.2 The overall shifts of microbial community

5.3.2.1 Microbial community Diversity

In this study, the microbial community in the CSTR corresponding to operational time was conducted based on 16S rRNA sequencing. The sequencing depth of five samples was more than 43898 reads, representing more than 2545 OTUs clusters (Tab. 5-2). The diversity of the microbial community in response to the shift of seawater proportions was evaluated and listed in Tab. 5-2. During the first stage, the microbial diversity decreased significantly even a shorter HRT was applied. Similar phenomena were obtained during the second stage that the microbial diversity decreased when the HRT was reduced from 7.8 h to 6.0 h. This result suggested that the microbial diversity in CSTR decreased with the operational time under same seawater proportion. Additionally, note that the microbial diversity increased significantly after 20% of seawater was added to the freshwater influent. Furthermore, the microbial diversity increased when the influent of CSTR contained 40% of seawater, while less contribution on the increasing of microbial diversity was observed compared with adding 20% of seawater. It should be noted that comparing to sample AS1, the microbial diversity of AS2, AS3, AS4 and AS5 were much less, though different proportions of seawater were added in the influent. These results suggested that the microbial diversity of CSTR responded to the shift of seawater proportions, however, it decreased with the operation.

Table 5-2 The diversity of microbial communities in different samples

Sample	Total No. of sequences	OTUs	invsimpson	shannon	chao	ace	coverage
AS1	90609	4407	19.20	4.22	9394.61	22008.61	0.96
AS2	99517	3225	5.76	3.17	5838.02	12097.02	0.97
AS3	86969	2642	10.50	3.36	5601.31	12088.63	0.98
AS4	75834	3499	5.07	3.15	9897.05	24674.5	0.96
AS5	43898	2545	6.97	3.47	8816.28	20324.74	0.96

The evolution of microbial community over time and, with respect to different seawater proportions was analyzed via principal components analysis (PCA) method (Fig. 5-2). A significant difference in community composition among AS3, AS4 and AS5 were observed comparing to AS1 and AS2, however, the community composition of AS1 and AS2 is similar. The significant difference between AS3 and AS4 mainly due to the enrichment of anammox bacteria, which relative abundance increased from 17.31% to 40.83% (Fig. 5-3b). However, the community composition of AS4 and AS5 was different obviously though the relative abundance of anammox bacteria is similar (Fig. 5-3b), indicating the seawater plays an important role on the community composition.

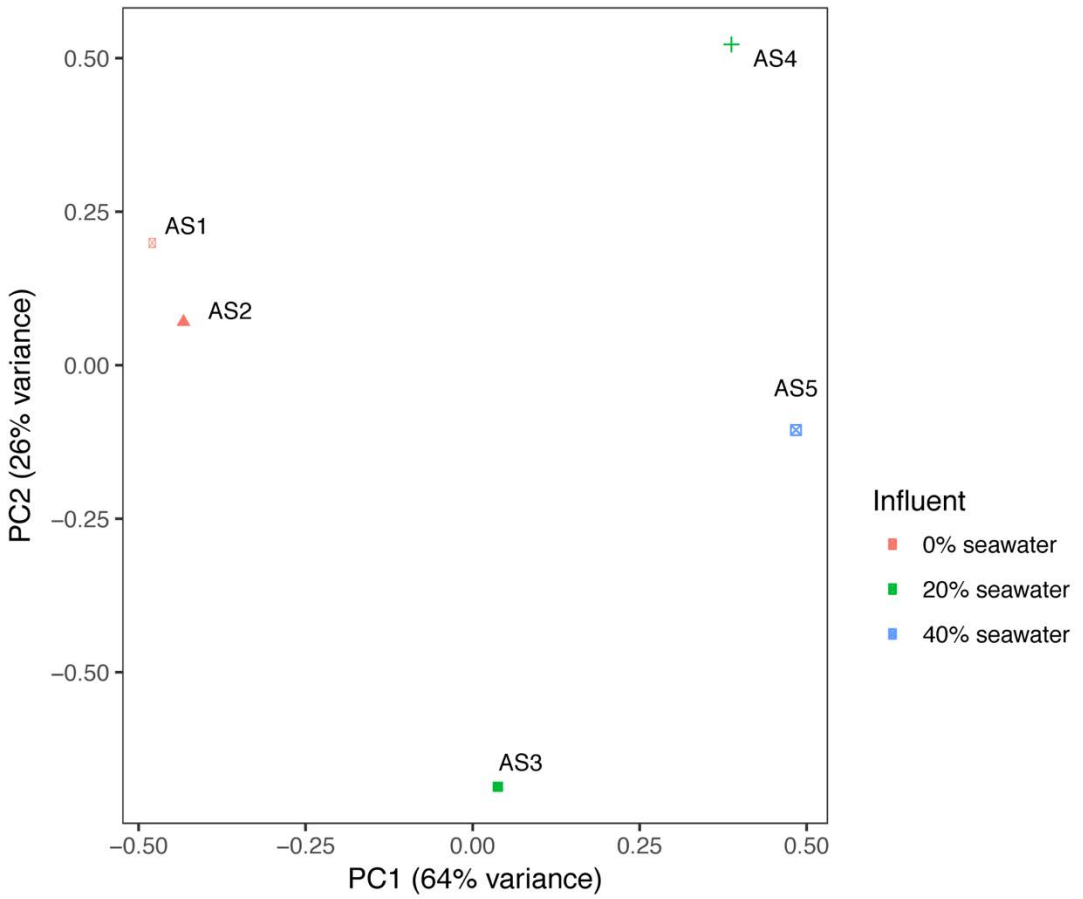


Fig. 5-2 Principal components analysis (PCA) of the microbial community using dominant OUT clusters (relative abundance>1%)

5.3.2.2 Microbial community structure

The taxonomic results showed that the microbial community belonged to *Planctomycetes*, *Proteobacteria*, *Bacteroidetes, Acidobacteria, Chloroflexi, Chloroflexi, Firmicutes* and *Nitrospirae* on phylum level, accounting for more than 82.51% of total microbes in all samples. The relative abundance of *Planctomycetes* increased and reached 40.84% on day 330 in the CSTR though the seawater proportion

increased from 0 to 20% (Fig. 5-3a). However, it should be noted that its relative abundance decreased slightly (35.44%) when the seawater proportion was improved to 40%. *Bacteroidetes* was enriched in freshwater environment, accounting for 43.20% of total microbial community on day 106, however, different from *Planctomycetes,* its relative abundance decreased significantly when seawater was added in the influent. The relative abundance of *Chloroflexi* increased from 2.70 % to 11.60% due to the addition of seawater, since it decreased in the freshwater condition. A phenomenon of negative impact on *Proteobacteria* was observed since its relative abundance decreased from 24.29% to 10.73%. Similar to *Proteobacteria*, the relative abundance of *Acidobacteria* also decreased from 7.49% to 4.90% due to the additional seawater. Additionally, *Firmicutes* was washed out from the reactor over time, however, *Nitrospirae* became one of the dominant species, accounting for a relative abundance of 12.79%. These results suggested that the changes of the microbial community in the CSTR affected by the introduction of seawater proportion.

At the genus level, significant differences in the dominant species and relative abundance had been observed. For example, from AS1 to AS5, the relative abundance of *Candidatus Brocadia*, *Nitrospira*, *Nitrosomonas*, and *Denitratisoma* were increased and became the dominant genus. Conversely, *Niabella*, *Bacillus*, and *Comamonas* were washed out from the reactor. Specially, anammox species identified as *Candidatus Brocadia* increased from 0.22% to 35.00% with increasing seawater proportions in the influent, and it became the most dominant genus at the end of the study (Fig. 5-3b).

Notably, *Candidatus Brocadia* accounted for over 99% of *Planctomycetes* in all samples. AOB affiliated with *Nitrosomonas* genus (3.29%) was enriched in AS5. Interestingly, although the relative abundance of *Nitrosomonas* decreased to 0.25% when the CSTR was fed freshwater, it increased along the increasing of seawater proportions. It should be noted that NOB affiliated with *Nitrospira* genus enriched with a relative abundance of 12.79% in AS5 when the seawater proportion was shift to 40%. Additionally, marine nitrite oxidizer identified to *Nitrospina* genus was detected in AS5, accounting for a relative abundance of 0.56%. In addition to nitrifying and anammox bacteria, *Denitratisoma* (1.55%) was fostered in the CSTR at the end of the study. Moreover, no obvious differences in the relative abundance of *Denitratisoma* between AS2 and AS3, even between AS4 and AS5, though the seawater proportion was increased.

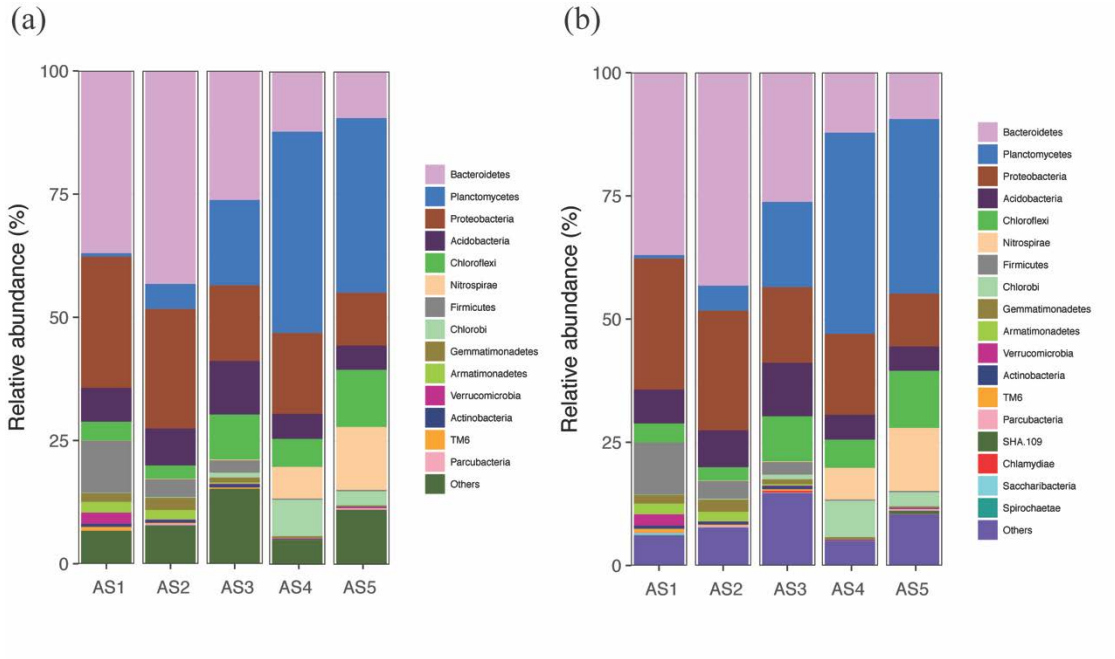


Fig. 5-3 Relative abundance of the major microbial community at the phylum and genus level by 16S rRNA sequencing. AS1: Day 51, AS2: Day 106; AS3: Day 207; AS4: Day 330; AS5: Day 408.

