Doctoral dissertation

博士学位論文

Anaerobic Treatment of *N*, *N*-Dimethylformamide (DMF)-Containing Industrial Wastewater

(*N*, *N*-ジメチルホルムアミド(DMF)含有産業排水の嫌気性処理)

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Abstract

With the rapid development of modern industrialization and economic globalization, the massive quantity of industrial wastewater discharged from a variety of chemical industries is becoming a worldwide formidable environmental issue which causes negative effects on ecosystem and also seriously endangers human health. The degradation-resistant organic wastes contained in the chemical wastewater own low BOD and are persistent to biodegradation. Conventional treatment processes are considered not suitable to the treatment of high strength chemical organic wastewater. Anaerobic digestion, as an efficient, eco-friendly, economic and energy-saving biological process, is extensively applied in the anaerobic treatment of a variety of wastewater streams and organic wastes. In this study, the author selected a common organic compound *N*, *N*-dimethylformamide which has been widely applied and implemented in the industries like textile, manufactory of synthetic leather and fiber, polymer dissolution, pesticides and pharmaceuticals as organic polar solvents. While DMF has excellent versatility, the excessive discharge of DMF-containing industrial wastewater is becoming a critical environmental issue. DMF is known for its hepatotoxicity and carcinogenicity, and its thermal stability and weak degradability make this compound obstinate and recalcitrant in nature. Few studies have focused on the anaerobic digestion of DMF because of its weak degradability. However, a great number of studies focused on the aerobic degradation of DMF. In this dissertation, by acknowledging the previous studies on aerobic degradation of DMF, the author initially cultivated DMF-degrading activated sludge and then mixed it with normal anaerobic digested sludge to establish a co-cultured sludge. With the cooperation of hydrolysis and methanogenesis, this new consortium could effectively degrade DMF into methane

under the anaerobic condition. The author then applied this consortium in both lab-scale UASB and SAnMBR to investigate the feasibility and stability of this consortium. Results indicated that this co-cultured consortium could only temporarily remain activated for a few weeks. The DMF-hydrolyzing bacteria kept decaying all the time because they could not grow under the anaerobic condition. By investigating the characterization and kinetics of the microbial community structure. The author found these DMF-hydrolyzing bacteria are in fact denitrifying bacteria, they are facultatively anaerobic and could grow aerobically and anaerobically. However, because the system in this study is a methanogenic system without any nitrate dosage, these denitrifying bacteria were unable to grow. As a result, the author also proposed some suggestions and improvements as future perspectives to enhance the hydrolysis of this co-system and maintain a stable anaerobic treatment of DMF-containing wastewater.

CONTENT

Chapter 1 Exordium and general introduction

1.1 Research background

1.1.1 Global warming and energy shortage

It is widely recognized that as the organic matter (biomass, zoomass and phytomass) such as food, meat, plant debris, livestock manure, wasted sewage sludge, and biodegradable portions of municipal solid waste - undergoes the decomposition or biodegradation process in natural environment, it produces mainly carbon dioxide (CO_2) or methane (CH_4) depending on whether the biodegradation had occurred under

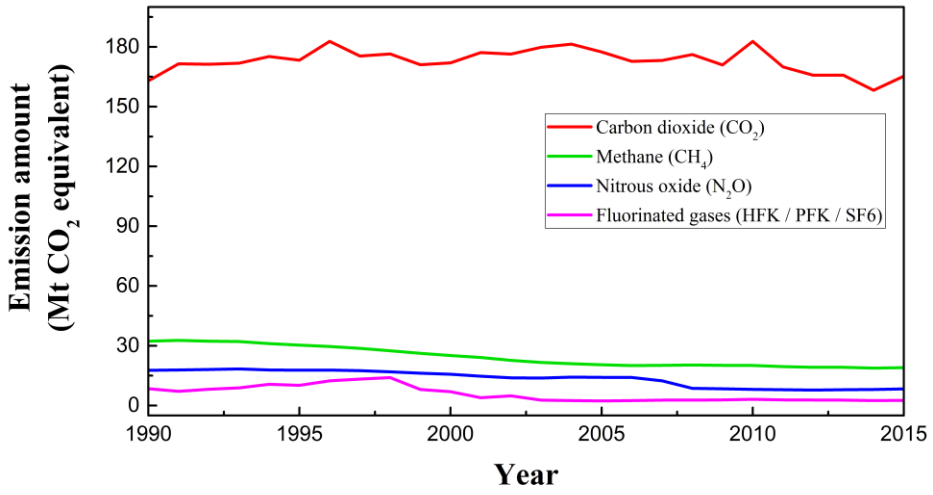

Fig. 1.1 Global greenhouse gases emissions from year 1990 - 2015.

aerobic or anaerobic conditions (Abbasi and Abbasi, 2010; Chynoweth, 2005; Ward et al., 2008). In absence of free oxygen, the biodegradation of organic matter normally generates a biogas which consists of 40 - 70% methane, the rest being mostly carbon dioxide with traces of other gases (Ferrer et al., 2011; Weiland, 2010). As shown in **Fig. 1.1**, the global warming is a worldwide concerned environmental issue due to the increasing emissions of greenhouse gases CO_2 and CH_4 (IEA, 2016). Although both

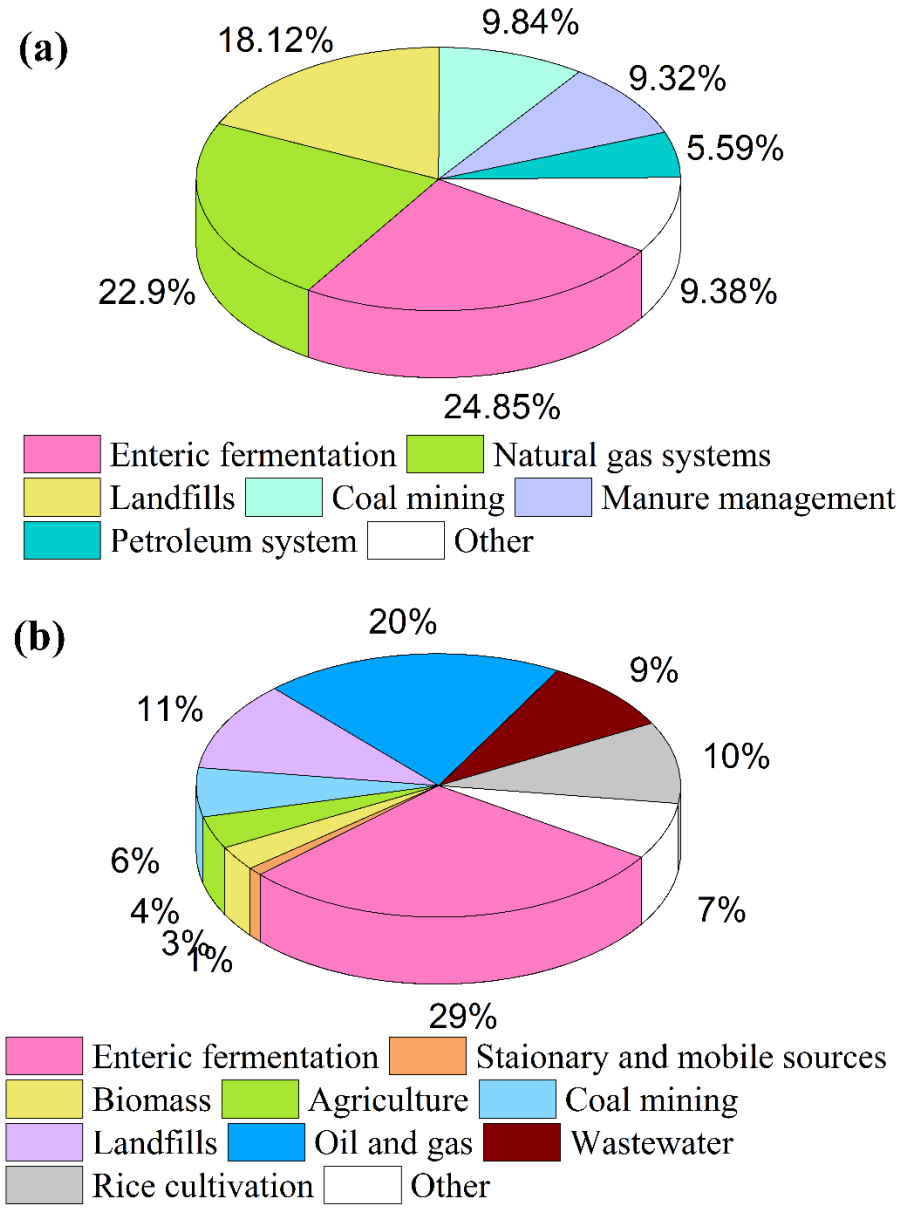

Fig. 1.2 Global methane emissions by source (a); estimated global anthropogenic methane emissions by source (b) in 2010.

the gases cause global warming, it is also now a well-accepted fact that methane is considered as a powerful greenhouse gas which brings about much serious damage to atmosphere than carbon dioxide, and each molecule of CH_4 causes about 25 times more global warming than a molecule of CO_2 (Abbasi et al., 2012). If we do not take steps to utilize organic wastes and recover methane from them but, instead, allow the waste to rot in the open, the naturally derived methane will escape into them atmosphere to

burden the global warming (Abbasi and Abbasi, 2010). As shown in **Fig. 1.2**, methane sources from a variety of organic matters (Pike, 2017), the dungs or dead bodies of animals lying in the open wild, the biodegradable portions of municipal solid waste which are abandoned everywhere; the dead plants decaying in the bottom mud of lakes, ponds and reservoirs; the human excreta or wasted sewage disposed and landfilled in soil, the wastewaters high in chemical oxygen demand (COD) of food processing, tanneries, distilleries and other industrial organic wastes discharged in public swears, etc., —all of these emit methane into the atmosphere and all contribute to global warming (Ramasamy et al., 2004; Sankar Ganesh et al., 2008). With the rapid industrialization, the developing countries emit more greenhouse gases, as listed in **Table 1.1**, statistics of the International Energy Agency (IEA) revealed that for the year 2005, China ranked the first in the world in methane emissions, then followed by India and the USA.

Table 1.1 Statistic data of top five methane-emitting countries or regions in 2005.

Country or region	**Kt of CO_2 equivalent**	**% of world in total**
China	1,333,098.10	18.7
India	583,977.60	8.2
United States of America	548,073.70	7.7
European Union	535,846.80	7.5
Brazil	492,160.70	6.9

1.1.2 Waste-to-energy strategy

Due to the rapid consumption and excessive utilization of fossil fuels, severe environmental and energy risks such as global climate change, world energy conflicts

and energy source shortages, have increasingly threatened world in past years. These global world problems can be summarized through the following three sections: 1) Decrease in fossil fuel reserves due to world population growth and increasing energy demand. 2) Global climate change due to the increase of CO_2 concentration in the atmosphere. 3) Increase in levels of wastes (solid/liquid) due to increase in population among world.

It is reported that world requirements for energy will increase by a factor of about six times by 2100 (Kothari et al., 2010). In the developed countries, there is no shortage of power, whereas, in the developing countries like China and India, the ratio of energy available to energy required is highly incompatible. This uneven energy distribution in the world, a technology needs to be developed to serve as a secondary source of energy and mitigate energy crisis. It would be wise to develop other fuels, which do not give so much carbon dioxide and can be easily produced with the use of environmental waste. Consequently, a growing interest in the development of anaerobic digestion (AD) has the potential to solve these problems. As given in **Table 1.2**, biogas produced from AD process is considered clean and sustainable energy and could be generated in large quantities using millions tons of organic wastes or wastewater produced all over the world (IEA, 2016).

Table 1.2 Statistic data of world renewables and waste for 2016.

	Municipal waste (TJ)	**Industrial waste (TJ)**	**Primary solid biofuels (TJ)**	**Biogases (TJ)**
Gross heat production	294983	166924	549507	37475
Production	1405862	1031186	49010564	1312646
Imports	28464	2529	373126	0

Exports	-2627	0	-232931	0
Stock changes	-22	-735	1202	0
Domestic supply	1431677	1032980	49151961	1312646
Statistical differences	0	-3500	24242	291
Transformation	1 296485	625343	10853616	807972
Electricity plants	673303	339267	3543510	454376
CHP plants	520051	147771	1520260	331609
Heat plants	103131	133462	299511	10684
Other transformation	0	4843	5490335	11303
Industry own use	3345	9570	515333	26879
Losses	0	0	483	1709
Final consumption	131847	394567	37806771	476377

1.1.3 Anaerobic digestion

Anaerobic digestion is applicable for a wide range of material including municipal, agricultural and industrial wastes, and plant residues (Chen et al., 2008; Khalid et al., 2011). Anaerobic digestion process typically occurs in a warmed, sealed anaerobic bioreactor (anaerobic digester), which establishes the ideal conditions for the bacteria and archaea to ferment the organic matters under the anaerobic condition. It is also essential for the digester tank to be warmed and mixed thoroughly in order to create the ideal conditions for the microorganisms to convert organic matters into biogas. Anaerobic digestion process takes place mainly by two types, the mesophilic condition (temperature range = 30 - 35 °C and retention time for 15 - 30 days) and the thermophilic condition (temperature range = 55 °C and retention time for 12 - 14 days). The production of biogas through anaerobic digestion offers significant advantages

over other forms of organic wastes and wastewater treatment, including: 1) less sludge yield in comparison to aerobic treatment technologies. 2) Successful in the treatment of wet wastes which contain less than 40% dry matter. 3) Effective pathogen and virus removal (Sahlström, 2003). 4) Minimization of odor emissions as 99% of volatile compounds are oxidatively decomposed upon combustion such as H_2S forms SO_2 (Smet et al., 1999). 5) Effective reduction the amount of biodegradable waste entering landfill. 6) The slurry and digestate produced from AD process is an improved fertilizer in terms of both its availability to plants (Tafdrup, 1995).

As illustrated in **Fig. 1.3**, AD process is divided into four phases: hydrolysis, acidogenesis, acetogensis and methanogensis (Mao et al., 2015). During the hydrolysis phase, large protein macromolecules, fats, lipids, and carbohydrates (such as cellulose and starch) are decomposed into water soluble monomers (amino acids, long-chain fatty acids, and sugars). This is functioned by exoenzymes (hydrolase) present in facultative and obligatory anaerobic bacteria. During the following acidogenesis phase, these products are then fermented to short-chain volatile fatty acids (VFAs), principally lactic, propionic, butyric, and valeric acid. During the acetogenesis, homoacetogenic microorganisms consume these fermentation products and produce acetic acid, carbon dioxide and hydrogen. During the final phase, methanogenic archaea, which are strictly anaerobic, consume the acetate, hydrogen, and some of the carbon dioxide to produce methane. Three metabolic pathways are functioned by methanogens to achieve the methanogenesis: acetotrophic pathway, hydrogenotrophic pathway and methylotrophic pathway.

The quantity of biogas (methane and carbon dioxide) and ammonium that will be generated from an anaerobic biodegradation of organic substrate (nitrogen-containing

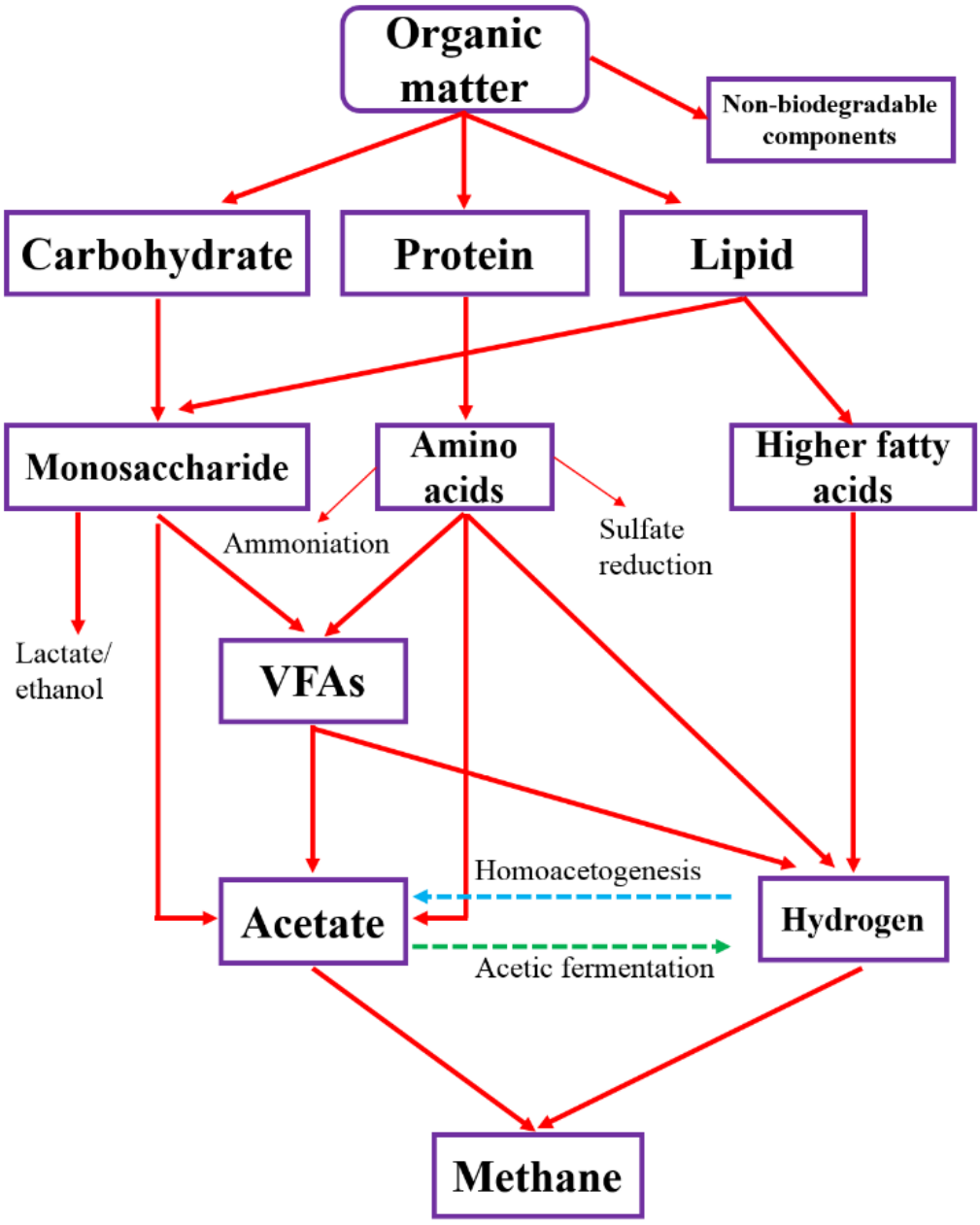

Fig. 1.3 Conceptual graph of the metabolic pathways in anaerobic digestion.

organics) can be estimated using the following stoichiometric equation (Fang and Zhang, 2015):

$$C_aH_bO_cN_d + \frac{4a-b-2c+7d}{4}H_2O \rightarrow \frac{4a+b-2c-3d}{8}CH_4 + \frac{4a+b+2c-5d}{8}CO_2 + dNH_4HCO_3 \quad (1)$$

1.2 Objectives and structure of dissertation

As shown in Fig. 1.4, this dissertation consisted of seven chapters along with acknowledgement and a list of published papers and participated conferences.

In Chapter 1, the author briefly introduced the some global environmental issues such as global warming and energy shortage that should be concerned, leading to the significance of the application of anaerobic digestion process for both wastewater treatment and bioenergy recovery.

In Chapter 2, statistical data were given and reviewed to indicate that the environmental pollutions caused by the excessive discharging of industrial wastewater are serious. The author introduced the degradation-resistant organic wastes derived from industrial wastewater streams in detail and also discussed the current challenges and barriers to the application of anaerobic digestion to industrial wastewater and specifically focused on the anaerobic treatment of chemical-industrial organic wastewater, and also provided some suggestions alternatives for the improvement of anaerobic digestion in future prospect.

In Chapter 3, the author selected a commonly used organic solvent *N*, *N*-dimethylformamide (DMF) as the research object, which is a kind of degradation-resistant amide widely applied in a variety of chemical industries. There is lack of information of the anaerobic digestion of this organic matter. With several attempts, the author successfully proposed a co-cultured sludge which could effectively degrade DMF under the anaerobic condition and produce methane. The functional microorganisms which could degrade DMF anaerobically were also investigated.

In Chapter 4, with the help of this co-culture method, the author combined normal anaerobic granular sludge and the co-cultured sludge as the seed sludge, and then inoculated them into a lab-scale up-flow anaerobic sludge blanket (UASB) reactor.

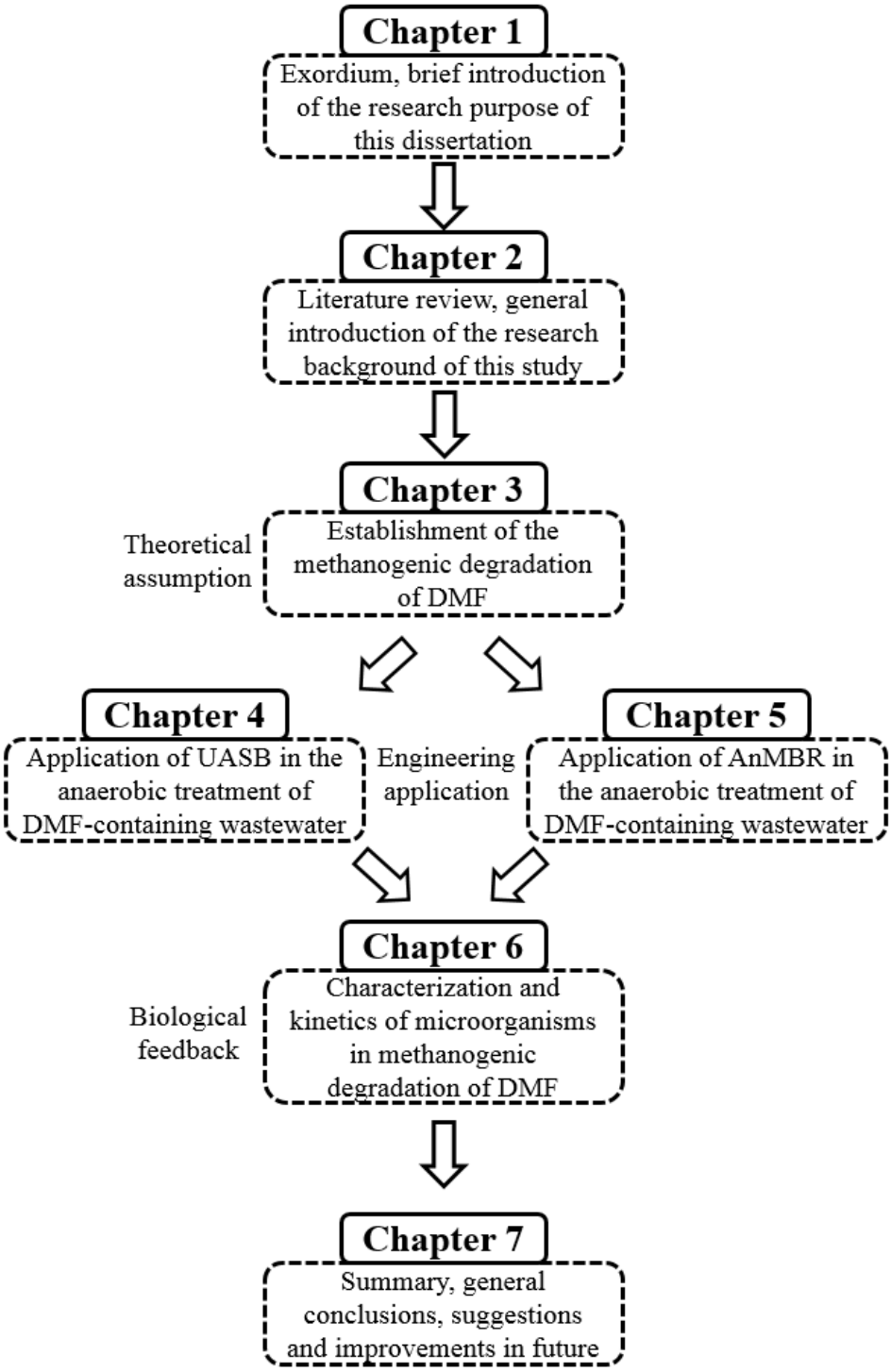

Fig. 1.4 Technical flow map of this dissertation.

The long-term performance and operation stability of the UASB were investigated in detail in this chapter.

In Chapter 5, differed from the UASB, the author combined normal anaerobic

digested sludge and the co-cultured sludge as the seed sludge, and inoculated them into a lab-scale submerged anaerobic membrane bioreactor (SAnMBR). The long-term performance and operation stability of the SAnMBR were investigated in detail in this chapter.

In Chapter 6, the author collected sludge samples from batch tests and from both UASB and SAnMBR to investigate the kinetics of bacteria and archaea in the anaerobic system of DMF methanogenic degradation by using molecular microbial technologies.

In Chapter 7, the author concluded all successful and failed results obtained in this study, and also proposed some suggestions and improvements in future work.

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Chapter 2 Preliminary review: anaerobic treatment of chemical-industrial wastewater

Abstract

Anaerobic digestion, as an efficient, eco-friendly, economic and energy-saving biological process, is extensively applied in the anaerobic treatment of a variety of wastewater streams and organic wastes. However, due to ineffective mineralization of those degradation-resistant organic wastes, this promising technology has not been generalized to the anaerobic treatment of chemical-industrial organic wastewater. This review represents the current challenges and barriers to the application of anaerobic digestion to the anaerobic treatment of chemical-industrial organic wastewater, and also provides some suggestions and alternatives for the improvement of anaerobic digestion in future prospect. In this work, the superiorities and merits of anaerobic digestion was highlighted in comparison with conventional physical, chemical and biological processes. Some specific obstacles and difficulties to the mineralization of degradation-resistant organic wastes were discussed in detail. Furthermore, some improvements such as co-culture, co-digestion and nitrate-reducing anaerobic digestion were proposed for the effective anaerobic treatment of chemical-industrial organic wastewater.

2.1 Introduction

With the rapid development of modern industrialization and economic globalization, the massive quantity of industrial wastewater discharged from a variety of chemical industries is becoming a worldwide formidable environmental issue which causes negative effects on ecosystem and also seriously endangers human health (Ennouri et

al., 2016; Kamali et al., 2016), and which is especially urgent in the developing countries. As shown in **Fig. 2.1**, a statistic of the world chemical sales is illustrated, in which China owned the largest chemical productions and sales in 2016 (Cefic, 2017), and correspondingly, approximately 18.64 billion tons of industrial wastewater was also reported as the largest discharge amount in the world that year (MEE, 2017). It should be noted that, among the diverse industrial wastewaters, the chemical-industrial organic wastewater (CIOW) should be distinguished from those wastewaters or organic wastes derived from common industrial streams, for example, natural biomass including sewage excessive sludge (Latif et al., 2017), livestock manure (Wallace et al., 2018),

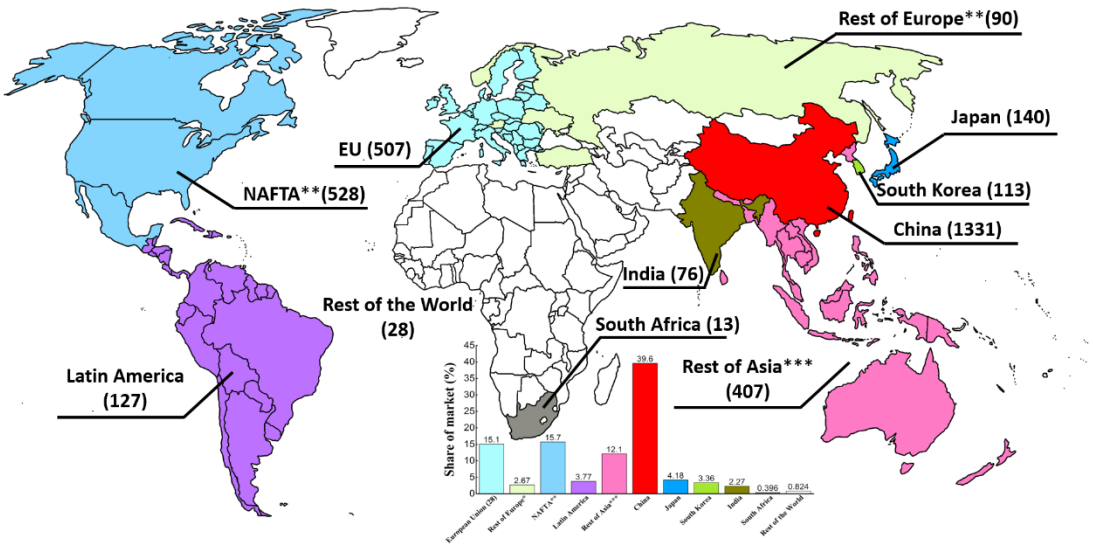

Fig. 2.1 A statistic graph of world chemicals sales in 2016 (Cefic, 2017), unit: Euro €. *Rest of Europe covers Switzerland, Norway, Turkey, Russia and Ukraine. **North America Free Trade Agreement, including U.S., Canada and Mexico. ***Asia excluding China, Japan, South Korea and India.

food waste (Zamanzadeh et al., 2016), agricultural wastes (Pardo et al., 2017) and pulp waste (Carvalheira et al., 2018) etc., most of which usually own high biochemical oxygen demands (BOD) and are considered conveniently biodegradable, and the biological treatments of these organic wastes and wastewaters have already been

investigated frequently and applied in engineering applications for decades (Ariunbaatar et al., 2014; Moraes et al., 2015). However, the organic wastes derived from CIOW are totally different. For one thing, as listed in **Table 2.1**, these organic wastes are usually toxic, mutagenic, teratogenic, or/and carcinogenic, and are much more hazardous and harmful to humans than those common organic contaminants. For another, the majority of those organic wastes are either extracted from natural substances or artificially synthesized as chemical compounds, and could be also defined as the degradation-resistant organic wastes (DROW) which usually own low BOD and are persistent to biodegradation. As shown in **Table 2.1** as well, the production of DROW mainly sources from the industries of pharmacy, petrochemicals, coal coking, pesticides, herbicides, textile and polymer synthesis etc. which are closely bound up to the human society and daily life, and most of the DROW are considered as wealth and treasures which could be either recycled or reused as carbon sources and energy instead of being abandoned as useless and hazardous wastes. Therefore, it stands to reasons that besides disregarding for the excessive discharge of CIOW to become a threat that puts both the environment and human health at risk, the improper treatment of CIOW is also running counter to the objectives of global low-carbon and energy conservation. Faced with tightening regulations, further studies of the effective treatment of CIOW should be not only environmentally friendly but also repaid with economic benefits.

Among all the state-of-art technologies, anaerobic digestion (AD) is considered one of the most reliable and cost-effective techniques for the treatment of wastewater and biomass (Bougrier et al., 2018; Kong et al., 2018a). With a series of synergistic biological degradation process performed by a microbial consortium in the absence of air, organics are converted to methane and carbon dioxide in the AD process (Jain et al., 2015; Monlau et al., 2015). AD also enables much higher loading rates than normal

aerobic process, and a greater destruction of pathogens (Chen et al., 2014), and is convenient to manage waste and reduce greenhouse gas emissions (Harris and McCabe, 2015). Consequently, benefited from its numerous significant advantages, such as low sludge production, low energy requirement and high energy recovery, various industries have embraced AD for the effective anaerobic treatment of high-strength wastewater and organic wastes (Fernández-Rodríguez et al., 2015; Suksong et al., 2016). Nevertheless, the refractory and obstinate characteristics of DROW along with its poor operational stability prevents AD process from being widely commercialized in the anaerobic treatment of CIOW streams (Rajagopal et al., 2013).

In view of the rapidly increasing demand of the AD technology, a number of review papers have been published in the recent past on the anaerobic treatment of industrial wastewater streams. However, the perspectives and investigations on the application of AD to CIOW are rarely discussed and overviewed. In the present paper, the authors have comprehensively reviewed the different aspects of AD for the anaerobic treatment of CIOW including the superiorities of AD, the difficulties and obstacles faced in the AD of CIOW, and the suggestions and new directions for the future prospects of AD in the anaerobic treatment of CIOW.

2.2 Significance of AD for the treatment of CIOW

2.2.1 Physical methods

Although a variety of physical technologies are currently available and have been used for the treatment of industrial wastewater, such as distillation (Quist-Jensen et al., 2017), adsorption (Han et al., 2017), extraction (Pereyra et al., 2015) and membrane filtration (Abdel-Shafy et al., 2016) etc., it is considered that the organic pollutants removed by those physical methods are just “transferred” rather than “converted” or

"decomposed". Despite that this kind of "mass transfer" is sometimes essential and helpful to the recovery or reclaim of some specific materials or pollutants (Rott et al., 2018), physical methods are still considered not the most suitable alternative for the treatment of CIOW, and are mainly applied in the pre-treatment or subsequent processing of the mainstream wastewater treatment technologies (De Gisi et al., 2016). Taking the adsorption method as an example, which is recognized as one of the most widespread and prevalent technologies for the wastewater treatment due to its effectiveness and low cost (Attari et al., 2017). Adsorption is classified into physical adsorption (physisorption) and chemical adsorption (chemisorption). Physisorption is majorly reversible because which is characterized by weak van der Waals intraparticle bonds between adsorbate and adsorbent, while chemisorption is generally irreversible because which is formed by the exchange of electrons with strong chemical associations between molecules or ions of adsorbate to adsorbent surface (Yagub et al., 2014). Among different adsorbent materials, activated carbons have been predominantly used for removing both organic and inorganic pollutants from industrial wastewater due to its high-surface-area, pore volume, and porosity, while other natural materials like clays, zeolites, volcanic rocks and coal fly ashes and artificially modified absorbents are also used for removing specific pollutants (Gupta et al., 2016). Although a majority of DROW could be effectively removed using the absorption method, the regeneration of adsorption capacity, modification of new absorbents, the disposal of the end-of-life adsorbents, and the post-treatment of transferred absorbates still elevate the cost of this technology (De Gisi et al., 2016). Therefore, adsorption is considered much more compatible just to the recovery of heavy metals from industrial wastewater or the decolorization of low-strength dye wastewater (Sohni et al., 2018).

2.2.2 Chemical methods

Comparatively, chemical methods are more effective and reliable due to the thorough degradation and mineralization of some DROW which are performed by a series of enhanced chemical reactions. The state-of-art chemical techniques for the treatment of CIOW and decomposition of DROW are flocculation and precipitation (Mauchauffée and Meux, 2007), electrolysis (Khatibikamal et al., 2010), ozonation (Ciardelli and Ranieri, 2001), catalysis (Melero et al., 2009), advanced oxidation processes (AOPs) (Oller et al., 2011), etc., and recently, much more attention has been paid to the technique of AOPs due to its effectiveness in the degradation of DROW. Among the AOPs, Fenton oxidation process is the one of the most prevalent and promising oxidative techniques for the removal of those toxic and refractory DROW and has been extensively used for the treatment of CIOW (Cabrera-Reina et al., 2019; Papoutsakis et al., 2016). The high removal and degradation ability of this technique can be explained by the formation of strong hydroxyl radical (·OH) from the decomposition of H_2O_2 with oxidation of ferrous (Fe^{2+}) to ferric ions (Fe^{3+}). With strong standard oxidation potential, high bimolecular reaction rate constants and non-selective reactivity, hydroxyl radicals fiercely attack and oxidize the organic radicals produced by organic matters present in the wastewater (Babuponnusami and Muthukumar, 2014), finally realizing a thorough mineralization of organic compounds to the end products of CO_2 and H_2O. As a result, Fenton process has been proved capable of effectively degrading a variety of DROW derived from dye, textile, pesticides and pharmaceutical wastewater (Amin et al., 2017; Hayat et al., 2015), and which is far more suitable to the treatment of CIOW than other chemical methods in both efficiency and engineering scale. However, this advanced technique is still limited by the cost of chemical reagents. For one thing, considering the mass balance of chemical reaction, more DROW contained in wastewater certainly

demands higher cost of hydrogen peroxide and ferrous (Gar Alalm et al., 2015), which is also considered as a kind of "equivalent exchange". This suggests that advanced oxidation technologies may not satisfy the large-scale treatment of millions tons of CIOW. For another, both ferrous and ferric ions can serve as coagulants and precipitate as ferric hydroxide ($Fe(OH)_3$) sludge, the Fe-sludge disposal is also a troublesome issue (Bokare and Choi, 2014). Besides, it is precisely because that AOPs could realize an effective and thorough mineralization, high-strength DROW contained in wastewater are all degraded to the large quantities of CO_2, burdening the global warming.

2.2.3 Activated sludge process

Apparently, it is widely recognized that biological methods are fit for the treatment of CIOW in large scales, and among all the biological techniques, activated sludge is the most widely implemented process for effectively treating industrial wastewaters and has been applied for decades (Radjenović et al., 2009). Activated sludge process (ASP) exhibited its significant superiority as a pure biological wastewater treatment technology which derives from natural microbial consortia, and is praised as an effective and eco-friendly method which could also realize the thorough degradation and mineralization of a variety of organic pollutants in large scales (Bengtsson et al., 2008; Roslev et al., 2007). Besides, distinguished from those chemical methods which usually consume tons of one-time disposable reagents for the effective degradation of DROW, ASP is also considered a repeatable method for the wastewater treatment due to the proliferation of microorganisms as long as sufficient organic wastes are fed to the sludge. With the development of this technology, the conventional ASP has been improved and upgraded into a series of new configurations and derivatives such as sequential batch reactor (SBR) (Papadimitriou et al., 2009), oxidation ditch (OD)

(Hashimoto et al., 2007) and anaerobic/anoxic/oxic (A^2/O) process (Y. Chen et al., 2011), etc. However, ASP is gradually becoming out of fashion as its drawbacks aggravate the global warming, more or less conflicting with the principles of energy conservation, emission reduction and low-carbon economy. Because for one thing, the biodegradation of organic wastes and the behaviors and activities of those aerobic microorganisms in ASP are significantly influenced by dissolved oxygen (DO), and an enough oxygen supply requires a high flow rate of aeration, which finally leads to a high energy consumption and may also deteriorate the sludge quality. It is also reported that the electricity cost for the aeration generally accounts for 45 - 75% of the total energy expenditure in wastewater treatment plants applied with the ASP (Rosso et al., 2008). The high energy consumption of aeration makes ASP unfeasible to effectively treat CIOW, because in which, the concentrations of those DROW are usually very high (Mutamim et al., 2012). Therefore, to maintain the DO for the aerobic treatment of the high-strength wastewater, the electricity cost in the aeration correspondingly elevates. For another, as ASP is a metabolism of aerobic microorganisms, due to a large amount of organic uptake and rapid growing rate of cells under the aerobic condition, 25 - 50% of the influent chemical oxygen demand (COD) converts into primary or secondary sludge during the treatment process (Leite et al., 2016), which results in a shortened sludge retention time (SRT) and a huge amount of excess sludge. It should be noted that the wasted excess activated sludge turns out to be another organic contaminant and usually contains pathogens, and must to be stabilized and minimized before any land disposal or combustion (Ennouri et al., 2016). Similar to the aeration, the cost for further treatment and disposal of excess sludge accounts for 25 - 40% of the total cost in a wastewater treatment plant, sometimes even up to 60% (Wu et al., 2014). As a result, the proper technologies for minimizing the excess activated sludge are becoming

much more concerned recently (Kavitha et al., 2015). Thirdly, due to the thorough conversion from organic wastes to carbon dioxide, the massive emission of this non-renewable greenhouse gas is another shortcoming of ASP (Hartley and Lant, 2006).

Comparatively, contributing from the highlights of bioenergy recovery from organic waste, low biomass (excess sludge) yield and strong tolerance to high organic loading, AD basically overcomes all drawbacks of the conventional ASP, and has become the mainstream process for the treatment of both wastewater and organic solid wastes (Kamali et al., 2016). Moreover, it is commonly known that compared with municipal wastewater, whose COD concentration is usually 300 - 500 mg L^{-1}, whereas organic pollutants contained in industrial wastewater streams especially CIOW are usually with high strength, as shown in **Table 2.1**, their COD concentrations are reported to typically exceed 1000 mg L^{-1} or even several folds higher. Therefore, aerobic treatment of such high strength of industrial wastewater is considered unfeasible and ineligible. To date, in some developed countries, AD and its derivatives have being gradually replacing the conventional ASP due to its remarkable and distinguishing merits, whereas, in developing countries, the dominating processes and technologies applied in wastewater treatment plants are still ASP and its derivatives or upgrades, and the excess sludge disposal strategies are usually combustion or landfill. Therefore, to generalize and popularize the AD technology is helpful and essential for preventing global warming and saving energy in the developing countries.

2.2.4 Applications of AD for the anaerobic treatment of CIOW

2.2.4.1 Up-flow anaerobic sludge blanket

Despite that the up-flow anaerobic sludge blanket (UASB) has been early introduced in 1970s, it is still by far the most popular and preferred anaerobic digester for the

anaerobic treatment various types of wastewater all over the world (Kong et al., 2019a; Wu et al., 2018). Varying in diverse shapes and designs, as illustrated in **Fig. 2.2**, an UASB reactor is typically constituted of four compartments: (1) the granular sludge bed; (2) the fluidized zone, (3) the gas-water-solid three-phase separator, and (4) the settling device. The anaerobic digestion of organic matters relies on the establishment of a dense sludge bed in the bottom of the reactor, which is consisted of anaerobic granular sludge (AGS) and serves as the core component of an UASB reactor (Schmidt and Ahring, 1996). The AGS is a mixture of aggregated flocs and granules in the system

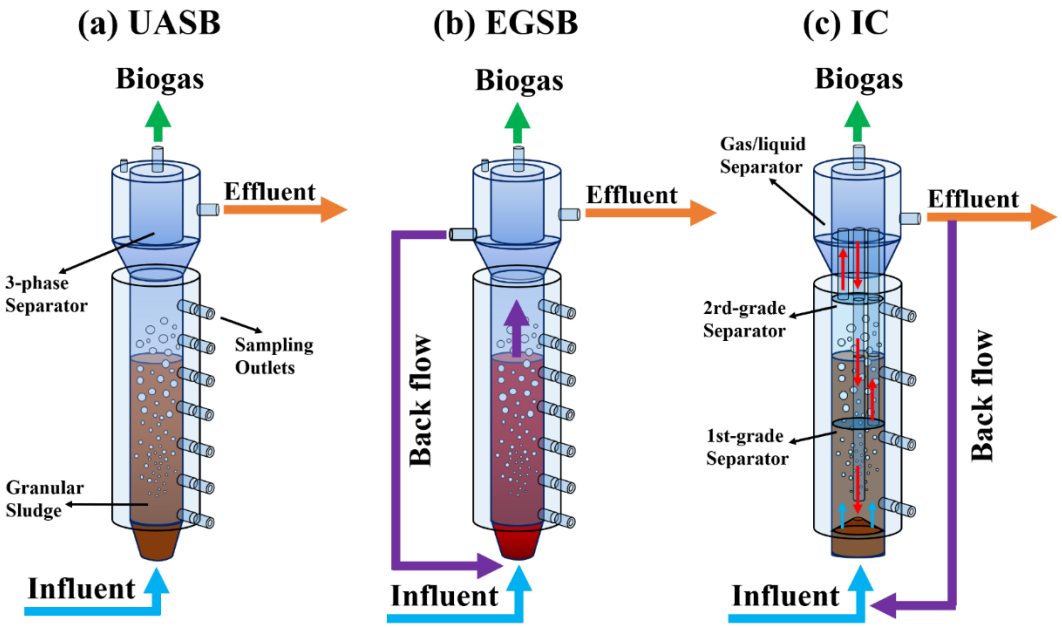

Fig. 2.2 A conceptual graph of up-flow anaerobic sludge blanket (UASB) reactor (a) and its derivatives expanded granular sludge bed (EGSB) reactor (b) and internal circulation (IC) reactor (c).

and formed by natural turbulence and hydrodynamic shear force caused by the influent up-flow velocity and the biogas production, and the granulation process in UASB reactor was generally favored by the combination of high up-flow velocity and short hydraulic retention time (HRT). Benefited from its high removal efficiency, small footprint, flexibility and tolerance of high organic loading rate, UASB has been

extensively adopted in the effective treatment of various types of industrial wastewater streams (Kamali et al., 2016). And currently, as listed in **Table 2.2**, there has been increased interest in applying UASB to the anaerobic treatment of CIOW. However, UASB still faces some drawbacks such as long start-up duration, low pathogen and nutrient removal, and suspended solids (SS) contained in the effluent. Therefore, the derivatives and upgrades of UASB such as expanded granular sludge bed (EGSB) reactor and internal circulation (IC) reactor have already been adopted to improve the performance of AD (Delforno et al., 2014; Luo et al., 2016).

2.2.4.2 Anaerobic membrane bioreactor

It is well known that conventional AD process generally requires a long HRT and long sludge retention time (SRT) in order to ensure a sufficient metabolism of microorganisms and enough reaction time for the degradation of organic matters (Maharaj and Elefsiniotis, 2001), especially for the digestion of organic solid wastes and DROW. Besides, without a sufficient HRT, the biomass can be easily washed out with the effluent due to its poor settling properties, further resulting in an inferior degradation, which is commonly emerged in UASB system (Wang et al., 2013). The anaerobic membrane bioreactor (AnMBR), which is a combination of anaerobic digester and a series of advanced membrane modules, uses a membrane as a physical barrier to facilitate a short HRT coupled with long SRT (An et al., 2009). The SRT in AnMBR can also be controlled much easier than other types of anaerobic digesters and it is completely independent from the HRT. Contributed to the membrane modules, the sludge can be completely retained in the reactor under a short HRT, and the biomass could proliferate at a relatively low growth rate to ensure a much longer STR, which is benefit to more biogas production due to the improved stabilization of organic matter

in AnMBR (Dereli et al., 2014). Since its first introduction in 1980s, AnMBR has been comprehensively investigated and extensively applied in AD systems and characterized by its diverse merits such as higher removal efficiency, higher methane production, and better effluent quality without SS, and can scale down the reactor volume to approximately 1/5 of the conventional anaerobic digesters (Qiao et al., 2013).

