Development of bioreactors for methane-driven nitrogen removal in anaerobic wastewater treatment

嫌気性廃水処理におけるメタン駆動の窒素除去のためのバイオリアクターの開発

By

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A dissertation submitted in partial fulfilment of the requirement for the degree of Doctor of Engineering

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Achievement

Publications

Journal Articles

1. **Thao Tran P.**, Hatamoto M., Aoki M., Watari T., Syutsubo K., Yamaguchi T., Effect of inoculum sources on autotrophic nitrogen removal in anaerobic hollow fiber membrane reactors, Environmental Technology & Innovation, 26, 102375, 2022. https://doi.org/10.1016/j.eti.2022.102375

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Awards

1. Best Oral Presentation Award in VANJ 2021 (conference No.1) 4-5/12/2021
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ABBREVIATION

Abbreviation	Explaination
ABR	anaerobic baffled reactor
AD	anaerobic digestion
Anammox	anaerobic ammonium oxidation
AnMBR	anaerobic membrane bioreactor
ANME	anaerobic methane-oxidizing archaea
AOA	ammonia-oxidizing archaea
AOB	ammonia-oxidizing bacteria
AS	activated sludge
BNR	biological nitrogen removal
BOD	biochemical oxygen demand
COD	chemical oxygen demand
DHS	downflow hanging sponge
DIN	dissolved inorganic nitrogen
DNB	heterotrophic denitrifying bacteria
DNRA	dissimilatory nitrate/nitrite reduction to ammonium
DO	dissolved oxygen
EGSB	expanded granular sludge bed
FISH	fluorescence in situ hybridization
GHG	greenhouse gas
HRT	hydraulic retention time
MDD	methane-driven denitrification
MBR	membrane bioreactor
NOB	nitrite-oxidizing bacteria
NRPW	natural rubber processing wastewater
OLR	organic loading rate
ORP	oxidation-reduction potential
OTU	operational taxonomic unit
PCA	principal component analysis
PICRUSt	phylogenetic investigation of communities by reconstruction of unobserved states
PN	partial nitrification
SBR	sequencing batch reactor
SRT	sludge retention time
SS	suspended solid
UASB	upflow anaerobic sludge blanket
VFA	volatile fatty acid
WWTP	wastewater treatment plant

CHAPTER 1. GENERAL OUTLINES AND OBJECTIVE

1.1. Background

The increasing population has posed serious environmental threats by discharging excessive nutrients and organic contaminants into water bodies. There are a variety of physical, biological, and/or chemical technologies applied in wastewater treatment plants (WWTPs) to remove these contaminants. Above all, biological treatment is proved as the most sustainable approach because it utilizes the natural metabolisms of microorganisms to remove the contaminants from water effectively and turns them into resources (Puyol et al., 2017). This approach is especially attractive in circular economy, where WWTPs shift to resource recovery plants by using microorganisms to remedy the disruption of carbon and nitrogen cycles and conserve water environment.

Biological treatment in the absence of oxygen, so-called anaerobic digestion (AD), has long been practiced for high-strength industrial wastewater, focused on recovery methane-rich biogas as fuel (Abbasi et al., 2012). Natural rubber processing wastewater (NRPW) is one typical example of high-strength industrial wastewater. Despite the differences in natural rubber manufacturing process in each country, the wastewater generally has low pH and high concentrations of organic matter and nitrogen compounds (Jawjit et al., 2015; Mohammadi et al., 2010; Nguyen & Luong, 2012). Besides, the conventional open treatment causes air pollution with greenhouse gases (GHGs) and strong odor of volatile fatty acids from organic matters degradation. Therefore, several configuration reactors of AD were developed to resolve pollution issues in NRPW and surrounding environment.

However, the remaining nitrogen contents in the effluent of AD are still a concern before discharging to receiving water bodies. The low C/N ratio in the AD effluent is a bottleneck in denitrification step converting nitrogen pollutants in water completely into harmless dinitrogen gas. As a result, an external organic carbon supplement would be used to

address this issue (Bonassa et al., 2021; Tanikawa et al., 2020). Still, beside the extra cost, there is some concern regarding the toxic excess organic carbon (e.g., methanol) remaining in treated water. Thus, an alternative electron donor for denitrification is needed to investigate.

In the last few decades, denitrification process using CH_4 as carbon and electron sources was discovered and combined with other anaerobic autotrophic denitrification processes to remove nitrogen pollutants. Although methane gas produced from anaerobic digester is an environmental-friendly alternative energy source, the uncaptured CH_4 via gas leakage along transporting chain or gas strip from the effluent poses a risk of global warming effect 30 times higher than CO_2. Using CH_4 gas as electron has several advantages: on-site accessible in WWTPs, easy to strip from treated wastewater, and nontoxic for human health. Moreover, methane-driven denitrification (MDD) also occurs under anaerobic condition as main biological unit for methane recovery, so that it is easy to add-on in WWTPs. So far, the application of these processes is limited due to two main drawbacks: the slow growth rate of autotrophic microbes and the poor solubility of CH_4.

1.2. Objectives

This thesis employed anaerobic treatment to remedy both nitrogen and carbon cycles. Anaerobic reactors were implemented to firstly convert carbon matter to methane gas (Chapter 3), then use methane for MDD process to transform dissolved inorganic nitrogen compounds and methane to harmless nitrogen gas and CO_2, respectively (Chapter 4, 5) as illustrated in Fig. 1.1. This thesis focused on MDD process's application by developing anaerobic reactors to resolve the two mentioned drawbacks. The two main chapters 4 and 5 have objectives:

i) Investigate the effects of hydraulic retention times and a reducing agent on nitrogen removal and N_2O emission of a closed-type downflow hanging sponge (DHS) reactor applying MDD process.

ii) Compare the nitrogen removal and dynamic changes in microbiota inside anaerobic upflow hollow fiber membrane reactors inoculated with either enriched sludge from previous reactors or natural paddy soil.

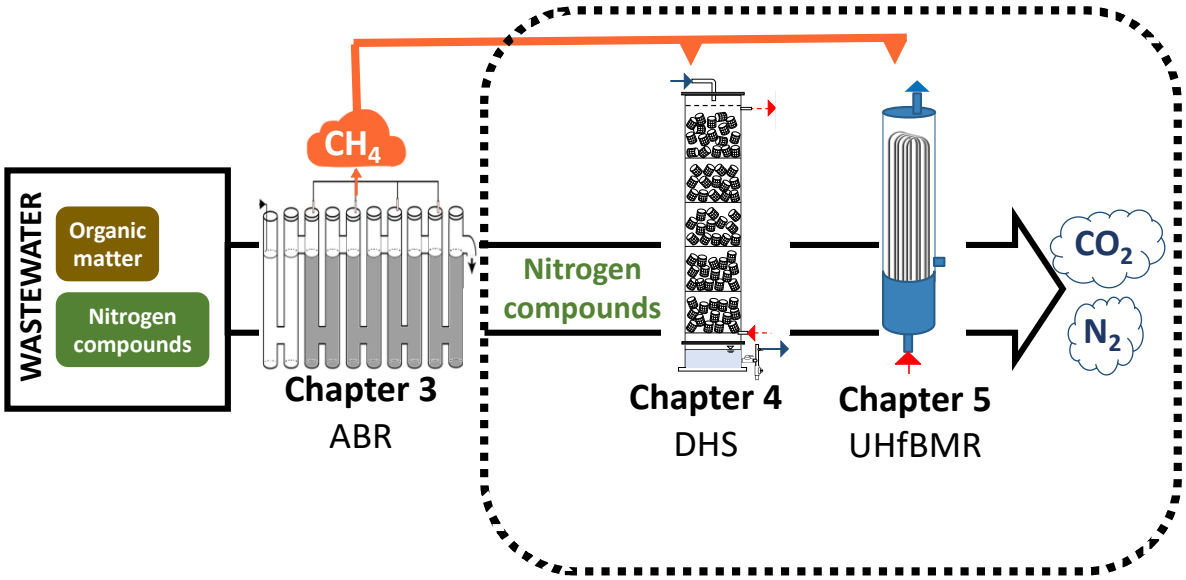

Fig. 1.1 Demonstration of anaerobic reactors' application to remedy both nitrogen and carbon cycles and this thesis's main focus on MDD bioreactor

Thesis outline

This thesis consists of six chapters as shown in Fig. 1.2. Chapter 1 introduces the background of this study and the overall structure of this thesis. Chapter 2 provides a literature review about the application of AD in treating wastewater containing organic and nitrogen pollutants, especially the MDD process in reducing nitrogen compounds and GHGs emission. Chapter 3 shows the results of the anaerobic baffled reactor (ABR) in treating natural rubber processing wastewater, an example for AD in treating industrial wastewater contains high concentrations of carbon and nitrogen. Chapter 4 and Chapter 5 present the results of the DHS reactor and the upflow reactors applying MDD process for nitrogen removal. Chapter 6 summarizes the key findings from this study and recommendations for future work.

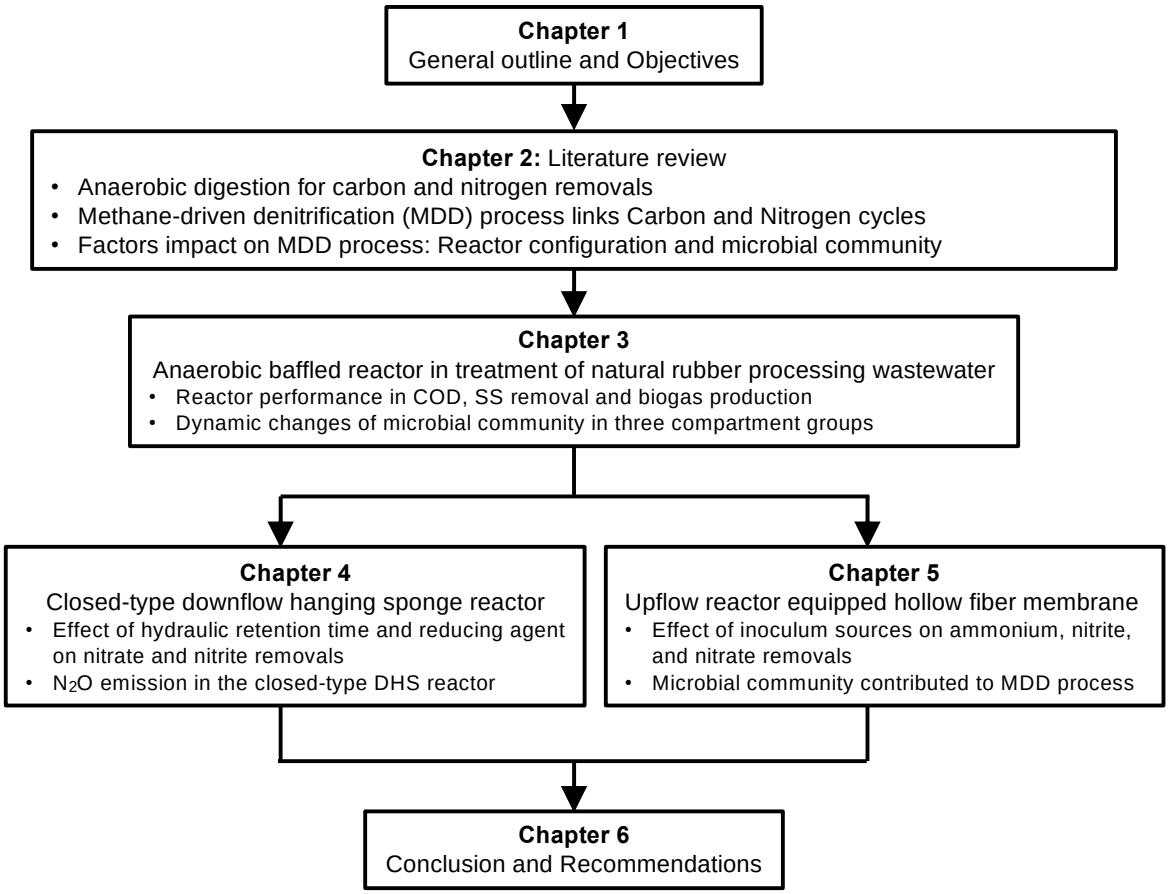

Fig. 1.2 Outline structure of the thesis

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CHAPTER 2. LITERATURE REVIEW

2.1. Anaerobic digester for carbon and nitrogen-rich industrial wastewater

2.1.1. Anaerobic digestion in carbon-rich wastewater treatment

In recent years, the circular economy has been leading the role shift of anaerobic digestion (AD) technology, from a platform removing contaminants to a platform recovering energy-resources (Ghimire et al., 2021). Given its proven operational reliability, no aeration, less sludge handling requirement, AD reduce the two major electricity consumers within the activated sludge (AS) process. Furthermore, the methane-rich biogas from AD can be used as a fuel source in a combined heat and power system, which produces electricity and heat simultaneously, hence transforms wastewater treatment plants (WWTPs) from being an energy consumer to an energy producer (Sarpong and Gude, 2021).

The conversion from organic matters to methane gas in AD consists of four processes in the absence of oxygen, i.e., hydrolysis, acidogenesis, acetogenesis, and methanogenesis driven by a complex network of microorganisms belonging to hydrolytic bacteria, fermenting bacteria (acidogens), syntrophic bacteria (acetogens), and methanogenic archaea (methanogens), respectively (Cabezas et al., 2015). As shown in Fig. 2.1, hydrolysis is considered as the pre-treatment to degrade complex high molecular-weight compounds, such as lignocellulosic

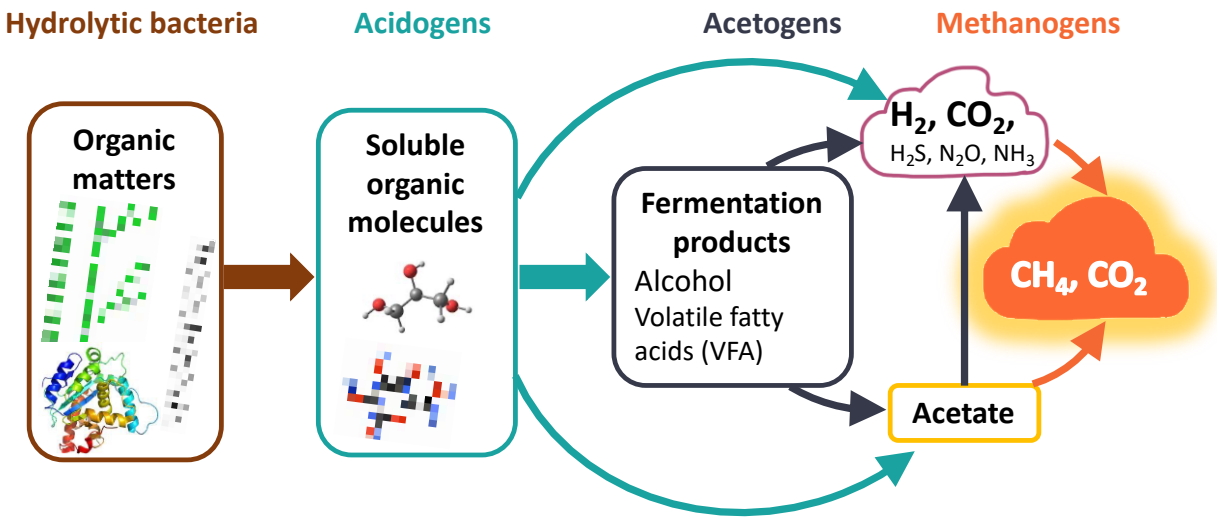

Fig. 2.1 Schematic view of the anaerobic degradation of organic matter

biomass, solid food waste, to soluble molecules. Depending on the initial characteristic of complex organic matters, the hydrolysis step is carried out by extracellular enzymes (e.g., cellulases, proteinases and lipases) from microorganisms in anaerobic digestors, or can be supported by chemical or mechanical methods (Vavilin et al., 2008). The soluble products (e.g., long-chain fatty acids, glycerol, amino acids, monosaccharides) of hydrolysis are then transformed mainly to volatile fatty acids (VFA) (e.g., acetate, butyrate, propionate) and some other intermediates, such as alcohols, lactate, formate, H_2, and CO_2, by intracellular enzymes in acidogenic/acetogenic microorganisms. Detman et al. (2021) revealed that the dominant products of acidic fermentation led to two different pathways of final methanogenesis step: acetate and lactate determines the acetotrophic pathway (i.e., generate CH_4 from acetate), while butyrate and propionate determines the hydrogenotrophic pathway (i.e., generate CH_4 from H_2 and CO_2). Besides, the development of acidogens and acetogen producing VFA or the overload of wastewater may cause VFA accumulation, hence tend to lower pH, which can inhibit the methanogens to some extent (Wang et al., 2020). A drop of pH is more harmful to acetotrophic methanogens than to hydrogenotrophic methanogens because it inhibited both acetogen growth and acetate production (Shin et al., 2015). Wang et al. (2020) also observed that a sudden shock of low pH (i.e., from 7 to 6 or 6 to 5) reduce the abundance of acetoclastic methanogen, such as *Methanothrix,* whereas it increased hydrogenotrophic methanogens, such as *Methanospirillum* and *Methanolinea.* These results suggest a higher acid tolerance of hydrogenotrophic methanogens. Nonetheless, a gradually decrease in pH appears to be beneficial for improving the adaptability of acetoclastic methanogens to acidic conditions (Wang et al., 2020).

Despite of all advantages mentioned above, there are still some concerns about AD in wastewater treatment: (i) slow-growth rate of anaerobic microorganisms resulting in less sludge production but longer time to accumulate the optimal population in sludge as well, (ii)

H_2S and CH_4 mainly produced in AD can cause bad odors, or corrosion (with H_2S) and potential global warming risk (with CH_4 and N_2O) for surrounding areas without a proper handling of biogas, and (iii) necessity of post-treatment to polish the residual organic matter, nutrients and pathogens. Modification of reactor configuration is an engineering approach to overcome these issues. Many attempts has been made in designing anaerobic reactors, covering two main kinds: suspended-growth reactors (van Lier et al., 2016) and attached-growth reactors (Maaz et al., 2019).

The upflow anaerobic sludge blanket (UASB), which was invented by Lettinga and his co-workers in the Netherlands in 1970's, has been one of the most widely implemented high-rate sludge bed reactors for treatment from domestic to industrial wastewaters (van Lier et al., 2016). Later, Lettinga et al. (1997) introduced second generation of anaerobic sludge bed reactor, expanded granular sludge bed (EGSB), considered as a novel variation of UASB. Similar to UASB mechanism, this concept also relied on granular sludge with good settling properties. In addition, its high superficial velocity was facilitated by increase the reactor's height and the upflow liquid velocity, which eliminates dead zones and improves the internal mixing in reactor. Given this enhancement, EGSB can treat low strength wastewater and operate at low temperature, i.e., < 20 ^{o}C (Gomec, 2010). While the mechanism of UASB and EGSB reactors focuses on the internal mixing in reactors' volume, anaerobic baffled reactor (ABR) focuses on creating a plugflow in the waterline and staging the reactor following different steps of AD (van Lier et al., 2016). The biggest advantage of ABR over UASB and EGSB reactors is its ability to separate acidogenesis and methanogenesis steps longitudinally down the reactor, allowing the reactor to behave as a two-phase system without the associated control problems and high costs (Barber & Stuckey, 1999). Besides, ABR can avoids the risk of clogging and sludge bed expansion; higher tolerance to hydraulic and organic shock loads can be achieved by prolonging retention time of sludge and liquid. ABR is also simple to build

or to modify from conventional lagoons or tanks, it needs low maintenance and operational attentions (Gomec, 2010). With these mentioned advantages, ABR shows promising application for treating wastewater with high concentration of carbon and suspended solid (SS) (Kiflay et al., 2021; Putra et al., 2020). The typical features and challenges for UASB, EGSB, and ABR were summarized in Table 2.1.

Table 2.1 The typical features and limitations of three common high-rate anaerobic reactors based on suspended-growth. Summarized from Barber and Stuckey (1999), Chong et al. (2012), Gomec (2010), and Seghezzo et al. (1998).

Reactor	Features	Challenges
UASB	•Granular sludge combined different groups cross-feeding •High biomass content, enabling a wide range of OLRs, short hydraulic retention time (HRT), and high sludge retention time (SRT) •Produced rising gas bubbles are used for mixing instead of mechanical devices	•Long start-up or/and supplements are required for sludge granulation •Need gas-liquid-solid separator device to purify biogas and separate SS from effluent •Start-up is susceptible to temperature and organic shock load •Need post-treatment to polish COD, N, P, S, and pathogens
EGSB	•Better internal mixing than UASB due to higher reactor and faster upflow influent velocity •Sludge bed is expanded, sludge is always granular with high activity and settleability •Higher removal efficiency for soluble contaminants and higher gas production yield •Can treat wider range of loading rates and even in low temperature	•Long start-up or/and supplements are required for sludge granulation •Need gas-liquid-solid separator device to purify biogas and separate SS from effluent •Need post-treatment to polish N, P, S, and pathogens •Low suspended solid removal. Due to high upflow velocity, flocculant solid is easy to wash-out
ABR	• Baffles are placed to compartmentalize reactor and to force the liquid flow up and down from one to next compartment; simple design, inexpensive construction	• Need post-treatment to polish N, P, S, and pathogens • Requirement to build shallow reactors to maintain acceptable liquid and gas upflow

	• Separate acidogenesis and methanogenesis longitudinally down the reactor, allowing each bacterial groups to develop under the most favorable conditions • No requirement for biomass with settling properties and special gas or sludge separator device • Can treat wastewater with high SS without clogging risk • Extremely stable to hydraulic shock loads, high tolerance to toxic, organic shocks	velocities, and maintain an even distribution of the influent • The sludge mass may slowly move with the liquid flow through the various compartments. So HRT is usually longer

Furthermore, another kind of anaerobic reactor is attached-growth reactor. One of the most advanced attached-growth reactors is anaerobic membrane bioreactors (AnMBR). The growing application experiences from aerobic membrane bioreactors in recent years has resulted in extending membrane application in AD as well. It is expected to utilize the advantage of membrane technology (i.e., reducing footprint, handling wide fluctuations in influent quality, retaining a large population of slow growing microorganisms) to improve AD's performance and effluent quality regardless of sludge settling and/or granulation properties. There are two strategies to implement membrane module into an anaerobic reactor: inside bioreactor or outside bioreactor. In the former strategy, membrane module is directly immersed in anaerobic digester (Submerged AnMBR) and treated water obtains under vacuum pump or gravity on permeate side of membrane and retained biomass presents in bioreactor. It is more favorable for low strength organic loads like municipal wastewater. In the latter strategy, membrane module is placed outside reactor as side-stream treatment and permeate obtains under pressure provided by recirculation pump in external cross flow configuration. Its advantage is membrane cleaning is easy because membrane can easily be taken out for cleaning purposes. However, the drawback of this approach is in form of energy consumption by recirculation pump to maintain transmembrane pressure (TMP) and elevated volumetric flow to keep cross flow velocity at certain level. Operating TMP and cross flow

velocity through membrane in external membrane module is relatively higher compared to the submerged AnMBR. (Maaz et al., 2019). Table 2.2 compares conventional aerobic treatment, anaerobic treatment, aerobic MBR and AnMBR in term of efficient operation. It is apparent from Table 2.2 that AnMBR technology has the advantages of both anaerobic treatment and MBR technology. Among these, the ones most often cited are: total biomass retention, excellent effluent quality, low sludge production, a small footprint and net energy production (Lin et al., 2013). Since an improved effluent quality might not always be required and the high cost for filter and fouling control, AnMBR can be used as a post-treatment following high-rate anaerobic reactor configurations such as UASB or EGSB in a hybrid system if high effluent quality is needed, but it has not been widely used as the primary bioreactor.

Table 2.2 Comparison of conventional aerobic treatment, anaerobic treatment, aerobic MBR and AnMBR (Lin et al., 2013)

Features	conventional aerobic treatment	anaerobic treatment	aerobic MBR	AnMBR
Start-up time	2–4 weeks	2–4 months	<1 week	<2 week
Energy requirement	High	Low	High	Low
Nutrient requirement	High	Low	High	Low
Alkalinity requirement	Low	High for certain industrial stream	Low	High to moderate
Temperature sensitivity	Low	Low to moderate	Low	Low to moderate
Organic loading rate	Moderate	High	High to moderate	High
Effluent quality	High	Moderate to poor	Excellent	High
Footprint	High	High to moderate	Low	Low
Sludge production	High	Low	High to moderate	Low
Biomass retention	Low to moderate	Low	Total	Total
Bioenergy recovery	No	Yes	No	Yes

2.1.2. Remained challenges in effluent of anaerobic digestion

Despite the widespread application of AD in WWTPs as a tool to recover energy, it produces effluent with high ammonia-nitrogen, organic-nitrogen and dissolved methane concentrations. As a result, additional processes to remove nitrogen compound are usually applied to meet the stringent discharge standards (Delgado Vela et al., 2015). Additionally, the emission risk of greenhouse gases (GHGs) from AD processes, such as CO_2, N_2O, and CH_4, is also a concern for surrounding atmosphere. Nitrous oxide (N_2O) having 300 times the warming potential of carbon dioxide can be produced in some conditions, such as (i) low dissolved oxygen concentration in the nitrification and the presence of oxygen in denitrification stages, (ii) high nitrite concentrations in both nitrification and denitrification stages, (iii) low COD/N ratio in the denitrification stage, (iv) sudden shifts of pH and dissolved oxygen and ammonia and nitrite concentrations, and (v) transient anoxic and aerobic conditions (Campos et al., 2016). Methane (CH_4), with a global warming potential 34-86 times higher than CO_2 (Guerrero-Cruz et al., 2021), is the main composition in AD's biogas and even dissolved in AD's effluent. It is reported that dissolved CH_4 concentrations in anaerobic effluents can account for about half of the total production, thus strategies for its recovery as energy source or direct use within the same treatment line, are the keys to approach energy-neutral anaerobic treatment, and to valorize the intrinsic features of such process to be economically feasible and environmentally friendly (Stazi & Tomei, 2021).