5.3.2.3 Anammox and nitrifying species

Three OTU clusters, five OTU clusters and four OTU clusters for anammox bacteria, AOB and NOB with relative abundance over 0.1% were select, respectively, and further aligned with reference species to construct neighbor-joining phylogenetic trees (Tab. 5-3 and Fig. 5-4). In the present study, the dominant anammox genera were not changed with the increase of seawater proportion. *Ca. Brocadia* and *Ca. Kuenenia* were the only two anammox species detected in this study, which was consistent with the reports conducted by Kuenen et al. (2008). However, their relative abundance had distinctly varied due to the shift of seawater proportion. Taxonomic results revealed that the

biodiversity of anammox species was reduced significantly because the dominant anammox bacteria (OTU00001), closed to *Ca. Brocadia sp* (98%), was enriched significantly (from 0.17% to 34.24%) and accounted for over 99% in all anammox species in AS 2-5 (Tab. 5-3 and Fig. 5-4a). In this study, *Ca. Brocadia sp* dominated the whole process, which was different from the results conducted by Gonzalez-Silva et al., (2017), who reported that the dominant anammox species were *Ca. Brocadia fulgida* or *Ca. Kuenenia stuttgartiensis* through adding NaCl as salinity. Another two anammox clusters (OTU00072 and OTU00182) identified as *Ca. Kuenenia stuttgartiensis* (99%) and *Ca. Brocadia fulgida* (98%) were also detected, respectively. Different from genus *Ca. Brocadia*, the relative abundance of anammox cluster OTU00072, similar to *Ca. Kuenenia stuttgartiensis*, decreased with the increasing seawater proportion. Additionally, it should be pointed out that no marine anammox species, i.e. *Ca. Scalindua*, were detected.

Table 5-3 Relative abundance of major OTUs sequences related to anammox bacteria, AOB and NOB

Species	OTUs	AS1	AS2	AS3	AS4	AS5
AMX	Otu00001	0.17	4.37	16.93	39.92	34.24
	Otu00182	0	0.01	0.02	0.04	0.12
	Otu00072	0.04	0.44	0.09	0.06	0.04
AOB	Otu00016	1.95	0.20	0.17	1.87	1.10
	Otu00088	0	0.01	0	0.05	0.80
	Otu00114	0	0	0	0	0.64
	Otu00054	0	0	0	0.87	0.48
	Otu00108	0.03	0.03	0.23	0.06	0.02
NOB	Otu00123	0	0	0	0	0.55
	Otu00007	0.01	0.08	0.15	6.24	12.33
	Otu00188	0	0	0	0	0.23
	Otu00053	0.45	0.27	0.20	0.01	0.03

Different from anammox species, the biodiversity of AOB and NOB increased obviously along the increase of seawater proportions. The shift of seawater proportions not only affect the species of AOB and NOB but also their relative abundance. The phylogenetic composition of AOB and NOB were also investigated (Tab. 5-3 and Fig. 5-4b). In total, five OTU clusters affiliated to *Nitrosomonas* and *Nitrosococcis* were

detected in AS5, however, only two OTU clusters affiliated to *Nitrosomonas* were observed in AS1.

Specifically, the AOB populations were almost dominated by the same one cluster (OUT00016), closely related to *Nitrosomonas* europaea (97%), except in AS3 (Tab. 5-3 and Fig. 5-4b). Hence, there was almost no shift of the dominant AOB species during the low strength wastewater treatment with different seawater proportions. In particular, the rapidly growing of AOB such as *Nitrosomonas* europaea could outcompete from the selective environment (Ahn et al., 2008, Reino et al., 2016). However, the phylogenetic analysis revealed that AOB affiliated with genus *Nitrosococcus* appeared along the operation, accounting for 0.8% of total reads in AS5. Previous study reported that some species affiliated with *Nitrosococcus* genus were obligate halophilic and detected in the marine environment (Ahn et al., 2008). Moreover, two more clusters (OTU00054 and OTU00114) affiliated with genus *Nitrosomonas* were also detected in AS5 (Tab. 5-3 and Fig. 5-4b), suggesting the biodiversity of AOB species was significantly affected by the 40% of seawater proportions. Notably, the specific AOB species of clusters OTU00054, 00088, 00108, 00114 could not be identified through phylogenetic analysis. Although the length of the 16S rRNA sequence might be one reason, it was possible that these AOB clusters were classified as novel AOB species due to the induction of seawater.

Similar situation as AOB had also been observed for NOB, whose OTU clusters increased from two to four. Two OTU clusters (OTU00007 and OTU00053) affiliated with *Nitrospira* and *Nirolance* genus were detected in all sludge samples, respectively (Tab. 5-3 and Fig. 5-4c). Two unique OTU clusters related to species *Nitrospira marina* and genus Nitrospina were only observed in AS5, suggesting the biodiversity of NOB increased suddenly when the seawater proportion was increased to 40%. Phylogenetic analysis revealed that cluster OUT00007, similar to *Nitrospira defluvii* (100%), became the most dominant NOB species along the increase of seawater proportion, accounting for 12.33% of total reads. Note that *Nitrospira marina* is regarded as one of the most prevalent NOB in some marine environments and its best growth is obtained under mixotrophic condition (Watson et al., 1986). Similar to *Nitrospira marina*, the *Nitrospina* genus was exclusively found in marine conditions (Ngugi et al., 2016). These results suggested the marine NOB species appeared due to the induction of seawater.

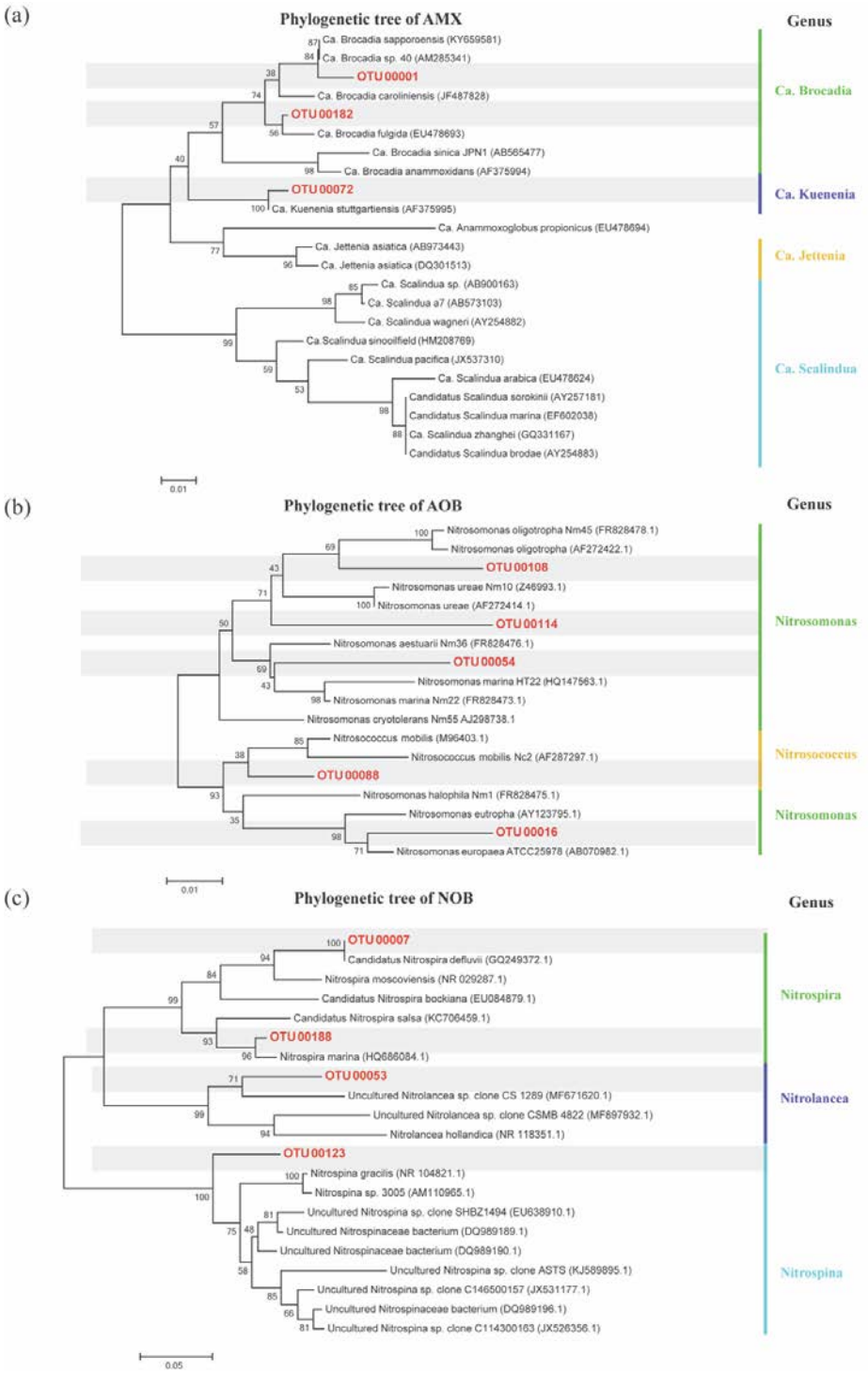


Fig. 5-4 Neighbor-Joining phylogenetic trees of major OTUs sequences (relative abundance > 0.1%) related anammox (a), AOB (b), and NOB (c) in CSTR. The trees based on Jukes-Cantor distance was constructed using Mega 7.0 with a bootstrap value of 1000.

5.4 Discussion

The anammox activity was affected by the introduction of seawater obviously since the NRR dropped suddenly at the point of the increase of seawater proportion (Fig. 5-1). However, the TNRE recovered back to 80% under low salinity condition (20% of seawater), indicating the anammox activity could recover through domestication. For long-term operation, *Ca. Brocadia* genus could adapt to low salinity environment (40% of seawater), which was also revealed by other studies (Dapena-Mora et al., 2007, Kartal et al., 2006, Ma et al., 2012). The reactor performance response to the salinity shock could be divided into the sensitive period, interim stable period and recovery period (Ma et al., 2012). Note that, the reactor performance recovered back to 80% of TNRE after around 150 days when the seawater proportion was shift to 20%. The recovery time was longer than the results conducted by Ma. et al (2012). The relative abundance of anammox bacteria may be the key point since high abundance of anammox species was first enriched in previous studies on salinity shock (Chen et al., 2014, Ma. et al., 2012, Tang et al., 2011). At the point of the first seawater proportion adjustment, the relative abundance of anammox bacteria in the CSTR was only 4.91%, resulting in its weak ability response to the salinity shock. Unlike the first stage of salinity shock, less time was required to recover back to the steady state when seawater proportion was increased from 20% to 40% (Fig. 5-1) since the relative abundance of anammox bacteria reached 40.52% before the salinity shock. Correspondingly, the nitrate concentration increased suddenly at the point of salinity shock, indicating NOB

was more adaptable to the salinity shock compared to anammox bacteria though its relative abundance was less than anammox bacteria. Besides, the over-production of nitrate is the main reason resulted in the decreasing of TNRE with the increase of seawater proportion. Whatever, the CSTR for low strength seawater-based wastewater (20% of seawater) was started up successfully (80% of TNRE) after 260 days through enriching and domesticating freshwater anammox bacteria synchronously. Therefore, this study could widen the application of anammox process for seawater-based wastewater treatment in coastal cities. Take Hong Kong for instance, the seawater toilet flushing may result the sewage consists about 20-30% of seawater (Leung et al., 2012), which is similar to the seawater proportion in this study.