The currently applied AnMBR configurations can be divided into two types: the internal submerged membrane module and the external (or side-stream) submerged membrane module, which means the membrane is either immersed directly into the bioreactor or immersed in a separate chamber, and both modules are illustrated in **Fig. 2.3**. In the AnMBR system, the liquid crossflow and biogas circulation are typically used to generate the surface shear to control the membrane fouling, while the permeate

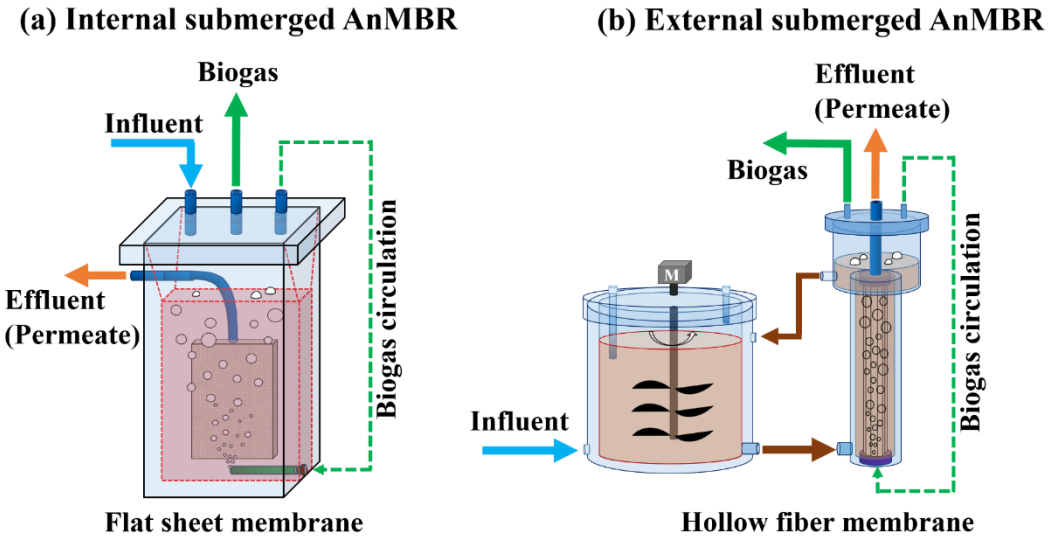

Fig. 2.3 A conceptual graph of anaerobic membrane bioreactor (AnMBR): (a) Internal submerged AnMBR; (b) external submerged AnMBR.

flow is driven by the crossflow generated by pump suction (Chang, 2014). It has been comparatively investigated that the internal submerged membrane module helps to cut the foot-print of the reactor, whereas, in which the observation and maintenance of membrane are not that convenient (Chen et al., 2017). The external membrane operation

is considered an appropriate alternative (Visvanathan and Abeynayaka, 2012), and which is commonly coupled with another anaerobic digester such as a continuously stirred tank reactor (CSTR). The flat sheet membrane and hollow fiber membrane are currently the most commonly used types of membrane modules (Chen et al., 2017; Sethunga et al., 2018). Advanced materials used for the construction of membrane were typically polymers such as poly-ethersulphone, poly-ethylene, poly-vinylidene fluoride and poly-tetra-fluoroethylene etc., or non-polymeric materials like ceramics (Skouteris et al., 2012). As the membrane separation technology significantly facilitates the elimination of SS in the effluent, AnMBR has been widely used in the AD of organic solid wastes such as food waste and paper waste (Jeong et al., 2017). However, the higher SS concentration is also likely to burden the membrane filtration and aggravate the membrane fouling. It is considered that AnMBR is greatly suitable to the anaerobic treatment of CIOW, because some of the DROW contained in CIOW are liquid or miscible to water which do not serve as SS in the feed. Therefore, it is also conceivable that AnMBR could obtain higher sludge concentration and much shorter HRT for the effective treatment of CIOW. Consequently, as listed in **Table 2.3**, the application of AnMBR to the anaerobic treatment of CIOW has been widely concerned.

2.3 Obstacles and challenges to the anaerobic treatment of CIOW

2.3.1 General obstacles and difficulties for the AD of CIOW

It is well known that the entire metabolic progress of AD is divided into four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis, and each phase is in the charge of the corresponding microorganisms (Ponsá et al., 2008). It has been widely reported that hydrolysis is considered as the rate-limiting step which plays the role of the depolymerization of organic matters (Adekunle and Okolie, 2015). Generally in AD

system, the majority of common biomass or organic wastes such as polysaccharides, proteins and fats/lipids are hydrolyzed by extracellular enzymes of bacteria to monosaccharides, amino acids and long chain fatty acids correspondingly. The following three phases could be integrated as the "methane fermentation". After stepping into the acidogenesis phase, these monomers further degrade to organic acids such as volatile fatty acids (VFAs) which are typically C_3 – C_6 compounds, or convert to alcohols. During the acetogenesis phase, VFAs and alcohols further degrade to acetate and H_2/CO_2, and which are the direct substrates for methane-producing archaea or methanogens in the final methanogenesis phase (Gonzalez-Fernandez et al., 2015). Therefore, in most of AD systems, the methanogenic types are acetotrophic or

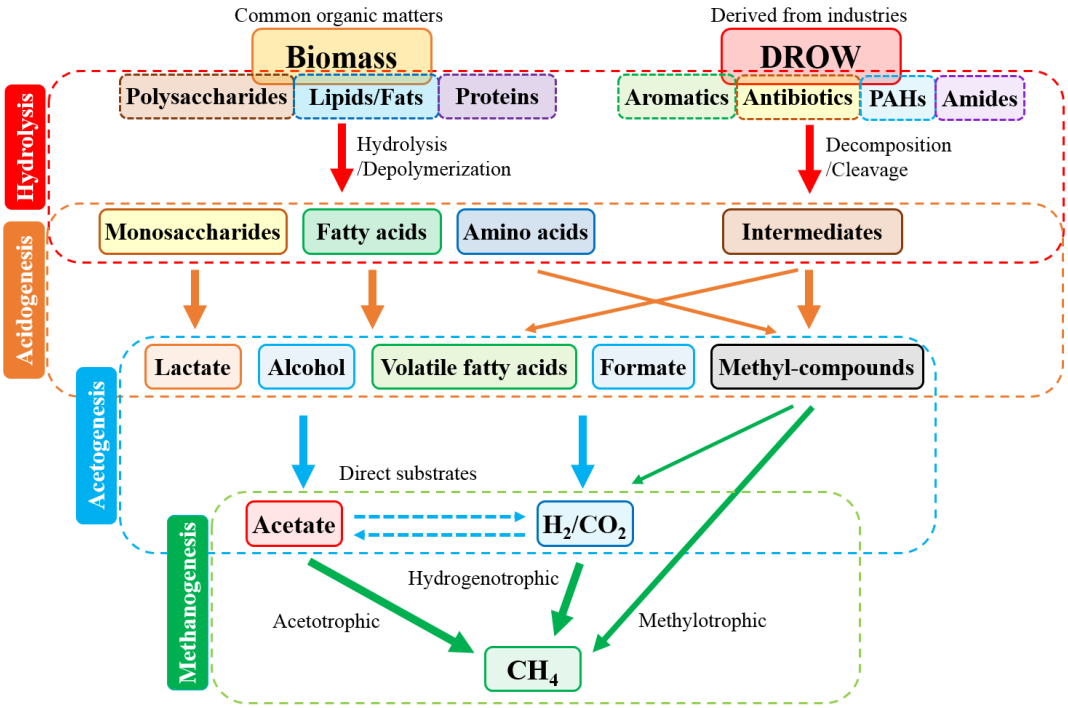

Fig. 2.4 Metabolic pathways of anaerobic digestion of biomass (common organic matters) and degradation-resistant organic wastes (DROW) derived from chemical-industrial organic wastewater (CIOW) streams.

hydrogenotrophic (Franke-Whittle et al., 2014; Ros et al., 2017). It should be also noted that another unique metabolic pathway, the methylotrophic methanogenesis, on which

methanogens feed on low-molecular-weight methyl-compounds such as amines from the rot of proteins (Jiang et al., 2005). However, methylotrophic methanogenesis is not that common in the majority of AD cases. Hence, as shown in **Fig. 2.4**, in order to produce methane through either acetotrophic or hydrogenotrophic pathways, we should establish a condition under which the direct substrates are provided to methanogens. In other word, no matter how high the molecular weight is obtained by a complicated polymeric compound, the methanogenesis should be realized as long as this compound is finally decomposed or depolymerized into acetate and H_2/CO_2. Furthermore, as the conversions from VFAs to acetate are convenient and functioned by abundant bacteria in most AD systems (Siegert and Banks, 2005), it is also conceivable that a degradation potential to VFAs indicates a high feasibility of AD from high-molecular-weight DROW to acetate and H_2/CO_2 and finally to methane. Similarly, the hydrolysis the rate-limiting step for the digestion of DROW, however, the hydrolysis of DROW is not that easy compared with common biomass or organic wastes. For one thing, the hydrolytic enzymes for DROW are rare and specific, and the corresponding bacteria which excrete such enzymes are not widespread in typical AD systems and usually require a long-term of domestication and acclimation for establishing the enriched microbial consortium (Carballa et al., 2007). For another, before successfully converting to C_2 – C_6 monomeric compounds via hydrolysis, some specific DROW like aromatic compounds demand for a series of reactions for ring cleavage, which are far more complicated than the hydrolysis of common biomass or organic wastes (Santos and Linardi, 2004).

2.3.2 Difficulties for some specific DROW

2.3.2.1 Aromatic compounds

Aromatic compounds can be defined as a species of organic compounds containing one or more aromatic rings. These compounds are the second most abundant organics (next to carbohydrates) which typically derive from three natural sources: petroleum and petroleum-derivatives (petrogenic), combustion processes such as volcanic eruption (pyrogenic), and animals and plants such as aromatic amino acids, lignin compounds and their derivatives (biogenic) (Fuchs et al., 2011; Seo et al., 2009). A majority of aromatic compounds are also known for their toxicity, mutagenicity and carcinogenicity, and are usually regarded as hazardous organic pollutants which are dangerous to both humans and environment (Ghosal et al., 2016). Due to the great stability caused by the resonance energy of C-H and C-C bonds, aromatic compounds are among the most the prevalent and persistent DROW contained in CIOW streams, and are considered difficult to achieve the degradation under the anaerobic condition (Boll et al., 2002). In general, aromatic compounds are divided into three major categories: monocyclic aromatics, heterocyclic aromatics and polycyclic aromatic hydrocarbons (PAHs).

Monocyclic (or homocyclic) aromatics are only consisted of only one aromatic ring connecting with at least one substituent group, such as benzene, toluene, ethylbenzene and xylene (BTEX), which are common monocyclic aromatic pollutants derived from petro-industries. Apparently, it is widely understood that oxygen, either as an electron acceptor or as a substituent, is convenient to the degradation of aromatic compounds. As a result, aerobic degradation of aromatic compounds has been extensively studied and summarized (Ramos et al., 2016). Under aerobic condition, monocyclic aromatics are normally attacked with the help of oxygen by monooxygenases and covert to central intermediates such as catechol (1, 2-dihydroxybenzoate) and protocatechuate (3, 4-dihydroxybenzoate) via a peripheral degradation, and are then followed by a cleavage

of the aromatic ring by ring-cleavage dioxygenases in a central degradation, the monooxygenases and dioxygenases are produced by some bacteria and fungi (Harwood and Parales, 1996; Lipscomb, 2008). However, due to the lack of oxygen and reductive agents with redox potentials required in the reduction of aromatic ring, the anaerobic degradation of aromatic compounds is considered relatively difficult and slow. It is commonly reported that a central intermediate benzoyl-coenzyme A (benzoyl-CoA) plays the most important role in the anaerobic degradation of aromatic compounds, which is further inserted with carboxyl groups from carbon dioxide or succinic acids for the ring cleavage and degraded to a chain intermediate 3-hydroxypimelyl-CoA, and finally degraded to acetate or succinate (Löffler et al., 2011; Pelletier and Harwood, 2000). With a mixed anaerobic consortium containing methanogens, these end intermediates (VFAs, hydrogen or lactate etc.) serve as the substrates in the process of methanogenesis to thoroughly realize a methanogenic degradation of aromatic compounds (Na et al., 2016; Ramos et al., 2016).

Heterocyclic aromatics are a type of cyclic compounds whose carbon chain contains one or more non-carbon atoms, these substituted atoms are typically nitrogen, sulfur or oxygen, such as carbazole, quinoline, pyridine, indole, thiophene and furan etc. Their heterocyclic structure makes them more soluble than their homocyclic analogues, and therefore they are much easier to be transported in aquatic environment (Kuhn and Suflita, 1989). These compounds widely exist in natural environment, and are also produced in large quantities of pharmaceuticals, dyes, pesticides and herbicides (Padoley et al., 2006). It is reported that under anaerobic conditions, the initial step in heterocyclic aromatics metabolism can be either ring reduction or ring hydroxylation, and the source of atomic oxygen for hydroxylation reaction can be molecular oxygen or water (Padoley et al., 2008). To date, investigations on the fermentation of

heterocyclic aromatic compounds have focused on the heterocyclic nitrogenous compounds such as pyridine, picoline, lutidine and quinolone, which are also the most common heterocyclic compounds, and the fermentative pathways for N-heterocyclic aromatic compounds are reported to involve an initial ring hydroxylation step prior to the ring reduction ring cleavage sequence (Berry et al., 1987). Fortunately, due to the electron-rich chemical property of these heteroatoms and their relatively weak bond energies and long bond lengths, some heterocyclic compounds exhibit weaker stability than homocyclics and have been suggested more easily degradable under the anaerobic condition for a thorough mineralization of CH_4 and CO_2 than homocyclic compounds like benzene and phenol (Licht et al., 1996).

Differed from those monocyclic aromatics, PAHs are typically consisted of at least two aromatic rings with or without substituent groups, such as naphthalene, anthracene, phenanthrene and pyrene etc., and relatively stable and recalcitrant in soils and less easy to degrade than many other organic compounds. The anaerobic degradation of PAHs is also much more complicated than monocyclic aromatics as which requires more steps of ring-cleavage reactions by enzymes and carboxylation (Carmona et al., 2009; Foght, 2008). To date, the methanogenic degradation of homocyclic and heterocyclic aromatics such as alkyl benzenes and phenolic compounds are commonly reported (Borja et al., 1993; García et al., 2005), however, considering the complicated and slow ring cleavage, only a limit number of studies have investigated the methanogenic degradation of PAHs in AD system (Fuchedzhieva et al., 2008; Sayara et al., 2010).

2.3.2.2 Azo dyes

The effective treatment of textile wastewater containing high strength dyes is another concern in developing countries. It is well known that a majority of dyes are azo

compounds (–N–N–), which are linked by an azo bridge and this is the most common chromophore of reactive dyes (Talarposhti et al., 2001). Interestingly, azo dyes are electron deficient and this property makes them less susceptible to aerobic degradation. However, decolorization of azo-dyes can be gratuitously achieved under the anaerobic condition (Manu and Chaudhari, 2002). As a result, after a successful cleavage of azo bond, the compound is separated into two aromatics, which are typically monocyclic or polycyclic aromatic amines. It should be noted that dyes are not normally cytotoxic, mutagenic or carcinogenic, but these amines derived from the cleavage of azo bond may possess these hazardous characteristics. As mentioned before, aromatic compounds are generally not that easy to be degraded under the anaerobic condition. Therefore, conventional treatment of azo-dyes is usually a cooperation of anaerobic and aerobic processes: decolorization in anaerobic stage and mineralization in aerobic stage (O'Neill et al., 2000; Zissi and Lyberatos, 1996). Currently, there is a growing interest in applying AD process to the treatment of dyes-containing textile wastewater because more researchers preferred a further methanogenic degradation of those aromatic amines under the same anaerobic condition as the decolorization (Georgiou et al., 2004; Liu et al., 2012), however, most of the AD studies on treating azo-dyes just handled a limited organic loading rates.

2.3.2.3 Antibiotics

Although antibiotics are probably the most successful family of drugs so far developed for improving human health since their first introduction, it is also regarded as an urgent environmental issue that the introduction of antibiotics into the environmental through anthropogenic sources, mainly pharmaceuticals, can constitute a potential risk for aquatic and terrestrial organisms, and may also cause resistance in

bacteria, resulting in feeble or useless treatments of several diseases in the future (Baquero et al., 2008; Martinez, 2009). Most of these antibiotics derives from microorganisms, but they may be also semi-synthetic or totally synthetic, and are typically with large molecular weight and consisted of complicated monocyclic or heterocyclic structures (Homem and Santos, 2011). A number of antibiotics are reported degradable under the anaerobic condition (Pan and Chu, 2016), however, scrupling the large molecular weight and low biodegradability of antibiotics, there are only a few studies applying AD process to the treatment of antibiotics-containing pharmaceutical wastewater (Álvarez et al., 2010; Feng et al., 2017).

2.3.2.4 Amides

Amides are generally small-molecular-weight DROW and could be defined either as the nitrogenous derivatives of carboxylic acids (-OH replaced by $–NH_2$ or $–NR^2$) or the derivatives of amines. The most typical amides are formamide, *N*-methlyformamide (NMF), *N*, *N*-dimethlyformamide (DMF) and *N*, *N*-dimethyacetamide (DMAc), which are widely used in the industries like textile, manufactory of synthetic leather and fiber, polymer dissolution, pesticides and pharmaceuticals as organic polar solvents (Peng et al., 2018; Yang et al., 2014). Take the DMF as an example, which remains persistent and stable in natural environment and is also hepatotoxic and carcinogenic (Chen et al., 2015). It has been reported that the cleavage of C-N bond could be realized by a specific enzyme under the anaerobic condition, formate and dimethylamine are produced from the hydrolysis, which could serve as the direct substrates to methanogens, and a large quantity of methane could be recovered from DMF (Kong et al., 2018a). However, only a limited studies considered the anaerobic treatment of high strength DMF-containing wastewater (Z. Chen et al., 2018; Kong et al., 2019a), the majority of researchers

scrupled the low biodegradability under the anaerobic condition and therefore simply focused on the aerobic treatment of DMF (Rahmaninezhad et al., 2016; Zheng et al., 2016).

2.4 Prospects and improvements

2.4.1 Co-culture

It should be emphasized that although many other good alternatives and advanced techniques have been widely reported to improve and enhance the anaerobic treatment of CIOW and the biodegradation of DROW, such as ion exchange (Rengaraj et al., 2001), electrochemicals (Gong et al., 2017) and photocatalysis (Deng et al., 2017) etc.,

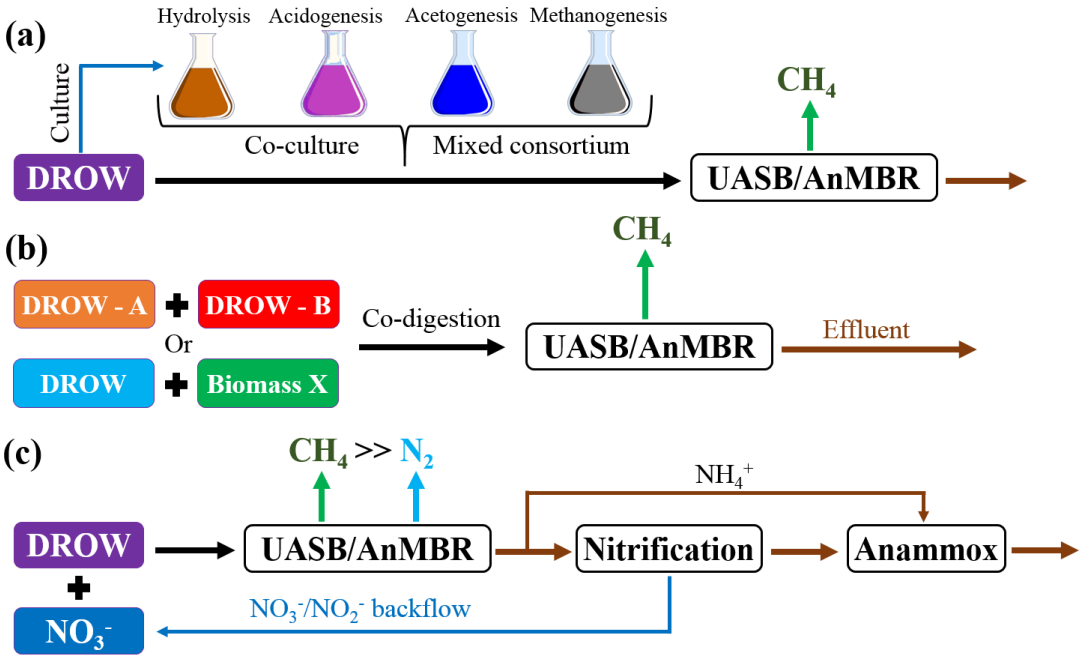

Fig. 2.5 Future prospects and improvements proposed by authors and their laboratory, including: (a) co-culture method to establish a mixed microbial consortium; (b) co-digestion of two (or more) kinds of DROW to enhance the anaerobic digestion; (c) nitrate-reducing anaerobic digestion (NRAD) to enhance the anaerobic digestion under redox conditions.

hereby, the authors and their laboratory prefer those "pure" biological anaerobic

technologies which should be eco-friendly and feasible, without extra exogenous addition of energy consumptions or economic costs, and could be also applied in large-scale of wastewater treatment engineering projects in order to be worthy and qualified to the merits of AD for harvesting bioresource and bioenergy. As discussed previously, from hydrolysis to methanogensis in AD process, each phase is in charge of its corresponding microoorganisms, in other word, the entire progress of AD is a synergetic metabolism of different microorganism. Therefore, as shown in **Fig. 2.5 (a)**, the co-culture of different bacteria (or fungi) and archaea is conceivable to establish a symbiotic mixed consortium which could achieve the corresponding steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis, and finally realize an entire anaerobic degradation of DROW (Mujtaba et al., 2017; Patel et al., 2018).

2.4.2 Co-digestion

Co-digestion has been extensively applied in the anaerobic treatment of solid organic wastes in a variety of AD studies (Astals et al., 2015; Edwards et al., 2017). Generally, the principle of co-digestion is to facilitate an appropriate C/N ratio in AD system by introducing a type of nitrogen-enriched biomass such as food waste which usually contains abundant nitrogenous proteins, to a nitrogen-deficient one such as paper waste which is majorly consisted of cellulose and lack of nitrogen (Li et al., 2019). Similarly, it is conceivable to take advantages from the co-digestion of solid organic wastes to the improvements on AD of DROW. As some specific hydrolyzing bacteria are probably unable to feed on just one kind of DROW as the sole substrate, therefore, the introduction of other organic matters is likely to enhance the degradability of this co-system. Currently, there is also a growing interest in the co-digestion strategy for the AD of DROW, such as co-digestion of DMF and antibiotics (Z. Chen et al., 2018) and

co-digestion of azo dyes and brewery grains (Gonçalves et al., 2015).

2.4.3 Nitrate-reducing anaerobic digestion

It is well known that nitrate (NO_3^-), as one of the most common inorganic contaminants contained in various kinds of wastewater streams, should be removed by denitrification process in order to prevent nitrate from causing eutrophication in aquatic environment or symptoms and illness of humans. Consequently, in the conventional denitrification process, organic matters are usually dosed as the additional carbon sources. Inversely, nitrate is also regarded as an excellent oxidant which is adapt at oxidizing a majority of organic matters under the anaerobic condition. A great number of studies have investigated the anaerobic degradation of DROW under the nitrate-reducing condition (Junghare et al., 2016; Wang et al., 2012), and the nitrate-reducing degradation is considered much more susceptible and facile than methanogenic degradation (Meckenstock et al., 2016). However, most of the previous studies focusing on nitrate-reducing degradation usually pursued a thorough mineralization of DROW by dosing excessive nitrate. As a result, although the DROW are completely removed biologically and anaerobically, they are converted to useless CO_2 instead of CH_4 as the bioenergy, the cost of large amount of nitrate reagents is somehow similar to those "chemical" methods. Therefore, as shown in Fig. 5 (c), from the perspective of our strategy, the nitrate dosage should be moderate and strictly controlled in order to realize a nitrate-reducing anaerobic digestion (NRAD), because excessive nitrate contained in the AD system significantly inhibits methanogenesis, and denitrifying bacteria overwhelmingly outcompete methanogens on organic matters as carbon source (Sakthivel et al., 2012). Which means the proper dosage of nitrate is to lead the nitrate-reducing degradation of DROW to the just right completion of hydrolysis or

acidogenesis phases, and the rest intermediates directly follow the metabolisms of methanogenic degradation for the recovery of methane from the DROW (Kong et al., 2019b). Although there are some other redox processes which have been comprehensively reported for the biodegradation of DROW such as sulfate-reducing (SO_4^{2-}), ferric-reducing (Fe^{3+}) or manganite-reducing (Mn^{4+}) etc. (Nzila, 2018), hereby, the authors and their laboratory more intensively recommend the NRAD as an appropriate solution for the anaerobic treatment of DROW, especially for the degradation of those N-containing compounds such as nitrogenous heterocyclic aromatics, azo dyes and amides. Because ammonium (NH_4^+) is one of the end products of these nitrogenous compounds after AD process, and then ammonium could be easily nitrified and totally reduced to nitrogen gas (N_2) by the anaerobic ammonia oxidation (anammox) process (Ma et al., 2018), while nitrate could just originate from those nitrogenous DROW themselves and further backflows to the AD process, realizing a literally "thorough" degradation and a recycle utilization.

2.5 Conclusions

Anaerobic digestion is considered suitable and promising to the effective treatment of chemical-industrial organic wastewater derived from a variety of industries, as those toxic and hazardous degradation-resistant organic wastes could be converted into bioenergy, and this process exhibits significant potential in both environmental and economic interests. Future improvements and upgrades on this process should be focused on the biological enhancement in the efficiency of cleavage and hydrolysis of the organic pollutants in order to realize a thorough methanogenic degradation by decomposing and degrading them to those fermentable intermediates which could be directly used to produce methane gas for bioenergy recycle.

Table 2.1 A list of some common DROW derived from different types of CIOW and their hazards and concentrations.

DROW categories	Representative DROW	Chemical formula	Molecular weight (g mol^{-1})	Hazards	DROW concentration (mg L^{-1})	COD concentration (mg L^{-1})	CIOW industrial origins	Reference
Antibiotics	Amoxicillin	$C_{16}H_{19}N_3O_5S{\cdot}3H_2O$	419.46		70 - 105	13000		(Zhaobo Chen et al., 2011)
	Ampicillin	$C_{16}H_{19}N_3O_4S{\cdot}3H_2O$	403.14	Bacterial toxicity	3.5	16240		(Zhou Ping et al., 2006)
	Aureomycin	$C_{22}H_{23}ClN_2O_8$	478.88	Toxicity	4.6	2300	Pharmaceutical	
	Berberine	$C_{20}H_{18}ClNO_4$	235.32	Irritability	1500	3800	Livestock manure	(Qin et al., 2015)
	Erythromycin	$C_{37}H_{67}NO_{13}$	733.93	Resistance	200	17200	Brewery	(Amin et al., 2006)
	Nalidixic acid	$C_{12}H_{12}N_2O_3$	232.24	Fungi infection	38	3400	Slaughterhouse	(Sirtori et al., 2009)
	Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	253.28		1 - 45	4400		(Cetecioglu et al., 2015)
	Tylosin	$C_{46}H_{77}NO_{17}H_3PO_4$	1982.31		20 - 200	7000		(Chelliapan et al., 2006)
Homocyclic aromatic compounds	Phenol	C_6H_6O	94.11	Toxicity/corrosivity	100 - 500	40000	Textile/petroleum	(Muñoz Sierra et al., 2018a)
	Benzene	C_6H_6	78.11	Toxicity	1300	4000	Petroleum	(Eke and Scholz, 2008)
	Toluene	C_7H_8	92.14	Mutagenicity	5 - 100	5000	Gasoline/oil	(Enright et al., 2007)
	Ethylbenzene	C_8H_{10}	106.16	Carcinogenicity	250 - 850	2700	Coking	(Aisien, 2013)
	Xylene	C_8H_{10}	106.17	Toxicity	600	1900	Pharmaceutical	(da Silva et al., 2012)
Heterocyclic aromatic compounds	Pyridine	C_5H_5N	79.1	Toxicity/irritability	1700	65000	Pharmaceuticals	(Padoley et al., 2011)
	Carbazole	$C_{12}H_9N$	167.2	Genotoxicity	12.5	1700	Pesticides	(Shi et al., 2014)
	Quinoline	C_9H_7N	129.16	Mutagenicity	28	1400	Coal	(Bai et al., 2011)
	Indole	C_8H_7N	117.15	Carcinogenicity	440	2600	Coking	(Lu et al., 2010)
Polycyclic aromatic hydrocarbons	Naphthalene	$C_{10}H_8$	128.18	Toxicity/irritation/ Mutagenicity	285.3	1361	Petroleum	(Panizza et al., 2000)
	Anthracene	$C_{14}H_{10}$	178.22		172.8		Pesticides	
	Phenanthrene	$C_{14}H_{10}$	178.23	Carcinogenicity	500	30000	Coal/coking	(Machate et al., 1997)
Azo dyes	Reactive Black 5	$C_{26}H_{21}N_5Na_4O_{19}S_6$	991.82	Toxicity	200	7250	Textile	(Türgay et al., 2011)

	Reactive Red 2	$C_{19}H_{10}Cl_2N_6Na_2O_7S_2$	615.32	Irritation	300	7200		(Balapure et al., 2015)
	Acid Red 14	$C_{20}H_{12}N_2Na_2O_7S_2$	502.42	Genotoxicity	20 - 60	1000		(Franca et al., 2015)
	Reactive Orange 16	$C_{20}H_{17}N_3Na_2O_{11}S_3$	617.54	Mutagenicity	100 - 1000	20393		(Ma et al., 2014)
	Acid Black 10B	$C_{22}H_{14}N_6O_9S_2Na_2$	616.49	Carcinogenicity	25 - 750	3000		(Naresh Kumar et al., 2015)
	Remazol Brilliant Violet 5R	$C_{20}H_{16}N_3Na_3O_{15}S_4$	735.58		10 - 2000	1000		(Yurtsever et al., 2015)
Amides	*N*,*N*-dimethylformamide	C_3H_7NO	73.09	Toxicity	2000	3000		(Kong et al., 2019a, 2018a)
	N,*N*-dimethylacetamide	C_4H_9NO	87.12		9910	18924	Pharmaceuticals	(Zhuo et al., 2018)
	Acetochlor	$C_{14}H_{20}ClNO_2$	269.77	Irritation	150	*N.A*	Textile	(Li et al., 2013)
	Propanil	$C_9H_9Cl_2NO$	218.08	Genotoxicity	48.2	*N.A*	Herbicides	(Herrera-González et al., 2013)
	Metolachlor	$C_{15}H_{22}ClNO_2$	283.8	Mutagenicity	40.6 - 45.3	1662 - 1960	Pesticides	(Vilar et al., 2012)
	Malathion	$C_{10}H_{19}O_6PS_2$	330.36	Carcinogenicity	10 - 11200	14 - 1220000		(Zhang and Pagilla, 2010)

Table 2.2 Current studies applying UASB to the anaerobic treatment of CIOW.

CIOW sources	DROW categories	Specific DROW	Concentration[a]	HRT (h)	OLR (g COD L^{-1} d^{-1})	Removal (%)	Reference
Pharmaceuticals	Antibiotics	Berberine	75 - 375 mg L^{-1}	24	0.07 - 0.38	57.6 - 99.0	(Qiu et al., 2013)
Pesticides/herbicides	Aromatics	Nitrophenol	0.72 mM L^{-1}	24	*N.A*[b]	90.9 - 100	(Shen et al., 2014)
Explosives/dyes	Aromatics	2, 4-dinitrochlorobenzene	0.5 mM L^{-1}	48 - 120	*N.A*	98.4 - 99.3	(Jiang et al., 2016)
Textiles/dyes	Azo dyes	Direct Black 22	1045 - 1143 mg COD L^{-1}	12	1.84 - 2.7	48.0 - 53.0	(Amaral et al., 2014)
Petroleum	Aromatics/PAHs	Chlorophenols/PAHs	950 - 2500 mg L^{-1}	9 - 24	0.95 - 2.5	84.2 - 99.6	(Moussavi and Ghorbanian, 2015)
Petrochemicals	Aromatics	Terephthalic acid	10250 mg COD L^{-1}	12 - 24	10.2 - 20	69.2 - 76.9	(Yen et al., 2016)
Pharmaceuticals	Antibiotics	Sulfamethoxazole	2012 - 7165 COD mg L^{-1}	24 - 72	0.5 - 5.0	36.0 - 57.0	(Y. Chen et al., 2018)
Textiles/pharmaceuticals	Amides	*N*, *N*-dimethylformamide	2000 mg L^{-1}	6 - 48	1.63 - 6.17	47.4 - 97.9	(Kong et al., 2019a, 2018b)
Pharmaceuticals	Antibiotics	Sulfamerazine	3000 mg COD L^{-1}	19.9	3.6 - 3.8	80.0 - 90.0	(Sponza and Demirden, 2007)
Pharmaceuticals	Antibiotics	Amoxicillin	19951 COD mg L^{-1}	16.8 – 40.3	12.57 - 21.02	39.0 - 85.0	(Zhiqiang Chen et al., 2011)

[a] Units of the DROW concentrations were not unified, some studies used COD concentrations, some measured the exact concentrations or mole concentrations of the DROW.

[b] Some studies did not provided the exact data of OLRs.

Table 2.3 Current studies applying AnMBR to the anaerobic treatment of CIOW.

CIOW sources	DROW categories	Specific DROW	Concentration[a]	HRT (h)	OLR (g COD L^{-1} d^{-1})	Removal (%)	Reference
Pharmaceuticals	Antibiotics	Penicillin	15365 mg COD L^{-1}	10.6 - 42.6	8.7 - 34.0	36.7 - 50.8	(Ng et al., 2014)
Textiles	Azo dyes	RO16	600 - 3200 mg L^{-1}	2.5	2.7	55.0 - 95.0	(Spagni et al., 2012)
Textiles	Azo dyes	Remazol Yellow Gold RNL	50 mg L^{-1}	24	0.53 - 0.59	73.0 - 94.0	(Baêta et al., 2013)
Pharmaceuticals	Antibiotics/amines	Tetrahydrofuran/tripropylamine	550 - 10600 mg COD L^{-1}	1.7 - 5	0.6 -14.2	44.4 - 94.0	(Svojitka et al., 2017)
Textiles/pharmaceuticals	Amides/antibiotics	DMF/m-Cresol/isopropyl alcohol	8053 - 22077 mg COD L^{-1}	18 - 48	3.9 - 12.7	94.7 - 98.9	(Z. Chen et al., 2018)
Pharmaceuticals	Antibiotics	Tetrahydrofuran	4250 - 5129 mg COD L^{-1}	12 - 48	2.0 - 10.0	81.0 - 97.0	(Hu et al., 2018)
Coal/coke/pulp-paper	Aromatics	Phenol	100 - 500 mg L^{-1}	223	1.86 - 4.37	61.3 - 72.7	(Muñoz Sierra et al., 2018b)
Pharmaceuticals	Antibiotics	β-lactams	4428 - 5118 mg COD L^{-1}	23.9 - 48.1	2.37 - 4.46	87.1 - 94.0	(Huang et al., 2018)
Pharmaceuticals	Antibiotics	Etodolac	2500 - 10000 mg COD L^{-1}	56	0.30 - 0.54	85.0 - 90.0	(Kaya et al., 2017)
Petrochemicals	Aromatics	2-chlorophenol	150 - 300 mg COD L^{-1}	5.2 - 13.4	*N.A*[b]	70.8 - 82.4	(Wang et al., 2015)

[a] Units of the DROW concentrations were not unified, some studies used COD concentrations, some measured the exact concentrations of the DROW.

[b] Some studies did not provided the exact data of OLRs.

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Chapter 3 Establishment of a co-cultured consortium for the methanogenic degradation of DMF

Abstract

The complete methanogenic degradation of N, N-dimethylformamide (DMF) was achieved in this study. Initially, DMF was found to be feebly degradable by a lab-scale submerged anaerobic membrane bioreactor (SAnMBR) using normal anaerobic digested sludge (ADS) even after 120-day's culturing. However, aerobic DMF-degrading activated sludge (AS) was rapidly cultivated in a continuous aeration reactor (CAR). A specially designed anaerobic co-cultured sludge (ACS) made by artificially mixing AS with ADS was successfully domesticated by a long term repeated batch experiment. The results demonstrated that ACS could effectively degrade over 5000 mg/L DMF for methane recovery. The metabolic pathway and stoichiometric equation of DMF methanogenic degradation were also revealed and verified in detail. We confirmed that under the anaerobic condition, with the help of enzyme, DMF converts into dimethylamine and formic acid, and the intermediates are effectively fermented through methylotrophic/hydrogenotrophic methanogenesis. Analysis of the microbial community suggested that some facultatively anaerobic bacteria played the key roles in methanogenic degradation due to their DMF-hydrolyzing ability. By co-culturing two sources of inoculum under the anaerobic condition, the symbiosis of facultatively anaerobic DMF-hydrolyzing bacteria and methylotrophic/hydrogenotrophic methanogens makes methanogenic degradation of DMF available. This study also provides a novel sludge cultivation method for anaerobic treatment of degradation-resistant organics.

3.1 Introduction

As a versatile organic polar solvent, *N*, *N*-dimethylformamide (DMF) [$(CH_3)_2NCHO$] has been widely employed in a variety of chemical and manufacturing industries due to its excellent water-miscibility (Bromley-Challenor et al., 2000a; Sanjeev Kumar et al., 2013; Swaroop et al., 2009). Consequently, a massive amount of industrial wastewater which usually contains high concentration of DMF was produced from chemical processing. According to statistics from the Ministry of the Environment in Japan, DMF ranks 9th on the Pollutant Release and Transfer Register, with approximately 5.36×10^3 tons of DMF either discharged or transferred in 2015 (JMOE, 2015). Moreover, it has been estimated that the global consumption of DMF will increase at an average annual rate of about 3.5% during 2013-2018 (CEH, 2014). DMF is poisonous and presents a human health hazard: it is both hepatotoxic and carcinogenic (Twiner et al., 1998). Also, because of its thermal stability and low degradability, it remains obstinate in natural environment (Sanjeev Kumar et al., 2012). As a result, the effective treatment of DMF-containing wastewater has become a challenge which has garnered the attention of researchers worldwide.

Most of the physical and chemical methods proposed for DMF removal (Das et al., 2006; Kamimoto et al., 2009; Sun et al., 2008; Ye et al., 2013) have been shown to be either unfeasible or unfriendly to environment. In the large-scale treatment of DMF-containing wastewater, biodegradation has been considered an appropriate alternative (Nisha et al., 2015; Sanjeev Kumar et al., 2013; Swaroop et al., 2009; Vidhya and Thatheyus, 2013). The principles of the two metabolic pathways in DMF aerobic biodegradation have been reported (Ghisalba et al., 1985). In Pathway I, an enzyme called *N*, *N*-dimethylformamidase (DMFase) hydrolyzes the DMF into dimethylamine (DMA) and formic acid (HCOOH), converting DMA into mono-methylamine (MMA).

Corresponding strains capable of producing DMFase have been identified and the feasibility of using DMF as the sole substrate has been shown (Dziewit et al., 2010; Sanjeev Kumar et al., 2013; Schär et al., 1986; Siddavattam et al., 2011; Urakami et al., 1990; Veeranagouda et al., 2006). In Pathway II, DMF loses methyl-groups and converts into *N*-methylformamide (NMF), formaldehyde (HCHO) and formamide (F) by repeated oxidative demethylation. Considering the toxicity and the inhibition effect of DMF on sludge, previous researchers have either not considered the possibility of DMF methanogenic degradation (Chen et al., 2015; Rahmaninezhad et al., 2016; Xiao et al., 2016; Yang et al., 2014), or have regarded DMF as a degradation-resistant compound under anaerobic conditions (Bromley-Challenor et al., 2000a; Vidhya and Thatheyus, 2013). Compared with aerobic process, anaerobic digestion (AD) has been considered as one of the most suitable processes to treat wastewater containing high concentration of organic waste owing to its merits of less biomass yield and energy production (De Vrieze et al., 2015; Jang et al., 2014; Le and Stuckey, 2017). An effective process for the degradation of toxic and obstinate DMF-containing wastewater for large-scale operations using methanogenic degradation for bioenergy recovery would be a significant step forward. However, the mechanisms have yet to be determined and the concept of methanogenic degradation has not been applied to the treatment of DMF-containing wastewater.

On the other hand, DMA and MMA, the intermediate products of DMF produced in Pathway I which serve as substrates in methylotrophic methanogenesis, have been well documented (Ferguson et al., 2000; Naumann et al., 1984; Yeliseev et al., 1993). By assuming that DMF can be degraded anaerobically conforming to Pathway I, the following methanogenesis phases of DMA and MMA are theoretically conceivable. Besides, another intermediate product, HCOOH, is also a general substrate for

hydrogenotrophic methanogenesis (Angelidaki and Batstone, 2010; Pan et al., 2016). Comprehensively, the metabolic pathway of DMF methanogenic degradation can be hypothesized by the following stoichiometric equations.

Hydrolysis of DMF by DMFase (Ghisalba et al., 1985; Sanjeev Kumar et al., 2013; Schär et al., 1986; Siddavattam et al., 2011; Urakami et al., 1990):

$$(CH_3)_2NCHO + H_2O \xrightarrow{DMFase} (CH_3)_2NH + HCOOH \quad (1)$$

The methane fermentation of intermediate products (Ferguson et al., 2000; Naumann et al., 1984; Yeliseev et al., 1993):

$$4(CH_3)_2NH + 2H_2O \longrightarrow 4CH_3NH_2 + CO_2 + 3CH_4 \quad (2)$$

$$4CH_3NH_2 + 3H_2O + 3H^+ \longrightarrow 4NH_4^+ + HCO_3^- + 3CH_4 \quad (3)$$

$$\mathrm{HCOOH} \longrightarrow CO_2 + H_2 \quad (4)$$

$$CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O \quad (5)$$

The theoretical stoichiometric equation of DMF methanogenic degradation:

$$(CH_3)_2NCHO + 2.5H_2O \longrightarrow 1.75CH_4 + 0.25CO_2 + NH_4^+ + HCO_3^- \quad (6)$$

To initiate the inception of DMF methanogenic degradation, the influence of toxicity, and the biodegradability were systematically investigated, and careful consideration was given to the possible intermediate products and functional microorganisms. The objectives of this study were: 1) to investigate the possibility and effectiveness of DMF methanogenic degradation; 2) to seek and design a novel co-cultured DMF-degrading consortium if DMF could not be degraded by normal anaerobic digested sludge; 3) to identify DMF-degrading microorganisms and to determine the metabolic pathway of DMF methanogenic degradation.

3.2 Materials and methods

3.2.1 Experimental methods and analytical reagents

A submerged anaerobic membrane bioreactor (SAnMBR) which has an operating volume of 7 L and contains a flat sheet membrane was used for culturing the anaerobic digested sludge (Qiao et al., 2016). Fresh anaerobic digested sludge (ADS) collected from a local wastewater treatment plant was used as the seed sludge. SAnMBR was initiated with 5 L ADS and 2 L of distilled water containing sufficient nutrients. The recipe of nutrients was provided in the supplementary file. Initially, the reactor was dosed with approximately 350 mg DMF L^{-1} for batch cultivating. After Day 70, continuous operation with 48 h HRT was launched and DMF concentration was elevated to 500 mg L^{-1} and then 1000 mg L^{-1}. A continuous aeration reactor (CAR) was used for culturing activated sludge (AS) with an operating volume of 10 L. The seed sludge of CAR consisted of 5 L activated sludge and 5 L distilled water with nutrients. CAR was initiated by batch cultivation at the beginning with a DMF dosage starting at 1000 mg L^{-1}. Both reactors operated at mesophilic temperature around 35 °C.

A long term batch test was conducted with 220 mL serum vials for the domestication of an anaerobic co-cultured sludge (ACS), while 120 mL vials were used for the specific methanogenic activity (SMA) and biomethane potential (BMP) tests. All vials were initially sparged with pure nitrogen gas for 30 min and were placed in a water bath shaker under conditions of 100 rpm and 35 °C. Raw enzyme solution was extracted by smashing 200 mL of concentrated sludge with ultrasonic (MUS-20D, EYELA, Japan) for 30 min, then centrifuging the broken cells at 10000 rpm for 20 min (Kubota 6000 Centrifuge, Japan). The supernatant was then filtered by a 0.45 μm PES filter head (Millex®, Merck Millipore Ltd., Ireland).

All analytical reagents were purchased from Wako Co. Ltd., Japan. DMF, *N*-methylformamide (NMF) and formamide (F) were determined by using Waters

ACQUITY UPLC H-class system (Milford, USA) at 25 °C. The system was equipped with an Xselect® CSH C18 column (130Å, 1.7 ~ 5 μm, 2.1 ~ 4.6 mm × 50-150 mm, Waters Co.), protected by a VanGuard pre-column (130 Å, 1.7 μm, 2.1 mm × 5 mm, Waters Co.). Equipment control, data acquisition and integration were performed by Empower 3.0. The mobile phase was prepared with 25% methanol (v/v) (A), 70% ultrapure water (B) and 5% acetonitrile (C). The total running time was 10 min. The flow rate was 1.0 mL min^{-1} and the injection volume was 10 μL. NH_4^+, DMA, MMA and HCOOH were determined by 7100 Capillary Electrophoresis (Agilent Technologies, USA). COD and protein concentrations were determined by a DR5000 spectrophotometer (HACH, USA) using standard potassium dichromate method and Lowry's method respectively (APHA, 1998). Biogas production and temperature were recorded by a wet gas meter (Sinagawa, Japan). Gas components were measured by a GC-8A gas chromatograph (Shimadzu, Japan) with 0.4 mL injection volume, and pH was measured by a HM-30R pH meter (DKK-TOA, Japan).

3.2.2 Samples preparation and experimental procedure

Water samples were taken from the outlet of each reactor and then filtered by a 0.45 μm PES filter head. Water samples from the batch tests were taken from vials by 1 mL plastic injectors and filtered by a 0.45 μm PES filter head. Biogas production was measured by glass injectors with volumes varying from 5 mL to 50 mL. Biogas produced from specific methanogenic activity and biomethane potential tests were standardized and calculated as follows:

$$\Delta V_{n,standard} = \frac{A_n T_0 [\gamma_n (V_g + \Delta V_n) - \gamma_{n-1} V_g]}{A_0 (T_n + T_0)} \quad (7)$$

$$P_n = \frac{1}{CV_a} \sum_{i=1}^{n} \Delta V_{n,standard} \quad (8)$$

Where ΔV_n stands for the volume of biogas production (mL) measured by the injectors

each time, γ_n refers to the CH_4 or CO_2 content (%), A_0 and T_0 refer to the standard atmosphere (1013.25 hpa) and temperature (273.15 K) respectively, while A_n and T_n refer to the real atmosphere and temperature recorded under laboratory conditions. V_g refers to the volume of gaseous phase of vials (40 mL), while V_a refers to the volume of aqueous phase (80 mL or 180 mL). P_n and C represent the biogas accumulation (mL g^{-1} VSS) and sludge concentration (g VSS L^{-1}), respectively. Standardized results were then processed by Origin 2016 software using a non-linear fitting of Gompertz equation (Li et al., 2015):

$$P = P_{max} \exp\left\{-\exp\left[\frac{\mu_{max} \times e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (9)$$

Where t is the cultivation period (day), P_{max} indicates the maximum potential of biogas production (BMP, mL CH_4 g^{-1} VSS), λ means lag phase (day) and μ_{max} is represented as the maximum slope of curve (SMA, mL CH_4 g^{-1} VSS d^{-1}).

The toxicity inhibition experiment was conducted with a group of SMA tests. Anaerobic digested sludge (ADS) collected from a continuous stirred tank reactor (CSTR) capable of digesting food waste was used as inoculum. Into 120 mL vials, 30 mL sludge with 50 mL substrate solution was filled. SMAs were determined by dosing DMF concentrations from 0 to 30000 mg COD L^{-1} with increments of 5000 mg COD L^{-1} along with the three substrates added to represent the activities of three general metabolic pathways of methanogenesis: acetic acid (HAc), methanol (MeOH) and H_2/CO_2 (or HCOOH). Non-DMF groups were regarded as blank controls with 100% activity.

For the long-term batch experiment, after adequately mixing same volume of concentrated DMF-degrading AS (14.89 ± 0.39 g VSS L^{-1}) with ADS (19.96 ± 0.14 g VSS L^{-1}), a specially designed novel anaerobic co-cultured sludge (ACS) was produced for application to long term batch tests. Into a 220 mL serum vial, 40 mL of ACS was

inoculated with 140 mL distilled water containing approximately 5000 mg L^{-1} DMF and sufficient nutrients. Another group of vials was inoculated with 40 mL ADS in parallel. Each group has four controls, from two of which water samples were taken and gas samples were taken from other two. All vials had been sparged with pure nitrogen gas for 30 min to ensure the anaerobic condition.