2.2. Biological nitrogen removal

2.2.1. Nitrogen cycles and metabolic pathways

Nitrogen pollutants in wastewater consist of dissolved organic nitrogen - DON (e.g., urea, amino acids, peptides, amino sugars, purines, pyrimidines, and amides) and dissolved inorganic nitrogen - DIN (i.e., ammonium, nitrate and nitrite). A comprehensive review by Kuypers et al. (2018) summarized the global inventories of nitrogen compounds, six distinct nitrogen transforming processes in the biogeochemical nitrogen cycle, and the annual nitrogen fluxes for a number of these processes from the available literature as in Fig. 2.2. Ammonia bound in rocks and sediments, with 1.8×10^{10} Tg nitrogen, is the largest global nitrogen inventory. On the other hand, N_2 gas, with 3.9×10^{10} Tg nitrogen, is the largest freely accessible global nitrogen inventory followed by organic nitrogen (9×10^5 Tg), nitrate (6×10^5 Tg), NH_3 (terrestrial inventory is unknown, and marine inventory is estimated about 340-3600 Tg), and

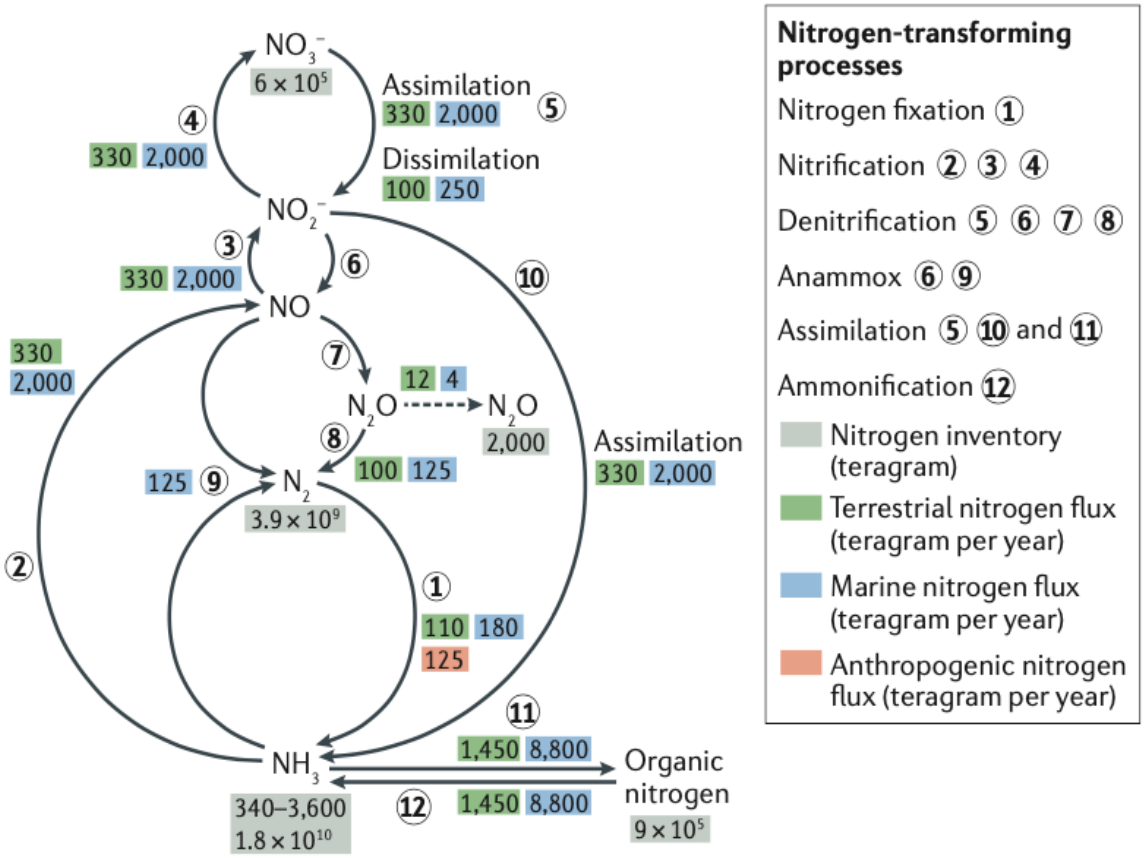

Fig. 2.2 Summary of the global inventories of nitrogen compounds, six distinct nitrogen transforming processes, and the annual nitrogen fluxes (Kuypers et al., 2018)

N_2O (2000 Tg). Among six distinct nitrogen transforming processes, the interconversion of organic nitrogen and ammonia accounts for the biggest nitrogen fluxes (Kuypers et al., 2018). In particular, organic nitrogen is converted to ammonia in hydrolysis process, so DIN becomes the main issue in wastewater treatment.

DIN removal can be executed by physicochemical processes such as ion exchange, adsorption, and reverse osmosis, but it needs high cost for machinery and maintenance. Besides, it only can concentrate nitrogen compounds in brine, which required secondary treatment. Therefore, biological nitrogen removal (BNR), converting DIN to harmless denitrogen gas by using microorganisms' enzymes, is a more cost-effective and environmentally friendly approach. Nitrogen-transforming microorganisms are generally classified according to metabolic pathway they are involved in, e.g., nitrifiers in nitrification, denitrifiers in denitrification, nitrogen-fixers in nitrogen fixation. However, genomic data collected during the past decade have revealed versatile nitrogen-related functional genes or protein within microorganisms. Thus, owing to their metabolic versatility, it has become nearly impossible to objectively classify nitrogen-transforming microorganisms according to the six classical processes (Kuypers et al., 2018). Instead of that, more attention is paid to and biochemical pathways and responsible enzymes (Fig. 2.3).

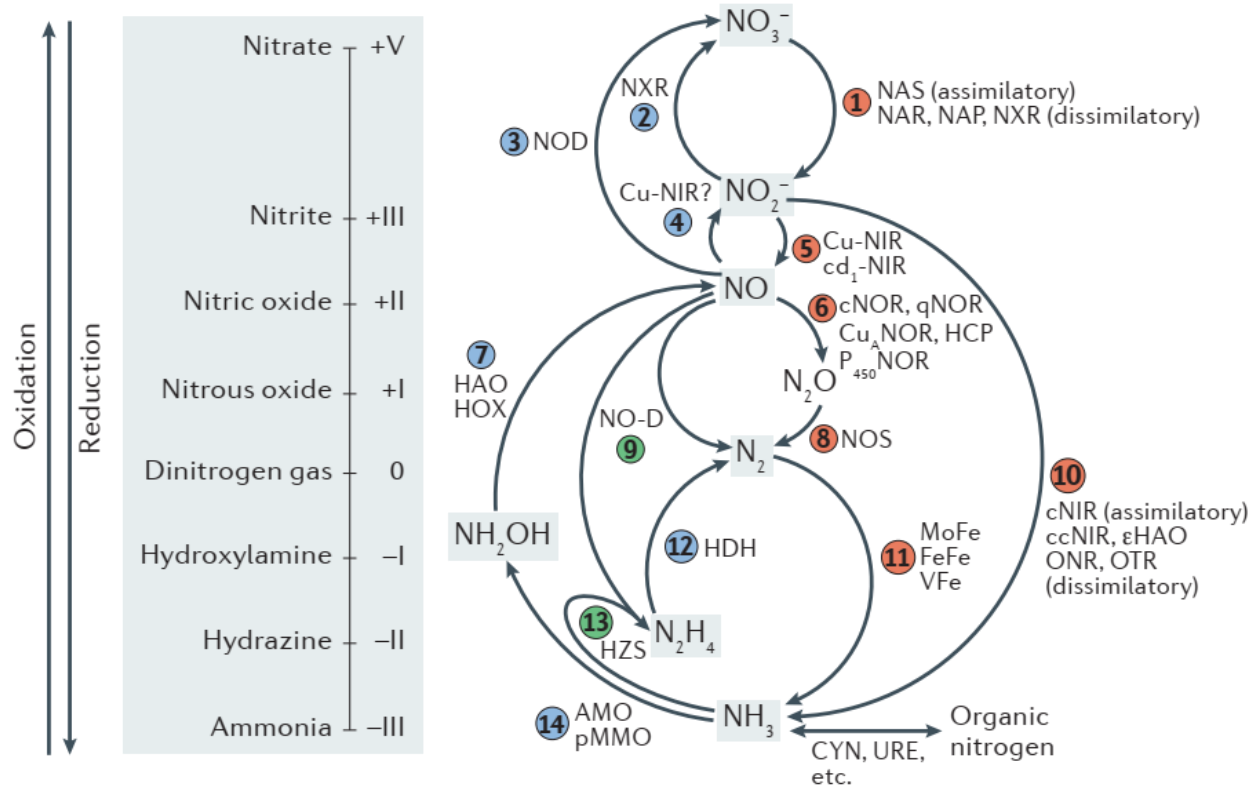

Fig. 2.3 Biochemical pathways and responsible enzymes (Kuypers et al., 2018)

The reactions involve reduction (red), oxidation (blue) and disproportionation and comproportionation (green). Abbreviations for enzymes: ① assimilatory nitrate reductase (NAS), membrane-bound (NAR), and periplasmic (NAP) dissimilatory nitrate reductases; ② nitrite oxidoreductase (NXR); ③ nitric oxide oxidase (NOD); ⑤ copper-containing (Cu-NIR) nitrite reductases and haem-containing (cd1-NIR); ⑥ cytochrome c-dependent (cNOR), quinol-dependent (qNOR), copper-containing quinol-dependent (CuANOR), NADH-dependent cytochrome P450 nitric oxide reductase (P_{450}NOR) and hybrid cluster protein (HCP); ⑦hydroxylamine oxidoreductase (HAO) and hydroxylamine oxidase (HOX); ⑧ nitrous oxide reductase (NOS); ⑨ nitric oxide dismutase (NO-D); ⑩ assimilatory nitrite reductase (cNIR), dissimilatory periplasmic cytochrome c nitrite reductase (ccNIR), ε-hydroxylamine oxidoreductase (εHAO), octahaem nitrite reductase (ONR), and octahaem tetrathionate reductase (OTR); ⑪ molybdenum-iron (MoFe), iron-iron (FeFe), and vanadium-iron (VFe) nitrogenases; ⑫ hydrazine dehydrogenase (HDH); ⑬ hydrazine synthase (HZS); ⑭ ammonia monooxygenase (AMO); particulate methane monooxygenase (pMMO); cyanase (CYN); urease (URE)

2.2.2. Novel biological nitrogen removal processes

The conventional processes to remove nitrogen pollutants need to go through autotrophic aerobic nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) and heterotrophic anaerobic denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). This approach is a high-energy-consuming and resource-demanding process: aeration for nitrification and additional organic carbon for denitrification. In addition, excessive sludge produced from activated sludge and GHGs emission (especially N_2O) are other disadvantages of nitrification-denitrification method. These limitations have been urged engineer and biologist to discover alternative methods, which is more energy-resource efficient.

In the past decades, to alleviate these problems of conventional biological nitrogen removal processes, novel nitrogen removal processes have been discovered. There are several comprehensive literature reviews summarizing kinetic and stoichiometric parameters of these new metabolisms and related microorganisms (Delgado Vela et al., 2015), evaluating in the technical-economic points (Rahimi et al., 2020), and their coupling with gas emissions abatement in wastewater treatment facilities (Chan-Pacheco et al., 2021). Among them, anaerobic ammonium oxidation (Anammox) process, utilizing NO_2 instead of oxygen as electron acceptor for ammonium oxidation to N_2 without additional organic carbon, revolutionized the paradigm that nitrification and denitrification were the only BNR processes. The stoichiometric equation of Anammox process is shown in Eq. (2.1) (Strous et al., 1998):

$$1NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \qquad (2.1)$$

There are two main challenges in Anammox process: provide NH_4^+ and NO_2^- supply to fit with Anammox ratio and eliminate NO_3^- accumulation in Anammox effluent (Ma et al., 2020). Thus, the combination of Anammox and partial nitrification (so-called nitritation, $NH_4^+ \rightarrow NO_2^-$) or partial denitrification processes ($NO_3^- \rightarrow NO_2^-$) offers an enormous opportunity to achieve

sustainable BNR due to less aeration and organic carbon demand as well as less sludge production. In partial nitrification process, to keep nitrite at a sufficient level for Anammox, nitrite-oxidizing bacteria (NOB) is suppressed by controlling dissolved oxygen (DO) or oxygen/ammonium flux ratio, treating with free nitrous acid or free ammonia as inhibitors, shortening aerobic sludge retention time, and augmenting Anammox bacteria and ammonia oxidizing bacteria (Cao et al., 2017).

The increase in nitrate contamination leads to serious eutrophication in water bodies, harming aquatic life, and put human heath at risk. Traditional denitrification process removing nitrate faces several challenges, especially for high-strength nitrate removal, including the high consumption of organic carbon source, huge production of waste sludge, and long acclimatization period. And the emerging Anammox process cannot be applied for direct nitrate removal. Since the last decade, considerable attention is given to partial denitrification, which can generate nitrite for Anammox process and mitigate N_2O emission (Du et al., 2019). Furthermore, recently, autotrophic denitrification has been received more interest to reduce the additional cost for organic carbon and the risk of inhibitory effect of organic matter on Anammox bacteria. There are several alternative electron donor (e.g., methane, hydrogen, reduced sulfur compounds, ferrous iron, and iron sulfides) for autotrophic denitrification (Di Capua et al., 2019; Pang & Wang, 2021). However, there is some concern regarding the toxic of excess organic carbon (e.g., methanol) or metals (e.g., iron, arsenic) remain in treated water or the corrosion of H_2S gas, or the competitive usage of H_2 gas in the energy industry. Thus, the utilization of methane gas as an electron donor for denitrification process has been attractive in the last two decades because methane is easy to strip from treated wastewater and nontoxic for human health.

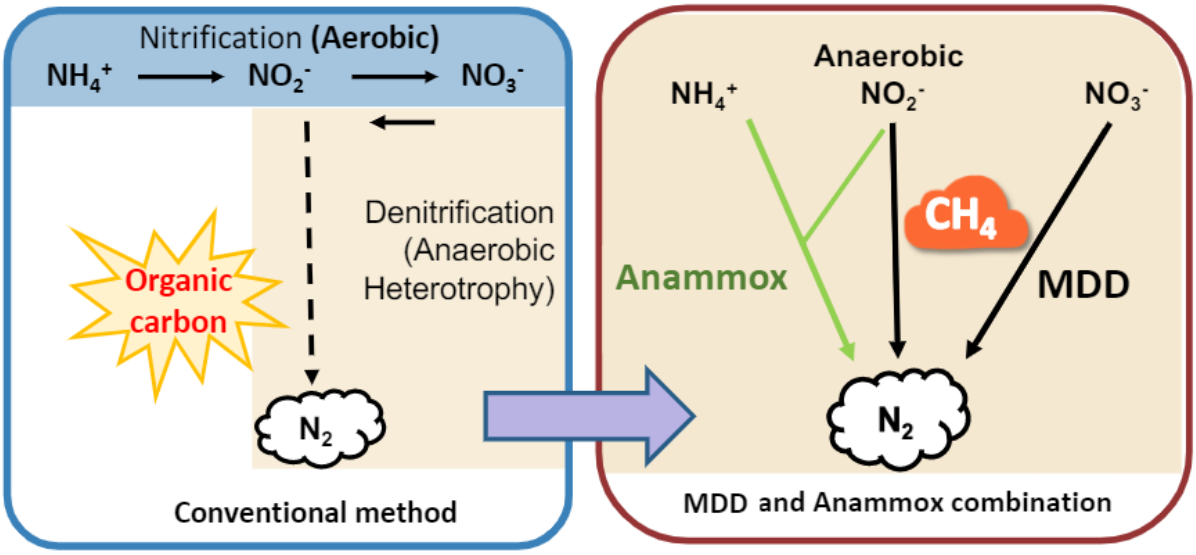

Fig. 2.4 The combination of MDD and Anammox processes can remove nitrogen completely under anaerobic condition

2.3. Methane-driven denitrification

2.3.1. Microbial community

Methanotrophs including bacteria and archaea oxidizing methane with and without the presence of oxygen and their contribution in denitrification process (known as methane-driven denitrification – MDD) have been discovered for more than 115 years. Some milestones of methane-driven denitrification process are demonstrated in Fig. 2.4.

The first methane-oxidizing microorganism was aerobic bacterium isolated in 1906 (Leadbetter and Foster, 1958), then over 100 strictly aerobic methanotrophic bacteria were isolated and classified into five groups (named: *Methylosinus, Methylocystis, Methylomonas, Methylobacter*, and *Methylococcus*) based on morphology, fine structure, and type of resting stage formed by Whittenbury et al. (1970). Later, based on the formaldehyde assimilation pathways (i.e, RuMP pathway and Serine pathway), cell morphology, and culture temperature, aerobic methanotrophic bacteria was divided into three assemblages: type I (including the genera *Methylomonas* and *Methylobacter*) utilizes ribulose monophosphate (RuMP) as the primary pathway for formaldehyde assimilation, type II (including the genera Methylosinus

and Methylocystis) follows Serine pathway, and type X (*Methylococcus*) has similar cell shape as type I but follow both RuMP pathway and Serine pathway and grows at higher temperatures than both type I and type II (Hanson & Hanson, 1996). It was assumed that aerobic methanotrophic bacteria belonged to only Alphaproteobacteria (type II) or Gammaproteobacteria (type I and X) classes of Proteobacteria phylum, till studies discovering extremely acidophilic methanotroph of the phylum *Verrucomicrobia* in 2007 (Dunfield et al., 2007; Pol et al., 2007). The role of aerobic methanotroph in nitrogen cycles was initially reported to oxidize ammonia, or assimilate nitrogen (i.e., either ammonia or nitrate), or cross-feed to associated heterotrophic denitrifers by product from methane oxidation (Modin et al., 2007).

Anaerobic methane oxidation was discovered later than aerobic, it was first discovered in 1975 (Reeburgh, 1976), and the responsible anaerobic methane-oxidizing archaea (ANME1, ANME-2abc, and ANME-3) were successfully identified in marine and mud volcano consortia with sulfate-reducing bacteria based on the carbon isotope signature of specific lipid biomarker and 16S rRNA sequences in more than 20 years later (Boetius et al., 2000; Hinrichs et al., 1999; Niemann et al., 2006). These ANME performs "reverse methanogenesis", which means they consume methane to produce cellular carbon and energy (Hallam et al., 2004); ANME converts CH_4 to CO_2 and reduced by-products to cross-feed sulfate-reducing bacteria, where sulfate is used as an electron acceptor (later known as the character for ANME-1, ANME-2abc, and ANME-3) (Timmers et al., 2017). The combination of anerobic methane oxidation (by mostly archaea, proteobacteria was less than 5% of the community) and denitrification (by bacteria) was first proposed by Raghoebarsing et al (2006). The bacteria/archaea cells ratio in this consortium was observed at approximately 8:1 by fluorescence in situ hybridization (FISH), higher than the ratio in previous anerobic methane oxidation coupled with sulfate reduction consortium (at about 2:1), which is likely due to the higher energy yield of denitrification

compared to sulfate reduction (Raghoebarsing et al., 2006). The discovery of anaerobic bacterial *Candidatus Methylomirabilis oxyfera* (Ettwig et al., 2008, 2010), *Candidatus Methylomirabilis sinica* (He et al., 2016), and *Candidatus Methylomirabilis lanthanidiphila* (Versantvoort et al., 2018) belonging to NC10 phylum and archaeal *Candidatus Methanoperedens nitroreducens* belonging to ANME-2d group (Haroon et al., 2013) possessing both the methane oxidation and nitrite/nitrate denitrification abilities within themselves has opened a new era for denitrification by anaerobic methanotrophs. ANME-2d use "reverse methanogenesis" as other ANME groups for methane oxidation and nitrate as the electron acceptor for denitrification, whereas NC10 members use a novel "intra-aerobic" pathway for methane oxidation and nitrite as the electron acceptor for denitrification. Based on the complete genome data of *M. oxyfera* and the isotopic labeling experiments, Ettwig et al. (2010) proved the "intra-aerobic" pathway for oxygen production in anaerobic

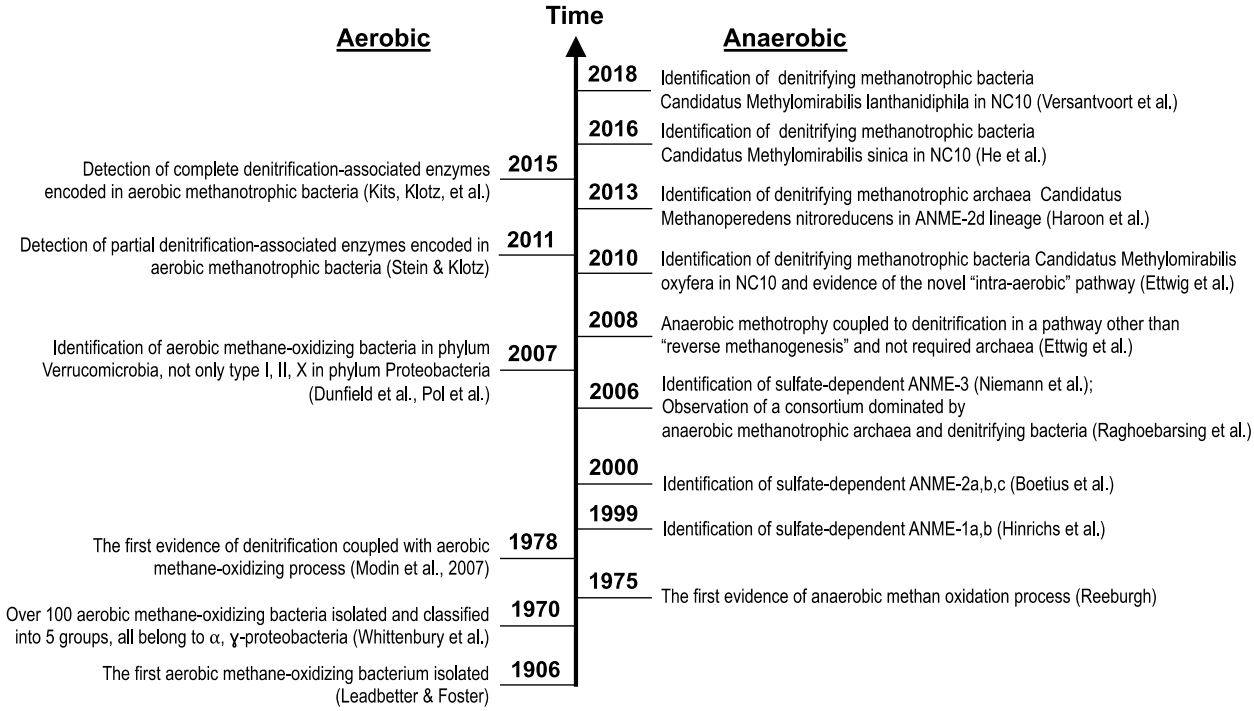

Fig. 2.5 Milestones in the development of methanotrophy and methane-driven denitrification process and responsible microorganism

methanotrophic bacteria, in which nitrite-denitrification provides oxygen for methane oxidation and the methane oxidation provides electron for nitrite reduction.

The development of metagenomic analysis and molecular techniques for environmental samples has revealed the ubiquity of aerobic methanotrophic bacteria in not only the oxic-anoxic interfaces in lake or coastal sediments or paddy soil (Bessette et al., 2017; He et al., 2021; Reim et al., 2012), but micro-oxic conditions (DO concentration at nM range) (Dalcin Martins et al., 2021; Stolpera et al., 2010). In addition, recent studies with metagenomic technology revealed nitrification and partial denitrification-associated enzymes (Boden et al., 2011; Khadem et al., 2012; Stein and Klotz, 2011) and complete denitrification-associated enzymes (Dam et al., 2013; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015) encoded within methanotrophic groups in *Gammaproteobacteria*, *Alphaproteobacteria*, and *Verruccomicrobia.* These findings suggest direct denitrification capability by aerobic methanotrophs, thus open application potentials of aerobic methanotrophs in nitrogen removal in anaerobic wastewater treatment.