The introduction of seawater induced the succession of heterotrophic microorganisms in anammox consortia during the start-up of reactor for low-strength seawater-based wastewater treatment. The phyla of *Chloroflexi* has been found in various anammox reactors, which can interact with anammox bacteria through organic matters exchange such as extracellular polymeric substances (EPS) or Vitamin B12 (Lawson et al. 2017). In the present study, the seawater had a positive impaction on the relative abundance of *Chloroflexi*, which is different to the results conducted by Gonzalez-Silva et al., (2017). Gonzalez-Silva et al., (2017) found the relative abundance of *Chloroflexi* decreased in anammox consortia when NaCl was added as salinity in the influent. In this present CSTR system, the additional seawater introduces not only sodium, but also sulfate, organic matters etc. These extra matters might involve in the potential metabolism of

Chloroflexi, for instance, *Chloroflexi* possibly benefits from the introduced organic matters in seawater for cell synthesis or energy generation. Therefore, *Chloroflexi* might act as a more important role in pilot-scale anammox reactors since the real mainstream seawater-based wastewater contains more organic matters than synthetic seawater-based wastewater, which needs to be further explored. Moreover, the presence of sulfate was the potential electron acceptor for denitrification mediated by *Chloroflexi,* resulting in the sufficient substrates for its metabolism. The presence of *Acidobacteria* has also been reported in previous anammox systems. The *Acidobacteria* can utilize various organic matters, even can degrade xylan, chitin, cellulose and hemicellulose (Costa et al., 2014). Similar to *Chloroflexi*, *Acidobacteria* might also play an important role in the cross-feeding in anammox consortia. However, the introduction of seawater results a negative effect on *Acidobacteria,* which are less resistant or susceptible to salinity (Zheng et al., 2017). *Bacteroidetes* is one of the common phyla in anammox systems, possibly interacting with other microorganisms by organic matters supply (Costa et al., 2014). In this study, the relative abundance of *Bacteroidetes* decreased significantly due to the introduction of seawater. This contrasts with the result by the addition of NaCl as salinity (Gonzalez-Silva et al., 2017). The salinity may not the key point since *Bacteroidetes* is a phylum resistant to salt. It is possible that *Bacteroidetes* cannot compete for substrates with *Chloroflexi* among the interaction with anammox bacteria. However, further study is needed to explore it.

In the present CSTR, two anammox genus *Ca*. *Brocadia* and *Ca*. *Kuenenia* were enriched during the freshwater treatment period. Previous studies revealed that *Ca*. *Kuenenia* was the dominant anammox species treating saline wastewater (Gonzalez-Silva et al., 2017, Kartal et al., 2006), however, it was washed out with the increase of seawater proportion in this study. Conversely, high relative abundance of anammox bacteria close to *Ca*. *Brocadia sp* (34.24%) was enriched (Tab. 3 and Fig. 4). One hypothesis is that the growth rate of *Ca*. *Brocadia sp* is higher than *Ca*. *Kuenenia stuttgartiensis* (Zhang et al., 2017), suggesting *Ca*. *Brocadia sp* has a stronger potential for substrate competition than *Ca*. *Kuenenia stuttgartiensis* under the shortage of substrate condition (low-strength wastewater). Additionally, *Ca*. *Brocadia* was assumed to be mainly accumulated in floc and *Ca*. *Kuenenia* tended to present in biofilm or granular (Guo et al., 2016, Park et al., 2015). Therefore, *Ca*. *Brocadia* could compete for more substrates than *Ca*. *Kuenenia* due to the well-mixing. It could be proved during freshwater treatment period that the relative abundance of *Ca*. *Brocadia sp* increased faster than *Ca. Kuenenia stuttgartiensis*. Recently, Almeida et al. (2016) proposed that *Ca*. *Kuenenia stuttgartiensis* utilized a sodium-motive force (*smf*), which might support the cell synthesis. In this study, anammox species might benefit from the introduction of seawater. The additional seawater would drive the enzyme encoding sodium pumping NADH: quinone oxidoreductase (sodium-NQR) and Na^+-ATPases to transport sodium iron into anammox cell with energy generation and establish the *smf* (Paparoditis et al., 2014). Next, enzyme encoding Na^+-translocating (RNF) is activated by the *smf* and pump Na^+ out from cell with reduction of ferredoxin for carbon

metabolism (Almeida et al. 2016). In this study, the anammox species affiliated with *Ca. Brocadia* genus has the potential for salinity-tolerance and they may benefit from the additional salinity. Though the *smf* has not been reported in genus *Ca. Brocadia* till now, based on these results conducted in this study, it is possible the dominant anammox species could apply the *smf* for energy harvest and cell synthesis. However, further study still needs to be conducted to explore its metabolic pathways associated with sodium pumps.

The relative abundance and biodiversity of nitrifying bacteria (AOB and NOB) increased with the increase of seawater proportion. The presence of dissolved oxygen might not the key factor since their relative abundance decreased when the reactor was fed with freshwater media. Hence, they possibly are driven by the addition of seawater. Take the reactor performance and taxonomic results together, the relative abundance of dominant NOB (OTU0007), similar to *Ca. Nitrospira defluvii*, increased along with the increase of seawater proportion. It is possible that the dominant NOB could tolerant to the salinity concentration conducted in this study. Similar reason was proposed by Wang et al. (2017) that some NOB species might tolerant to salt. However, opposite point of views was also reported by other studies (Bassin et al., 2011, Cui et al., 2009, Hunik et al., 1993, Moussa et al., 2006, Pronk et al., 2014, Vredenbregt et al., 1997). Since significant relative abundance of *Ca. Nitrospira defluvii* was obtained even in anaerobic condition when seawater was introduced, two possibilities have been proposed as below. One hypothesis is that sulfate contained in the seawater may serve

as an electron acceptor for ammonia oxidation by *Ca. Nitrospira defluvii* for metabolic flexibility development. Another possibility is that sodium ion in the seawater may also induce sodium-ATPase for energy harvest and support its cell synthesis, which is similar to *Ca*. *Kuenenia stuttgartiensis*. However, a firm conclusion needs to be made after further investigation. Whatever, the increase of seawater proportion in this study resulted in the accumulation of NOB and nitrate.

5.5 Conclusion

In the present study, mainstream seawater-based anammox process was conducted. The CSTR anammox process was successfully started up and achieved 80% of TNRE (0.180 kg-N/(m^3•d) of NRR) treating low-strength wastewater containing 20% of seawater. High relative abundance of anammox species related to *Ca*. *Brocadia sp* (34.24-39.92%) was enriched and acclimated with the introduction of seawater. The seawater resulted in the accumulation of nitrifying oxidizing bacteria even in anaerobic conditions, which might be the main reasons for the excess production of nitrate. Especially, the significant relative abundance of *Ca. Nitrospira defluvii* in the saline environment indicated that it might benefit from the substrates in seawater, such as sulfate or sodium iron. The introduce of seawater also caused the appearance of marine nitrifying species, which aggravated the deterioration of reactor performance.

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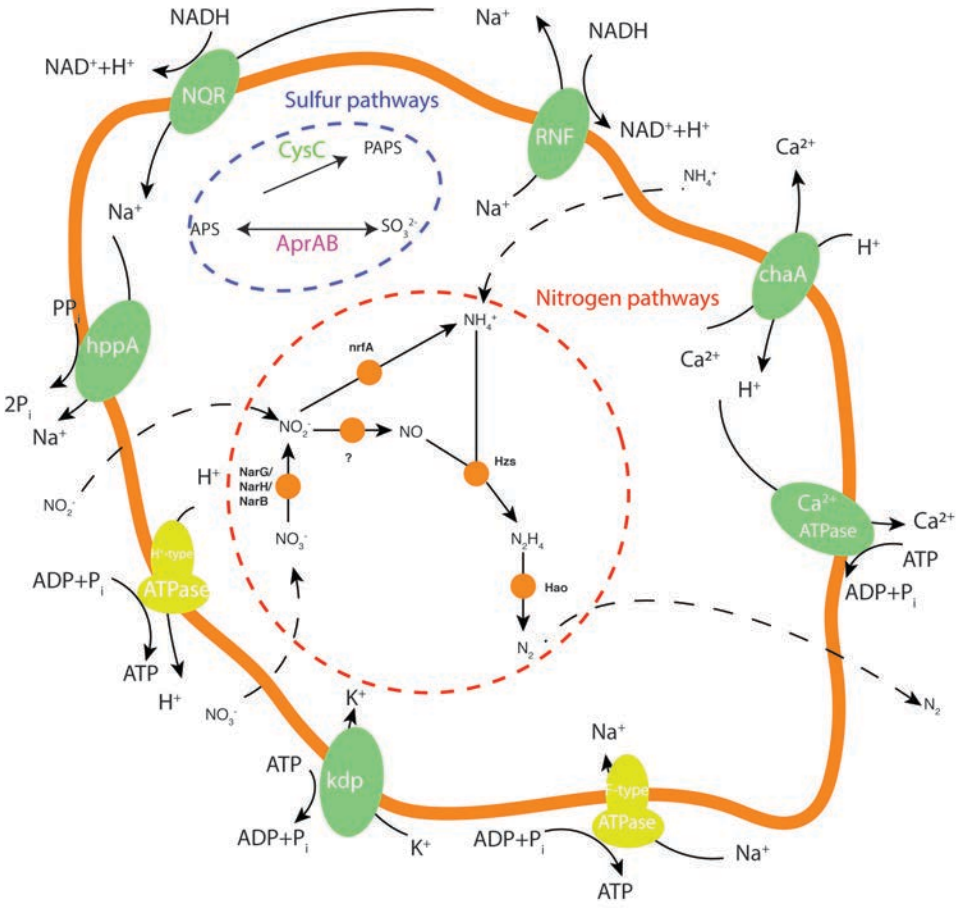
Chapter 6 Saline effect on anammox bacteria: sodium pump and energy conservation

GRAPHIC ABSTRACT



6.1 Introduction

Anaerobic ammonium oxidation (anammox) has been widely applied for ammonium-containing wastewater treatment, which converts ammonium and nitrite to dinitrogen gas with its energy-saving advantages (Lawson et al. 2017, Speth et al. 2016a, Van Teeseling et al. 2015). The anammox-based processes were successfully implemented for sidestream treatment with high ammonium concentration, however, the potential possibility of anammox on mainstream wastewater treatment was still under investigation(Ali and Okabe 2015, Lackner et al. 2014). In addition to the unfavorable environment such as low temperature and low nitrogen concentration, the salinity addition in mainstream wastewater due to the seawater flushing toilets and industry wastewater increased the difficulty on anammox process application (Lackner et al. 2014, Li et al. 2018). In recent, studies suggest that anammox has the potential for nitrogen removal from saline wastewater (Li et al. 2018). Relevant anammox processes for saline wastewater treatment have been investigated such as freshwater anammox species (FAB) acclimatization and marine anammox species (MBA) enrichment (Li et al. 2018). Among them, the FAB acclimatization is regarded as a realistic solution due to most anammox species belonging to FAB (Dapena-Mora et al. 2010, Li et al. 2018). Previous studies revealed that FAB could adapt to a salinity environment of 30 g/L NaCl with an NRR of 4.5 ± 0.1 kg N/(m^3·d) (Dapena-Mora et al. 2010, Jin et al. 2011, Kartal et al. 2006, Liu et al. 2009, Yang et al. 2011), however, the discrepancy to salinity adaptation exist in different anammox species (Carvajal-

Arroyo et al. 2013, Hou et al. 2013, Kartal et al. 2004, Malovanyy et al. 2015). However, although the impact of salinity has been conducted on FAB, the potential mechanism of sodium on FAB is still elusive.