3.2.3 DNA extracting, PCR and Illumina sequencing and data processing

DNA was extracted with ISOIL for Beads Beating kit (Nippon gene, Japan), then the concentration was measured by NanoDrop 2000 (Nanodrop Inc., USA). V3-V4 fragment of 16S rRNA gene were amplified with forward primer 341F (5'-CCTAYGGGRBGCASCAG-3') and mixed reverse primer 806R/806R-P (30:1) (5'-GGACTACHVGGGTHTCTAAT-3'/5'-GGACTACCAGGGTATCTAAG-3'). PCR condition was as follows: 30 cycles of 94 °C for 5 sec, 50 °C for 30 sec, 68 °C for 10 sec, and a final extension at 68 °C for 7 min with Low DNA Ex Taq® (TaKaRa, Japan). Amplified products were purified with Agencourt® AMPure® XP (Beckman Coulter, Inc., USA) according to the manufacturers' instructions. Purified DNA was used as template for second PCR to add barcode sequence. The PCR products were sequenced by Illumina Miseq platform. Sequences were analyzed by using QIIME software (version 1.8.0). OTUs were generated based on 97% identity, then Chimeras were removed with ChimeraSlayer. Singleton OTUs were removed and sequences were randomly selected to unify the sequence number of each sample to 40,000.

3.3 Results and discussion

3.3.1 Toxicity influence of DMF on methane fermentation

The toxicity inhibition of DMF on methanogenesis was performed by a group of

SMA tests. The influence of different DMF dosage concentrations on SMAs of acetic acid (HAc), methanol (MeOH) and H_2/CO_2 (or HCOOH) were investigated respectively. Although it has been reported that DMF had a toxic effect on activated sludge (Yang et al., 2014), the results of this study shown in **Fig. 3.1** suggested that SMA remained even after high doses of concentrated DMF. IC_{50} and IC_{10} were 28.32 g COD L^{-1} and 39.91 g COD L^{-1} respectively. The high inhibition concentrations of DMF suggested that the inhibition effect of DMF on methanogenesis was indistinctive. Therefore, the attempt on methanogenic degradation of DMF was considerable in this study.

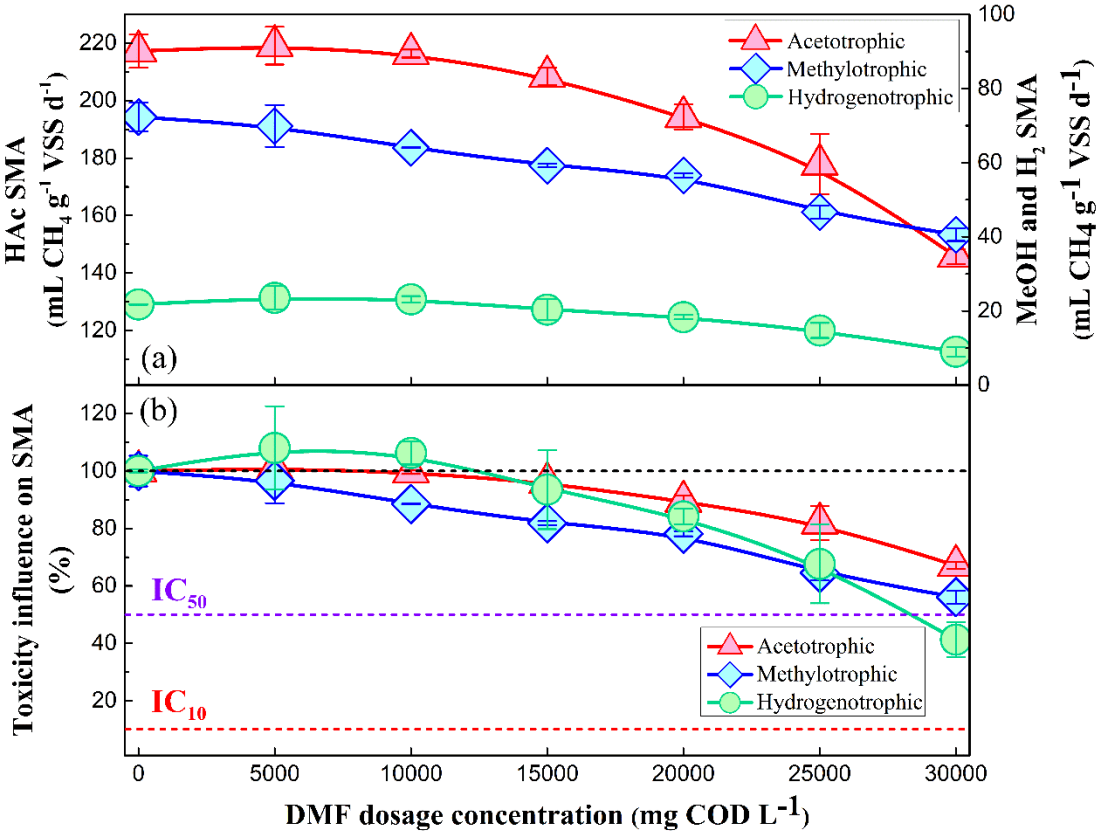

Fig. 3.1 Toxicity influence of DMF concentration on acetotrophic, methylotrophic and hydrogenotrophic methanogenesis. (a) Variation of specific methanogenic activities (SMA) influenced by DMF concentration; (b) SMA inhibition ratio standardized by defining the blank control as 100%. Inhibition concentrations (IC50 and IC10) were calculated by a non-linear Slogistic1 model.

3.3.2 Biodegradability of DMF and anaerobic sludge cultivation by SAnMBR

As illustrated in Fig. 3.2, during the batch cultivation period of SAnMBR, the DMF concentration slightly decreased from 328.64 mg L^{-1} to 269.63 mg L^{-1}, indicating weak

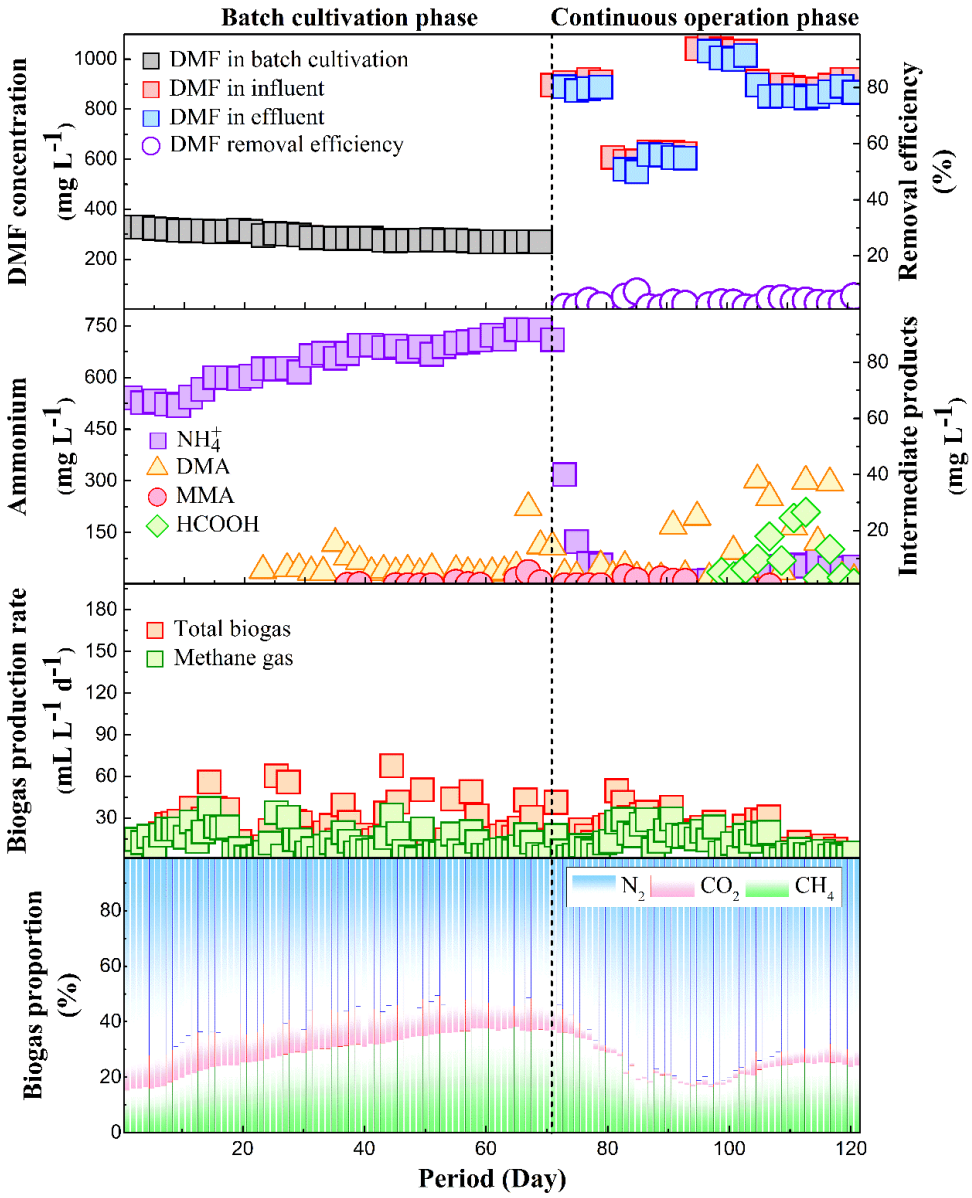

Fig. 3.2 A failed cultivation of DMF-degrading anaerobic digestion sludge (ADS) by a lab-scale submerged anaerobic membrane bioreactor (SAnMBR). (a) DMF concentration and DMF removal efficiency; (b) ammonium concentration and intermediate products concentrations; (c) total biogas production rate and methane production rate; (d) volume proportion of biogas produced from SAnMBR.standardized by defining the blank control as 100%.

DMF removal. Even though an increase in the NH_4^+ concentration was observed, it was likely that the decay and lysis of sludge released NH_4^+. During the continuous operation period, synthetic wastewater containing 500 ~ 1000 mg DMF L^{-1} was pumped into the SAnMBR and effluent was permeated by a flat membrane. Both the influent (7.31 ± 0.22) and effluent pH (7.42 ± 0.32) remained stable. However, there was barely no DMF degradation being observed: DMF removal efficiency was consistently below 6%. Besides, low NH_4^+ concentration also indicated that little substrate had decomposed. A small amount of biogas was produced during the entire period with a CH_4 content of less than 40%. However, the low concentrations of possible intermediate products DMA, MMA and HCOOH detected since Day 23 demonstrated that a small quantity of DMF was indeed degraded under anaerobic condition, yet this biodegradability of DMF was weak and unreliable even after 120-days of cultivation. It should be noted that while it has been reported that DMF anaerobic digestion was observed in a previous study, no detailed information of the seed sludge or evidence of intermediates was provided (Stronach et al., 1987), but another study suggested that the seed inoculum contains wasted activated sludge (Lausund, 2014), which perhaps helps to degrade DMF. However, we could not repeat the results of previous studies by using the normal ADS from a local wastewater treatment plant, because the ADS in this study seemed to lack DMF-degrading microorganisms. The results of our experiment suggested that the biodegradability of DMF may depend on the type of seed sludge, therefore, an effective cultivation method for DMF-degrading sludge was clearly required.

3.3.3 Cultivation of DMF-degrading activated sludge by CAR

Since the aerobic degradation of DMF has been widely reported (Rahmaninezhad et al., 2016; Sanjeev Kumar et al., 2012; Xiao et al., 2016; Yang et al., 2014), the

cultivation of DMF-degrading activated sludge (AS) by CAR was considered a feasible option. As shown in Fig. 3.3, a rapid decrease in the DMF concentration was observed within one week and high concentrations of DMA and MMA were detected. During the continuous operation period, both the influent (7.28 ± 0.12) and effluent pH (8.34 ± 0.37) remained stable. Although the DMF concentration in synthetic wastewater was

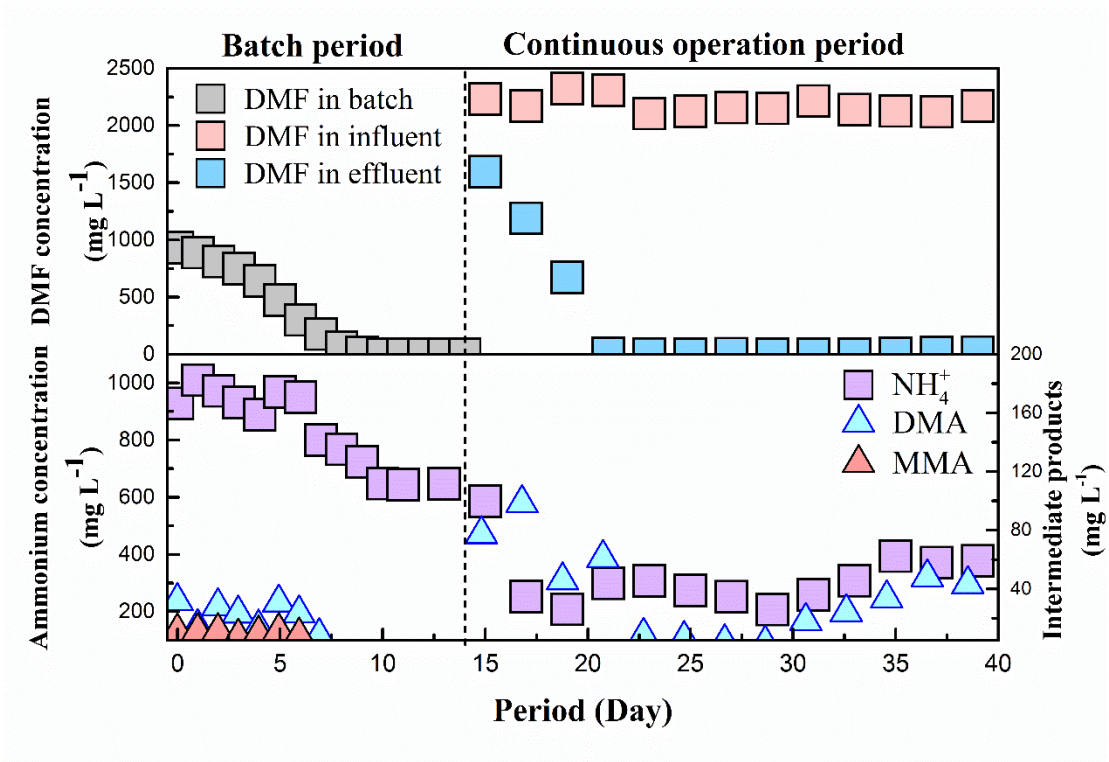

Fig. 3.3 A successful cultivation of DMF-degrading activated sludge (AS) by a lab-scale continuous aeration reactor (CAR). (a) DMF concentration in both cultivation period and continuous operation period; (b) final product (ammonium) and intermediate products (DMA and MMA, while HCOOH was BDL) concentrations.

elevated to 2000 mg L^{-1}, the effluent DMF was consistently below 20 mg L^{-1} and the concentrations of the intermediate products were also low, indicating a remarkable removal efficiency of almost 100%. The results demonstrated that DMF can be effectively degraded under the aerobic condition and that DMF-degrading activated sludge was successfully cultured by continuous aeration. Nevertheless, according to the metabolic pathway of DMF mentioned above, if anaerobic hydrolysis of DMF can be

achieved, methane-producing archaea were able to directly utilize intermediates. Some previous studies enhanced the performance of anaerobic digestion or pre-treatment by combining AS with ADS (Bromley-Challenor et al., 2000b; Peces et al., 2016). Similarly, we also made an attempt on co-culturing a mixed sludge. Therefore, we supposed that there might be facultatively anaerobic DMF-hydrolyzing microorganisms in the AS. As normal ADS from a wastewater treatment plant always contains abundant methane-producing archaea, the mixture of AS with ADS under anaerobic condition suggests those facultatively anaerobic DMF-hydrolyzing microorganisms can effectively hydrolyze DMF. This provides a thorough methanogenic degradation process for DMF.

3.3.4 Methanogenic degradation of DMF by a co-culture of DMF-degrading AS and ADS

The entire batch test was conducted for 62 days and the concentrations of DMF, NH_4^+ and the intermediate products (DMA, MMA and HCOOH) are given in **Fig. 3.4** with the biogas accumulation. For the ACS group, a rapid decrease in the DMF was observed with a high amount of biogas production during the adaptive phase. The increase in NH_4^+ and observation of high intermediate products concentrations demonstrated that DMF was successfully and effectively degraded by the ACS anaerobically. However, because the ACS consisted of AS and ADS, it was possible that the aerobic microorganisms may only temporarily remain activated and not survive under anaerobic condition during the adaptive phase. Consequently, in order to eliminate that risk, the ACS should be tested and qualified repeatedly. Hence, when the production of biogas was low, a new phase was begun by centrifuging ACS at 10000 rpm for 10 min and abandoning the old supernatant and refilling with a new substrate containing the

same DMF concentration and nutrients as well as sparging with pure nitrogen gas for 30 min. From Day 23 to Day 63, repeated batch tests were conducted under the same condition 4 times. Apparently, steeper slopes of DMF concentrations demonstrated the degradation of DMF was more rapid in Phase 2, with 5000 mg L^{-1} DMF consumed thoroughly within 10 days and a corresponding increase in the NH_4^+ concentration.

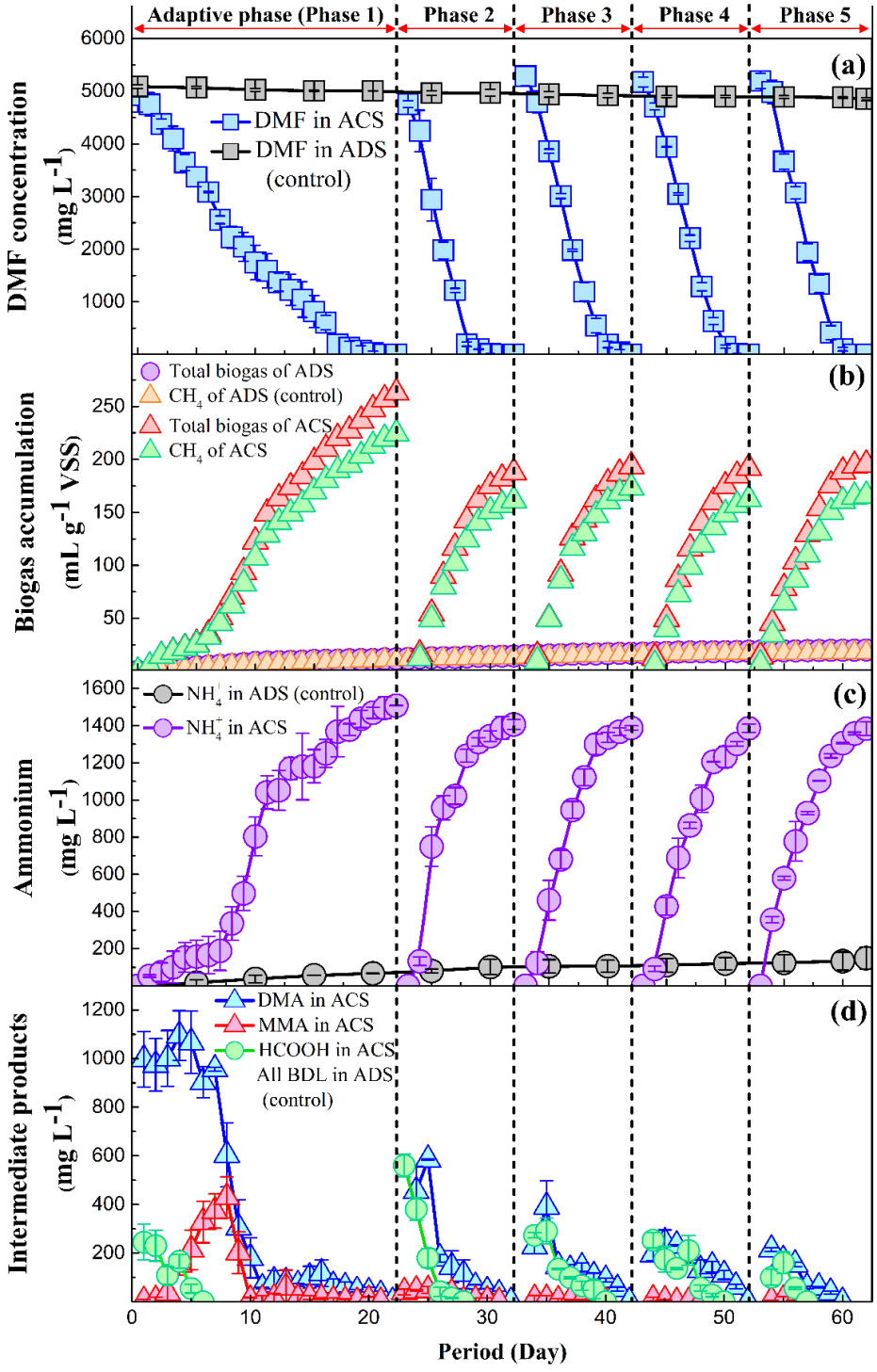

Fig. 3.4 A long term batch test using anaerobic co-cultured sludge (ACS) for the methanogenic degradation of DMF. (a) Variation of DMF concentrations; (b) total biogas and methane accumulation; (c) ammonium concentration; (d) potential intermediate products concentrations including dimethylamine (DMA), mono-methylamine (MMA) and formic acid (HCOOH).

After Phase 2, the concentrations of the intermediate products including DMA, MMA and HCOOH was obviously lower than that in Phase 1, and were almost determined during the early period of each phase, which suggested intermediates were effectively utilized and ACS was gradually becoming acclimated and specific to DMF. It should be noted that in the ADS control, the concentration of DMF concentration changed only slightly and almost no biogas or NH_4^+ was produced during the entire period, with no intermediate products detected. The results of ADS control accorded with our failed SAnMBR, suggesting again that the ADS from the wastewater treatment plant was unable to effectively degrade DMF. The results indicated that a specially designed ACS was successfully domesticated by combing two sources of inoculum (DMF-degrading AS and normal ADS) under the anaerobic condition. It is also possible that some facultatively anaerobic microorganisms played key roles in the anaerobic hydrolysis of DMF, helping to degrade DMF anaerobically.

3.3.5 Mechanism of DMF methanogenic degradation: DMF-hydrolyzing enzyme

In order to determine the significance of facultatively anaerobic DMF-hydrolyzing microorganisms, raw enzyme solutions of both DMF-degrading AS and ACS were extracted. An enzymatic specific activity (ESA) test and a BMP test were respectively performed to verify the role of the DMF-hydrolyzing enzyme in methanogenic degradation. In the ESA test, serum vials were filled with 80 mL of raw enzyme extractions and dosed with DMF. As shown in **Fig. 3.5 (a)** and **(b)**, both raw enzyme

extractions from AS and ACS (after the long-term batch experiment) directly revealed DMF-hydrolyzing ability with ESAs of 2.76 U mg^{-1} and 1.94 U mg^{-1}, respectively. In the BMP test, serum vials were inoculated with 30 mL ADS and 50 mL enzyme solution (diluted to 100%, 50%, 20%, 10% and 0%) respectively, then dosed with 2000 mg COD L^{-1} DMF. As raw enzyme extraction contains impurities like VFAs, as well as proteins

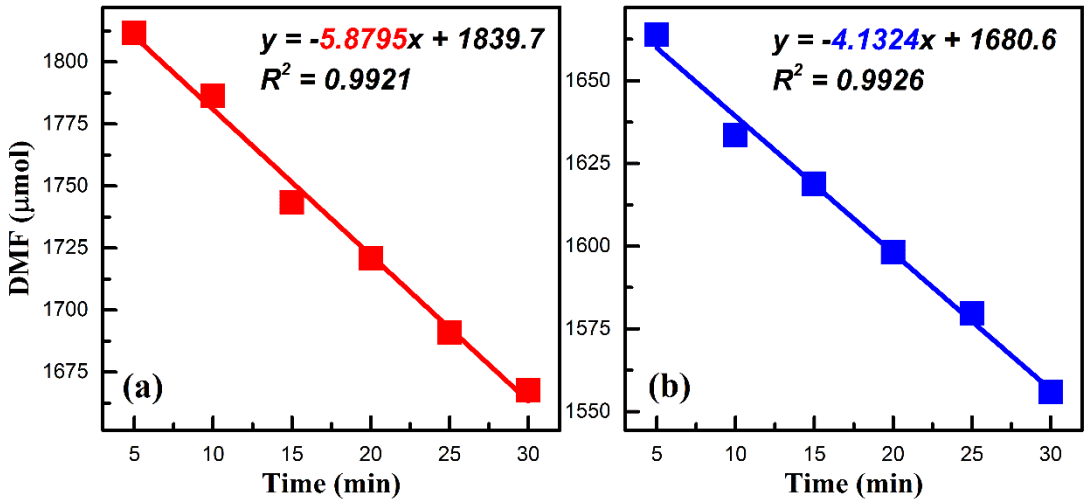

Fig. 3.5 An enzymatic specific activity (ESA) test using raw enzyme solutions extracted from both DMF-degrading activated sludge (AS) and anaerobic co-cultured sludge (ACS); the ESAs of AS (a) 2.76 U mg^{-1} and ACS (b) 1.94 U mg^{-1} were respectively calculated by the linear fitting with the corresponding total protein concentrations (26.55 mg L^{-1} and 21.63 mg L^{-1}) in the extractions.

and carbohydrates which were simultaneously extracted from the smashed cells along with enzyme. These organic residuals could serve as substrates and be further fermented by ADS, therefore a control group without DMF was conducted in parallel to eliminate extra biogas production. The results shown in **Fig. 6 (a)** and **(b)** indicated that the ADS previously shown to be unable to degrade DMF began to produce methane from DMF after dosing with the enzyme extracted from AS. The results also revealed that dosing with the enzyme significantly influenced methane production: more methane was produced when the enzyme dosage was increased from 0% to 100% even though all groups were fed with the same DMF concentration. Similarly in Fig. 6 (c) and (d),

enzyme extraction of the acclimated ACS also exhibited DMF-hydrolyzing ability. This is further evidence that facultatively anaerobic microorganisms surviving in ACS indeed produced DMF-hydrolyzing enzymes, playing a key role in the hydrolysis step of DMF methanogenic degradation.

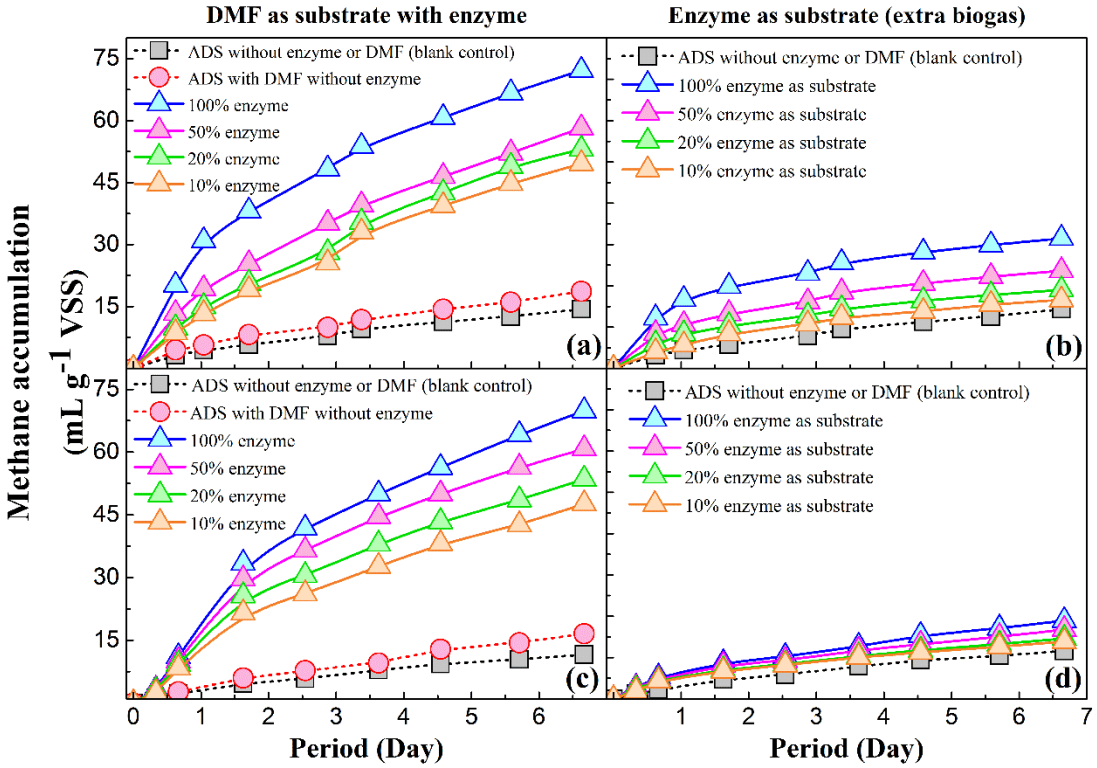

Fig. 3.6 Biomethane potential (BMP) test of DMF on ADS with the dosage of raw enzyme solutions extracted from DMF-degrading activated sludge (AS) and anaerobic co-cultured sludge (ACS) respectively; raw enzyme extractions of AS (a) and ACS (c) were respectively dosed with substrate DMF in ADS, while the extra biogas blank controls (b) and (d) which use raw enzyme extractions as substrates

3.3.6 Verification of stoichiometric equation of DMF methanogenic degradation

The theoretical stoichiometric equation of DMF methanogenic degradation below is based on empirical formula when neglecting biomass growth (Fang and Zhang, 2015):

$$C_nH_aO_bN_c + \left(\frac{4n+7c-a-2b}{4}\right)H_2O \longrightarrow \left(\frac{4n-3c+a-2b}{8}\right)CH_4 + \left(\frac{4n-3c+a-2b}{8}\right)CO_2 + cNH_4^+ + cHCO_3^- \quad (10)$$

In order to verify this equation by a real case, another BMP test was conducted by

respectively dosing DMF with increments of 500 mg L^{-1} into four serum vials. The initial DMF concentrations, total CH_4 and CO_2 productions, and final NH_4^+ concentrations (neglecting biomass growth) were recorded and manipulated into mole concentrations. The correlations of DMF between CH_4, CO_2 and NH_4^+ were respectively obtained by linear fittings with slopes representing 1 mole of DMF converted to a number of moles of CH_4, CO_2 and NH_4^+. As shown in **Fig. 3.7**, the four DMF concentration gradients fitted well with the final products. The real mole coefficients calculated by the BMP test were 1.67 CH_4, 0.26 CO_2 and 0.93 NH_4^+, which were in fairly good agreement with the theoretical values of 1.75, 0.25 and 1 according to Eq. 6, demonstrating the accuracy and reliability of this stoichiometric equation and a thorough conversion from DMF to methane.

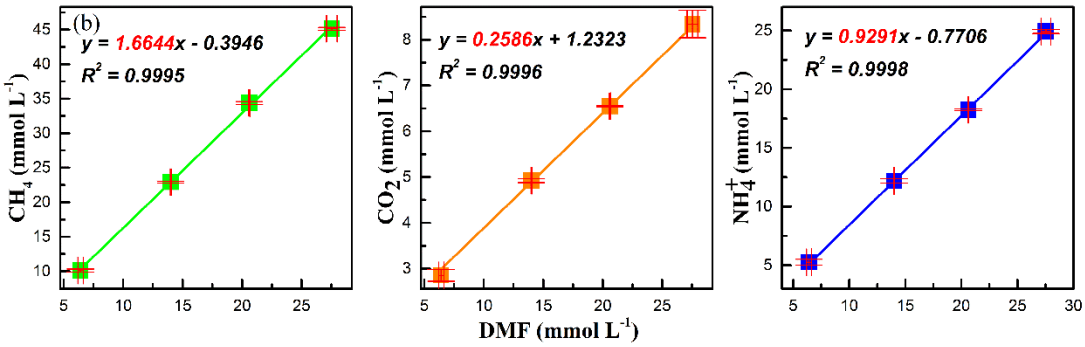

Fig. 3.7 Verification of stoichiometric equation of DMF methanogenic degradation. Mole numbers of final products CH_4, CO_2 and NH_4^+ were obtained from the slopes of the linear fittings.

3.3.7 Metabolic pathway, intermediate products and functional strains identification

DMF was reported to be metabolized by two pathways, and that the intermediate products produced on these two pathways were different. Although it was much more likely that DMF would convert into DMA, HCOOH and MMA under anaerobic

condition since all these intermediates are methanogenic substrates, it was considered possible that another pathway existed. An SMA test was conducted to determine whether Pathway II (*N*-methylformamide, formamide and formaldehyde) was relevant to the metabolization of DMF or whether it could be eliminated. All the potential intermediate products (including HAc and MeOH for basic methanogenesis) were fed into the ACS to test whether they could be fermented. The results of the SMA test illustrated in **Fig. 3.8** indicated that while the *N*-methylformamide (NMF) and formamide (F) were utilized by ACS, no methanogenic ability was found for HCHO (weaker than blank control, recorded as zero). As NMF and F are amides with

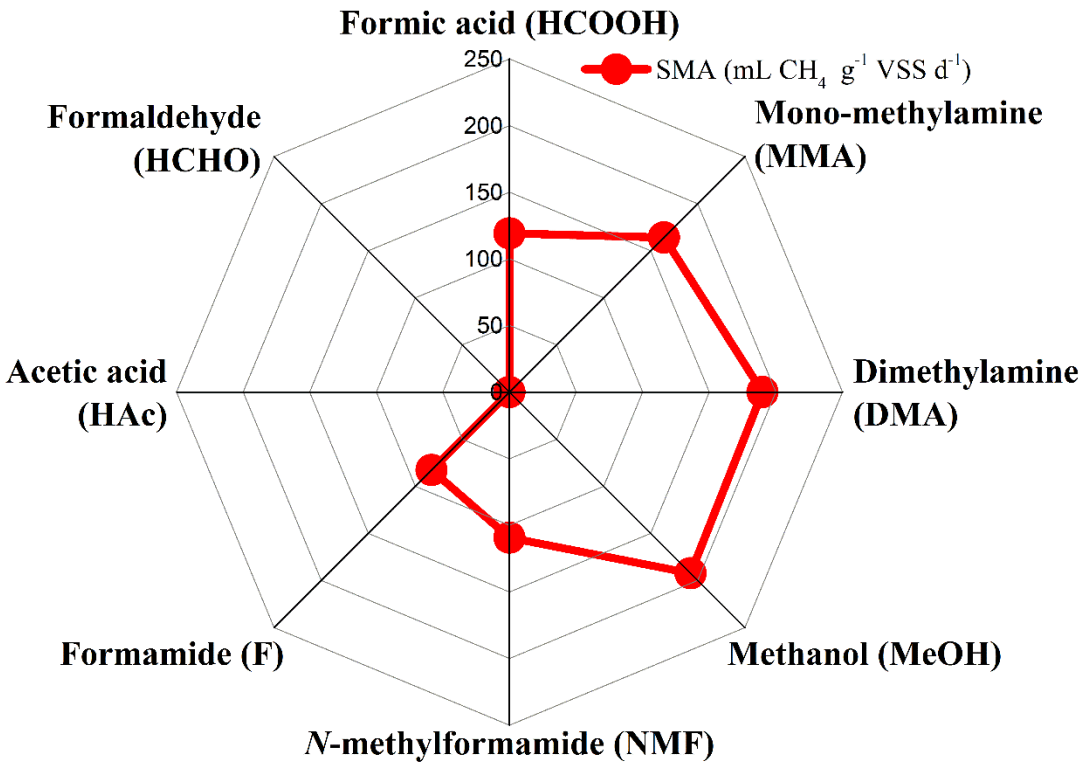

Fig. 3.8 Verification of all potential intermediate products of DMF methanogenic degradation by a specific methanogenic activity (SMA) test.

properties were similar to DMF, they were also potential substrates to ACS. Nevertheless, the unavailability of HCHO suggests that DMF does not anaerobically decompose into HCHO. Besides, the high activities of MeOH, DMA, MMA and

HCOOH and inactivation of HAc indicate that the methanogenic pathways of DMF were methylotrophic and hydrogenotrophic rather acetotrophic. Moreover, the UPLC spectrogram shown in **Fig. 3.9** provides clear evidence that after 12 h, 24 h and even 48 h, no sign of NMF or F peak was observed in the batch samples. From these results, it was concluded that Pathway II did not exist under the anaerobic condition and the

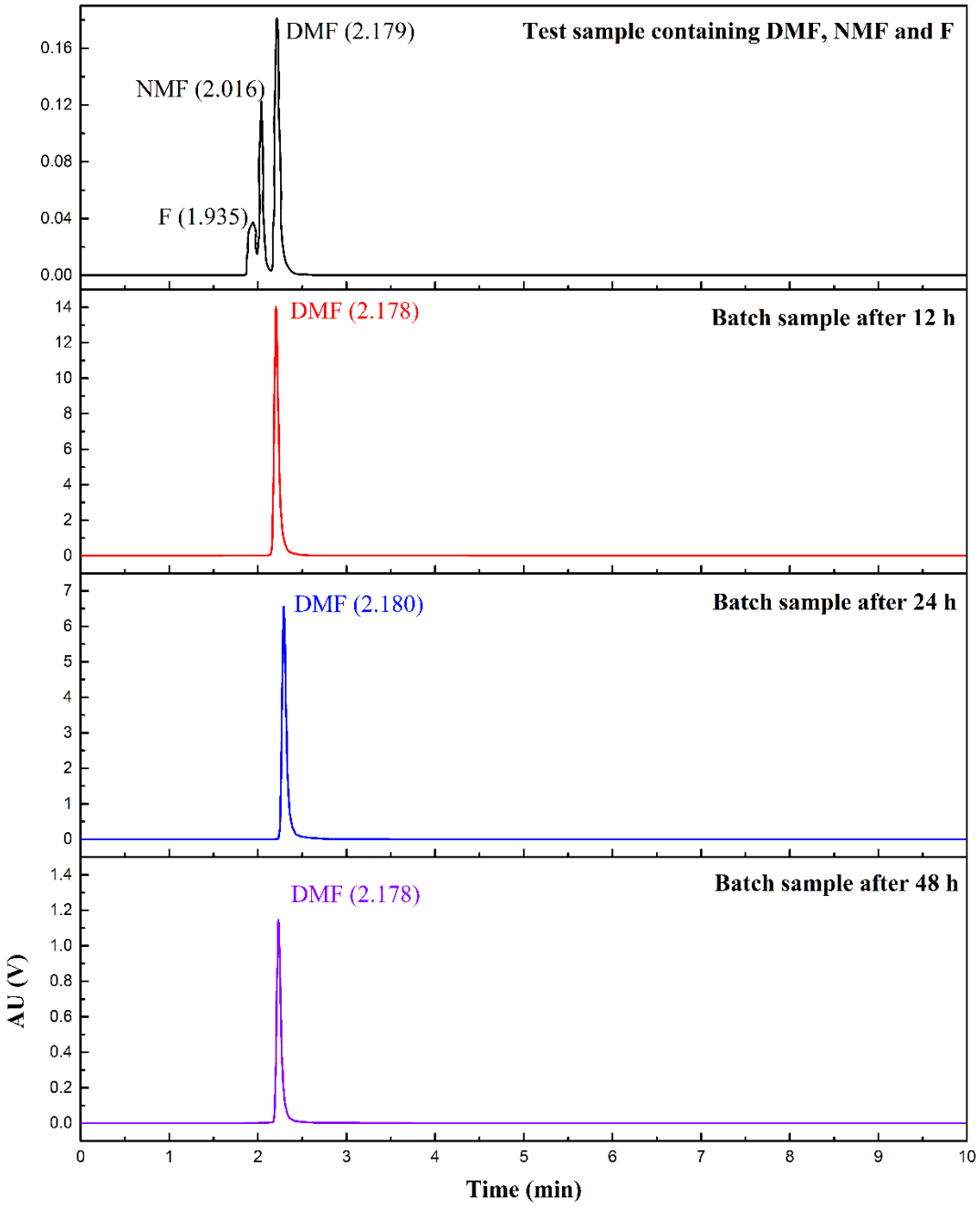

Fig. 3.9 A spectrogram obtained from an UPLC system by determining the existence of *N*, *N*-dimethylformamide (DMF), *N*-methylformamide (NMF) and formamide (F) in batch samples.

metabolic pathway of DMF methanogenic degradation was as follows: DMF converts into DMA (further into MMA) and HCOOH under anaerobic condition, and intermediates convert to CH_4, CO_2 and NH_4^+.

The microbial community structures of both bacteria and archaea in the sludge were analyzed by high throughput sequencing. Samples were taken from seed ADS of SAnMBR (ADS-0), AS on Day 30 (AS-30), ADS of SAnMBR on Day 120 (ADS-120) and ACS on Day 54 (ACS-54) respectively. Genera which occupied relative abundance ≥0.5% were illustrated as representatives in **Fig. 3.10** and uncultured sequences were classified as others.

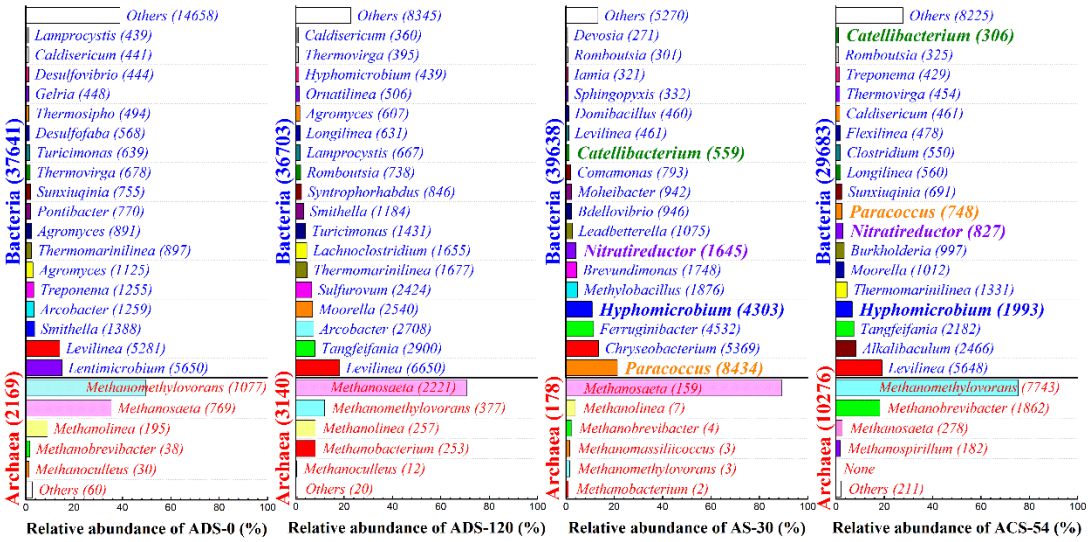

Fig. 3.10 Microbial community structures of bacteria and archaea in the sludge samples; seed anaerobic digestion sludge of SAnMBR (ADS-0), DMF-degrading activated sludge on Day 30 (AS-30), anaerobic digestion sludge of SAnMBR on Day 120 (ADS-120) and anaerobic co-cultured sludge on Day 54 (ACS-54) were illustrated respectively. The number in parentheses represents the total number of sequences for each genus (F) in batch samples.

In the case of bacteria, *Paracoccus* was clearly predominant in AS-30 with 21.29%

abundance, but rare in both ADS-0 and ADS-120, indicating its excellent ability to degrade DMF under the aerobic condition, as reported in previous studies (Nisha et al., 2015; Sanjeev Kumar et al., 2013; Siddavattam et al., 2011), and *Paracoccus* also accounted for 2.52% in ACS-54. However, the representative bacteria in ADS-0 and ADS-120 did not seem either DMF-degradable or DMF-related, which somehow explains why the ADS of SAnMBR in this study was unable to anaerobically degrade DMF. Consequently, the predominant bacterial strains in both AS-30 and ACS-54 which were subordinated in ADS-0 and ADS-120 were selected as potential candidates for facultatively anaerobic DMF-hydrolysis. These are given in **Table 3.1**. The results indicate that members of genus *Paracoccus* were targeted, among which *Paracoccus huijuniae* (Sun et al., 2013), *Paracoccus denitrificans* and *Paracoccus kocurii* (Ohara et al., 1990), all aligned as facultative anaerobes, were considered the most likely key DMF-hydrolyzing strains due to their known ability to utilize amides or amines, especially in the case of *P. denitrificans*, which has been documented as a DMF-degrading strain in previous studies (Sanjeev Kumar et al., 2013; Siddavattam et al., 2011). However, since *Paracoccus* only remained 2.52% in ACS-54, other bacteria might also be responsible for the hydrolysis of DMF: *Catellibacterium nanjingense*, which accounted for 1.03% in ACS-54, was also considered a possible facultative candidate since it has been reported capable of degrading propanil-amide (Zhang et al., 2012). Besides, *Nitratireductor aquibiodomus* (Labbe, 2004) and *Hyphomicrobium zavarzinii* (Jérôme et al., 2007), accounting 2.79% and 6.71% respectively in ACS-54, were also considered likely to be facultative anaerobes. Although there is no direct evidence of their ability to degrade DMF, they have been reported capable of degrading C_1-sources such as MMA, which are DMF-related. The hydrolyzing ability of *Nitratireductor aquibiodomus* and *Hyphomicrobium zavarzinii* needs to be determined

in a future study.

In the case of archaea, it was obvious that acetotrophic genus *Methanosaeta* (Lü et al., 2016; Venkiteshwaran et al., 2016; Zhang et al., 2009) predominated in ADS-120 with 70.73% abundance. Because DMF was so ineffectively degraded, the ADS in the SAnMBR self-digested, with acetotrophic methanogens feeding on decayed cells. It should be noted that the total number of archaeal sequences of ACS-54 was much larger than other samples, indicating that methanogens in ACS smoothly grew with the substrates, among which, methylotrophic *Methanomethylovorans* (Liu et al., 2017; Padmasiri et al., 2007) obtained overwhelming superiority (75.35%) in ACS-54 and formate/hydrogen-utilizing *Methanobrevibacter* (Cerrillo et al., 2017; Fang et al., 1995; Gagliano et al., 2015; Li et al., 2014) also predominated (18.12%). The results are further evidence that the intermediate products of DMF are DMA, MMA and HCOOH, which are responsible for methylotrophic and hydrogenotrophic methanogens, and also show that the shift in the archaeal community from acetotrophic to methylotrophic/hydrogenotrophic was significantly influenced by the facultative anaerobic DMF-hydrolyzing bacteria. This suggests that a specially designed consortium consisting of facultative anaerobic DMF-hydrolyzing bacteria, such as *Paracoccus* along with methanogens *Methanomethylovorans* and *Methanobrevibacter,* has potential for a thorough methanogenic degradation of DMF.

3.4 Conclusions

The complete methanogenic degradation from DMF to methane was firstly achieved by introducing a co-cultured sludge in this study. The possibility and feasibility of DMF anaerobic treatment were systematically complemented and verified in detail by investigating stoichiometry and metabolism of DMF methanogenic degradation, also

leading the following key conclusions of significant enlightenments for the anaerobic treatment of DMF-containing wastewater:

- DMF exhibited weak biodegradability when fed to normal anaerobic digested sludge as in which the lack of anaerobic DMF-hydrolyzing microorganisms restricted DMF from hydrolyzing into fermentable intermediates (DMA and HCOOH).
- The inoculation of DMF-hydrolyzing consortium (or enzyme) into anaerobic digested sludge makes the conversion from DMF to methane available and effective.
- A mixed consortium which consists of facultative anaerobic DMF-hydrolyzing bacteria along with methylotrophic/hydrogenotrophic methanogens has the potential for the methanogenic degradation of DMF, and the consortium could be easily co-cultured with activated sludge and anaerobic digested sludge.

