Conductive materials stimulated direct interspecies electron transfer and application in anaerobic digestion

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**CONDUCTIVE MATERIALS STIMULATED DIRECT
INTERSPECIES ELECTRON TRANSFER AND
APPLICATION IN ANAEROBIC DIGESTION**

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SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
CM	Conductive materials
CNT	Carbon nanotube
COD	Chemical Oxygen Demand
DIET	Direct interspecies electron transfer
DOC	Dissolved organic carbon
DOM	Dissolved organic matters
EET	Extracellular electron transfer
EPS	Extracellular polymeric substances
FPC	Feed phenol concentration
GAC	Granular activate carbon
GC-MS	Gas Chromatography coupled to Mass
HMW PN	High molecular weight protein
HMW PS	High molecular polysaccharides
HPLC	High performance Liquid Chromatography
HS	Humic-like substances
LB EPS	Loosely bound extracellular polymeric
LMW acids	Low molecular weight acids
LMW neutral	Low molecular weight neutral
LMW PN	Low molecular weight protein
LMW PS	Low molecular weight polysaccharide
PBS	Phosphate-buffered saline
R	Specific methane production rate

<i>R_{max}</i>	Maximum methane production rate
SB EPS	Soluble extracellular polymeric substances
TB EPS	Tightly bound extracellular polymeric
TOC	Total organic carbon
TSS	Total suspended solid
VFA	Volatile fatty acids
VSS	Volatile suspended solids

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ABSTRACT

Methanation of complex organic substances needs cooperation between syntrophic microorganisms. During this process, electrons and protons or other reducing equivalents are transferred from bacteria to archaea by intermediate shuttles, such as hydrogen. However, this process cannot be completed unless intermediates are readily removed. The speed of intermediates consumption is related to electron transfer efficiency between functional communities. Thus, the strategy to facilitate intermediates consumption and interspecies electron transfer could play an important role in promoting organics degradation.

Direct interspecies electron transfer (DIET) is a newly discovered electron transfer process within anaerobic system, where conductive materials (CM) act as electron conduits between syntrophic partners. Theoretical calculation revealed that the electron transfer rate via DIET is substantially higher than that via interspecies molecular transfer. Therefore, DIET is beneficial to facilitate intermediates consuming rate, and remove inhibition factor such as H₂ accumulation.

This thesis focuses on the role and impact of CM in four application cases of anaerobic digestion (AD), i.e. thermophilic reactor start-up, toxic compounds (organic matter - phenol or inorganic matter - ammonia) degradation, and organic compounds transformation. The results exhibited CM could greatly promote methanogenic performance, enhance system stability, and improve organic compounds converting efficiency.

In thermophilic AD start-up study, methane generation rate increased more than two times resulting in 50% shortened start-up time needed in CM group compared with Control group. The less affected AD performance under high hydrogen partial pressure, greater conductivity and healthy sludge morphology suggested CM trigger DIET between syntrophic partners. Similarly, one-fold higher phenol degradation rate was observed after dosing CM in a phenol degradation system. In CM groups, the electron shuttles, i.e. protein and humic substances, were greatly enriched in

extracellular polymeric substances (EPS). In particular, around 2.3 to 20 folds higher protein was observed in CM group. As for organics transformation study, the addition of CM promoted 35% to 86% more methane generation. The study revealed the improved methane generation was attributed to the stimulated hydrolysis of organic matters and greater degradation of humic-like substances. Microbial community analysis showed electro-active strains, and bacteria that are capable of degrading complex organics were greatly enriched in CM groups.

However, negative effects of carbon-based CM on methanogenic performance were observed in high ammonia system. This phenomenon was evidenced by a strong negative correlation between specific methane production rate and carbon nanotube (CNT) concentration ($R_s = -0.972$, $p < 0.01$). Further investigation found that potassium transportation process of the anaerobes was disturbed at the presence of CNT, and intracellular energy level was reduced probably due to the microbial detoxification activity and decreased ATP production capability.

This thesis investigated the role of CM in alleviating the common obstacles of AD technology. The working principles were carefully investigated. The results indicated CM promoted organic degradation via promoting sludge conductivity, acting as electron conduit for interspecies electron transfer, enriching electron shuttles within EPS, and altering microbial community. However, the negative effects of CNT on high ammonia inhibited AD system also alerted that CM may disturb the energy transfer system and worsen inhibition on anaerobic microorganisms. This study provides a basis for future research on application of CM in AD system.

CHAPTER 1

Introduction

Compared with aerobic digestion, anaerobic digestion (AD) has clear superiority in economy due to low sludge production and high energy recovery instead of consumption. However, the slow digestion rate and instability greatly inhibit its application. In AD process, some microorganisms, typically methanogens, can only use limited simple substances and rely on their upstream species to degrade more complex organic compounds. Anaerobic syntrophic metabolism has evolved to adapt this situation. In natural environment, there are mainly four reaction steps within the anaerobic syntrophic process i.e. hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The energy transfer is fulfilled by interspecies electron transport, where intermediates (like hydrogen) act as electron carrier. The efficiency of electron transfer determines the anaerobic digestion rate and efficiency. A strategy to accelerate the interspecies electron transfer can play an important role in stimulating substances degradation, maintain system stability, and potentially promote AD application.

Growing evidences show electron transfer among syntrophic community does not exclusively rely on intermediates. Direct interspecies electron transfer (DIET) may widely exist in the natural environment as well. Reguera et al. (2005) reported the pili of *Geobacter sulfurreducens*, which are the predominant Fe(III) reducers in many environments, can transfer electrons from the cell to the surface of Fe(III) oxides directly (Reguera et al. 2005). Electron transfer through pili indicates possibilities for other cell-surface and cell–cell interactions. Recently, CM, i.e. activated carbon, magnetite nanoparticles, carbon cloth, carbon nanotube (CNT) are reported to be with the capability of serving as conduits for electron transfer,

enriching electro-active bacteria, and accelerating the methanogenesis process (Cruz Viggi et al. 2014, Liu et al. 2012a, Zhao et al. 2016b). Hence, there is a great potential of bioengineering DIET via applying CM as an alternative to pili (Rotaru et al. 2014b, Yang et al. 2015) or cytochromes to facilitate extracellular electron transfer. On the basis mentioned above, this thesis aimed to study the possibility to engineer DIET by investigating the role of CM in thermophilic anaerobic reactor start-up, toxic compounds degradation and organics transformation. The inorganic inhibitory compound - ammonia was also chosen to broaden the knowledge about the application of CM and DIET in AD system.

Compared with mesophilic condition, there is superiority for biological systems operated under thermophilic condition, such as higher reaction rates, higher hydrolysis efficiency of complex compounds and increased destruction of pathogenic organisms. However, as methanogens are sensitive to temperature change, the start-up of thermophilic AD reactors from mesophilic condition often lead to uncoordinated syntrophic process (De la Rubia et al. 2005, Lyberatos and Skiadas 1999, Shen et al. 2013). The interrupted AD systems are often characterized with accumulated volatile fatty acids (VFAs) and hydrogen, which, in return, will inhibit acidogenesis and acetogenesis as the metabolism are thermodynamically unfavorable under high hydrogen partial pressure or products quantity (Schmidt and Ahring 1995). Consequently, the whole AD process will cease and even result in system failure. DIET can be a solution to solve the instability based on the following reasons. Firstly, hydrogen is not involved in as well as inhibits DIET process, since electron can be delivered to syntrophic partner directly. Secondly, the electron transfer speed via DIET is 10^6 faster than interspecies hydrogen transfer (IHT) (Cruz Viggi et al. 2014), which allows much more rapid substrate conversion. Thirdly, DIET is energy efficient as intermediates generation and diffusion are unnecessary. The substrates and intermediates can be consumed much faster and more energy efficiently via DIET

pathway. Thus, the addition of CM into AD system can be a potential solution to accelerate and stabilize the theomorphic AD start-up process by removing VFA immediately.

Another potential application of CM is to promote the degradation of toxic compound, for example phenol. Li et al. (2013) suggested that phenols could be completely degraded with the cooperation of phenol-degrading bacteria and hydrogen-utilizing methanogen. This syntrophic phenomenon was also found in pure culture study, where *Syntrophorhabdus aromaticivorans* cooperated with hydrogenotrophic methanogens was capable of degrading phenol (Qiu et al. 2008). It was also found that the phenol degradation performed better in microbial fuel cells (Luo et al. 2009). Moreover, benzoate, the common intermediate in anaerobic metabolism of aromatic compounds, could be degraded via applying iron minerals as conduits (Zhuang et al. 2015). These studies support the possibility of stimulating phenol degradation via triggering DIET in AD system.

Currently, the biggest obstacles of AD application are slow digestion rate and low organics converting efficiency. As a faster syntrophic electron transfer pathway, DIET could be a solution to this problem. To date, some studies indicated CM could promote the degradation of complex substrate and increase methane generation (Yin et al. 2017, Zhao et al. 2017a). However, little study compared the role of CM in influencing the transformation efficiency of different organic fractions. In fact, the promoting efficiency varied with specific compounds. For example, studies indicated CM can greatly promote the degradation of propionate and butyrate (Zhao et al. 2016c), whereas it did not work in case of glucose (Zhao et al. 2017a). The knowledge of CM on specific compounds transformation is important for directing their further application.

It is known that the performance of AD is closely related to organic loading and the

solids concentration in the feed (Mata-Alvarez et al. 2000, Rincón et al. 2008). To improve the loading and solids content, many solids pretreatment methods have been developed, e.g. ultrasound-alkaline (Liu et al. 2008, Tyagi and Lo 2011), thermal hydrolysis process (THP) (Xue et al. 2015) etc. Most of pretreatment methods, THP in particular, would yield high ammonia concentration, typically ranging from 1000 mg-N/L to 4000 mg-N/L (Abu-Orf and Goss 2012, Oosterhuis et al. 2014). Such high ammonia concentration may subsequently pose adverse effect on the down-stream AD process. Moreover, anaerobic digestion of livestock and food waste often impeded by high ammonia due to rich nitrogen content. DIET was proven to help reduce the accumulation of intermediates and mitigate the inhibition from toxic organic compounds in the feed in AD system. If the above DIET mechanisms work universally, the inhibition from inorganic compounds should be also mitigated efficiently. In fact, it is a quite different case from former studies of focusing on promoting organic degradation, thus study is needed to support the hypothesis. However, little study reported how carbon-based CM would interact with the inhibition from ammonia. Since ammonia inhibition is the most common case lead to AD system failure, it is important to explore the interactive effect of ammonia and CM on AD system.