In the cell, a proton- (or sodium-) motive force (*pmf* or *smf*) is conserved by the energy released in redox processed. Then, H^+ (or Na^+)-dependent membrane-bound ATP synthase (ATPase, complex V) synthesize ATP due to the driver of the force of *pmf* or smf. Recently, metagenomic approaches have been used to explore the microbial community structure of anammox consortia and anammox metabolism pathways (Bhattacharjee et al. 2017, Park et al. 2017, Zhao et al. 2018). In anammox bacteria, electron derived from the intrinsic anammox reaction in anammoxosome is transferred via the membrane-bound Respiratory Complexes (RCs), resulting a cyclic flow of electrons to establish the *pmf* for ATP synthesis (de Almeida et al. 2016). Interestingly, Paparoditis et al. (2014) found an aerobic marine halotolerant Planctomycete, *Rhodopirellula baltica*, has two F-type ATPases that has the potential to utilize the *smf* instead of the *pmf* for ATP synthesis. Additionally, the sequenced anammox genomes revealed that they contained complex RCs systems with four different ATPases (Gori et al. 2011, Hira et al. 2012, Hu et al. 2012, Oshiki et al. 2015, Strous et al. 2006). Almedia et al. (2016) found that *Ca. Kuenenia stuttgartiensis* could utilize an smf established by the Na^+- (sodium pumping) NADH:quinone oxidoreductase by using Na^+ as the coupling ion and possibly for energy production by sodium-pumping N-ATPases. Salinity concentration was increased due to the seawater addition in

mainstream wastewater, especially the Na^+ concentration. Therefore, the energy generation by anammox bacteria may be complex due to the salinity environment. Recently, an increasing number of whole-genome anammox bacteria has been sequenced and the nitrogen transformation pathways were investigated (Gori et al. 2011, Hira et al. 2012, Hu et al. 2012, Oshiki et al. 2015). Hence, it is possible to apply (meta)omics to explore the intrinsic mechanism of sodium on anammox bacteria. Simultaneously, the related pathways on nitrogen and sulfur in anammox bacteria are vital to be explored to deeply understand the reactor performance treating low strength wastewater containing seawater.

In this study, a continuously stirred tank reactor was set up for low-strength wastewater treatment which contained 40%, 50% and 0% of seawater. The objectives of this study were to explore the sodium pump in anammox bacteria and the nitrogen and sulfate-dependent metabolic pathways in saline environment. Metagenomic analysis was performed to identify the anammox bacteria enriched in CSTR and reconstruct the metabolic pathways. The expression values of related genes involving in the metabolic pathways were detected by metatranscriptomic analysis. Our results broaden the diversity of anammox species and provide deeper insights on the mechanism of sodium pump in anammox bacteria, which will widen the application of anammox-based processes for saline wastewater treatment.

6.2 Material and methods

6.2.1 Reactor set-up, inoculum and operation strategy

A lab-scale anammox Continuously Stirred Tank Reactor (CSTR) with an effective volume of 1.0 L was set up and operated with the continuous mode at room temperature (17-21℃). Magnetic stirring with 50 rpm was used for mixing in the reactor. Biomass was intercepted through the inclined plate installed in the sedimentation area. Before this study, The CSTR had been operating for more than one years at room temperature (17-21℃) treating low-strength wastewater with different seawater proportions. The reactor had reached at steady state with a total nitrogen removal rate (TNRE) of over 70%. Taxonomic results based on 16S rRNA sequencing revealed that *Candidatus Brocadia* was the dominant anammox species, accounting for a relative abundance of 40.83%, and *Candidatus Kuenenia* was also detected with a relative abundance of 0.04%.

The reactor was fed with synthesis wastewater contained NH_4Cl, $NaNO_2$, KH_2PO_4, $MgSO_4 \cdot 7H_2O$ and 1 mL/L of trace element solution as described by Van de Graaf et al. (1996). The influent concentration of ammonium and nitrite were configured around 30 mg/L and 30 mg/L. The influent pH was adjusted to 8.0 using $KHCO_3$, and it was not controlled during the operation. Real seawater was generated from the Hong Kong Victoria Harbour without any pre-treatment process, which the salinity was 3.1-3.3% at the point of collection. The seawater proportion in the influent was adjusted to 40%

at the beginning of this study. Three operational phases with different seawater proportions, representing for 40% (75 days), 50% (24 days), and 0% (61 days), were conducted in this study, respectively.

6.2.2 Analytical methods

The collected influent and effluent water samples were filtered (0.45μm) before measurement. The water quality parameters of NH_4^+-N, NO_2^--N, NO_3^--N from influent and effluent were measured according to standard methods (APHA 2002). The pH and water temperature were done using a pH meter (Leici Company, China).

6.2.3 Sludge sampling and DNA extraction

Biomass samples were collected from the reactor by centrifugation (5 min, 10000 g, 4 °C) on day 75 and 150 represented by AS1 and AS2, respectively. DNA extraction for AS1 was performed using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's instruction, and the DNA quality and concentrations were measured using NanoDrop® ND-1000 (NanoDrop Technologies, Wilmington, DE).

6.2.4 Whole-genome metagenomics analysis

Genomic DNA sample (50 μg) collected from AS1 was used for shotgun metagenomic sequencing on the Illumina HiSeq platform by sending sample to BGI company (Shenzhen, China). The raw metagenomic reads obtained were filtered to remove adapters, low-quality data and N bases through trimming progress, which ensured the length of the clean read was over 150 bases. Contigs were then assembled from the clean reads using SPAdes Genome Assembler (ver. 3.10.1) by setting kmer parameters of 19,33,47,61,75 (Nurk et al. 2013). QUAST was used to access the quality of assembled contigs (Gurevich et al. 2013). The generated contigs were binned into draft population genomes using MaxBin (Wu et al. 2014). The completeness and contamination of the draft genomes were evaluated using CheckM based on the marker gene sets with 111 essential single-copy genes (Parks et al. 2015). Prokka was performed to annotate the functional genes in the draft genomes (Seemann 2014), which packages used were described as Park et al. (2017). JSpeciesWS was used to calculate the pairwise average nucleotide identity (gANI) as well as the alignment frequency (AF) between the draft and reference genomes based on BLASTP+ (Richter et al. 2015). The relative abundance of each metagenome assembled genomes (MAGs) was calculated using BBMap with command script_split. The paired clean reads were split based on the recovered draft genomes and the relative abundance was calculated using the ratio between the mapped reads of each MAGs and the total paired clean reads. The functional genes in the draft genomes were further queried based on the Kyoto

Encyclopedia of Genes and Genomes (KEGG) pathway database and LocalBlastp. The NCBI database (nr) was used for LocalBlastp.

The phylogenetic tree was constructed using Phylophlan to identify the relationship between the recovered anammox genomes and reference anammox genomes. Ten published draft anammox genomes from JGI and NCBI databases were used as templates for phylogenetic tree construction; (1) *Ca. Brocadia fulgida* (SAMN03103735), (2) *Ca. Brocadia sinica JPN1* (SAMD00036619), (3) *Ca. Brocadia sp R4W10303* (PRJNA383754), (4) *Ca. Brocadia sp 40* (SAMN05817825), (5) *Ca. Brocadia sp UTAMX1* (SAMN06342761), (6) *Ca. Brocadia sp UTAMX2* (SAMN06342762), (7) *Ca. Kuenenia stuttgartiensis* (SAMEA104335229), (8) *Ca. Jettenia caeni* (SAMD00036615), (9) *Ca. Scalindua rubra* (SAMN05368398), and (10) *Ca. Brocadia caroliniensis* (SAMN04504644).

6.2.5 Metatranscriptomics library construction and analysis

Metatranscriptomics analysis was performed to access the expression value of functional genes in recovered anammox genomes. About 5 mL mixed liquor were collected from AS1 and AS2 and mixed with 10 mL buffer to protect the total RNA, respectively. The mixed liquor samples were sent to BGI company (Shenzhen, China) for RNA extraction and library construction, and was sequenced on the Illumina HiSeq4000 platform. Clean reads with an average length of 100 bp were generated

through removing adaptor sequences, contamination and low-quality reads from raw reads using Trimmomatic. Next, the clean reads were *de novo* assembled into contigs using Trinity v2.5.1 (Grabherr et al. 2011). The observed contigs were mapped to the recovered genomes using BBMap with the parameters "minid = 95%" and "ambig = random" to investigate the expression value of functional genes (Konstantinidis and Tiedje 2005). The number of clean reads mapped for each potential functional gene in recovered genomes was normalized to reads per kilobase per million reads mapped (RPKM) (Mortazavi et al. 2008).

6.3 Results and discussion

6.3.1 Reactor performance

The overall nitrogen removal performance was shown in Fig. 6-1. According to the seawater proportions, the operation of the CSTR was divided into three phases in this study. In phase I (40% seawater proportion), both of the NH_4^+-N and NO_2^--N removal rates reached more than 94% at the steady state (From day 15 to 75). Meanwhile, the NO_3^--N concentration in the effluent accounted for 23.09 mg/L on day 75, resulting in a total nitrogen removal efficiency (TNRE) of 60.03% (Fig. 6-1). In this phase, the reactor achieved a nitrogen removal rate (NRR) of 0.245 kg-N/(m^3•d) at HRT of 3.6 hr. In phase II (50% seawater proportion), the reactor performance deteriorated in first nine days due to the sudden increase in seawater proportion, with NRR and TNRE decreasing from 0.245 to 0.163 kg-N/(m^3•d) and from 60.03% to 41.95%, respectively.

Specifically, the NH_4^+-N and NO_2^--N removal rate decreased to 74.96% and 80.68%, respectively, while the NO_3^--N concentration kept stable. The results suggested that the inhibition on anammox activity by seawater was the main reason for the deterioration of reactor performance. The NRR further increased to 0.202 kg-N/(m^3•d), representing for a TNRE of 48.29% at the end of phase II. In phase III, the seawater was removed from the influent to avoid its inhibition on anammox activity. As except, the TNRE recovered back to 61.00% rapidly in six days. Furthermore, almost all NH_4^+-N and NO_2^--N were removed at steady state (from day 117 to 159). The nitrogen loading rate (NLR) was increased to 0.506 kg-N/(m^3•d) from day 99 due to the shortening of HRT from 3.6 hr to 3.0 hr. As a result, the reactor achieved an TNRE of 83.77%, representing for an NRR of 0.418 kg-N/(m^3•d), at the end of this phase. Interestingly, the NO_3^--N concentration decreased from 22.42 mg/L to 10.12 mg/L in the effluent, revealing the NO_3^--N concentration increased with the increase of seawater proportion. Whatever, the reactor performance for low-strength wastewater treatment observed at the end of this study was similar to the results conducted by other researchers (Liu et al. 2017, Sun et al. 2018, Lotti et al. 2015), suggesting the effect of seawater on anammox activity was temporary.