2.3.2. Reactor configuration and inoculum source

Although aerobic and anaerobic methanotrophs are ubiquitous and detected in various many natural (sediment in lake or ocean, paddy soil, volcanoes) and artificial ecosystems (WWTPs' sludge) all over the world (Knief, 2015; Wen et al., 2017), their slow growth rates is challenging for practical application in denitrification treatment. The HRT in start-up period should be long enough to accommodate the MDD microorganisms and avoid biomass washout (Harb et al., 2021). Sequencing batch reactor (SBR) was first used to cultivate these microbes because it can retain biomass in a separated settlement phase (Ettwig et al., 2010; Haroon et al., 2013; He et al., 2016; Raghoebarsing et al., 2006). But the batch operation is difficult to scale up and the treatment capacity is limited. Because continuous operation is preferable and

membrane technology has been strongly developing recently, it raises the interest in attached growth reactors, such as downflow hanging sponge (DHS) reactor and membrane bioreactor (MBR) configuration. The DHS reactor packed with porous bio-carrier can retain the biomass and provide large surface area for gas - liquid interaction. One the other hand, membrane's function is not only a filter to eliminate tiny elements from effluent, but a gas diffusor without forming bubble in solution to enhance the solubility of methane gas and a bio-carrier supporting biofilm formation. Table 2.3 shows some previous studies installing SBR, DHS and MBR to facilitate methane-driven denitrification (MDD) and their operational conditions, substrates sources, denitrification performance, and presenting microbial communities.

Since autotrophic microorganisms have relatively lower growth rates than heterotrophic microorganisms, the enrichment in autotrophic nitrogen removal reactors usually takes months (Harb et al., 2021). The inoculum plays a vital role in reactor performance; thus, a common approach is to use a well-established culture as inoculum to shorten the start-up period and/or achieve high performance (Cai et al., 2015; Fu et al., 2017; Shi et al., 2013; Xie et al., 2018). However, the dependence of the enriched inoculum may limit the applicability of this process. Therefore, researchers have been exploring and testing various alternative inocula, such as freshwater and coastal sediments, anaerobic sludge from digesters, activated sludge, and paddy soils (Ding & Zeng, 2021). Among these alternatives, paddy soil has been previously reported to house diverse microbial communities, including methanotrophs, Anammox bacteria, and denitrifiers (Ding et al., 2016; Vaksmaa et al., 2017; Zhou et al., 2014); thus, it has been used as an inoculum in several methane-driven denitrification or Anammox reactors (Hatamoto et al., 2014; He et al., 2015; Xu et al., 2018). Indeed, He, Cai, et al., (2015) reported that paddy soil was the optimal inoculum among the three inocula (methanogenic sludge, paddy soil, and freshwater sediment) considered for their sequencing batch reactor experiment, which enriched nitrite-dependent anaerobic methane-oxidizing bacteria.

Table 2.3 Bioreactor for MDD process application

Inocula	Reactors	Substrates	Nitrogen removal	Microbial community analysis	Ref
Activated sludge from WWTP	MBR with V 86mL (diameter 1.1 cm, length 30 cm); HRT 12h; ~ 25°C; 192 days	NO_3^-:20 ± 1.2 $mgN.L^{-1}$; CH_4: 12.9 ± 0.19 $mg.L^{-1}$ (1.48 atm)	$NO_{3\ eff}^-$ = 3.3 ± 0.98 $mgN.L^{-1}$	Sequencing of 16S rRNA genes (Pro341F/Pro805R): bacteria 85.4% (*Methylocystaceae*-21%); archaea 0.003%	Alrashed et al. (2018)
Parent reactors originated from return activated sludges, sediments from farmland ditch, anaerobic granular sludges treating paper mill wastewater	MBR with V 685mL; HRT 8 days; 200 days	NH_4^+: 800 $mgN.L^{-1}$, NO_3^-: 1200 $mgN.L^{-1}$; CH_4:20 kPa	rNH_4^+ = 85 $mgN.L^{-1}.d^{-1}$, rNO_3^- = 140 $mgN.L^{-1}.d^{-1}$ NO_2^- :ND	Sequencing of 16S rRNA genes (926F/1392R): archaea Ca.Methanoperedens 43.43%, Anammox bacteria 25.03%*, bacteria Ca. Methylomirabilis 2.03% qPCR for quantification of the functional genes (Anammox: hzsA1597F/hzsA1857R, ANME-2d: McrA159F/McrA 345R, NC10: cmo182/cmo568, total bacteria and archaea: 926F/1392R)	Nie et al. (2020)
Parent reactors originated from methanogenic sludge and activated sludge in WWTP	MBR with V 1260mL; HRT 5days; 35°C; 200 days	NH_4^+: 215 $mgN.L^{-1}$, NO_3^-: 428 $mgN.L^{-1}$; CH_4:0.1-0.5 atm	rNH_4^+ = 37.95 $mgN.L^{-1}.d^{-1}$, rNO_3^- = 85.33 $mgN.L^{-1}.d^{-1}$	Sequencing of 16S rRNA genes (341b4F/806R): bacteria 25.7% and archaea ANME-2d 74.3% FISH observation for identification the microbial structure (Anammox: Cy5-AMX-368, ANME-2d: FITC-DARCH-872, NC10: Cy3-DBACT-0193/1027, total archaeal ARCH-915, total bacteria EUBmix)	Fu et al. (2017)
Freshwater lake sediment, paddy soil, and methanogenic	SBR with V 1.5L; exchanged 500mL supernatant every	NO_3^- 50-150 $mgN.L^{-1}$; CH_4 was flushed into the reactors every 2–5 days	rNO_3^- = 21.91 ± 0.73 $mgN.L^{-1}.d^{-1}$, NO_2^-: ND	qPCR for quantification of M. oxyfera bacteria (qP1F-qP1R) and M. nitroreducens archaea (345F-541R) Phylogenetic analysis of M. oxyfera and	Li et al. (2018)

sludge in WWTP	month; 30°C; > 600d			M. nitroreducens via 16S rRNA genes	
Parent reactors (nitrite and nitrate MDD reactors) originated from paddy soil	Two DHS reactors with V_{sponge} 0.12 L inside 1.25 L column; 30°C; HRT 3h; 448d	Phase 1: simultaneously supply NO_3^- 0.7 mM, NO_2^- 0.7 mM; Phase 2: only NO_3^- 1.0 mM; CH_4 supply at 2.0 mL/min	Phase 1: rNO_3^- = 39.0 ± 2.2 $mgNO_3^-N.L^{-1}.d^{-1}$, rNO_2^- = 70.4 ± 7.6 $mgNO_2^-N.L^{-1}.d^{-1}$; Phase 2: 84.4 $mgNO_3^-N.L^{-1}.d^{-1}$	Cloning 16S rRNA and pmoA genes then sequencing and do phylogenetic analysis. NC10 in group b was dominant, not group a as in other enrichment. FISH observation (bacteria: EUBmix; NC10: NC10–1162, DBACT-193, DBACT-447; archaea: ARC344; aerobic methanotrophic bacteria: Mα450, Mγ705): NC10 was 50-70% bacteria; archeae < 5% of DAPI stained cell; alpha-proteobacterial aerobic methane-oxidizing bacteria were also present	Hatamoto et al. (2017)
Parent reactors (contain Anammox and MDD microbes)	MBR with V_{total} 2356 mL; ambient temperature ~20.8 ºC; HRT 4h; 730d	NH_4^+: 22.6 $mgN.L^{-1}$, NO_2^-: 28.9 $mgN.L^{-1}$; CH_4 pressure in lumen 300kPa	$NO_{2\ eff}^-$ = 0.20 ± 0.03 $mgN.L^{-1}$, $NH_{4\ eff}^+$ = 0.43 ± 0.08 $mgN.L^{-1}$, $NO_{2\ eff}^-$ = 2.82 ± 0.19 $mgN.L^{-1}$, rTN = 280 $mg.L^{-1}.d^{-1}$; MDD archaea used 30-60% nitrate produced by the anammox reaction; MDD bacteria used < 10% and Anammox bacteria used > 90% NO_2^-	Sequencing of 16S rRNA (universa;l primer 926F and 1392R): NC10 sequences belonged to family *Methylomirabiliaceae* (18%), archaea sequence belonged to family *Methanoperedenaceae* (3%), anammox bacteria is *Brocadiae* (3%); FISH observation (NC10: S-*-NC10-1162-a-A-18, Anammox: S-*-Amx-820-a-A-18, MDD archaea: S-*-Darc-872-a-A-18)	Xie et al. (2018)

ND: not detectable
* calculated from given data

2.4. Next generation sequencing for investigating microbial dynamics

2.4.1. Advancement of metagenomic technologies

Molecular analysis has played a distinguished role in the elucidation of MDD process and community since the early years. There are two main approaches for microbial dynamics investigation: one is detected MDD microorganisms using their specific probes and primers (i.e., FISH and qPCR) designed based on 16S rRNA gene and functional genes' sequences, such as pmoA (particulate methane monooxygenase), mcrA (methyl-coenzyme M reductase) genes; another one is detected MDD microorganisms in coexistence with other microorganisms by genome sequencing. The advent of metagenomics in the 21st century brings us a powerful tool to understand the microbial profile of many environmental samples without culture-dependent analysis.

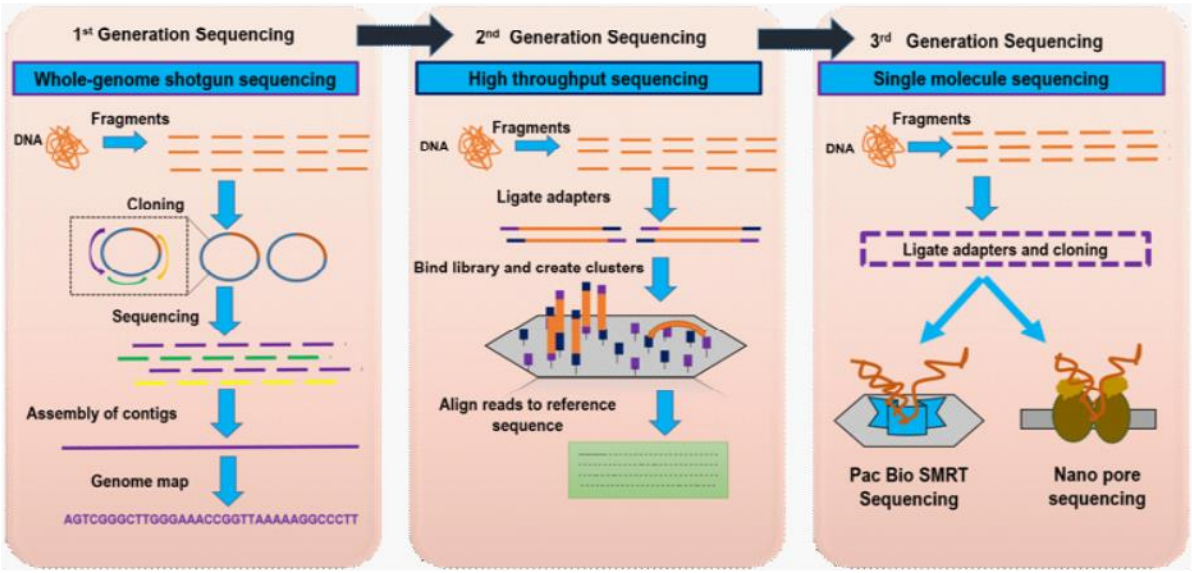

Fig. 2.6 Advancements in microbial genome sequencing technologies (Ali et al. 2018)

There are three major revolutions in sequencing technologies: the first-generation sequencing (whole genome shotgun sequencing), the second-generation sequencing (high throughput sequencing), and the third-generation of sequencing (single molecule long read sequencing). Fig. 2.5 shows these three advancements from Ali et al. (2018): whole genome shotgun sequencing requires laborious sample preparation; high throughput sequencing (or

next generation sequencing-NGS) gives high accuracy but short read lengths; while single molecule sequencing gives low accuracy with long read lengths.

2.4.2. Application of NGS to have an insight into microbial functionality

Stable isotope probing (SIP), tracking the availability of heavy stable isotopes as biomarkers, such as ^{13}C, ^{15}N, ^{18}O, ^{2}H, is widely used approaches at the moment to get a direct insight into metabolic pathways. However, the scarcity and high cost of labeled substrates are the major drawback of this approach. Besides, considering current techniques and infrastructure, SIP is still labor-intensive and low throughput (Uhlik et al., 2013). Therefore, other more efficient methods are in demand. As mentioned in section 2.4.1, NGS providing huge amount of data with high accuracy and combining with the support from bioinformatics tools is a promising approach. The output data of NGS can be analyzed and visualized via various online genome browsers and open-source software (Ali et al., 2018), which gives researcher a tool for hypothesis-making and even prove assumptions in some extent. For example, PICRUSt2 (phylogenetic investigation of communities by reconstruction of unobserved states) is a computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes (Douglas et al., 2020).

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CHAPTER 3. ANAEROBIC BAFFLED REACTOR IN TREATMENT OF NATURAL RUBBER PROCESSING WASTEWATER

3.1. Introduction

Natural rubber manufacturing industry have an adverse effect on the environment by discharging a large amount of high-strength wastewater. This wastewater may primarily pollute water bodies with high concentrations of ammonia, organic matters, and residual rubber particles; and secondarily pollute the surrounding air with volatile fatty acids (VFA) from organic degradation. Considering these pollution risks, anaerobic digestion is an optimal wastewater treatment due to its great tolerance of high-strength industrial wastewater and good control of odor emission. Many reactor modifications have been developed to adapt with a wide range of industrial wastewater in full-scale (van Lier et al., 2016). The advantages of anaerobic sludge reactor over the activated sludge process are listed as low set up and operational costs, energy recovery via methane production, low production of excess sludge, and no requirement for high-tech equipment. Conventional WWTPs for natural rubber processing wastewater in Vietnam also include anaerobic digestion, but mainly an upflow anaerobic sludge blanket (UASB) reactor (Nguyen & Luong, 2012). Although the UASB reactor has demonstrated excellent performance and stability for numerous full-scale operations worldwide, it needs pre-treatment for wastewater containing high concentration of suspended solid (Aiyuk et al., 2010). A considerable amount of residual rubber particles in natural rubber processing wastewater is usually removed by rubber trap prevent it from accumulating and clogging pipes and machinery during long-term operation (Nguyen et al., 2016; Tanikawa, 2017; Watari et al., 2015; Watari et al., 2017b). Additionally, single-configuration reactors need a long start-up period to form the granular sludge, which has higher retention capacity then flocculant sludge, thus stable performance. Therefore, an efficient alternative anaerobic technology that can overcome these drawbacks is still required.

Barber and Stuckey (1999) have introduced anaerobic baffled reactor (ABR) with a wide range of applications and modifications, which can be a promising alternative to conventional single-configuration reactors. ABR is a modified anaerobic sludge bed reactor, which contains vertical baffles dividing the reactors into a series of upflow units without internal gas–liquid–solid separation devices. These baffles create plug flows in the waterline by permitting the liquid to flow under and over these baffles, thereby decreasing the risk of clogging machinery parts or connecting pipes. The reactor has a high void volume, which enhances the solid retention time; thus, no post-treatment for clarifying is required. Moreover, the compartmentalization may promote phase separation (hydrolysis, acidogenesis and methanogenesis) longitudinally down the reactor. There have been studies on the use of ABR for the treatment of natural rubber processing wastewater in laboratory-scale (Saritpongteeraka and Chaiprapat, 2008; Watari et al., 2017b) and pilot-scale (Tanikawa et al., 2016b; Watari et al., 2017a). Different profiles of the wastewater discharged from natural rubber processing are observed because of the chemical used during the coagulation of rubber latex, for example, sulfuric acid (Saritpongteeraka and Chaiprapat, 2008; Tanikawa et al., 2016a) or organic acids (Nguyen et al., 2016; Watari et al., 2017b) in Thailand or Vietnam, respectively. As a result, ABR treated natural rubber processing wastewater in each country exhibits different performance and characteristics. Besides, few studies have reported the microbial communities in the retained sludge from the individual compartments in the ABR, particularly in the ABR-treated this wastewater.

Therefore, this study aims to investigate the microbial communities present in the different compartments of the ABR using metagenomics technology and evaluate the tolerance of a laboratory-scale ABR under a high organic loading rate (OLR) with the stepwise increase in the chemical oxygen demand (COD) concentration of the influent. This chapter gave an

example of anaerobic reactor applied in highly polluted wastewater treatment to recover methane-rich biogas as renewable resources.

3.2. Materials and methods

3.2.1. Wastewater preparation

Raw natural rubber processing wastewater was collected by the laboratory-scale coagulation of concentrated rubber latex using acetic acid following the coagulation method used in an actual natural rubber processing factory in North Vietnam. The characteristics of this raw wastewater were analyzed soon after the sample collection, which were a pH of 4.9 ± 0.1, a total COD of 19,200 ± 1,100 $mg.L^{-1}$, a total biochemical oxygen demand (BOD) of 12,600 ± 2,000 $mg.L^{-1}$, a total suspended solid (TSS) of 108 ± 74 $mg.L^{-1}$, and a total nitrogen (TN) of 1,960 ± 490 $mgN.L^{-1}$. The influent consisted of raw wastewater diluted to the desired COD concentration by tap water, was added continually to the influent tank.

3.2.2. Setup and operation of the ABR

This study was performed in a ten-compartment ABR. This ABR was made of polyvinyl chloride (PVC) pipes (diameter of 110 mm and height of 1 m) and a working volume of 68 L. The 2nd to 10th compartments were covered by PVC lids to maintain anaerobic conditions, except for the first compartment, which was used to feed the influent. The 3rd to 10th compartments of the ABR were equally inoculated with a total of 20 L of sludge collected from a household biogas system treating livestock manure. The 1st and 2nd compartments were used as a rubber trap; thus, these compartments were not inoculated. The influent was continually made, stored in the influent tank, and fed into the reactor by a variable-speed peristaltic pump

(Masterflex® L/S, Vernon Hills, USA). Biogas was collected via a network of pipes leading to a gas meter. Fig. 3.1 shows the schematic diagram of the whole system.

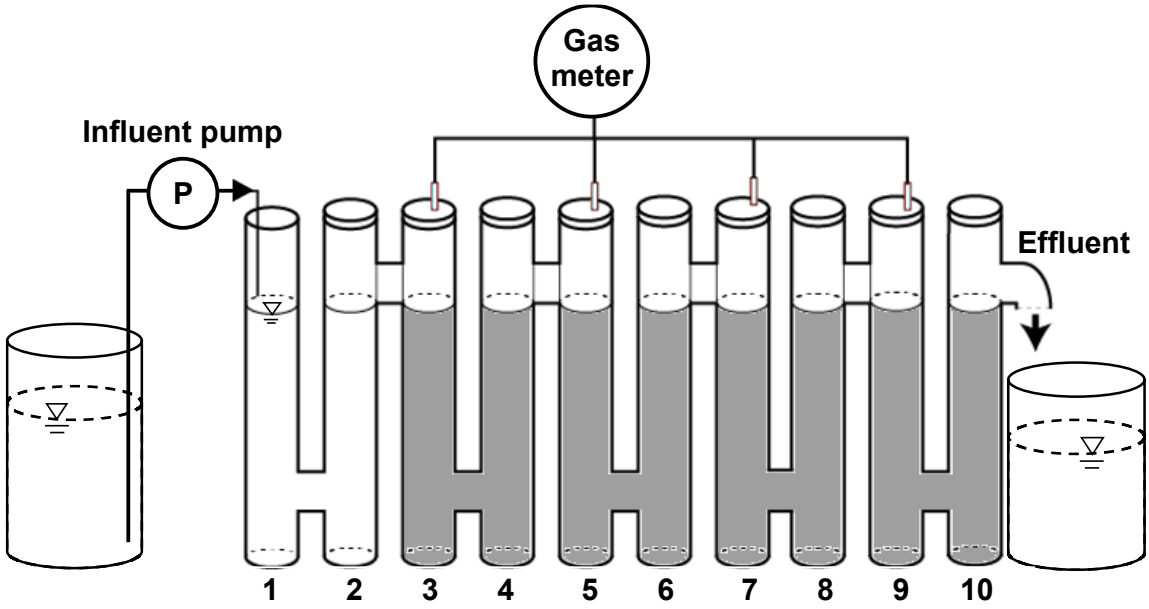

Fig. 3.1 The schematic diagram of the ten-compartment ABR in this study

The ABR reactor was operated at an ambient temperature of 27.1 ± 4.7°C for 224 days, divided into three phases (Table 3.1). In order to acclimate the sludge to this wastewater, the reactor was run with the stepwise increase in the COD influent during start-up period (phase 1). After the acclimatization period, the reactor's efficiency was evaluated in the next two phase 2 and phase 3. The hydraulic retention time (HRT) was calculated on the basis of the working volume and flow rate. The COD influent was increased in a stepwise manner; thus, the OLR increased during three phases of the experiment.

Table 3.1 Operational conditions during three phases operation of the ABR

Parameters	**Unit**	**Phase**		
		1	2	3
Time period	days	83 (1st - 83rd)	60 (84th - 143rd)	81 (144th - 224th)
Flow rate	$L.d^{-1}$	20.3 ± 5.2	19.4 ± 3.3	15.9 ± 2.2
HRT	days	3.2 ± 1.0	3.2 ± 0.7	3.9 ± 0.6
OLR	$kgCOD.m^{-3}.d^{-1}$	1.1 ± 0.3	1.4 ± 0.3	2.1 ± 0.1

3.2.3. Analytical methods

The wastewater was sampled and the pH, COD, BOD, TSS, VFA, and TN were analyzed. pH was measured using a portable pH meter (B-712, Horiba, Kyoto, Japan). The total COD and TN were measured by the HACH method using a spectrophotometer (DR-2800, HACH, Colorado, USA). BOD and SS were measured by standard methods (American Public Health Association-APHA, 2012). Samples were filtered using 0.4 µm glass fiber filter paper (Advantec GB-140, Vernon Hills, USA) prior to the determination of the VFA concentrations by high-performance liquid chromatography (L-2000, Hitachi, Tokyo, Japan). The amount of the produced biogas was calculated on the basis of the volume changes using a wet gas meter (WS-1A, Shinagawa, Tokyo, Japan) over a period of time. The biogas composition was analyzed by a gas chromatograph equipped with a thermal conductivity detector (GC-8A, Shimadzu, Kyoto, Japan). The performance of the ABR was evaluated by determination of removal efficiencies of the total COD, TSS and produced biogas, particularly methane.

3.2.4. Massively parallel sequencing of 16S rRNA genes

Illumina high-throughput sequencing of the 16S rRNA genes was employed to analyze the microbial community in the retained sludge from the bottom of each of the ABR compartments on the 143rd day. The sludge samples were gently washed with phosphate buffered saline and stored at -20°C until DNA extraction. Total genomic DNAs extraction from all samples was performed using a FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, USA) following the manufacturer's instructions. The universal primers for bacteria and archaea, Univ515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and Univ806R (5′-GGA CTA CHV GGG TWT CTA AT-3′), and the PCR solution Premix Ex Taq Hot Start (Takara Bio, Otsu, Japan) were used to amplify the V4 region of 16S rRNA genes from the extracted DNA (Caporaso et al., 2012). PCR fragments were purified from primers, nucleotides, polymerases, and salts using a MinElute PCR purification kit (Qiagen, Hilden, Germany) according to the

manufacturer's specifications. After normalizing purified PCR products to a DNA concentration of 0.5 ng.μL^{-1}, they were used as templates for pair-end sequencing by MiSeq system (Illumina, Inc., San Diego, California, USA) with MiSeq Reagent Kit v2.

Microbial analysis based on the 16S rRNA sequencing data was carried out using the QIIME software package v.1.9.1 (Caporaso et al., 2010). The input sequences with over 97% identity were clustered into one operational taxonomic unit (OTU), with chimeric sequences removed using ChimeraSlayer. Taxonomic classification was based on the Greengenes Database v.13_8. The closest relative genera of the unclassified sequences were identified by the nucleotide BLAST search in the NCBI database. Correlations among different communities were analyzed by principal component analysis (PCA) and visualized by STAMP software (Parks et al., 2014).

3.3. Results and discussion

3.3.1. Reactor Performance

The ABR was operated in three phases in different OLRs for 224 days (Table 3.1). Fig. 3.2 shows the influent and effluent concentrations of total COD and TSS, as well as removal efficiencies of the ABR. The reactor was started up with an influent COD of 3,420 ± 660 mg.L^{-1}, the COD removal efficiency was gradually improved and reached 92.4% on the 72nd day. However, the overall COD removal efficiency during phase 1 was low at 56.2 ± 18.5%, leading to an effluent COD concentration of 1,500 ± 620 mg.L^{-1} under an OLR of 1.1 ± 0.3 kgCOD.m^{-3}.d^{-1}. After phase 1, this reactor exhibited higher tolerance for the COD pollutant compared with that reported in a previous study for the treatment of concentrated rubber latex wastewater using an ABR under the same OLR. A COD removal efficiency of 92.3 ± 6.3% and a COD effluent concentration of 311 ± 218 mg.L^{-1} were observed during phase 2 under an OLR of 1.4 ± 0.3 kgCOD.m^{-3}.d^{-1}. On the other hand, in the previous study, only 75.2 - 75.7% of COD is

removed under an OLR of 1.16 kgCOD.m^{-3}.d^{-1} (Saritpongteeraka and Chaiprapat, 2008). The high removal efficiency in our study is related to the higher number of compartments, facilitating the contact between the anaerobic microbial consortium, overcoming the low pH of the influent, and utilizing more COD pollutants without adjusting the pH using NaOH or parawood ash as used in the previous study (Saritpongteeraka and Chaiprapat, 2008).