Based on the needs and potentials mentioned above, this thesis investigated the mechanisms of DIET in promoting thermophilic AD reactor start-up, phenol degradation, organic compounds transformation, as well as the interactive effect of ammonia and CM on AD system. The roles of CM on methanogenesis, organics and intermediates degradation, and alteration of microbial communities were studied. To further understand the response of microbe toward CM, microbial EPS were also investigated to explore microbial activity and their correlation with direct extracellular electron transfer.

Following this chapter is a comprehensive literature review of the historical and fundamentals of AD, AD application in wastewater treatment, AD application obstacles, the fundamentals of DIET and theoretically analyzing the potential of solving these problems by dosing CM. Detailed analytical methods are presented in Chapter 3. Chapter 4 to 6 examined the effects of CM in promoting thermophilic AD reactors start-up, phenol degradation and organics transformation. Chapter 7 explored the interactive effects of CM and ammonia inhibition on AD system. Finally, the outcomes of the thesis, conclusion discussion and recommendations for future study are presented in Chapter 8.

CHAPTER 2

Literature Review

2.1 Introduction

This literature review began with a brief of anaerobic digestion development, its fundamental mechanisms and application in wastewater treatment plant. The historical facts revealed it was the system instability and low transformation efficiency that greatly hindered the application of AD in industry. The inhibition mechanism and application obstacles were then explored in detail, with special attention focused on cases of thermophilic AD reactors start-up, toxic compound inhibition and degradation, and low organics degradation efficiency. In addition, a review of bioengineering DIET and its effects on AD was present. Two representative conductive materials, namely granular activated carbon and magnetite nanoparticles, were chosen as examples to explain the mechanism of stimulating DIET, their effects on AD performance, and current knowledge achieved in this area. The following chapters will address defined topics encompassed in this literature review.

2.2 Historical aspects of anaerobic digestion

The recognition of anaerobic biological process could date back to as early as 1776, when Barker (1956) firstly reported waste organic matters could convert into methane effectively. In the beginning of 1890s, the first biological treatment system was constructed and could produce clear effluent and inoffensive gases (McCarty 2001). Shortly afterwards, a simple and practical septic tank was launched, thereafter AD system has been widely known by public. However, the application of anaerobic wastewater treatment did not take off as little knowledge about its microbiology and biochemistry. In 1970s, the development of up flow anaerobic sludge bed (USAB) reactor first successfully applied in treating high-strength industrial wastewater, since

then the status of AD had changed dramatically (Lettinga 1995).

However, the application of anaerobic treatment was often neglected in industry in favor of aerobic biological method, which is easier to operate and obtains faster organic degradation rate (Arden and Lockett 1914). “Anaerobic wastewater treatment did not enjoy the popularity it truly deserves” said by McHugh et al. (2003). Compared with aerobic method, AD exhibited superiority in many aspects, such as low sludge production, more economical and energy saving, etc. In addition, the need for a more sustainable society drive the development of AD process, and it is estimated thousands of anaerobic wastewater treatment plants have been built all over the world (Tchobanoglous et al. 2003). Some wastewater treatment plants applied AD alone for treating high-strength industrial and domestic wastewater. As the most important and eco-friendly technology, the fundamentals of anaerobic processes and the application efficiency should be improved further.

2.3 Fundamentals of syntrophic anaerobic digestion

In methanogenic process, many microorganisms, typically methanogens, can only use a few simple substances as carbon source. Therefore, they rely on other microorganism to degrade complex organics into small compounds for metabolism, which is called syntrophic metabolism in anaerobic digestion (Stams and Plugge 2009). As shown in Fig. 2.1, the syntrophic process includes 4 stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Gujer and Zehnder 1983). Each of the stage obtains individual metabolic characteristic and is carried out by different microorganisms. Table 2.1 exhibits the representative genera of each stage.

Table 2.1 Summary of the representative genera within methanogenic process

Anaerobic Stages	Products	Representative genera	References
Hydrolysis	Mono-hydrocarbon, CO ₂	<i>Streptococcus</i> , <i>Enterobacterium</i> , <i>Staphylococcus</i> , <i>Peotococcus</i> , <i>Micrococcus</i> , <i>Clostridium</i>	Bryant (1979), Smith (1966), Zehnder (1988).
Acidogenesis	Acids, H ₂	<i>Pseudomonas</i> , <i>Bacillus</i> , <i>Clostridium</i> , <i>Micrococcus</i> , <i>Flavobacterium</i>	Ali Shah et al. (2014)
Acetogenesis	CH ₃ COOH	<i>Syntrophomonas</i> , <i>Syntrophobacter</i> , <i>Methanobacterium propionicum</i>	Schink (1997)
Methanogenesis	CH ₄	<i>Methanosaeta</i> , <i>Methanosarcina</i> , <i>Methanobacterium</i> , <i>Methanoculleus</i>	Lee (2018); (Luo and Angelidaki 2012)

Hydrolysis stage

Hydrolysis is the first step reaction in AD. In this stage, larger organic compounds break down into small and simple substances for further digestion. It is an extracellular reaction. A series of extracellular enzyme such as protease, lipases and cellulases take a role in hydrolyzing the macromolecules into smaller compounds. When large quantity of particulates or polymeric organic compounds exist, hydrolysis is often the rate-limiting step and this reaction is thermodynamically unfavorable at the accumulation of its products such as glucose, amino acid, as well as VFAs.

Figure 2.1 Schematic representation of the decomposition of organic compounds by AD (Gujer and Zehnder 1983).

Acidogenesis stage

The acidogenic bacteria can convert sugars, amino acids, fatty acids, and alcohols into VFAs, ketones, alcohols, carbon dioxide and hydrogen. Acidogenic bacterium are fast growing strains with doubling time of 30min or less. The acidogenesis was very sensitive to external environment, and the end products of this stage strongly depend on AD system stability. For example, at stable state, carbon source mainly go through acetate (70%), carbon dioxide and hydrogen. When AD is under unstable status, longer chain intermediates (e.g. C3-C7 fatty acids) will accumulate (Schink 1987). The following reactions takes glucose degradation as an example for

illustrating purpose (Mosey 1983).

There are three fermentative pathways and preferred one depends on the concentration of hydrogen (McCarty and Mosey 1991). At low hydrogen concentration, the acetate producing reaction is preferred, while the hydrogen consumption pathway will be stimulated at high hydrogen partial pressure. In addition, as competition exists between propionic and butyric bacteria, substrate concentration and pH also play an important role in selecting the glucose fermentation pathway. For instance, butyrate and hydrogen will be produced by butyric acid bacteria for adapting to low-substrate concentration and hydrogen partial pressure. In contrast, propionate will be produced by propionate-forming bacteria in the present of high substrate, hydrogen partial pressure and neutral pH (Zheng and Yu 2004).

Acetogenesis stage

The acetogenesis process functioned in oxidizing of VFAs and alcohols into acetate, hydrogen and carbon dioxide based on the reactions below (Mosey 1983). Thermodynamically, the acetogenic process can start only when hydrogen partial pressure is below 10^{-4} atm, as the reaction is energy demanded under standard condition.

Methanogenesis stage

Microorganisms capable of producing methane have been identified only from the

domain Archaea. Methanogens can only use limited types of substance namely hydrogen, acetate, formate, methanol, carbon dioxide, monoethylamine, dimethylamine, trimethylamine, methyl mercaptans. As methanogens are sensitive to external environment and they have relatively slower metabolism rate compared to the upstream microbial community, the degradation of acetate is commonly reported as the rate-limiting step of the overall methanogenic reaction. There are two pathways of methane production as following reactions (Petersen and Ahring 1991).

Acetolactic cleavage

Non-acetolactic oxidation

The first pathway is completed by acetolactic methanogenic archaea (Eq.2.7), which degrade acetate to CH₄ and CO₂ directly. Only members of the *Methanosarcinales* including two well-known genera - *Methanosarcina* and *Methanosaeta*, are reported with acetolactic capability (Fournier and Gogarten 2008, Hattori et al. 2000, Vanwonterghem et al. 2016). They are slow growing organisms with doubling time of 2-3 days and unaffected by high hydrogen partial pressure. It is estimated that around 70% of methane is produced through acetolactic cleavage pathway (Jeris and McCarty 1965). The second methanogenic pathway is via non-acetolactic oxidation (Eq.2.8-2.9). The co-metabolic pathway includes acetate-oxidizing bacteria and hydrogenotrophic methanogens, and sensitive to factors, such as substrate composition, temperature, organic loading rate and even reactor type.

2.4 Syntrophic electron transfer via hydrogen production and its correlation with system stability

The energy transfer within AD system is fulfilled by interspecies electron transfer.

During the process, intermediates, typically hydrogen, act as electron carrier between syntrophic partners. Briefly, during the fermentative process, electron is accepted by proton with the production of hydrogen. The hydrogen producing process is coupled with the transformation of redox mediator, such as NAD^+/NADH , FAD/FADH_2 , and $\text{Fd}_{(\text{ox})}/\text{Fd}_{(\text{red})}$ (Ferredoxins). However, as the redox point of H^+/H_2 is very low ($E^\circ = -414\text{mV}$), the free energy is thermodynamically unfavorable under standard condition (Stams and Plugge 2009). Thus, hydrogen scavenger, i.e. hydrogenotrophic methanogens, is needed to maintain a low hydrogen partial pressure for making the fermentative reaction thermodynamically possible.

As the process of hydrogen consumed by hydrogenotrophic methanogens often couples with $\text{F}_420/\text{F}_420\text{-H}_2$, the enzyme activity of F_420 is often adopted as an indicator to show the methanogens activity. Different from the fermentative process, hydrogen is the reactant in methanogenesis process, thus it must be maintained above certain level to make the reaction of methane generation thermodynamically possible. In addition, the hydrogen threshold varies among methanogens. The threshold for methanogens without cytochromes was larger than 10 Pa, but hydrogen partial pressure could be lower than 10 Pa for those obtained cytochromes (Thauer et al. 2008).

Therefore, both fermentative and methanogenic reactions are regulated by hydrogen partial pressure. Under high H_2 partial pressure, activity of syntrophic bacteria will be inhibited, while the hydrogen consumption process is stimulated. In the case of low H_2 partial pressure, H_2 producing process will be stimulated, but its consumption is inhibited (Harper and Pohland 1986) .