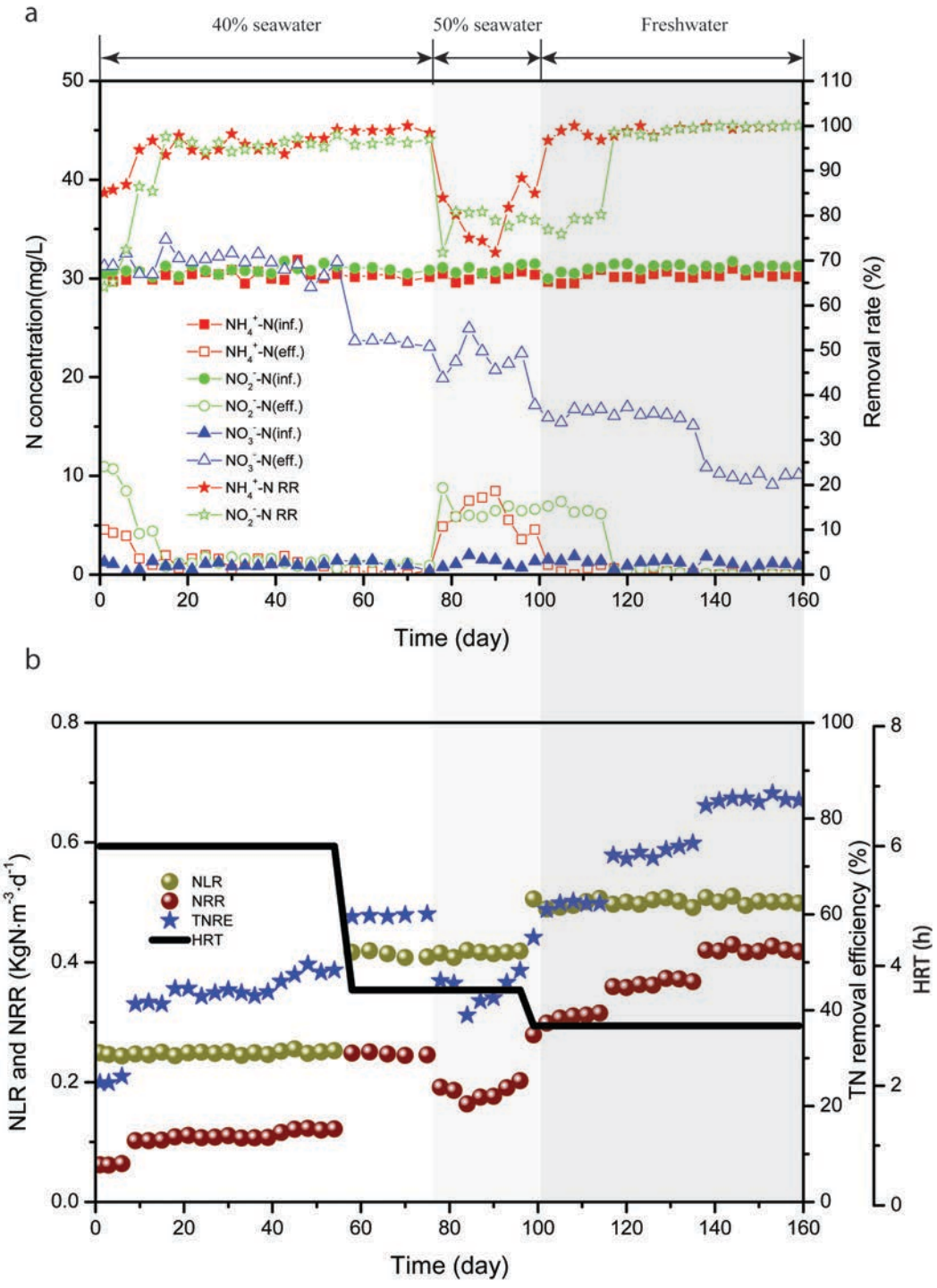


Fig. 6-1 The performance of nitrogen removal in CSTR treating low strength wastewater. (a): Variation of nitrogen compounds, ammonium, and nitrite removal rate; b: Time courses of total nitrogen removal efficiency (TNRE), Nitrogen loading rate (NLR) and Nitrogen removal rate (NRR), and hydraulic retention time (HRT).

6.3.2 Metagenomic sequencing, binning and annotation

In total, metagenomic sequencing yielded 37,679,194 bp raw data and 27,203,609 bp clean data were generated after read quality filtration. The obtained clean reads were assembled into 318,334 contigs with an N50 of 2442 bp (Tab. S6-1). Contigs were further binned into population draft genomes, which were mainly affiliated with the phyla *Planctomycetes, Bacteroidetes, Chlorobi, Chloroflexi, Nitrospirae, Acidobacteria*, and *Proteobacteria*, etc. Among them, two high-quality anammox-related metagenome bins (AMX1 and AMX2) were observed. The retrieved AMX1 and AMX2 had high completeness and low contamination of 98.90%, 1.65% and 98.90%, 8.19%, respectively, and accounted for a relative abundance of 31.52% and 3.96% (Tab. 6-1). The average GC content of AMX1 and AMX2 were 43.54% and 42.39%, similar to the reported GC content of other anammox bacteria (Ali et al. 2016, Gori et al. 2011, Park et al. 2017, Oshiki et al. 2015).

Table 6-1 Anammox genome statistics recovered in the CSTR

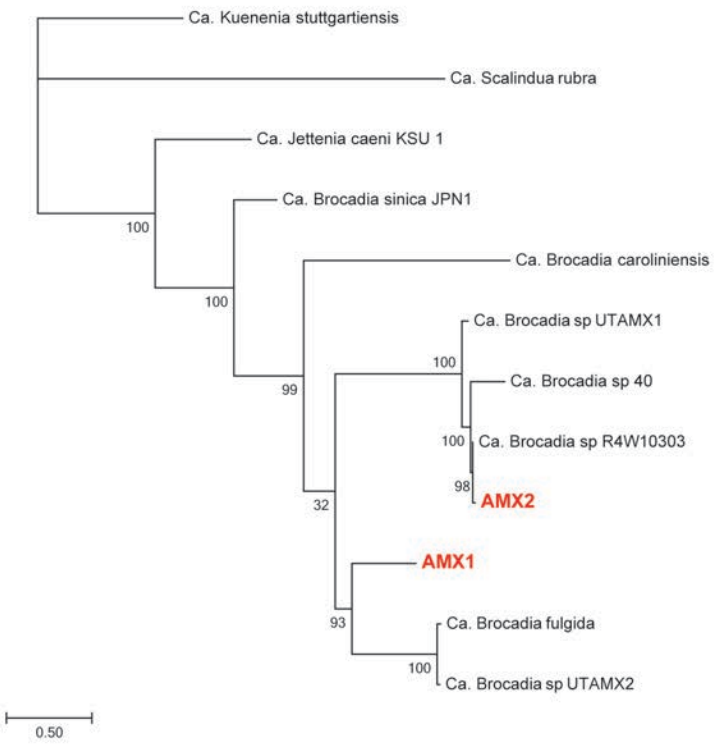
Bins	AMX1	AMX2
Mapped size (bp)	8571582	1077251
Relative abundance (%)	31.51	3.96
Genome size (bp)	4029957	4324011
GC (%)	43.56	42.39
# Contigs	166	487
Completeness (%)	98.90	98.90
N50 (bp)	51592	22200
Contamination (%)	1.65	8.19
Predicted genes	3789	4300

Phylogenetic tree was constructed to investigate the relatedness between the reported anammox species and the recovered anammox genomes (Fig. 6-2a). The results revealed a high similarity (>99%) between AMX2 and *Ca. Brocadia sp R4W10303.* Furthermore, the taxonomic position of the recovered AMX2 was demonstrated by the alignment of average nucleotide identity (ANI) as well as fraction (AF) against five reference anammox genomes affiliated to *Ca. Brocadia sp.* (Fig. 6-2b). The ANI and AF result reveals the relatedness of AMX2 to *Ca. Brocadia sp R4W10303.*

Therefore, these results reflected that AMX2 was one strain of *Ca. Brocadia sp.* However, the phylogenetic relationship between AMX1 and the reported anammox

species was far away (Fig. 6-2a). Additionally, the highest ANI value between AMX1 and the strains of *Ca. Brocadia sp* was only 81.82%, which was lower than the confidence value (~94%) for species definition. Based on the analysis performed, it speculated that AMX1 was one new anammox species belonging to the genus *Ca. Brocadia* and its whole-genome had not been generated until now. Additionally, as AMX1 dominated the anammox population, therefore, the recovered anammox genome (AMX1) was used for the downstream analysis to explore its metabolic mechanism.

a



b

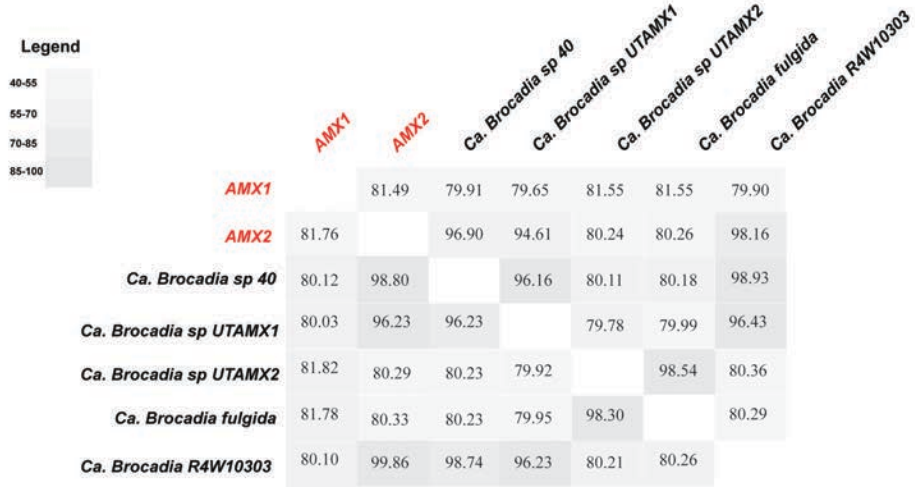


Fig. 6-2 (a) Phylogenetic relationships of the recovered anammox genomes in CSTR and published anammox genomes using PhyloPhlAn. (b) Average nucleotide identity (ANI) and aligned nucleotides (%) between closed *Ca. Brocadia* and anammox genomes recovered in this study using BLASTP+. The reference anammox genomes were obtained from JGI and NCBI database.

6.3.3 Nitrogen metabolism

To identify the anammox bacteria, as well as to investigate the reactor performance, it is vital to understand the nitrogen transformation pathways in anammox bacteria. It was known that anammox bacteria took up nitrogen substrates (ammonium and nitrite) and converted them into dinitrogen gas. The retrieved AMX1 genome was found to contain the nitrogen-related transporters, formate and nitrite transporter *focA* (1 copy), nitrite transporter *nirC* (2 copies), ammonium transporter *amtB* (5 copies) and nitrate transporter *NRT* (2 copies) (Fig. 6-3a and Tab. S6-2). The observed *focA* gene in AMX1 indicated its potential to adapt in the oligotrophic environments by behaving in low half-saturation nitrite coefficients (Lam and Kuypers 2011, Hu et al. 2012, van de Vossenberg et al. 2013). Transcripts represented in Fig. 6-3b revealed that *focA* gene was only expressed in saline environment. In this study, the NO_3^--N concentration was much higher under seawater condition than that under freshwater condition (Fig. 6-1a), which might be caused by nitrite oxidizing bacteria (NOB). Therefore, limited nitrite was available for AMX1 under seawater condition. Noted that all *amtB* gene expressed under freshwater condition, while only one expressed *amtB* was observed under saline condition (Fig. 6-3b). Similar to the reason proposed for *focA*, AMX1 need to compete for limited ammonium with aerobic oxidizing bacteria under saline condition due to the inhibition on anammox activity. The obtained *NRT* genes with their highly expressed RPKM value in AMX1 genome indicated that it had the potential to reduce nitrate by using organic electron donors.

In anammox reaction, nitrite is reduced to nitric oxide (NO) by cytochrome cd1-type nitrite reductase (*nirS*) or copper-based nirK-type nitrite reductase (*nirK*) (Shu et al. 2016). However, no genes encoding *nirS/K* were annotated in AMX1, which was similar to *Ca. Brocadia fulgida* and *Ca. Brocadia sp 40*. Previous studies revealed that *Ca. Brocadia* genus might use some unidentified genes to reduce nitrite and produce the essential intermediate NO. Moreover, one copy of gene encoding hydrazine synthase (HZS) and seven copies of genes encoding hydroxylamine oxidoreductase (HAO)-like were detected in AMX1. Different from the expressed gene *hzsB*, significant differences in the expression was observed for genes hao-like. Specially, all hao-like genes expressed under the saline condition, while two of them were not expressed under freshwater condition (Fig. 6-2b). Furthermore, genes encoding nitrate reductase (*narG* and *narH*), ferredoxin-nitrate reductase (*narB*) and nitrite reductase cytochrome c-552 (*nrfA*) were identified in AMX1 for dissimilatory nitrate reduction to ammonium (DNRA) and assimilatory nitrate reduction. Moreover, these genes were highly expressed under both conditions (40% seawater proportion and freshwater) (Fig. 6-2b). Therefore, the observed NRT genes and key expressed genes encoding nitrate reduction pathways revealed that AMX1 developed the metabolic flexibility to generate nitrite from nitrate reduction. It should be noted that RPKM values of *narG, narB, narH* and *nrfA* genes detected under saline condition were higher than that under freshwater condition, which might be due to the high nitrate concentration under saline condition. Recently, the interactions between anammox and heterotrophic bacteria have been widely reported (Lawson et al. 2017, Speth et al. 2016b, Zhao et al. 2018). In this study,

microbes might release more extracellular polymeric substances (EPS) to protect them from the unfavorable environment (salinity) (Xing et al. 2015), which possibly act as electronic donor to promote the DNRA and assimilatory nitrate reduction pathways in anammox bacteria.