Table 3.1 Representative strains which were selected as potential facultatively anaerobic DMF-degrading bacteria in this study.

Relative abundance (%)[a]				Aligned bacterial strains	Similarity	Accession Number	Reported Function
ADS-0	ADS-120	AS-30	ACS-54				
0.02	0.11	10.86	6.71	*Hyphomicrobium zavarzinii* strain ZV-622	99%	NR_026429.1	C_1-source-fed (Jérôme et al., 2007)
0	0.02	4.15	2.79	*Nitratireductor aquibiodomus* strain NL21	98%	NR_117929.1	Methanol-fed (Labbe, 2004)
0.17	0.10	21.28	2.52	*Paracoccus huijuniae* strain FLN-7	100%	NR_108224.1	Amides-hydrolyzing (Sun et al., 2013)
				Paracoccus denitrificans strain NBRC 102528	99%	NR_114145.1	DMF-degrading (Siddavattam et al., 2011)
				Paracoccus kocurii strain NBRC 16713	99%	NR_029129.1	Amines-fed (Ohara et al., 1990)
0.78	0.37	1.41	1.03	*Catellibacterium nanjingensis* strain Y12	99%	NR_108188.1	Propanil-degrading (Zhang et al., 2012)

[a] The total bacterial sequences for each sample: ADS-0 (37,641), ADS-120 (36,703), AS-30 (39,638) and ACS-54 (29,683). The total sequences for each sample were unified to 40,000 (including both archaea and bacteria) by removing singleton OTUs.

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Chapter 4 Anaerobic treatment of DMF-containing wastewater by UASB reactor

Abstract

Wastewater containing *N*, *N*-Dimethylformamide (DMF) was treated by artificially mixing the anaerobic granular sludge (AGS) with DMF-degrading activated sludge (DAS) in this study. An up-flow anaerobic sludge blanket (UASB) successfully treated wastewater containing approximately 2000 mg L^{-1} DMF during an operation period of 215 days. An inoculation of DAS brought about remarkable results: a rapid start-up with effective DMF methanogenic degradation on the first day, and under a low organic loading rate (OLR) of 1.63 ~ 4.22 g COD L^{-1} d^{-1}, the UASB maintained excellent DMF removal efficiency at over 96% along with the high methane production rate (MPR). However, when the OLR increased to 9.24 g COD L^{-1} d^{-1}, DMF removal efficiency and MPR dropped to 47.36% and 1.05 L L^{-1} d^{-1}. A further increase in the OLR to 13.25 g COD L^{-1} d^{-1} resulted in a sharp deterioration in the DMF-degrading ability, at merely 19.19% and a low MPR of 0.38 L L^{-1} d^{-1}. The excessive elevation of OLR resulted in the insufficient hydrolysis of the DMF, and the further weakening of the conversion from DMF to intermediates and an acceleration the decaying of DMF-hydrolyzing bacteria. Methane-producing archaea was starved of intermediates when hydrolysis was inadequate. Since the DAS can be massively domesticated, and the OLR should be kept lower than 6.17 g COD L^{-1} d^{-1}, the timely replenishing of the DAS to the UASB may be a solution to maintain a stable and effective DMF hydrolysis for long-term operation. The results of this study provide insight for the development of a new concept and an improved method for the effective treatment of wastewater containing degradation-resistant organics.

4.1 Introduction

Industrial wastewater from the agrochemical, pharmaceutical and textile industries usually contains high concentrations of toxic, carcinogenous or degradation-resistant organic compounds and is hazardous and harmful to both human and environment (Aravind et al., 2016; Segura et al., 2015; Sun et al., 2014). Among those organics is *N*, *N*-dimethylformamide (DMF) [$(CH_3)_2NCHO$], a common solvent known for its excellent miscibility to both water and various kinds of organic matters. DMF is widely used in a variety of chemical industries and is discharged in high concentration in industrial wastewater (Bromley-Challenor et al., 2000; Chen et al., 2018; Das et al., 2006; Kamimoto et al., 2009; Swaroop et al., 2009). DMF is also a refractory compound resistant to decomposition or degradation in nature, and is both hepatotoxic and carcinogenic to humans (Twiner et al., 1998; Vidhya and Thatheyus, 2013). Therefore, the effective treatment of wastewater with high concentrations of DMF has become a worldwide concern.

Biodegradation has been considered an appropriate process for the large scale treatment of DMF-containing wastewater since the metabolism of DMF degradation was elucidated (Bromley-Challenor et al., 2000; Fan et al., 2017; Swaroop et al., 2009; Vidhya and Thatheyus, 2013). DMF has been reported to follow two pathways under the aerobic condition (Ghisalba et al., 1985): it is either hydrolyzed into dimethylamine (DMA) and formic acid (HCOOH) and then mono-methylamine (MMA) on Pathway I or degraded into *N*-methylformamide (NMF), formaldehyde (HCHO) and formamide (FA) by repeated oxidative demethylation on Pathway II. The function of the DMF hydrolytic enzyme *N*, *N*-dimethylformamidase (DMFase) in Pathway I has been documented in numerous reports (Hasegawa et al., 1999; SCHÄR et al., 1986) and the corresponding bacteria which live on DMF as the sole energy and nitrogen source have

also been identified (Dziewit et al., 2010; Nisha et al., 2015; Siddavattam et al., 2011; Swaroop et al., 2009; Urakami et al., 1990; Veeranagouda et al., 2006; Zhou et al., 2018). Even though some aerobic methods have been investigated for treating DMF-containing wastewater (Rahmaninezhad et al., 2016; Sanjeev Kumar et al., 2012; Zheng et al., 2016), anaerobic digestion (AD) would have some distinct advantages. AD recovers methane as bioenergy from organic waste, lowers biomass yield and is capable of handling high organic loading (Grimberg et al., 2015; Kamali et al., 2016; Tao et al., 2017). The anaerobic digestion of DMF-containing wastewater would also mark a significant improvement in energy saving and eco-friendliness. Among the many types of digesters, the up-flow anaerobic sludge blanket (UASB), has been extensively adopted in the treatment of various types of wastewater due to its high efficiency, small footprint, flexibility and energy recovery (Bassani et al., 2016; C. Chen et al., 2017; Rico et al., 2017). Currently, there has been increased interest in applying UASB to the treatment of industrial wastewater (Artsupho et al., 2016; Cairns and Mead, 2017; Li et al., 2015). It would follow that the application of UASB to the treatment of DMF-containing wastewater should also be a topic of interest: however, no experimental study on the use of the anaerobic digester for treating DMF-containing wastewater has been reported. In fact, in some reports, it has been claimed that DMF is a degradation-resistant compound under the anaerobic condition (Bromley-Challenor et al., 2000; Vidhya and Thatheyus, 2013). Therefore, an experimental study on the use of the AD to treat the hazardous and obstinate DMF-containing wastewater would be a significant step forward. In our experiment, because the start-up of anaerobic granular sludge (AGS) is always extremely time-consuming, we decided to mix normal AGS with DMF-degrading activated sludge (DAS) in an attempt to realize a rapid start-up. The theoretical stoichiometric equation of the methanogenic degradation of DMF is as

follows (Kong et al., 2018):

$$(CH_3)_2NCHO + 2.5H_2O \longrightarrow 1.75CH_4 + 0.25CO_2 + NH_4^+ + HCO_3^- \quad (1)$$

In the present study, a lab-scale UASB for culturing DMF-degrading consortium and performing the methanogenic degradation of DMF-containing wastewater by a long-term operation is investigated. Our aim was to achieve the first real case of the effective treatment of high concentration DMF-containing wastewater by UASB with a mixture seed sludge for biogas regeneration. We evaluated the feasibility and stability over the long term operation period, and provide feedback for future studies on biodegrading refractory organics in industrial wastewater.

4.2 Materials and methods

4.2.1 Reagents and experimental apparatus

All analytical reagents were purchased from Wako Co. Ltd., Japan. The real concentration (926.68 mg mL^{-1}) of DMF reagent was calibrated and standardized by measuring its total organic carbon (TOC) and total nitrogen (TN) concentrations. An up-flow anaerobic sludge blanket (UASB) with an operational volume of 6 L was used in this study for the anaerobic treatment of DMF-containing wastewater. In order to culture DMF-degrading activated sludge (DAS), a continuous aeration reactor (CAR) with an operational volume of 10 L was used as well.

4.2.2 Analytical methods

COD, protein and carbohydrate concentrations were measured by a spectrophotometer (DR5000, HACH, USA) using the standard spectrophotometric methods. TOC and TN were measured by a TOC-L analyzer equipped with a TNM-L unit (Shimadzu, Japan). Biogas production and temperature were recorded by a wet gas

meter (Sinagawa, Japan). Gas components were detected by a GC-8A gas chromatograph (Shimadzu, Japan), and pH was measured by a HM-30R pH meter (DKK-TOA, Japan). The DMF concentration was determined using a Waters ACQUITY UPLC H-class system (Milford, USA) at 25 °C. The system was equipped with an Xselect® CSH C18 column (130Å, 1.7 ~ 5 μm, 2.1 ~ 4.6 mm × 50-150 mm, Waters Co.), protected by a VanGuard pre-column (130 Å, 1.7 μm, 2.1 mm × 5 mm, Waters Co.). Equipment control, data acquisition and integration were performed by Empower 3.0. The mobile phase was prepared with 25% methanol (v/v) (A), 70% ultrapure water (B) and 5% acetonitrile (C). The total running time was 10 min. The flow rate was 1.0 mL min-1 and the injection volume was 10 μL. Ions including NH_4^+, DMA, MMA and HCOOH were determined by 7100 Capillary Electrophoresis (Agilent Technologies, USA). The concentrations of VFAs were measured by a 6890 Series GC system (Agilent Technologies, USA).

4.2.3 Inoculum sludge and synthetic DMF-containing wastewater

To pursue a rapid start-up and shorten the period of sludge granulation, a bucket of 10 L anaerobic granular sludge (AGS) was directly collected from a wastewater treatment plant in Tokyo. The UASB was inoculated with AGS, which previously fed on starch wastewater, as the seed sludge. Another bucket of 10 L normal anaerobic digestion sludge (ADS) was collected from a local domestic wastewater treatment plant and added to the CAR for culturing the DMF-degrading activated sludge (DAS). Synthetic industrial wastewater, which contains approximately 2000 mg L^{-1} DMF with sufficient nutrients, was prepared in a 120 L substrate tank. Both the nutrients recipe and detailed information of the inoculum sludge are listed in **Table 4.1**.

4.2.4 Experiment procedures and samples preparation

The schematic diagram of UASB and CAR system is illustrated in Fig. 4.1. Three

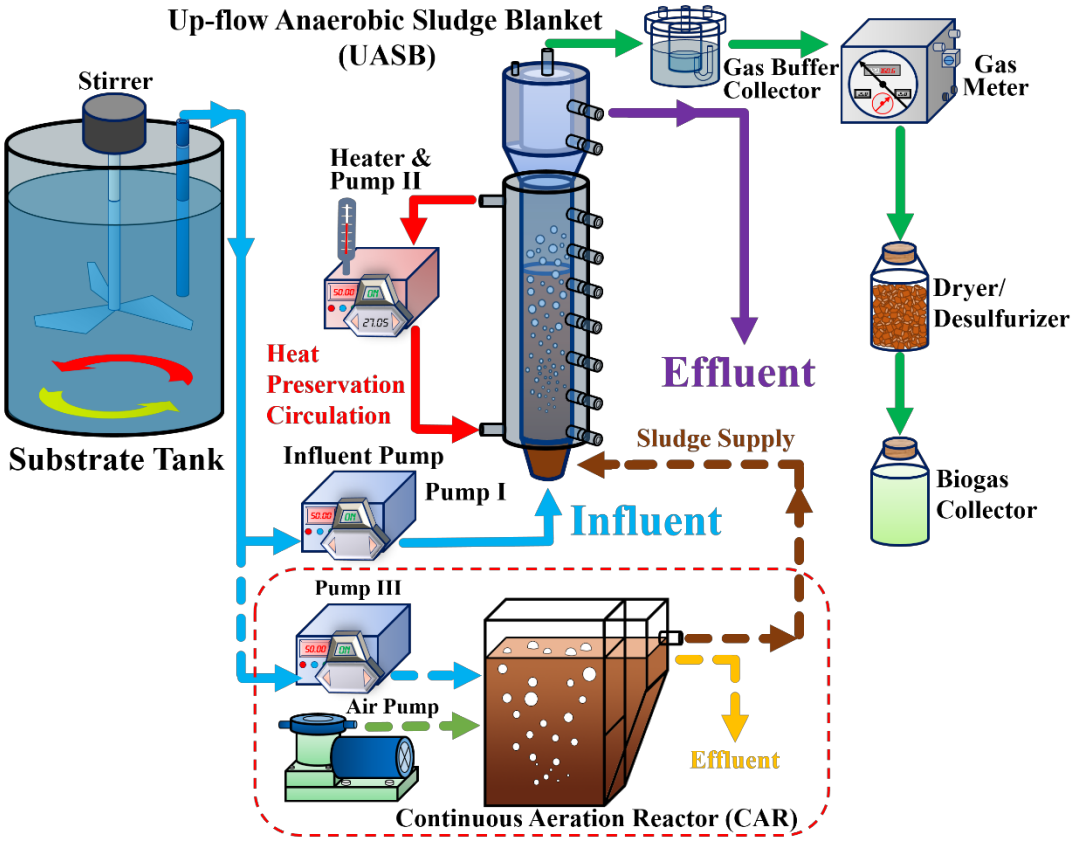

Fig. 4.1 A schematic diagram of the whole up-flow anaerobic sludge blanket (UASB) system including a continuous aeration reactor (CAR) system for the cultivation and supplement of DMF-degrading activated sludge (DAS).

stages of long-term experiments were performed in turn with the UASB. In the first stage, the UASB was solely inoculated with 6 L AGS as the seed sludge and operated for 180 days. A batch cultivation was attempted in the first month, and after that, a continuous operation was started with an HRT of 48 h, which was then lowered to 24 h. The CAR was continuously aerated by an air pump with an aeration rate of 7.5 L min^{-1}. In the second stage, the UASB was initially inoculated with 3 L AGS and fed with DMA instead of DMF for batch pre-culturing in order to enhance the methylotrophic activity. After 51-day of cultivation, the UASB was inoculated with 3 L of concentrated DAS and fully mixed with AGS. It should be noted that the DAS had

been simultaneously cultured in the CAR for 51 days. The third stage was initiated by this step. In the third stage, the UASB was continuously operated for 215 days and the HRT was shortened from 48 h to 6 h. Both the UASB and CAR operated at the mesophilic temperature of 35 °C. A group of specific methanogenic activity (SMA) tests were conducted to evaluate the methane-producing ability of AGS. Water samples were taken from the outlet of each reactor and then filtered by a 0.45 μm PES filter head (Millex®, Merck Millipore Ltd., Ireland).

While the following equations neglect cell growth, they are used to describe COD and nitrogen mass balance:

$$\gamma_{eff-COD} = \gamma_{eff-DMF} + \gamma_{eff-DMA} + \gamma_{eff-MMA} + \gamma_{eff-HCOOH} + \gamma_{CH_4} + \gamma_{CO_2} \quad (2)$$

$$\gamma_{eff-N} = \gamma_{eff-DMF-N} + \gamma_{eff-DMA-N} + \gamma_{eff-MMA-N} + \gamma_{eff-NH_4^+} \quad (3)$$

Where γ stands for the residual content of COD or nitrogen (%), γ values in the influent are all define as 100%, γ of COD in the effluent is the sum of residual DMF, DMA, MMA, HCOOH along with produced methane and carbon dioxide, while γ of nitrogen in the effluent is the sum of all N-containing matters including residual DMF, DMA, MMA and produced ammonium.

4.3 Results and discussion

4.3.1 Stage I: a long-term operation of UASB solely inoculated with AGS

The performance of a 180-day long term experiment was illustrated in **Fig. 4.2**. During the entire period, the temperature of the reactor was kept at 34.9 ± 0.7 °C, with relatively stable influent pH of 7.14 ± 0.23 and an effluent pH of 7.26 ± 0.34. To begin with, a 33-day batch cultivation was initially conducted with low DMF concentration. However, almost no DMF was removed as the concentration merely dropped from 276.83 mg L^{-1} to 249.69 mg L^{-1}. Besides, little biogas was produced: the methane

production rate (MPR) was only 8.53 ± 5.50 mL L^{-1} d^{-1}. The ammonium concentration increased from 492.90 mg L^{-1} to a high 700.92 mg L^{-1}. This high concentration of ammonium could not be produced from the degradation of DMF. It is likely that rather

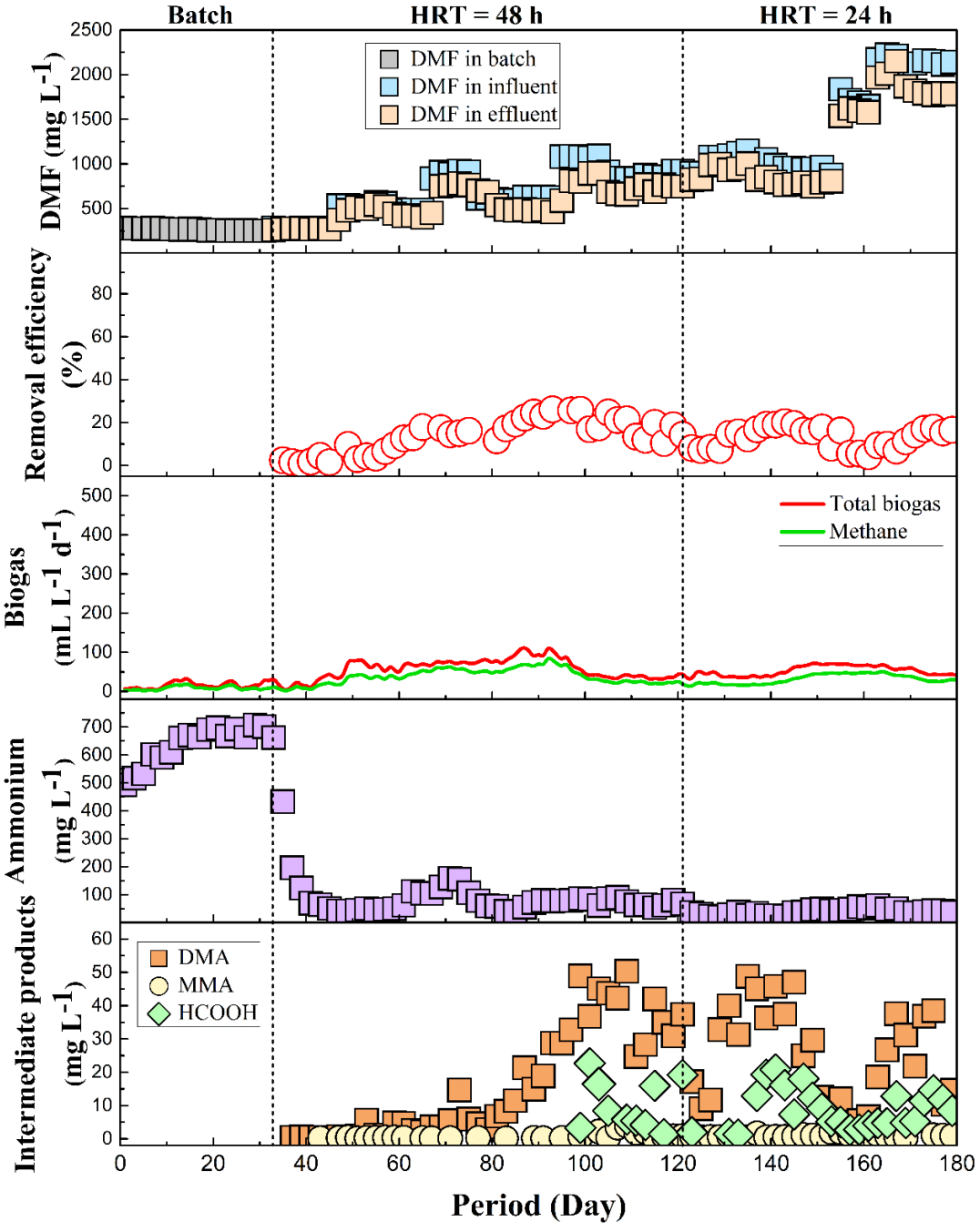

Fig. 4.2 A failed long-term cultivation by UASB solely inoculated with normal anaerobic granular sludge (AGS).

than feed on DMF, the AGS gradually self-digested, generating a little biogas, and the

decayed cells released a mass of ammonium.

After this stage, the UASB began to continuously operate under an HRT of 48 h and the influent DMF concentration shifted between 300 mg L^{-1} to 1000 mg L^{-1}. Despite the increasing production of biogas, the DMF removal efficiency remained low. During the period from Day 35 to Day 120, even though the MPR increased to 38.18 ± 20.48 mL L^{-1} d^{-1}, the average DMF removal efficiency was only 14.07%. After Day 35, after continuous operation was initiated, the ammonium accumulated during the batch period was flushed out by new influent, resulting in a sharp drop in the ammonium concentration from 661.69 mg L^{-1} to 69.49 mg L^{-1}. Even though the ammonium concentration fell to an average 73.83 ± 28.38 mg L^{-1}, it was still much higher than the theoretical ammonium concentration generated from just 14.07% DMF removal. This mismatch was evidence that the degradation of DMF was still inferior and ineffective during the continuously operational period. The inavailability of DMF likely led to the continued decaying of the AGS and most of the biogas and ammonium produced could be attributed to self-digestion. Even though the HRT was shortened to 24 h after Day 121, the elevation of the OLR did not stimulate or improve the degradation of DMF. The average MPR decreased to 31.71± 12.26 mL L^{-1} d^{-1}, the DMF removal efficiency remained at a low 13.20%. The ammonium concentration of 38.56 ± 10.50 mg L^{-1} in the effluent was even lower than the previous phase, indicating the very poor degradation of DMF.

In terms of intermediate products, it should be noted that low concentrations of DMA, one of the intermediate products of DMF, were observed since Day 37 and began to increase gradually, and that the other intermediates, HCOOH and MMA, were also occasionally detected after that point. The observation of DMF intermediate products was evidence that at least some of the DMF was indeed being hydrolyzed by AGS.

However, the biodegradability of DMF was so feeble that even after cultivated for 180 days of cultivation, the AGS was still unable to completely degrade DMF.

In summary, solely inoculated with AGS as the seed sludge, the UASB failed to effectively treat synthetic DMF-containing wastewater. It was considered that normal AGS could not feed on DMF even when cultivated for 180 days due to a lack of DMF-degrading bacteria in the original inoculum. Therefore, it was clearly necessary to inoculate the UASB with a new source of effective inoculum capable of degrading DMF.

4.3.2 Stage II: cultivation of DAS and pre-culturing of AGS

After consulting the extensive literature available on the aerobic degradation of DMF (Rahmaninezhad et al., 2016; Sanjeev Kumar et al., 2012; Xiao et al., 2016; Yang et al., 2014), it was considered possible that cultivating activated sludge would be an appropriate and convenient solution. Therefore, we launched a continuous aeration reactor (CAR) to culture a source of DMF-degrading activated sludge (DAS) which could specifically feed on DMF. On the other hand, from the results of Stage I, we found that AGS was also inferior at utilizing the intermediate products of DMF. According to the thumbnail **Fig. 4.3 (c)**, the SMA test on seed sludge indicated that AGS was adept at acetotrophic and hydrogenotrophic methanogenesis but poor at methylotrophic methanogenesis. As DMA and MMA are methyl-compounds which share the same methanogenic metabolism as methanol (Yeliseev et al., 1993), in order to enhance the methylotrophic ability, a pre-culturing period for AGS by UASB was simultaneously initiated with DMA as the substrate instead of DMF.

The DAS was cultivated for over 51 days (the sludge was taken on Day 51, but the reactor was kept operating all the time in case of lack of inoculum), and the performance of the 51-day cultivation is illustrated in **Fig. 4.3 (a)**. Day 1 to Day 14 was the batch

cultivation period, during which approximately 1000 mg L^{-1} DMF was dosed at the beginning. A decrease in the DMF concentration was clearly observed within 14 days, and the detection of the intermediate products DMA and MMA indicated that DMF was gradually being degraded under the aeration. Since Day 15, the CAR was switched to the continuous operation at the HRT of 48 h. During this period, even though the influent DMF concentration was elevated to about 2000 mg L^{-1}, the CAR had an excellent DMF removal efficiency of almost 100% since Day 21. The HRT was shortened to 24 h after Day 35, and with the increased organic loading, the DMF removal efficiency of CAR remained at nearly 100%. From this, it can be concluded that the successful cultivation of DAS with enriched DMF-degrading bacteria had been achieved.

Meanwhile, the pre-culture of AGS was performed by the UASB for 51 days. Approximately 1000 mg L^{-1} DMA was dosed into the reactor for three times of batch cultivation. When the substrate was exhausted, the AGS was washed with tap water and refed with DMA. As shown in **Fig. 4.3 (b)**, it was apparent that the DMA concentration and pH decreased as the biogas and ammonium concentration increased. Since the DMA shows alkalinity, the decrease in the pH indicated that DMA was gradually consumed in the UASB. The massive quantities of methane and ammonium produced were evidence of the thorough methanogenic digestion of DMA. It should be noted that during Phase 1, the much higher ammonium concentration detected was attributed to the eventual weeding out and decay of microorganisms which rejected DMA. In Phases 2 and 3, however, methylotrophic microorganisms were gradually cultured and enriched. The results of another SMA test conducted using the AGS which survived from the 51-day pre-culturing are shown in the thumbnail **Fig. 4.3 (d)**. As shown in **Fig. 4.3 (c)** and **(d)**, in comparison to the seed sludge, the methylotrophic activity of AGS

on Day 51 was enhanced due to the elevated SMAs of methanol, DMA and MMA from pre-culturing.

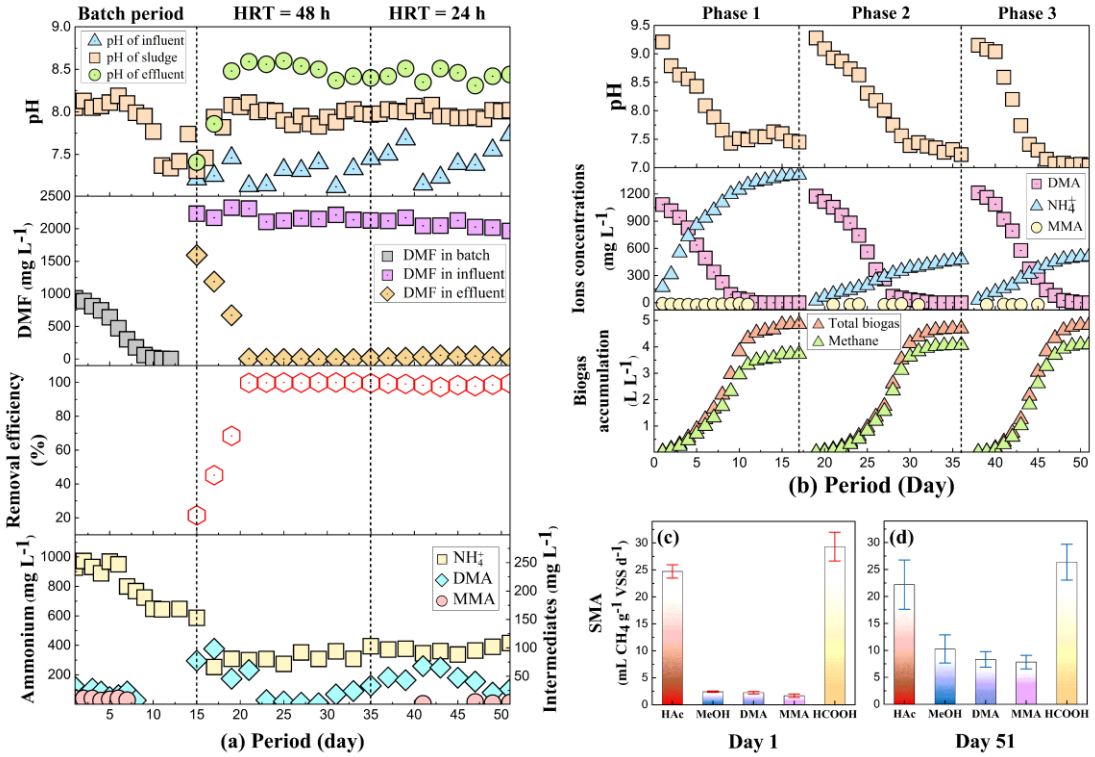

Fig. 4.3 A successful cultivation of DMF-degrading activated sludge (DAS) by a continuous aeration reactor (CAR) and the performance of aerobic degradation of DMF-containing wastewater (a); a batch cultivation fed by dimethylamine (DMA) in order to enhance the methylotrophic methane-producing ability of anaerobic granular sludge (AGS) (b); the specific methanogenic activities (SMA) of seed AGS on different substrates measured on the first day (c); the SMAs of AGS which had been fed with DMA for 51 days (d).

Based on the knowledge of DMF hydrolysis and methanogenesis (Ferguson et al., 2000; Sanjeev Kumar et al., 2013; Siddavattam et al., 2011; Urakami et al., 1990; Yeliseev et al., 1993), it is possible that DAS initially hydrolyzes DMF into DMA and HCOOH, then enhanced AGS makes use of the intermediate products to produce methane. Therefore, the complete methanogenic digestion of DMF could be achievable by mixing these two sources of sludge in the UASB.

4.3.3 Stage III: a long term continuous operation of UASB with mixed inoculum of DAS and AGS

4.3.3.1 Methanogenic degradation of DMF

All of the operational parameters for the different phases of the UASB in Stage III are given in **Table 4.2**. As shown in **Fig.4.4 (a)** and **Table 4.3**, the performance of DMF, COD removals and biogas production were investigated in detail over the entire

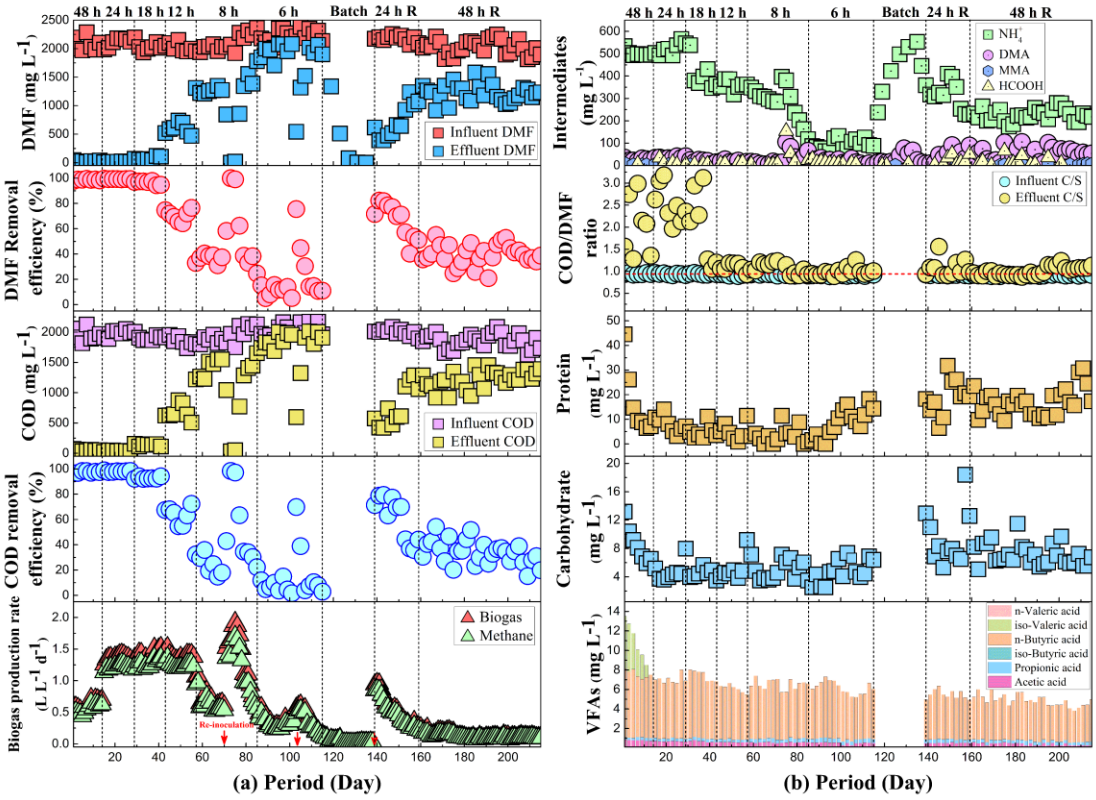

Fig. 4.4 Performance of UASB for treating DMF-containing wastewater in a 215-day long-term operation, variations of DMF and COD concentrations, DMF and COD removal efficiencies and biogas production rate were illustrated in (a), while the concentrations of intermediate products, protein, carbohydrate and volatile fatty acids (VFA) along with COD/DMF ratio were illustrate in (b).

experimental period of 215 days. Inoculated with the mixed sludge (DAS and AGS), the UASB successfully realized a rapid start-up without further cultivation in Stage III: 97.89% DMF removal efficiency and 96.48% COD removal efficiency were obtained on the first day along with a 0.52 L L^{-1} d^{-1} methane production rate (MPR). Stage III

was obviously much more effective than in Stage I: the UASB spent 180 days on domesticating DMF-degrading ability, but its eventual failure was attributed to the UASB being solely inoculated with AGS. From Day 1 to Day 28, under HRTs of 48 h and 24 h, the DMF was effectively degraded by the mixed sludge with remarkable removal efficiencies of 98.82% and 99.24%, respectively, and the efficiency was maintained at 96.53% when the HRT was shortened to 18 h. Correspondingly, the MPRs were 0.56 L L^{-1} d^{-1}, 1.218 L L^{-1} d^{-1} and 1.270 L L^{-1} d^{-1}, respectively. The thorough DMF removal and excellent methane recovery demonstrated that the methanogenic digestion of DMF was achievable when inoculating with a mixed inoculum consisting of DAS and AGS, and the cooperation of hydrolysis and methanogenesis (methylotrophic and hydrogenotrophic) were smoothly functioning by DAS and AGS, respectively. Among the bacterial strains considered to have the potential DMF-hydrolyzing ability are *Paracoccus huijuniae* (Sun et al., 2013), *Nitratireductor aquibiodomus* (Labbe, 2004) and *Hyphomicrobium zavarzinii* (Jérôme et al., 2007) etc. These bacterial strains hydrolyze DMF into DMA and HCOOH, which are then directly used by the methylotrophic archaea *Methanomethylovorans* and hydrogenotrophic archaea *Methanobrevibacter*. These results also suggested that using a mixed inoculum is feasible and practical for the anaerobic treatment of DMF-containing wastewater. One important point is that the DAS is quickly and massively domesticated by CAR within weeks. Another important point is that the methanogenic degradation of DMF produces methane with high purity. As shown in **Fig. 4.4 (a)**, methane accounted for over 90% of total biogas, which was much higher than that in other cases of AD reported in the literature, for chicken manure waste (Li et al., 2014), starch wastewater (Antwi et al., 2017) and food waste (Xiao et al., 2018), etc. In these studies, methane content was reported at around 50% ~ 70%.

Although DMF was effectively removed under the HRT 18 ~ 48 h, during these periods, the OLRs were just 1.63 ~ 4.22 g COD L^{-1} d^{-1}. Such OLRs were ordinary when compared to AD systems in other studies (Dhar et al., 2016; Qin et al., 2018). Therefore, in this study, the entire UASB system inoculated with the mixed sludge was considered easy to stabilize when fed with the low OLRs of DMF. Also, a period of two weeks was considered sufficient to estimate the stability of the removal performance under low OLRs. However, as DMF is difficult to be degraded and the real concentrations of DMF from factories are usually much higher, in order to qualify whether this system is capable of dealing with high OLRs for the practical applications, the OLR should be further elevated and the HRT should be shortened. When the HRT was further lowered to 12 h from Day 43 on, the DMF removal efficiency dropped to 70.64% and the MPR decreased to 1.250 L L^{-1} d^{-1}. After this, both the efficiency and the MPR sharply dropped to 36.58% and 0.56 L L^{-1} d^{-1} when the HRT was further shortened to 8 h. On the one hand, because both hydrolysis and methanogenesis were restrained and weakened when the OLR was greatly elevated and the duration of the reaction was shortened, the DMF-degrading ability of the mixed sludge eventually deteriorated. On the other hand, since DAS was domesticated under the aerobic condition, it was considered that those strictly aerobic microorganisms capable of producing the DMF-hydrolyzing enzyme gradually died and decayed over time while only a small proportion of facultative anaerobes survived in the UASB. The deterioration of the DAS may result in the weakening of DMF degradation. It should be noted that 0.5 L concentrated DAS was reloaded in the UASB on Day 70 in order to recover the DMF-degrading ability. Although DMF removal efficiency recovered to almost 100%, the MPR reached was at a high 1.71 L L^{-1} d^{-1}, and these values were maintained for just a week under such a high OLR of 9.24 g COD L^{-1} d^{-1}. This reloading of DAS demonstrated that the hydrolysis of DMF is the

key point determining whether the complete DMF methanogenic digestion could be achieved. The newly inoculated DAS contained fresh DMF-hydrolyzing bacteria and enzymes which helped the UASB to regain DMF removal, however, fresh aerobic sludge only survived temporarily under the condition of high OLR. When the HRT was further reduced to 6 h, the DMF removal efficiency fell dramatically to just 10.81% with a low MPR of just 0.33 L L^{-1} d^{-1}. Even though another portion of 0.5 L DAS was inoculated to the UASB again, under the highest OLR of 13.25 g COD L^{-1} d^{-1}, the DMF was still unable to be effectively degraded within the short reaction duration of 6 h. The DMF removal efficiency was temporarily regained to 75.54% before plunging to 10.86% within two weeks. From then on, the influent pump was stopped and the UASB was kept in a batch recovery period for 22 days. After injecting 0.5 L DAS into the UASB again on Day 139, the HRT was lengthened to 24 h. During this period, the DMF removal efficiency rebound to 67.99% while the MPR recovered to 0.53 L L^{-1} d^{-1}, however, the values decreased again after these improvements. Despite elevating the HRT to 48 h until the end, neither the removal efficiency nor the MPR regained during the final phase, and were barely maintained at 39.87% and 0.12 L L^{-1} d^{-1}, respectively. The deterioration of DMF removal indicated that DMF-degrading bacteria were insufficient in the UASB, and only a small proportion of facultative anaerobes survived in the end. This also suggests that only when sufficiently inoculated with DAS inoculum can the thorough anaerobic degradation of DMF be achieved in the UASB.

4.3.3.2 COD removal

The performance of COD removal by the UASB is shown in both **Fig. 4.4 (a)** and **Table 4.3**. It should be noted that although the COD concentration measured by the standard method is defined as the real COD concentration, for some degradation-

resistant organics, the measured COD concentration was much lower than its theoretical oxygen demand (ThOD) according to the stoichiometry (Baker et al., 1999). In this case, the ratio of real COD concentration to DMF concentration was measured as 0.96, and

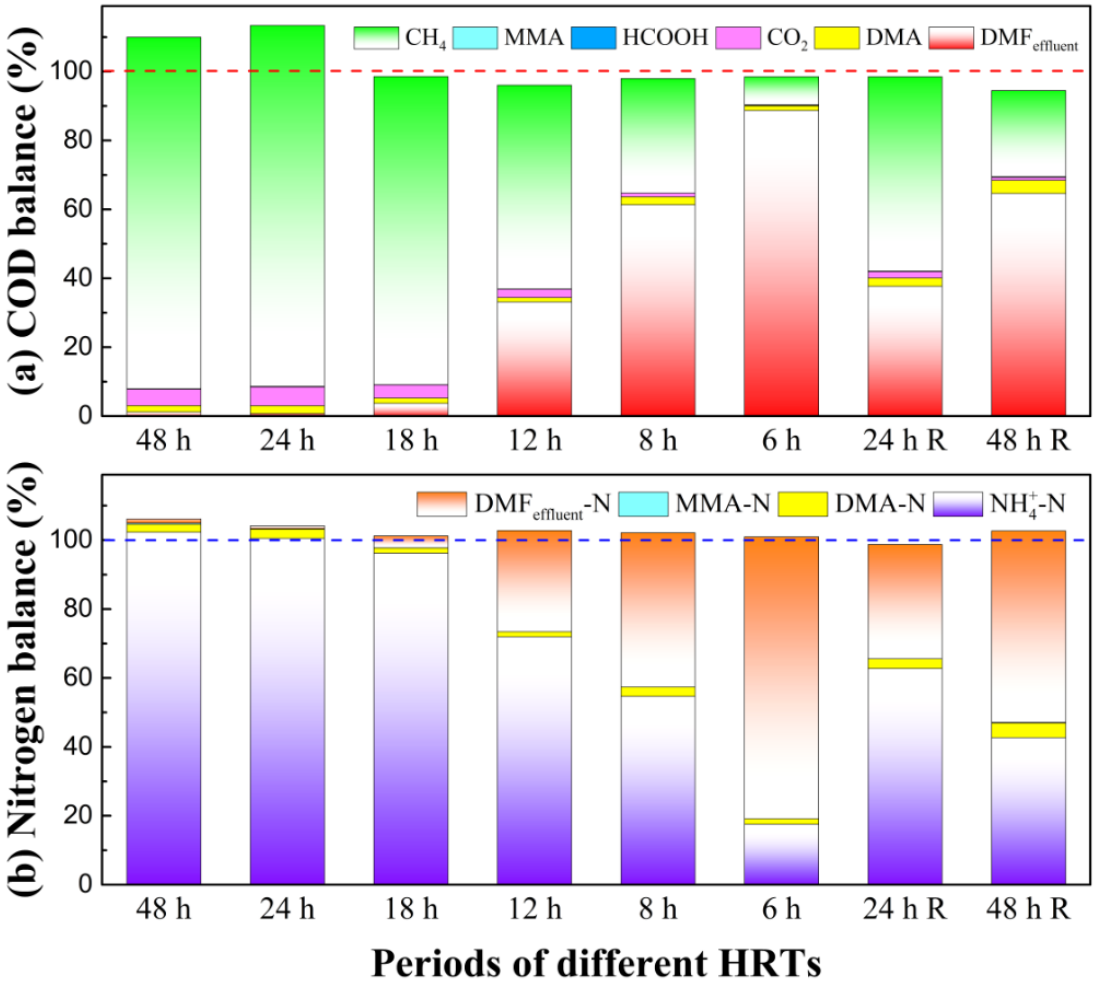

Fig. 4.5 Variation of COD balance and nitrogen balance along with the changing of hydraulic retention time (HRT). Notably, the COD value of all organics were calculated with the value of theoretical oxygen demand (ThOD).

a previous study reported about 0.98 (Kamimoto et al., 2009), while the ThOD to DMF is 1.53. The COD concentration measured in **Fig. 4.4 (a)** was considered suitable to evaluate the overall removal performance (Chen et al., 2016). When it comes to the COD balance, the real COD concentration was inadequate. As shown in **Fig. 4.5 (a)**, the COD balance of the substrate, intermediate and final products should be calculated with their ThOD values, and the results were close to 100%. Besides, the COD/DMF

ratio given in Fig. 4.4 (b) could be defined as an indicator for the existence of intermediate products or cellular metabolic products, as mentioned before. The measured ratio of COD/DMF should be closed to 0.96, the fact that COD/DMF ratios exceeded 0.96 suggests the existence of other organics such as intermediates, VFAs or protein in the effluent.

4.3.3.3 Ammonium and intermediate products

Ammonium, one of the final products, represents the degree of completion of DMF degradation. As shown in Table 4.3, Fig. 4.4 (b) and Fig. 4.5 (b), the ammonium concentration in the effluent was in accordance with the degradation of the DMF and was in good agreement with the nitrogen balance: when the DMF was thoroughly degraded, ammonium was the main source of nitrogen balance in the effluent; when DMF was insufficiently degraded, ammonium concentration decreased and the main source of nitrogen balance switched to the residual of the DMF. It should be also noted that most of the time, the real ammonium concentration was a little higher than the theoretical amount: this is likely due to the ammonium released from the lysis of dead cells.

In terms of intermediate products, on the one hand, high concentrations of which demonstrated DMF was indeed hydrolyzed. However, on the other hand, it was also considered that the relative low concentrations, and even absence of DMA, HCOOH and MMA, could be considered indications of excellent methanogenic ability when DMF was fully hydrolyzed. On the contrary, under the circumstance of low DMF removal, the relatively high concentrations of intermediate products could be indications that the methanogenic ability of sludge was inhibited or weakened. As shown in Fig. 4.3 (b) and Table 4.3, all the intermediates produced during the entire

experimental period were kept at low concentration levels, suggesting that most of the intermediate products were utilized in the process of methanogenesis. However, the average concentrations of DMA and HCOOH in the period from Day 139 to Day 215 were much higher than that in previous phases. This was probably because the DMF-hydrolyzing ability of the sludge had deteriorated since Phase VI when the DMF removal efficiency sharply dropped to 12.44%. The deterioration in DMF-hydrolyzing ability also significantly inhibited the following methanogenesis process. Consequently, the methane-producing archaea (MPA) were starved of usable substrates when just 12.44% DMF was converted into DMA and HCOOH during this period. When only 0.5 L DAS was re-inoculated to the UASB again on Day 139, the feeble MPA were still unable to effectively utilize the intermediate products despite the slight enhancement in the DMF-hydrolyzing ability.

4.3.3.4 Cellular metabolic products

Besides the DMF and intermediate residuals, the effluent of UASB contained some cellular metabolic products such as proteins, carbohydrates or VFAs (volatile fatty acids). Hence, COD removal efficiency was consistently lower than DMF removal efficiency. Results of the cellular metabolic products are provided in **Fig. 4.3 (b)**. It should be noted that although VFAs are commonly considered as the intermediates of the majority of organic wastes during the AD process (Huang et al., 2015; Jin et al., 2017; Wang et al., 2014), the extremely low concentrations of VFAs (in total < 20 mg L^{-1}) detected in this study suggested that they were likely from the self-digestion of decayed cells rather than the degradation of DMF, since DMF is a C_3 organic compound which follows methylotrophic and hydrogenotrophic metabolisms (Kong et al., 2018). As a result, in the anaerobic treatment of DMF-containing wastewater, the

accumulation of VFAs or pH drop is of no concern. Similarly, protein and carbohydrate, usually defined as soluble microbial products (SMP) in anaerobic treatment (R. Chen et al., 2017; Noguera et al., 1994), were obviously biomass-associated products (BAP) from metabolism or cell decay in this study, with low concentrations detected during the entire experimental period. However, these concentrations were relative higher during the period when the DMF was insufficiently degraded and the sludge was deteriorating. This could perhaps be attributed to the excretion of more proteins and carbohydrates on the occasion of a stress response performed by sludge during their interacting with a new environment, or cell lysis due to starving (Barker and Stuckey, 1999; Laspidou and Rittmann, 2002).