In fact, the stability of AD relies on the metabolism balance between acids and methane forming microorganisms. However, AD instability is commonly occurred as the syntrophic partners differ greatly in terms of nutritional needs, growth kinetics,

physiology, and sensitivity to environmental conditions. Among the anaerobes, methanogens are generally accepted as the most sensitive genera to the changed environment. Normally, the inhibition on methanogens lead to increase in hydrogen partial pressure, which will, in return, impact the upstream metabolism. Consequently, acids accumulation and less methane production will occur in the AD system. Thus, the strategy to strengthen the syntrophic electron transfer is the main way to solve the problem of anaerobic digestion instability.

2.5 Syntrophic electron transfer via direct interspecies electron transfer (DIET)

2.5.1 Definition of DIET

Growing evidences suggest that syntrophic electron transfer does not rely exclusively on the exchange of diffusible molecules and energy carriers such as hydrogen, rather microorganisms have the capability to exchange metabolic electrons directly (Morita et al. 2011b, Rotaru et al. 2014a, Zhuang et al. 2015).

Extracellular electron transfer (EET) is a latest found electron transfer pathway (Liu et al. 2012a, Morita et al. 2011b). Lovley et al. (1987) firstly reported that microorganism could deliver electron to extracellular space with Fe (III) as electron acceptor. Since then, more and more microorganisms were reported with the capability of completing respiratory of applying extracellular inorganic compounds as electron acceptor to complete oxidation of organic donor inside cell. There are mainly four types of EET mechanisms. (1) EET via cytochrome on the cell membrane. Summers et al. (2010) reported *Geobacter metallireducens* and *Geobacter sulfurreducens* could form electronic flocs with the existence of cytochrome on the surfaces. Latest study found that the cytochromes C of *G. sulfurreducens* decrease would lead to incomplete EET, which demonstrated the necessity of cytochromes within the EET process (Estevez-Canales et al. 2015). (2) EET via pili. By using Atomic force microscopy (AFM), Reguera et al. (2005) measured the conductivity of

pili, and suggested that interspecies electron transfer could achieve via applying pili as conduit, which was later confirmed by study of Malvankar et al. (2014). (3) EET via redox electron shuttle. During the process, the electron shuttles accept electrons from the metabolism of organic compounds and become reduction static, then return to oxidation static after transferring electron to its acceptor at extracellular space (Marsili et al. 2008, Newman and Kolter 2000, Rabaey et al. 2005). (4) EET via electro kinesis (Harris et al. 2010). Electron is preserved at the extracellular membrane temporally with formation of bio-condenser. Then, through “touch and go” process, electron is delivered to the adjacent microorganisms.

In the case of syntrophic methanogenesis, the reported EET is mainly the first two mechanisms i.e. EET via cytochrome (Shi et al. 2009, Summers et al. 2010) and pili (Malvankar and Lovley 2012, Reguera et al. 2005, Vargas et al. 2013). Direct contact between cells is needed to complete the electron transfer through the two extracellular accessories. Therefore, the two pathways are called direct interspecies electron transfer (DIET).

2.5.2 DIET superiority over interspecies hydrogen transfer

Free energy determines the occurrence of biological reactions; thus it is the primary parameter when comparing DIET and interspecies hydrogen transfer. Thermodynamically, DIET is superior over hydrogen mediated electron transfer, as metabolite generation and diffusion are not necessary when electrons are transferred via DIET. However, energy lose also exists when electrons conduct from the donor to acceptor. In addition, it is difficult to quantify the difference as the free energy of DIET is not as easy as interspecies hydrogen transfer that can be measured. The energy gained via interspecies hydrogen transfer can be calculated via thermodynamic equation easily. As for DIET, it is driven by voltage difference between syntrophic partners, which varies among partner microorganisms (Cheng

and Call 2016). Contrary to this point, Storck et al. (2016) reported that DIET also involved energy loss as activation energy required for redox cofactors (e.g. cytochromes) to process electrons transfer. Overall, it is still controversial on which types of electron transfer is more energy favorable.

Although undermined idea about energy transmission efficiency, consensus are achieved about the superiority of DIET over interspecies hydrogen transfer in other aspects which are summarized as follows.

- The speed of electron transfer via DIET is about 10^6 times faster than molecular mediated electron transfer based on theoretical calculation, thus faster substance consumption and biogas production speed can be obtained via DIET.
- The inhibition factor, typically hydrogen, does not involve in DIET process. Therefore, DIET might aid in resisting to system instability.
- It is likely that the uncoordinated syntrophic process within AD can be narrowed via DIET, because of the promoted interspecies electron transfer rate.
- Benefiting from the faster electron transfer rate via DIET, microorganism involved in DIET may gain greater metabolic speed as well as growth rate.

2.5.3 Engineering DIET

Significant research efforts of engineering DIET have been focused on adding non-biological CM (Barua and Dhar 2017). Recently, carbon-based materials (like granular activated carbon (GAC), carbon nanotube (CNT), carbon cloth) and iron-based materials (like magnetite particles and hematite) were reported with the capability of serving as conduits for the direct electron transfer between syntrophic partners (Cruz Viggi et al. 2014, Dang et al. 2016b, Lei et al. 2016, Zhao et al. 2016a). For instance, the supplement of GAC successfully established DIET in co-culture of *Methanosarcina Bakeri* and pilin-deficient *Geobacter Metallireducens* (Rotaru et al. 2014a). This is because CM can substitute microbial pili or cytochrome for fulfilling

direct electron transfer process in defined coculture (Liu et al. 2012a, Rotaru et al. 2014b, Yang et al. 2015). Thus, the addition of CM has a great potential to be a sustainable approach for engineering DIET. The following content of this section will take GAC/ magnetite nanoparticles as the representative of carbon/iron-based CM to illustrate their fundamental roles in stimulating DIET and the research progress in this area.

Granular Activated Carbon

Due to electrical conductivity and high surface area, GAC has been widely used as electrodes in microbial electrochemical systems. Deployment of GAC for biocathode has suggested that many electrogenic bacteria can accept electrons from GAC directly (Kalathil et al. 2012, Liu et al. 2014a). Liu et al. (2012a) first reported GAC had substantially reduced lag phase and enhanced methane production. Microscopic analysis showed that both methanogens and their upstream bacteria were tightly attached on the surface of GAC without forming any electrical aggregates via conductive pili (Liu et al. 2012a). This observation indicates that the formation of DIET-active microbial community onsite GAC could alleviate the role of outer membrane c-type cytochromes and conductive pili.

Currently, the application of GAC has been studied in both batch and continuous digesters fed with various organic substrate (Dang et al. 2016a, Lee et al. 2016, Liu et al. 2012a). It is evidenced from these study that stimulating DIET with GAC could greatly enhance methanogenesis rate. For example, GAC-amended anaerobic digester significantly increased the methane production rate by 1.8 times, and the GAC attached biomass exhibited 3.7 times higher specific methane production rate (Lee et al. 2016). However, few studies optimized the GAC loading and specific surface area that required for improving DIET kinetics. Hence, more studies were required to optimize the GAC application for efficiently stimulating DIET kinetics in

anaerobic digesters.

Magnetite nanoparticles

Magnetite particles are abundant in nature and can stimulate the extracellular electron transport at the presence of iron-reducing microorganisms (Kato et al. 2012a, Liu et al. 2014a). Kato et al. (2012a) first reported the stimulated methanogenic process due to DIET based syntrophy promoted by magnetite nanoparticles. Those studies suggested the mechanism of stimulating DIET by iron-based nanoparticles could be different from carbon-based CM (Baek et al. 2016, Liu et al. 2015a). The syntrophic microorganisms can attach to surfaces of relatively larger CM, such as GAC, biochar, etc. In comparison, magnetite nanoparticles attached to conductive pili and acted as an alternative to multiheme c-type cytochromes for DIET (Liu et al. 2015a). Microscopic analysis observed that magnetite nanoparticles amended digesters exhibited complex aggregate biomass due to extensive colonization (Baek et al. 2017). Therefore, it maybe not possible to promote distant DIET between syntrophic partners.

To date, many studies have shown that iron-based nanoparticles can greatly accelerate DIET and promote the methanogenesis (Jing et al. 2017, Yamada et al. 2015, Zhuang et al. 2015). However, most of these studies were conducted in batch mode as it could be engineering challenge to retain these nanoparticles during continuous operation.

2.6 Obstacles of anaerobic digestion (AD) application and feasibility to be alleviated by DIET

2.6.1 Anaerobic digestion application in industry

Currently, the objectives of AD applications are to increase organic mineralization, decrease the waste volume and reduce the operational cost. Traditionally, the application of AD benefits from its flexible design and capability to degrade various organic fractions. In full-scale application, the AD system can optimize its

configuration to suit various occasions with different style, size and organic compositions. Recently, the technology of AD has been regarded as great potential to support sustainable development in many countries. However, poor operational stability as well as low organic transformation efficiency still exist and limit its widespread commercialization.

The drawbacks of AD can be concluded to either decoupled or slow syntrophic metabolism, both of which are associated with interspecies electron transfer efficiency. Theoretical calculations revealed that DIET allows electrons to be transferred among syntrophic partners at speed which is substantially higher than those attainable via interspecies H_2 transfer. This would enhance the syntrophic electron greatly and improve the methanogenic rate tremendously. Therefore, enhancement of syntrophic electron transfer could be a potential strategy to alleviate the application problems of AD. The following content discussed the three main application obstacles of AD technology and feasibility to solve the problems via introducing DIET.

2.6.2 Thermophilic AD start-up

Anaerobic technology can be mainly divided into mesophilic and thermophilic digestion. Compared to mesophilic AD, thermophilic AD exhibits better methanogenic and organic matter removal efficiency (Ahring et al. 2002, Gavala et al. 2003, Palatsi et al. 2009). In addition, thermophilic digestion can achieve a faster digestion rate and better pathogens reduction effects (Zabranska et al. 2002).