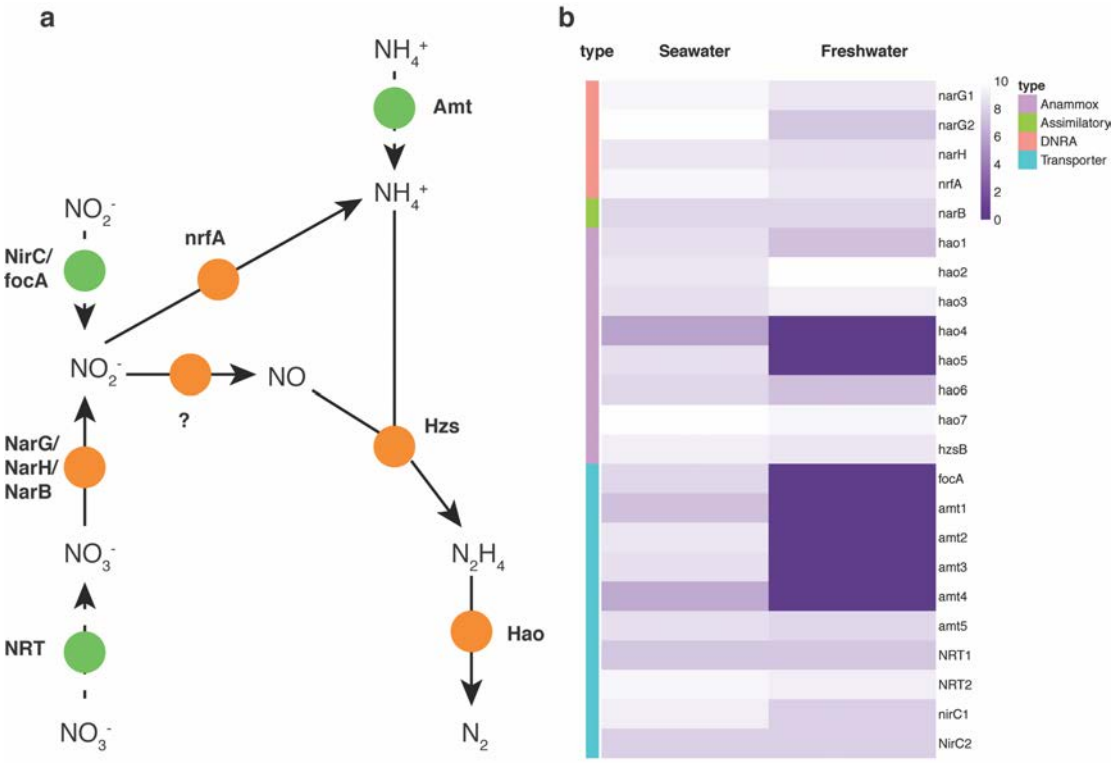


Fig. 6-3 (a) Overall of nitrogen metabolism of AMX1 genome. (b) The expression value of genes (RPKM log2) related to nitrogen metabolism. The dotted black line indicates the transportation of nitrogen compounds and the question mark means the unidentified gene in this study. The transporter genes are labeled green and the nitrogen metabolic genes are labeled orange. *focA*: nitrite/formate transporter, amt: ammonium transporter, *nirC*: nitrate transporter, NRT: nitrate transporter, Hzs: hydrazine synthase, *narG/H*: nitrate oxidoreductase complex, *nrfA*: nitrite reductase cytochrome c-552, *hao*: hydroxylamine oxidoreductase like, *narB*: ferredoxin-nitrate reductase

6.3.4 Sodium pump and energy generation

Based on the survival conditions of anammox bacteria, the sodium, calcium, potassium transport systems in AMX1 genome were investigated. Metagenomic analysis revealed that Na^+-translocating NAD^+:ferredoxin oxidoreductase RNF (7 copies) and sodium pumping NADH quinone oxidoreductase NQR (4 copies) were observed in AMX1 (Fig. 6-4a and Tab. S6-3), whereas NQR is restricted found in marine and pathogenic microorganisms and NADH dehydrogenase NUO is observed in bacteria universally (Reyes-Prieto et al. 2014). Remarkably, these two types of genes recovered in AMX1 were also reported in *Ca. K. stuttgartiensis* (Kartal et al. 2011, Strous et al. 2006). RNF is encoded by the gene cluster of RnfA (1 copy), RnfB (1 copy), RnfC (2 copies), RnfD (2 copies) and RnfE (1 copy). Na^+-NQR is encoded by the gene cluster of nqrE (2 copies)and nqrF (2 copies).

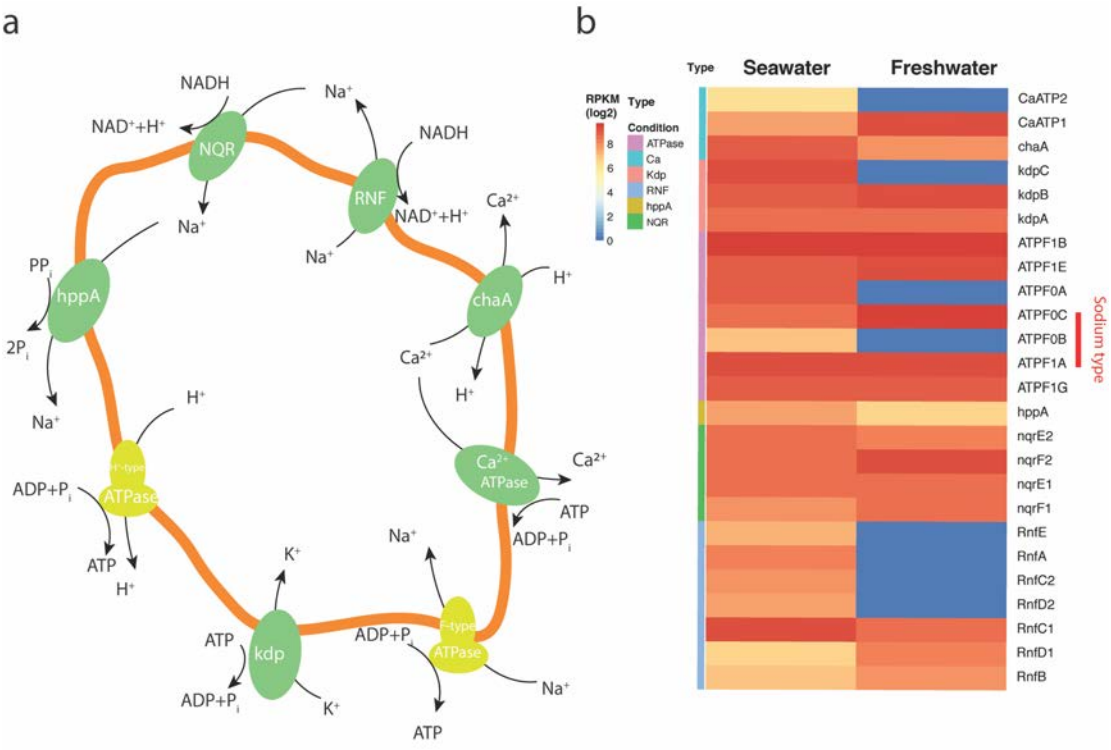


Fig. 6-4 (a) Overview of sodium, calcium, potassium transport systems in AMX1 genome and (b) The expression value of related genes (RPKM log2). CaATPaes: calcium-transporting ATPase, chaA: Ca(2+)/H(+) antiporter, hppA: K(+)-stimulated pyrophosphate-energized sodium pump, kpb: potassium-transporting ATPase, RNF: Na^+-translocating NAD^+:ferredoxin oxidoreductase, NQR: (sodium pumping) NADH:quinone oxidoreductase, ATPase: ATP synthase.

Among the ATPase synthesis, H^+-dependent F1F0-ATPase was the major ATPase for AMX1 under both saline and freshwater conditions (Fig. 6-4b), implying that AMX1 thrive on a *pmf*, which is in agreement with the results conducted in *Ca. K. stuttgartiensis* (de Almeida et al. 2016). Although the RPKM values of expressed H^+-dependent F1F0-ATPase are similar under both conditions, one more copy of H^+-

dependent F1F0-ATPase expressed in the saline environment. It is possibile that AMX1 required more energy to support its survival in unfarvort environmment.This finding suggests the seawater induces AMX1 to develop metabolic flexibility for the ATP synthesis by *pmf*.

The observation of the expressed sodium-NQR and Na^+-ATPases in AMX1 suggested the *smf* might be established on anammoxosome membrane(Fig. 6-4b), which was also found in *Ca. K. stuttgartiensis* (de Almeida et al. 2016). The salinity gradient between cell body and environment created the force to drive Na^+ across anammoxosome membrane through Na^+-ATPases for ATP synthesis. It aslo contributed to the estabilishment of *smf* for RNF complex. The established *smf* may support the reduction of ferredoxin for cell carbon metabolism by the expressed RNF complex. Therefore, AMX1 may benefit from the salinity gradient not only for energy harvest but also for cell synthesis. Interestingly, the observed Na^+-ATPases expressed under both conditions, revealing AMX1 developed the metabolic flexibility for ATP synthesis in different environment. Notably, one more copy of sodium-pumping F-type ATPases highly expressed under the saline condition than that under freshwater condition. The salinity gradient may be the main reason for the marked differentiation of the expression of sodium-pumping F-type ATPases. A much greater salinity gradient is created by the introduction of seawater compared to that in the freshwater environment, resulting in a stronger driven force for Na^+ crossing anammoxosome membrane. Thus,

the created driven force due to the addition of seawater activates more sodium-pumping F-type ATPases to synthesize ATP.

The ability of AMX1 to extrude Na^+ using RNF allows it to adapt the saline environment while protecting itself from the potentially negative effects of high intracellular Na^+. The expressed RNF were observed in this study (Fig. 6-4), which might couple with the sodium-NQR and Na^+-ATPases in AMX1. In addition, it is worth to note that significant differences in the RNF expression were detected in different environments (Fig. 6-4b). All copies of Rnf genes expressed under the saline condition, conversely, less than half of Rnf genes expressed under freshwater condition. Therefore, it could be speculated that the Na^+ extrusion mechanism in AMX1 is more flexible under saline condition. It should be noted that there are almost no differences in the expression of sodium-NQR under both conditions (Fig. 6-4b). Hence, the marked differentiation in Rnf genes expression after the addition of seawater might be due to the activating of sodium-pumping F-type ATPases. Moreover, potassium-transporting ATPase (*kdp*) highly expressed in both environments, which transported potassium into the cytoplasm using ATP hydrolysis as an energy source. It coupled with RNF to maintain the resting potential of the cell. Similar to the expression of RNF, one more copy of kdp expressed in saline environment.

In addition to sodium extrusion by RNF, intriguingly, one copy of K(+)-stimulated pyrophosphate-energized sodium pump (hppA) was observed in AMX1. It is regarded

as one type of Membrane-bound pyrophosphatase (PPase) and utilizes Pyrophosphatase (PPi) instead of ATP as an energy source. Membrane-bound pyrophosphatase (PPase) are ubiquitously found in the cytoplasmic membrane of bacteria that transport Na^+ or H^+ across the membrane couple Pyrophosphatase (PPi) hydrolysis (Baykov et al. 2013, Kellosalo et al. 2012). They are auxiliary device to help cell resist stress conditions by maintaining cation gradients (Baykov et al. 2013). The previous study stated that the pumping mechanism of membrane-bound pyrophosphatase is different from the other primary pumps (Tsai et al. 2014). To date, three different ion-pumping mechanisms for membrane-bound pyrophosphatase have been proposed i.e. two based on hydrolysis drives ion-pumping and one based on ion-pumping drives hydrolysis (Baykov et al. 2013, Kellosalo et al. 2012, Lin et al. 2012).