4.3 Significance of the discoveries

According to the results of the 215-day operation of the UASB and the relationship between the OLR and removal performance illustrated in **Fig. 4.6**, it is clear that with the increase in the OLR, or the shortening of the HRT, the hydrolysis of the DMF (the

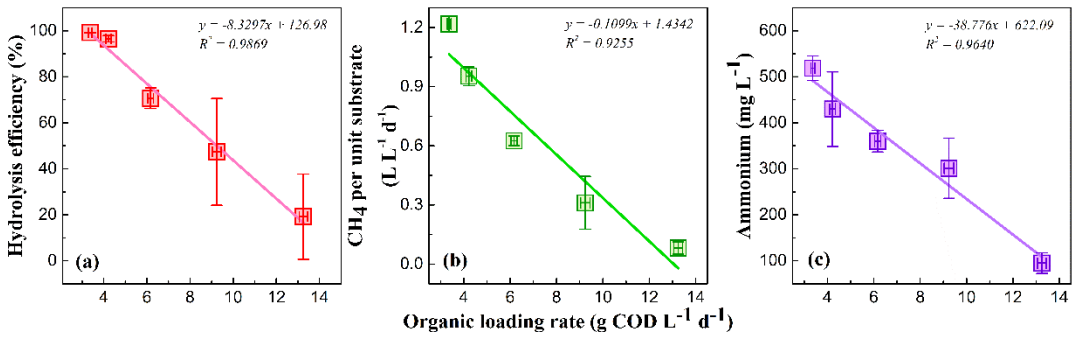

Fig. 4.5 Negative correlations of organic loading rate (OLR) to DMF hydrolysis efficiency (a), methane-producing ability (b) and ammonium production (c).

DMF removal efficiency) gradually weakened, finally resulting in a feeble methanogenesis with low methane production and low ammonium concentration. A comprehensive evaluation of the toxicity influence of DMF on SMA was carried out in

our previous study (Kong et al., 2018). The results of this evaluation showed that the DMF exhibited indistinct inhibition on methanogenesis, which means even when the system was suffering due to a high concentration of DMF, the MPA maintained tolerance to the high concentration of DMF. However, it should be noted that because DMF has been reported to be toxic to the activated sludge at a relatively low concentration of 200 mg L^{-1} (Chen et al., 2015; Yang et al., 2014), it is possible that the high concentration of DMF had a negative influence on the bacterial community and led to further deterioration in the hydrolyzing ability of those functional bacteria. In this study, however, even though the DAS was initially aerobic, after mixing with AGS in the UASB, the majority of the aerobes gradually died and only a small fraction of the facultative anaerobes survived under the anaerobic condition. It was these provided the key hydrolysis function. It was apparent that those aerobes which could have experienced massive growth under the aerobic condition turned into facultative anaerobes in the UASB, exhibiting much slower growing rates than their aerobic counterparts (Clements et al., 2002). It was also considered that these facultative anaerobes may have been fragile and sensitive to high OLR. With the shortening of the HRT, excessive DMF was not hydrolyzed sufficiently within the short interaction time and placed further pressure on the sludge. This presents the risk of irreversible inhibition and the continuous decay of facultative DMF-hydrolyzing bacteria. Therefore, a reasonable solution is to inoculate the UASB with enough fresh DAS at appropriate times to maintain the stable long-term operation of effective degradation of DMF in practical application.

4.4 Conclusions

In this study, the anaerobic treatment of DMF-containing wastewater was

successfully achieved by a UASB. A new source of inoculum was introduced to achieve the effective methanogenic degradation of DMF for practical application. The specific conclusions are as follows:

- The artificially mixed sludge of DAS and AGS could effectively degrade DMF and achieve an extraordinary methane recovery along with a quick start-up of UASB.
- DMF is hydrolyzed into DMA and HCOOH by DMF-hydrolyzing bacteria (or enzyme) contained in DAS, and then intermediates are fermented into methane by AGS.
- Although DAS plays key role in the hydrolysis of DMF, the facultative anaerobes grow slowly under the anaerobic condition, and are fragile and sensitive to the elevating of OLR.
- DAS was easily and rapidly cultured in massive quantities. With an OLR at no more than 6.17 g COD L^{-1} d^{-1}, the timely addition of DAS to the UASB is highly recommended to maintain a stable, effective performance for long-term operations.

Table 4.1 Information of inoculum sludge and recipe of nutrients for the substrate.

Information of inoculum sludge				**Nutrients (mg L^{-1})**	
	ADS[a]	**DAS[b]**	**AGS[c]**	NaCl	200
TS (g L^{-1})	12.73 ± 0.23	11.63 ± 1.43	-	KH_2PO_4	150
VS (g L^{-1})	9.91 ± 0.08	9.71 ± 1.21	-	$MgCl_2 \cdot 6H_20$	10
SS (g L^{-1})	11.02 ± 0.35	11.34 ± 0.40	-	$NiCl_2 \cdot 6H_20$	10
VSS (g L^{-1})	8.8 ± 0.11	9.59 ± 0.31	-	$CoCl_2 \cdot 6H_20$	10
Moisture content (%)	-	-	91.81 ± 0.46	$FeCl_2 \cdot 6H_20$	20
VSS/SS (%)	-	-	86.81 ± 0.93	$ZnSO_4$	20
Granular size (mm)	< 1	< 1	2.8 ~ 5	$CaCl_2$	20

[a] Anaerobic digestion sludge (ADS): collected from a local domestic wastewater treatment plant, inoculated into the CAR as seed sludge.

[b] DMF-degrading activated sludge (DAS): cultivated from ADS by continuously aerating for 51 days, then inoculated into the UASB.

[c] Anaerobic granular sludge (AGS): collected from a wastewater treatment plant which treats starch wastewater, inoculated into the UASB as seed sludge.

Table 4.2 Parameters of operation for different phases of the UASB in Stage III.

Phase	Periods (d)	HRT (h)	OLR[a] (g COD L^{-1} d^{-1})	DMF concentration (mg L^{-1})	Temperature (°C)	Influent pH	Up-flow flux (mL min^{-1})	Up-flow velocity (cm min^{-1})
I	1-14	48	1.63 ± 0.07	2127.70 ± 92.21	35.8 ± 0.5	7.65 ± 0.14	2.08	0.03
II	15-28	24	3.37 ± 0.08	2204.21 ± 53.74	35.2 ± 0.7	7.26 ± 0.16	4.17	0.06
III	29-42	18	4.22 ± 0.12	2116.11 ± 72.97	35.5 ± 0.7	7.21 ± 0.15	5.56	0.08
IV	43-56	12	6.17 ± 0.13	2059.02 ± 63.38	34.8 ± 0.4	7.43 ± 0.26	8.33	0.12
V	57-84	8	9.24 ± 0.20	2013.91 ± 60.10	34.9 ± 0.5	7.41 ± 0.17	12.50	0.18
VI	85-116	6	13.25 ± 0.19	2206.63 ± 117.47	35.3 ± 0.4	7.41 ± 0.22	16.67	0.24
VII (Batch Recovery)	117-138	∞[b]	-	-	35.4 ± 0.4	-	0	0
VIII	139-156	24	3.34 ± 0.10	2182.84 ± 53.64	35.1 ± 0.8	7.28 ± 0.20	4.17	0.06
IX	157-215	48	1.52 ± 0.07	2035.48 ± 127.69	35.3 ± 0.8	7.33 ± 0.15	2.08	0.03

[a] The organic loading rates (OLR) in this study was calculated by the real influent DMF concentrations ($COD_{therotical}$: DMF = 1.53).

[b] During the batch recovery period, HRT was regarded as infinite because influent was stopped.

Table 4.3 Performance of the UASB for treating DMF-containing wastewater in Stage III.

Periods (d)	Effluent pH	DMF removal efficiency (%)	COD removal efficiency (%)	Total biogas production rate (L^{-1} L^{-1} d^{-1})	Methane production rate (L^{-1} L^{-1} d^{-1})	Ammonium concentration (mg L^{-1})	DMA concentration (mg L^{-1})	HCOOH concentration (mg L^{-1})	MMA concentration (mg L^{-1})
1-14	7.74 ± 0.14	98.82 ± 0.57	97.63 ± 0.85	0.62 ± 0.08	0.56 ± 0.07	502.23 ± 14.13	26.56 ± 5.63	1.06 ± 2.82	2.86 ± 3.67
15-28	7.67 ± 0.12	99.24 ± 0.10	97.98 ± 0.33	1.36 ± 0.05	1.22 ± 0.04	518.82 ± 26.53	34.63 ± 3.31	8.97 ± 12.51	2.19 ± 2.15
29-42	7.56 ± 0.13	96.53 ± 1.37	92.87 ± 0.91	1.42 ± 0.07	1.27 ± 0.06	429.77 ± 80.91	22.61 ± 10.53	1.25 ± 1.18	0.45 ± 1.18
43-56	7.69 ± 0.15	70.64 ± 4.49	63.72 ± 6.68	1.40 ± 0.05	1.25 ± 0.05	359.98 ± 23.50	17.90 ± 5.59	2.22 ± 2.17	0
57-84	7.55 ± 0.20	47.36 ± 23.22	39.83 ± 26.29	1.05 ± 0.46	0.93 ± 0.40	301.21 ± 65.57	28.69 ± 29.88	0.38 ± 0.77	0
85-116	7.23 ± 0.15	19.19 ± 18.56	12.44 ± 18.51	0.38 ± 0.14	0.33 ± 0.13	95.02 ± 22.53	21.17 ± 15.37	3.34 ± 3.12	0
117-138	-	-	-	0.04 ± 0.03	0.04 ± 0.02	428.36 ± 123.81	32.86 ± 23.88	0.33 ± 0.86	0.13 ± 0.41
139-156	7.54 ± 0.21	67.99 ± 14.53	62.70 ± 17.16	0.59 ± 0.23	0.53 ± 0.21	337.45 ± 47.91	37.37 ± 29.11	12.53 ± 18.76	0.26 ± 0.78
157-215	7.35 ± 0.15	39.87 ± 8.45	34.13 ± 9.40	0.15 ± 0.06	0.12 ± 0.05	226.10 ± 25.95	54.21 ± 27.16	9.79 ± 17.28	1.65 ± 2.65

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Chapter 5 Anaerobic treatment of DMF-containing wastewater by SAnMBR

Abstract

The anaerobic treatment of wastewater containing approximately 2000 mg L^{-1} *N*, *N*-dimethylformamide (DMF) was conducted by a lab-scale submerged anaerobic membrane bioreactor (SAnMBR). The inoculum consisted of aerobic DMF-hydrolyzing activated sludge (DAS) and anaerobic digested sludge (ADS). A rapid start-up was achieved with thorough DMF methanogenic degradation on the first day. The results of a 250-day long-term experiment demonstrated that under a low organic loading rate (OLR) of 3.14 ~ 4.16 g COD L^{-1} d^{-1}, SAnMBR maintained excellent DMF removal efficiency along with high methane conversion. However, the elevation of OLR significantly limited DMF hydrolysis. When OLR exceeded 6.54 g COD L^{-1} d^{-1}, both removal efficiency and methane production dramatically dropped. The DMF-hydrolyzing bacteria originating from the DAS gradually decayed under the anaerobic condition, resulting in the weak hydrolysis of DMF. The shortening of hydraulic retention time (HRT) is not recommended for the SAnMBR because severe membrane fouling emerged when HRT was shortened to 8 h. To handle high OLRs, an appropriate solution is to maintain a low F/M ratio by increasing both the influent DMF concentration and sludge concentration. The high CH_4 content in the biogas, exceeding 85%, was shown to be the reason for the suitability of anaerobic treatment to DMF. Some improvements which would help to maintain the effective hydrolysis are propose: a side-stream system to replenish DAS to the SAnMBR is helpful; slight dosage of nitrate could also help to enrich the DMF-hydrolyzing bacteria; and the co-digestion of DMF and other organics might be convenient to establish a stable DMF-degrading

consortium.

5.1 Introduction

N, *N*-dimethylformamide (DMF) [$(CH_3)_2NCHO$] is widely used as a water-miscible polar solvent in a wide variety of chemical industries (Nisha et al., 2015; Sanjeev Kumar et al., 2013; Swaroop et al., 2009). While DMF has excellent versatility, the excessive discharge of DMF-containing industrial wastewater is becoming a critical environmental issue. DMF is known for its hepatotoxicity and carcinogenicity (Kim and Kim, 2011; Twiner et al., 1998), and its thermal stability and weak degradability make this compound obstinate and recalcitrant in nature (Sanjeev Kumar et al., 2012; Vidhya and Thatheyus, 2013). As a result, the effective treatment of DMF-containing wastewater is now becoming a worldwide concern.

Although many costly physical and chemical methods have been brought forward (Das et al., 2006; Ye et al., 2013), the biodegradation treatment of DMF-containing wastewater is considered the most suitable large scale and low cost solution (Kamimoto et al., 2009; Rahmaninezhad et al., 2016; Xiao et al., 2016; Zheng et al., 2016). As illustrated in **Fig. 5.1**, the principles of the two metabolic pathways of DMF aerobic biodegradation were elucidated many years ago (Ghisalba et al., 1986, 1985): with the help of *N*, *N*-dimethylformamidase (DMFase) on Pathway I, DMF is hydrolyzed into dimethylamine (DMA) and formic acid (HCOOH), and then DMA is further converted into mono-methylamine (MMA). Those bacteria which produce DMFase and feed on DMF as the sole substrate have been identified and documented in previous studies (Dziewit et al., 2010; Siddavattam et al., 2011; Zhou et al., 2018). On Pathway II, DMF loses its methyl-groups and is converted into *N*-methylformamide (NMF), formaldehyde (HCHO) and formamide (FA). This explains the focus of the majority of

previous studies on the aerobic treatment, and why the possibility of the anaerobic treatment of DMF has been largely neglected to date (Chen et al., 2016; Rahmaninezhad et al., 2016; Xiao et al., 2016; Yang et al., 2014), and also why DMF has been regarded

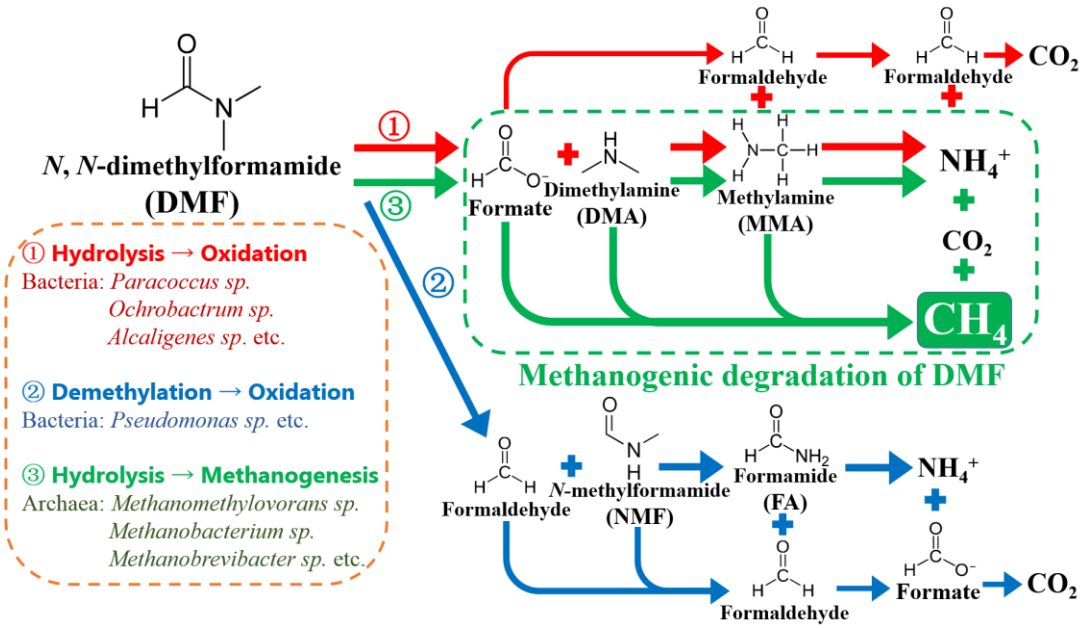

Fig. 5.1 Metabolic pathways of DMF biological degradation.

as a refractory compound under the anaerobic condition (Bromley-Challenor et al., 2000; Vidhya and Thatheyus, 2013). Actually, DMA and HCOOH, the intermediates of DMF on Pathway I, are common substrates for methylotrophic (Ferguson et al., 2000) and hydrogenotrophic methanogenesis (Pan et al., 2016), respectively. It would be a significant step forward if the hazardous DMF-containing wastewater could be effectively treated by methane fermentation in large quantities. The hypothesis for Pathway III proposed by our group is given in Fig. 1: DMF is also hydrolyzed anaerobically to DMA and HCOOH, and then the intermediates are further fermented to methane. This hypothesis was confirmed in our previous study on a co-cultured consortium consisting of both DMF-hydrolyzing bacteria and methylotrophic/hydrogenotrophic methanogens: the thorough methanogenic degradation of DMF was realized (Kong et al., 2018a). The stoichiometric equation of

DMF methanogenic degradation is described as follows:

$$(CH_3)_2NCHO + 2.5H_2O \longrightarrow 1.75CH_4 + 0.25CO_2 + NH_4^+ + HCO_3^-;\ \Delta G^0 = 349.07\ kJ\ mol^{-1} \quad (1)$$

Still, the feasibility and practicability of the anaerobic treatment of DMF must be qualified by an actual anaerobic digester. Among all the state-of-art processes, the submerged anaerobic membrane bioreactor (SAnMBR) which fulfills an effective solid/liquid separation with highly-enhanced effluent quality, smaller footprint, less sludge yield and longer solids retention time, is considered one of the most popular alternatives for the application of anaerobic treatment (Huang et al., 2011; Xia et al., 2016). Growing interest in the application of the SAnMBR to the treatment of industrial wastewater has been expressed in recent years (Kaya et al., 2017; Yurtsever et al., 2017). The feasibility of applying the SAnMBR to the treatment of DMF-containing wastewater, considering that DMF is an inherently miscible solvent, has also been explored (Chen et al., 2018; Hu et al., 2017).

To the best of our knowledge, this is the first attempt to apply SAnMBR to the treatment of high strength DMF-containing wastewater with the DMF as the sole substrate. The purpose was to evaluate the long-term behavior and stability for the practical application of SAnMBR on treating DMF-containing wastewater. We investigated the performance of a lab-scale SAnMBR during 250 days of operation with an inoculum of anaerobic co-cultured sludge (ACS) consisting of DMF-degrading activated sludge (DAS) and normal anaerobic digested sludge (ADS). The objectives of this study were to qualify the long-term feasibility and stability of SAnMBR and the shift of microbial community using ACS as the inoculum, and briefly evaluate the membrane performance when treating high strength DMF-containing wastewater; and also to provide new directions and suggestions for the applications and improvements

on the anaerobic treatment of wastewater containing degradation-resistant organic wastes.

5.2 Materials and methods

5.2.1 Experimental apparatus

As illustrated in Fig. 5.2, a submerged anaerobic membrane bioreactor (SAnMBR) made of polytetrafluoroethylene (PTFE) was used with an operational volume of 7 L.

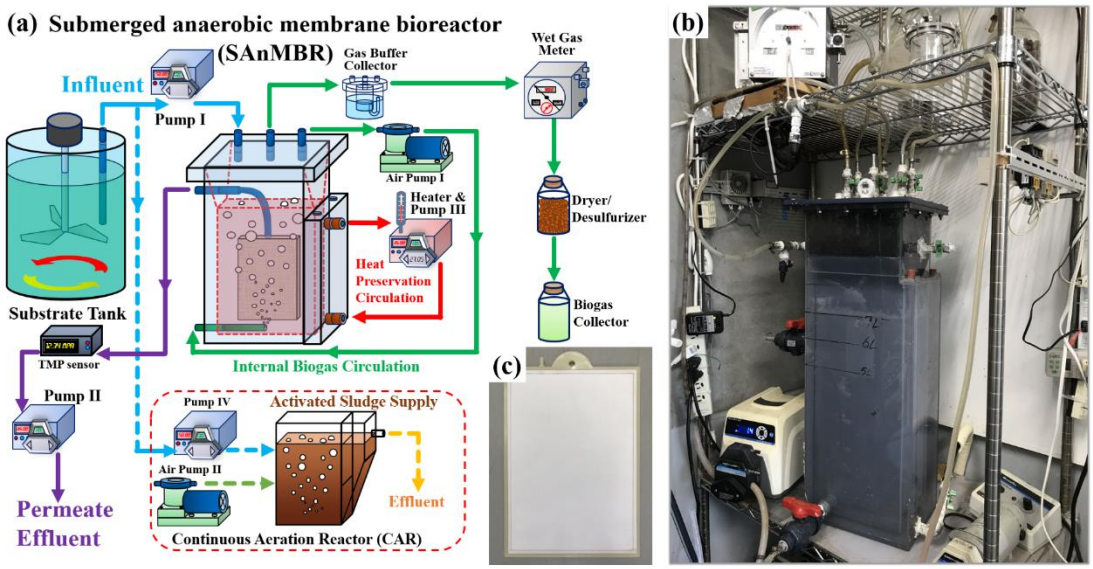

Fig. 5.2 Conceptual graphs of submerged anaerobic membrane bioreactor (SAnMBR) system in this study: (a) A schematic diagram of the SAnMBR system; (b) a photograph of the SAnMBR when operating; (c) a photograph of the new flat sheet membrane used in this study.

A flat sheet membrane with a pore size of 0.2 μm and a total area of 0.122 m^2 (21 cm × 29 cm × 2), made of chlorinated polyethylene (CPE) (Kubota Membrane Cartridge, Japan) was inserted into the reactor. The SAnMBR was kept at the mesophilic condition of 35 °C by thermostatic water circulation, and aerated by its own biogas with a flow rate of 5 L min^{-1} to adequately stir the sludge and to prevent membrane fouling. The hydraulic retention time (HRT) was gradually shifted from 24 h to 8 h during the entire

experimental period. The permeate circle of the flat membrane was 4 mins for operation and 1 min for relax. A continuous aeration reactor (CAR) made of PVC was usedto culture activated sludge on a large scale with an operational volume of 10 L. The CAR was also operated at 35 °C with a HRT of 48 h along with the SAnMBR in order to supply sludge if necessary.

5.2.2 Analytical reagents and methods

All analytical reagents were purchased from Wako Co. Ltd., Japan. Measurements of pH, temperature, DMF concentration, ions concentrations, and biogas components of N_2, CH_4 and CO_2 were the same as those employed in our previous study (Kong et al., 2018a). The membrane fouling-related substances, soluble microbial products (SMP) and extracellular polymeric substances (EPS) were defined as protein and carbohydrate. EPS was extracted using a cation exchange resin (DOWEX R Marathon C, Na^+ form, Sigma-Aldrich, USA) extraction method (Chen et al., 2017). Transmembrane pressure (TMP) was recorded by a digital pressure sensor (Keyence, AP-V85) which was joint to the permeate pipe of SAnMBR. Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectra of the sludge EPS were measured using a luminescence spectrometer (F-7000, Hitachi, Japan).

5.2.3 Feedstock, inoculum and experimental procedure

Synthetic wastewater containing approximately 2000 mg L^{-1} DMF and sufficient nutrients was stored in 120 L substrate tank. The SAnMBR inoculum consisted of 3 L of DMF-degrading activated sludge (DAS) cultivated for over two months by the CAR, and 3 L of normal anaerobic digested sludge (ADS) taken from a local wastewater treatment plant. Because the DAS could be repeatedly cultured in large quantities with

ease using the CAR (Kong et al., 2018a), the CAR was kept in continuous operation to supply DAS for the SAnMBR when needed. Details of the seed sludge and nutrients dosed in the synthetic wastewater are given in **Table 5.1**. Water samples were directly collected from the permeate outlet of the SAnMBR while sludge samples were taken from the discharge outlet.

The specific methanogenic activities (SMA) and biomethane potential (BMP) were calculated using the Gompertz equation described as follows (Kong et al., 2018a):

$$P = P_{max} \exp\left\{-\exp\left[\frac{\mu_{max} \times e}{P_{max}}(\lambda - t) + 1\right]\right\} \quad (2)$$

Where t is the cultivation period (day or hour), P_{max} indicates the maximum potential of biogas production (BMP, mL CH_4 g^{-1} VSS), λ represents the lag phase (day) and μ_{max} represents the maximum slope of the curve (SMA, mL CH_4 g^{-1} VSS d^{-1}).

The COD balance was standardized by stoichiometrically converting the concentrations of all output organic compounds derived from 1 L of substrate to their theoretical oxygen demands (ThOD) (Kong et al., 2019a), and the sum of the ThOD in the effluent was compared to the influent substrate by its percentage using the following equation:

$$\gamma_{COD} = \frac{1.53C_{DMF-eff} + 2.13C_{DMA} + 1.55C_{MMA} + 0.35C_{HCOOH} + 2.86V_{CH_4} + 1.43V_{CO_2}}{1.53C_{DMF-inf}} \% \quad (3)$$

Where C is the concentration (mg L^{-1}), and V is the volume (mL L^{-1}) of biogas produced from 1 L of substrate. The numbers before C and V are the conversion coefficients of the corresponding organic matter from its concentration and volume to ThOD.

The nitrogen balance was standardized by calculating the sum percentage of nitrogen concentrations of all output N-containing inorganic and organic matters derived from 1 L of substrate, and was compared with the influent nitrogen using the following equation:

$$\gamma_N = \frac{0.78C_{NH_4^+} + 0.19C_{DMF-eff} + 0.31C_{DMA} + 0.45C_{MMA}}{0.19C_{DMF-inf}} \% \quad (4)$$

Where C is the concentration (mg L^{-1}) and the number before C is the nitrogen content coefficient of the corresponding inorganic or organic matter calculated from its molecular weight. A close to 100% γ represents a good COD or nitrogen balance.

The utilization efficiencies of the intermediate products of DMF were calculated as follows:

$$\varphi_{DMA} = \frac{0.62(C_{DMF-inf} - C_{DMF-eff}) - C_{DMA}}{0.62(C_{DMF-inf} - C_{DMF-eff})} \% \quad (5)$$

$$\varphi_{HCOOH} = \frac{0.44(C_{DMF-inf} - C_{DMF-eff}) - C_{HCOOH}}{0.44(C_{DMF-inf} - C_{DMF-eff})} \% \quad (6)$$

$$\varphi_{MMA} = \frac{0.69(0.62(C_{DMF-inf} - C_{DMF-eff}) - C_{DMA})) - C_{MMA}}{0.69(0.62(C_{DMF-inf} - C_{DMF-eff}) - C_{DMA}))} \% \quad (7)$$

Where the number before the parenthesis is the ratio of molecular weight (DMA: DMF = 0.62, HCOOH: DMF = 0.44 and MMA: DMA = 0.69).

5.2.4 Sludge samples preparation, DNA extraction, sequencing and data processing

A total of 16 sludge samples were collected from the bottom outlet of the SAnMBR at different periods. The methods and procedures of DNA extraction, sequencing and data processing were consistant with those used in our previous study (Kong et al., 2019b). The operation taxonomic units (OTUs) were generated on the basis of 97% similarity. Singleton OTUs which only appeared once in all samples were removed and the total sequence number of each sample was unified to 65,000 to remove the errors of sequencing depth caused by DNA concentration. The α-diversity such as Chao1, the Shannon index, the Simpson index, good's coverage, observed species and singles were also calculated and listed in **Table 5.2**.

5.3 Results and discussion

5.3.1 Anaerobic treatment of DMF-containing wastewater

5.3.1.1 Long-term performance of the methanogenic degradation of DMF

The overall performance of the SAnMBR during the entire 250-day operation is

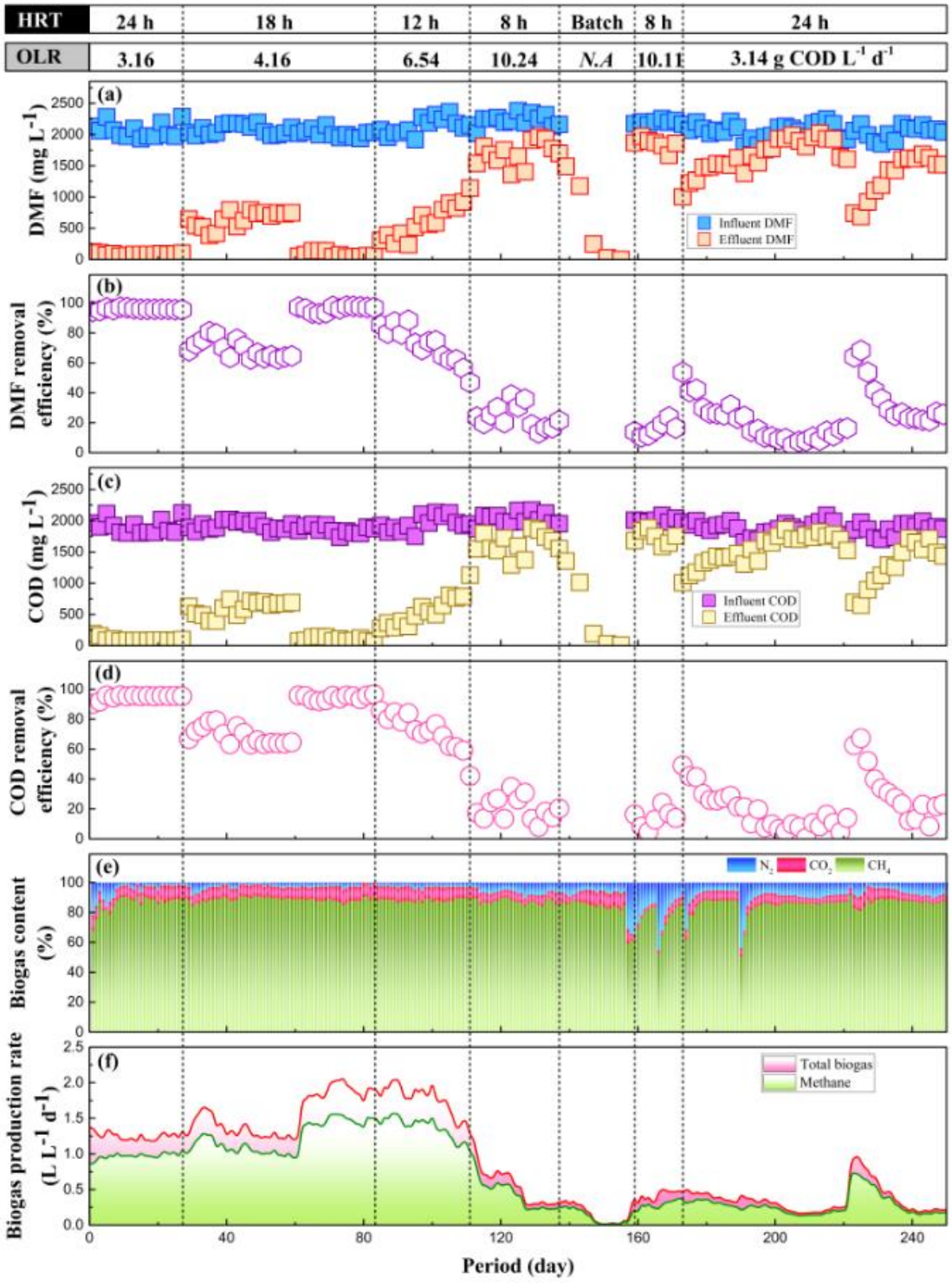

Fig. 5.3 Long-term performance of the SAnMBR during 250 days. (a) Influent and effluent DMF concentrations. (b) DMF removal efficiency; (c) Influent and effluent COD concentrations; (d) COD removal efficiency; (e) Biogas components (N2, CH4 and CO2); (f) Biogas production rates of total biogas and methane.

given in **Fig. 5.3**, **Table 5.3** and **Table 5.4**. At the beginning, the SAnMBR was initiated with a HRT of 24 h and an organic loading rate (OLR) of 3.16 g COD L^{-1} d^{-1}. As shown in **Fig. 5.3 (a)** and **(b)**, with the inoculation of ACS, the SAnMBR exhibited excellent DMF-degrading ability immediately: A high DMF removal efficiency of 94.45% and a high methane production rate (MPR) of 0.863 L L^{-1} d^{-1} were obtained on the first day. In our previous study, we found that some facultatively anaerobic bacteria could be enriched in the DAS. These bacteria were considered likely to have the potential to hydrolyze DMF into DMA and HCOOH, and then the methylotrophic and hydrogenotrophic methanogens directly utilized the intermediates (Kong et al., 2018a). The rapid establishment of a DMF-degrading consortium demonstrated that the co-culture of DAS and ADS was effective and feasible (Kong et al., 2018a, 2019b), and was also a repeatable and convenient cultivation method which could be easily applied to the quick start-up of anaerobic digesters (Kong et al., 2019a). From Day 1 to Day 28, as shown in **Fig. 5.3 (b)** and **(f)**, the reactor exhibited stable and excellent DMF removal efficiency and a high MPR. The nearly 100% removal indicated that the co-culture of DAS and ADS could achieve an effective methanogenic degradation of DMF under the low OLR.

However, when the HRT was shortened to 18 h and the OLR was elevated to 4.16 g L^{-1} d^{-1} from Day 29, the SAnMBR immediately showed the response by a slight decrease in the removal of DMF: from Day 29 to Day 59, the removal efficiency dropped to an average of 69.13%. It should be noted that the volatile suspended solids (VSS) concentration on Day 15 was 5.71 g L^{-1}, as shown in **Fig. 5.4 (d)**, and the

corresponding feed to microorganism (F/M, DMF to VSS) ratio was calculated as 0.34, and was then elevated to 0.46 on Day 29 when the OLR lifted. Apparently, DMF was not thoroughly degraded within 18 h with the higher DMF loading. This suggests that the sludge concentration was insufficient to maintain a thorough degradation of DMF. The sludge was replenished on Day 60 by re-inoculating 1 L of concentrated DAS. After that, the F/M ratio decreased to 0.33 and the reactor regained excellent removal efficiency and maintained a stable operation.

With the further shortening of HRT to 12 h, the OLR correspondingly rose to 6.54 COD L^{-1} d^{-1}. From Day 85 to Day 112, the SAnMBR exhibited a continuously decreasing trend in both DMF removal and MPR, suggesting that the degrading ability kept weakening with the increase in OLR, which was similar to our previous study (Kong et al., 2019a). When the HRT was lowered to 8 h, the OLR was elevated to the maximum level of 10.24 g COD L^{-1} d^{-1}. From Day 113 to Day 140, the DMF removal efficiency dramatically dropped to an average of 23.89%. It was clear that under such a high OLR, the sludge could no longer handle the excessive DMF within 8 h. Unlike the first re-inoculation on Day 60, the system could not regain its high removal efficiency even though another 1 L of concentrated DAS was replenished to the SAnMBR on Day 126. This was probably due to the shock of the sludge caused by the high OLR and the deactivation of the DMF-hydrolytic enzyme.

The reactor was then temporarily stopped from Day 141 to Day 157 for a batch period in order to observe whether the sludge was capable of recovering from the shock. As shown in **Fig. 5.3 (a)** and **(c)**, a decline in the DMF and COD concentrations were observed during this period. However, the low degrading rate suggests that rather than the expected recovery, the DMF-degrading ability of the ACS was further weakened. The reactor was then restarted on Day 159, and the HRT was still 8 h. The removal

efficiency further dove to its lowest average at 15.89%. This period only lasted for two weeks, and the HRT was soon lengthened to 24 h. The DMF removal efficiency slightly recovered to 53.66% on Day 173 due to the lowered OLR, however, after that, the efficiency maintained a steady decrease, finally dropping again to a low average of just 19.57% by Day 222. Without a high OLR, the DMF removal efficiency kept weakening. This phenomenon indicates a high likelihood that those DMF-hydrolyzing bacteria originating from the DAS kept decaying all the time even though the HRT was recovered to 24 h. The third re-inoculation of 1 L of concentrated DAS was performed on Day 222. The improvements were visible on the following day: The removal efficiency was immediately lifted up to 53.66% and the MPR raised to 0.368 L L^{-1} d^{-1} on Day 223. However, this re-inoculation was not sufficient to regain the thorough degradation of DMF to nearly 100% even when the HRT was 24 h. The low removal of DMF suggested that most of the DMF-hydrolyzing bacteria had decayed and the newly replenished 1 L of DAS was still insufficient to handle a normal OLR of 3.14 g COD L^{-1} d^{-1}. It should be restated that at the very beginning, it was the initial inoculation of 3 L DAS that realized a thorough methanogenic degradation of DMF. The removal efficiency and MPR then gradually decreased rather than remaining stable, which indicated that the newly inoculated DAS still kept decaying with time, and also suggested again that these DMF-hydrolyzing bacteria contained in DAS could not grow under the anaerobic condition (Kong et al., 2019b).

5.3.1.2 Ammonium, intermediate products and mass balance,

As one of the end products of DMF, ammonium was in correspondence with the completion of DMF methanogenic degradation. As illustrated in **Fig. 5.4 (b)**, the variation of ammonium concentration was quite similar to the DMF and COD

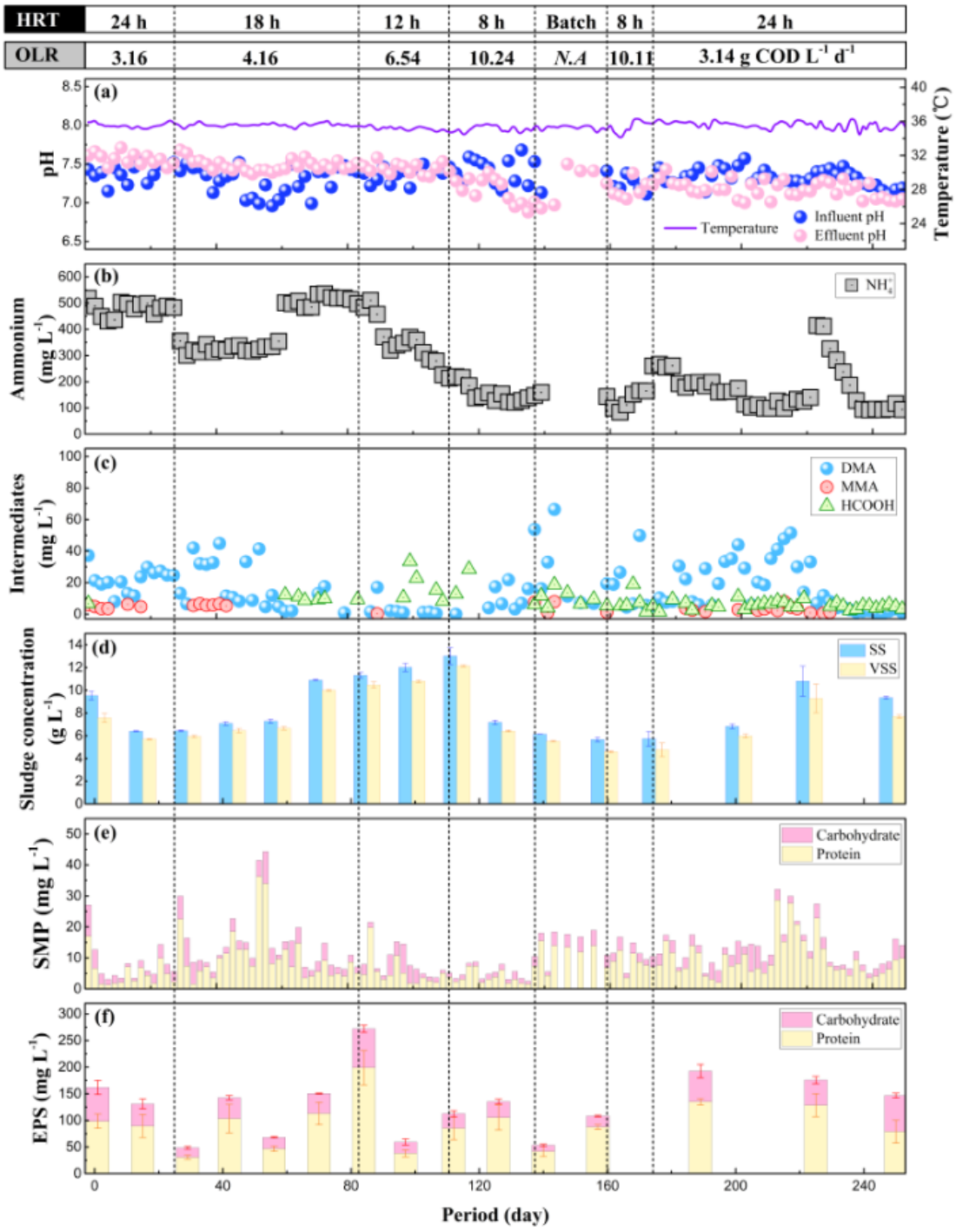

Fig. 5.4 Metabolic performance of the SAnMBR during 250 days. (a) Variations of influent and effluent pH and temperature. (b) Variation of ammonium (NH4+) concentration; (c) Intermediate products (DMA, MMA and HCOOH) concentrations; (d) Variation of sludge concentration (SS and VSS); (e) Variation of soluble microbial products (SMP, defined as protein and carbohydrate); (f) Variation of extracellular polymeric substrates (EPS, defined as protein and carbohydrate).

removal efficiencies given in **Fig. 5.3 (b)** and **(d)**. A high ammonium concentration

represented a relatively thorough degradation of DMF and a high MPR. Besides, as ammonium provides alkalinity, the variation of pH given in **Fig. 5.4 (a)** also reflected the production of ammonium. When DMF was effectively degraded, more ammonium was produced, and the effluent pH was higher than that of the influent. It has been confirmed that the intermediate products of DMF methanogenic degradation are DMA, HCOOH and MMA (Kong et al., 2018a): the residual concentrations of all intermediate products are illustrated in **Fig. 5.4 (c)**. As these intermediates are direct substrates for methanogenesis, once DMF was hydrolyzed into DMA and HCOOH, the methylotrophic and hydrogenotrophic methanogens could quickly utilize them. Therefore, the residual concentrations of intermediate products were usually very low.

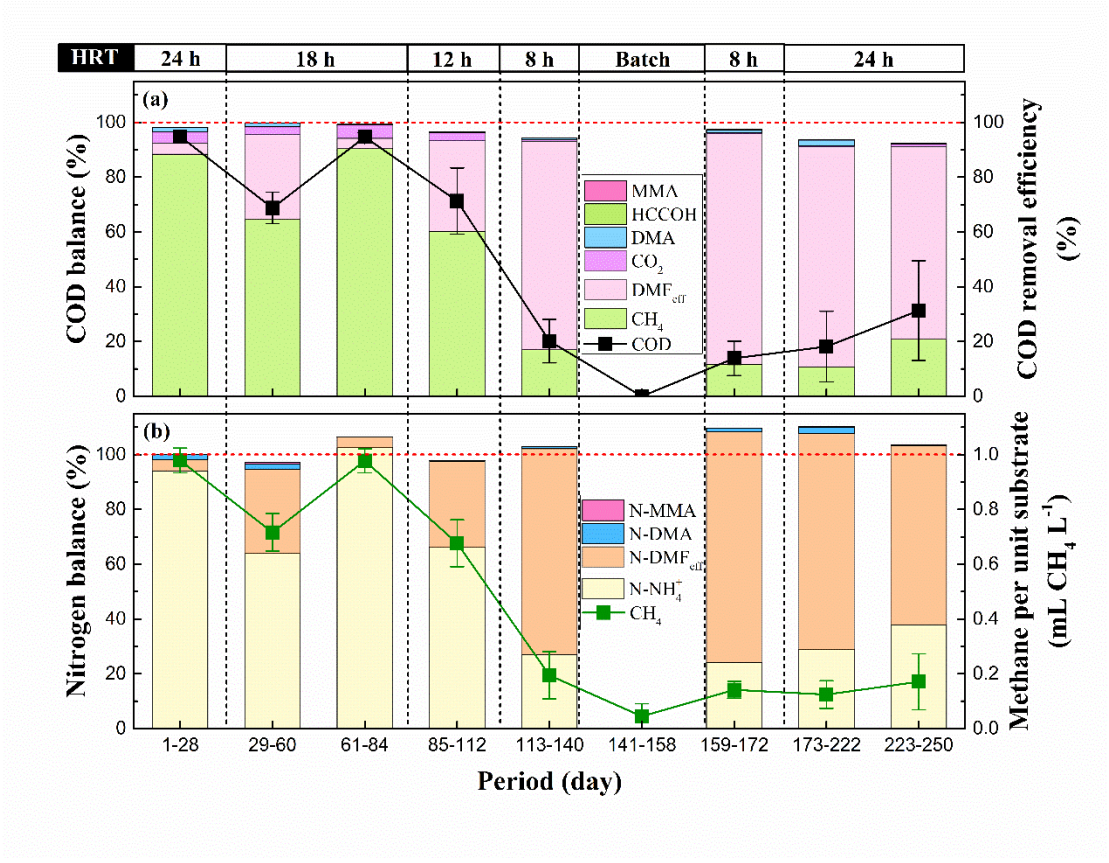

Fig. 5.5 Mass balances of (a) COD and nitrogen (b) in different periods of the long-term operation.

As shown in **Fig. 5.5 (a)**, the COD balance was in good accordance with all

components including total biogas (CH_4 and CO_2), residual DMF and residual intermediate products. All of them constituted a total effluent COD stack, and which was basically equivalent to a 100% of total influent COD. The largest component when the system obtained the excellent COD removal efficiency of nearly 100% was methane. When the DMF-degrading ability weakened, the residual DMF turned out to be the major component. Similarly in **Fig. 5.5 (b)**, the nitrogen balance was also in good accordance with all N-containing components. Ammonium became the largest source of nitrogen balance when DMF was effectively degraded to methane, and was replaced by residual DMF when the DMF hydrolysis completion was low.

It was also demonstrated that the methanogenic degradation of DMF can be divided into two phases: hydrolysis and methanogenesis (Kong et al., 2018a). Therefore, the DMF removal efficiency represents the completion of hydrolysis, while the removal efficiencies of intermediate products represent the completion of methanogenesis. As shown **Fig. 5.6**, a group of SMA tests were conducted at time intervals to evaluate the performance of methanogenesis. The overall SMA of ACS was represented by feeding ACS with DMF as the sole substrate, and the results are given in **Fig. 5.6 (b)**. The DMF-driven SMA was initially high with an ability of 113.60 (unit: mL CH_4 g^{-1} VSS d^{-1}) at the beginning, and reached its highest at 264.38 after the first re-inoculation on Day 61. After that, the SMA continued to decrease to its lowest point on the final day. Apparently, the variation of the DMF-driven SMA was in accordance with the variation of DMF removal efficiency (or hydrolysis completion) given in **Fig. 5.6 (a)**: high SMAs were obtained when the DMF removal efficiency was high. For the intermediate products, on the one hand, the DMF-driven, MMA-driven and methanol-driven (MeOH) SMAs directly reflect the abilities of methylotrophic methanogenesis. As shown in **Fig. 5.6 (c)**, the high removal efficiencies of DMA and MMA during the entire experiment

indicate that the methanogens could effectively utilize DMA and MMA produced from the hydrolysis process, and all the methyl-compounds-driven SMAs indicated in **Fig. 5.6 (d)** basically remained stable at a high ability of 100 ~ 150. The highest SMAs were achieved when re-inoculation was conducted. On the other hand, the HCOOH-driven SMA directly represents the hydrogenotrophic ability. An effective utilization of HCOOH was also performed by the methanogens, as the removal efficiency of HCOOH was closed to 100%, as given in **Fig. 5.6 (e)**, and the HCOOH-driven SMA given in **Fig. 5.6 (f)** was basically higher than the initial SMA of over 50. The results indicated that the methanogenic degradation of DMF relies on both hydrolysis and methanogenesis, and that hydrolysis is the key process which decides whether a thorough methanogenic degradation of DMF can be achieved. The high SMAs of all corresponding intermediates obtained during the entire operation show that the methanogenesis process was not significantly influenced even when the hydrolysis

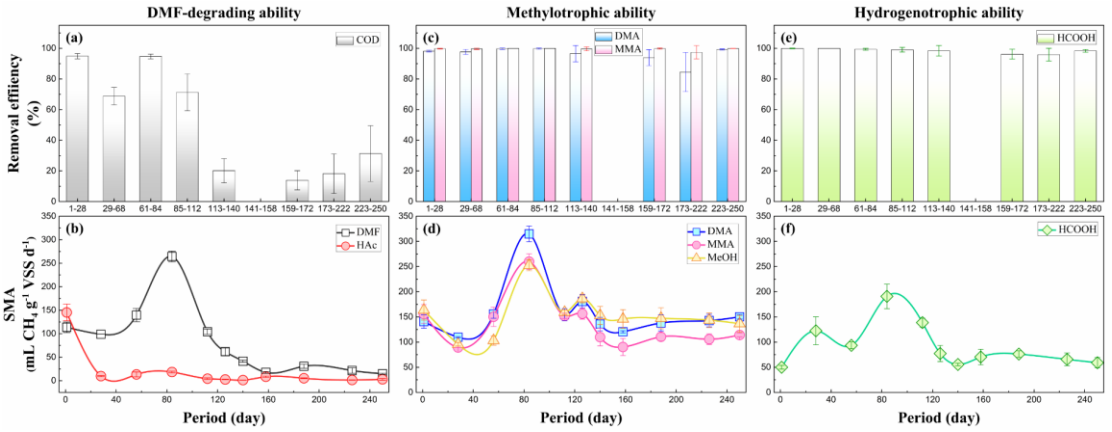

Fig. 5.6 Evaluation of DMF-degrading ability (a), methylotrophic ability (c) and hydrogenotrophic ability (e), along with the variations of specific methanogenic activities (SMAs) of DMF-driven and HAc-driven activities (b), methyl-compounds-driven activities (d) and HCOOH-driven activity (f).

process was weakened. However, the deterioration in the hydrolysis process significantly weakened the overall methanogenic degradation of DMF when the methylotrophic and hydrogenotrophic abilities of methanogens remained at high levels.