The start-up of thermophilic AD reactor is a sensitive process. Its success depends on various factors such as the inoculum characterization, the digester design, the organic loading rate and the start-up strategy (Palatsi et al. 2009). Thermophilic inoculum often come from mesophilic sludge, which, however, contain little thermophilic strains (Kim et al. 2002b). Therefore, thermophilic AD start-up requires the transient

from mesophilic microorganism to thermophilic population, and the key to success rely on creating environment in favoring the growth of the initially minor thermophilic population (Kim et al. 2002b). Currently, three thermophilic start-up operational strategies have been reported to increase the temperature from mesophilic to thermophilic status, namely one step increase (Ahring et al. 2002, Bolzonella et al. 2003, Rimkus et al. 1982), gradual increase (Rimkus et al. 1982), and stepwise increase (Boušková et al. 2005, De la Rubia et al. 2005). The strategy of increasing temperature directly from mesophilic to thermophilic condition was reported as the optimal strategy with the shortest time needed to system stable and highest ratio of real thermophilic strains (Boušková et al. 2005). However, still more than 30 days was needed for stabilizing the system, and a high risk of start-up failure as the VFAs were much higher than the other two strategies (Palatsi et al. 2009). Therefore, a fast and more stable thermophilic reactor start-up strategy is urged for the application of thermophilic AD technology.

Generally, methanogenic activity is more susceptible to temperature change than acidogenic stage. Therefore, the sudden increase of temperature can lead to uncoordinated syntrophic process presenting with accumulation of VFAs and hydrogen (De la Rubia et al. 2005, Lyberatos and Skiadas 1999, Shen et al. 2013). On the other hand, the accumulated intermediates will, in return, inhibit acidogenesis and acetogenesis, which are thermodynamically unfavorable under high hydrogen partial pressure (Schmidt and Ahring 1995). Consequently, the whole AD process will stop, and even result in system failure. DIET might be a solution to solve the instability based on the following reasons. Firstly, hydrogen is not involved in DIET pathway, as electron can be delivered to syntrophic partner directly. Secondly, the faster interspecies electron transfer rate enables rapider organics conversion (Cruz Viggi et al. 2014). Thirdly, DIET is energy efficient, since intermediates generation and diffusion are unnecessary. The advantages of DIET function in removing

intermediates readily, and aid in decreasing inhibitors. Thus, it can be a potential solution to accelerate and stabilize the thermophilic AD reactors start-up process.

2.6.3 Toxic organics degradation

Lots of organic compounds are reported of being degraded by anaerobic microorganisms after acclimation, such as phenol and alkyl benzenes (Fang et al. 1995, Soto et al. 1991), alkanes (Mormile and Suflita 1996), esters (Hayward and Lau 1989), halogenated aliphatic compounds (Boucquey et al. 1995) etc. However, toxic chemicals can also pose inhibition on AD systems, especially for chemicals being lack of solubility that can be absorbed onto cell surface. As a result, the accumulated toxic compounds cause cell swelling and leakage, disrupt enzyme activity and ion gradients, and eventually result in cell lysis (Heipieper et al. 1994, Sikkema et al. 1994).

Microbial acclimation is critical to relieve the extent of inhibition and enhance toxicant degradation (Stuckey et al. 1980, Wu et al. 1993). Four mechanisms have been reported in improving the inhibitory tolerance, namely enrichment of functional organisms; introduction of degradation related enzyme; exhaustion of preferential carbon source before change to xenobiotic substrate; genetic engineering (Spain et al. 1980, Spain and Van Veld 1983, van der Meer 1994). Currently the most common and easier acclimation strategy is to increase the concentration of toxic compounds gradually. During the acclimation period, anaerobic microbial community could enhance both their toxic tolerance as well as degradation capability, via enriching functional strains that can resist or degrade toxic compounds (Stuckey et al. 1980, Wu et al. 1993). However, the acclimation process is a slow and unstable process. New strategies are required to narrow the acclimation period and improve AD system stabilization. The dosing of CM into anaerobic system would play a role in alleviating the toxic compounds degradation. For example, phenol is a typical toxic compound

in industry discharged effluent. Li et al. (2013) suggested that phenol could be completely degraded with the cooperation of phenol-degrading bacteria and hydrogen-utilizing methanogen (Li et al. 2013). This syntrophic phenomenon was also found in pure culture study, where the coculture of *Syntrophorhabdus aromaticivorans* and hydrogenotrophic methanogens was capable of phenol degradation (Qiu et al. 2008). Nevertheless, it was also found that phenol degradation performed better in microbial fuel cells (Luo et al. 2009). These studies suggested electron transfer of phenol degradation could be carried out via both interspecies hydrogen transfer and direct extracellular electron transfer. Figure 2.2 proposes the two pathways of anaerobic phenol degradation, and different CM are adopted to show how electron transfer can be carried out via various conduits.

It is noteworthy differing from thermophilic AD start-up case, the rate-limiting step is phenol oxidation rather than the interspecies electron transfer. Both electron shuttles and intermediates are the products during phenol oxidation process. DIET could play a role in promoting the consumption of intermediates, thus facilitate the whole co-metabolism process indirectly.

Figure 2.2 Proposed synergy between phenol-degrading bacterial and methanogen (A) via hydrogen transfer, (B) electrons transfer and (C) electrons transfer via CM

2.6.4 Low organics converting efficiency

As an economical and environmental friendly technology, the application of AD has experienced an annual growth rate of 25% in treating solid wastes, in particular waste

active sludge (Buffiere et al. 2008). Traditionally, the hauling cost of extra biomass could constitute up to 50% of operational fee in wastewater treatment plant (Appels et al. 2010). After AD treatment, the volume of sludge can reduce by 30% - 40%, thus greatly save the transportation cost (Appels et al. 2008). Other beneficial features of AD include the improvement of sludge dewaterability, the stabilization of sludge, and the potential for reducing and inactivating pathogens (Appels et al. 2008). However, AD technology is known with the drawbacks of long retention time and low converting efficiency for degrading solid organic matters, attributing to the slow hydrolytic rate (Appels et al. 2011). Therefore, a series of pre-treatments have been developed to improve the solid wastes solubilization and hydrolytic potential. These pretreatment technologies include mechanical, thermal, chemical and biological methods. They are used independently or combined and can often achieve great improvement in anaerobic performance. For instance, a thermal pretreatment could lead to 60% increase in methane generation, specifically increasing from 115 mL/g COD feed to 186 mL/g COD feed (Haug et al. 1978). An ultrasound pretreatment (64s at 31 kHz) could reduce the sludge retention time from 22 days to 8 days achieving the same volatile solids removal efficiency (Tiehm et al. 1997).

Although pretreatment can greatly improve the methanogenic efficiency, recent studies reported the effluent from AD tanks remained a great quantity of recalcitrant compounds (Lu et al. 2018a, Lu et al. 2018b). These recalcitrant compounds include polar metabolites (like benzene, dipeptide) as well as non-polar lipids (like alkenes, flavonoids, long chain fatty acids, and diacylglycerols). The effluent may bring potential environmental problem if discharged directly. However, till now, little studies have been conducted on how to further enhance the converting efficiency and remove the remained recalcitrant compounds within the AD tank.

Many studies reported iron-based materials can greatly stimulate the hydrolytic

reaction (John et al. 2017, Mu and Chen 2011, Saito et al. 2017, Suanon et al. 2016, Zhao et al. 2017a). Metaproteomic analysis revealed the existence of iron-based CM increase enrich the abundance of hydrolytic bacteria and enzymes such as hydrolases, peptidases, proteases and endoglucanase (Yin et al. 2018). In addition, the materials could greatly enrich iron reducing bacteria, which was reported capable of degrading complex compounds (Marozava et al. 2018, Niedzwiecka et al. 2017, Silva E et al. 2018, Weber et al. 2006, Yang et al. 2016). Therefore, it is highly possible the existence of iron-based materials can enhance organics efficiency via promoting hydrolysis as well as complex compounds degradation for further improving the quality of effluent from anaerobic tank.

Furthermore, studies reported CM could promote anaerobic digestion of various carbon source, such as propionate (Cruz Viggi et al. 2014), alcohol (Dubé and Guiot 2017), tryptone-based high-strength wastewater (Yin et al. 2017), and waste active sludge (Zhao et al. 2017a). However, no study compared the stimulating efficiency of CM on different types of organic compounds. In fact, the enhancement efficiency varied with specific compounds. Our previous studies indicated CM can greatly promote the degradation of phenol, whereas it did not work in the case of glucose under stable condition. In addition, many researchers also reported that CM could enable greater methane production from complex carbon source (Feng et al. 2015, Liu et al. 2015b, Zhao et al. 2017a). For example, Zhao et al. (2017a) reported the addition of magnetite enable 6-fold higher methane production than Control group under the same organic loading rate. However, it is difficult to determine whether it is because of the faster metabolism rate or greater degradation degree of certain organics or both happened. This is because most studies limited to specific configuration, where supernatant discharge happened. These types of configurations could not provide sufficient time for the degradation of recalcitrant compounds. Therefore, it is important to study the effects of CM on the degradation of complex

carbon source in reactors, which allow enough degradation time for both easily and recalcitrant compounds for better understanding the promoting mechanism and application of CM in industry.

2.6.5 Inhibition of ammonia on CM supplemented AD system

Ammonia is the end-products of anaerobic digestion of protein, nucleic acids and urea. Unlike the importance of ammonia for bacteria growth at low concentration, high concentration of ammonia may cause a severe inhibition on the microbial activities. Free ammonia, rather than total ammonia, is the main cause of inhibition of methanogenic microflora due to the high permeability to bacterial membrane (Muller et al. 2006) . This is because ammonia molecule can diffuse passively into bacterial cell, causing proton potassium deficiency or proton imbalance (Gallert et al. 1998). Briefly, a fraction of ammonia molecules that enter into cells can cause a pH change due to its conversion into ammonia, while absorbing protons during the process. The cells must then invest energy in proton balancing by activating a potassium pump to maintain the intracellular pH, thus increasing maintenance energy requirements and potentially causing inhibition of specific enzyme reactions (Wittmann et al. 1995).

It is known that the performance of AD is closely related to organic loading and the solids concentration in the feed (Mata-Alvarez et al. 2000, Rincón et al. 2008). To improve the loading and solids content, many solids pretreatment methods have been developed, e.g. ultrasound-alkaline (Liu et al. 2008, Tyagi and Lo 2011), thermal hydrolysis process (THP) (Xue et al. 2015) etc. Most of pretreatment methods, THP in particular, would yield high ammonia concentration, typically ranging from 1000 mg-N/L to 4000 mg-N/L (Abu-Orf and Goss 2012, Oosterhuis et al. 2014). Such high ammonia concentration may subsequently pose adverse effect on the down-stream AD process. Moreover, anaerobic digestion of livestock and food waste often

impeded by high ammonia due to high nitrogen content.