In this study, sodium ions were able to be extruded out from the cell by hppA without consuming ATP for an energy source, which is vital for bacteria to relieve stress caused by low energy level during unfavorable environment. It is known that, based on thermodynamic analysis, the energy generated from anammox reaction is limited. Meanwhile, cell synthesis and environment adaption of anammox bacteria required energy. Hence, more energy is required to extruse sodium ions by freshwater anammox bacteria to maintain the sodium concentration in the cell when they are in saline environment. Transcripts represented of the hppA gene in Fig. 6-4b suggested that it was possible to play an important role in sodium extrusion in AMX1, especially under saline conditions (Luoto et al. 2013).

In this study, we also observed the calcium transport systems, which contained one copy of gene encoding Ca(2+)/H(+) antiporter (chaA) and two copies of gene encoding calcium-transporting ATPase (CaATPaes) in AMX1. The observed CaATPases indicated that anammox bacteria pumped calcium from the cell by consuming ATP as an energy source. As except, the expression value of chaA genes was much higher under saline condition than that under freshwater condition. Meanwhile, two copies of CaATPases were expressed under the saline condition, while only one was expressed under freshwater condition. These results revealed that AMX1 behaved more activity to extrude calcium ions from the cell under seawater condition in order to maintain intracellular cation stress.

6.3.5 Sulfur metabolism

Based on the calculation of the Gibbs free energy (negative $\Delta G^{0'}$), sulfate-dependent anammox reaction is feasibility, in which anammox bacteria used sulfate as electron acceptors instead of nitrite (Gao and Tao 2011). It is known that the sulfate concentration in seawater is higher than that in freshwater (Van Den Brand et al. 2015), therefore, anammox bacteria is possible to metabolic sulfate due to the shortage of nitrite. In this study, we observed the key genes involving in assimilatory sulfate reduction in the AMX1 genome. In addition, similar genome evidence for *Ca. Kuenenia stuttgartiensis* had also been reported by Strous et al. (2006). Gene encoding sulfate adenylyltransferase (*sat*) was recovered for sulfate reduction to adenylyl sulfate (Aktan

et al. 2015). However, it was not expressed in this study, indicating the sulfate reduction was not performed by AMX1 even under seawater condition (Fig. 6-5a and Tab. S6-4). Furthermore, genes encoding bifunctional enzyme CysN/CysC (*cysC*) and phosphoadenosine phosphosulfate reductase (*cysH*) were highly expressed under seawater condition, suggesting AMX1 could reduce APS reduction to 3'-phosphoadenylyl sulfate (PAPS) and further to sulfide. It revealed that AMX1 possessed the potential metabolic activity by using sulfate-dependent substrates (APS and PAPS) as electron acceptors. Note that no transcripts were detected for gene *cysH* under freshwater condition. In addition to assimilatory sulfate reduction, two genes encoding adenylylsulfate reductase (*aprA* and *aprB*) involving in dissimilatory sulfate reduction and oxidation were also recovered, which could reduce APS to sulfide or oxide sulfide to APS directly. AMX1 was more activity on dissimilatory sulfate reduction and oxidation under saline condition than under freshwater condition (Fig. 6-5b), in which gene *aprA* was only expressed under saline condition. Though AMX1 had not shown the activity on sulfate reduction in this study, it could utilize the sulfur reduction product as electron acceptors that produced by other microbes in the reactor (Aktan et al. 2015), since microbial interaction has been widely reported in previous studies (Lawson et al. 2017, Speth et al. 2016a, Zhao et al. 2018). Furthermore, the expression of key genes indicated that the sulfur-dependent metabolic activity in AMX1 increased with the increase of sulfate concentration (Fig. 6-5b).

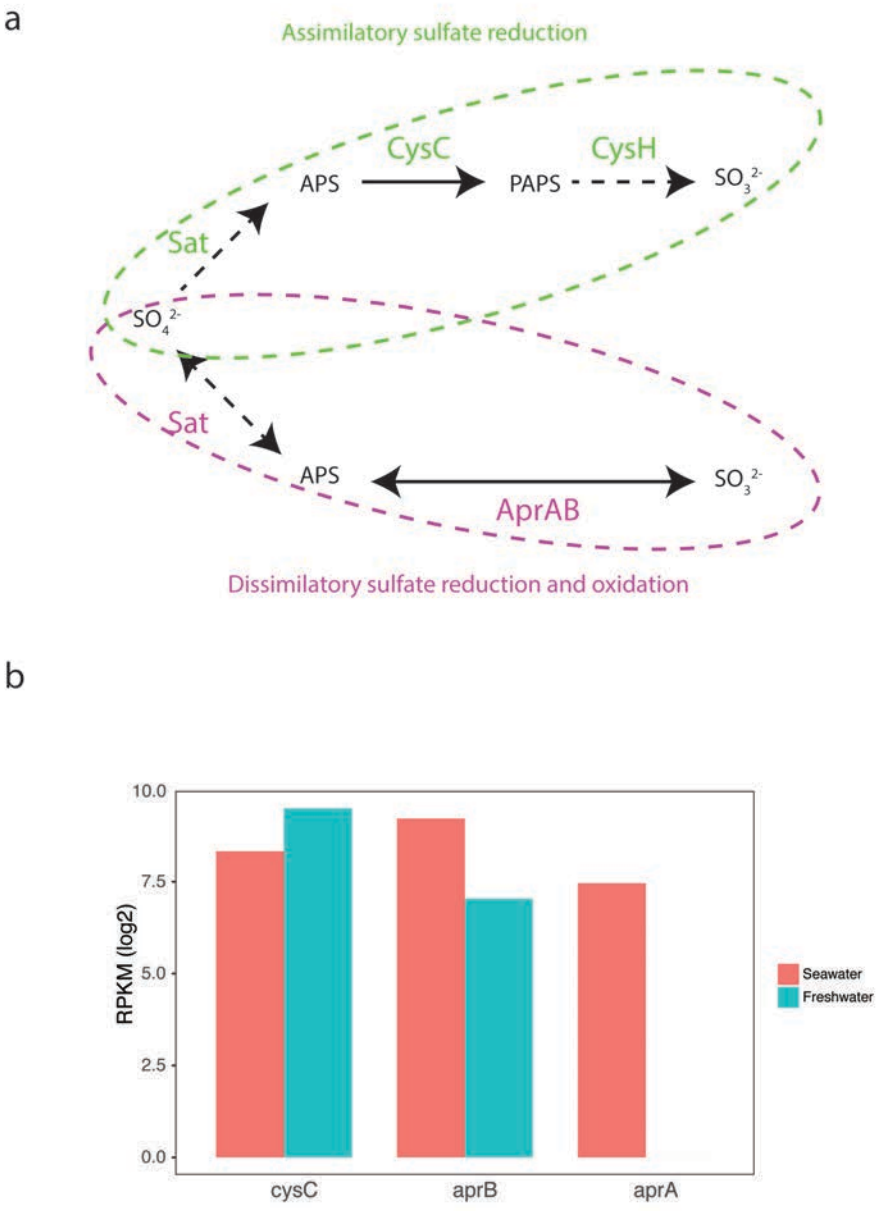


Fig. 6-5 Temporal expression profiles for selected genes associated with (a) S metabolism, and (b) the expression value of related S genes. The dashed line of pathway represents no expression of genes. APS: adenylyl sulfate, PAPS: 3'-phosphoadenylyl sulfate, *sat*: sulfate adenylyltransferase, *cysH*: phosphoadenosine phosphosulfate reductase, *aprA/B*: denylylsulfate reductase, *cysC*: bifunctional enzyme CysN/CysC.

6.4 Significance of this study

The anammox species and its metabolism ability related to nitrogen transformation, sodium pump, and sulfate-dependent conversion are important for salinity wastewater treatment. This manuscript provided a better understanding of the gene responses of relative metabolic pathways to complex bioreactor conditions. In this study, one new anammox species with high relative abundance (35.10%) affiliated to genus *Ca. Brocadia* was observed in CSTR treating low strength wastewater containing seawater, which broadened the understanding on the anammox species. Our studies revealed that this anammox bacteria was more active on nitrogen transformation under the saline condition, especially the DNRA and assimilatory nitrogen reduction pathway. This study provided a detailed analysis of the sodium pump in anammox bacteria for the first time, which was predicted and confirmed by Kartal et al (2013) and Almeida et al (2016). In fact, this anammox species has the potential on energy generation based on Na^+-ATPases. Meanwhile, it developed the metabolic flexibility to maintain the cation stress in the cell and thereby supported its survival in salinity environment. Furthermore, the sulfate-dependent reduction was confirmed in anammox bacteria and the activity was affected by the salinity concentration. Taken together, our study expands our knowledge on related metabolic mechanisms in salinity environment and confirmed the versatility of sodium pump in anammox bacteria. This study provides a comprehensive understanding on anammox metabolism and its mainstream applications.

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Table S6-1 The information of assembled contigs from metagenomics analysis by QUAST

Assembly	contigs
# contigs (>= 0 bp)	1190255
# contigs (>= 1000 bp)	110012
# contigs (>= 5000 bp)	12210
# contigs (>= 10000 bp)	5095
# contigs (>= 25000 bp)	1368
# contigs (>= 50000 bp)	482
Total length (>= 0 bp)	777698976
Total length (>= 1000 bp)	371565765
Total length (>= 5000 bp)	193406255
Total length (>= 10000 bp)	144615010
Total length (>= 25000 bp)	88473014
Total length (>= 50000 bp)	58438595
# contigs	318334
Largest contig	952263
Total length	512727295
GC (%)	57.91
N50	2442
N75	927
L50	30916
L75	123506
# N's per 100 kbp	0.00

Table S6-2 Lists the expressed nitrogen metabolism and transporter genes in the AMX1 genome

CDS	Length	Coverage	Log2 (RPKM)	Length	Coverage	Log2 (RPKM)	Ab.
	40% of seawater			freshwater			
Bining2.001_01340 nitrate reductase / nitrite oxidoreductase, alpha subunit	3450	13.24	9.64	3450	7.43	9.00	*narG1*
Bining2.001_01343 nitrate reductase / nitrite oxidoreductase, beta subunit	1212	10.06	8.93	1212	2.70	8.51	*narH*
Bining2.001_02909 Nitrate reductase	2130	1.71	8.34	2130	0.47	8.28	*narB*
Bining2.001_03528 nitrate reductase / nitrite oxidoreductase, alpha subunit	3450	9.43	9.74	3450	1.94	7.58	*narG2*
Bining2.001_00048 Hydroxylamine oxidoreductase	1677	4.02	8.46	1677	0.44	7.04	*hao1*
Bining2.001_00482 Hydroxylamine oxidoreductase	2535	14.00	8.74	2535	2.96	10.03	*hao2*
Bining2.001_02042 Hydroxylamine oxidoreductase	1746	15.13	8.40	1746	7.66	9.30	*hao3*
Bining2.001_02478 Hydroxylamine oxidoreductase	1704	7.26	5.85	-	-	-	*hao4*
Bining2.001_03174 Hydroxylamine oxidoreductase	1452	3.82	8.67	-	-	-	*hao5*
Bining2.001_03525 Hydroxylamine oxidoreductase	1611	0.96	8.25	1611	2.84	7.10	*hao6*
Bining2.001_03640 Hydroxylamine oxidoreductase	1608	13.55	10.02	1608	5.37	9.68	*hao7*
Bining2.001_02564 hydrazine synthase subunit	1026	3.07	9.17	1026	1.03	8.75	*hzsB*
Bining2.001_03486 nitrite reductase, formate-dependent, cytochrome	1455	24.72	9.54	1455	3.57	8.83	*nrfA*
Bining2.001_00037 putative formate transporter 1	903	5.04	8.35	-	-	-	*focA*
Bining2.001_01964 Ammonia channel	1320	1.01	7.22	-	-	-	*amt1*
Bining2.001_02440 Ammonia channel	1554	4.97	8.98	-	-	-	*amt2*