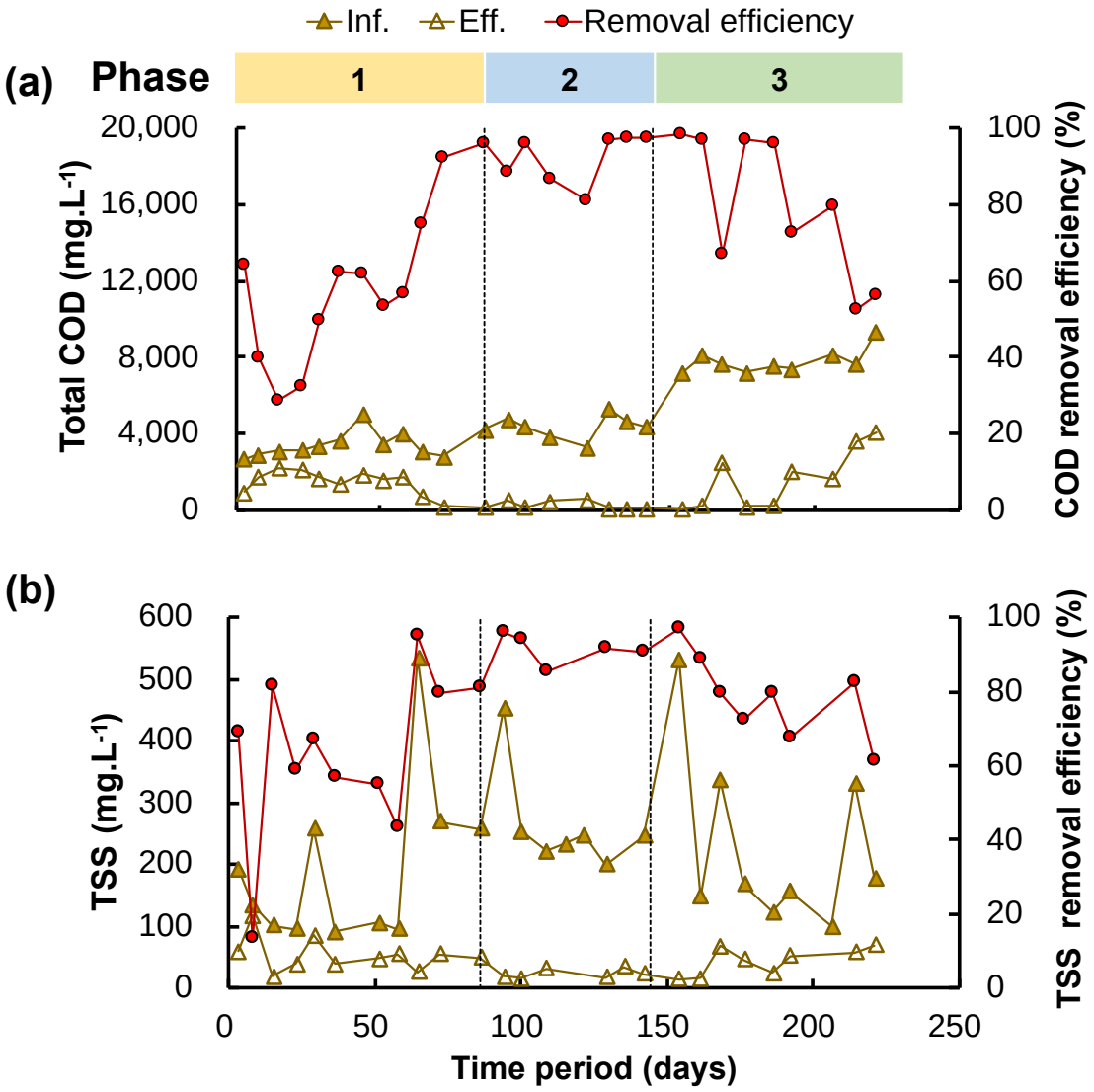

Fig. 3.2 The ABR's performance in total COD removal (a) and TSS removal (b)

Recently, UASB and ABR are the frequently used anaerobic process for the treatment of wastewater from natural rubber processing. Some laboratory-scale UASB reactors achieved a high organic removal efficiency and a high methane recovery rate (Nguyen et al., 2016; Watari

et al., 2015). However, the pilot-scale UASB reactor requires the pretreatment of wastewater because raw wastewater contains high concentrations of sulfate or residual natural rubber particles (Tanikawa et al., 2016a; Watari et al., 2017b). Tanikawa et al. (2016a) have used a pilot-scale UASB reactor following the rubber trap to treat the wastewater containing high sulfate from natural rubber processing and reported a total COD removal efficiency of 72.6 ± 3.9% under an OLR of 1.5 $kgCOD.m^{-3}.d^{-1}$. In addition, total COD and BOD removal efficiencies of 55.6 ± 16.6% and 77.8 ± 10.3%, respectively, were obtained after the removal of the residual natural rubber particles from the pilot-scale UASB reactor for the treatment of natural rubber wastewater under an OLR of 1.7 ± 0.6 $kgCOD.m^{-3}.d^{-1}$ (Watari et al., 2017b). Residual rubber hindered the scale-up operation of UASB reactors for this wastewater treatment. Previously, baffled reactors without sludge have been used as a rubber trap for the pretreatment of rubber wastewater (Tanikawa et al., 2016a; Watari et al., 2015). Therefore, the number of compartments in our ABR was increased and the back compartments were inoculated with sludge for exploiting its characteristics as a rubber trap in the two first compartments, as well as the role of anaerobic treatment in the latter compartments. During phase 1, the wash-out sludge lead to high TSS concentration in the effluent; thus, a low TSS removal efficiency of 62.0 ± 22.8% was obtained. The reactor exhibited good performance for TSS removal, with an efficiency of 90.0 ± 6.0% and a TSS effluent concentration of 27 ± 12 $mg.L^{-1}$ during phase 2. Fig. 3.2 shows the rubber particles accumulated on the surface of the 1st compartment at the end of the experiment. Particulate rubber was not removed through the experiment, then gradually accumulated into a thick scum layer, which may cover the liquid from exposure to air, leading to anaerobic condition in these compartments. The capacity of the ABR for TSS removal was greater than that of the UASB reactor. Nguyen et al. (2016) have reported the influent and effluent TSS concentration of 279 ± 128 $mg.L^{-1}$ and 72.4 ± 58.5 $mg.L^{-1}$, respectively, using the UASB reactor for treating wastewater. On the other hand, the influent

and effluent TSS concentration of 225 ± 125 $mg.L^{-1}$ and 43 ± 25 $mg.L^{-1}$, respectively, were obtained for the ABR in this study.

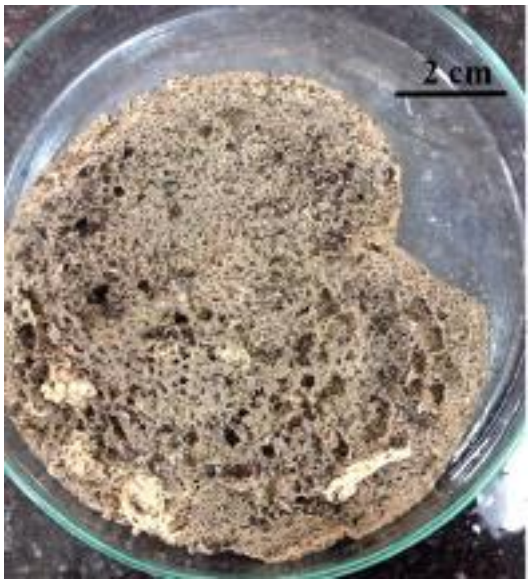

Fig. 3.3 A thick layer of rubber accumulated on the surface of the 1st compartment at the end of our experiment

The biogas produced from ABR during phase 2 consisted of 73.7 ± 5.1% methane, 23.8 ± 5.5% carbon dioxide, and 2.5 ± 2.4% nitrogen. The methane recovery ratio based on the total COD removal was 52.4 ± 33.6% during phase 2, while the removal efficiency for total COD in the form of methane improved to 81.3 ± 14.3% using the UASB reactor treating the same type of wastewater (Nguyen et al., 2016). The maximum methane gas production of 29.8 $NL.d^{-1}$ was observed on day 177th. Different operational conditions, such as longer HRT and an uncontrolled temperature, as well as construction specifications (Nelting et al., 2015) with high number of compartments and gas ports in the ABR, may lead to the low methane recovery ratio in our study. On the other hand, some parts of the total COD in the influent comprised residual rubber particles accumulated in the reactor, not degradable COD; thus, the methane recovery ratio was low.

With the increase in the OLR of up to 2.1 ± 0.1 $kgCOD.m^{-3}.d^{-1}$, the process performance of ABR deteriorated. During phase 3, the influent and effluent COD values were 7,890 ± 680

mgCOD.L^{-1} and 1,840 ± 1,520 mgCOD.L^{-1}, respectively. At the end of phase 3, the reactor reached its tolerance limit of OLR, and foam was observed on the water surface of the reactor in the 7th, 8th, and 9th compartments. The COD removal efficiency and methane recovery ratio of the ABR significantly decreased to 57% and 20%, respectively. In addition, when foam observed in the final compartments was used as a clarifier, the TSS removal efficiency decreased to 60%. These results indicated that the acceptable maximal OLR of this wastewater should be between 1.4 to 2.1 kgCOD.m^{-3}.d^{-1} for the ABR operation.

On the one hand, only anaerobic treatment stage could not completely convert high concentration of ammonia in this wastewater, and the TN removal efficiency was less than 23% (data not shown). These results indicated that although the ABR exhibits better ability for the total COD and TSS removal compared to the UASB reactor, further post-treatment is required to satisfy the industrial standards for the TN and COD.

3.3.2. Variation of VFA in each compartment

Fig. 3.4 shows the concentration of VFA in the liquid phase from all of the compartments analyzed on the 103rd day (phase 2) and 199th day (phase 3). Acetic acid was used in the coagulation process; hence, almost 80% of the soluble COD is acetate. The concentrations of propionate, as well as acetate in particular, longitudinally decreased down the reactor. On the 103rd day, acetate, a key intermediate product in methane digestion, significantly decreased in the 3rd and 4th compartments (Fig. 3.4a). Hence, methanogen was possibly dominant in these compartments and it used acetate to produce biogas. This result indicated that, in an ABR, different microorganisms are developed in different compartments, leading to phase separation.

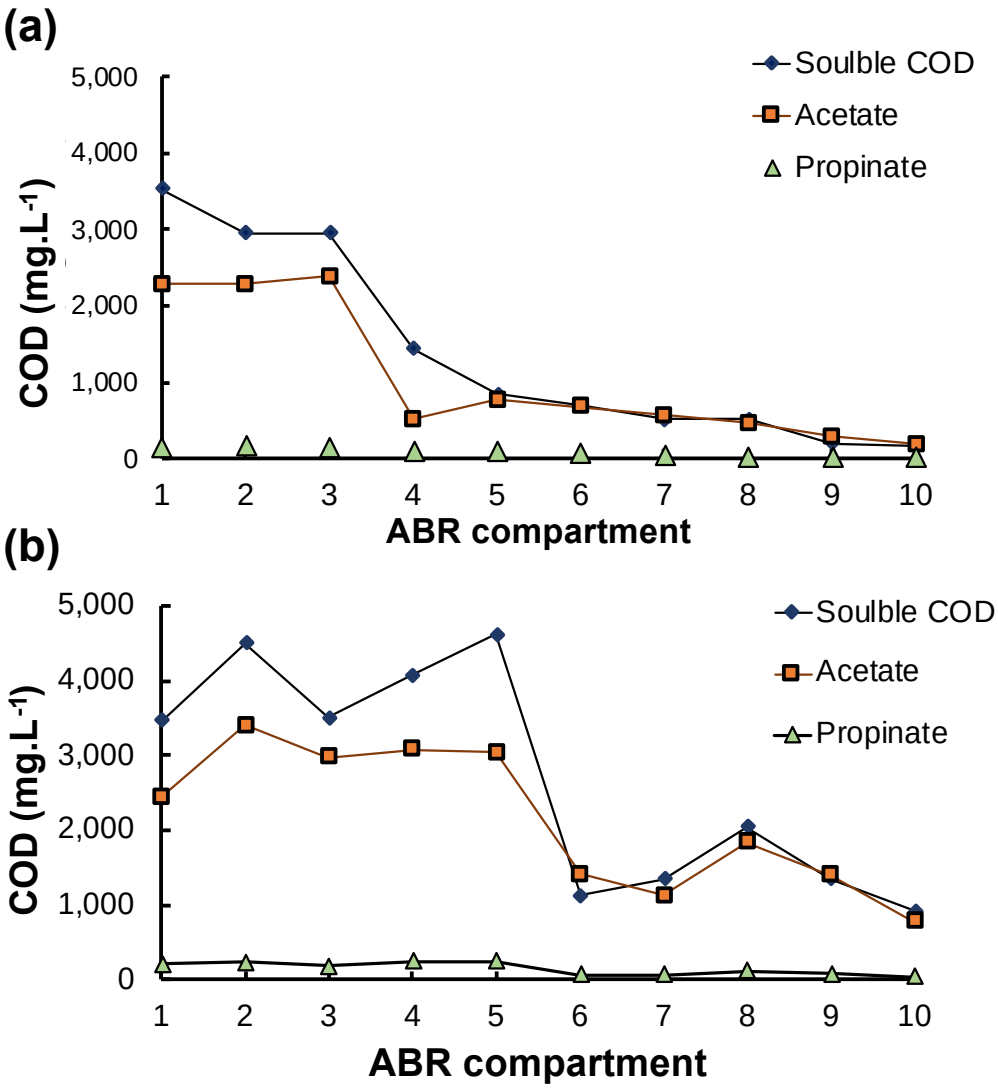

Fig. 3.4 The VFA **c**ompositions in each ABR compartment on the 103th day (a) and 199th day (b)

Under high OLR operation during phase 3, the acidification of wastewater occurred in the 1st to 5th compartments. The highest acetate concentration was observed in the 2nd compartment. Watari et al. (2015) have reported increased concentrations of acetate and propionate in the baffled reactor treating wastewater discharged from natural rubber processing because of the acidification by the accumulated sludge. The removal of acetate was clearly observed clearly in the 5th and 6th compartments on this day. In the 6th and 7th compartments, the acidification of wastewater was observed again and acetate was accumulated in the 8th, 9th, and 10th compartments (Fig. 3.4b). The accumulation of acetate has been reported to cause

foaming (Ganidi et al., 2009). Thus, the accumulation of acetate leaded to the low COD removal efficiency and forming in our ABR during high OLR operation.

3.3.3. Microbial community structure in the ABR

The microbial community structure in the ABR-retained sludge in each compartment on the 143rd day was analyzed by 16S rRNA gene sequencing using Illumina MiSeq. A total of 190,260 sequence reads were determined, and median sequence length of the 16S rRNA genes was 251 bp. Approximately 12,000 - 28,000 sequence reads per sample were analyzed, and 523 - 369 OTUs per sample were found at 97% identity. Fig. 3.5 shows the predominant phyla, as well as dominant genera belonging to each phylum in the ABR-retained sludge on the 143rd day. The principle microbial groups in the ABR-retained sludge were the bacterial phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Chloroflexi*, and archaeal phylum *Euryarchaeota*. These phyla have been frequently reported in mesophilic methanogenic sludge (Narihiro et al., 2009). The content of these phyla in each sample was different, leading to the division into three groups as shown in Fig. 3.6 by PCA analysis. After the 143-day operation, there was shifts in the microbial community structure in the retained sludge in the first two compartments (labeled as 1 and 2 in group I) and that in the 3rd, 4th, and 5th compartments (labeled as 3, 4, and 5 in group II) and that in the last compartments (labeled as 6 to 10 in groups III). The sludge in group I was derived from the accumulated TSS in the influent, while the sludge in the remaining compartments was inoculated with the anaerobic seed sludge. The detection rate of the phylum *Chloroflexi* found in the groups II and III were 6.75 - 14.20%, while that in group I was only 0.06 - 0.25%. On the other hand, although the phylum *Euryarchaeota*, comprising all of the known methanogens, was detected in groups II and III, the detection rates varied at a rate of greater than 10% in the 3rd, 4th, and 5th compartments (group II) and less than 6.4% in the 6th to 10th compartments (group III). The high concentration of acetate in the front compartments may promote the growth of acetate-utilizing

methanogens in the seed sludge to produce methane in group II, as well as the acetate-utilizing methanogens to gain biomass, but not produce methane accumulated in group I.

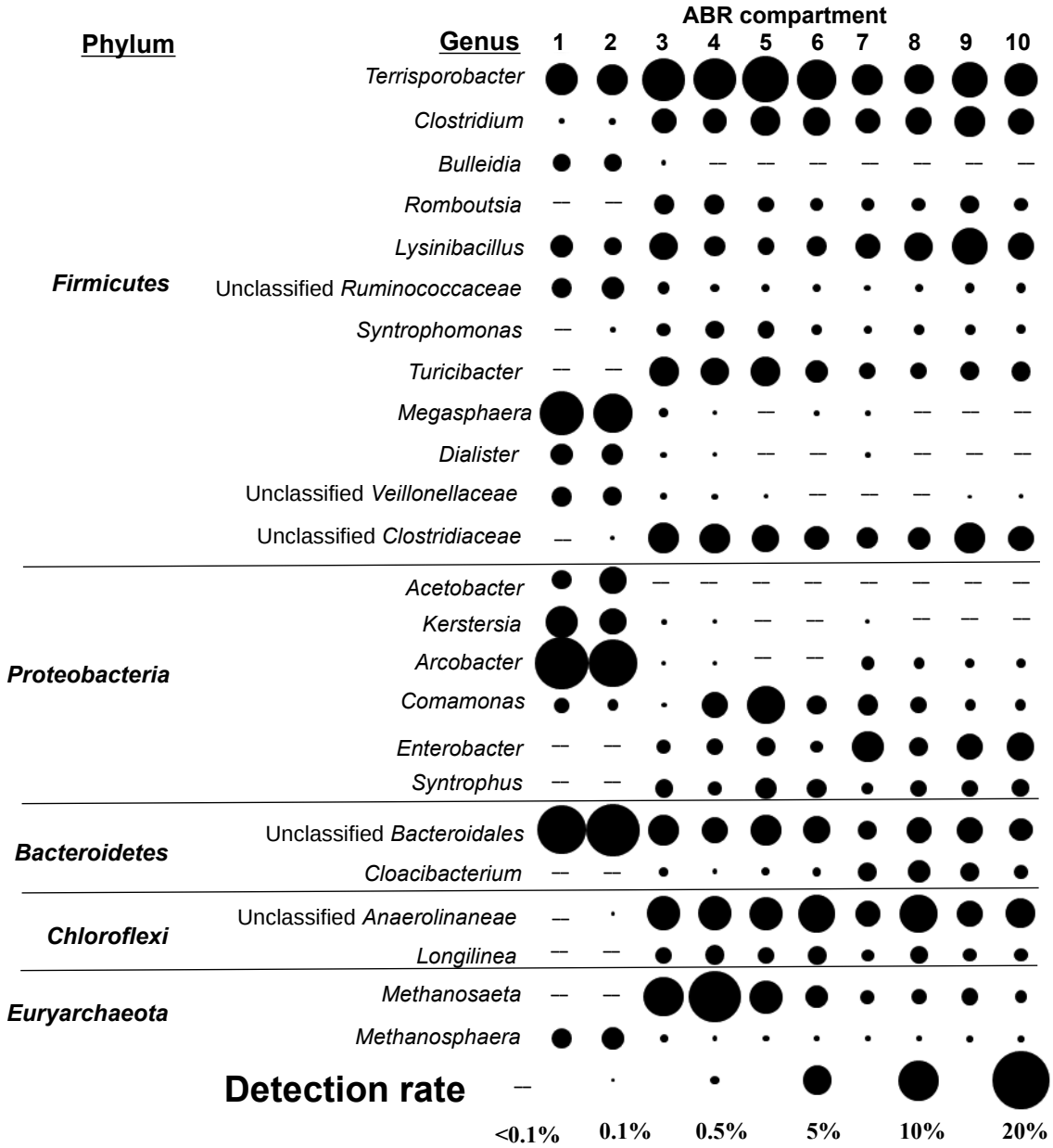

Fig. 3.5 Predominant genera in the ABR's sludge on the 143rd day

Differences in the sludge inoculation, COD concentration and influent pH led to the variation in the microbial detection rate in each group. With respect to bacteria, the genera *Bulleidia, Megasphaera, Dialister,* unclassified *Ruminococcaceae,* and unclassified *Veillonellaceae* belonging to the phylum *Firmcutes*; genera *Acetobacter, Kerstersia*, and

Arcobacter belonging to the phylum *Proteobacteria*; and unclassified *Bacteroidales*, belonging to the phylum *Bacteroidetes*, were more abundant in group I than in groups II and III (Fig. 3.6). *Bulleidia* and *Dialister*, which play a role during hydrolysis, have been reported to be predominant in the first chamber of the ABR (Gulhane et al., 2017). *Megasphaera* was mainly detected in group I, under an optimal pH of 4.1 - 4.5 (Juvonen and Suihko, 2006). *Bacteroidetes* exhibits cellulolytic, hemic-cellulolytic, and proteolytic properties, which are responsible for the initial degradation of organic substances into soluble products (Ariesyady et al., 2007). *Arcobacter*, which grows under microaerophilic conditions, is a facultative anaerobic bacterium that reduces nitrate to nitrite (Saia et al., 2016). In addition, Tanikawa et al. (2016b) have reported that ammonia was oxidized to nitrate and nitrite at the surface of an open-type anaerobic baffled lagoon. Therefore, ammonia in the wastewater can be oxidized or retained in the influent and utilized by *Arcobacter*. The genera *Kerstersia* and *Acetobacter*, belonging to the phylum *Proteobacteria* were mainly found in group I. *Kersteria* and *Acetobacter* were previously found under aerobic conditions, indicating that oxygen was still present in the 1st and 2rd compartments. The dominant genus in group I seemed to be well-adapted to low pH, aerobic or anoxic conditions, and abled to degrade complex compounds, while those in groups II and III were likely to adapt to anaerobic conditions and participate in the treatment process as acetogen, hence partner with methanogens. The genera *Terrisporobacter*, *Turicibacter*, and *Clostridium* belonging to the phylum *Firmcutes*, known as acetogens, were predominant in the 3rd and 4th compartments. Several species of *Clostridium* can grow at a pH of 5.0, and syntrophic bacteria oxidize acetate to H_2 and CO_2 (Valdez-Vazquez and Poggi-Varaldo, 2009). Besides, considerable concentrations of VFA-oxidizing bacteria, for example, *Syntrophomonas* and *Syntrophus* were detected in the 3rd, 4th, and 5th compartments (group II). Strictly anaerobic bacteria, such as the genera *Romboutsia* and *Longilinea*, were also detected in groups II and III.

In archaea, the detection rate of the genus *Methanosphaera* was 2.56 - 3.31% in group I, while that in the other groups was only 0.19 - 0.31%. Besides the high concentration of acetate in the influent, the presence of acetate-oxidizing *Acetobacter* and *Clostridium* producing carbon dioxide served as carbon sources for this genus to gain biomass but not produce methane (Miller and Wolin, 1985). The 3rd to 10th compartments were inoculated with seed sludge, leading to a large percentage of the archaeal detection rate, as well as more various archaeal genera. Acetate-utilizing *Methanosaeta* was the most predominant methanogen in the 3rd, 4th, and 5th compartments with abundance of 9.8%, 16.4% and 6.8%, respectively. Narihiro et al. (2009) have reported that *Methanosaeta*, with good removal efficiencies for acetate and propionate, has been frequently detected in the reactor. According to water quality profile, acetate from the coagulation process was significantly removed between the 3rd and 4th compartments (Fig. 3.4a). The proliferation of *Methanosaeta* in group II showed correlation between the microbiomes and the degradable COD in terms of the VFA variation and gas production. Although, among acetoclastic methanogens *Methanosarcina* typically predominates over *Methanosaeta* at high acetate concentrations because of their considerably higher maximum specific utilization rate (Mussati et al., 2005), *Methanosaeta* predominated over *Methanosarcina* in the sludge of the anaerobic reactors treating rubber wastewater (Nguyen et al., 2016; Tanikawa et al., 2016b; Watari et al., 2015; Watari et al., 2017b). The result from the relative rates between these two methanogens in this study is in good agreement with those reported previously. *Methanosaeta* was predominant, while the detection rates of *Methanosarcina* were low (0.08 - 0.46%). In addition, as a result of the acetogenesis by the acetogenic bacteria mentioned above, hydrogen is released, thus creating a favorable condition for hydrotrophic methanogens, including genera *Methanobacterium* and *Methanomassiliicoccus*. These genera were detected at the total rate of 0.78 - 2.21% in the 3rd to 10th compartments (data not shown).