In addition, different factors are involved in inorganic inhibition (high ammonia) and organics degradation process, which are reported could be facilitated by adding CM. Therefore, it is vital to understand if CM again can mitigate the negative impact caused from ammonia inhibition. To date, it is not clear how carbon-based CM would interact with ammonia on AD performance.

2.7 Summary

The main conclusions from the literature review are as follows:

Anaerobic degradation has attracted increasing attention during the last decades, attributing to its advantages of low sludge production, energy saving, flexible scale, economics etc. Anaerobic digestion is mainly composed of four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Methanation of complex organic substances needs cooperation between syntrophic microorganisms. During this process, electrons and protons or other reducing equivalents are transferred from bacteria to archaea by intermediate electron shuttles such as hydrogen. However, this degradation could not be completed unless intermediates are readily removed. The rate of intermediates consumption is related to electron transfer efficiency between functional communities. Thus, the strategy to facilitate intermediates consumption and interspecies electron transfer could play an important role in promoting organics degradation.

DIET is a newly discovered electron transfer process within anaerobic system, where CM act as electron conduits between syntrophic partners. Theoretical calculation revealed that electron transfer rate via DIET is substantially higher than that via interspecies molecular transfer. Therefore, DIET exhibits great potential in facilitating intermediates consuming rate, and removing inhibition factor.

In response of the energy saving policy, AD applications experienced significant

development within recent years. However, its application has been greatly hindered by the drawbacks of system instability, slow degradation rate and low organic compounds converting efficiency, especially in the cases of thermophilic AD reactors start-up and toxic compounds degradation. Studies found CM could introduce DIET, which enable faster consumption of metabolism intermediates. Base on the superiority of DIET, there is a great potential of adding CM into anaerobic system to balance the syntrophic metabolism, thus solving the obstacles met in AD application. Anaerobic digestion promoted by CM varies with the carbon sources. It is significant to figure out which types of organic compounds are stimulated by CM for bettering understanding its role in AD system. The effects of CM on the refractory compounds were often ignored by most studies, as most of the experimental configures did not allow enough time for their degradation. The study on the transformation of organic compounds, especially refractory compounds, would provide fundamental knowledge about the role of CM on anaerobic digestion.

Lastly, most studies were focus on exploring the effects of CM on promoting the degradation of organic compounds. However, little studies investigated its role in case of inorganic inhibition. Ammonia inhibition is commonly occurred in AD process, and even lead to system failure. Before widespread application of CM in AD process, it is important to figure out the potential interaction between CM and ammonia in anaerobic system.

2.8 Research objectives

Based on the needs and potentials mentioned above, this thesis focuses on investigating the application of three representative CM, i.e. GAC, CNT and magnetite nanoparticles, in solving application obstacles of AD technology. The mechanism of CM triggering DIET and how CM effect AD performance will be explored in specific case studies (i.e. thermophilic AD system start-up, phenol

degradation, organic compounds transformation in AD system, and interactive effects of ammonia and CM on AD system). The direct effects of CM would be confirmed primarily through monitoring the rates of methanogenesis, compounds degradation and intermediates accumulation. The response of AD performance to high hydrogen partial, sludge conductivity, and microbial community would be investigated to explore the existence of DIET and their correlation with methanogenic performance. CM acting as electric currents could be presented visually via SEM and TEM. Bacterial responses to CM will be explored by analyzing EPS fractions from different EPS layers.

The specific objectives are listed as follows:

- To investigate the role of GAC and CNT on alleviating AD application obstacles, i.e. thermophilic AD reactors start-up instability, toxicity organics degradation difficulty, and slow organics converting efficiency.
- To study and compare the role of magnetite nanoparticles and iron ions on the transformation of different organic compounds
- To assess the interaction of CNT and inorganic inhibitory factors on AD performance.
- To explore the role of CM (i.e. GAC, CNT and magnetite nanoparticles) in bridging electron transfer, as well as the specific impact on each AD stage (i.e. hydrolysis, acidogenesis, acetogenesis, methanogenesis).
- To determine the effects of GAC, CNT and magnetite nanoparticles on microbial community, especially the exoelectrogens.
- To compare the effects of GAC, CNT and magnetite nanoparticles on AD performance and possible metabolic pathways.
- To study the effects of GAC, CNT and magnetite nanoparticles on EPS fractions and explore the mechanism behind the change of each EPS fraction.

This study thoroughly investigated the effects of three CM (i.e. GAC, CNT and magnetite nanoparticles) on the performance of anaerobic digestion from macro to micro level. Firstly, it studied the effects of CM on basic parameters of AD performance, i.e. methane generation, intermediates accumulation and organic compounds degradation. The results provided a possibility for the achievement of stable operating thermophilic anaerobic systems, faster and stable removal rate of phenols and greater organics converting efficiency. Further study on the interactive effects of carbon-nanotube and ammonia on AD performance revealed the drawbacks of applying CM in AD system. Secondly, the effects of CM on anaerobic digestion were studied from the molecular level by tracking the transformation of individual compounds in AD system, which provide the direct information about the role of CM in anaerobic digestion from molecular level. Thirdly, the secretion of EPS influenced by carbon-nanotube and magnetite nanoparticle, and their effects on DIET were explored, which provide a new perspective of understanding the role of CM on anaerobic system. Lastly, microbial community analysis provided basic information on the effects of CM on anaerobic digestion. This study not only revealed the role of CM on methanogenic system, provided basis for removing recalcitrant compounds, but also potentially promoted the application of anaerobic biotechnology.

CHAPTER 3

Materials and methods

3.1 Synthesis and pretreatment of CM

The single-walled CNT was purchased from Huangqiu Graphene Technology Co. Ltd. (Jiangsu, China) with purity >90%, outside diameter 1-2 nm, inside diameter 0.8-1.6 nm, length 5-30 μm , surface area 380 m^2/g , and electric conductivity > 100 $\mu\text{S}/\text{cm}$. The pretreatment method of single-walled CNT followed the method described by Li et al. (2015). Briefly, single-walled CNT was suspended in distilled water, followed by ultrasonic treatment for 30 min to produce a homogeneous mixture before added into anaerobic reactors.

GAC was purchased from Sigma-Aldrich with bulk density 0.44-0.49 g/mL , specific surface 500-1500 m^2/g , and diameter 1.22-1.43 mm.

Magnetite nanoparticles were synthesized according to Kang et al. (1996). The reaction steps for synthesis Fe_3O_4 nanoparticles process are as follows:

Briefly, 5.2 g of FeCl_3 and 2.0 g of FeCl_2 were dissolved into 25.85 mL acidic aqueous solution (0.85 mL of 12.1 N HCl and 25 mL of deoxygenated water). Then, the solution was drop-wisely added into 250 mL of 1.5 M NaOH solution. The instant black precipitation would be observed. A magnet was placed near the black precipitate to check the paramagnetism. X-ray diffraction (XRD) and transmission electron microscope (TEM) were employed to confirm the product as magnetite nanoparticles.

3.2 Automatic methane detection

The methane production profile was recorded by Automatic Methane Potential Test System (AMPTS) II automatically. The system was composed of 3 units. Anaerobic

inoculums were incubated in unit A. Biogas produced will pass through unit B, where CO₂ will be absorbed by sodium hydroxide solution (3 mol/L). The volume of CH₄ released from unit B will be measured by a wet gas flow measuring device, which is based on liquid displacement and buoyancy. The resolution was 10mL.

3.3 Anaerobic seed sludge and trace element compositions

The seed sludge for anaerobic batch reactors was collected from an anaerobic reactor in a local wastewater treatment plant.

The composition of the trace element was as follows: NH₄HCO₃, 410 mg/L; K₂HPO₄·3H₂O, 210 mg/L; MgCl₂·6H₂O, 100 mg/L; AlCl₃·6H₂O, 4.5 mg/L; CaCl₂·2H₂O, 50 mg/L; NaCl, 10 mg/L; MnCl₂·4H₂O, 5 mg/L; FeCl₂·4H₂O, 39 mg/L; (NH₄)₆Mo₇O₂₄·4H₂O, 5 mg/L; CuSO₄·5H₂O, 5 mg/L; NiCl₂·6H₂O, 5 mg/L; ZnCl₂, 5 mg/L; CoCl₂·6H₂O, 5 mg/L; H₃BO₃, 8 mg/L.

3.4 General analysis

3.4.1 pH analysis

pH was measured by pH meter (Mettler- Toledo Seven Compact™), and the obtained values were accurate to within ± 0.05.

3.4.2 Volatile fatty acids (VFAs)

The concentration of VFAs was analyzed by using GC (Agilent, USA), equipped with DB-FFAP column (Phenomenex, USA) and a flame ionization detector. The filtered sample (0.9 mL) was acidified with 0.1 mL formic acid (10%, V/V) before analysis. The column operational parameters were 80 °C for 1min, increased to 120 °C with 20 °C/min, further to 205 °C at 10 °C/min, then hold for 2 min. The detector and injector temperature were 260 °C.

3.4.3 Biogas composition

The composition of biogas was analyzed by using a Shimadzu GC-2010plus equipped

with a thermal conductivity detector. Gas fractions were separated by a select permanent gases/CO₂ column. Helium was used as the carrier gas. The coefficient of variance (COV) for 5 samples was within $\pm 3\%$.

3.4.4 Total organic carbon (TOC)

The dissolved organic compound was characterized by total organic carbon (TOC). Samples were filtered through 0.45 μ m filter, then 1 mL sample were taken and diluted to concentration within 1 to 100ppm. The prepared samples were then analyzed by a TOC analyzer (Shimadzu, Japan).

3.4.5 Total and volatile suspended solids

The total and volatile suspended solids (TSS/VSS) were measured according to the Standard Methods for the Examination of Water and Wastewater (Federation and Association 2005). For TSS/VSS analysis, 2 mL of sludge was filtered, then the intercepted sludge was added to a pre-weighted foil tray and put into 105 °C oven for drying till the weight was stable. After cooling down in a desiccator, the sample weight was recorded for TSS measurement. The samples were then placed into a 550 °C for 1 hours followed by cooling in a desiccator. The reduction in samples mass was recorded as VSS. All samples were measured in triplicates, and the SD was within $\pm 5\%$.