It should also be noted that the acetotrophic ability illustrated in **Fig. 5.6 (b)** was obtained by feeding the ACS with acetate (HAc) as the sole substrate. The quick drop in the HAc-driven SMA to an extremely low level approaching zero demonstrated again that the methanogenic degradation of DMF was irrelevant to the acetotrophic methanogenesis since DMF is a C_3 compound and there is no possibility of producing C_3 to C_6 volatile fatty acids (VFAs) on the pathway of DMF methanogenic degradation (Kong et al., 2018a, 2019a).

5.3.1.3 Shift of microbial community structure

It has been proved that DAS contains abundant DMF-hydrolyzing bacteria such as such as genera *Pseudomonas* (Schär et al., 1986), *Ochrobactrum* (Veeranagouda et al., 2006), *Paracoccus* (Zhou et al., 2018), *Alcaligenes* (Hasegawa et al., 1999) and *Bacillus* (Vidhya and Thatheyus, 2013), and some bacteria which potentially hydrolyze DMF were also proposed in our previous studies such as *Hyphomicrobium*, *Nitratireductor*, *Burkholderia*, *Rhodobacter* and *Catellibacterium* (Kong et al., 2018b, 2018a, 2019b), while ADS collected from wastewater treatment plant originally contains abundant methanogens, including the genera *Methanomethylovorans* and *Methanobacterium*, which are responsible for the methanogenesis. Similarly, in this study, we also focused on the variation of those bacteria and archaea which play a role in the hydrolysis and methanogenesis of DMF. As shown in **Fig. 5.7**, the variation of the prokaryotic community structure of the ACS consortium was characterized and represented by a total of 16 samples taken at various intervals during the entire operation. As illustrated in **Fig. 5.7 (b)**, the variation of the total sequence number of 5 genera of bacteria was in good accordance with the DMF removal efficiency. As a matter of fact, in our previous study, we found that these candidate DMF-hydrolyzing

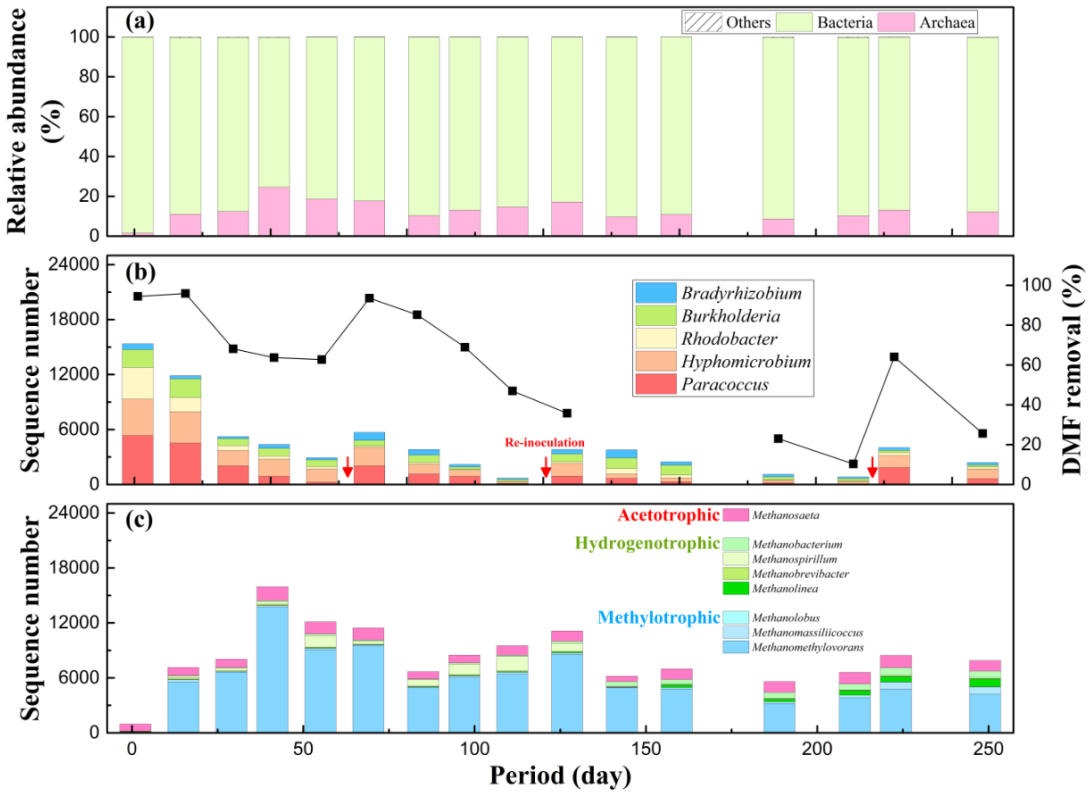

Fig. 5.7 Characterization and shift of the prokaryotic community: (a) A comparison of entire bacterial and archaeal abundances; (b) Variation of the total sequence number of some bacteria which are selected as the potential DMF-hydrolyzing bacteria; (c) Variation of the total sequence number of all methanogens in the prokaryotic community.

bacteria were facultatively anaerobic denitrifying bacteria (Kong et al., 2019b). They are aerobic and can be enriched in activated sludge, but also survive under the anaerobic condition. However, these bacteria were decaying all the time from the beginning, an effective hydrolysis of DMF seemed to be "temporary" and only lasted for weeks. It was considered that these denitrifying bacteria might cause a "niche overlap" with the methanogens, due to the lack of nitrate or nitrite in our system, and that these denitrifying bacteria could not perform the "denitrification process" without nitrate as the electron acceptor. Therefore, they became hydrogenotrophic bacteria to assimilate hydrogen (or formate) or methylotrophic to assimilate C_1 compounds such as MMA:

both of these are feed to methanogens (Kong et al., 2019b). Consequently, they are outcompeted by methanogens. Due to the insufficient formate and MMA for these hydrolyzing bacteria, they continued to decay over time, and the hydrolysis of DMF gradually weakened and could not be recovered unless new sludge was re-inoculated.

Methylotrophic genus *Methanomethylovorans* was the most predominant genus of archaea, as shown in **Fig. 5.7 (c)**. Although this methanogen was rare at the beginning, it proliferated in great proportions with time, demonstrating again that DMF largely follows the path of methylotrophic methanogenesis (Kong et al., 2018a, 2019a). Four genera including *Methanobacterium*, *Methanolinea*, *Methanobrevibacter* and *Methanospirillum* were aligned as hydrogenotrophic methanogens and their total abundance was the second predominant in the archaeal community. Only one acetotrophic genus *Methanosaeta* was found in this study, and was relatively rare in the archaeal community.

5.3.2 Performance of membrane

5.3.2.1 Flux and transmembrane pressure

The overall variations of flux and the maximum TMP during the entire operation are illustrated in **Fig. 5.8 (a)** and **(b)**, respectively. The flux was kept constant during each period. Details of the filtration behaviors of real time TMP within a permeate circle are provided in **Fig. 5.8 (c)** to **(h)**. It has been shown that with higher permeated flux, higher TMPs were obtained (Shang et al., 2015; Zhang et al., 2016). As shown in **Fig. 5.8 (a)** and **(b)**, under the HRTs of 24 h and 18 h, the performance of the flat membrane was good due to the consistently low and stable TMP averaging just 0.24 MPa and 0.47 MPa, respectively. It should be noted that after the first re-inoculation, the TMP for 18 h slightly increased to 0.80 MPa due to the artificially increased SS. The real time TMP

performance under 24 h and 18 h are given in **Fig. 5.8 (c)**, **(d)** and **(e)**. The maximum TMP was obtained at the very beginning of each permeate circle with constant flux (He et al., 2017; Miller et al., 2014). However, when the HRT was further lowered to 12 h, the TMP significantly increased by about 10 folds to an average of 7.00 MPa. A

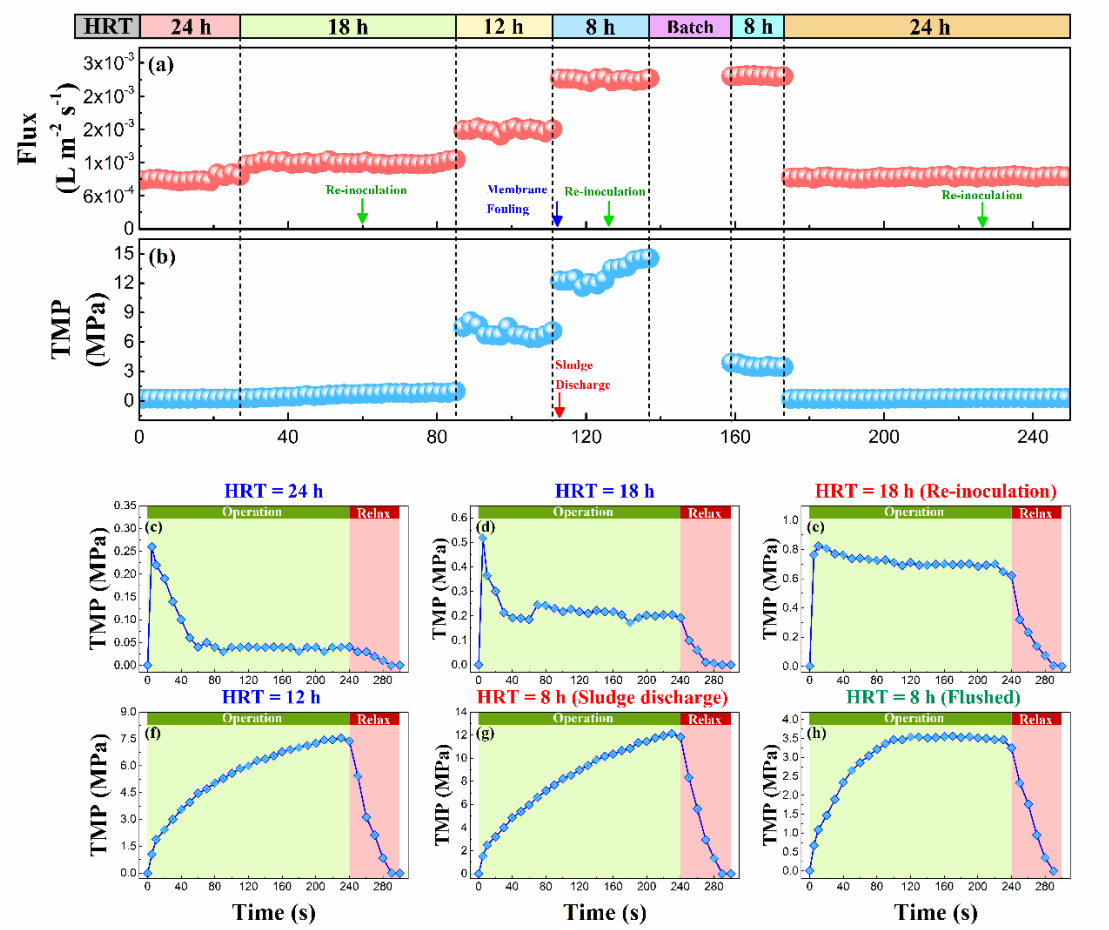

Fig. 5.8 Overall performance of the membrane fouling: Variations of flux (a) and TMP (b). The real-time filtration performance of the membrane: (c) HRT = 24 h; (d) HRT = 18 h; (e) HRT = 18 h (after re-inoculation); (f) HRT = 12 h; (g) HRT = 8 h (after sludge discharging) and (h) HRT = 8 h (after flushed).

continuously increasing trend was observed during the permeate phase, with a maximum TMP obtained at the end of permeating. This indicated the flat membrane was burdened and that membrane fouling was gradually occurring due to the shortening of the HRT. It should be noted that a fatal fouling was observed on Day 112 under the HRT of 8 h. At this point, the permeate flux dropped dramatically to a point where it no

longer maintain constant value even though the membrane was then flushed by the biogas stripping system for 12 hours. To recover the permeate flux, we immediately discharged a portion of sludge from the SAnMBR. However, the maximum TMP for 8 h was still elevated to an average of 12.99 MPa. During the batch period, the membrane was flushed for more than two weeks, and the TMP dropped to an average of 3.58 MPa. When the HRT was finally lengthened to 24 h, the TMP recovered again to a low level of just 0.25 MPa, which was similar to the initial period with HRT 24 h.

5.3.2.2 Sludge concentration and cellular metabolic products

The suspended solid (SS) concentration of the sludge is usually considered the dominant direct factor influencing the TMP and membrane fouling (Tan and Li, 2016; Xu et al., 2015). In this study, the variation of the sludge concentration of SAnMBR during the entire operation was attributed to the assimilation and decay of cells, re-inoculation and discharge. The overall results are provided in **Fig. 5.4 (d)**. Since the seed inoculum consisted of DAS and ADS, those aerobic bacteria which were abundant in DAS quickly died and decayed resulting in a decrease in the SS concentration from 9.54 g L^{-1} to 6.41 g L^{-1} on Day 28. After that, due to the assimilation and proliferation, the SS slightly increased from 7.29 g L^{-1} on Day 56. Although the re-inoculation on Day 60 significantly lifted the SS to 10.91 g L^{-1}, the TMP still maintained at a low 0.47 MPa under the HRT of 18 h. With the shortening of HRT, the SS gradually increased to 13.02 g L^{-1}, resulting in serious fouling. The effluent could not permeate thorough the flat membrane to realize a HRT of 8 h when the SS concentration was higher than 13.02 g L^{-1}. After sludge discharge, the SS dropped to 6.77 g L^{-1} and the smooth permeate was recovered. Then the sludge continued to decay even though a re-inoculation of a tiny amount of DAS was conducted on Day 126. The SS concentration kept decreasing

from 6.42 g L^{-1} to 5.74 g L^{-1} on Day 174. While the third re-inoculation elevated the SS to 10.81 g L^{-1} again, the SS concentration decreased to 9.35 g L^{-1} by the end with newly inoculated DAS.

The overall variation of membrane fouling-related substances, SMP and EPS, are given in **Fig. 5.4 (e)** and **(f)**, respectively. It can be seen that the most abundant component was protein and that carbohydrate was minor. SMP are defined as soluble cellular components and are divided into utilization-associated products (UAP) and biomass-associated products (BAP) (Namkung and Rittmann, 1986). Because UAP are

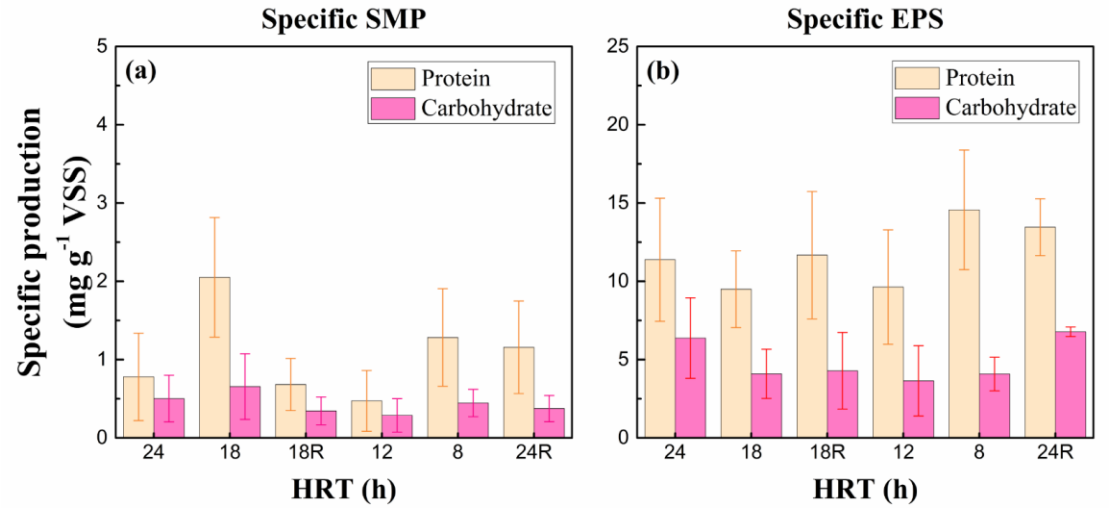

Fig. 5.9 Variation of specific SMP (SMP mg L^{-1}/VSS g L^{-1}) and EPS (EPS mg L^{-1}/VSS g L^{-1}) in different periods.

directly produced during the metabolism and associated with substrate uptake and biomass growth, the possibility that SMP component depends on different carbon sources has been considered (Barker and Stuckey, 2001). The carbohydrate percentage of SMP presented from some earlier studies was higher than protein when treating municipal wastewater (Salazar-Peláez et al., 2011) and carbohydrate-rich wastewater (Arabi and Nakhla, 2008). In this study, however, the carbohydrate occupancy in SMP was lower. This can most likely be attributed to the use of DMF as the sole substrate. It has been reported that BAP are associated with biomass decay and are also defined as

dissolved EPS (Luna et al., 2014), and that the protein content is also much higher than carbohydrate when the sludge is decaying (Laspidou and Rittmann, 2002). It should be also noted that the DMF-hydrolytic enzyme, DMFase, is the most important protein synthesized for the degradation of DMF, and the enzyme is more likely associated with the EPS (Hasegawa et al., 1999).

Although the total SMP and EPS changed significantly from one period to the next, as shown in **Fig. 5.4**, no close relationship was found between them and the TMP or fouling. Note that in the literature, the total SMP and EPS have been widely reported as the key foulants to membrane fouling. As shown in **Fig. 5.9**, the specific SMP and EPS were calculated for the purpose of comparison, and that the variations of specific SMP and EPS were irregular with the shortening of HRT, and no correlation was found with the increase in TMP. Conversely, the SMP and EPS appear more inclined to be related to the decay of sludge. It was found that relatively higher concentrations of SMP and EPS were observed during these "deterioration periods" when DMF removal efficiency was low, as shown in **Fig. 5.4 (e)** and **(f)**. On the one hand, more metabolic products

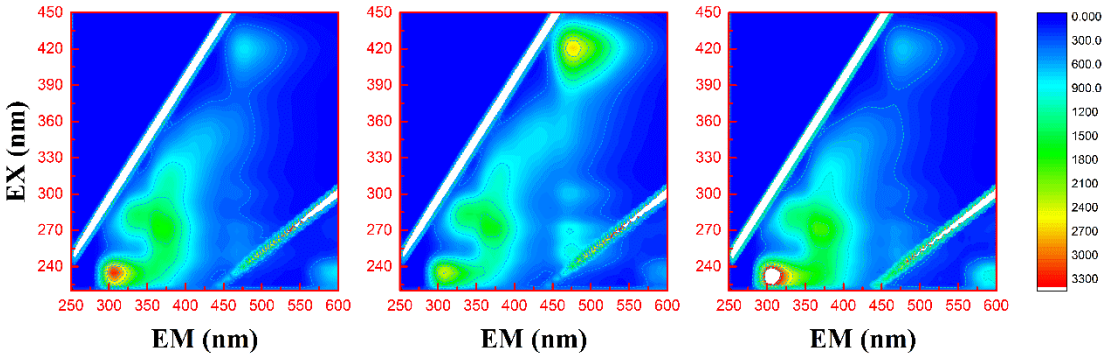

Fig. 5.10 Three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectra of EPS extractions during different periods. (a) EPS extracted on Day 15, stable operation with the highest DMF removal efficiency; (b) EPS extracted on Day 85, DMF removal began to weaken; (c) EPS extracted on Day 159, end of batch recovery period, with low DMF removal efficiency.

were excreted when a stress response was performed by sludge during the starvation

(Barker and Stuckey, 2001; Laspidou and Rittmann, 2002). On the other hand, as mentioned in the section focused on the microbial community, those DMF-hydrolyzing bacteria did not grow consistently under the anaerobic condition. Instead, they gradually decayed and released cellular metabolic products which also form SMP and EPS (Kong et al., 2019a). Besides, as illustrated in **Fig. 5.10**, the 3D-EEM fluorescence spectra of EPS extracted during different periods also suggested that the EPS in this study was likely sourced from the sludge decay and cell lysis, as the intensity of the humic acid-like peak was high (Zhu et al., 2012). Peak A (Ex/Em ≈ 230/310) and Peak B (Ex/Em ≈ 280/360) represent the aromatic protein-like EPS, whose intensities are usually the highest ones, suggesting that protein might be the main component of EPS in this study. While Peak C (Ex/Em ≈ 420/475) represents the humic acid-like EPS which mainly originates from the sludge decay and cell lysis, the intensity of Peak C is also high, especially during the period when the DMF removal efficiency was deteriorating.

5.4 Significance of findings and prospects

5.4.1 Superiority on methanogenic degradation of DMF

The results of this study indicated that the methanogenic degradation was perfectly suitable to the treatment of DMF-containing wastewater. For one thing, anaerobic treatment could tolerate a much higher OLR and realize the energy recovery by methane production. A comparison to other similar studies on the treatment of DMF is listed in **Table 5.5** to emphasize the superiority in this study. Another point is that the methanogenic degradation of DMF obtained a much purer biogas as the CH_4 content in this study: the CH_4 content was basically over 85%, while the CH_4 content in the anaerobic digestion of other organic wastes was commonly 50% ~ 75% (Antwi et al.,

2017; Xiao et al., 2018). This is shown in **Fig. 5.3 (f)** and **Table 5.4**.

5.4.2 F/M ratio and HRT

A challenging issue is that in order to maintain an effective hydrolysis of DMF under a high OLR, the SAnMBR should retain enough sludge which contains sufficient DMF-hydrolyzing bacteria. However, the increase in the sludge concentration also inevitably

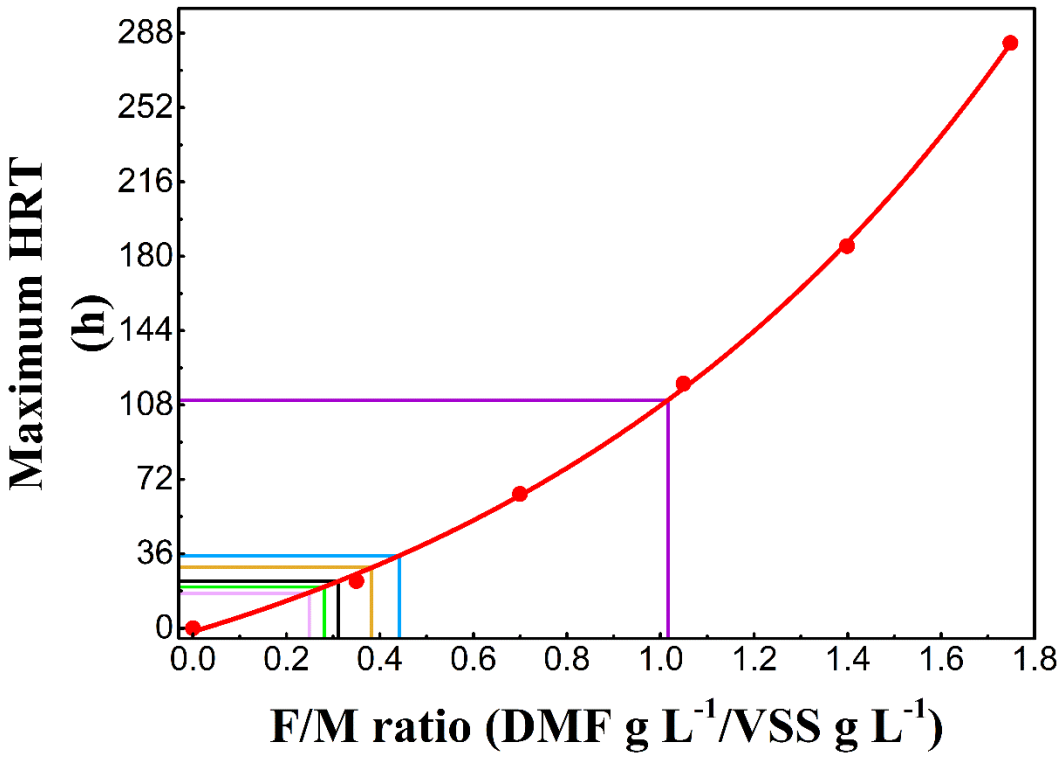

Fig. 5.11 Correlation between F/M ratio and maximum HRT. Raw data was manipulated using a non-linear fitting of an exponential model: $y = y_0 + Aexp(Rx)$, $R^2 = 0.9997$. Lines with different colors represent the real F/M ratios of the SAnMBR during different periods. Black: initial HRT = 24 h, F/M = 0.32; Blue: HRT = 18 h, F/M = 0.44; Green: HRT = 18 h with re-inoculation, F/M = 0.28; Orange: HRT = 12 h, F/M = 0.38; Violet: HRT = 8 h with sludge discharging, F/M = 1.03; Pink: final HRT = 24 h, F/M = 0.24.

burdens the membrane and causes fouling. Therefore, an optimum F/M ratio should be proposed and investigated by a BMP test using DMF concentration gradients from 1000 – 5000 mg L^{-1}. The overall results of the BMP test and the calculation of maximum

HRT are based on Eq. (1), while the correlation between F/M ratio and maximum HRT is given in **Fig. 5.11**. Apparently, to effectively degrade DMF within the HRT of 24 h, the F/M ratio in the SAnMBR must be lower than 0.35, and a higher F/M definitely requires a much longer reaction time. In our SAnMBR, the average F/M ratio of 0.32 initially conducted under the HRT of 24 h, met the theoretical value, making it possible to obtain a nearly 100% removal of DMF. However, when the HRT was then shortened to 18 h or even lower levels, the OLR and F/M also correspondingly increased, it was obvious that the ACS could not thoroughly degrade more DMF within a shorter HRT unless more sludge is replenished to the system to lower the F/M. Nevertheless, the increased sludge concentration is a significant burden on the membrane filtration, with the membrane fouling occurring when the HRT was reduced to 8 h with a high SS of 13.02 g L^{-1}. However, the membrane could be smoothly permeated under the HRT of 24 h even with a high SS. These results indicate that a short HRT is not feasible for the anaerobic treatment of high strength DMF-containing wastewater by SAnMBR. If we pursue an effective degradation under higher OLRs of DMF, an appropriate solution is to increase both the influent DMF concentration and sludge concentration to keep a low F/M ratio under a longer HRT.

5.4.3 Improvements to the anaerobic treatment of DMF-containing wastewater

As discussed in the character of microbial community, by maintaining an effective and stable methanogenic degradation of DMF, the abundance of DMF-hydrolyzing bacteria is maintained. From this perspective, we haveproposed some further improvements and strategies, as indicated in **Fig. 5.12**.

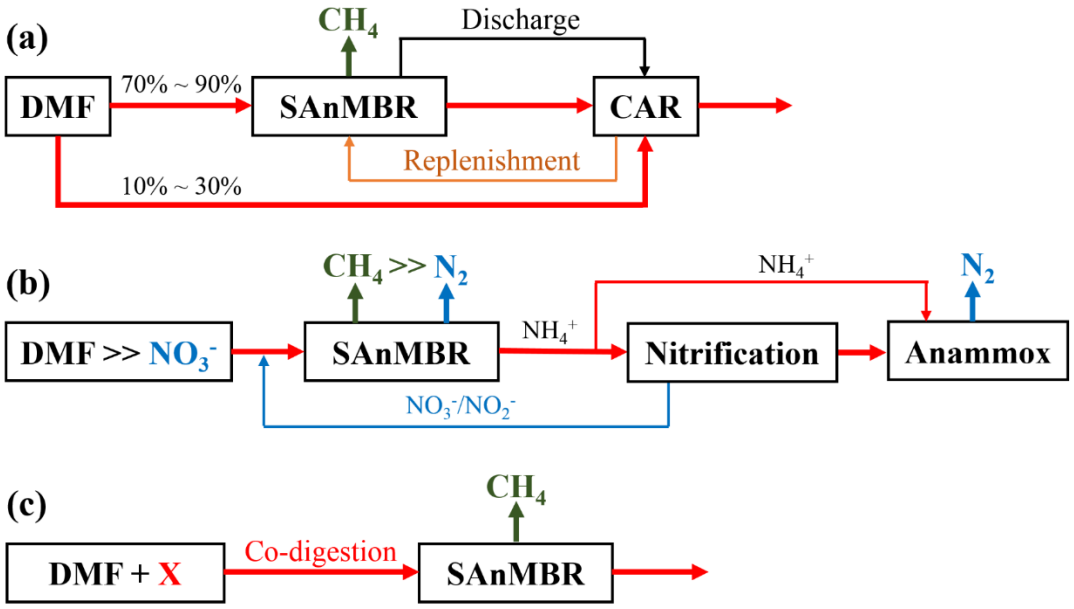

Fig. 5.12 Improvements and prospects on the anaerobic treatment of DMF-containing wastewater: (a) Side-stream DMF-degrading activated sludge (DAS) replenishment strategy; (b) nitrate-reducing anaerobic digestion of DMF strategy; (c) co-digestion of DMF and other organic matters strategy.

1) Because the DMF-hydrolyzing bacteria provide hydrolytic enzymes while methanogens perform the methanogensis, the cooperation of both is required for the thorough methanogenic degradation of DMF, even though the DMF-hydrolyzing bacteria do not grow consistently under the anaerobic condition. Therefore, as shown in **Fig. 5.12 (a)**, a side-stream CAR could be used to cultivate DAS. As aerobic DAS is enriched quickly when only fed with a little proportion of DMF (< 30% perhaps, extract percentage should be verified by long-term operation), it can be continuously replenished to SAnMBR to maintain the hydrolysis of DMF, while excessive anaerobic sludge can be discharged to CAR. However, the drawback of this strategy is that CAR consumes electricity for aeration which perhaps counteracts the majority of bioenergy produced from methane fermentation.

2) As mentioned previously, these DMF-hydrolyzing bacteria are actually denitrifying bacteria. In other words, the addition of nitrate (or nitrite) as the elector

acceptor is likely to improve or stimulate the growth of these DMF-hydrolyzing bacteria to establish anaerobic digestion under the nitrate-reducing condition (Keller et al., 2018; Mechichi et al., 2005). As illustrated in **Fig. 5.12 (b)**, the dosage of nitrate to the DMF degradation system appears to improve the abundance of these DMF-hydrolyzing bacteria for producing more hydrolytic enzymes. Since DMF contains nitrogen and ammonium is the end product, this strategy is probably the most suitable alternative to the anaerobic treatment of DMF-containing wastewater. Therefore, the nitrate may be sourced from DMF itself through a nitrification stage and then flow back to the SAnMBR. Moreover, excessive ammonium and nitrate could be further removed by the Anammox process to indeed realize a "thorough" biodegradation of DMF. However, for this method, the nitrate dosage must be strictly controlled, because with excessive nitrate, denitrifying bacteria are known to compete with methanogens as a carbon source, resulting a redox system instead of methanogenesis (Sakthivel et al., 2012; Sheng et al., 2013).

3) As shown in **Fig. 5.12 (c)**, the co-digestion of DMF and other compounds is also a good alternative. If these DMF-hydrolyzing bacteria do not grow anaerobically with DMF as the sole substrate and compete with methanogens on intermediates, replacing DMF with other organics for the growth of these bacteria is required. This will result in the DMF-hydrolyzing bacteria feeding on other carbon sources rather than DMF but still provide enzymes for hydrolysis, and the methanogens will utilize the intermediates. Some previous studies succeeded in the co-digestion of DMF and other antibiotics and obtained a stable performance (Chen et al., 2018; Hu et al., 2017). However, the co-digestion of different organics significantly changes the metabolism and the content and proportion of functional microorganisms, whose mechanism is totally different from the methanogenic degradation of DMF as the sole substrate. This,

therefore, requires further investigation.

5.4 Conclusions

This study is the first time the SAnMBR has been applied to the anaerobic treatment of high strength DMF-containing wastewater. The long-term performance of methanogenic degradation of DMF, membrane filtration and microbial community were investigated in detail, leading the following key conclusions and significant enlightenments into the anaerobic treatment of DMF-containing wastewater:

1) The co-culture of DAS and ADS could realize a rapid start-up which facilitates the thorough methanogenic degradation of high strength DMF under a low OLR. However, the elevation of OLR significantly limited the hydrolysis of DMF.
2) DMF-hydrolyzing bacteria originating from DAS brought about a niche overlap in the prokaryotic community. DMF-hydrolyzing bacteria was gradually outcompeted by methanogens in the competition of intermediates and continuously decayed.
3) Membrane fouling was influenced by high SS concentration and the shortening of HRT, while no direct correlation was found between SMP/EPS and the variation of TMP. SMP and EPS were more likely sourced from cell lysis.
4) To handle higher OLRs of DMF, increases in both the influent DMF concentration and sludge concentration are recommended to keep a low F/M ratio under a longer HRT rather than shortening the HRT.

Table 5.1 Information of seed sludge and recipe of nutrients and trace elements in this study.

Information of seed sludge			**Nutrients**	**Concentration (mg L^{-1})**
	Anaerobic	DMF-degrading	NaCl	200
Sludge	digested	activated	KH_2PO_4	150
	sludge	sludge	K_2HPO_4	50
Abbreviation	ADS	DAS	$MgCl_2·6H_20$	10
TS	13.00 ± 0.23	10.06 ± 0.68	$NiCl_2·6H_2O$	10
VS	9.91 ± 0.08	8.36 ± 0.10	$CoCl_2·6H_2O$	10
SS	11.02 ± 0.35	9.80 ± 0.37	$FeCl_2·4H_2O$	20
VSS	8.80 ± 0.11	7.84 ± 0.38	$ZnSO_4$	20
Volume (L)	3.0	3.0	$CaCl_2$	10

Table 5.2 Statistical data derived from high-throughput sequencing of prokaryotes in all 16 samples of this study.

Sampling day	Observed species	Singles	Chao1	Good's coverage[a]	Shannon	Simpson
1	2101	816	2969.20	0.9874	7.10	0.9751
15	1752	751	2787.39	0.9884	6.64	0.9690
29	1471	646	2409.45	0.9901	6.07	0.9599
42	1289	547	2050.89	0.9916	5.81	0.9416
56	1284	579	2213.62	0.9911	5.95	0.9602
70	1199	503	1912.29	0.9923	6.07	0.9600
84	1370	641	2315.25	0.9901	5.90	0.9578
98	1247	562	2163.52	0.9914	5.92	0.9596
112	1221	537	2062.61	0.9917	5.78	0.9573
126	1082	428	1679.24	0.9934	5.82	0.9544
141	1266	546	2017.44	0.9916	5.94	0.9567
156	1543	634	2372.18	0.9902	6.65	0.9736
189	1963	801	2942.82	0.9877	7.08	0.9766
210	2045	844	3097.50	0.9870	7.13	0.9788
224	1836	828	3008.53	0.9873	6.66	0.9714
250	1541	645	2417.33	0.9901	6.46	0.9667

[a] Good's coverage was calculated as $G = 1-(n/N)\cdot 100$, where n is the number of singleton OTU and N is the total number of sequences in all samples, in this study, N was unified to 65,000.

Table 5.3 Operational parameters of the SAnMBR during the entire 250-day long-term continuous experiment.

Phase	Period (d)	Substrate and influent parameters						Hydraulic parameters	
		Temperature (℃)	pH	DMF (mg L^{-1})	ThOD[a] (mg L^{-1})	COD[b] (mg L^{-1})	OLR[c] (g L^{-1} d^{-1})	HRT (h)	Flow (mL min^{-1})
Ⅰ	1 - 28	35.5 ± 0.3	7.39 ± 0.11	2066.03 ± 117.98	3161.03 ± 180.50	1907.92 ± 112.98	3.16 ± 0.18	24	6.11 ± 0.34
Ⅱ	29 - 84	35.6 ± 0.2	7.29 ± 0.18	2055.71 ± 77.44	3145.23 ± 118.48	1895.13 ± 72.60	4.16 ± 0.26	18	8.02 ± 0.16
Ⅲ	85 - 112	35.1 ± 0.3	7.37 ± 0.10	2136.92 ± 133.31	3269.49 ± 203.96	1955.42 ± 118.45	6.54 ± 0.41	12	11.99 ± 0.26
Ⅳ	113 - 140	35.1 ± 0.4	7.40 ± 0.18	2229.89 ± 95.80	3411.73 ± 146.58	2033.78 ± 88.78	10.24 ± 0.44	8	18.10 ± 0.16
Ⅴ	141 - 158	35.3 ± 0.3	*N.A*	*N.A*	*N.A*	*N.A*	*N.A*	∞[d]	*N.A*
Ⅵ	159 - 172	35.3 ± 0.8	7.25 ± 0.11	2201.62 ± 34.97	3368.47 ± 53.51	2015.21 ± 37.48	10.11 ± 0.16	8	18.55 ± 0.06
Ⅶ	173 - 250	35.6 ± 0.5	7.32 ± 0.11	2054.73 ± 112.70	3143.73 ± 172.43	1877.04 ± 101.21	3.14 ± 0.17	24	6.31 ± 0.11

[a] Theoretical oxygen demand (ThOD) was calibrated by the real DMF concentration (ThOD : DMF = 1.53).

[b] COD concentrations represent the real COD values which were measured using the standard potassium dichromate method.

[c] OLR was calculated on the basis of ThOD.

[d] During the batch recovery period, because both influent and effluent pumps were stopped, the HRT was considered as infinity.

Table 5.4 Long-term performance and results of the SAnMBR during the entire 250-day continuous experiment.

Phase	Period (d)	Gas production		Final and intermediate products (mg L^{-1})				Removal efficiency (%)				
		MPR (L L^{-1} d^{-1})	CH_4 (%)	NH_4^+	DMA	MMA	HCOOH	DMF	COD[c]	DMA	MMA	HCOOH
I	1 - 28	0.975 ± 0.045	86.35 ± 5.13	479.07 ± 25.94	21.95 ± 7.59	2.05 ± 2.56	0.49 ± 1.82	95.94 ± 0.74	94.87 ± 1.70	98.21 ± 0.62	99.76 ± 0.31	99.95 ± 0.21
II	29 - 60	0.719 ± 0.068	89.05 ± 1.58	327.95 ± 14.97	21.14 ± 14.91	2.21 ± 2.29	0	69.13 ± 6.11	68.77 ± 5.74	97.66 ± 1.65	99.66 ± 0.47	100
	61 – 84[a]	0.963 ± 0.082	88.19 ± 1.28	509.68 ± 17.34	2.95 ± 5.89	0	5.05 ± 5.35	96.23 ± 1.87	94.68 ± 1.57	99.75 ± 0.50	100	99.41 ± 0.63
III	85 - 112	0.677 ± 0.086	88.25 ± 1.08	348.27 ± 88.44	1.91 ± 4.43	0.02 ± 0.07	6.46 ± 10.65	71.67 ± 12.24	71.30 ± 12.08	99.82 ± 0.40	100	99.05 ± 1.51
IV	113 - 140	0.194 ± 0.087	87.19 ± 1.43	154.39 ± 31.93	10.43 ± 14.63	0.54 ± 2.04	4.22 ± 8.33	23.89 ± 7.77	20.14 ± 7.90	96.57 ± 5.34	99.64 ± 1.31	98.42 ± 3.46
V	141 - 158	0.045 ± 0.046	84.41 ± 1.35	526.76 ± 50.77	24.94 ± 25.54	1.80 ± 3.46	10.45 ± 5.74	*N.A*	*N.A*	*N.A*	*N.A*	*N.A*
VI	159 - 172	0.144 ± 0.030	81.62 ± 3.22	147.56 ± 54.85	17.07 ± 15.64	0.15 ± 0.41	6.13 ± 5.67	15.85 ± 4.56	13.86 ± 6.27	93.93 ± 5.20	99.86 ± 0.38	96.20 ± 3.21
VII	173 - 222	0.123 ± 0.051	86.37 ± 1.64	160.87 ± 51.49	31.51 ± 23.30	1.69 ± 2.13	4.39 ± 3.63	19.58 ± 12.57	18.16 ± 12.90	84.53 ± 12.84	97.44 ± 4.32	95.88 ± 4.18
	223 - 250[b]	0.171 ± 0.102	86.98 ± 2.35	190.70 ± 91.92	3.67 ± 3.23	0.14 ± 0.36	4.07 ± 2.14	34.64 ± 16.11	31.31 ± 18.22	99.27 ± 0.33	99.97 ± 0.08	98.36 ± 0.91

[a, b] During these two phases, because the re-inoculation of DAS significantly influenced the performance, the results should be discussed evaluated separately before and after the re-inoculation.

[c] COD removal efficiency was calculated on the basis of the real COD concentrations which were measured using the standard potassium dichromate method.

Table 5.5 A comparison of anaerobic treatment of DMF-containing wastewater by SAnMBR to other similar studies on the treatment of DMF.

Apparatus	Process	Metabolism	DMF concentration (mg L^{-1})	Acceptable OLR (g COD L^{-1} d^{-1})	Removal efficiency	Energy recovery	Reference
Mesh-filtration bioreactor	Biological	Aerobic	1000	0.18 - 0.4	> 98	×	(Kamimoto et al., 2009)
Bio-trickling filter	Chemical/physical	*N.A*	38600	*N.A*[c]	96	×	(Chen et al., 2016)
Aeration tank	Biological	Aerobic	200 - 400	0.2 - 0.4	60 - 90	×	
Continuous packed bed reactor	Catalytic/biological	Aerobic	2.5%, v/v[a]	*N.A*	68.3 - 100	×	(Sanjeev Kumar et al., 2012)
Sequencing-batch attached biofilm reactor	Biological	Aerobic	500 - 3000	*N.A*	87 - 92	×	(Rahmaninezhad et al., 2016)
Mixed-bed biofilm reactor	Biological	Aerobic	1000 - 9000	1.0 - 9	55.6 - 89.8	×	
Fixed bed	Physical	*N.A*	5% - 20%, w/w[b]	*N.A*	> 99	×	(Ye et al., 2013)
Graphene oxide	Physical/biological	Aerobic	100 - 2000	*N.A*	> 90	×	(Zheng et al., 2016)
Eco-tank	Biological	Aerobic	75.4 - 161.1	*N.A*	68.3 - 91.7	×	(Xiao et al., 2016)
Up-flow anaerobic sludge blanket	Biological	Anaerobic	2000	1.63 – 6.17	47.4 - 97.9	√	(Kong et al., 2019)
Submerged anaerobic membrane bioreactor	Biological	Anaerobic	2000	3.14 – 6.54	77.3 - 96.2	√	This study

[a, b] These studies did not provide the exact concentration of DMF.

[c] These studies did not provide the OLR or HRT.

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Chapter 6 Functional microorganisms and their relationships in the methanogenic degradation of DMF

Abstract

The methanogenic degradation of N, N-dimethylformamide (DMF) was investigated using anaerobic digested sludge (ADS), aerobic activated sludge (AAS) and co-cultured sludge (CCS), respectively. Both the metabolic pathway and the corresponding microorganisms which function in the methanogenic degradation of DMF were elucidated. DMF was unable to be degraded anaerobically by ADS due to the lack of DMF-hydrolyzing bacteria. DMF can be effectively degraded by AAS, however, no methane was recovered under the aerobic condition. The co-culture of DMF-hydrolyzing bacteria and methanogens in the CCS allowed for both hydrolysis of DMF and methane production to proceed successfully under the anaerobic condition, realizing the complete conversion from DMF to methane. However, a niche overlap due to the competition for the intermediates lowered the abundance of DMF-hydrolyzing bacteria. A lab-scale UASB was operated successfully to anaerobically treat wastewater containing approximately 2000 mg L^{-1} *N*, *N*-dimethylformamide (DMF) by artificially mixing anaerobic granular sludge with DMF-degrading activated sludge**.** DMF was effectively degraded by the UASB under a low OLR of 1.63 ~ 4.22 g COD L^{-1} d^{-1}, with over 96% DMF removal efficiency and a high methane production rate. However, the DMF-degrading ability gradually weakened along with increases in the OLR. The analysis of the microbial community structure by high-throughput sequencing revealed a decline in the abundance of the facultatively anaerobic DMF-hydrolyzing bacteria originating from activated sludge with increasing OLR, further deteriorating the methanogenic degradation of DMF. When the OLR was lowered again,

the slow growth of those facultative anaerobes recovered, and slight improvements in the removal were noted. Methylotrophic methanogens utilized the intermediate products from the hydrolysis of DMF, which kept increasing in abundance throughout the entire experimental period. The introduction of nitrate, timely replenishment of AAS, micro-aeration and co-digestion were likely to maintain a high abundance of DMF-hydrolyzing bacteria to ensure an effective hydrolysis.

6.1 Introduction

Due to its excellent miscibility, *N, N*-dimethylformamide (DMF) has been widely employed as a versatile organic solvent in a variety of chemical industries, and particularly in pharmaceutical and textile industries (Bromley-Challenor et al., 2000; Swaroop et al., 2009; Veeranagouda et al., 2006). The wastewater discharged from these industries typically contains high concentrations of DMF which lead to water pollution and eutrophication (Chen et al., 2016). DMF is also a toxic and hazardous organic compound known for its hepatotoxicity and carcinogenicity that endanger human health (Das et al., 2006; Redlich et al., 1990). As a result, the effective treatment of DMF-containing wastewater has become a matter attracting research interest worldwide.