Bining2.001_02442 Ammonia channel	1410	2.90	8.45	-	-	-	*amt3*
Bining2.001_02444 Ammonium transporter NrgA	1341	0.78	6.20	-	-	-	*amt4*
Bining2.001_01469 Nitrite transporter NirC	891	9.26	9.11	891	0.90	7.95	*nirC1*
Bining2.001_01811 putative nitrate transporter NarT	1203	0.60	7.35	1203	0.21	7.52	*NRT1*
Bining2.001_03529 Nitrite transporter NirC	894	3.09	7.78	894	1.57	7.94	*nirC2*
Bining2.001_03690 putative nitrate transporter NarT	1224	12.65	9.50	1224	3.67	9.08	*narT*

Table S6-3 Lists the expressed sodium pump and energy related genes in the AMX1 genome

CDS	Length	Coverage	Log2 (RPKM)	Length	Coverage	Log2 (RPKM)	Ab.
	40% of seawater			freshwater			
Bining2.001_01130 Electron transport complex subunit RsxB	825	1.13	6.90	-	-	-	RnfB
Bining2.001_02400 Electron transport complex subunit RsxD	1104	0.41	6.48	-	-	-	RnfD1
Bining2.001_02401 Electron transport complex subunit RsxC	1035	8.70	9.16	-	-	-	RnfC1
Bining2.001_02889 Electron transport complex subunit RsxD	996	4.96	7.63	996	0.37	7.79	RnfD2
Bining2.001_02890 Electron transport complex subunit RnfC	1341	3.40	7.78	1341	0.38	8.36	RnfC2
Bining2.001_03346 Electron transport complex subunit RsxA	639	4.94	8.27	-	-	-	RnfA
Bining2.001_03347 Electron transport complex subunit RnfE	636	1.19	7.27	636	2.36	8.44	RnfE
Bining2.001_00460 Na(+)-translocating NADH-quinone reductase subunit F	1119	13.09	8.04	1119	3.38	8.62	nqrF1
Bining2.001_00461 Na(+)-translocating NADH-quinone reductase subunit E	531	1.47	8.53	531	1.88	8.70	nqrE1
Bining2.001_02395 Na(+)-translocating NADH-quinone reductase subunit F	1629	6.96	8.50	1629	5.20	9.08	nqrF2
Bining2.001_02398 Na(+)-translocating NADH-quinone reductase subunit E	1425	18.77	8.43	1425	9.40	8.27	nqrE2
Bining2.001_02748 Putative K(+)-stimulated pyrophosphate-energized sodium pump	2427	10.59	7.66	2427	4.81	6.50	hppA
Bining2.001_02534 ATP synthase gamma chain	846	2.14	8.86	846	1.65	9.02	ATPF1G
Bining2.001_02535 ATP synthase subunit alpha, sodium ion specific	1497	8.02	9.21	1497	1.02	9.20	ATPF1A
Bining2.001_02536 ATP synthase subunit b, sodium ion specific	741	0.49	7.05	-	-	-	ATPF0B
Bining2.001_02537 ATP synthase subunit c, sodium ion specific	267	0.90	8.52	267	1.69	9.69	ATPF0C

Bining2.001_02538 ATP synthase subunit a	666	3.80	8.79	-	-	-	ATPF0A
Bining2.001_02540 ATP synthase epsilon chain	405	1.45	8.92	405	0.56	9.09	ATPF1E
Bining2.001_02541 ATP synthase subunit beta	1365	8.22	9.49	1365	1.36	9.66	ATPF1B
Bining2.001_02702 Potassium-transporting ATPase potassium-binding subunit	1746	2.01	8.40	1746	3.68	8.56	kdpA
Bining2.001_02703 Potassium-transporting ATPase ATP-binding subunit	2076	8.76	8.74	2076	10.59	9.31	kdpB
Bining2.001_02704 Potassium-transporting ATPase KdpC subunit	597	3.43	9.36	-	-	-	kdpC
Bining2.001_02922 Ca(2+)/H(+) antiporter	1005	3.21	8.94	1005	1.18	7.78	chaA
Bining2.001_03154 Calcium-transporting ATPase 1	2769	14.52	7.74	2769	11.66	9.31	CaATP1
Bining2.001_02002 Calcium-transporting ATPase 1	2760	0.49	6.16	-	-	-	CaATP2

Table S6-4 Lists the expressed sulfur metabolism genes in the AMX1 genome

CDS	Length	Coverage	Log2 (RPKM)	Length	Coverage	Log2 (RPKM)	Ab.
	40% of seawater			freshwater			
Bining2.001_01667 putative adenylyl-sulfate kinase	612	6.75	8.33	612	0.70	9.49	*cysC*
Bining2.001_01569 Adenylylsulfate reductase subunit beta	330	7.35	9.22	1677	0.14	7.04	*aprB*
Bining2.001_01570 Adenylylsulfate reductase subunit alpha	1677	2.71	7.46	-	-	-	*aprA*
Bining2.001_00523 putative phosphoadenosine phosphosulfate reductase	741	0.88	7.05	-	-	-	*cysH*

Chapter 7 Conclusion and Recommendations

7.1 Rapid start-up partial nitrification-anaerobic ammonium oxidation fluidized-bed bioreactor treating low strength ammonium wastewater

A partial nitrification (PN)-anaerobic ammonia oxidation (anammox) fluidized-bed bioreactor (FBR) containing granular activated carbon (GAC) particles was built to treat low strength ammonium wastewater with inoculating conventional activated sludge. In the study, a rapid startup of single-stage PN/A system was achieved with over 50% of total nitrogen loss under a dissolved oxygen concentration of 0.7 mg/L. The overall microbial community revealed that the abundance of anammox species, affiliated to the genus *Candidatus* Brocadia, increased with the operation and reached 11.18% and 26.96% in the suspended sludge and biofilm at the end of the study, respectively. Metagenomics analysis revealed that the dominant anammox species were affiliated to the strain of *Candidatus* Brocadia sp. Additionally, the unique genes related to heterotrophic ability and dissimilatory nitrate reduction to ammonium (DNRA) were recovered in the anammox bacteria, which were associated with nitrite loop and heterotrophic cell synthesis in anammox consortia. Furthermore, the missing of genes encoding nitrite reductase and the detection of the gene encoding TonBdependent vitamin B12 transporter (*btuB*) in anammox bacteria suggested it might interact with other organisms through nitric oxide and vitamin B12 exchanging in PN/A FBR system. This study offers a comprehensive understanding on the unique genes in the dominant

anammox bacteria and their relationship with microbial interaction in the single-stage PN/A system treating low strength ammonium wastewater.

7.2 Oxygen detoxification and mixotrophic metabolism in anammox bacteria

The metabolisms of anaerobic ammonium oxidation (anammox) bacteria related to anammox reaction and cell synthesis with inorganic carbon have been extensively observed. However, little is known about the specific metabolic pathways associated with dissolved oxygen and organic carbon. Here, we obtained high abundance of anammox species (~50%) in a lab-scale continuous stirred-tank reactor (CSTR) at room temperature without strict anaerobic condition. The draft genome of the dominant anammox bacteria affiliated to the strain of *Ca. Brocadia sp* was recovered. Its metabolic pathways and genes expression were reconstructed and examined through metagenomic and metatranscriptomic analyses. Interestingly, the results suggested that this anammox lineage might be able to perform oxygen detoxification with genes encoding superoxide dismutase and cytochrome c peroxidase. It also revealed that anammox bacteria possibly beneficially used the intermediate product (hydrogen peroxide) as a terminal oxidant while simultaneously protecting it against the toxicity. Additionally, the observed carbon metabolism pathways also indicated the possibility of regulation on organics utilization by oxygen detoxification in anammox bacteria. This study offers a comprehensive understanding on the diverse metabolic activities in

anammox species affiliated to the strain of *Ca. Brocadia sp* and expanded the applicability of anammox process.

7.3 Anammox process for low-strength seawater-based wastewater treatment

The salinity effect on anammox bacteria has been widely reported, however, rare studies available describe the evolution of microbial community during the startup of anammox process for low-strength wastewater treatment with real seawater. In this study, anammox process was started up at room temperature with shifting it to saline environment and characterized the microbial community at different stages. The reactor achieved 80% of total nitrogen removal efficiency with 20% of seawater, representing for a nitrogen removal rate of 0.180 kg-N/(m^3·d). However, the total nitrogen removal efficiency decreased to 60% when the seawater proportion shift to 40% due to the over-production of nitrate. The bacterial biodiversity was significantly increased with the increase of seawater proportion. High relative abundance of anammox species closed to *Ca. Brocadia sp* (34.24-39.92%) was enriched and acclimated in the saline environment. Taxonomic results revealed that the increase of seawater proportion caused the enrichment of nitrite-oxidizing bacteria (*Ca. Nitrospira defluvii*) even in anaerobic conditions, which might be responsible for the deterioration of nitrogen removal efficiency. Meanwhile, the introduction of seawater also resulted in the enrichment of marine nitrifying bacteria. These results suggest the potential application

of anammox process for mainstream seawater-based wastewater treatment and highlight the importance of microbial diversity for anammox process under different environment.

7.4 Sodium pump associated with energy conservation in anammox bacteria

Two high-quality (completeness>90%) anammox genomes were recovered in a lab-scale continuous stirred-tank reactor (CSTR) treating low strength wastewater containing 40% of seawater. Among them, one draft anammox genome AMX1 (relative abundance 31.51%) affiliated with the genus of *Ca. Brocadia* was the dominant anammox bacteria. The related metabolic pathways (nitrogen transformation and sulfur-dependent metabolism) with genes expression values under different seawater proportions conditions were reconstructed and examined through metagenomic and metatranscriptomic analyses. The results revealed that AMX1 possessed the ability of dissimilatory nitrate reduction to ammonium (DNRA) and assimilatory nitrate reduction. The sodium pump systems with genes expression were obtained in AMX1 genome. It revealed that AMX1 has the potential to synthesize ATP using Na^+-dependent membrane-bound ATP synthase (ATPase, complex V) genes by the force of sodium motive force. Additionally, sodium extrusion may be performed by K(+)-stimulated pyrophosphate-energized sodium pump using energy from the hydrolysis of Pyrophosphatase (PPi) under salinity environment.

7.5 Recommendations for further work

Based on the achievement conducted in this endeavor, several efforts still need to be developed:

1) To optimized operating conditions for the rapid start-up partial nitrification-anaerobic ammonium oxidation process for low strength wastewater treatment without inoculating anammox sludge and maintain a stable long-term operation.
2) To explore the microbial interaction in anammox consortia in different environment such as mainstream condition and salinity condition.
3) To assembly high-quality whole-genome anammox bacteria and explore their potential metabolic mechanisms such as carbon metabolism.
4) To conduct experiments related to organic matters utilization by anammox bacteria, such as short-chain organic matters and pharmaceuticals and personal care products (PPCPs).
5) To characterize the proteins related to the expressed genes involving in oxygen detoxification and mixotrophic metabolism using metaproteomics analysis.
6) To seek salt tolerance mechanisms and energy generation ways in freshwater anammox bacteria.