3.5 System conductivity measurement

Three-probe electrical conductance measurement was conducted to analyze the sludge conductivity according to the method described by (Zhao et al. 2016c). Briefly, two gold electrodes (height 2.60 cm; width 1.31cm; depth 0.1mm) were separated by 0.5 mm nonconductive gap with glass as a base support. Sludge from all batch reactors was washed with 0.1 M NaCl and then placed on the non-conductive gap for conductivity test. A voltage of -0.3 - 0.3 V (against standard hydrogen electrode)

generated by an electrochemical workstation was applied on sludge in the ramp step of 0.025 V. The electric current gained from each applied voltage was used to establish current-voltage curve. The following equation was used to calculate the conductivity.

$$\sigma = L/(R * S) \quad \text{Eq. 3.2}$$

Where σ is conductivity (S/m); L is the width of the gap (m); R is reciprocal of the slope of the current-voltage curve (Ω); S is the cross-sectional area (m²) of the gap.

3.6 SEM samples preparation and analysis

Samples were fixed with formaldehyde (50%, V/V) and incubated for 6 h. Dehydrate with a graded ethanol series 25%, 50%, 75%, 95% of 1 × 5 minutes, followed with 100% anhydrous ethanol* 3 × 10 minutes. Critical point drying was used for dehydration samples. Subsequently, specimens were coated with platinum and glued to a metallic base, which was then used for analysis by using field- emission SEM system (NanoSEM 230, FEI, USA).

3.7 TEM samples preparation

The fixation method for TEM samples was the same as SEM samples. After that, 10uL sample was dropped on carbon coated TEM grids. Then, the samples on grid were taken for air drying, which were then used for TEM (TEOL, Tokyo, Japan) analysis.

3.8 ATP extraction and quantification

ATP quantification was conducted by using ATP Test Kit (QuenchGone21waster water test Kit-50, Luminultra). The analysis of ATP is based on using the firefly luciferase enzyme to catalyze the transformation of luciferin into oxyluciferin (Montanez et al. 2016), during which luminescence can be produced and further quantified by a microplate reader (model Infinite 200 PRO, TECAN, Switzerland) as relative light units immediately.

3.9 Membrane integrity analysis

Hydrophobic crystal violet was known for its weak penetration of outer membrane, but it can pass through the impaired membrane easily. Thus, it was applied for detecting bacterial or membrane integrity. A crystal violet assay method was modified to detect membrane damage (Vaara and Vaara 1981). Briefly, sludge sample was washed twice in deoxygenated Phosphate-buffered saline (PBS). The solids were then resuspended in crystal violet solution ($OD_{590} = 0.549$). After incubation at 37 °C for 10 mins, suspension was centrifuged at 13400 * g for 10 mins, and the OD of the supernatant was measured at the wavelength of 590 nm by using UV-vis spectrometer (Shimadzu UV 2450).

The absorption capability of anaerobic microorganisms was defined as following formula.

$$X_{Abs} = \left(1 - \frac{OD\ value}{0.549}\right) * 100\% \quad \text{Eq. 3.3}$$

Where X is the membrane integrity index, OD_{590} is the sample adsorption value at UV wavelength of 590nm.

3.10 Potassium analysis

Sludge samples for extracellular K^+ analysis was first centrifuged at 4000 g for 10 mins, and then the supernatant was collected and filtered through 0.45 um filter. Potassium concentration in the filtrate was measured by using microwave plasma atomic emission spectrometry (MP-AES) (Agilent model 4200). The coefficient of variance for ten identical samples was within $\pm 1\%$ with 0.01mg/L detection limit.

3.11 EPS extraction and analysis

A modified heat extraction method was applied to extract soluble extracellular polymeric substances (SB-EPS), loosely bound extracellular polymeric substances (LB-EPS), and tightly bound extracellular polymeric substances (TB-EPS) (Zhang

et al. 2016b). Briefly, sludge samples were centrifuged at 3000 gravitational acceleration (g) for 10min, and the supernatant was collected as SB-EPS. The sludge pellet was then re-suspended to its original volume by using 0.05% NaCl, which should be pre heated to 70 °C. Then the sample was vortexed for 1min, followed by centrifugation at 3000g for 10min. The supernatant was collected as LB EPS. The residual sludge pellet was resuspended in 0.05% NaCl solution to its original volume. The suspension was then put at 60 °C water bath for 30min. The sludge mixture was then centrifuged at 3000g for 15mins, and the supernatant was collected as TB-EPS.

3.12 Organic compounds analyzed by using Liquid chromatography-organic carbon-organic nitrogen detection (LC-OCD-OND) system

For analysis of the detailed compositions of EPS and dissolved organic carbon, the (LC-OCD-OND) system, equipped with three online detectors, namely organic carbon detector, UV detector, and organic nitrogen detector, was used by continuous analyze the relative signal response of organic carbon, UV and organic nitrogen. The identified organic fractions were mainly divided into two parts, i.e. hydrophobic and hydrophilic dissolved organic carbon (DOC). The hydrophilic DOC was subdivided into biopolymers, high molecular weight protein-like substances (including high molecular weight (HMW) protein, and carbohydrate), HS, building blocks, low molecular weight (LMW) neutrals, and LMW acids. The concentration of these fractions was quantified by a software (ChromCALC, DOC-LABOR, Karlsruhe, Germany), and the unit was mg equivalent carbon per liter (mg-C/L).

Total polymeric substance was measured with phenol-sulphuric acid method (DuBois et al. 1956). Total protein was determined with the modified Lowry method (Frolund et al. 1995). The concentration of LMW protein/carbohydrate was calculated by subtracting HMW protein/carbohydrate from total protein/carbohydrate. To unify the units, 1 g of polysaccharide or protein equals 0.444 and 0.497 g of equivalent carbon,

based on chemical formulae of polysaccharide $(C_6H_{10}O_5)_n$ and protein $C_{52.5}H_{6.65}N_{16}O_{21.5}S_2$.

3.13 DNA extraction and microbial community analysis

DNA for microbial community analysis was extracted using an automated DNA extraction kit (MP Biomedicals, Singapore) as per the manufacturer's protocol. Nano-Drop ND-1000 was used to check the purity of extracted DNA, and the DNA concentration was observed in the range of 50-200 ng/ μ L. The A260/280 ratios were approximately 1.8. Primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') were used for amplifying V4 regions of both bacteria and archaea 16S rDNA genes.

The PCR reactions and sequencing were conducted by Macrogen Inc. (Korea). The first-round PCR reactions were carried out in 50 μ L of PCR mixture. The condition for first-round PCR were as follows: 94 °C for 3 min, 5 cycles at 94 °C for 30 s, 45 °C for 20 s, 65 °C for 30 s, followed by 20 cycles at 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. Then, 20 ng DNA products from the first-round PCR was taken out for the second-round PCR reactions. The conditions of the second-round PCR were 95 °C for 30 s, 5 cycles at 95 °C for 15 s, 55 °C for 15 s, 72 °C for 5 min and 30 s.

The PCR products were then purified using a SanPrep DNA gel Extraction Kit, and then quantified using a Qubit2.0 DNA Assay Kit, followed by sequencing on the Miseq sequencing platforms (Illumina, USA). After removing the low-quality sequences, adapter primer, barcodes, the treated sequencings were imputed into microbial ecology community software program Mothur for data analysis. Operational taxonomic units (OTUs) were defined by clustering at a 0.03 distance level (97% similarity). Final OTUs were taxonomically classified using BLASTN against a curated database derived from Green Genes, RDPII and NCBI

(www.ncbi.nlm.nih.gov, <http://rdp.cme.msu.edu>). Based on OTUs, the Chao1 richness estimator, the abundance-based coverage estimator (ACE), the coverage percentage and the Shannon diversity indices were calculated to show the structure and diversity of microbial community.

3.14 Statistical analysis of methane production

To determine the performance of AD for biogas production, the cumulative methane production of each batch reactors was fitted to a modified Gompertz equation. According to CH₄ accumulation data, the following model was used to estimate the kinetic constants of CH₄ producing process within each feeding cycle (Poirier et al. 2016, Syaichurrozi and Sumardiono 2013).

$$M(t) = P \times e \left\{ -e \left[\frac{R_{max} \times e}{P} \times (\lambda - t) + 1 \right] \right\} \quad \text{Eq. 3.4}$$

Where $M(t)$ is cumulative methane production (mL) at time t (h); P is the maximum CH₄ yield (mL); R_{max} is the maximum CH₄ production rate (mL/hour); e is exponential constant; λ is the lag time (h).

3.15 Other statistical analysis

Statistical analysis of difference between various parameters was performed with the Student's t-test by using SPSS version 19.0. Spearman ranking correlation was adopted to analyze the correlation between different factors. p value < 0.05 was considered as statistically significant.

CHAPTER 4

The role of conductive materials in the start-up period of thermophilic anaerobic system

4.1 Introduction

Anaerobic digestion is normally carried out under either mesophilic (30-38°C) or thermophilic (49-57°C) conditions (Song et al. 2004). In comparison with mesophilic AD, thermophilic AD has the advantages of operation at high organic loading rate (OLR) and low sludge retention time (SRT), enhanced hydrolysis of complex organic materials, higher biogas production rate, reduction in foaming, and greater reduction of pathogens (Ahring 1994, Kim et al. 2002a). Recently, thermophilic AD was reported to be more efficient in removing organic micropollutants, which may cause substantial environmental and human health risks upon accumulation (Gonzalez-Gil et al. 2016). However, the broad application of thermophilic AD is often limited by its long and unstable start-up phase, where high VFAs are usually accumulated in the system and eventually lead to system failure (De la Rubia et al. 2005).

Two primary reasons cause such unstable phenomenon. Firstly, limited thermophiles are present in mesophilic seeding sludge. The strategy of adapting the mesophilic system to high temperature was often used to obtain thermophilic AD system (De la Rubia et al. 2013). However, thermophilic and mesophilic AD reactors are populated with entirely different microorganisms. According to Chen (1983), only 10% thermophiles present in mesophilic sludge. Therefore, the temperature change from mesophilic to thermophilic condition may result in a substantial deactivation of microorganisms and significant population shift, thus leading to a long acclimation time (Boušková et al. 2005). Another effect is due to the kinetic uncoupling within the biological reaction steps. A robust AD system requires the balanced reactions

among multi-step processes, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis. Methanogens are considered more sensitive than other anaerobes. For example, the sudden increase in temperature will bring out great inhibition on methanogenesis. (De la Rubia et al. 2013, Lyberatos and Skiadas 1999, Shen et al. 2013). On the other hand, the increase in temperature can improve hydrolysis, and it will not impose significant impact on the fermentation, thus aggravate the accumulation of intermediates (Alkarimiah et al. 2011, Schmidt and Ahring 1995, Song et al. 2004). In fact, syntrophy is crucial for methanation of complex organic substance (Qiu et al. 2008), where electron and proton or other reducing equivalents are transferred from one organism to another by intermediate shuttles such as hydrogen and formic acid. However, anaerobic oxidation of organic compounds is thermodynamically unfavorable, unless the intermediates, mainly hydrogen, are consumed and remain at low concentration (Schmidt and Ahring 1995). Based on these facts, the strategy of accelerating the substrates or intermediates consumption could result in promoting the growth of thermophiles and their co-metabolisms, which, in turn, plays a crucial role in alleviating the temperature shock during the thermophilic AD start-up period.