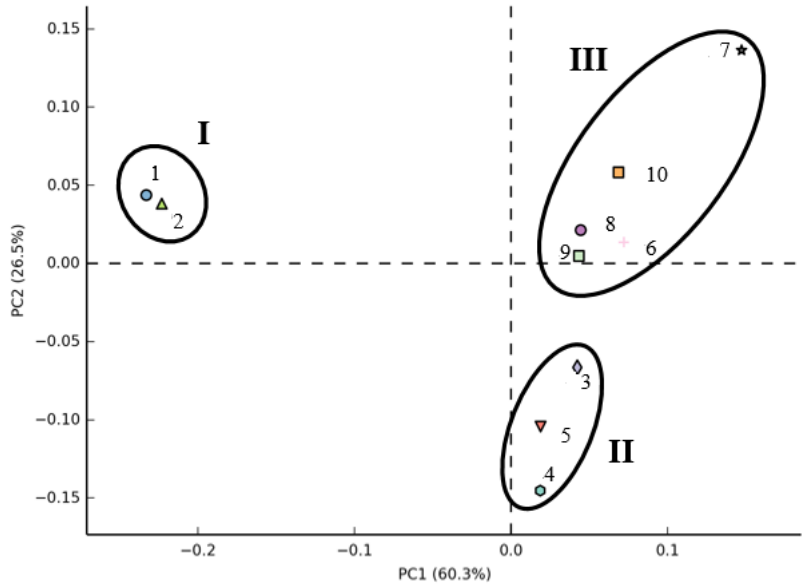

Fig. 3.6 PCA of the microbial community in different compartments of the ABR

3.4. Conclusions

In this study, ABR treating natural rubber processing wastewater operated best under OLR of approximately 1.4 kgCOD.m^{-3}.d^{-1} with COD removal efficiency and TSS removal efficiency of 92.3 ± 6.3% and 90.0 ± 6.0%, respectively. In addition, methane recovery ratio of 52.4 ± 33.6% was achieved. VFA analysis indicated that the middle compartments (3rd to 5th) play the main role in organic digestion, while the first two and last five compartments serve as rubber trap and clarifier, respectively. The inoculation of the seed sludge and the low pH of influent led to the shift in the content of the dominant phylum in three groups, corresponding to the VFA reduction and gas production. Several types of acetogens growing under low pH and ammonia-utilizing bacteria were detected in group I. Group II contained the highest amount of methanogens, particularly *Methanosaeta*, and VFA-oxidizing bacteria, which degraded almost the soluble COD, and then converted it into methane. These results demonstrated the potential of the ABR for the treatment of high-strength wastewater. However, a further posttreatment system is required to satisfy regulated total nitrogen concentration.

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CHAPTER 4. EVALUATION OF AUTOTROPHIC NITROGEN REMOVAL AND NITROUS OXIDE EMISSION IN AN ANAEROBIC DOWNFLOW HANGING SPONGE REACTOR

4.1. Introduction

Anthropogenic activities, such as the agriculture, aquaculture, and chemical industry, has been increasingly discharging dissolved inorganic nitrogenous (DIN) compounds (primarily NO_3^-, NO_2^-, and NH_4^+) to waterbodies. For example, China, the biggest producer and consumer of reactive nitrogen (Nr), was predicted to create 98.4 Tg $Nr.y^{-1}$ in 2050, double than that in 2010, in the forms of synthetic fertilizers, industrial goods, and fossil fuels. Meanwhile, only approximately 39% and 2% of the total Nr input in China in 2010 were finally released through denitrification in forms of N_2 and N_2O, respectively; furthermore, approximately 9% of the Nr was lost via water in 2010 (Gu et al., 2015). Nitrogen pollution is a challenge not only for developing countries, but for developed countries, e.g., the United States, where 71% of anthropogenic Nr leakage ended up in surface freshwater, groundwater, and coastal zones (Sobota et al., 2015). The DIN compounds presenting in the ecosystem, especially in groundwater, cause human health issues (e.g., blue baby syndrome caused by excess NO_3^- in drinking water), and environmental problems (e.g., eutrophication in freshwater). Because biological treatment is inexpensive, effective, and eco-friendly, many efforts have been put into enhancing the biological nitrogen removal processes, especially the denitrification processes, to convert DIN compounds to nitrogen gas. However, the conventional complete microbial process for DIN removal consists of aerobic autotrophic nitrification and subsequent anaerobic heterotrophic denitrification. The limitation of this pathway is its opposite requirements in each process. In the last few decades, Anammox and methane-driven denitrification (MDD) were discovered and combined as an efficient alternative for complete DIN removal. They possess remarkable advantages over the conventional microbial process,

such as co-occur in an oxygen-depleted environment, using methane gas instead of organic carbon substrate (e.g., methanol, acetic acid, starch), shortcuts of nitrous oxide gas emission, and producing less sludge (Kampschreur et al., 2009; Wang et al., 2014). In addition, methane gas (CH_4) is a promising electron donor for denitrification because it is on-site accessible in wastewater treatment plants (WWTPs), easy to strip from treated wastewater, and nontoxic for human health.

In this study, the focus was on improving the MDD processes residing in ANME-2d archaea and NC10 bacteria. These processes utilize methane gas to convert NO_3^- and NO_2^- to nitrogen gas following Eq. (4.1) from Caldwell et al. (2008) and Eq. (4.2) from (Ettwig et al., 2010), respectively.

$$CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^- + 2H_2O \ (\Delta G^o = -503.4 \text{ kJ.mol}^{-1} \ CH_4) \quad (4.1)$$

$$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O \ (\Delta G^o = -928 \text{ kJ.mol}^{-1} \ CH_4) \quad (4.2)$$

Because of the poor solubility of CH_4 in water and the slow growth rates of MDD microorganisms, which are reported at 1-2 weeks for *Candidatus Methylomirabilis oxyfera* (belong to NC10 bacteria – MDD bacteria) (Ettwig et al., 2010) and 1-2 months for *Candidatus Methanoperedens nitroreducens* (belong to ANME-2d – MDD archaea) (Vaksmaa et al., 2017), the use of a sequencing batch reactor (SBR) has been common in early studies. Its sequenced stages for methane purging and biomass settling extend the treatment time in the case of high nitrogen pollutants and eliminate biomass washout. However, the sequencing batch mode requires a complex timing unit and control, which hinders SBRs' application for large-scale. Thus, the continuous reactor is preferred for large-scale treatment plants. Among the continuous flow reactors, the downflow hanging sponge (DHS) reactor is a promising configuration for implementing the MDD processes due to its several benefits, such as a high mass transfer rate (which means methane gas easily diffuses to bulk liquid), long sludge retention time, flexibility, and low cost in setup and maintenance. The DHS reactor is a

trickling filter reactor using polyurethane sponges as media to retain biomass, and its modification for a wide range of application has been reported (Hatamoto et al., 2018). The DHS reactor has become popular in the treatment of nitrogen-polluted wastewater, initially for nitrification in an aerobic condition in the post-treatment (Onodera et al., 2014), and then, recently, for denitrification with modification in the design, such as the bypass of raw wastewater to the lower layer to encourage anoxia and alleviate carbon limitation (Bundy et al., 2017).

Crone et al. (2016) suggested that the majority of methane emissions in anaerobic wastewater treatment plants are dissolved methane in the effluent, which are stripped directly into the atmosphere in aerobic post-treatment. The MDD processes utilizing dissolved methane as an electron-donor instead of extra organic carbon sources in the anaerobic tertiary treatment is a promising option to cut CH_4 emission and minimize carbon footprints. Therefore, a closed-type DHS reactor with the MDD processes was conceived to be a sustainable alternative for denitrification following an anaerobic treatment. The MDD processes were successfully adopted in a laboratory-scale DHS reactor as reported in Hatamoto et al. (2017). This DHS exhibited a high removal rate of 70.4 ± 7.6 $mgNO_2^-\text{-}N.L^{-1}.d^{-1}$ and 39.0 ± 2.2 $mgNO_3^-\text{-}N.L^{-1}.d^{-1}$ when both nitrite and nitrate were fed as substrates; then, it gradually increased and reached a removal rate of 84.4 $mgN.L^{-1}.d^{-1}$ when nitrate was used as the sole substrate (Hatamoto et al., 2017).

Apart from CH_4 gas, N_2O is one of the significant greenhouse gases with a global warming potential of a 310-fold more than CO_2 and an atmospheric lifetime of 114 years, which produced from wastewater treatment plants, especially those treating nitrogen pollution (Forster et al., 2007). According to current literature, the MDD processes, similar to the Anammox process, is considered to bypass the production of N_2O in the pathway of conversion to nitrogen gas (Ettwig et al., 2010; Kartal et al., 2007). However, in previous

studies, a trace amount of N_2O generated during these processes was detected in SBRs (Ali et al., 2016) and continuous upflow anaerobic reactors (Ma et al., 2017; Okabe et al., 2011).

The NO_2^- concentration and dissolved oxygen (DO) concentration are operating factors that influence the release of N_2O in denitrification processes. It has been reported that operational parameters, such as hydraulic retention time (HRT), sludge retention time, and mode of operation, can cause NO_2^- accumulation. The high concentration of NO_2^- in wastewater causes an increase in free nitrous acid, which is a cytotoxin to a wide range of microorganisms and inactivate various enzymes in the metabolic processes rather than the nitrite (Zhou et al., 2011). Besides, DO is a key factor inhibiting denitrification rate. Preventing oxygen exposure in denitrifying reactor via reactor configuration designs and supplements of reducing agents is an approach to mainly enhance denitrification rate and minimize the N_2O release as well (Capodaglio et al., 2016). The inhibitions of the accumulation of NO_2^- and the presence of DO on both nitrogen removal and N_2O emission have been verified in several denitrification processes, such as heterotrophic denitrification (Chen et al., 2020; Wang et al., 2019), denitrifying phosphorus removing process (Miao et al., 2018), sulfide-oxidizing autotrophic denitrification (Lan et al., 2019), and hydrogenotrophic denitrification (Wang et al., 2018). Meanwhile, their impact on the MDD processes is lacking to date.

In this chapter, we operated a closed-type DHS reactor with gradually shortened HRT and a supplement of titanium(III) nitrilotriacetate (Ti(III)-NTA) as an oxygen scavenger to investigate their effects on MDD performance and nexus of nitrous oxide emission.

4.2. Materials and methods

4.2.1. Set up and operation of the DHS reactor

A laboratory-scale closed-type DHS reactor was set up to foster the MDD processes. This DHS reactor was made of a clear rigid polyvinyl chloride cylinder-shaped with a total

working volume, the diameter, and the height of 14.1 L, 15 cm, and 80 cm, respectively. The reactor was loaded with 300 pieces of polyurethane sponges G3 (square cubes with dimensions of 33 × 33 × 33 mm and packed inside a cylinder plastic carrier 33 mm in diameter and 33 mm in height) (Hatamoto et al., 2018) divided into five segments. The total volume of the sponge was 8.47 L and considered as the liquid volume inside the DHS reactor. The synthetic wastewater was continuously fed into the reactor via a peristaltic pump and then dripped down from a holed-plate distributor from the top through five sponge segments inside the DHS reactor. The effluent was collected at the bottom via a T-tube installed under the water level at the bottom to prevent air exposure. Also, the medium bottle was connected with a CH_4/CO_2 (95/5%, v/v) gas bag to balance the pressure inside the bottle, hence prevent air leak from the atmosphere. The input gas (CH_4/CO_2 = 95/5%, v/v) was supplied from beneath the lowest segment of sponge at a speed of 14.2 $L.d^{-1}$ via a flowrate regulator and collected into a gas bag at the top of the reactor. The DHS reactor was operated at a temperature of 35 °C during the experiment. A schematic of this system is shown in Fig. 4.1.

The compositions of the synthetic wastewater were $KHCO_3$ (500 $mg.L^{-1}$), KH_2PO_4 (50 $mg.L^{-1}$), $CaCl_2•2H_2O$ (300 $mg.L^{-1}$), $MgSO_4•7H_2O$ (200 $mg.L^{-1}$), an acidic trace element solution (0.5 $mL.L^{-1}$), and an alkaline trace element solution (0.2 $mL.L^{-1}$) (Hatamoto et al., 2014). The acidic and alkaline trace element solutions were prepared following the composition and method reported by Ettwig et al. (2009). Stock solutions of 1 M $NaNO_2$ and 1M $NaNO_3$ were filtered through 0.22 μm membrane (Advantec) to sterilize and stored in 4 ^oC until use. They were supplied individually or simultaneously as nitrogen sources at a final concentration of about 20 $mgN.L^{-1}$ for each compound. The synthetic medium was made in a 10 L bottle, flushed with argon gas for 30 min and then CH_4/CO_2 (95/5%, v/v) gas for 30 min

in every new batch to remove dissolved oxygen in the medium and oxygen in the headspace. The final pH of the medium pH was not adjusted and in range of 6.8 to 7.2.

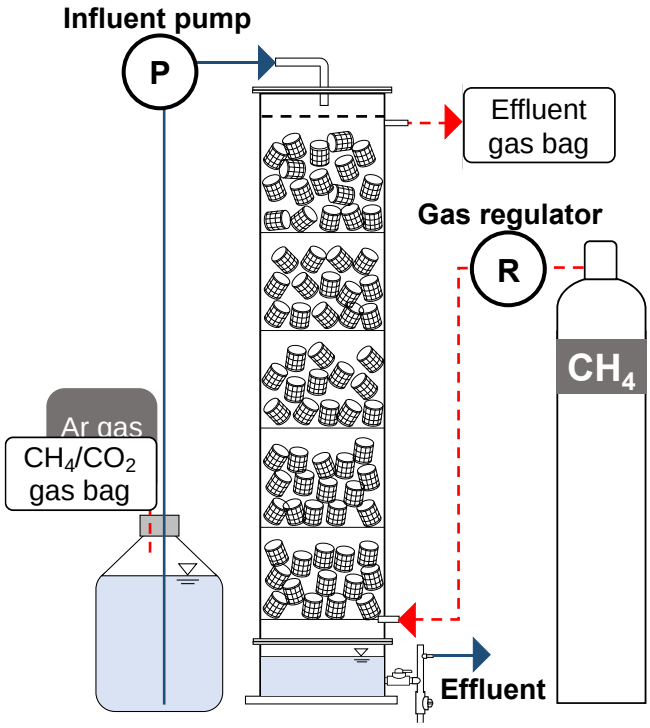

Fig. 4.1 The schematic diagram of the closed-type DHS reactor in this study

The titanium(III) nitrilotriacetate stock solution was made according to a previous research (Moench and Zeikus, 1983), in which titanium(III) (Ti(III) – 25 mM) was the main redox element and nitrilotriacetic acid (NTA – 100 mM) was a chelating reagent to stabilize and solubilize Ti(III) in solution. Firstly, one liter of distilled water was autoclaved and deoxygenated by a vacuum machine and ultrasonic vibration for 3 h. Then, 17.3 g NTA (Dojindo Molecular Technologies, Inc.) was weighed and dissolved into 500 mL of the sterilized and deoxygenated water under pH of 9.0 by adding solid NaOH. A total of 17.3 mL of titanium(III) chloride ($TiCl_3$ 20 wt. %, Wako Pure Chemicals) solution was added slowly to the NTA solution. Adding $TiCl_3$ solution decreased pH of NTA solution; therefore, successively add 1 mL of $TiCl_3$ and 1 mL of saturated Na_2CO_3 solution to the NTA solution to keep the pH maintaining at about 7.0. The solution was stirred continuously by using a magnetic stirrer and the pH was measured during the preparation. The final volume was

adjusted to 900 mL by the sterilized and deoxygenated water, subsequently divided into 100 mL glass vials and sealed by rubber stoppers and aluminum caps. Every vial was purged with N_2 gas for 5 min to remove air in headspace. The color of the Ti(III)-NTA stock solution is dark blue of Ti(III) ion. This solution becomes colorless when Ti(III) is oxidized to Ti(IV).

The seed sludge was collected from the previous study in which MDD microorganisms were enriched. The initial sludge was paddy field soil, inoculated in an upflow biofilm reactor using a coarse sponge sheet as the carrier material and feeding $NaNO_2$ and $NaNO_3$ as nitrogen sources (Hatamoto et al., 2014). The sponges used in this study were immersed in this seed sludge before being loaded into five segments of the reactor.

Table 4.1 Operational conditions during three phases operation of the DHS

	Time (days)	HRT (hours)	NO_2^- and NO_3^- supply	Ti (III)-NTA addition
1st stage	1 - 43	24	Both	No
	44 - 48	12	Both	No
	49 - 52	6	Both	No
2nd stage	53 - 112	12	Either NO_2^- or NO_3^-	No
	113 - 182	12	Either NO_2^- or NO_3^-	Yes
	183 - 204	12	Both	Yes

The experiment was conducted in two stages, as shown in Table 4.1. In the first stage, the HRT was shortened from 24 to 12 and 6 h by adjusting the speed of the influent pump during 52 days for evaluating the impact of HRT on the denitrification performance when nitrogen sources were both nitrate and nitrite at levels of approximately 20 $mg.L^{-1}$. The second stage focused on the effect of Ti(III)-NTA on nitrogen removal under either nitrate or nitrite supply from the 53rd to 182nd day, and subsequently under both nitrate and nitrite supplies from the 183rd to 204th day. The Ti(III)-NTA stock solution with final concentrations of 25 µM Ti(III) as a reducing agent was added to the medium after gas purging. Based on the result in the first stage, the HRT was kept at 12 h for the next stage. In this second stage, N_2O released

from the DHS reactor was as well determined during both periods that the reactor was operated with and without the Ti(III)-NTA supplement in order to assess the potential of the DHS reactor for not only favoring autotrophic denitrification but mitigating a release of N_2O gas.

4.2.2. Analytical methods

The influent DO concentration was measured by an oxygen probe OXROB10 connected to a FireStringO2 oxygen meter (PyroScience GmbH). The probe was calibrated in 1-point calibration mode in the air at room temperature (controlled at 25 ^oC) and its detection limit is 0.01 $mg.L^{-1}$. The medium pH was measured by a portable pH meter D-52 (D-25; Horiba, Kyoto, Japan). The concentrations of NO_2^- and NO_3^- in the influent and effluent were determined by a high-performance liquid chromatograph CTO-20A equipped with an IC-Pak A HC column (4.6 × 150 mm, Waters) and a UV–Vis detector (SPD-20AV, Shimadzu) after filtering through a 0.22 μm membrane (Advantec). The mobile phase was 10 mM KCl at flow rate of 1.0 $ml.min^{-1}$ and column oven temperature was 40 ^oC.

Since the ambient air was prevented entering the DHS reactor by water seal on the effluent port of the reactor and the input gas was purged continuously into the reactor, the produced N_2O was easily stripped off into the off-gas and collected into the gas bag on top of the reactor. The percentage of N_2O in effluent biogas was analyzed by a gas chromatograph (GC-2014, Shimadzu) equipped with an electron capture detector and a packed column Porapak-Q 50/80 (GL science). The carrier gas was ultra-high purity N_2 gas (> 99.999 vol%, TAIYO NIPPON SANSO).

The ratio of N_2O production rate in off-gas and NO_x^- (i.e., NO_2^- and NO_3^-) consumption rate (Fig. 4.4) was calculated as the equations below.

$$\frac{N_2O}{NO_x}(\%) = \frac{N_2O \text{ production } (gN.d^{-1})}{NO_x^- \text{ consumption } (gN.d^{-1})} \times 100\ (\%) \quad (4.3)$$

$$N_2O \text{ production } (gN.d^{-1}) = \frac{N_2O \text{ concentration} \times V_{air} \times 28\ (gN.mol^{-1})}{22.4\ (L.mol^{-1}) \times ART} \quad (4.4)$$

$$NO_x^- \text{ consumption } (gN.d^{-1}) = NO_x^- \text{ consumption rate} \times V_{liquid} \quad (4.5)$$

where the N_2O concentration (%) is measured in the effluent gas, the NO_x^- consumption rate ($mgN.L^{-1}.d^{-1}$) is calculated based on the N change in the influent and effluent,

$V_{air} = V_{reactor} - V_{sponge} = 5.63$ (L),

$V_{liquid} = V_{sponge} = 8.47 \times 10^{-3}$ (m^{-3}), assuming that all the sponges were filled by liquid

Air retention time (ART) $= \frac{V_{air}\ (L)}{\text{air flowrate } (L.d^{-1})} = \frac{5.63}{14.2} = 0.40$ (d).

4.3. Results and discussion

4.3.1. Nitrogen removal performance under different HRTs

The DHS reactor was started at an HRT of 24 h for the first 43 days. Since the removal rates were stable at 2.1 ± 1.5 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ and 2.9 ± 1.8 $mgNO_2^-$-$N.L^{-1}.d^{-1}$, the HRT was shortened to 12 and then 6 h in the next few days. When the HRT was halved to 12 h, the nitrate and nitrite removal efficiencies on the 45th day reduced from 10.2 ± 7.0% to 4.8% and from 16.6 ± 8.4% to 7.5%, respectively. As Fig. 4.2 shows, the nitrogen removal rates gradually increased from the 44th to the 48th day and reached 2.6 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ and 5.6 $mgNO_2^-$-$N.L^{-1}.d^{-1}$, which were higher than the removal rates during the HRT of 24 h. The nitrate-dependent denitrification requires more steps, via Eqs. (4.1) and (4.2), than nitrite-

dependent denitrification, via only Eq. (4.2), under similar N levels, which might have caused the nitrite removal to be consistently higher than the nitrate removal.

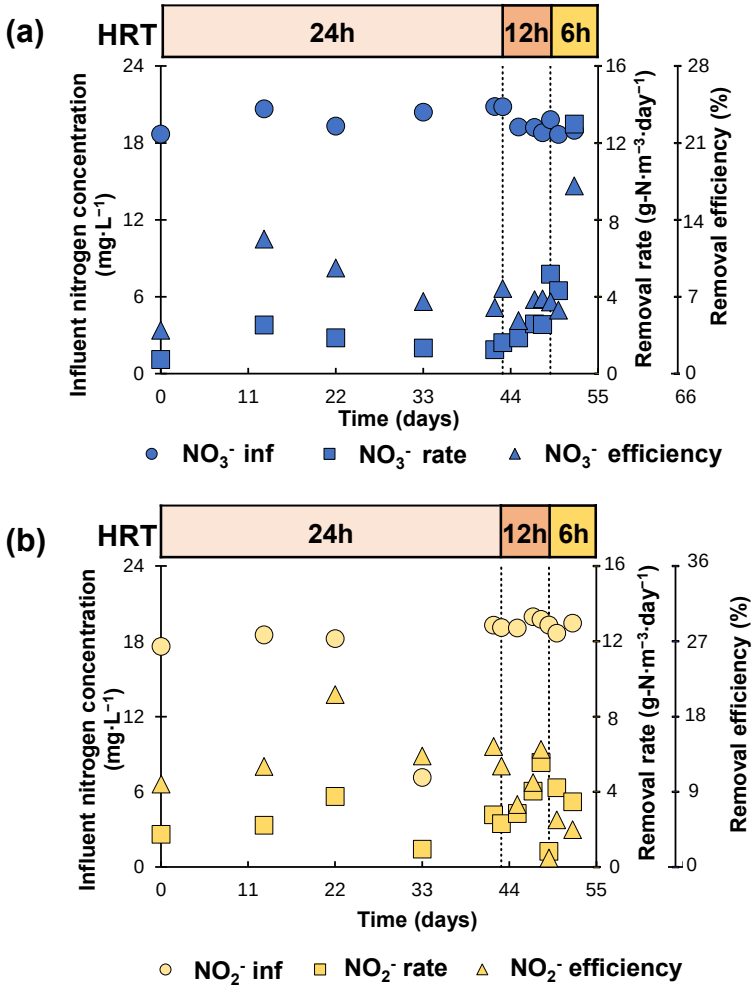

Fig. 4.2 The DHS's performance in nitrate removal (a) and nitrite removal (b)

However, this pattern was not witnessed at HRT of 6 h from the 49th to the 52nd day. During that time, the nitrate removal rate rapidly increased to 7.5 ± 4.8 mgNO_3^--N.L^{-1}.d^{-1}, whereas the nitrite removal rate dropped to 2.8 ± 1.8 mgNO_2^--N.L^{-1}.d^{-1}. Lu et al. (2019) estimated the affinity constant for nitrate to be 2.1 ± 0.4 mgN.L^{-1} in *C. Methanoperedens nitroreducens*, which is much lower than the affinity constant for nitrite at 12.74 ± 1.26 mgN.L^{-1} (calculated from He et al., 2013). Therefore, nitrate tends to be consumed faster than nitrite with the same concentration presenting in the influent. Also, the shorter HRT might result in the partial denitrification of NO_3^- in Eq. (4.1) instead of complete denitrification

through both Eqs. (4.1) and (4.2). These two reasons likely contributed to the accumulated nitrite in the effluent.

4.3.2. Reducing agent Ti(III)-NTA improved performance of the DHS reactor

This closed-type DHS was operated with the continuous purge of CH_4/CO_2 (95/5%, v/v) gas; however, the DO in the medium can inhibit the MDD activity, unless Ti(III)-NTA as a reducing agent was added to remove trace amount of DO. Ti(III)-NTA solution as a reductant was first developed by Moench and Zeikus (1983); then, it was used in later studies to eliminate the DO in media for both batch (Temme et al., 2017) and continuous experiments (Aoki et al., 2014). In this study, the medium supplemented with Ti(III)-NTA solution, in which the final concentration of Ti(III) was 25 µM, was also applied to accelerate the MDD processes. The Ti(III) solution was reported to have a lower redox potential. Hence, it is highly effective in reducing the redox potentials of media compared with other reducing agents, such as cysteine, dithionite, and Fe(III) (Herbel et al., 2007; Jones and Pickard, 1980; Lesage et al., 1998). Additionally, NTA was chosen to chelate Ti(III), because it is not consumed in the anaerobic metabolism (Moench and Zeikus, 1983); thus, it did not interfere with this study as a carbon source, but only methane did.