To effectively treat DMF-containing wastewater in large quantities and at low cost, biodegradation is usually considered an appropriate solution. As the metabolic pathways of DMF under the aerobic condition were comprehensively elucidated many years ago (Ghisalba et al., 1986, 1985; Schär et al., 1986), the majority of the studies to date have been concentrated on the aerobic degradation of DMF (Chen et al., 2016; Kamimoto et al., 2009; Sanjeev Kumar et al., 2012; Zheng et al., 2016). The aerobic process, however, comes with serious shortcomings: it requires high electricity

consumption and yields large amounts of excess sludge. The anaerobic digestion (AD) process, on the other hand, has benefited from its high organic loading resistance, energy recovery and lower sludge yields: it has been widely employed in the treatment of various industrial wastewater for decades (Astals et al., 2015; Banks et al., 2011; Bayr and Rintala, 2012). However, few studies on the anaerobic treatment of DMF are available in the literature. Some have reported the observations of the anaerobic degradation of DMF, roughly describing the degradation phenomenon with no further detailed microbial analysis (Lausund, 2014; Stronach et al., 1987). Some achieved the co-digestion of DMF and other antibiotic solvents, however, neither the specific functional strains nor the mechanisms was elucidated (Chen et al., 2018; Hu et al., 2017). In another study, while the bacteria which grow on DMF were systematically identified, the degradation of DMF was not observed under the anaerobic condition (Bromley-Challenor et al., 2000). The ambivalent results and lack of knowledge in this area make the anaerobic treatment of DMF-containing wastewater a great challenge. Although the specific bacterial strains which degrade DMF have been extensively documented, such as *Pseudomonas* DMF 3/3 (Schär et al., 1986), *Ochrobactrum* sp. DGVK1 (Veeranagouda et al., 2006), *Paracoccus aminophilus* and *Paracoccus aminovorans* (Urakami et al., 1990), *Paracoccus* sp. strain DMF-3 (Zhou et al., 2018), Alcaligenes sp. KUFA-1 (Hasegawa et al., 1999), *Bacillus subtilis* (Vidhya and Thatheyus, 2013), and *Bacillus cereus* (Okazaki et al., 1995) etc., and all of them are aerobic bacteria and produce an enzyme called *N, N*-dimethylformamidase (DMFase) which hydrolyzes DMF into dimethylamine (DMA) and formic acid (HCOOH). However, those bacteria which could degrade DMF anaerobically are still unknown and their metabolisms and cooperation/competition relationship under the anaerobic condition are unclear. Recently, there has been heightened interest in the application of

the up-flow anaerobic sludge blanket (UASB) to the treatment of industrial wastewater (Artsupho et al., 2016; Cairns and Mead, 2017; W. Li et al., 2015). The suitability of the UASB to the treatment DMF-containing wastewater remains to be clearly determined. However, the granulation of the anaerobic granular sludge (AGS) in the start-up of an UASB is known to be a time-consuming process, and normal anaerobic sludge is not obtained by the degrading ability to DMF. In our previous study, we succeeded in the methanogenic degradation of DMF by the co-culturing of two sources of inoculum (Kong et al., 2018). In this study, we artificially mixed normal AGS with DMF-degrading activated sludge (DAS) to realize a quick start-up. Although it has been widely reported that the DMF is the sole energy and nitrogen source which feeds bacteria (Dziewit et al., 2010; Nisha et al., 2015; Siddavattam et al., 2011; Swaroop et al., 2009; Urakami et al., 1990; Veeranagouda et al., 2006), the variation and response of the microbial community structure of the entire DMF-degrading consortium during long-term operation has never been investigated. Besides, the functions of some candidate strains capable of anaerobically degrading DMF need to be clarified by the long-term operation.

In this study, the aim was to cultivate the anaerobic DMF-degrading consortium from the normal anaerobic digested sludge (ADS), aerobic activated sludge (AAS) and anaerobic co-cultured sludge (CCS). Microbial samples were taken from each process during the cultivation period for the purpose of the following: 1) to elucidate the metabolic pathways and the corresponding prokaryotes which play roles in the degradation of DMF under different conditions; 2) to investigate the evolution direction of the prokaryotic community and the cooperation/competition relationship between functional bacteria and archaea in the anaerobic DMF-degrading consortium; 3) to investigate the shift and variation of bacterial and archaeal communities in a lab-scale

UASB along with the change in operational conditions for the anaerobic treatment of DMF-containing wastewater in a long-term operation, and to reveal all potential functional microorganisms for DMF degradation by high-throughput sequencing. We focused on evaluating the stability and feasibility of the artificially mixed DMF-degrading consortium when treating a high concentration of DMF, and sought candidate anaerobic DMF-degrading strains through the response of microbial community to the long-term performance of the UASB. This study also provides detailed biological directions and suggestions for the application of the anaerobic treatment of DMF-containing wastewater from ecological and phylogenic angles.

6.2 Materials and methods

6.2.1 Experimental apparatus, operational procedure and samples preparation

There were four groups of experiments conducted in this study, and a total of 14 samples of sludge were taken for the microbial analysis. The procedure of the experiments were briefly listed in **Table 6.1**.

Group-A was a long-term continuous operation conducted by a submerged anaerobic membrane bioreactor (SAnMBR) in order to cultivate the DMF-degrading anaerobic digested sludge (ADS). The SAnMBR has an operational volume of 7 L. Synthetic industrial wastewater containing approximately 300 ~ 1000 mg L^{-1} DMF was pumped to the SAnMBR and the effluent was permeated by a flat sheet membrane under the HRT of 48 h. The seed inoculum of the ADS for the start-up of the SAnMBR was collected from a local wastewater treatment plant with a volatile suspended solids (VSS) concentration of 9.60 g L^{-1}. Three samples labeled An-0, An-60 and An-120 were correspondingly taken on Day 0, Day 60 and Day 120, respectively.

The purpose of Group-B was to cultivate the DMF-degrading aerobic activated

sludge (AAS) by a one-stage continuous aeration reactor. A reactor with an operational volume of 10 L was inoculated with normal ADS. Synthetic industrial wastewater containing approximately 1000 ~ 2000 mg L^{-1} DMF was introduced to the continuous aeration reactor under the HRT of 48 h. After being continuously aerated with an aeration rate of 7.5 L min^{-1} for 35 days, the ADS gradually became AAS. Sludge samples were taken from the reactor every 7 days and assigned the following sample IDs: Ae-12, Ae-24 and Ae-36 on Day 12, 24 and 36, respectively. It should be noted that the seed inoculum of the continuous aeration reactor was also ADS, and that it was collected on a different day. Therefore, the inoculum sample in Group-B was marked Ae-0.

Group-C was a long-term batch experiment whose inoculum was artificially mixed by the same volume as the cultivated AAS (same as Ae-36) and the seed ADS (same as An-0), and this co-cultured sludge (CCS) had a VSS concentration of 17.42 g L^{-1}. It was marked as sample ID Cc-0. The long-term batch experiment was conducted for 62 days under the anaerobic condition to verify whether DMF can be degraded by this co-cultured consortium. The batch experiment was performed using 220 mL serum vials in which 60 mL sludge and 120 mL substrate solution containing approximately 5000 mg L^{-1} DMF were dosed together as the aquatic phase. Sludge samples were taken at the end of each repeated phase before renewing DMF solution to the batches. During the long-term batch experiment, 5 samples, labeled Cc-22 ~ Cc-62 according to the day of sampling, were collected on Day 22, 32, 42, 52 and 62, respectively.

Group-D was a comparative group which was conducted in parallel to the long-term batch experiment of Group-C under the same culturing condition for 62 days. However, the seed sludge was also inoculated with the original ADS (An-0) as the blank control of the co-cultured sludge (Cc-0). Only one sample (marked Bc-62) was taken in the end

of the batch experiment, that is, on the same day as Cc-62. The evolutionary relationship of all 14 samples is shown in Fig. 6.1. All sludge samples were stored at -20 °C in a refrigerator until further processing. All water samples collected from reactors and

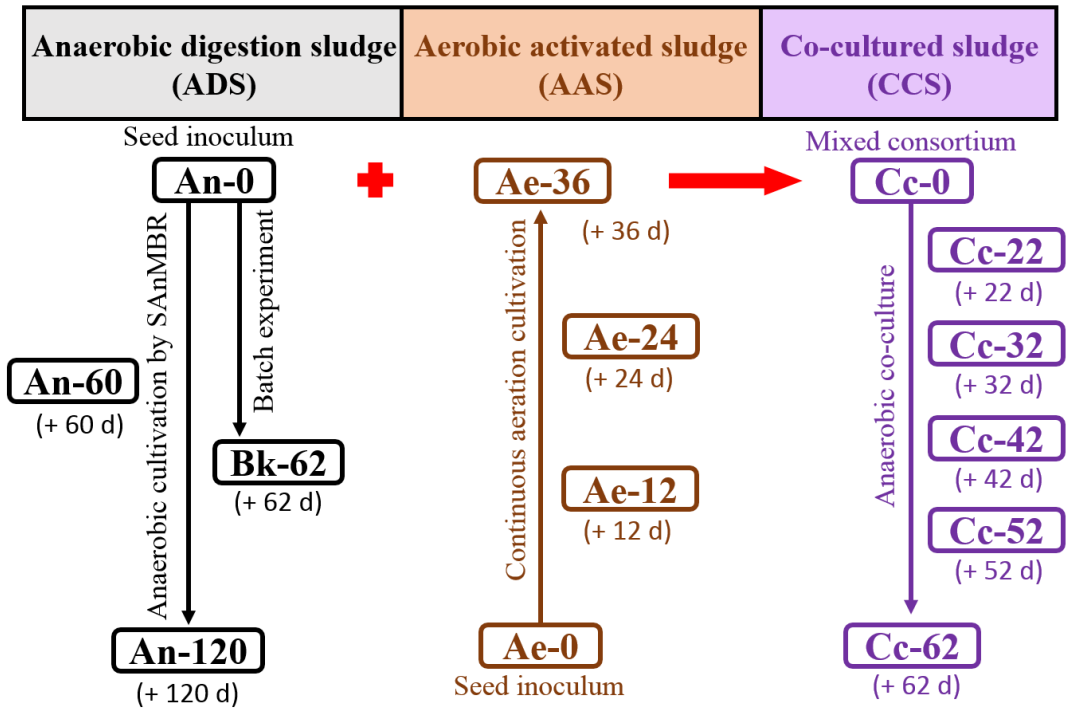

Fig. 6.1 The evolutionary relationship of all 14 samples in this study. Samples of An-0, An-60, An-120 and Bk-62 belong to the anaerobic digested sludge (ADS); samples of Ae-0, Ae-12, Ae-24 and Ae-36 belong to the aerobic activated sludge (AAS); samples of Cc-0, Cc-22, Cc-32, Cc-42, Cc-52 and Cc-62 belong to the co-cultured sludge (CCS). The number written in the sample name represents the sampling day.

batches were filtered by 0.45 μm PES filter head (Millex®, Merck Millipore Ltd., Ireland) before analyzing.

For the UASB samples, an up-flow anaerobic sludge blanket (UASB) with an operational volume of 6 L was used for the anaerobic treatment of DMF-containing wastewater. In order to culture a large quantity of DMF-degrading activated sludge (DAS), a continuous aeration reactor (CAR) with an operational volume of 10 L was used as well. In order to realize a rapid start-up and to avoid the lengthy period of sludge

granulation, a bucket of 10 L anaerobic granular sludge (AGS) was collected from a wastewater treatment plant in Tokyo. The AGS (granular size: 2.8 ~ 5 mm, moisture content: 91.81 ± 0.46%, VSS/SS = 0.87), previously used in the treatment of starch wastewater, was inoculated as the seed sludge to the UASB. Another 10 L of normal anaerobic digestion sludge (ADS, SS = 11.02 ± 0.35 g L^{-1}, VSS = 8.8 ± 0.11 g L^{-1}) was collected from a local domestic wastewater treatment plant and inoculated to the CAR for the culturing of DMF-degrading activated sludge (DAS). Synthetic industrial wastewater containing approximately 2000 mg L^{-1} DMF with sufficient nutrients was prepared in a 120 L substrate tank.

The continuous aeration reactor (CAR) was continuously aerated by an air pump with an aeration rate of 7.5 L min^{-1}. The UASB was initially inoculated with 3 L of AGS and fed with DMA instead of DMF for a batch pre-culture to enhance the methylotrophic activity. During the pre-culture period, a group of specific methanogenic activity (SMA) tests were conducted on Day 1 and Day 51 respectively to evaluate the methane-producing ability of the AGS. After 51-days of culturing, 3 L concentrated DAS (SS = 11.34 ± 0.40 g L^{-1}, VSS = 9.59 ± 0.31 g L^{-1}) was collected and then inoculated into the UASB and fully mixed with the AGS. The DAS had also been simultaneously cultured in the CAR for 51 days, and the DAS was proved to effectively degrade DMF (Kong et al., 2018). With the mixed sludge, the continuous operation of the UASB was started for 215 days and the HRT was gradually shortened from 48 h to 6 h. Both the UASB and CAR were operated at the mesophilic temperature of 35 °C. A total 15 samples of sludge were analyzed for the UASB, including 4 samples of pre-cultured AGS, 10 samples of mixed sludge, and one sample of DAS. Both the AGS samples and mixed sludge samples were collected from the bottom of the UASB in different periods, and the DAS sample was collected from the mixed liquor of the CAR. All samples were

stored in the refrigerator at -20 °C before extracting DNA.

6.2.2 Genome DNA extracting, PCR and Illumina sequencing

DNA was extracted with the ISOIL for Beads Beating kit (Nippon gene, Japan), then the concentration was measured by NanoDrop 2000 (Nanodrop Inc., USA) and diluted to 10 ng μL^{-1} for PCR. The V3~V4 fragments of the 16S rRNA gene were amplified with the former primer 341F (5'-CCTAYGGGRBGCASCAG-3') and mixed reverse primer 806R/806R-P (30:1) (5'-GGACTACHVGGGTHTCTAAT-3'5'-GGACTACCAGGGTATCTAAG-3'). The PCR condition was as follows: 30 cycles of 94 °C for 5 secs, 50 °C for 30 secs, 68 °C for 10 secs, and a final extension at 68 °C for 7 mins with Low DNA Ex Taq® (TaKaRa, Japan). The purity and concentration of amplified DNA were verified by the DNA chip with Bioanalyzer 7500 (Agilent Technologies, USA) and then purified with Agencourt® AMPure® XP (Beckman Coulter, Inc., USA) according to the manufacturers' instructions. Purified DNA was then measured by Qubit 3.0® (Life technologies, USA) and diluted to 2 ng μL^{-1}. The barcode with PCR products was sequenced by the Illumina Miseq platform.

6.2.3 Data processing

Raw data were filtered to remove short and poor-quality sequences, and de-multiplexing was carried out with split_libraries_fastq.py in QIIME (version 1.8.0). The operational taxonomic units (OTUs) were generated on the basis of 97% similarity, then Chimeras were removed with ChimeraSlayer. Singleton OTUs were removed and sequences were randomly selected to unify the sequence number of each sample to 40,000 as the standard number. The principal coordinate analysis (PCoA) was conducted with the weighted calculation method. The linear discriminant effect size

(LEfSe) analysis was proceeded online on the Galaxy website.

6.2.4 Analytic methods

The COD concentration was measured by a spectrophotometer (DR5000, HACH, USA) using the standard spectrophotometric methods. Biogas production and the temperature of the reactors were recorded by a wet gas meter (Sinagawa, Japan). Biogas production of the batch experiment was measured by glass injectors of various volumes ranging from 5 mL to 50 mL. The gas components of the biogas including N_2, CH_4 and CO_2, were detected by a GC-8A gas chromatograph (Shimadzu, Japan) with a 0.4 mL injection volume. The DMF concentration was determined using the Waters ACQUITY UPLC H-class system (Milford, USA) at a wavelength of 210 nm and a temperature of 25 °C. The system was equipped with an Xselect® CSH C18 column, protected by a VanGuard pre-column. The mobile phase was prepared with 25% methanol (v/v) (A), 70% ultrapure water (B) and 5% acetonitrile (C). The total running time was 10 min. The flow rate was 1.0 mL min^{-1} and the injection volume was 10 μL. All ions including NH_4^+, DMA, MMA and HCOOH were determined by 7100 Capillary Electrophoresis (Agilent Technologies, USA).

6.3 Results and discussion

6.3.1 Performance of DMF degradation in different groups

The performance of DMF degradation and the sampling dates in the four groups are all illustrated in **Fig. 6.2**, and their comparative results are given in **Table 6.2** in detail. As shown in **Fig. 6.2 (a)**, the results of Group-A suggested that the normal ADS was unable to be cultivated into DMF-degrading consortium. It was apparent that even

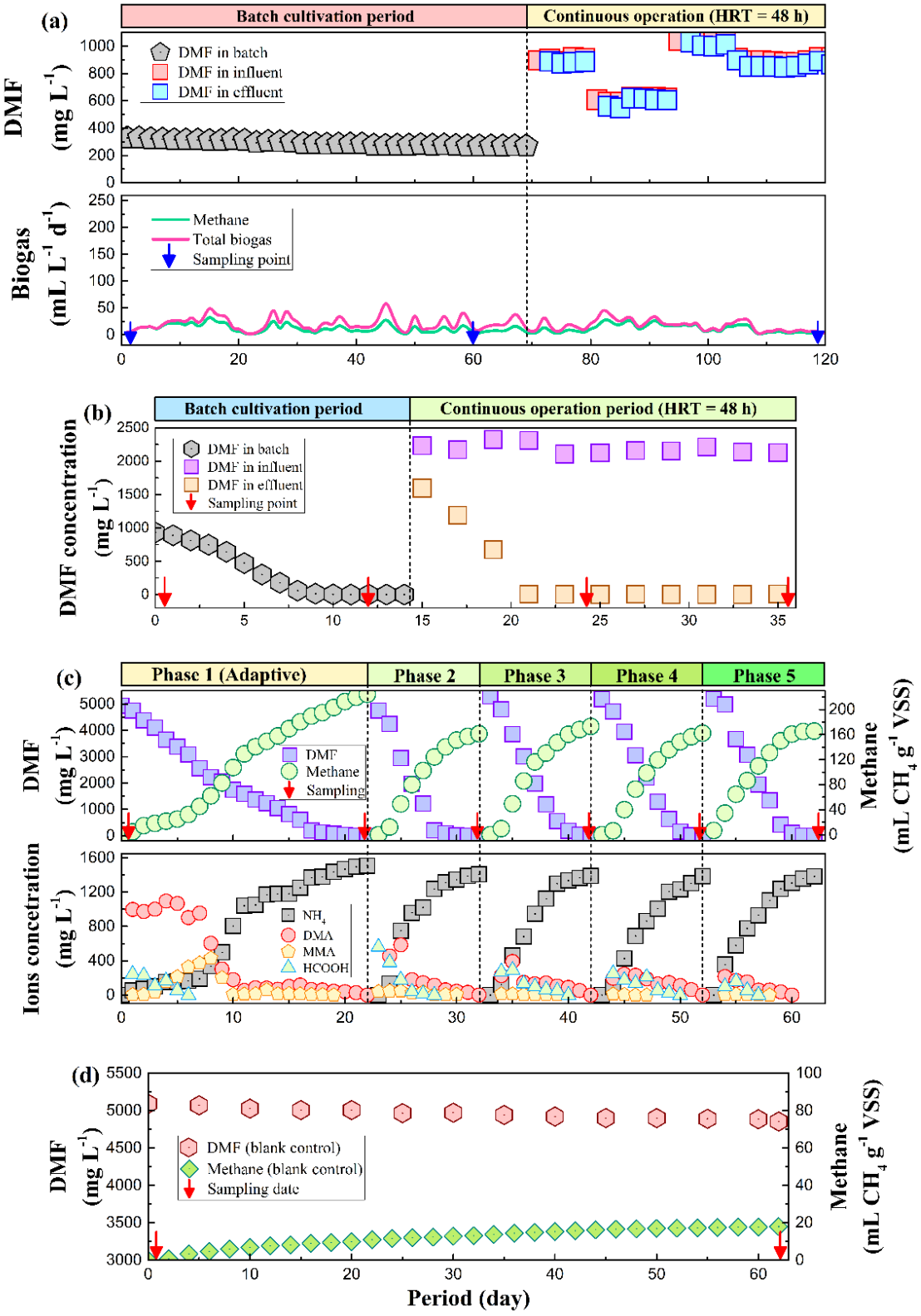
(a)
Batch cultivation period
Continuous operation (HRT = 48 h)
DMF (mg L-1)
DMF in batch
DMF in influent
DMF in effluent
Biogas (mL L-1 d-1)
Methane
Total biogas
Sampling point
(b)
Batch cultivation period
Continuous operation period (HRT = 48 h)
DMF concentration (mg L-1)
DMF in batch
DMF in influent
DMF in effluent
Sampling point
(c)
Phase 1 (Adaptive)
Phase 2
Phase 3
Phase 4
Phase 5
DMF (mg L-1)
DMF
Methane
Sampling
Methane (mL CH4 g-1 VSS)
Ions concetration (mg L-1)
NH4
DMA
MMA
HCOOH
(d)
DMF (mg L-1)
DMF (blank control)
Methane (blank control)
Sampling date
Methane (mL CH4 g-1 VSS)
Period (day)

Fig. 6.2 Overall performance of DMF degradation and the sampling dates in the four groups: (a) Group-A, anaerobic cultivation by a SAnMBR solely inoculated with anaerobic digested sludge; (b) Group-B, aerobic cultivation by a continuous aeration reactor inoculated with anaerobic digested sludge to acclimate aerobic activated sludge (c) Group-C, anaerobic co-culturing cultivation by a repeated long-term batch experiment inoculated with the mixture of anaerobic digested sludge and aerobic activated sludge; (d) Group-D, the blank control to the co-cultured group, solely inoculated with anaerobic digested sludge.

though being cultured for 120 days with both batch cultivation and continuous operation, the ADS in the SAnMBR acquired an extremely weak DMF removal efficiency of 1.55 ~ 7.29% and a low methane production rate 2.79 ± 2.03 mL L^{-1} d^{-1}. As the vials were also solely inoculated with ADS, the results of Group-D were similar to Group-A, which are given in **Fig. 6.2 (d)**, the DMF concentration almost remained unchanged and the methane production was very low during the entire 62-day batch experiment. Conversely, as shown in **Fig. 6.2 (b)**, the AAS cultured by the continuous aeration reactor in Group-B exhibited an excellent DMF removal efficiency of almost 100%, however, because AAS was cultured under the aerobic condition, it was obviously that no methane was produced. It should be noted that the CCS, which consisted of both AAS and ADS in Group-C, was the only consortium that realized both the thorough DMF removal and the high methane production. The rapid decrease of the DMF concentration and the corresponding accumulation of methane are clear in **Fig. 6.2 (c)**, where the results are provided. Moreover, high concentrations of the intermediate products (DMA and HCOOH) of DMF and their consuming trends were also observed. The remarkable results of Group-C also demonstrated that the feasibility and repeatability of DMF methanogenic degradation could be verified by the CCS.

6.3.2 Proportion analysis of prokaryotes in batch samples

After the filtering process, a total of 40,000 high-quality sequences were reserved from each sample for further analysis. It should be noted that, with the exception of Ae-36 (4.96), all the samples showed a relatively high Shannon diversity index from 7.22 to 5.74: the results for Ae-36 were due to the aerobic consortium after being cultivated by aeration, which was quite different from that of the other samples. As shown in **Table 6.3**, the Shannon diversity index also reveals a declining trend in the aerobically cultured samples or anaerobically cultured samples. This decline suggests that the evolution of the microbial community structure is towards a specific direction as it acclimatizes to the new habitat provided by the aerobic or anaerobic conditions.

The overall proportions of prokaryotes are shown in **Fig. 6.3 (a)**, it was apparent that there are obvious changes in the proportions of both bacteria and archaea. In Group-A and Group-D, the relative abundance of archaea in the ADS samples (also including Bk-62) of An-0, An-60 and An-120 remained low and stable between 4.30% ~ 7.88% even after the 120-day anaerobic cultivation. This was because that with insufficient hydrolysis of DMF, there was no substrates (the intermediate products DMA and HCOOH) for the growth of archaea. The low abundance of archaea was in correspondence with the low methane production during the operation of SAnMBR. Conversely, in Group-C, the abundance of archaea substantially increased in the process of anaerobic cultivation for the co-cultured sludge, which increased from 1.69% in Cc-0 to a high 25.75% in Cc-62. This result was consistent with the high methane production in the long-term batch experiment of Group-C. However, for the aerobic cultivation in Group-B, the abundance of archaea sharply decreased from 4.40% (Ae-0) to merely 0.47% (Ae-36), suggesting that the methanogens were almost eliminated due to continuous aeration, and the ADS gradually evolved to an aerobic consortium.

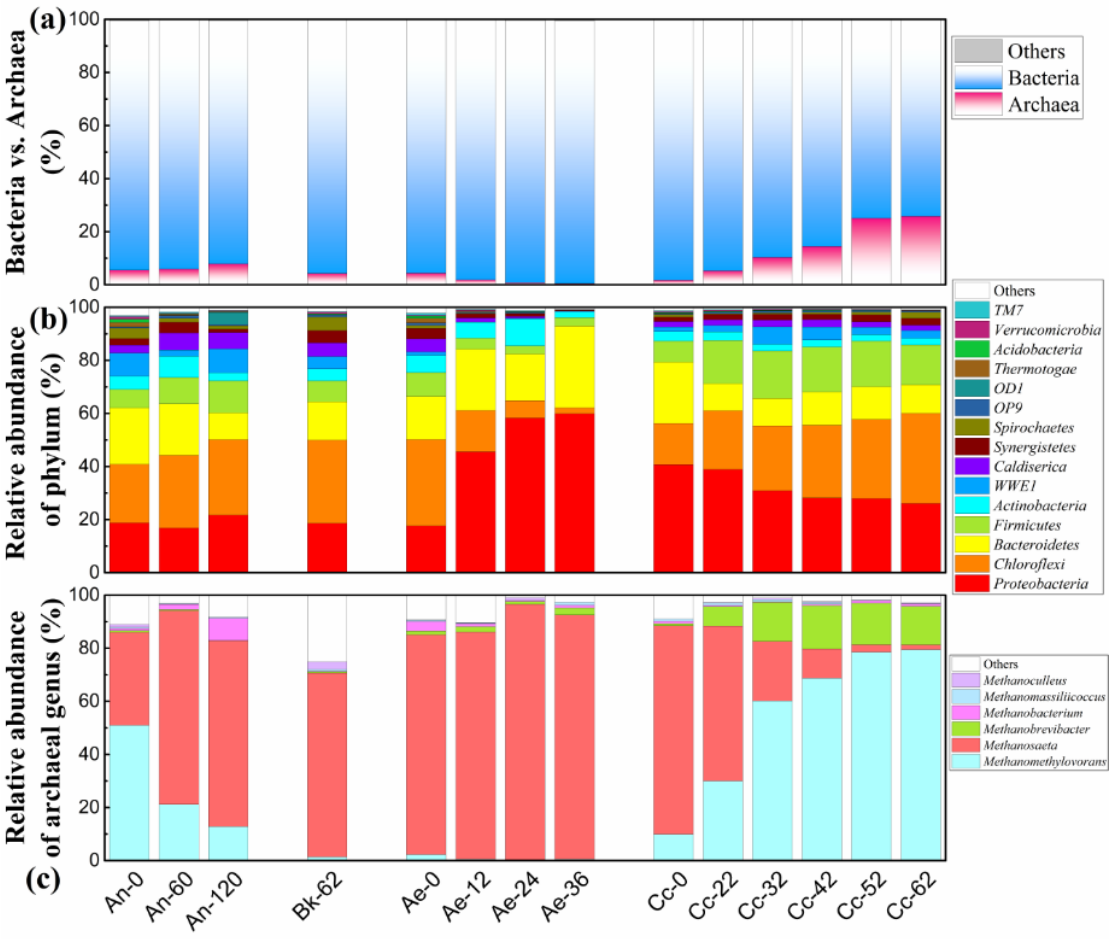

Fig. 6.3 Overall variation in the proportions of prokaryotes in all samples. (a) The comparison of the entire bacterial and archaeal communities; (b) the shift of the bacterial community at the level of phylum; (c) the shift of the archaeal community at the level of genus.

In Group-A and Group-D, because both the SAnMBR and serum vials were solely inoculated with the normal ADS, the microbial community structure barely changed from the seed inoculum sample An-0 to An-120 even after the 120-day anaerobic cultivation (also no significant change from An-0 to Bk-62). The result indicated that inoculating with ADS alone was not sufficient to degrade DMF anaerobically, which also accorded with the performance of the SAnMBR and blank control batch: the biogas production was extremely low, and the concentration of DMF remained basically unchanged. Therefore, only the CCS was capable of degrading DMF to methane under the anaerobic condition.

6.3.2.1 Shift of bacteria community structure

Based on 97% similarity, a total of 3,860 OTUs were generated from the final 513,296 high-quality bacterial reads. In which, a total of 335 genera affiliated to 47 phyla were identified referring to the GreenGenes database. It should be noted that significant variations of the bacterial community were observed in the samples of Group-B and Group-C. For the aerobic cultivation in Group-B, whose results are shown in **Fig. 6.3 (b)**, the abundance of phylum *Proteobacteria* increased rapidly from 17.56% (Ae-0) to 59.84% (Ae-36). Meanwhile, *Bacteroidetes* also increased from 16.25% to 30.76%, while *Chloroflexi* sharply decreased from 32.62% to 2.28%. However, for the 62-day anaerobic cultivation of CCS in Group-C, the abundance of *Proteobacteria* conversely decreased from 40.66% to 26.11%, and *Bacteroidetes* also decreased from 23.07% to 10.74%, but *Chloroflexi* increased from 15.62% to 33.91% and *Firmicutes* also increased from 7.29% to 11.69%. Compared to the seed inoculum sample of An-0 at the very beginning, the relative abundances of the major phyla *Proteobacteria*, *Chloroflexi* and *Firmicutes* increased by 7.18%, 6.56% and 5.64%, respectively, however, *Bacteroidetes*, *WWE1*, *Caldiserica* and *Actinobacteria* decreased by 5.58%, 3.26%, 3.22% and 2.58%, respectively.

On the level of genus, as listed in **Table 6.4**, in the anaerobic samples from An-0 to An-120 (also including Bk-62), no significant shift was observed in the entire bacterial community. Some major genera such as *Levilinea*, *Lentimicrobium*, *Thermomarinilinea* and *Treponema* etc. are also common bacteria which exist in normal anaerobic digestion system (Li et al., 2015; Palatsi et al., 2011; Yang et al., 2018), and were likely considered to be unrelated to the degradation of DMF. However, it should be noted that in the aerobic samples Ae-0 to Ae-36, the abundances of some bacterial genera

increased obviously. The major genera in sample Ae-36 were *Paracoccus* (21.33%), *Chryseobacterium* (11.48%), *Hyphomicrobium* (10.94%), *Chitinophagaceae* (7.46%), *Methylobacillus* (4.78%), *Mycoplana* (4.55%), *Nitratireductor* (4.27%), *Chitinophagaceae* (2.96%), *Bdellovibrio* (2.42%), *Moheibacter* (2.21%) and *Leadbetterella* (2.08%) etc. All of these bacteria are rare or even absent in the ADS samples, suggest these bacteria are suitable to the aerobic condition and might be related to the degradation of DMF. Among these bacteria, some genera such as *Paracoccus* (1.41%), *Hyphomicrobium* (6.06%), *Nitratireductor* (2.52%) and *Burkholderia* (1.56%) etc. (Jérôme et al., 2007; Labbe, 2004; Lee et al., 2012; Sun et al., 2013), still remained in relatively high abundance in the CCS sample of Cc-62 after being cultivated under the anaerobic condition for 62 days. The relative high predominance of these genera in both aerobic and anaerobic samples suggested that they were likely considered to be facultative anaerobes and the candidate bacteria capable of degrading DMF. Some other bacteria were also aligned as facultative anaerobes such as *Rhodobacter* and *Catellibacterium* (Wen et al., 2016; Zhang et al., 2012), although they were rare in the CCS samples and accounted for less than 1%, they were still considered the candidate bacteria for playing roles in the degradation or hydrolysis of DMF.

6.3.2.2 Shift of archaeal community structure

All archaea identified in this study all belong to the phylum *Euryarchaeota*, and the majority of them are methanogens. All archaea identified in this study are listed in **Table 6.5**. The results suggest that the archaeal community structure was much more simplified than bacteria, because only 61 OTUs of archaea were generated with 97% similarity in all samples. As shown in **Fig. 6.3 (c)**, the predominant genus in the

inoculum samples An-0 and Ae-0 was *Methanosaeta*, which is the most common genus of acetotrophic methanogens in the anaerobic digestion system (Patel and Sprott, 1990). While the abundance of *Methanosaeta* slightly increased in the ADS samples of An-60, An-120 and Bk-62, it significantly decreased in the CCS samples from Cc-0 to Cc-62.

In the CCS samples, the abundance of the genus *Methanomethylovorans* gradually increased until it became the most predominant genus (CCS-62). This is a genus of methylotrophic methanogens which utilizes such methyl-compounds as methanol, MMA, DMA and also trimethylamine (TMA) to produce methane (Lomans et al., 1999). The second predominant genus was *Methanobrevibacter*, which is a genus of hydrogenotrophic methanogens which utilizes hydrogen or formic acid to produce methane (Gräwert et al., 2014). It should be noted that from Day 32 (CCS-32) of the CCS, the total number of methylotrophic *Methanomethylovorans* and hydrogenotrophic *Methanobrevibacter* exceeded the acetotrophic *Methanosaeta* and became the most abundant genera. This variation demonstrated that the methanogenesis type of the archaeal community was gradually shifted from acetotrophic methanogesis to the methylotrophic and hydrogenotrophic types: this indicates that the methanogens in the CCS began to specifically feed on methyl-compounds and hydrogen (or formate) instead of acetate. At the end of the co-culture cultivation, due to the available substrates were just DMA and HCOOH, the acetotrophic methanogen *Methanosaeta* was totally outcompeted by methylotrophic *Methanomethylovorans* and hydrogenotrophic *Methanobrevibacter*, the abundance of *Methanosaeta* finally dropped to a low level of 1.86% on Day 64 (CCS-64). At that time, the enriched CCS did not show any activity on the acetotrophic methanogenesis (almost no methane production when CCS was fed with acetate, data not shown).Therefore, it is concluded that in the process of DMF methanogenic degradation, acetate is not the intermediate product of DMF. The

corresponding intermediate products of DMF under the anaerobic condition should be DMA and HCOOH which were the feed to *Methanomethylovorans* and *Methanobrevibacter*, respectively. This is also in accordance with the metabolic pathway of DMF that DMF is hydrolyzed to DMA and HCOOH by enzyme (Ghisalba et al., 1985). Besides, as previously shown in **Fig. 6.2 (c)**, at the beginning of the co-culture cultivation, high concentrations of DMA (including MMA, the secondary intermediate of DMA) and HCOOH were detected, however, after the adaptive phase, the lowering of the concentrations of intermediate products suggests that the consumption of intermediate products became more rapid during the late period. These results show that the methylotrophic and hydrogenotrophic archaea gradually became predominant in the consortium when feeding on DMA and HCOOH.

6.3.3 Clustering analysis by PCoA and LEfSe

The PCoA was conducted with the 14 samples in this study in order to investigate the evolution direction of the microbial community, and the results are shown in **Fig. 6.4 (a)**. It should be noted that significant changes took place in the different types of samples. The clustering of the anaerobic samples An-0, An-60, An-120 (Group-A) and Bk-62 (Group-D) suggests that they were similar and the anaerobic cultivation did not bring any significant structural differences. However, in Group-B, due to continuous aeration, the anaerobic sludge Ae-0 gradually transformed into Ae-36: the obvious evolution direction of the microbial community followed the red arrow in **Fig. 6.4 (a)**. After that, we mixed the An-0 and Ae-36 to establish the CCS consortium of Group-C, and the point of Cc-0 was rightly located between An-0 and Ae-36. In the following 62-day anaerobic cultivation for the CCS, the mixed community structure of Group-C evolved in another direction, gradually becoming a relatively stable co-cultured system.

This indicates the establishment of a symbiosis relationship in the co-cultured consortium capable of effectively degrading DMF anaerobically.

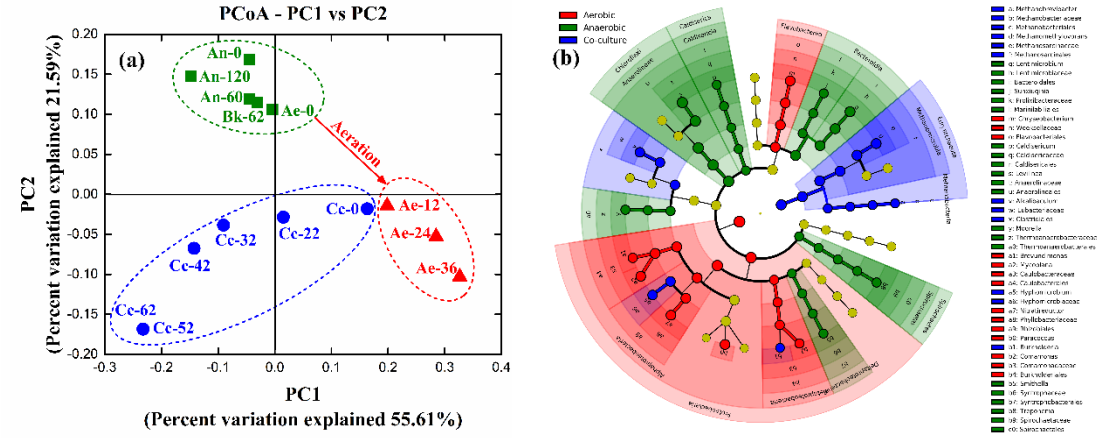

Fig. 6.4 The clustering analysis of all samples based on PCoA (principal coordinate analysis) and LEfSe (linear discriminant effect size). (a) PCoA revealing the evolution direction of the sludge at the OTU level. Samples were in three clusters: anaerobic digested sludge (green), aerobic activated sludge (red) and co-cultured sludge (blue); (b) a LEfSE cladogram representing differentially abundant taxa based on the top 30 OTUs of each sample in this study. A range of prokaryotic taxa from phylum to genus level were associated with the aerobic activated sludge group (red), the anaerobic digested sludge group (green) and the co-cultured sludge (blue) ($\alpha = 0.1$, size of circles is proportionate to each taxon's mean relative abundance).

The LEfSe analysis was conducted on the basis of the top 30 OTUs, which represent 80% of the total sequence from each sample, and these OTUs were clustered and aligned according to their phylogenetic levels. The cladogram of LEfSe is shown in **Fig. 6.4 (b)**. All of the samples were clustered into 3 groups of leaf: the Aerobic group (including Ae-12 ~ Ae-36) whose leaves were indicated in red color, the Anaerobic group (including An-0 ~ An-120 along with Ae-0 and Bk-62) indicated in green color, and the Co-culture group (including Cc-0 ~ Cc-62) indicated in blue color. The yellow clades represent those genera which did not show significant variation in their abundances, and the other colored clades (blue, red and green) were defined as biomarkers which represent their predominance in the corresponding leaf (group). For

the blue clade points a5 (***Hyphomicrobium***), b1 (***Burkholderia***) and v (*Alkalibaculum*) are bacteria, and d (*Methanomethylovorans*) and a (*Methanobrevibacter*) are archaea. It should be noted that while b1 and a5 were indicated in blue in the red clades, other bacteria considered likely to have DMF-degrading ability were still indicated in red, including a7 (***Nitratireductor***) and b0 (***Paracoccus***). This suggests that the genera ***Hyphomicrobium*** and ***Burkholderia*** were much more adaptive to the anaerobic condition even though all the potential DMF-degrading bacteria were aligned as facultative anaerobes in this study. It was also apparent that d (*Methanomethylovorans*) and a (*Methanobrevibacter*) are methylotrophic and hydrogenotrophic methanogens, respectively, and were predominant in the blue leaves. It should be also noted that v (*Alkalibaculum*) was a genus of hydrogenotrophic acetogens absent in the red (aerobic) and green (anaerobic) leaves but present in the blue leaf, although this genus is not considered likely to be involved with the degradation of DMF (Liu et al., 2012), its hydrogen-feeding property is evidence that hydrogen (or formate) was produced in the CCS due to the degradation of DMF.

Based on the results of PCoA and LEfSe, it is proposed that with CCS, the mixture of AAS and ADS, the thorough methanogenic degradation of DMF can be realized under the anaerobic condition. This is because the AAS offer some facultatively anaerobic DMF-degrading bacteria which produce DMFase to hydrolyze DMF, and the ADS contains abundant methylotrophic and hydrogenotrophic methane-producing archaea which could directly utilize DMA and HCOOH (equivalent to H_2 and CO_2), intermediate products of DMF hydrolysis.

6.3.4 Metabolic pathways and ecological relationship between functional bacteria and archaea

According to the description in previous studies, under the aerobic condition, DMF initially hydrolyzes to DMA by the enzyme DMFase, and then DMA further degrades to MMA with NADPH due to the aerobic respiration and releases energy for the growth of cells (Ghisalba et al., 1986, 1985; Schär et al., 1986). However, in this study, the methanogenic degradation of DMF was performed under the anaerobic condition. The hydrolysis products DMA and HCOOH are common substrates for methylotrophic and hydrogenotrophic methanogens (Ferguson et al., 2000; Pan et al., 2016). Therefore, it is reasonable to assume that the thorough methanogenic degradation of DMF can be realized only after DMF is hydrolyzed anaerobically. Apparently, due to the lack of anaerobic hydrolyzing bacteria, the normal ADS in Group-A and Group-D was unable to hydrolyze DMF to fermentable intermediate products. After continuous aeration, abundant DMF-hydrolyzing bacteria grew in the AAS of Group-B. Therefore, in Group-C, the CCS, which is a mixture of AAS and ADS, contains both DMF-hydrolyzing bacteria (cultured in Ae-36 with aeration) and methane-producing archaea (originated from An-0), and finally succeeds in the complete methanogenic degradation of DMF.

As mentioned, 6 genera of facultatively anaerobic bacterial genera including *Paracoccus*, *Hyphomicrobium*, *Nitratireductor*, *Burkholderia*, *Rhodobacter* and *Catellibacterium* were selected as the candidates which potentially provide the hydrolyzing ability of DMF under the anaerobic condition. Based on the existence of DMF-hydrolyzing bacteria, the metabolic pathways of DMF methanogenic degradation were proposed. All of these are indicated in **Fig. 6.5**. It should be noted that under the anaerobic condition, due to the lack of oxygen, the respiration on DMA by bacteria was likely considered to be directly interrupted (Ghisalba et al., 1986, 1985). On the one hand, with the existence of methanogens, DMA is preferentially utilized by the

methylotrophic methanogens and further degraded into MMA and ammonium, methane was also produced in this process. On the other hand, hydrogenotrophic methanogens

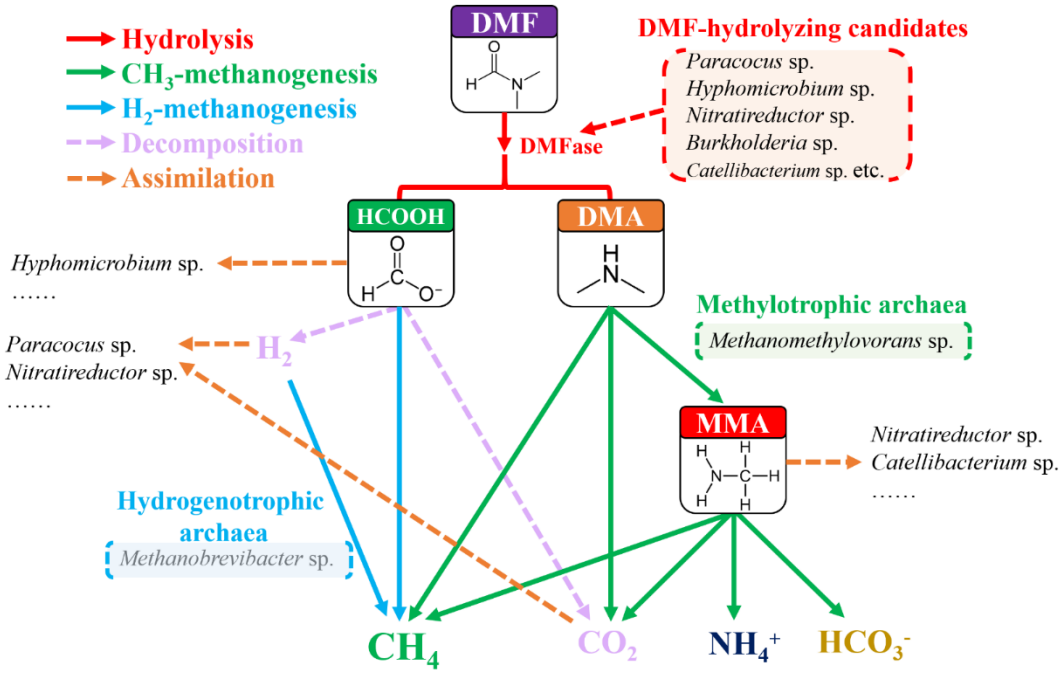

Fig. 6.5 Metabolic pathways of DMF methanogenic degradation proposed in this study.

also utilize formate (or CO_2 and H_2) to produce methane. In this investigation, it was apparent that the growth rates of these facultatively anaerobic DMF-hydrolyzing bacteria were significantly lower due to the switch in the major metabolism from aerobic respiration to methanogenesis. It should be noted that apart from the potential DMF-degrading ability, members of *Paracoccus* are also reported as hydrogenotrophic denitrifying bacteria which grow in both the heterotrophic and autotrophic metabolism (Hartop et al., 2017; Zhang et al., 2018). When the aerobic heterotrophic growth feeding on DMA is interrupted under the anaerobic condition, *Paracoccus* begins to use hydrogen as an electron donor to reduce CO_2 and produce C_{-1} organic matter in order to realize assimilation and allow for cell growth. Similarly, the other DMF-degrading candidate genera, *Hyphomicrobium*, *Nitratireductor* and *Catellibacterium*, etc., aligned

in this study are all denitrifying bacteria and are also methylotrophic bacteria which use methanol, MMA, formate or other C_1 organic matters as the carbon and energy sources (Manickam et al., 2012; Martineau et al., 2015; Zhang et al., 2012). As a result, even though the heterotrophic pathway feeding on DMA was interdicted, those functional bacteria were still able to use the intermediate products of DMF, such as HCOOH, H_2 and CO_2 as well as MMA to remain alive.