Growing evidence shows electron transfer among syntrophic community does not exclusively rely on intermediates, and DIET may widely exist in the natural environment as well (Dubé and Guiot 2015). It is highly possible that DIET could be a solution to solve the instability caused by the temperature shock based on the following reasons. Firstly, in DIET process, hydrogen is not involved, and hydrogen does not interact and impose inhibition on the DIET metabolism process. Hence, the microbial community can have an alternative way for electron transfer. Secondly, the electron transfer speed via DIET is 10^6 faster than interspecies hydrogen transfer (Cruz Viggi et al. 2014), which allows much more rapid substrate conversion. The above-discussed advantages of DIET indicate that substrates and intermediates could

be consumed via a faster and energy efficient way. Thus, it can be an effective solution to accelerate and stabilize the thermophilic AD start-up process.

Nevertheless, the key challenge is how to create a DIET environment in the anaerobic system. Some anaerobic microorganisms can release electrons from oxidase in the respiratory chain to extracellular solid surface, which enables their unique cell-surface electron process. The introduction of conductive material as conduit can substitute microbial pili or cytochrome for electron transfer. Recently, CM, i.e. activated carbon, magnetite particles, carbon cloth, are reported to be capable of serving as conduit for electron transfer, enriching electro-active bacteria, and accelerating the methanogenesis process (Cruz Viggi et al. 2014, Dang et al. 2016b, Lei et al. 2016, Zhao et al. 2016a). Hence, there is a great potential to use the CM in the anaerobic system to establish the DIET metabolism pathway (Rotaru et al. 2014c, Yang et al. 2015).

In order to shorten the start-up phase of thermophilic anaerobic system and mitigate inhibition from the intermediates, this study dosed CM - granular activated carbon (GAC) or carbon nanotube (CNT) into anaerobic reactors under mesophilic conditions and elevated the temperature to thermophilic temperature. The system performance and microbial community dynamics were closely monitored. The feasibility of creating DIET, and its effects in enhancing the microbial co-metabolisms were also studied. To reveal the robustness of established conductive systems against other operating condition variations, the effects from one of the important intermediates, H_2 , on the system performance were also studied.

4.2 Operation details

4.2.1 Thermophilic anaerobic reactors set-up

The seed sludge for anaerobic batch reactors was collected from a lab-scale mesophilic (35 °C) continuous stirred tank reactor (CSTR) fed with glucose as carbon

source. The volatile suspended solids (VSS) concentration of CSTR was 6.83 g/L with an SRT of 30 days.

Three batch experiment groups, namely CNT group, magnetite nanoparticles supplemented group and Control group, were setup in triplicates each. All batch experiments were conducted using AMPTS II system (Bioprocess Control Company) to measure the methane production. Each batch reactor had a working volume of 360 mL with final VSS concentration of 2.89 ± 0.67 g/L. CNT (1 g/L) or GAC (10 g/L) was added into corresponding batch reactors, then purged with nitrogen for 10 min to remove dissolved gases. All batch reactors were operated at 35 °C for 20 days with semi-continuous stirring (10 s on, and 90 s off) and a stirring speed of 80 rpm. From Day 21, the operating temperature increased to 55 °C for all the batch reactors. In each cycle, four milliliter feed (100 g/L glucose and 10 g/L NaHCO₃) was added into the reactor resulting in an initial glucose concentration of 1100 mg COD/L. Once the methane generation stopped, another four milliliters feed was added to initiate another cycle. During the second cycle, two milliliter samples were taken out from the reactors for VFAs analysis, and an equal volume of medium, without carbon source, was added back into the reactors to keep the constant working volume. In total, nine feeding cycles were conducted before the below hydrogen inhibition tests started. The biogas generation was documented within each feeding cycle.

4.2.2 H₂ inhibition experiment

The H₂ concentration in the headspace is an important indicator of AD system performance, and high H₂ partial pressure will inhibit the whole AD process (Schmidt and Ahring 1995). H₂ concentration was monitored closely during the mesophilic, thermophilic start-up and recovery phases. After the systems had been entirely recovered from the temperature shock (day 51), sludge from each batch reactor was transferred into serum bottles for system robustness tests. Nitrogen was purged into

serum bottles to keep anaerobic condition. Serum bottles were then sealed with the rubber seal. H₂ was manually injected into each serum bottles with final H₂ partial pressure 0.25 atmosphere (atm) at 328 K. Then, the same amount of feed (4mL of 100 g/L glucose + 10 g/L NaHCO₃) was added into each serum bottles.

4.3 Results and discussion

4.3.1 Anaerobic reactors performance in different stages

All reactors were operated for 20 days under mesophilic conditions to obtain a stable baseline. Fig. 4.1 was the methane generation profile of last cycle under mesophilic condition, which showed little statistical difference between control and CM groups (Fig. 4.1). Glucose consumption rates were also similar in each batch reactor, and no VFAs accumulation was observed in all the reactors. It suggests that glucose degradation metabolism reached a stable state, and CM did not have a noticeable effect on the metabolism under mesophilic conditions, which is similar to the findings of Zhao et al. (2017b). The temperature of 9 batch reactors was changed from 35 °C to 55 °C in one step on day 21. Since less than 10% of thermophiles were presented in mesophilic sludge, a long acclimation period is needed to adapt mesophilic AD to thermophilic AD (Boušková et al. 2005). In the case of sludge digestion, 30 to 70 days were required for thermophilic AD to be recovered when the temperature directly increased from 35 °C to 55 °C (Boušková et al. 2005). Similarly, in this study, a recovery time of 13 to 21 days was required after the sudden temperature shock in all reactors (Fig. 4.2).

Figure 4.1 Methane production profile at mesophilic condition

Figure 4.2 Methane production profile during thermophilic recovery phase.

(A) CNT-supplemented group, (B) GAC-supplemented group and (C) Control group.

The performance of all the reactors started to recover from day 27 (the 7th day after increased the temperature), when methane production was resumed (Fig. 4.2). However, the methane production rates were different among the control and CM groups (Fig. 4.2 and Fig. 4.3A). The data of the first thermophilic feeding cycle was used to fit the modified Gompertz equation. Fig. 4.3B, 4.3C, and 4.3D display the experimental and fitting curves. With regression $R^2 > 0.99$, the modified Gompertz equation is reliable to calculate parameters associated with the methane production process. According to the results obtained from the modified Gompertz equation, the maximum specific methane production rate of GAC and CNT groups were 0.67 and 0.48 mL/g VSS/h), respectively, which were almost two-fold of that in the Control group (0.25 mL/g VSS/h) (Table 4.1). It is notable that hydrogen could reach as high as 546 ppm in some of the reactors (Table 4.2).

Figure 4.3 Methane production profile in the first thermophilic feeding cycle

Note: (A) comparison of CNT, GAC and control reactors. (B) methane producing process in CNT group: actual process VS modeled process (C) methane producing process in Control group: actual process VS modeled process (D) methane producing process in GAC supplemented group: actual process VS modeled process.

Table 4.1 Compare methane producing kinetics of different feeding cycles

Experiment group	Parameter	Mesophilic	First	Second	Thermophilic
		Stable	thermophilic	thermophilic	stable
		(day 20)	feeding cycle	feeding cycle	(day 50)
CNT	R_{max}/VSS	1.77 ± 0.06	0.48 ± 0.01	1.79 ± 0.08	2.46 ± 0.14
	R^2	0.994	0.997	0.981	0.983
	Duration time (h)	30 ± 4	270 ± 8	36 ± 3	30 ± 2
GAC	R_{max}/VSS	1.81 ± 0.06	0.67 ± 0.02	2.36 ± 0.08	2.20 ± 0.14
	R^2	0.994	0.995	0.992	0.979
	Duration time (h)	30 ± 3	258 ± 6	35 ± 2	30 ± 5
Control	R_{max}/VSS	1.76 ± 0.05	0.25 ± 0.01	0.99 ± 0.06	2.16 ± 0.09
	R^2	0.993	0.998	0.949	0.977
	Duration time (h)	30 ± 4	357 ± 11	52 ± 4	30 ± 3

Note: R_{max}/VSS : Maximum methane production rate (mL/g VSS/h)

R^2 : Correlation coefficient Duration time. The “during time” refers to the time that had lasted during each cycle.

Table 4.2 The hydrogen concentration under thermophilic condition.

First Thermophilic Feeding cycle				Second Thermophilic Feeding cycle			
Time	CNT	GAC	Control	Time	CNT	GAC	Control
(h)	(mg/L)	(mg/L)	(mg/L)	(h)	(mg/L)	(mg/L)	(mg/L)
90	546 ± 13	389 ± 19	491 ± 15	6	383 ± 18	275 ± 13	343 ± 15
196	366 ± 17	337 ± 8	470 ± 16	22	156 ± 7	378 ± 16	143 ± 14
254	105 ± 12	78 ± 3	146 ± 7	48	84 ± 13	375 ± 14	103 ± 13

Note: The recorded time started at the beginning of each feeding cycle.

As soon as the first feeding cycle completed, a second thermophilic feeding cycle was initiated. Since the systems started to recover in the first feeding cycle, the methane production rates in the second cycle were higher, and no lag phase was observed in all the groups (Fig. 4.2). Similarly, two-fold methane production rate was observed in CM groups (Table 4.1). The calculated specific methane production rates were 1.79, 2.36, and 0.99 mL/g VSS/h) in CNT, GAC, and Control group, respectively (Table 4.1). In comparison with the first thermophilic feeding cycle, the maximum specific methane producing rate was increased substantially in all groups, which showed the rapid recovery process. The performance of CM groups was comparable to that of mesophilic conditions, while Control group were still slow. The CM groups were fully recovered during the third feeding cycle. However, the Control group achieved full recovery in the fifth cycle.