In the second stage, the HRT of 12 h was chosen to operate the reactor due to its good effect reported in the first stage in section 4.3.1. Fig. 4.3 shows that the supplementation of Ti(III)-NTA increased the reactor's performance in terms of both nitrate and nitrite removals. When the influent comprised either NO_3^- or NO_2^-, the addition of the reducing agent increased the nitrate and nitrite removal rates from 1.4 ± 0.6 $mgNO_3^-N.L^{-1}.d^{-1}$ to 4.1 ± 1.9 $mgNO_3^-N.L^{-1}.d^{-1}$ and from 3.2 ± 2.8 $mgNO_2^-N.L^{-1}.d^{-1}$ to 6.6 ± 3.3 $mgNO_2^-N.L^{-1}.d^{-1}$, respectively. When both nitrate and nitrite were simultaneously supplied from the 183rd to the 204th day, the doubled nitrogen loading rate resulted in the deterioration in the DHS reactor's performance

at the beginning. After that, the DHS reactor quickly recovered within 9 days and reached higher removal rates of 6.0 mgNO_3^--N.L^{-1}.d^{-1} and 10.7 mgNO_2^--N.L^{-1}.d^{-1}.

4.3.3. The DHS reactor mitigated N_2O emission

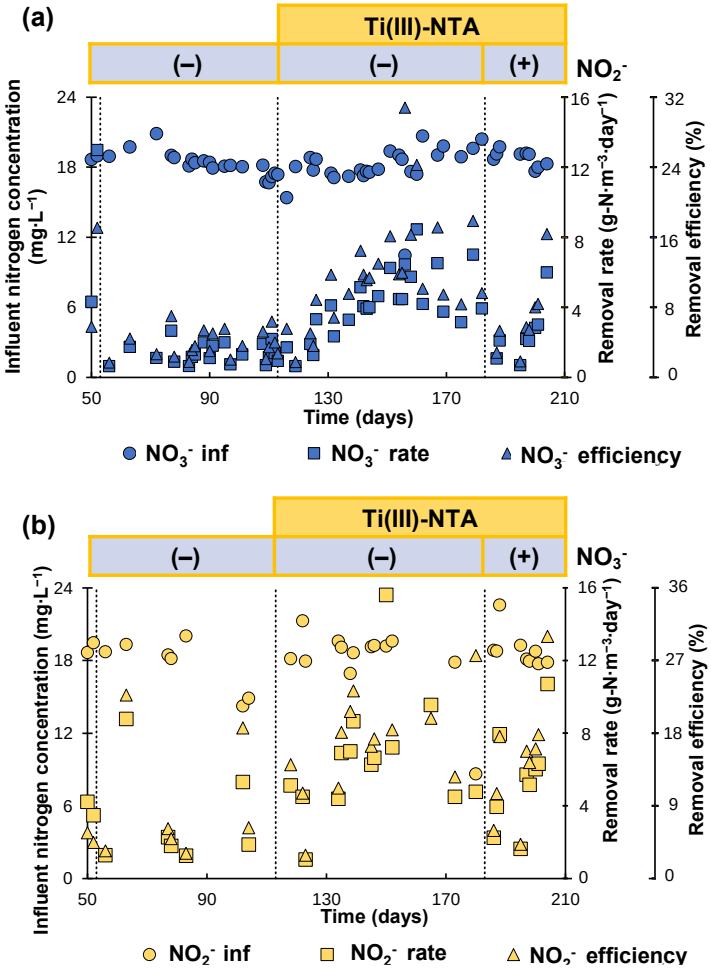

Fig. 4.3 Positive effect of 25 µM Ti(III) on nitrate removal (a) and nitrite removal (b)

Mitigation greenhouse gas emissions, particularly nitrous oxide, is one of the reasons to consider autotrophic processes, e.g., MDD, as a sustainable alternative for nitrogen removal. Studies have shown that the MDD processes occur in ANME-2d archaea using a reverse methanogenesis process to reduce nitrate to nitrite and in NC10 bacteria using an intra-aerobic methane oxidation pathway to reduce nitrite to nitrogen gas without N_2O as an intermediate product (Haroon et al., 2013; Wu et al., 2011). To date, there has been no study in which pure

MDD microorganisms (i.e., NC10 bacteria and ANME-2d archaea) were cultivated; therefore, it has been possible to measure N_2O in few MDD reactors (Ettwig et al., 2010; Ma et al., 2017). This phenomenon was also observed in this study. The ratio of N_2O emission per NO_x^- removal was calculated from the 83rd to the 182nd day as shown in Fig. 4.4.

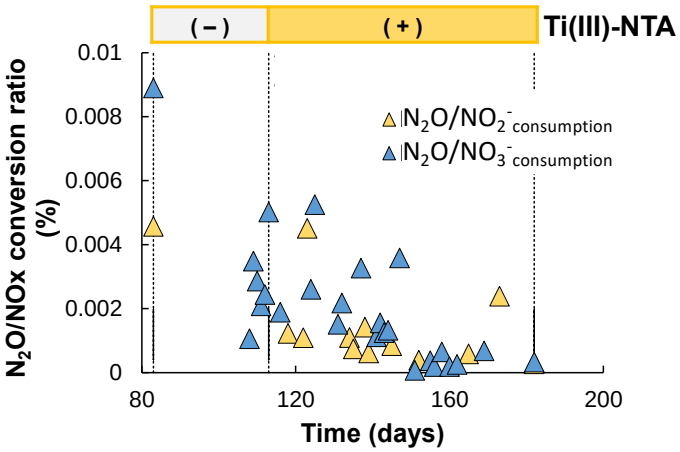

Fig. 4.4 Ratio of N_2O production/NO_x^- consumption declined under Ti(III)-NTA supplement

The DHS reactor converted 4.6 × 10^{-3}% of consumed NO_2^- and 8.9 × 10^{-3}% of consumed NO_3^- to N_2O at first without a Ti(III)-NTA supplement. Consequently, the addition of Ti(III)-NTA as a reducing agent from the 113th to the 182nd day intensified the anaerobic condition; thus, both N_2O/consumed NO_2^- and N_2O/consumed NO_3^- gradually decreased to extremely low levels of 3.8 × 10^{-4}% and 7.2 × 10^{-5}%, respectively. Ettwig et al. (2010) used an isotope in a batch experiment and found that 7% of the consumed $^{15}NO_2^-$ was converted to N_2O. Ma et al. (2017) reported a smaller amount of 0.03% removed NO_2^- converted to N_2O. The number of studies that have focused on N_2O emission in MDD reactors is still limited compared with other biological nitrogen removal methods (Kampschreur et al., 2009; Massara et al., 2017; Sun et al., 2015), and most of them used an isotope tracer to evaluate the N_2O production or performed measurements in batch experiments. This study is the first to consider the N_2O emission in a continuous reactor applying the MDD processes. The

results indicate that the N_2O emission in this DHS is more 100 times lower than the previously reported results for the MDD reactions (Ettwig et al., 2010; Ma et al., 2017).

The low level of N_2O content in the biogas was stable during the experimental period, regardless of the increases in the NO_2^- and NO_3^- consumption rates (Fig. 4.5), which indicates that the N_2O content seems to be irrelevant to the MDD processes' development. Recent research showed that the N_2O is released by either heterotrophic denitrification or nitrifier denitrification (Massara et al., 2017). In these processes, NO_2^- is the key factor triggering the N_2O formation (Desloover et al., 2012), which was consistent with the increase of N_2O concentration in the effluent gas from 2.2×10^{-6} g.m^{-3} to 9.4×10^{-6} g.m^{-3} when the NO_2^- consumption rate increased from approximately 2.2 mgNO_2^--N.L^{-1}.d^{-1} to 7.0 mgNO_2^--N.L^{-1}.d^{-1} (Fig. 4.5). Meanwhile, this pattern was not observed in nitrate consumption.

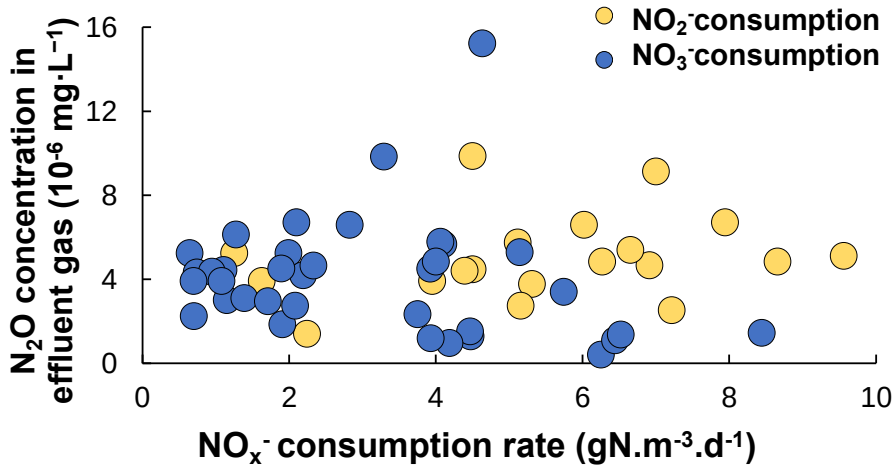

Fig. 4.5 Effect of nitrate–nitrite consumption rates on the concentration of N_2O in effluent gas

The coexisting heterotrophic denitrification process ascribed to the small amount of N_2O emission was reported in an upflow anaerobic sludge blanket reactors facilitating a nitrite-MDD process. Although there was no carbon supply into the autotrophic reactor, the heterotrophic denitrifying bacteria was detected. They might use organic matters from biomass decay to reduce NO_3^-. But the initial product is NO_2^-, which was consequently

converted to N_2O before completely denitrifying to N_2 gas (Ma et al., 2017). Furthermore, a gammaproteobacterial methanotroph, *Methylomonas denitrificans* sp. strain FJG1, was discovered to be able to couple methane oxidation to nitrate reduction and release N_2O as a terminal product under oxygen limitation by its reaction products (Kits et al., 2015) and, recently, by its revealed complete genome (Orata et al., 2018). Another autotrophic denitrification process utilizing only DIN compounds as electron donors and acceptors for denitrification, i.e., Anammox, has been reported to release N_2O at a level as low as for MDD in previous studies (Ali et al., 2016; Okabe et al., 2011). Considering that the two main components for Anammox, NO_2^- is not commonly available in wastewater, a partial nitrification (PN) step converting NH_4^+ to NO_2^- is usually performed prior to the Anammox step. It was reported that the PN reactor produced much more N_2O than the Anammox reactor in the continuous two-stage upflow reactors (Okabe et al., 2011). Authors reported that the larger part (97.5%) of N_2O, corresponding to 9.6 ± 3.2% of the removed nitrogen, was converted to N_2O in the PN reactor, while only 0.14 ± 0.09% of the removed nitrogen was converted to N_2O in the Anammox reactor. It was suspected that ammonium-oxidizing bacteria (AOB) produced N_2O during denitrification of NO_2^- with NH_4^+ as electron donor under high NH_4^+ concentration in the PN reactor under the oxic condition. On the other hand, it was proved that the N_2O released in the latter Anammox reactor was most likely from putative heterotrophic denitrifying bacteria under the anoxic condition. Their results also indicate that the Anammox activity and N_2O production are spatially separated (Okabe et al., 2011). Despite the difference in reactor configurations, Ali et al. (2016) operated a SBR for nitritation-Anammox reactions also reported about 70% of N_2O was produced in the oxic zone, where nitrifiers (nitrifier-denitrification) were predominant, and the rest of N_2O was produced in the anoxic zone by coexisting putative heterotrophic denitrifiers and some other unknown pathway(s). In comparison with the previous reports, the source of N_2O in the

closed-type DHS reactor is possible for heterotrophic denitrifiers or specific methanotrophs such as *M. denitrificans* sp. strain FJG1. This could be elucidated by further microbial community analysis in future.

4.4. Conclusions

This chapter proposed a closed-type DHS reactor as a simple configuration to facilitate nitrate/nitrite – denitrification coupled to anaerobic methane oxidation. The DHS reactor achieved the best nitrate and nitrite removal at 4.1±1.9 mgNO_3^--N.L^{-1}.d^{-1} and 6.6 ± 3.3 mgNO_2^--N.L^{-1}.d^{-1}, respectively under HRT of 12h and an addition of of 0.025 mM Ti(III). Our findings indicated that shortening HRT caused the partial denitrification of NO_3^- instead of complete denitrification, hence the accumulation of NO_2^-, which likely to be toxic for MDD microorganisms. Supplementary of Ti(III)-NTA intensified the anaerobic condition resulted in not only the improvement of nitrogen removal but also significant reduction of N_2O releasing per NO_x^- removal from this DHS reactor. In this study, N_2O per consumed NO_2^- and NO_3^- were detected at extremely low levels of 3.8×10^{-4}% and 7.2×10^{-5}%, respectively, which is more 100 times lower than the previously reported results for the MDD reactions. This DHS reactor demonstrates the potential application of MDD processes for autotrophic denitrification in post-treatment, in which CH_4 produced from primary anaerobic bioreactor is consumed to reduce residual nitrite/nitrate to N_2 with a trace amount of N_2O compared to heterotrophic denitrification. However, further study is required to improve the nitrogen removal rate because despite the same inoculum source this DHS had low nitrogen removal rate compared to the previous smaller DHS (Hatamoto et al., 2017) under similiar operational conditions, such as temperature and nitrogen concentrations.

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CHAPTER 5. EFFECT OF INOCULUM SOURCES ON AUTOTROPHIC NITROGEN REMOVAL IN ANAEROBIC HOLLOW FIBER MEMBRANE REACTORS

5.1. Introduction

Despite our attempt at reactor installation and supplement addition, the nitrogen removal rate by methane-driven denitrification (MDD) process has not reached practical use as described in chapter 4. Therefore, this chapter employ another approach to understand the microorganism inside reactors. Nitrogen-transforming microbial network is a natural solution to remedy nitrogen imbalance in the ecosystem. Microbes can utilize NH_4^+ to build biomass via assimilation, or anaerobically oxidized to N_2 via anaerobic ammonium oxidation (Anammox) by Anammox bacteria (AnAOB), or aerobically oxidized to NO_2^- and NO_3^- via nitrification by ammonia-oxidizing bacteria/archaea (AOB/AOA) and nitrite-oxidizing bacteria (NOB), respectively. Subsequently, NO_2^- and NO_3^- also can be assimilated to biomass or converted completely to harmless N_2 via denitrification by heterotrophic denitrifying bacteria (DNB). Besides, dissimilatory nitrate/nitrite reduction to ammonium (DNRA) process appears to be favored over denitrification in case of an excess supply of electron donors (i.e., high C/NO_3^- ratio), which retain dissolved inorganicDIN in water instead of removing it (Kuypers et al., 2018).

In addtion, microbes utilizing methane or single-carbon compounds as their carbon and energy source are known as methanotrophic (i.e., microbes utilize sole CH_4) and methylotrophic (i.e., microbes utilize single-carbon compounds like methanol, methylamine, formate, and methane as well). They are phylogenetically diverse and available in both natural and artificial ecosystems. Apart from their significant role in atmospheric methane sink, their

denitrification ability has been observed (Modin et al., 2007). An anaerobic methanotrophic microbial consortium couple to denitrification was firstly enriched by (Raghoebarsing et al., 2006). Later, bacteria and archaea in this consortium were assigned to NC10 bacterial phylum and anaerobic methanotrophic archaea (ANME)-2d cluster, respectively (Ettwig et al., 2009; Haroon et al., 2013). Since then, their genomic structures, ecological availability, and physiological performance in direct denitrification have been deeply investigated. Their combination with Anammox process for DIN removal even reached practical rates (Cai et al., 2015; Nie et al., 2020). However, the crucial role of enriched inoculum from parent reactors and the requirement of strict anaerobic with low redox potential limit the niche habitat for anaerobic microorganisms (Liu et al., 2013).

On the other hand, aerobic methanotrophic bacteria involved in MDD process are ubiquitous in both oxygen-rich and oxygen-depleted environments. They exhibit certain advantageous traits to thrive in micro-oxic environments, e.g., its proteins with high affinities for oxygen to scavenge trace oxygen available in a surrounding area or its synergistic interaction with the biotic environment for carbon metabolism (Guerrero-Cruz et al., 2021). Aerobic methane-oxidizing bacteria were formerly known to supply organic compounds or donate electrons to co-existing DNB for denitrification (Zhu et al., 2016). Recent metagenomic studies have revealed the nitrification and partial denitrification-associated enzymes (Boden et al., 2011; Khadem et al., 2012; Stein & Klotz, 2011), and complete denitrification-associated enzymes (Dam et al., 2013; Kits, Campbell, et al., 2015; Kits, Klotz, et al., 2015) that are encoded within methanotrophic groups in *Gammaproteobacteria, Alphaproteobacteria*, and *Verruccomicrobia.* Dalcin Martins et al. (2021) still detected high levels of aerobic methanotrophs in their reactor even after 2.5 years of operation under dissolved oxygen concentration between 0.27 and 4.7 μM. These findings suggest direct denitrification capability of aerobic methanotrophs, which reveals the possibility of using aerobic methanotrophs for

wastewater treatment. However, to date, limited number of research have demonstrated the ecophysiological roles of aerobic methanotrophs in anaerobic DIN removal (Alrashed et al., 2018; Lee et al., 2019).

Furthermore, recently, hollow fiber membrane reactors have been widely applied to shorten the enrichment of autotrophic nitrogen-removing microbes by increasing the gas-liquid interaction and retaining the biomass during continuous operation (Cai et al., 2015; Fu et al., 2017; Xie et al., 2018). As mentioned in Chapter 2, inoculum source and microbial consortium inside play crucial role in reactors' performance, which has been proved in SBR system. There is limited information on effects of inoculum on nitrogen removal performance of hollow fiber membrane reactors. Therefore, our study aimed to compare the DIN removal performance in the two reactors inoculated with specialized sludge from parent reactors and non-specialized paddy soil. To elucidate our aim, we considered two objectives: i) evaluating reactors' DIN removal performance by denitrification of either NO_3^- or NO_2^-, and by simultaneous removal of the two most common nitrogen pollutants, that is, NO_3^- and NH_4^+; and ii) determining the microbial community dynamics between inocula and reactors' biomass after long-term operation.

5.2. Materials and methods

5.2.1. Inoculum preparation

Our experiment used two sources of inoculum: mixed sludge from parent reactors and paddy soil, representing specialized denitrifying methanotrophic consortium and non-specialized natural consortium, respectively. The specialized sludge was collected from our three laboratory-scale upflow reactors described in Hatamoto et al. (2018), separately operated under methane-driven nitrate and nitrite denitrification and Anammox conditions. The paddy soil was collected in a paddy field located in Nagaoka, Japan, at 5-10cm depth. The soil was

stirred in a bottle with 400mL of medium containing NO_3^- and NH_4^+ (14 mgN.L^{-1} each) as nitrogen sources and CH_4/CO_2 (95/5%, v/v) gas sparging for acclimation in three days. The medium's general compositions follow the synthetic wastewater's compositions as mentioned in section 4.2.1, chapter 4.

5.2.2. Reactor set-up

Two laboratory-scale upflow reactors were set up with a plastic cylinder equipped with a U-shaped hollow fiber membrane as a gas diffuser inside. Each hollow fiber contains a bundle of 1000 polysulfone hollow fibers with the inner diameter of 0.25 mm, the outer diameter of 0.5 mm, the pore size of 0.1 µm, and the surface area of 0.5m^2 (Kankyo Technos, Wakayama, Japan). Both reactors have a total and working volumes of 1 L and 500mL, respectively. The space inside of reactors were separated into interior and exterior of the hollow fiber. Gas (i.e., Ar or CH_4:CO_2) was supplied from the fiber's interior and passed through the fiber's pores into the bulk liquid in the exterior space without bubble formation. The influent gas and liquid were supplied separately upflow from gas bags and an influent bottle, respectively, by peristaltic pumps (Masterflex® L/S, Vernon Hills, USA). The effluent gas and liquid were discharged in the same ports at the top of reactors. R1 is the reactor inoculated with the mixture of sludge from the parent reactors, R2 is the reactor inoculated with the paddy soil after acclimation.

5.2.3. Operational conditions

The operation of these reactors consists of two stages: the enrichment stage in recirculation flow and the activity assessment stage in continuous flow. The recirculation stage was conducted for 50 days with an extreme condition of 400 mgN-NO_3^-.L^{-1} and 300 mgN-NH_4^+.L^{-1} as nitrogen-sources, and gas mixture CH_4/CO_2 (95/5%, v/v), used as a sole carbon and electron donor, was supplied with a gas flow rate of 25.2 L.d^{-1}. After 50-day recirculated operation, the medium inside reactor columns and the influent bottle was totally replaced with

a fresh medium to remove all the suspended sludge; only bundles of fiber with attached biofilm were used in the next continuous stage.

The continuous stage was carried out for 240 days with various operational conditions differing in nitrogen sources and gas supply, in order to evaluate the performances of R1 and R2. In this stage, the temperature and hydraulic retention time were kept at 30°C and 9.5 hours, respectively. The gas flow rate was reduced to 3.6L.d^{-1}. The influent was prepared in a 10 L bottle using the mineral compositions described in section 4.2.1, Chapter 4. Argon and CH_4/CO_2 gases flushing and Ti(III)-NTA supplement created anaerobic condition. The influent bottle was sealed with a rubber stopper and connected with a gas bag filled CH_4 to minimize air intrusion into the reactors. Stock solutions of 1 M $NaNO_3$, 1 M $NaNO_2$, and 0.5 M $(NH_4)_2SO_4$ solution were added into the influent to achieve the desired concentration of nitrogen sources. The detailed conditions are described in Table 5.1.

Table 5.1 Operational conditions during 240-day continuous operation of the reactors

Phase (days)	N sources (mg N/L)	Gas supply
1 (0 – 50)	NO_3^-(28) and NH_4^+(13)	CH_4:CO_2, 95:5 (vol:vol)
2 (51 – 58)	NO_2^-(10) and NH_4^+(14)	Ar
3 (59 – 68)	NO_2^-(10)	CH_4:CO_2, 95:5 (vol:vol)
4 (69 – 75)	NO_2^-(10)	Ar
5 (76 – 83)	NO_3^-(13)	Ar
6 (84 – 87)	NO_3^-(12)	CH_4:CO_2, 95:5 (vol:vol)
7 (88 – 90)	NO_3^-(28)	CH_4:CO_2, 95:5 (vol:vol)
8 (91 – 147)	NO_3^-(29) and NH_4^+(13)	CH_4:CO_2, 95:5 (vol:vol)
9 (148 – 240)	NO_3^-(30) and NH_4^+(9)	CH_4:CO_2, 95:5 (vol:vol)

5.2.4. Analytical methods

The influent and effluent of both reactors were taken daily to measure pH, oxidation-reduction potential (ORP), nitrate, nitrite and ammonia concentrations during the continuous stage. The pH was measured by a portable pH meter (D-25; Horiba, Kyoto, Japan). After filtration through cellulose acetate membrane filter unit with 0.2 μm pore size (ADVANTEC, Tokyo, Japan), the concentration of NO_2^- and NO_3^- were determined by a HPLC equipped with

an IC-Pak A HC column (Waters, Milford, USA) and UV–VIS detector (SPD-20AV, Shimadzu Co., Japan) under the operation condition described in section 4.2.2 in Chapter 4. The ammonia nitrogen concentration was measured using Nessler method 8038 (DR6000 spectrophotometer Hach, USA). Dissolved oxygen (DO) was occasionally measured by inserting optical oxygen sensor OXROB10-OI (FireSting O2, Pyro Science, Aachen, Germany) into the reactor columns. ORP in effluent and influent were measured by an ORP meter (RM-30P, TOADKK, Japan).

Biomass collecting from reactors was limited due to the small scale of reactors and the slow growth rate of autotrophic biomass. Therefore, we collected three samples from each reactor during the experimental period: a1 and a2 were inocula from parent reactors and paddy soil, respectively; b1 and b2 were biomass after 50-day recirculation of medium in R1 and R2, respectively; c1 and c2 were biomass at the end of 240-day continuous period in R1 and R2, respectively. These samples were stored at -20 ^{o}C before extracting DNA for sequencing.

5.2.5. Microbial community analysis

Total DNA from all six samples were extracted and amplified the V4 region of the bacterial and archaeal following procedures described in section 3.2.4, Chapter 3.

Downstream analysis of forward and reverse sequences from MiSeq output data was performed using the Mothur bioinformatics package version 1.44.1 (Schloss et al., 2009), according to MiSeq standard operating procedures (MiSeq SOP; https://mothur.org/wiki/miseq_sop/). Firstly, two sets of forward and reverse reads in each sample were combined using the make.contigs command. Secondly, low-quality and chimeric sequences were filtered and removed using the screen.seqs, unique.seqs, and chimera.vsearch (VSEARCH algorithm) commands. Thirdly, the effective sequences were assigned to different bacterial and archaeal taxonomic levels (from phylum to genus) in SILVA version 138.1 reference database with at least 80% confidence level, using the Bayesian classifier with the

classify.seqs command. Finally, these sequences were clustered using the classify.otu command into operational taxonomic units (OTUs) with at least 97% sequence similarity for further data analysis. The top 10 predominant genera of each sample were assembled into 34 genera and visualized in a heatmap according to their relative abundance using superheat R package (Barter & Yu, 2018) in Fig. 5.4.

Alpha diversity in Table 5.3 were calculated from OTU-based dataset. Since the number of sequences varied from 8073 in b2 to 20153 in b1, the smallest of 8073 sequences were randomly selected from each sample for 1000 times and calculated the average of these indices to standardize the calculation. Table 5.3 contains observed species (so-called observed OTU), abundance-based coverage estimator (Ace) and Chao indices for the richness (i.e., number of taxonomic groups), Shannon and Simpson indices for the evenness (i.e., (distribution of abundances of the groups), and Good's coverage value for the percentage of total OTUs covering in each sample.