As can be seen in **Fig. 6.6**, the total sequence numbers of those candidate DMF-

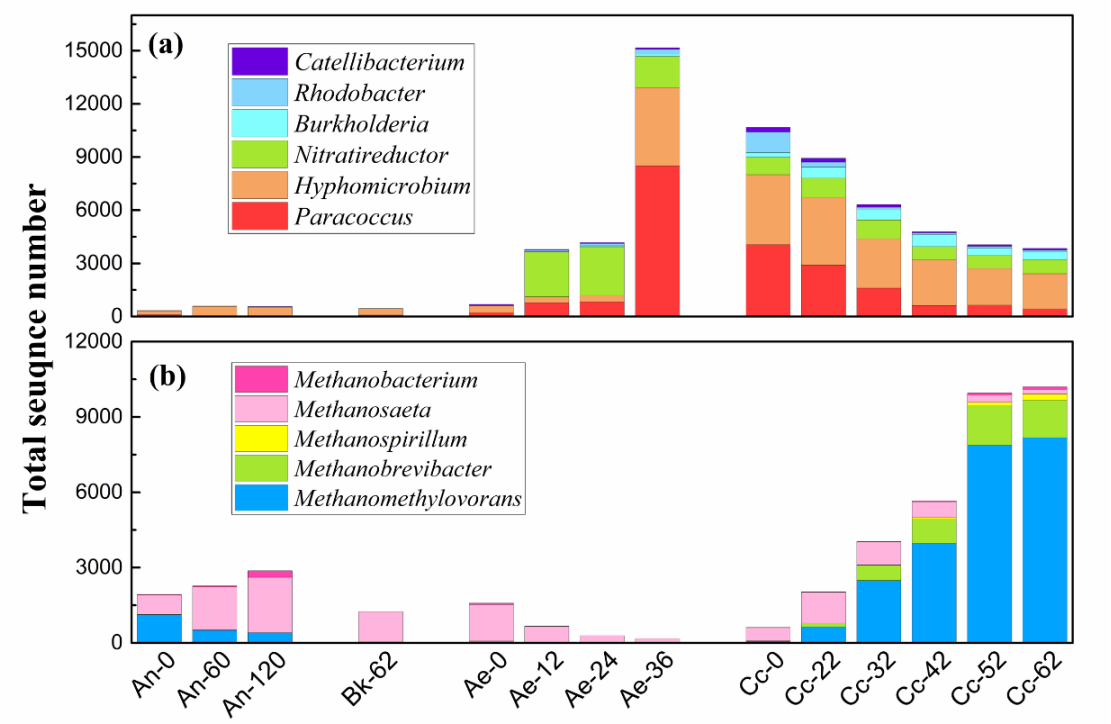

Fig. 6.6 Variation of the total sequence numbers of those functional bacteria and archaea which were considered to have the potential of degrading DMF or to play roles on the metabolic pathways of DMF methanogenic degradation. Both bacteria and archaea were aligned at the level of genus. (a) Variation of potential DMF-hydrolyzing bacteria including 6 genera of candidates *Paracoccus*, *Hyphomicrobium*, *Nitratireductor*, *Burkholderia*, *Rhodobacter* and *Catellibacterium* in all samples; (b) variation of methane-producing archaea in all samples. Methylotrophic genus *Methanomethylovorans*, hydrogenotrophic genus *Methanobrevibacter* and acetotrophic genus *Methanosaeta* were specifically concerned.

hydrolyzing bacteria gradually decreased while the methylotrophic and hydrogenotrophic methanogens increased significantly during the 62-day co-culture cultivation of Group-C. Under the anaerobic condition, apparently, there was a niche overlap between the DMF-hydrolyzing bacteria and methane-producing archaea in CCS. These DMF-hydrolyzing bacteria were initially the most predominant occupants in the AAS, while there was an absence of archaea. After mixed with ADS under the anaerobic condition, they could no longer utilize DMA due to the interruption of respiration, and the competitions on these intermediates HCOOH, MMA, H_2 and CO_2 occurred between bacteria and archaea. Obviously, after DMF was hydrolyzed into DMA and HCOOH by enzyme, there was no competitor to the methylotrophic methanogen *Methanomethylovorans* for sharing DMA, therefore, the sequence number of *Methanomethylovorans* kept increasing until this methanogen became the most predominant genus in both archaeal community and the entire prokaryotic community. Conversely, the decline in the number of the DMF-hydrolyzing bacteria such as *Paracoccus*, *Hyphomicrobium*, *Nitratireductor* and *Catellibacterium* were also clearly shown in **Fig. 6.6 (a)**. It was likely that the H_2 in the co-cultured consortium was basically sourced from the decomposition of HCOOH (Pan et al., 2016), and that the lack of H_2 resulted in a low abundance of *Paracoccus*. On the one hand, hydrogenotrophic methanogen *Methanobrevibacter* was in competition with *Paracoccus* on H_2, whereas, on the other hand, methylotrophic *Methanomethylovorans* further competed with *Hyphomicrobium*, *Nitratireductor* and *Catellibacterium* on MMA, which is the secondary intermediate of DMF. As shown in **Fig. 6.6 (b)**, the continuously increasing sequence numbers of both *Methanomethylovorans* and *Methanobrevibacter* demonstrated that archaea were much more competitive for intermediate products than bacteria during the co-culture cultivation. As a result, the

DMF-hydrolyzing bacteria grew anaerobically at a much slower rate and their proportion remained small in the prokaryotic community.

6.3.5 Dynamics of microbial community structures of UASB

The dissection of the microbial community structures of the 15 sludge samples were investigated at the phylum level. Phyla with a relative abundance of over 0.5% in the total sequence were specifically aligned and are given in Fig. 6.7, while the phyla with abundance of less than 0.5% were classified as others. The microbial community structure shown in Fig. 6.7 was quite different from the other anaerobic samples

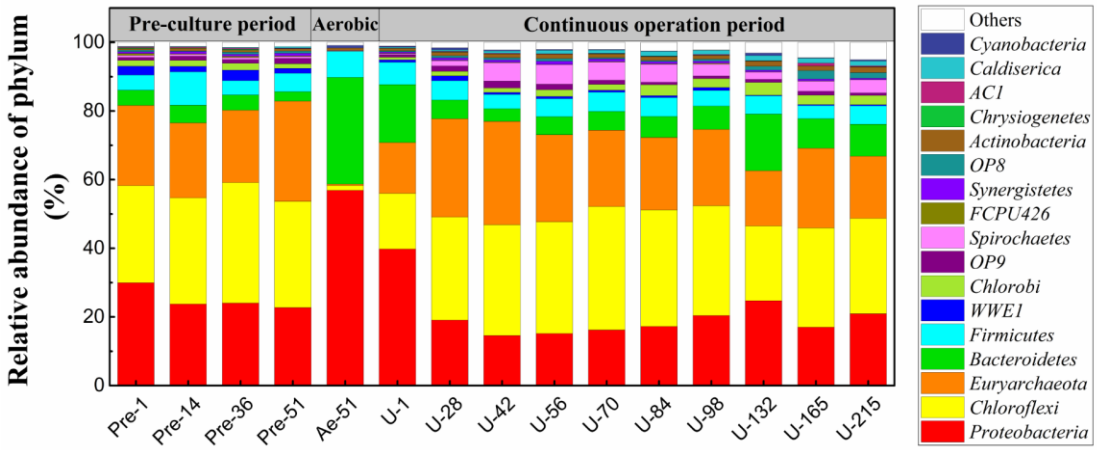

Fig. 6.7 An overview of the variation of prokaryotic community structure in 15 samples of sludge at the phyla level.

because sample Ae-51 was collected from the DAS. In sample Ae-51, the most abundant phylum was *Proteobacteria*, which overwhelmingly accounted for 56.85% of the total sequence. The second predominant phylum was *Bacteroidetes*, with a relative abundance of 31.09%, and *Firmicutes* ranked third in this sample with 7.58% abundance.

On the other hand, the variation of the microbial community structure was not that significant in the other 14 samples. Among the all assigned phyla, the most predominant of them was *Chloroflexi*, whose abundance remained at the range of 28.27% ~ 35.11%

in the 4 pre-culturing AGS samples, and slightly fluctuated from 27.73% to 35.93% in the 10 samples of mixed sludge during the continuous operation period. The phylum *Euryarchaeota*, to which all archaea identified in this study were assigned, was the second predominant phylum in the 14 samples, accounting for 16.11% ~ 30.09%. However, this phylum was extremely rare in the activated sludge sample Ae-51 with just 0.46% relative abundance. The following phylum was *Proteobacteria*, and accounted for 14.56% ~ 29.94%: note that this was the most abundant phylum in the aerobic sample. While *Bacteroidetes* and *Firmicutes* were also major phyla in all 14 samples, the abundance of each was less than 10%. Most of the phyla mentioned above have been commonly reported in various anaerobic digestion systems (Cerrillo et al., 2017; L. Li et al., 2015). This is to be expected since the seed sludge inoculated to the UASB originally contains a large quantity of those protogenous anaerobes, and they do not change significantly at the level of phylum. It should be also noted that significant variations were observed in the phyla *Chlorobi* (1.35% ~ 3.77%), *Spirochaetes* (0.52% ~ 5.68%), *Actinobacteria* (0.43% ~ 1.66%) and *Caldiserica* (0.14% ~ 1.51%). Although these were rare and minor in the community, their relative abundances significantly increased by several times from the pre-culture period to the continuous operation period. It is therefore likely that these changes are related to the degradation of DMF.

6.3.6 Characterization and variation of archaeal communities

As shown in **Fig. 6.8**, the characterization and variation of archaeal communities were determined at the level of genus along with the change in the methane production during the continuous operation period. The MPR per unit substrate illustrated in **Fig. 6.8 (a)** demonstrated that the completion of DMF degradation gradually weakened along with the increase in OLR and the decrease in HRT. It was apparent that with the

high OLR, excessive DMF could not be sufficiently hydrolyzed into the intermediate products DMA and HCOOH, therefore, the lack of intermediate products resulted in low methane production. In total, 14 genera of archaea were identified, including 12 genera of methanogens, as illustrated in Fig. 6.8 (b). Because the DAS contains little

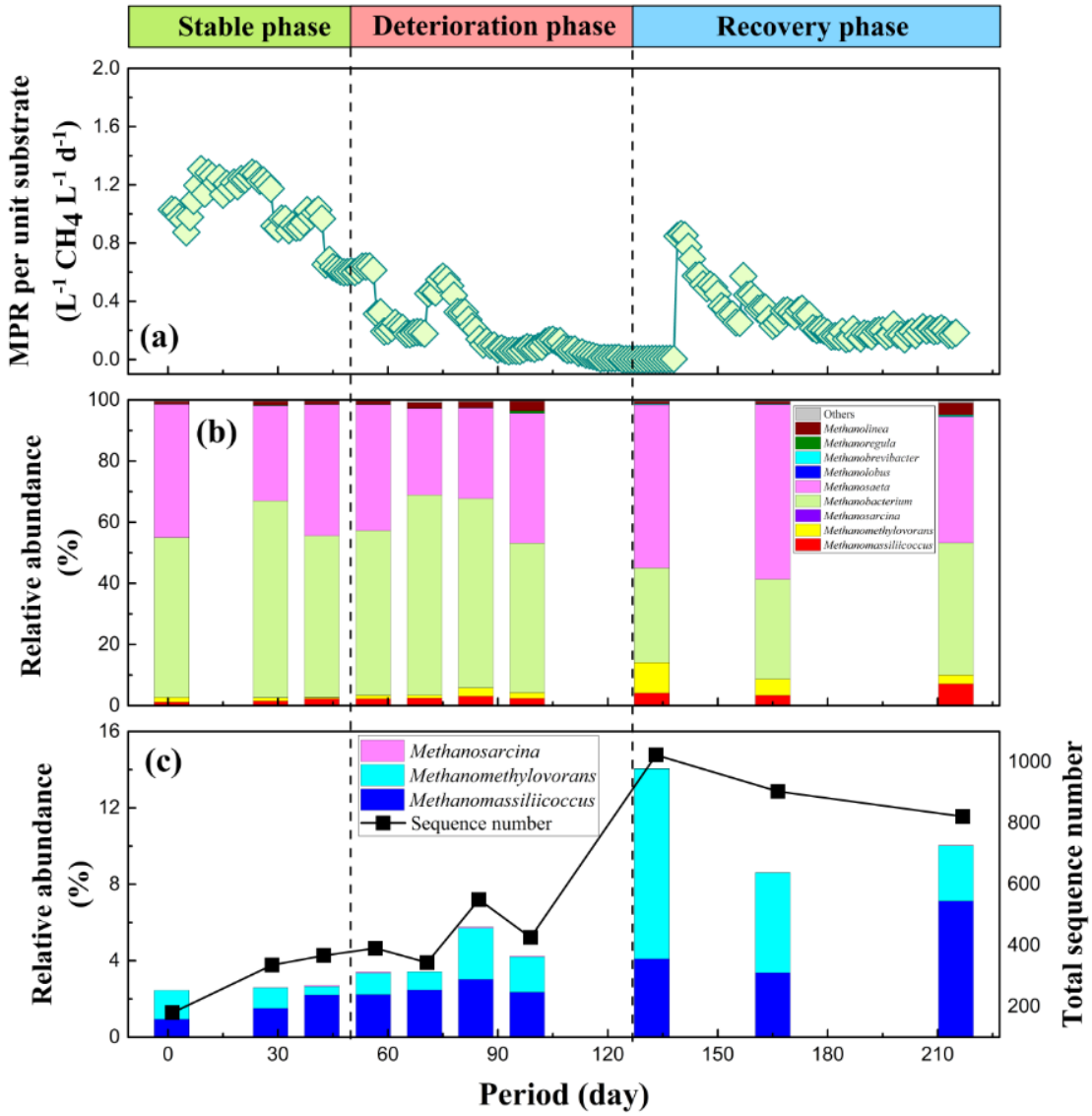

Fig. 6.8 Variation and response of archaeal community to changes in the methane production ability during the continuous operation period at the level of genus: (a) variation of methane production rate from unit substrate; (b) shift in the archaeal community structure at the level of genus; (c) specific change in the relative abundance and sequence number of methylotrophic archaea.

archaea, it can be concluded that the majority of archaea originated from the AGS. As

was the case with the 4 samples in the pre-culture period, the most abundant genera in the 10 samples during the continuous operation period were hydrogenotrophic *Methanobacterium* and acetotrophic *Methanosaeta* (Lee et al., 2017; Patel and Sprott, 1990), and the total abundance of these two genera accounted for almost 90% of the entire archaeal community. The other hydrogenotrophic archaea accounted for less than 5%. The AGS originally contained abundant hydrogenotrophic archaea, and because archaea these utilize both hydrogen and formate, HCOOH could be sufficiently utilized and the relative abundance of *Methanobacterium* was the most predominant genus during both the stable phase and the deterioration phase. However, during the recovery phase, *Methanosaeta* gradually turned into the most abundant genus. This was probably because most of the microorganisms of DAS were aerobic and, as such, unable to survive in the anaerobic condition. Therefore, after being inoculated to the UASB, they gradually died, and the decayed and lysed cells self-digested and released proteins, carbohydrates and VFAs, which are common feed for acetotrophic archaea. While acetotrophic methanogesis is the most common metabolism in the majority of anaerobic digesters, in a digester which is specific to methylotrophic or hydrogenotrophic methanogenesis such as the degradation of DMF, it may well be that the superiority of acetotrophic metabolism is an indicator of the deterioration of the sludge. However, it should be noted that although the MPR gradually declined, the methylotrophic archaea still exhibited a growing trend in the relative abundance during the entire experimental period. The variation of relative abundance of methylotrophic genera and total sequence numbers is shown in **Fig. 6.8 (c)**. The abundance of *Methanomassiliicoccus*, *Methanomethylovorans* and *Methanosarcina* increased from 2.45% to the highest 14.06% along with the increase in the total sequence number from 180 to 1032 during the continuous operation period, which was further elevated in comparison with the pre-

culture period. Among the methylotrophic archaea, *Methanosarcina*, which accounted for less than 1%, was rare in these samples, however, it has been shown capable of utilizing both acetate and methyl-compounds (Shimizu et al., 2011). The increasing abundance of methylotrophic archaea demonstrated that the intermediate product DMA was efficiently utilized by the mixed sludge. It is also suggested that with the help of both hydrogenotrophic and methylotrophic methanogens, the intermediate products of DMF could be used to realize effective methanogenesis. However, the extent to which the concentrations of intermediate products can be converted from DMF depends on the hydrolysis completion of DMF, which is performed by bacteria.

6.3.7 Shift of bacterial communities and potential candidate strains for DMF degradation

As shown in **Fig. 6.9 (a)**, the shift in the bacterial communities occurred at the level of genus along with the change in DMF removal efficiency during the continuous operation period. It was obvious that under the low OLR of 1.63 ~ 4.22 g COD L^{-1} d^{-1}, a high DMF removal efficiency was obtained. With the further elevating of the OLR, the removal efficiency sharply decreased. The DMF removal efficiency also represents the completion of DMF hydrolysis. When a lower proportion of DMF was hydrolyzed, the concentrations of the intermediate products produced were lower, finally resulting in the feeble methanogenic degradation of DMF. Therefore, the hydrolysis of DMF as performed by the bacterial community is considered the key factor that decides whether the thorough methanogenic degradation of DMF can be realized. The bacterial genus with a relative abundance of over 1% is shown in **Fig. 6.9 (b)**, and genera with less than 1% abundance were all classified as others. However, in the 10 samples of mixed sludge, diverse genera were aligned, suggesting that the major genera were those bacteria which

commonly exist in normal anaerobic digestion systems, such as *Levilinea*, *Geobacter*, *Tangfeifania*, *Longilinea*, *Thermomarinilinea* and *Syntrophobacter* etc. (Antwi et al., 2017; Gagliano et al., 2015; Ma et al., 2015). The shift of these common bacteria did not clarify the mechanism of DMF degradation. The growth and decay of those DMF-

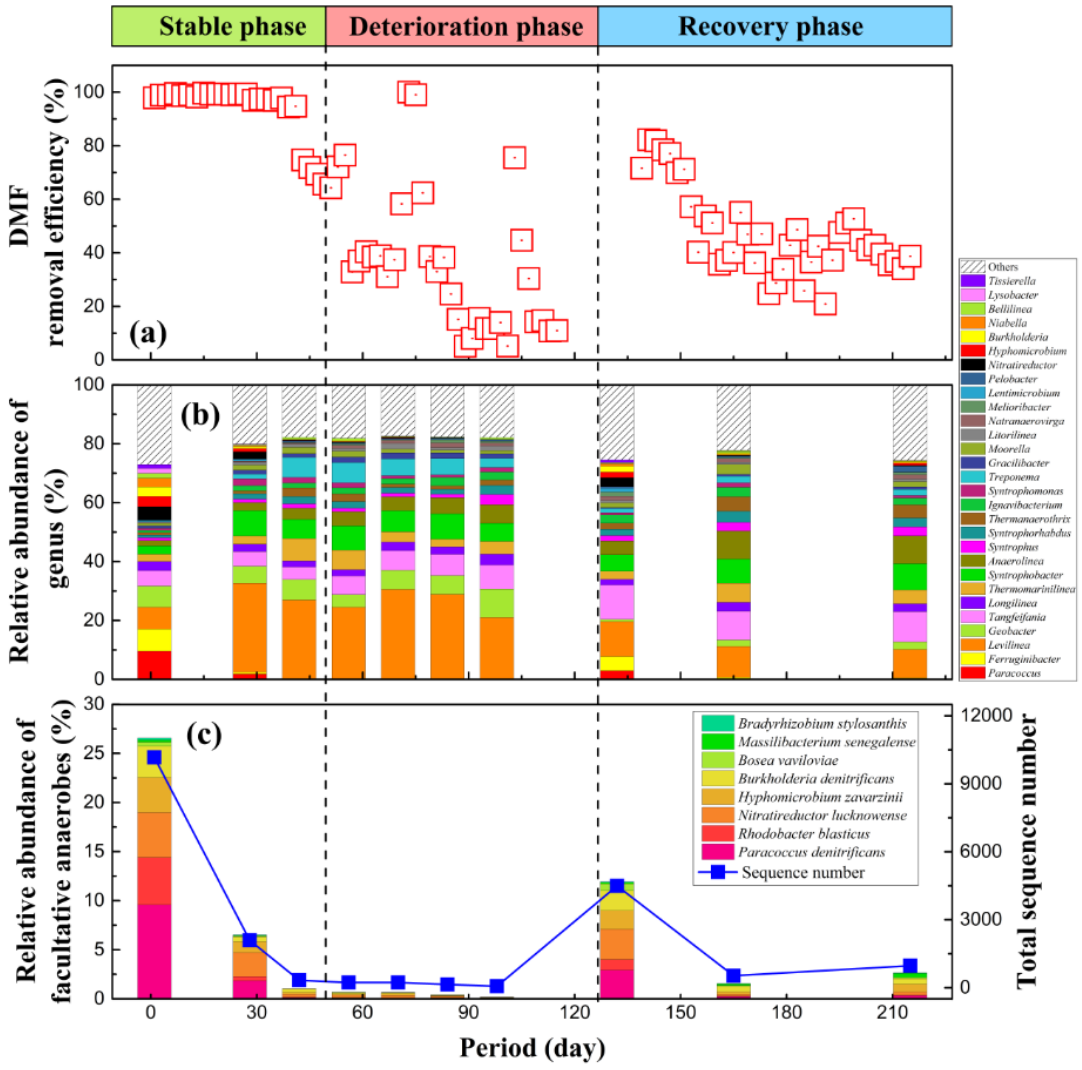

Fig. 6.9 Variation and response of the bacteria community to the change in DMF hydrolysis ability during the continuous operation period at the level of genus: (a) variation of DMF removal efficiency; (b) shift in the bacterial community structure at the level of genus; (c) specific changes in the relative abundance and sequence numbers of facultative anaerobes likely related to the degradation of DMF.

hydrolyzing bacteria appear to be the most important clues because they are responses to the change in the performance of the UASB.

As is mentioned above, normal AGS is unable to degrade DMF anaerobically, indicating that the AGS does not contain those DMF-concerned bacteria. Only after being mixed with DAS was the methanogenic degradation of DMF achieved. That is, those functional bacteria were brought into effect in the UASB by the DAS. However, because the DAS is aerobic sludge cultured under the anaerobic condition, most of the aerobic bacteria gradually died and decayed, and only a tiny proportion of facultative anaerobic bacteria could survive. Therefore, a group of bacterial strains of particular interest were selected, and these are listed in **Table 6.5**. These bacteria were rare in the 4 samples of AGS during the pre-culture period but abundant in the aerobic sample of Ae-51, and also remained in a low proportion in the rest 10 samples of mixed sludge during the continuous operation period. Among them, 8 strains were screened as DMF-related candidates since they were reported as facultative anaerobes. *Paracoccus denitrificans* has been directly reported as a DMF-degrading strain (Sanjeev Kumar et al., 2013), and was the most abundant bacteria in the samples Ae-51 and U-1, but its levels gradually decreased along with increasing OLR in the following periods. Similarly, *Hyphomicrobium zavarzinii* and *Nitratireductor lucknowense*, also found in our previous study, were reported to degrade dye and pesticide (Jérôme et al., 2007; Manickam et al., 2012), and is likely to be closely related to the degradation of DMF. *Burkholderia denitrificans* was also reported able to degrade refractory organics such as aromatic compounds (Lee et al., 2012). *Rhodobacter blasticus* is photosynthetic bacterium reportedly able to further degrade the organic residuals in the anaerobically digested wastewater (Wen et al., 2016). *Bradyrhizobium stylosanthis* was reported as a nitrogen-fixing symbiont capable of utilizing nitrogen-containing DMF (Delamuta et al., 2016). Although *Massilibacterium senegalense* and *Bosea vaviloviae* are facultative

anaerobes (Alou et al., 2016; Safronova et al., 2015), there was no direct evidence that they could degrade DMF. Therefore, further studies should be focused on these bacteria in pure culture tests. The variations of the total abundance and the sequence number of the DMF-related candidates are illustrated in **Fig. 6.9 (c)**. It was apparent that the variation of DMF degradation corresponded to changes in the abundance of these facultative anaerobes. During the stable phase, although the facultative anaerobes quickly decayed and the abundance dropped from 26.56% to 1.04%, the DMF removal efficiency was maintained at over 70%. However, with the increasing OLR, the abundance declined to its lowest level, at 0.19%, and the DMF removal efficiency dropped to a mere 12.44% during the deterioration phase. After re-inoculating the UASB with DAS, the HRT was lengthened to 48 h during the recovery phase, and the DMF removal efficiency slightly recovered to 34.13% along with the increase in the relative abundance of facultative anaerobes from 1.55% to 2.64%. This demonstrates that these facultative anaerobes do indeed grow slowly under the anaerobic condition, however, they seem sensitive to the elevating of the OLR, which significantly inhibited their growth rate. Therefore, it is reasonable to conclude that, in order to realize excellent DMF removal, an OLR of less than 6.17 g COD L^{-1} d^{-1} is optimal. Besides, because the growth rate of facultative anaerobes was relative slow, and the DAS can be cultivated in a large amount quickly, the timely addition of DAS to the UASB is also highly recommended to maintain a stable and effective performance for long-term operation.

6.3.8 Suggestions to the anaerobic treatment of DMF-containing wastewater

By co-culturing AAS and ADS, the effective methanogenic degradation of DMF was effectively realized using CCS. Because large quantities of AAS were easily cultured

with the continuous aeration, it is reasonable to suggest that CCS can be used as the seed inoculum to establish a quick start-up for the anaerobic treatment of DMF-containing wastewater. It seems, however, that the slow growth rate and tiny proportion of those DMF-hydrolyzing bacteria under the anaerobic condition was not likely to be capable of handling a high loading rate of DMF for the long-term operation. Therefore, appropriate solutions for the enhancement of the growth rate or maintaining a high relative abundance of the DMF-hydrolyzing bacteria should be considered. The results from the four groups of experiments have been considered to formulate the following ideas and suggestions: 1) because these facultatively anaerobic DMF-hydrolyzing bacteria are also known as denitrifying bacteria (Hartop et al., 2017; Manickam et al., 2012; Martineau et al., 2015; Zhang et al., 2012), the addition of nitrate (or nitrite) as an elector acceptor was considerable to improve the growth rate of these DMF-hydrolyzing bacteria by establishing a nitrate-reducing mineralization process under the anaerobic condition (Keller et al., 2018). However, a competition was also likely to extend to the organic carbon source between denitrification and methanogenesis, which would lower the methane production and suppress the process of methanogenesis (Sakthivel et al., 2012). As such, the nitrate dosage should be strictly controlled; 2) considering the application of the CCS in the anaerobic treatment of DMF-containing wastewater, on the one hand, the timely addition of AAS to the co-cultured consortium is highly recommended to ensure a large abundance of these DMF-hydrolyzing bacteria remained in the community in order to realize the effective hydrolysis of DMF, whereas, on the other hand, direct inoculation with AAS as the seed sludge was also an alternative to realize the start-up of the DMF-degrading consortium with more purified inoculum; 3) a two-stage aerobic/anaerobic system with micro-aeration well-suited to the treatment of some degradation-resistant organic matters should also be taken into

consideration (Sawatdeenarunat et al., 2017; Tsapekos et al., 2017); 4) the result of this study also suggest that DMF, as the sole substrate in the process of anaerobic digestion, is considered difficult to be degraded anaerobically due to the inactivation of these DMF-hydrolyzing bacteria under the anaerobic condition and the competition on intermediates between bacteria and archaea. Some previous studies achieved the anaerobic treatment of DMF with other antibiotic solvents (Chen et al., 2018; Hu et al., 2017), therefore, the co-digestion of DMF and other organic solvents seems to be a good alternative to improve the degradation ability, however, the degradation mechanisms and metabolic pathways of the co-digestion were quite different from the mono-digestion of DMF, which should be further investigated and elucidated.

6.4 Conclusions

Due to the lack of DMF-hydrolyzing bacteria, anaerobic digested sludge is unable to degrade DMF effectively. The co-cultured consortium containing DMF-hydrolyzing bacteria and methylotrophic/hydrogenotropic archaea allow for the methanogenic degradation of DMF. It is feasible that this co-cultured consortium could be applied in the anaerobic treatment of DMF-containing wastewater. However, those DMF-hydrolyzing bacteria also feed on some of the intermediate products of DMF, such as HCOOH and MMA. Outcompeted by archaea, DMF-hydrolyzing bacteria eventually resulted in the low abundance. It is considered that a sufficient abundance of DMF-hydrolyzing bacteria is the key to realize a thorough methanogenic degradation of DMF. The anaerobic treatment of DMF-containing wastewater was achieved in this study by the cooperation of DMF-hydrolyzing bacteria and methylotrophic methanogens in a UASB. Some facultative anaerobes originating from the DAS play an important role in the hydrolysis of DMF. Although they could grow slowly under the anaerobic condition,

at an OLR higher than 6.17 g COD L^{-1} d^{-1}, their growth was irreversibly inhibited. Therefore, the OLR should be kept at a low level, and the timely addition of DAS to the UASB is recommended to maintain a highly activated mixed consortium capable of hydrolyzing DMF efficiently for long-term operation.

Table 6.1 A brief introduction of experimental procedure in four groups.

Group	Cultivation		Operational parameters					
	Metabolism	Inoculum	Apparatus	Mode	Substrate renew	HRT (h)	Aeration	Sludge sampling (day)
A	Anaerobic	ADS	SAnMBR	Batch/Continuous	Artificial/Auto	∞→48	+	0, 60, 120
B	Aerobic	ADS	OCAR	Batch/Continuous	Artificial/Auto	∞→48	+	0, 12, 24, 36
C	Anaerobic	CCS	Serum vials	Batch	Artificial	∞	-	0, 22, 32, 42, 52, 62
D	Anaerobic	ADS	Serum vials	Batch	Artificial	∞	-	0, 62

[a] Anaerobic digested sludge (ADS) collected from a local wastewater treatment plant.

[b] ADS in Group-B was collected on the different day.

[c] Co-cultured sludge (CCS) was mixed by anaerobic digested sludge and aerobic activated sludge.

[d] OCAR means one-stage continuous aeration reactor.

[e] Artificial renew of substrate is to dose substrate to vials or reactors by injectors, the sludge in the vials was centrifuged and then the supernatant was abandoned and refilled with new substrate.

[f] Aeration for the SAnMBR is to use its own biogas internal circulation to fully stir and mix the sludge, while for the OCAR, which is to aerate the sludge by air.

Table 6.2 Comparative results of the DMF degradation ability and methane production ability in four groups.

Group	DMF dosage (mg L^{-1})		DMF degradation (hydrolysis)			Methane production (methanogenesis)		
	Batch	Continuous	Batch (%)	Continuous (%)	Ability	Batch (mL L^{-1} d^{-1})	Continuous (mL L^{-1} d^{-1})	Ability
A	328.64	591.05 - 1056.20	4.57	1.55 - 7.29	-[b]	7.28	11.04	-
B	932.17	2104.98 - 2323.98	100	99.74	+	*N.A*	*N.A*	*N.A*
C	5065.76	*N.A*[a]	100	*N.A*	+	138.53 - 267.19	*N.A*	+
D	5090.65	*N.A*	4.63	*N.A*	-	2.42	*N.A*	-

[a] Not available.

[b] + means excellent ability, - means weak ability.

Table 6.3 Statistical data derived from high-throughput sequencing of prokaryotes in all 14 samples of this study.

Items	Group-A			Group-B				Group-C						Group-D
	An-0	An-60	An-120	Ae-0	Ae-12	Ae-24	Ae-36	Cc-0	Cc-22	Cc-32	Cc-42	Cc-52	Cc-62	Bk-62
Good's Coverage[a]	98.55	98.67	99.00	98.55	99.17	99.27	99.35	98.78	98.92	99.01	98.95	98.87	99.00	98.89
Shannon	7.22	6.66	6.07	6.82	6.60	6.61	4.96	6.77	6.49	6.37	6.31	5.92	5.74	6.27
Simpson	0.98	0.97	0.95	0.96	0.97	0.97	0.92	0.97	0.97	0.97	0.97	0.95	0.94	0.94
Chao1	2165.04	1982.53	1508.51	2270.74	1353.67	1224.16	950.84	1865.19	1691.90	1531.10	1615.92	1669.75	1500.83	1723.32
Observed species	1541	1341	1075	1488	1083	974	722	1353	1184	1087	1084	1054	975	1184
Singles	581	533	401	582	332	293	259	488	434	397	422	454	399	446

[a] Good's coverage was calculated as $G = 1-(n/N)\cdot 100$, where n is the number of singleton OTU and N is the total number of sequences in all samples, in this study, N was unified to 40,000.

Table 6.4 (a) Identification of bacteria in Group-A and Group-B at the level of genus.

Group-A (Anaerobic cultivation by SAnMBR)			**Group-B (aerobic cultivation by continuous aeration reactor)**			
An-0	**An-60**	**An-120**	**Ae-0**	**Ae-12**	**Ae-24**	**Ae-36**
Levilinea (12.20)[a]	*Levilinea* (17.57)	*Levilinea* (18.20)	*Levilinea* (25.15)	*Levilinea* (10.03)	*Mycoplana* (12.68)	*Paracoccus* (21.33)
Lentimicrobium (9.51)	*Lentimicrobium* (10.88)	*W22* (7.22)	*Lentimicrobium* (7.06)	*Chryseobacterium* (8.29)	*Nitratireductor* (6.30)	*Chryseobacterium* (11.48)
W22 (3.18)	*Caldisericum* (3.23)	*Moorella* (6.60)	*Thermomarinilinea* (2.83)	*Thermomonas* (6.93)	*Chryseobacterium* (4.30)	*Hyphomicrobium* (10.94)
W5 (2.80)	*Allochromatium* (2.99)	*Sulfurovum* (6.16)	*Caldisericum* (2.78)	*Nitratireductor* (6.17)	*Levilinea* (2.85)	*Ferruginibacter* (7.46)
BHB21 (2.32)	*Thermovirga* (2.85)	*Caldisericum* (4.05)	*Thermovirga* (2.75)	*Brevundimonas* (5.56)	*Brevundimonas* (2.83)	*Methylobacillus* (4.78)
Thermomarinilinea (2.21)	*Thermomarinilinea* (2.71)	*Thermomarinilinea* (5.05)	*Sunxiuqinia* (2.37)	*Comamonas* (2.63)	*Marinimicrobium* (2.78)	*Mycoplana* (4.55)
Treponema (2.16)	*Microbacter* (2.22)	*Smithella* (2.73)	*Gelria* (1.91)	*Taibaiella* (2.33)	*Pseudoflavitalea* (2.68)	*Nitratireductor* (4.27)
Blvii28 (1.95)	*Syntrophorhabdus* (2.10)	*Lachnoclostridium* (2.62)	*Allochromatium* (1.83)	*Paracoccus* (1.81)	*Sphingopyxis* (2.45)	*Parasediminibacterium* (2.96)
Smithella (1.80)	*Caldisericum* (2.09)	*Lentimicrobium* (2.33)	*Romboutsia* (1.82)	*Sorangium* (1.64)	*Thermomonas* (2.28)	*Bdellovibrio* (2.42)
Sunxiuqinia (1.78)	*Moorella* (2.08)	*Syntrophorhabdus* (2.20)	*Microbacter* (1.73)	*Moheibacter* (1.56)	*Bacillus* (2.22)	*Moheibacter* (2.21)
Thermovirga (1.47)	*Gelria* (2.05)	*Romboutsia* (1.83)	*SC103* (1.64)	*Pseudoflavitalea* (1.46)	*Moheibacter* (2.05)	*Leadbetterella* (2.08)
Caldisericum (1.42)	*Blvii28* (1.80)	*Sphaerisporangium* (1.73)	*Longilinea* (1.41)	*Stenotrophomonas* (1.41)	*Devosia* (2.04)	*Pseudoflavitalea* (1.81)
SC103 (1.31)	*Romboutsia* (1.76)	*Longilinea* (1.71)	*Caldisericum* (1.38)	*Romboutsia* (1.39)	*Ferruginibacter* (1.99)	*Domibacillus* (1.26)
Microbacter (1.20)	*Sunxiuqinia* (1.27)	*W5* (1.68)	*Syntrophorhabdus* (1.32)	*Thermomarinilinea* (1.34)	*Paracoccus* (1.87)	*Levilinea* (1.07)
Luteitalea (1.20)	*W22* (1.26)	*Allochromatium* (1.55)	*Smithella* (1.16)	*Thermovirga* (1.28)	*Ilumatobacter* (1.65)	-
Allochromatium (1.10)	*Aciditerrimonas* (1.21)	*Microbacter* (1.34)	*Acidothermus* (1.01)	*Sphingopyxis* (1.23)	*Comamonas* (1.54)	-
Longilinea (1.09)	*Longilinea* (1.17)	*Thermovirga* (1.00)	-	*Lentimicrobium* (1.12)	*Bradyrhizobium* (1.41)	-
Caldisericum (1.05)	-	-	-	*Pseudobacter* (1.11)	*Taibaiella* (1.34)	-
Gelria (1.04)	-	-	-	*Ochrobactrum* (1.02)	*Acidothermus* (1.20)	-
Desulfofaba (1.00)	-	-	-	*Longilinea* (1.00)	*Allochromatium* (1.19)	-
Others (48.23)	Others (40.76)	Others (32.02)	Others (41.86)	Others (40.70)	Others (42.35)	Others (21.42)

[a] The number in the parenthesis means the relative abundance (%) of this genus in the entire bacterial community.

Table 6.4 (b) Identification of bacteria in Group-C and Group-D at the level of genus.

Group-C (anaerobic cultivation by long-term batch test with co-cultured sludge)						**Group-D (blank control)**
Cc-0	**Cc-22**	**Cc-32**	**Cc-42**	**Cc-52**	**Cc-62**	**Bk-62**
Paracoccus (10.09)[a]	*Levilinea* (15.98)	*Levilinea* (16.57)	*Levilinea* (18.79)	*Levilinea* (18.52)	*Levilinea* (23.99)	*Levilinea* (25.88)
Hyphomicrobium (9.47)	*Hyphomicrobium* (9.45)	*Alkalibaculum* (7.85)	*Hyphomicrobium* (6.87)	*Alkalibaculum* (7.83)	*Alkalibaculum* (6.25)	*Lentimicrobium* (7.87)
Levilinea (8.36)	*Paracoccus* (7.51)	*Hyphomicrobium* (7.18)	*Alkalibaculum* (6.84)	*Hyphomicrobium* (6.31)	*Hyphomicrobium* (6.06)	*Treponema* (4.06)
Ferruginibacter (3.67)	*Alkalibaculum* (5.98)	*BHB21* (5.71)	*Lentimicrobium* (6.48)	*Lentimicrobium* (5.77)	*Lentimicrobium* (4.93)	*Thermovirga* (3.63)
Rhodobacter (2.95)	*Tissierella* (3.03)	*Lentimicrobium* (4.53)	*BHB21* (3.47)	*Thermomarinilinea* (3.84)	*Thermomarinilinea* (4.35)	*Moorella* (3.08)
Nitratireductor (2.42)	*Nitratireductor* (2.75)	*Paracoccus* (4.32)	*Thermomarinilinea* (3.20)	*Moorella* (3.13)	*Moorella* (3.32)	*Caldisericum* (2.85)
Parasediminibacterium (2.28)	*Thermomarinilinea* (2.34)	*Thermomarinilinea* (3.25)	*Moorella* (2.39)	*Nitratireductor* (2.38)	*Sunxiuqinia* (2.70)	*W5* (2.38)
Lentimicrobium (2.02)	*Lentimicrobium* (2.21)	*Nitratireductor* (2.80)	*Nitratireductor* (2.06)	*Sunxiuqinia* (2.38)	*Nitratireductor* (2.52)	*W22* (2.26)
Ferruginibacter (1.96)	*Burkholderia* (1.68)	*Tissierella* (1.88)	*Burkholderia* (2.03)	*Paracoccus* (1.97)	*Treponema* (2.07)	*Sunxiuqinia* (1.94)
Thermomarinilinea (1.65)	*BHB21* (1.68)	*Thermovirga* (1.76)	*Longilinea* (1.89)	*Thermovirga* (1.79)	*Longilinea* (2.03)	*Romboutsia* (1.72)
Chryseobacterium (1.60)	*Lentimicrobium* (1.65)	*Burkholderia* (1.60)	*Sunxiuqinia* (1.74)	*Longilinea* (1.75)	*Thermovirga* (1.97)	*Syntrophus* (1.67)
Levilinea (1.38)	*Thermovirga* (1.57)	*Longilinea* (1.51)	*Paracoccus* (1.71)	*BHB21* (1.49)	*Burkholderia* (1.56)	*Caldisericum* (1.59)
Niabella (1.32)	*Romboutsia* (1.23)	*Moorella* (1.40)	*Tissierella* (1.58)	*Burkholderia* (1.33)	*W22* (1.48)	*Thermomarinilinea* (1.30)
Tissierella (1.20)	*Caldisericum* (1.20)	*Caldisericum* (1.36)	*Caldisericum* (1.45)	*Caldisericum* (1.30)	*Caldisericum* (1.44)	*Smithella* (1.27)
Thermovirga (1.07)	*Sunxiuqinia* (1.15)	*Sunxiuqinia* (1.22)	*Thermovirga* (1.41)	*Ruminiclostridium* (1.19)	*Paracoccus* (1.41)	*Syntrophorhabdus* (1.11)
Sunxiuqinia (1.05)	*Longilinea* (1.05)	*Romboutsia* (1.21)	*Romboutsia* (1.15)	*Microbacter* (1.12)	*Ruminiclostridium* (1.23)	-
Levilinea (1.00)	*Smithella* (1.02)	*Smithella* (1.00)	*Smithella* (1.11)	-	-	-
-	*Moorella* (1.00)	-	-	-	-	-
-	-	-	-	-	-	-
-	-	-	-	-	-	-
Others (46.50)	Others (38.52)	Others (34.84)	Others (35.85)	Others (37.92)	Others (32.71)	Others (37.39)

[a] The number in the parenthesis means the relative abundance (%) of this genus in the entire bacterial community.

Table 6.5 Bacterial strains selected as the potential candidates which might be concerned with DMF degradation from all 15 samples of sludge in this study. These bacteria were abundant in the aerobic samples Ae-51, but rare in the pre-culture samples (Pre-1 ~ Pre-51), and also remained in a little quantity in the mixed sludge samples (U1 ~ U215).

Description of candidate functional strains				**Total sequence number of each strain in all samples**														
Candidate species	**Similarity**	**Accession Number**	**Facultative Anaerobic**	**Pre-culture period**				**Aerobic**	**Continuous operation period**									
				Pre-1	**Pre-14**	**Pre-36**	**Pre-51**	**Ae-51**	**U-1**	**U-28**	**U-42**	**U-56**	**U-70**	**U-84**	**U-98**	**U-132**	**U-165**	**U-215**
Azohydromonas riparia strain UCM-11	99	NR_149203.1	-	0	0	0	0	471	236	53	12	6	9	0	0	14	3	4
Bosea vaviloviae strain Vaf-18	99	NR_136423.1	+	0	2	0	1	333	167	25	5	3	10	11	6	274	31	59
Bradyrhizobium stylosanthis strain BR 446	98	NR_151930.1	+	0	0	0	0	144	72	26	1	2	6	3	2	32	10	20
Burkholderia denitrificans strain KIS30-44	95	NR_108706.1	+	10	4	3	8	2409	1219	157	112	40	26	14	11	764	187	195
Comamonas testosteroni strain KS 0043	100	NR_029161.2	-	0	0	5	5	148	77	1	3	35	33	6	18	27	26	49
Ferruginibacter profundus strain DS48-5-3	95	NR_148259.1	-	0	0	1	0	5741	2871	201	23	1	9	14	9	1818	157	62
Gemmatimonas phototrophica strain AP64	95	NR_136770.1	-	0	0	0	0	143	72	45	6	5	2	4	2	38	2	7
Heliophilum fasciatum strain DSM 11170	86	NR_117585.1	-	0	0	0	0	327	164	37	7	5	9	4	1	84	7	13
Hyphomicrobium zavarzinii strain ZV-622	99	NR_026429.1	+	0	0	0	1	2719	1360	356	53	51	71	24	15	705	81	295
Lentimicrobium saccharophilum strain TBC1	93	NR_149795.1	-	0	0	0	0	312	156	204	28	7	8	15	10	465	61	30
Lysobacter sediminicola strain 7C-9	95	NR_147745.1	-	2	0	3	4	1273	639	69	5	12	8	3	0	46	35	50
Massilibacterium senegalense strain mt8	99	NR_144721.1	+	5	2	0	2	166	84	18	3	10	2	2	2	44	54	146
Niabella terrae strain ICM 1-15	97	NR_132698.1	-	0	0	0	0	2247	1124	123	4	2	5	8	1	303	29	9
Nitratireductor lucknowense strain IITR-21	99	NR_118014.1	+	0	1	0	0	3507	1754	780	101	83	91	53	14	1165	76	119
Paracoccus denitrificans strain NBRC 102528	99	NR_114145.1	+	0	0	1	2	7321	3662	591	46	32	21	36	15	1099	83	121
Parapedobacter soli strain DCY14	87	NR_044119.1	-	3	5	4	0	277	139	12	34	61	13	21	54	84	41	147
Rhodobacter blasticus strain KC2138	99	NR_043735.1	+	0	0	2	2	3696	1849	138	6	7	2	4	0	409	10	13
Rubrimonas shengliensis strain SL014B-28A2	90	NR_133044.1	-	0	0	0	0	235	118	25	11	8	8	3	2	225	2	8
Sphingopyxis panaciterrulae strain DCY34	99	NR_116164.1	-	0	0	2	0	369	185	34	6	1	16	5	1	56	11	41

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Chapter 7 General conclusions and future perspectives

Abstract

The anaerobic digestion process has been shown to be suitable to the treatment of high strength DMF-containing wastewater derived from chemical industrial wastewater. With the help of co-cultured consortium consisting of DMF-hydrolyzing bacteria and methanogens, DMF can be effectively degraded under the anaerobic condition and recovered as methane. The stable operation and microbial community should be improved and enhanced in order to realize a stable long-term operation in engineering application. Some suggestions and perspectives are also proposed in this section.

7.1 General conclusions

7.1.1 Methanogenic degradation of DMF

Due to the lack of DMF-hydrolyzing bacteria, DMF has been shown to be difficult to degrade under the anaerobic condition. However, DMF can be effectively degraded under the aerobic condition with continuous aeration. That is also why few studies have reported the methanogenic degradation of DMF. Only with the cooperation of both DMF-hydrolyzing bacteria and methylotrophic/hydrogenotrophic methanogens can DMF be degraded under the anaerobic condition.

7.1.2 Application of co-cultured consortium in UASB and AnMBR

Although the co-cultured consortium consisting DMF-degrading activated sludge and normal anaerobic digested sludge indeed demonstrated the excellent ability in the methanogenic degradation of DMF. This artificially established microbial consortium only remains its activity temporarily for a few weeks, because those DMF-hydrolyzing

bacteria originating from activated sludge could not grow under the anaerobic condition. Therefore, they kept decaying all the time and the DMF hydrolysis gradually weakened. With new inoculation of fresh activated sludge into the UASB and AnMBR, both the removal efficiency of DMF and the methane production rate recovered immediately. However, the high efficiency and biogas production only remained for a while, and continued to drop to a low level. Frequently inoculating activated sludge into the UASB and AnMBR is not an optional solution.

7.1.3 Kinetics and functions of microorganisms

For archaeal community, it has been proved that the methanogenic degradation relies on both the methylotrophic and hydrogenotrophic methanogenesis. From samples of batch experiment, samples of UASB and AnMBR, a number of bacteria were selected as the candidates which have potential to hydrolyze DMF into DMA and formic acid. However, these bacteria were all enriched in aerobic activated sludge and gradually decayed after inoculated into the UASB or AnMBR under the anaerobic condition. The reason for the decay is that there bacteria are all aligned as denitrifying bacteria. Although they are facultative anaerobes, they need nitrate as the electron acceptor under the anaerobic condition. In our study, both UASB and AnMBR are methanogenic conditions where no nitrate has been dosed, and neither the substrate contains nitrate. These facultative anaerobes could not grow under the anaerobic condition. They could only utilized the $C\text{-}_1$ substrates such as MMA and formic acid, however, these $C\text{-}_1$ substrates would be further consumed by methanogens, resulting in a “niche overlap”. These facultative DMF-hydrolyzing bacteria were gradually outcompeted by methanogens under the condition without nitrate.

7.2 Improvements in future study

7.2.1 Co-digestion

As these DMF-hydrolyzing bacteria are probably unable to feed on DMF as the sole substrate, the introduction of other organic matter is likely to enhance the degradability of this co-system. The co-digestion strategy could be either the combination of DMF and other chemicals, or the combination of DMF with other common organic matter or biomass. Some previous studies succeeded in the co-digestion of DMF and antibiotics and obtained a stable operation, however, the mechanisms and functional microorganisms would be changed in the co-system. If we use co-digestion strategy in future study, the entire mechanism of DMF degradation and community structures of both archaea and bacteria would be all changed.

7.2.2 Nitrate-reducing anaerobic digestion

Because these DMF-hydrolyzing bacteria are denitrifying bacteria, the addition of nitrate (or nitrite) as an elector acceptor was considerable to improve the growth rate of these DMF-hydrolyzing bacteria by establishing a nitrate-reducing mineralization process under the anaerobic condition. However, a competition was also likely to extend to the organic carbon source between denitrification and methanogenesis, which would lower the methane production and suppress the process of methanogenesis. As such, the nitrate dosage should be strictly controlled.

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