PICRUSt2 pipeline version 2.4.1 was applied to predict the functional composition of all microbial consortia based on the obtained OTU sequences (Douglas et al., 2020). The prediction refers to Kyoto encyclopedia of genes and genomes (KEGG) orthologs (KO) and enzyme classification numbers (EC numbers). The abundance of predicted enzymes directly contributing to nitrogen metabolism and methane metabolism were compared in Fig. 5.5.

5.2.6. Data analysis

The MDD and Anammox reactions co-occurring in phases 1, 8, and 9 were hypothesized to follow as Eq. (5.1) – (5.3) under an anaerobic environment, where NO_3^-, NH_4^+, and CH_4 are provided (Fu et al., 2015).

$$NO_3^- + 2/8\, CH_4 \rightarrow NO_2^- + 2/8\, CO_2 + 4/8\, H_2O \quad (5.1)$$

$$NO_2^- + 1/1.32\, NH_4^+ \rightarrow 1.02/1.32\, N_2 + 0.26/1.32\, NO_3^- \quad (5.2)$$

$$NO_2^- + 3/8\, CH_4 + H^+ \rightarrow 1/2\, N_2 + 3/8\, CO_2 + 10/8\, H_2O \quad (5.3)$$

Table 5.2 was calculated with two assumptions: i) the calculation was applied when ammonification process did not happen (i.e., effluent concentrations of NH_4^+ were lower than influent); ii) the reactions' rates were estimated based on the measured removal rates (mmol.d^{-1}) of NO_3^-, NH_4^+, and NO_2^- (so-called rNO_3^-, rNH_4^+, and rNO_2^-) over time in phase 1, 8, and 9.

r1 represents nitrite reduction rate (mmol.d^{-1}) by Anammox in Eq. (5.2),

r2 represents nitrite reduction rate (mmol.d^{-1}) by MDD in Eq. (5.3),

r3 represents nitrate reduction rate (mmol.d^{-1}) by MDD in Eq. (5.1).

NH_4^+ was removed via only Eq. (5.2), so $r1 = rNH_4^+ \times 1.32$.

NO_3^- was produced via Eq. (5.2) and removed via Eq. (5.1), therefore

$rNO_3^- = r3 - rNO_{3\,produced\,from\,(2)}^- = r3 - 0.26 \times rNH_4^+$ or $r3 = rNO_3^- + 0.26 \times rNH_4^+$.

NO_2^- produced from only Eq. (5.1) was always undetected in our operation, therefore

$r3 = r1 + r2$, or $r2 = r3 - r1$.

5.3. Results and discussion

5.3.1. Effects of inoculum on reactors' performance

5.3.1.1 Ammonium removal

Ammonium removal conventionally includes two steps under two opposite conditions: nitrification occurs under high oxygen and low organic carbon conditions, then denitrification occurs under low oxygen and high organic carbon conditions. A combination of Anammox and MDD processes under the same anaerobic autotrophic condition was a promising alternative to remove both ammonium and nitrate in a single configuration. Thus, this study attempted to adopt this combination in phases 1, 8, and 9. In contrast to our expectation, ammonium removal was very low with the presence of nitrate and methane in these phases, as shown in Fig. 5.1. Despite similar ammonium loading rates of 33.7 ± 1.8 mgNH_4^+-N.L^{-1}.d^{-1} and 33.9 ± 1.7 mgNH_4^+-N.L^{-1}.d^{-1} in phases 1 and 8, respectively, the ammonium removal in both reactors

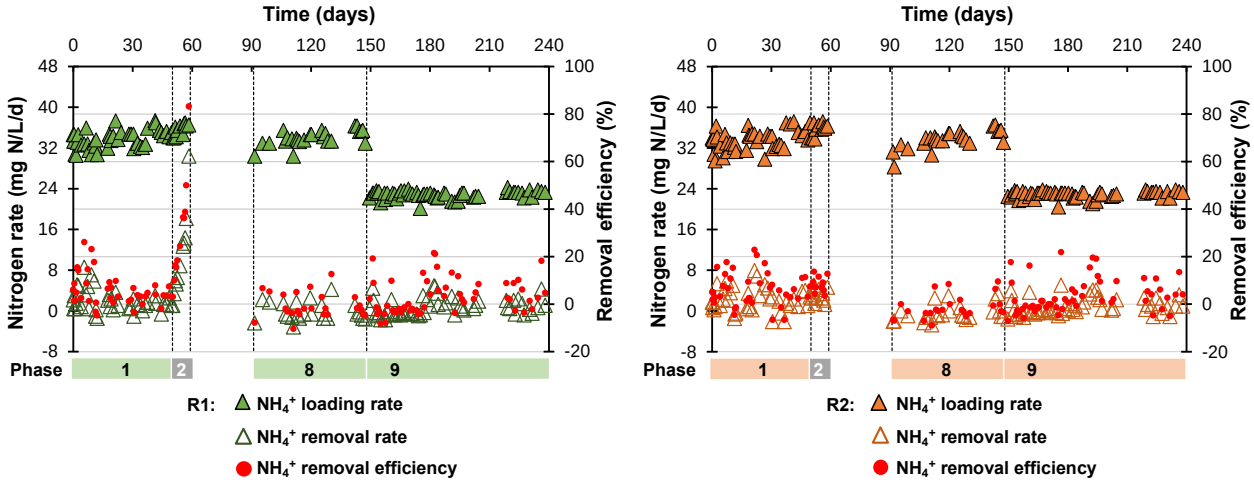

Fig. 5.1 Ammonium removal performance of R1 and R2 in Anammox-favoring condition (Phase 2) and MDD-favoring condition (Phases 1, 8, and 9)

even aggravated. The removal rates of R1 and R2 reduced from 1.9 ± 2.2 mgNH_4^+-N.L^{-1}.d^{-1} (5.6 ± 6.6%) and 2.1 ± 2.3 mgNH_4^+-N.L^{-1}.d^{-1} (6.3 ± 6.9%) to -0.2 ± 1.7 mgNH_4^+-N.L^{-1}.d^{-1} (-0.6 ± 5.2%) and -0.42 ± 1.6 mgNH_4^+-N.L^{-1}.d^{-1} (-1.41± 4.9%), respectively. In other words, Anammox activity was deficient for ammonium removal in both reactors, and DNRA process or ammonification may have occurred in both reactors. We reduced the ammonium loading rate to 22.9 ± 0.7 mgNH_4^+-N.L^{-1}.d^{-1} in phase 9 to alleviate the poor ammonium removal. Both reactors recovered, but after 90 days, the removal rates were still at a low range of 0.7 ± 1.7 mgNH_4^+-N.L^{-1}.d^{-1} (3.2 ± 7.3%) for R1 and 0.8 ± 1.6 mgNH_4^+-N.L^{-1}.d^{-1} (3.5 ± 7.0%) for R2. The insufficient concentration of NO_2^- solely originated from the nitrate denitrification might be the reason for the fluctuating and impractical Anammox activity in both reactors (Talan et al., 2021). Qiao et al. (2017) reported K_m value of nitrite at 4.18 – 7.66 mmol.L^{-1} for AnAOB, while He et al. (2013) obtained K_m value at only 0.91 ± 0.09 mmol.L^{-1} for nitrite-MDD bacteria at 30 ^oC. This considerably lower K_m of MDD bacteria explained their superiority over AnAOB in a nitrite-limited environment. In addition, as shown in Table 5.2, the higher values of nitrite reduction rates by nitrite-MDD activity (r2) over nitrite reduction rates by Anammox activity

(r1) in both reactors were confirmed. Indeed, the decrease in r1 values in phases 8 and 9 agreed with the deterioration of Anammox activity regarding ammonium removal.

To test the Anammox activity without nitrite competition, we created the Anammox-favoring condition in a short period of phase 2 by substituting NO_2^- for NO_3^- and Ar gas for CH_4 gas. As a result, with nitrite supply, a surge of ammonium removal rate to 31.4 $mgNH_4^+$-$N.L^{-1}.d^{-1}$ (83.2%) was confirmed, and the presence of Anammox activity in R1 was indicated by the production of a small amount of nitrate in the. Meanwhile, R2 showed only a slight increase in ammonium removal rate to 3.0 ± 1.3 $mgNH_4^+$- $N.L^{-1}.d^{-1}$ (8.4 ± 3.5%) and no nitrate was detected in the effluent. Under the condition favoring only Anammox (Phase 2), R1 inoculated with the sludge from parent Anammox reactors showed a remarkably higher ammonium removal, ten times higher than R2. However, the nitrate produced from the Anammox process resulted in an insignificantly higher value in R1's TN removal compared to R2 in phase 2.

5.3.1.2 Nitrite removal

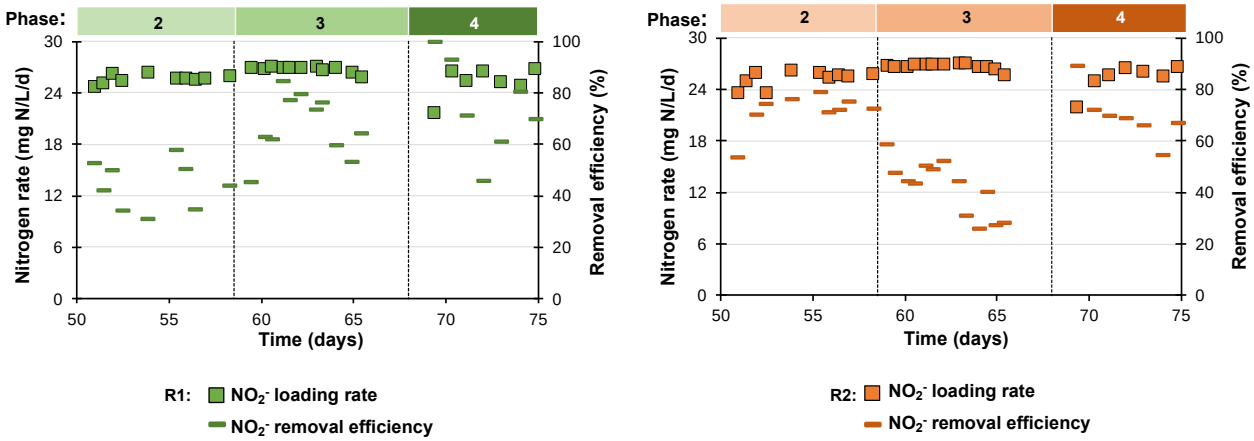

Fig. 5.2 Nitrite removal performance of R1 and R2 in Anammox-favoring condition (Phase 2), MDD-favoring condition (Phases 3), and without ammonium-CH_4 supply (Phase 4)

Nitrite can be removed from wastewater via denitrification and Anammox processes to nitrogen gas, or it can remain in wastewater in the form of ammonium via DNRA process.

When NO_3^- and NH_4^+ were fed as substrates, NO_2^- was always undetectable in both reactors. Therefore, we investigated the effect of inoculum on nitrite removal under the same loading rate of 25.9 ± 1 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ with the presence of either ammonium (phase 2), or methane gas (phase 3), or none of these supplements (phase 4) in Fig. 5.2. Under Anammox-favoring condition (phase 2), contrary to the behavior with ammonium, nitrite removal of 11.2 ± 2.4 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (44.1 ± 9.3%) in R1 was lower than that of 18.1 ± 2.3 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (71.7 ± 7.4%) in R2. While the removed NO_2^-/removed NH_4^+ ratio in R1 was near the theoretical Anammox ratio of 1.32 in Eq. (5.2), this ratio in R2 notably elevated at 6.0 ± 1.9. On the other hand, we observed a reverse pattern in nitrite removal under the nitrite-MDD-favoring condition with CH_4 supply in phase 3. R1's nitrite removal increased to 21.4 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (79.8%) in the first three days, then decreased to 14.0 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (53.35%) in 3 days later. Meanwhile, R2's nitrite removal gradually declined from 15.7 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (58.9%) to 7.18 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (27.2%) during this period. When none of the favored conditions was applied in phase 4, R1 witnessed a fluctuated nitrite removal at 18.7 ± 3.8 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (74.2 ± 17.1%) and a small amount of nitrate appeared in effluent at a concentration of 1.5 ± 1.2 $mgNO_3^-$-$N.L^{-1}$; while R2 observed a more stable nitrite removal at 17.6 ± 1.6 $mgNO_2^-$-$N.L^{-1}.d^{-1}$ (69.8 ± 9.5%) and no nitrate in the effluent. Besides AnAOB, MDD microbes and other DNB are likely to co-exist and support R1's performance in phases 3 and 4. AnAOB and MDD microbes surpassed DNB under their favored condition (phases 2 and 3, respectively), but without these conditions, DNB became the major denitrifier of R1 in phase 4. There was no organic substrate fed for heterotrophic denitrifier, so the organic matter might be secreted by microorganisms or released from biomass decay at an uncontrollable level. It resulted in the fluctuating nitrite removal. As for R2's performance, we did not observe high specific activities as in R1 due to the diversity of microorganisms in paddy soil inoculated in R2. In contrast, we noticed that R2 achieved a stably higher nitrite removal in phase 4 and an unexpectedly high

nitrite removal in phase 2. In these phases, the higher nitrite removal rate suggests outstanding heterotrophic denitrifying activity in the paddy soil. It was retained throughout the operation and only suppressed when methane gas was provided in phase 3. The existence of heterotrophic bacteria in autotrophic bioreactors seems inevitable due to the syntrophic relationship of growth factors and cross-feed, as proposed by He et al. (2015). Although adding ammonium in phase 2 did not harm the heterotrophic denitrification activity, methane addition probably inhibited it. Also, the original MDD activity in paddy soil was at a minor level compared to enriched sludge, which leads to R2's diminution in nitrite removal in phase 3.

5.3.1.3 Nitrate removal

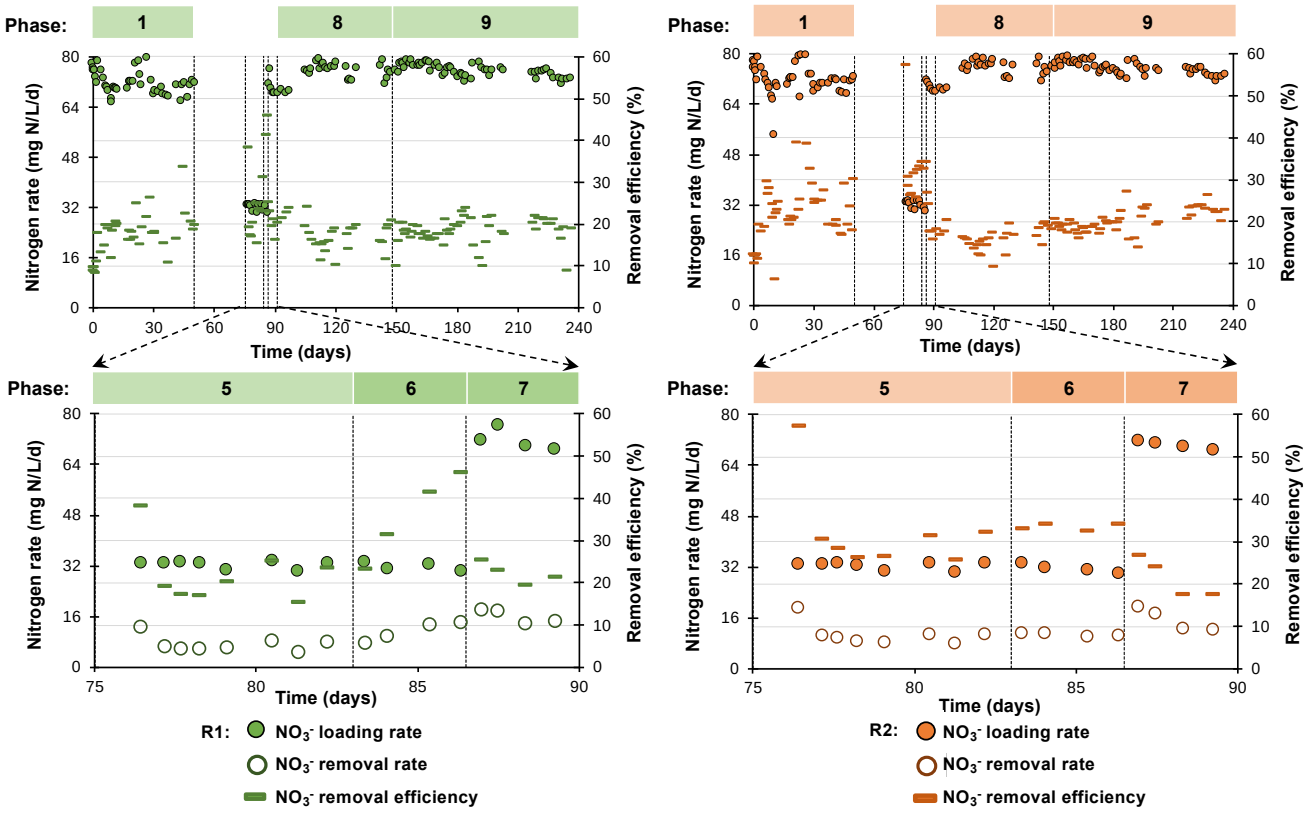

Fig. 5.3 Nitrate removal performance of R1 and R2 when solely supply nitrate (Phase 5 – without CH_4 and Phase 6, 7 – with CH_4) and when supply with ammonium (Phases 1, 8, and 9 with CH_4)

Treatment of nitrate, the most widespread nitrogen pollutant, was our focus in most of the operation period. Nitrate was supplied to the reactors as the sole nitrogen source in phases

5 – 7 and as the major substrate coupling with ammonium in phases 1, 8, and 9, as described in Table 5.1 and Fig. 5.3. As mentioned in section 5.3.1.1, we expected a synergistic interaction of Anammox and MDD processes to remove both nitrate and ammonium. Despite our expectation, nitrate removal outperformed ammonium removal at all phases, whereas nitrate loading rate was 74.1 ± 3.6 $mgNO_3^-$-$N.L^{-1}.d^{-1}$, 2-3 times higher than ammonium loading rate. Similar to ammonium removal, nitrate removal of R2 is relatively higher than that of R1 in phases 1 and 9, but lower in phase 8. In phase 1, R2 removed 14.9 ± 5.2 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (21.7 ± 6.9%), while R1 removed 12.6 ± 3.9 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (17.4 ± 5.2%). Then both reactors slightly improved their performance in phase 9, R2 and R1's removal rate rose to 15.2 ± 1.8 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (20.1 ± 2.7%) and 13.0 ± 2.4 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (17.5 ± 3.5%), respectively. Before that, R2 suffered a downturn in both nitrate and ammonium removals in phase 8, its nitrate removal rate reduced to 11.9 ± 1.8 mg NO_3^--$N.L^{-1}.d^{-1}$ (15.9 ± 2.6%). The effluent nitrate concentration depends on both removed nitrate by denitrification and produced nitrate by Anammox reaction. Thus, our reactors' actual nitrate removal rates were lower than the estimated value (r3) in Table 5.2. This result also indicates MDD reactions in our reactor were likely to be attributed to other microorganisms than ANME-2d and NC10 assumed in Table 5.2. The increase in MDD activity of R1 and R2 at the latter phases suggests prolonged operation and proper ammonium concentration are required to improve the denitrification rate in this Anammox-MDD combination.

We also monitored the nitrate removal capacity of both reactors without ammonium supplement in phases 5-7. Although nitrate in phases 5 and 6 was used at a comparable level to nitrite in phases 3 and 4, nitrate removal rates were significantly and consistently lower than that of nitrite. We also observed the same pattern in Chapter 4. The DNB in R2 functioned better not only in removing nitrite but nitrate as well. As a result, R2's nitrate removal of 10.6 ± 3.3 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (32.5 ± 9.8%) was higher than R1 of 7.2 ± 2.3 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (22.3

± 6.9%) in phase 5. Methane gas did not hinder heterotrophic nitrate reduction as much as the heterotrophic nitrite reduction (phase 3), so that R2 removed nitrate at the same level in phases 5 and 6. Meanwhile, R1 possessing MDD microbes in enriched sludge doubled the nitrate removal from 7.2 ± 2.3 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (22.2 ± 6.9%) in phase 5 to 14.0 $mgNO_3^-$-$N.L^{-1}.d^{-1}$ (46.1%) at the end of phase 6 with methane gas supply. Subsequently, we restored the nitrate loading rate in phase 7 at a level as high as at the period couple to ammonium. Despite a moderate increase in nitrate removal rate, a drop in nitrate removal efficiency happened in both reactors.

The R1's behavior of nitrate, nitrite and ammonium removal consolidate the contribution of Anammox and MDD activities in the enriched sludge to its greater performance under Anammox and MDD-favored conditions. Nevertheless, the diversity of microorganisms in the paddy soil appears to adapt quickly and performs moderately better in nitrogen removal in general under unspecific conditions.

Table 5.2 Relationship of Anammox and MDD processes in denitrification performance in R1 and R2

Phase (days)	R1				R2			
	r1	r2	r3	rTN (%)	r1	r2	r3	rTN (%)
1 (0 – 50)	0.11 ± 0.10	0.33 ± 0.17	0.45 ± 0.13	13.51 ± 3.72	0.14 ± 0.11	0.53 ± 0.18	0.68 ± 0.22	16.88 ± 5.99
[Actual]			[0.43 ± 0.13]				[0.65 ± 0.22]	
8 (91 – 147)	0.09 ± 0.05	0.42 ± 0.09	0.51 ± 0.10	13.00 ± 7.35	0.07 ± 0.07	0.36 ± 0.07	0.43 ± 0.09	10.60 ± 2.31
[Actual]			[0.46 ± 0.08]				[0.42 ± 0.06]	
9 (148 – 240)	0.09 ± 0.07	0.44 ± 0.13	0.52 ± 0.09	15.70 ± 5.35	0.08 ± 0.08	0.52 ± 0.15	0.60 ± 0.15	16.23 ± 2.53
[Actual]			[0.50 ± 0.08]				[0.56 ± 0.13]	

r1 is the calculated nitrite reduction rate (mmol.d^{-1}) by Anammox in Eq. (5.2)

r2 is the calculated nitrite reduction rate (mmol.d^{-1}) by MDD in Eq. (5.3)

r3 is the calculated nitrate reduction rate (mmol.d^{-1}) by MDD in Eq. (5.1)

[Actual] is the measured nitrate removal rate (mmol.d^{-1})

rTN is the measured total nitrogen removal efficiency (%)

Table 5.3 Alpha diversity indices of microbial community

Indices		Observed species	Chao	Ace	Shannon	Simpson	Good's coverage
R1	a1 (sludge)	353.6 ± 6.6	587.3 ± 43.8	694.5 ± 54.2	3.43 ± 0.01	0.10 ± 0.001	0.98
	b1 (day 0)	237.6 ± 8.0	463.2 ± 59.7	624.6 ± 83.0	2.61 ± 0.02	0.16 ± 0.002	0.99
	c1 (day 240)	610.3 ± 10.9	1027.1 ± 61.6	1300.0 ± 97.6	4.26 ± 0.02	0.06 ± 0.001	0.97
R2	a2 (paddy soil)	2627.7 ± 18.2	6923.5 ± 248.3	11432.1 ± 439.6	6.87 ± 0.01	0.0 ± 0.0	0.79
	b2 (day 0)	316.0 ± 0.0	716.9 ± 0.0	1142.0 ± 0.0	2.64 ± 0.0	0.22 ± 0.0	0.98
	c2 (day 240)	283.6 ±5.1	351.7 ± 20.0	404.0 ± 31.1	3.73 ± 0.01	0.07 ± 0.001	0.99

5.3.2. Microbial community dynamics

A total of 81070 16S rRNA sequence reads were obtained from the six samples of two reactors and clustered into 4560 OTUs. After normalization to the smallest reads of 8073 (in b2), alpha diversity indices of all samples were calculated and shown in Table 5.3. The Good's coverage index in all samples was over 0.97, except for soil sample (a2) at only 0.79 because of its high diversity. Biomass in R1 and R2 witnessed an opposite trend in alpha diversity metrics. R2 had the highest values of observed species Chao, Ace, and Shannon in a2, but decreasing values in b2 and c2; whereas R1's inoculum (a1) has low values, increasing the richness in b1 and c1. These diverging results on alpha diversity suggest specialized and non-specialized inocula behaved in two opposite ways under the same operational condition. Less nutrient in the influent than natural environment (i.e., paddy soil) led to lower diversity in R2, whereas the different compositions from previous parent reactors sightly increased the diversity in R1.

More than 98% of total reads in all samples were assigned to bacterial phyla. The bacterial populations in all samples were dominated by phylum *Proteobacteria* with classes *Gammaproteobacteria* (7.09 – 53.08%) and *Alphaproteobacteria* (5.08 – 39.64%), phylum *Bacteroidota* – formerly *Bacteroidetes* (7.11 – 45.01%), phylum *Planctomycetota* – formerly *Planctomycetes* (0.41 – 18.26%), phylum *Acidobacteriota* – formerly *Acidobacteria* (0.90 – 19.36%), phylum *Chloroflexi* (5.24 – 14.41%), phylum *Actinobacteriota* – formerly Actinobacteria (0.44 – 8.08%), phylum *Verrucomicrobiota* – formerly *Verrucomicrobia* (0.79 – 8.45%), phylum *Desulfobacterota* – formerly referred to class *Deltaproteobacteria* of phylum *Proteobacteria* (0 – 12.41%), phylum *Cyanobacteria* (0.01 – 4.61%), and phylum *Nitrospirota* – formerly *Nitrospirae* (0 – 7.93%) (Fig. 5.4). Our microbial community tally with the major phyla of denitrifying communities in wastewater treatment summarized by Lu et al. (2014) and denitrifying methanotrophic communities in previous studies (Alrashed et al., 2018; Guerrero-

Cruz et al., 2021; Lee et al., 2019; Osaka et al., 2008). *Proteobacteria* consistently had the highest relative abundances across all samples, increased from 58.50% to 67.50% in R1, and increased from 19.05% to 66.46% in R2. This phylum was found to be prominent in both natural and bioreactors due to their versatile nitrogen and carbon metabolisms (Zhang et al., 2021), especially on denitrification and methane oxidation (Guerrero-Cruz et al., 2021; Zhu et al., 2016). Phyla *Desulfobacterota* and *Nitrospirota* were detected at high percentages of 12.4% and 7.9% in soil inoculum (a2), respectively, but very few at 0.81% and 0.01% of them, respectively, in sludge a1, and almost undetected in reactors' biomass (b1, b2, c1, and c2). They are commonly known for sulfate reduction and nitrite oxidation in paddy soil (Han et al., 2018; Zecchin et al., 2018), whereas we did not feed sulfate and nitrite in the medium. That may be the reason why they diminished in the reactors' microbial community.

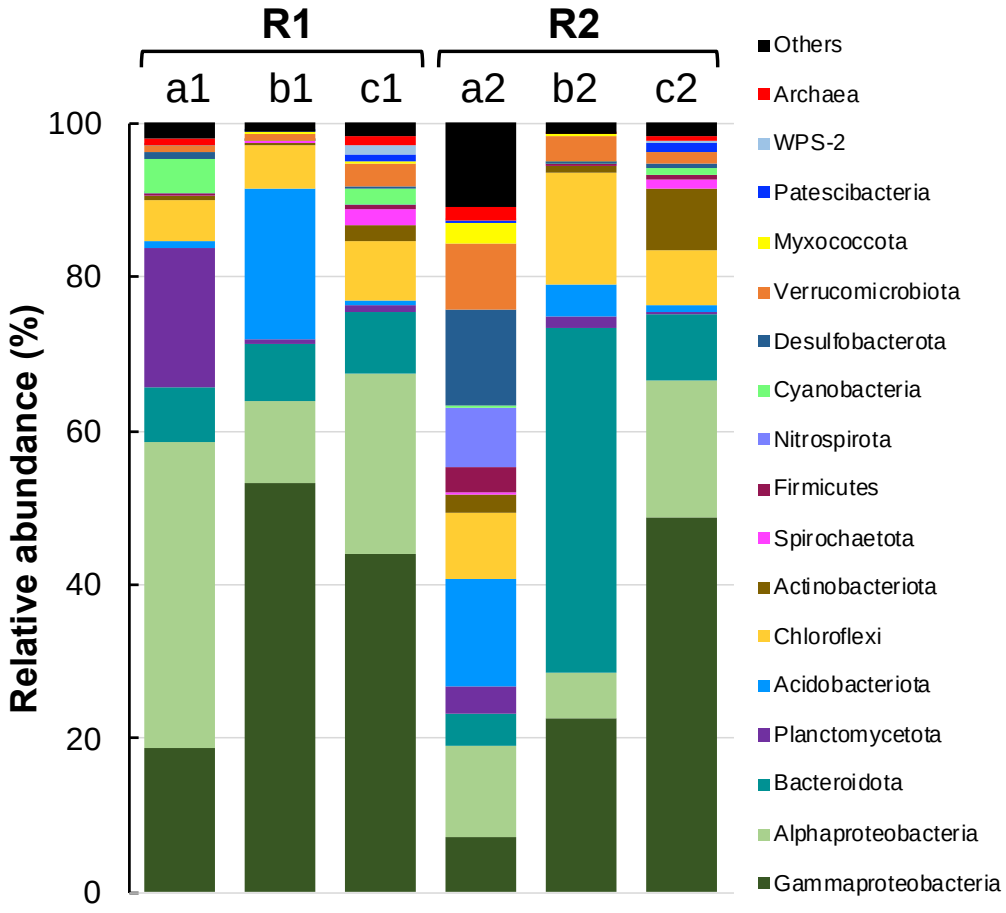

Fig. 5.4 Detection rates of archaea, predominant bacterial phyla (>1% frequency in at least one sample), and classes (α and γ) in phylum Proteobacteria in six samples.

Archaea merely accounted for an extremely low abundance, less than 1.8% of total sequencing read in each sample, even no detection in b1. The relative abundance of archaea in R1 increased from 0.86% of total reads in a1 to 1.13% in c1, whereas that in R2 decreased from 1.79% of total read in a2 to 0.74% in c2. This underweight figure is different from other previously reported MDD reactors dominated by ANME-2d group, which is believed to have a critical role in reducing nitrate into nitrite, a substrate for complete denitrification in MDD bacteria and ammonium removal in Anammox bacteria (Ding et al., 2014; Fu et al., 2017; Hu et al., 2015). Other studies operated with methane and nitrate, ammonium obtained a high amount of archaea range from 35.1% microorganisms observed by fluorescence in situ hybridization in Ding et al. (2014) to 71% community analyzed by Illumina HiSeq2000 in Hu et al. (2015) or 74.3% community in (Fu et al., 2017).

The outnumber of the bacterial population over archaeal population in all samples indicates its crucial role in reactors' nitrogen removal performance. The top 10 genera having the highest relative abundance in each sample were summarized into a total of 34 genera in Fig. 5.5. As mentioned in the phylum level, we observed some genera predominant in the soil sample, a2, (i.e., *ADurb.Bin063-1* – 2.75%, unclassified genus of class *Thermodesulfovibrionia* – 5.10%, *4-29-1_ge* – 2.44%, unclassified genus of order *Acidobacteriales* – 3.72%, unclassified genus of family *Geobacteraceae* – 4.26%, *Desulfobacca* – 3.68%), but no detection in neither R2's b2, c2 and R1's a1, b1, c1. Methane was the only carbon source supplied to both reactors, so methanotrophic and methylotrophic are predominant in biomass samples (i.e., b1, b2, and c1, c2). Most of them are capable of complete or partial denitrification, such as family *Pedosphaeraceae* of phylum *Verrucomicrobiota* (Dalcin Martins et al., 2021), and methylotrophic genus *Hyphomicrobium* paired with methanotrophic genus *Methylocystis* in class *Alphaproteobacteria* (Dam et al., 2013; Jeong and Kim, 2019; Martineau et al., 2015). The genera *Methylosarcina* and *Methylocaldum* within the class *Gammaproteobacteria* can

utilize both methane and methanol for carbon sources and both nitrate and ammonia for nitrogen sources (Takeuchi et al., 2014; Wise et al., 2001). The gammaproteobacterial methanotroph *Methylomonas denitrificans* can couple methane oxidation to nitrate reduction and releasing nitrous oxide (Kits, Klotz, et al., 2015). Recently, the metabolic versatility of methanotrophs in *Gammaproteobacteria* has been reported (Grinsven et al., 2020; Guerrero-Cruz et al., 2021). They could be active both aerobic and anoxic methane/nitrate-rich conditions. *Methylosarcina* was the most predominant in c1 and c2 with 21.02% and 21.82%, respectively; however, *Methylocaldum* had significantly lower relative abundances at 0.33 % and 0.03% in c1 and c2, respectively.

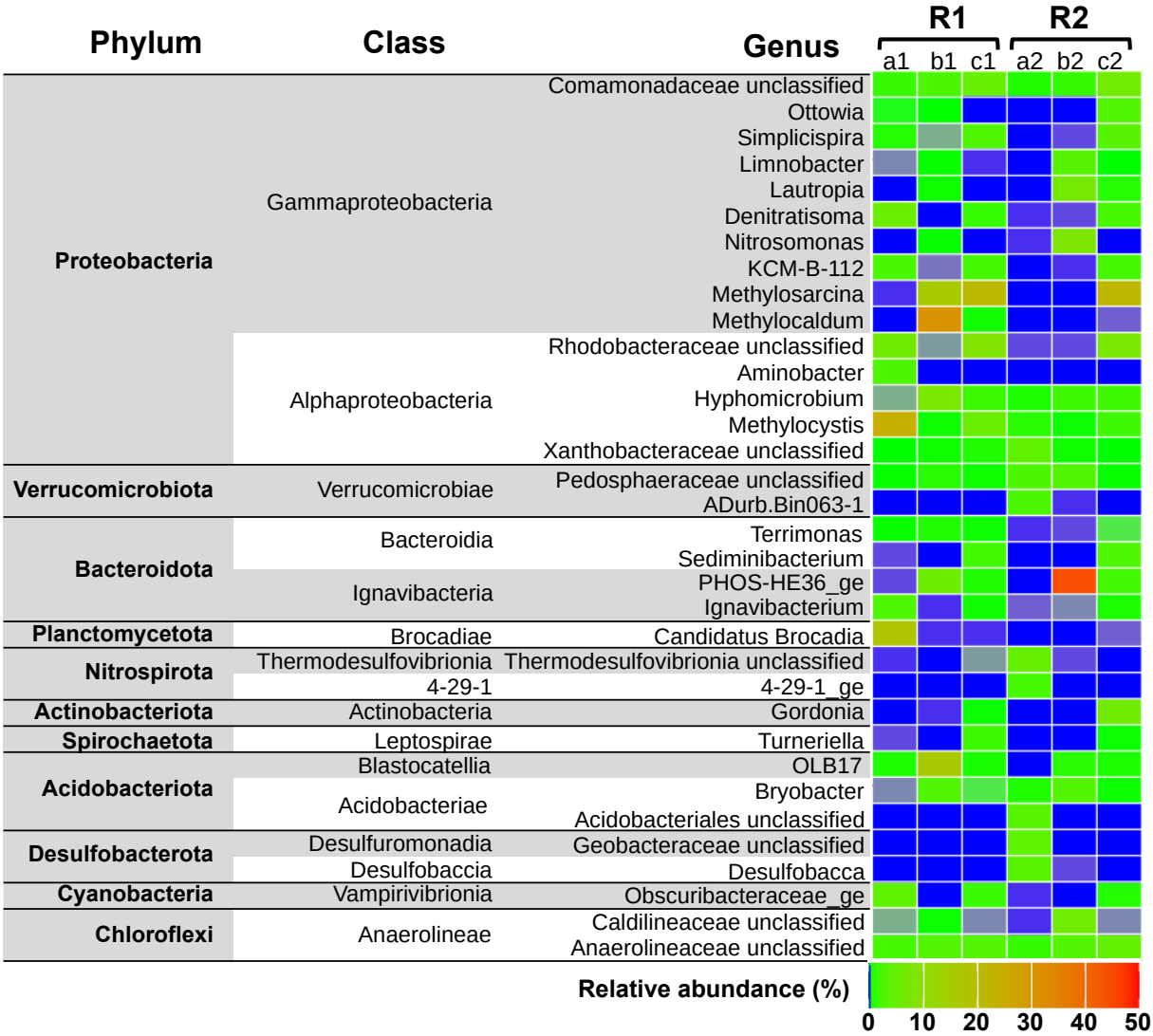

Fig. 5.5 The top 10 predominant genera in each sample (a total of 34 genera) and their taxonomy at class and phylum levels

These methanotrophs may potentially perform denitrification in the reactor, but more detailed analyses are required to determine their involvement in denitrification. The others, such as methylotrophic unclassified genera in families *Rhodobacteraceae* (Karwautz et al., 2018) and *Xanthobacteraceae* (Kappler and Nouwens, 2013), may indirectly contribute to denitrification by providing organic substrate for heterotrophic DNB. In the predicted enzymes by PICRUSt2 analysis, we found Mcr – archaeal methane-oxidizing enzyme in only a2, but Pmo – bacterial particulate methane monooxygenase in all reactors' biomass. These aerobic methanotrophic bacteria accounted for outstanding abundance values compared to ANME or NC10 in all samples. Although our reactors' dissolved oxygen was kept under detectable level (0.01mg.L^{-1}) by purging N_2 into the influent bottle in medium preparation and continuously purging mixture of CH_4/CO_2 (95/5%, v/v) gas into reactors, the ORP values of influent and effluent were high at about 114 – 319 mV, which is likely the reason why aerobic methanotrophs dominated in both reactors. Instead of *Methylomirabilis oxyfera*, ANME-2d archaea, and AnAOB, aerobic methanotrophic bacteria were found to be the key player in hypoxic condition adding methane and nitrate when inoculum is dominated by *Proteobacteria* (Alrashed et al., 2018; Lee et al., 2019). Modin et al. (2007) reported the extremely low C/N ratio of 0.625 for denitrification by anaerobic methanotroph, but much higher C/N ratio of 1.27 – 8.3 for denitrification by aerobic methanotroph, which implies that aerobic methanotroph requires more methane for denitrifying the same amount of nitrogen. This could explain our lower nitrogen removal rates compared to reactors possessing ANME-2d or NC10 (Ding et al., 2014; Fu et al., 2017; He, Wang, et al., 2015).

Methanotrophic enzymes, Pmo and Mdh, producing methanol and formaldehyde, respectively, were also predicted in all samples. It may indicate the role of methanotrophic/methylotrophic bacteria in providing carbon sources for syntrophic DNB in consortia; thus, we also found many heterotrophic denitrifiers in reactors' biomass as shown in

Fig. 5.5. Unclassified bacteria in family *Comamonadaceae* accounted for the highest detection rates of 5.20% and 6.51% in c1 and c2, respectively, followed by genus *Simplicispira*, genus *Denitratisoma*, genus *Gordonia*, genus *Ignavibacterium*, genus *Terrimonas*, and unclassified bacteria in family *Caldilineaceae*. These DNB were detected in both reactors, while denitrifying genera *Lautropia* and *Ottowi*a were detected in only R2's c2 at 0.79% and 3.20%, respectively. In addition, heterotrophic bacteria, such as genera *Bryobacter*, *Sediminibacterium*, *Aminobacter*, and *Limnobacter*, can grow on organic carbon produced by methanotrophs or produce growth factors to stimulate the development of methanotrophs (He, Wang, et al., 2015; Wang et al., 2018).

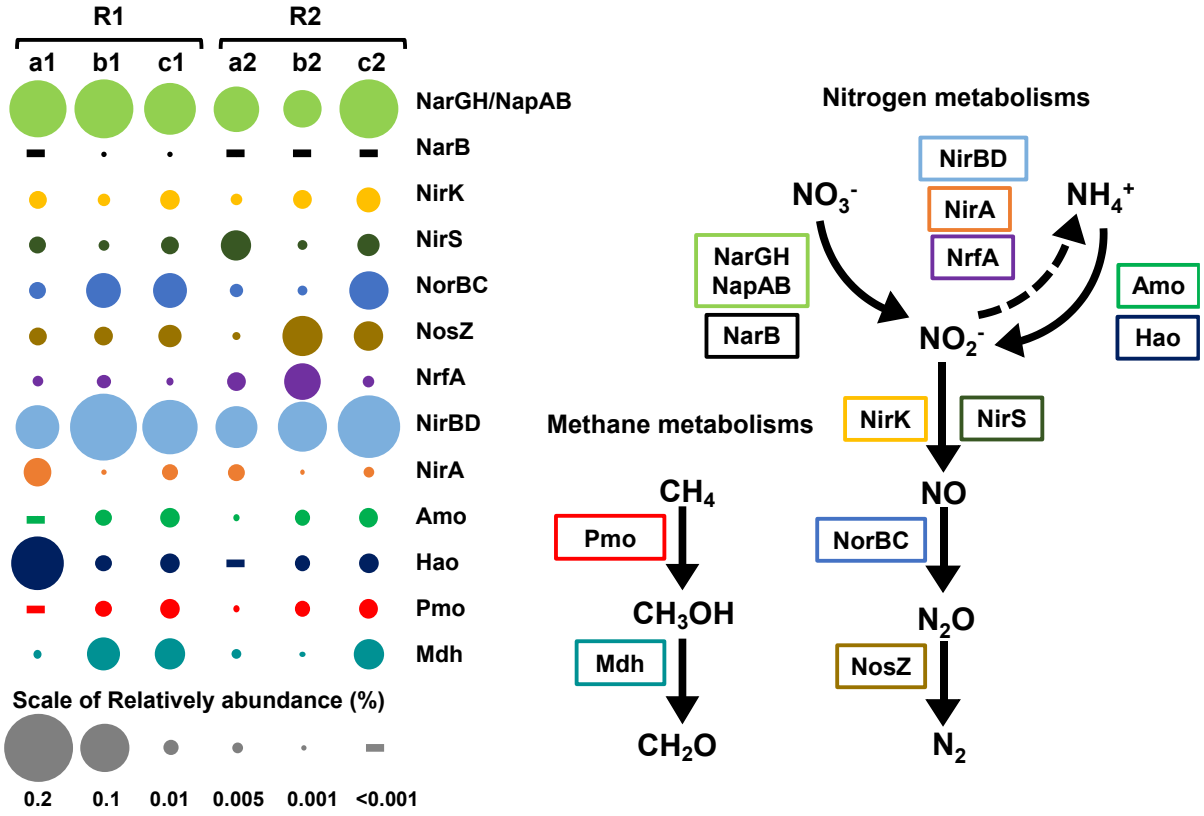

Fig. 5.6 Relative abundances of predicted enzymes contribute to nitrogen metabolism and methane oxidation from 16S rRNA genes by PICRUSt2

Nitrogen metabolism occurs in all microorganisms, and was not restricted to only the dominant genera listed in Fig. 5.5. Therefore, Fig. 5.6 that summarizes the relative abundances

of enzymes in nitrogen and methane metabolism pathways can provide an overview of microbial functions and elucidate reactor behavior. Complete denitrification enzymes (NarGH/NapAB, NirK, NorBC, NosZ) and anammox's marker enzyme (Hao) were predicted at a higher percentage in mixed sludge (a1) than that in paddy soil (a2); however, after a long operating period without organic carbon and nitrite, mixed sludge gradually lost its advantages. Meanwhile, paddy soil (a2) with a higher abundance of methanotrophic enzymes (Pmo, Mdh) was able to better utilize CH_4 and convert it to organic matter for the growth of syntrophic DNB in b2 and c2. Nevertheless, enzymes involved in the DNRA process (NirBD, NirA, NrfA) were predicted to be comparable to nitrate reductase (NarGH/NapAB) in both R1 and R2. This result and the disappearance of *Nitrosomonas* (an AOB) and *Candidatus* Brocadia (an AnAOB) as shown in Fig. 5.5 could explain the poor ammonium and nitrate removal efficiencies in our reactors.

5.4. Conclusions

The effect of inoculum on autotrophic nitrogen removal behaviors was investigated in two reactors. R1 inoculated with mixed sludge from parent reactors showed better performance in conditions favoring Anammox (phase 2), nitrite MDD (phase 3), and nitrate MDD (phase 6) processes with the higher removal rates of 31.4 $mgNH_4^+$-$N.L^{-1}d^{-1}$, 21.4 $mgNO_2^-$-$N.L^{-1}d^{-1}$, and 14.0 $mgNO_3^-$-$N.L^{-1}d^{-1}$, respectively. On the other hand, paddy soil was proved to be a feasible inoculum, which adapted fast to several changes in operational conditions and performed higher nitrogen removal in unspecialized phases. The higher abundance of DNB (e.g., genera *Gordonia, Ignavibacterium, Denitratisoma, Ottowia,* and *Lautropia*) in R2 is likely to contribute to its higher adaptation. In addition, our reactors' microbial communities were dominated by bacteria, especially aerobic methanotroph (e.g., genera *Methylocystis, Hyphomicrobium,* and family *Rhodobacteraceae* in class *Alphaproteobacteria* and genus *Methylosarcina* in class *Gammaproteobacteria*). They can directly denitrify or indirectly

contribute to denitrification via syntrophic DNB. Anaerobic denitrifying methanotrophs (i.e., ANME-2d and NC10) were not detected in our reactors' biomass. This study provides evidence for the denitrification capacity of aerobic methanotrophic bacteria, which can be applied in hypoxic environments. Further investigation into its practical use and control strategies is needed in future research.

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CHAPTER 6. Summary and recommendation for future research

6.1. Summary

This study focused on developing anaerobic bioreactor for treating major pollutants in wastewater, i.e., organic matter and nitrogen compounds. Firstly, an anaerobic baffled reactor (ABR) was implemented to treat natural rubber processing wastewater, an example of carbon-rich industrial wastewater. The ABR could converted organic compounds into methane-rich biomass; however, the remaining dissolved inorganic nitrogen (DIN) still needs to be polished prior to discharging to receiving water bodies. Next, methane-driven denitrification (MDD) process was investigated in laboratory-scale experiment using a closed type downflow hanging sponge (DHS) and upflow reactors equipped hollow fiber membranes to remove DIN without any additional organic carbon source. The findings of each study are summarized as follows:

Chapter 3 reported the application of ABR in natural rubber processing wastewater, containing high concentrations of organic compounds, mainly formic or acetic acid, and residual rubber particles. The ABR's continuous compartments is a promising configuration for an integrated system, which prevented low pH shock for methanogens and eliminated the clogging risk from suspended solid (SS), especially rubber particle in influent by extended front compartments. The highest COD and total SS removal efficiencies were observed at 92.3 ± 6.3% and 90.0 ± 6.0%, respectively, under an OLR of 1.4 ± 0.3 kg-COD.m^{-3}.d^{-1}. The microbial analysis of biomass in each compartment revealed most of the methanogens, particularly acetate-utilizing methanogens, were predominantly distributed in the 3rd, 4th, and 5th compartments, where volatile fatty acid concentration considerably decreased, and the highest biogas production was observed. In front compartments, several acetogens growing under low pH and ammonia-utilizing bacteria were detected, which is adapted to the influent characteristics.

Chapter 4 described a modified DHS reactor for anaerobic MDD application. The adjustment of hydraulic retention time (HRT) and the supplement of titanium(III) nitrilotriacetate (Ti(III)-NTA) as a reducing agent were applied to (i) improve nitrogen removal performance and to (ii) eliminate N_2O emission. Under the most optimal operational conditions including HRT of 12 h, the addition of 25 µM Ti(III) as final concentration, the nitrogen removal rates doubled to 4.1 ± 1.9 mgNO_3^--N.L^{-1}.d^{-1} and 6.6 ± 3.3 mgNO_2^--N.L^{-1}.d^{-1}; and the N_2O emission was 0.7×10^{-4}% to 61.4×10^{-4} % of removed NO_x^-. The closed-type DHS reactor emitted only a trace amount of N_2O compared to previous MDD studies using other reactors.

Chapter 5 demonstrated the application of upflow reactor equipped with U-shaped hollow fiber membrane as gas diffusors in MDD process. With the same inoculum as the DHS in chapter 4, hollow fiber membrane improved the nitrogen removal performance at 2-3 times. In addition, the effects of inocula on reactors' performance were determined during 240-day operation with various substrates' combination. The reactor inoculated with sludge mixture from parent reactors showed better performance in specialized conditions (i.e., Anammox, or nitrite MDD, or nitrate MDD processes) with the higher removal rates of 31.4 mgNH_4^+-N.L^{-1}.d^{-1}, 21.4 mgNO_2^--N.L^{-1}.d^{-1}, and 14.6 mgNO_3^--N.L^{-1}.d^{-1}. On the other hand, the reactor inoculated with paddy soil could quickly adapt to any changes and reached higher nitrogen removal under non-specialized conditions. The microbial communities were dominated by bacteria, especially aerobic methanotroph. They may directly denitrify or indirectly contribute to denitrification via syntrophic association with heterotrophic denitrifying bacteria (DNB). The higher abundance of DNB in the reactor with paddy soil is likely to contribute to its high adaptation.

Overall, this study demonstrated some anaerobic bioreactor configurations to facilitate MDD process as a novel autotrophic denitrification, which requires no extra organic supply

and mitigates N_2O emission as in conventional denitrification process. Also, the microbial analysis provided an insight that under oxygen-limited condition, microbial community predominated by aerobic methanotrophic bacteria were able to denitrify without well-known anaerobic methanotrophs.

6.2. Recommendation for future research

The nitrogen removal performance in Chapter 4 and 5 were still low regardless some attempts in changing operation strategy, such as changing HRT, changing substrate composition, and adding reducing agent. But these changes seem not to resolve the primary issue, i.e., the low activity of microorganism presenting inside reactors. Understanding aerobic methanotroph was the main population in charge for denitrification in these reactors, some recommendation for further research in MDD application are as follows:

- Screening composition of inoculum must be done in advance, even bioaugmentation with high-performance biomass would be considered to achieve a high abundance of MDD population.
- The oxidation-reduction potential (ORP) would be kept negative to ensure denitrification process can happen, dissolved oxygen in wastewater is not enough to evaluate the anaerobic condition.
- Nitrite was always undetectable in effluent despite its essential role in triggering Anammox or nitrite-MDD process, which is the final step to convert nitrogen compound completely to N_2 gas. Therefore, a sufficient addition of NO_2^- would enhance the whole performance.

Furthermore, in this study, the dynamic of DIN was the main focus, but information about methane consumption is lacking due to gas collection ability (chapter 5). Some modification in

reactor configuration is needed to improve liquid-gas separation in future research as well, thus it can obtain more comprehensive information about MDD process.