As shown in Fig. 4.2, GAC groups took two thermophilic feeding cycles (296 ± 8 h) to reach the stable state, and CNT group used 309 ± 11 h. However, four thermophilic feeding cycles (504 ± 14 h) were needed for the Control group to achieve a stable state. The results revealed that with supplementation of CM, thermophilic start-up period could be shortened by around 40%. The performance of all the groups during recovered state can be found in Fig. 4.2 and Table 4.1. It is noteworthy that there was no significant difference regarding maximum activity among all the groups under the same temperature conditions once the systems were stabilized. This is possible because glucose is an easily biodegradable substrate. Therefore, under stable state, the phenotypic kinetic parameter tends to be substrate dependent. Furthermore, R_{max} was around 20% higher in thermophilic conditions as compared to mesophilic conditions (Table 4.1). No significant variation in VSS concentration was observed during the recovery process. This fact implicates that the VSS difference could not be the reason for the stimulatory effect of CM.

4.3.2 VFAs profiles

VFAs profile is a vital parameter to show the stability of the anaerobic system. Acetate and propionate were the major VFAs accumulated during the thermophilic recovery stages. The acids formation and degradation processes within the second thermophilic feeding cycle were carefully monitored to disclose the activity difference. The results showed that the maximum quantity of acetate was similar in all groups (Fig. 4.4A), but peaked earlier and the degradation rates were much faster in the CM groups. It is notable that the accumulated acetate was removed by 80.16% and 81.08% in GAC and CNT group, respectively, while only 8.46% of acetate was consumed in Control group within 22 h. As for propionate, its concentration reached the peak values (1.66 ± 0.14 mM CNT group, and 0.65 ± 0.17 mM in GAC group) in 3 h after feeding (Fig. 4.4B). Then, its quantity decreased more than 70% after 22h degradation. However, in the Control group, high propionate acid level was maintained at around 2 ± 0.21 mM.

Figure 4.4 Acetate (A) and propionate (B) concentration profile in the second thermophilic feeding cycle.

Cruz Viggi et al. (2014) reported that upon addition of magnetite into AD, the establishment of DIET could facilitate the syntrophic pathway of methanogenic propionate degradation. Similarly, this study observed the lower concentration of acetate and propionate in the CM groups. The CM used in this study may also act as electron conduits and contribute to the establishment of DIET process. Consequently, the accelerated electron transfer alleviated the impact of temperature shock on the system. More discussion will be detailed in the following sections.

4.3.3 Mitigation of inhibition from high H₂ partial pressure by CM

NADH is a typical energy storage intermediate in anaerobic reactions. Under anaerobic condition, the process of oxidizing NADH into NAD⁺ is accompanied with hydrogen and ATP generation. However, this reaction only occurs under low hydrogen partial pressure, thus hydrogen scavenging partners are needed (Lyberatos and Skiadas 1999, Thauer et al. 1977). In fact, both acidogenesis and acetogenesis process are regulated by the hydrogen producing process. For acidogenesis process, under high hydrogen partial pressure, electron sink will change from hydrogen to more reduced end products, such as butyric, propionate and lactic acids. While acetogenesis process can only become active when the hydrogen partial pressure is below the range of 10⁻⁴ to 10⁻⁶ atm, as the free energy for the metabolic reactions of the acetogenic bacteria is very small. Thus, any significant accumulation of hydrogen may cease the conversion of propionate or butyric acids to acetic acid. Therefore, a stable syntrophic process requires low H₂ partial pressure to make the co-metabolism thermodynamically possible.

When DIET phenomenon exists in the system and partially replaces H₂ as electron transfer media, it may aid the anaerobic system to resist the high H₂ partial pressure, and maintain the function of syntrophic metabolism (Cruz Viggi et al. 2014). Under stable mesophilic conditions, no hydrogen was detected. However, during the first

thermophilic recovery process, hydrogen can be detected in all the groups. The accumulated hydrogen could be consumed gradually during each feeding cycles. It should be noted that the hydrogen concentrations often exceeded the inhibition threshold after each feeding during the recovery period, which may potentially inhibit the recovery.

Figure 4.5 Methane production profiles under high H_2 partial pressure

Interestingly, when the H_2 partial pressure increased to 0.25 atm with external injection of H_2 , methane production rates in the CM groups were remarkably higher than that in the Control group (Fig. 4.5). Glucose was completely consumed in 30 h after feeding in CM reactors, and about 140 mL methane was produced. The methane production rates in these CM reactors were comparable with the stable CM thermophilic system, in which no hydrogen accumulation was noticed. However, only half of that volume of methane was produced in the Control group under the same reaction period (Fig. 4.5). It confirms that CM groups were less affected by high

H₂ partial pressure, which implicates that interspecies hydrogen transfer was likely insignificant in CM groups. Instead, another electron transfer mode, e.g. DIET, was probably triggered by the presence of CM.

4.3.4 Sludge morphology and conductivity

On Day 54, sludge samples were taken out from the serum bottles for SEM observation. The sludge from the three groups showed entirely different structural characteristics (Fig. 4.6). Microorganisms were dispersed loosely in the Control group (Fig. 4.6 A), while there were more aggregates with denser sludge structure in CNT groups (Fig. 4.6 B). Fig. 4.6 C shows that CNT formed a network structure, which can integrate and pack microorganisms within the structure. As for GAC group, many microorganisms attached to the porous sites of GAC (Fig. 4.6 D). Some microorganisms associated with the GAC with pili like structure (Fig. 4.6 E).

CM could interact with c-type cytochromes directly and establish short electron transfer distance among microorganisms. Also, the thermodynamic analysis shows the possibility of electron transfer via CM, as the interaction energy of cytochrome-CM would decrease from reduction state to oxidation state (Liu et al. 2014b). The direct contact between CM and microorganism is necessary for establishing DIET. As observed, CNT could form a network-like structure to facilitate the syntrophic community aggregation. In GAC-supplemented groups, a significant number of bacteria grew on the porous sites of GAC, among which some contacted the granular activate carbon directly with pili like structure. All the evidence shows that CM could form direct contact with microorganisms and create the necessary conditions for DIET.

Figure 4.6 SEM images of (A) control anaerobic sludge, (B, C) sludge exposed to Single-walled CNTs and (D, E) biofilm on GAC.

It has been reported that the conductivity of biofilm or microbial aggregates can be improved via the electron exchange through DIET (Malvankar et al. 2011). DIET is a type of interspecies electron transfer pathway. It was observed that pili could pose metal-like characteristics and play a crucial role in the electron transfer, as it could form matrix structure with sufficient conductivity to account for electron flow between cells (Malvankar et al. 2011). CM could compensate pili to facilitate DIET (Liu et al. 2015a). In this study, the conductivity of CM sludge increased 27 and 3.5 times for CNT and GAC supplemented groups, respectively (Fig. 4.7). These results suggest that the CM formed direct electron transfer circumstance and allowed efficient extracellular electron localization. Furthermore, the significantly improved conductivity of CM sludge may indicate the establishment of DIET pathways.

Figure 4.7 The conductivity of suspended sludge under thermophilic condition

4.3.5 Microbial community transformation

Temperature certainly plays a significant role in altering microbial population. When the temperature was changed from mesophilic to thermophilic condition, the bacterial community in bulk sludge changed considerably (Fig. 4.8). *Actinobacteria*, which was predominant at the moderate temperature, decreased from more than 20% to less than 13% in all the reactors. Meanwhile, *Thermogae Fervidobacterium*, a common hydrogen producing fermentative thermophilic bacteria, increased substantially to 18.56%, 9.89% and 8.50% in control, CNT-supplemented and GAC-supplemented reactors, respectively (Figure 4.8). The doubled hydrogen-producing fermentative population in Control group indicates that hydrogen producing process may be more active than that in CM groups.

It is notable that after the first thermophilic recovery cycle, the abundance of *Caloramator* was 12.89%, 2.63% and 1.20% in CNT-supplemented, GAC-supplemented and control reactors, respectively. *Caloramator* is a type of

fermentative bacteria belonging to *Clostridiaceae*, which was the dominant exoelectrogenic bacterial group in soil (Jiang et al. 2016a). A study on thermophilic microbial fuel cell (MFC) reported that the genus *Caloramator* related bacteria played a crucial role in electricity generation (Fu et al. 2013). Further study confirmed that *Caloramator australicus* strain RC3 was a new thermophilic exoelectrogen (Fu et al. 2013). In this study, the uncultured *Caloramator sp.* was observed, which may display the similar electrochemical activity with *Caloramator australicus*, as species within the same genus normally have similar phenotypes and biochemical activity (Baron 1996). Further study will be required to identify the exact function of the discovered *Caloramator sp.* According to Figure 4.8, the number of *Actinobacteria*, *Thermogae* *Fervidobacterium* and *Caloramator* were slightly increased (< 5%) over 20 days incubation period under the mesophilic condition with minor differences within all the groups. However, after temperature shock, the microbial populations were altered considerably with high distinctions among control and CM groups within 12 days.

Figure 4.8 Relative abundances of phylogenetic groups in source sludge, Control group, CNT group and GAC groups.

Note: Microbial communities are presented in taxonomic phylum and genus levels. Phylogenetic groups accounting for $\leq 2.0\%$ of all classified sequences are summarized in the artificial group “others”.

Figure 4.9 Proportion of main archaea species under different conditions

The methanogenic community in the bulk sludge was also changed significantly after 12 days under thermophilic condition. The abundance of total archaea in original sludge was around 7.58%, and it increased up to 25% in all the batch reactors (Fig. 4.9). At the genus level, *methanothermobacter*, *methanosaeta*, and *methanosarcina* were the predominant methanogens in the thermophilic reactors. *Methanothermbacter*, a typical hydrogenotrophic methanogen was dominant in all reactors. It is noteworthy that the total ratio of *Methanosaeta* and *Methanosarcina* in CM groups were almost two-fold than that in Control group.

Among all the methanogens discovered so far, only *Methanosarcina* and *Methanosaeta* have been reported of obtaining membrane-bound cytochromes (Rotaru et al. 2014c, Thauer et al. 1977), and equipped with the extracellular electron

exchange capability. Another study showed that both *Methanosarcina* and *Methanosaeta* could receive electrons from syntrophic partners through bioelectrical connections directly (Rotaru et al. 2014c), and CM can stimulate the enrichment of these two types of electro-active methanogens to carry out DIET (Chen et al. 2014, Liu et al. 2012a). It is likely that CNT and GAC in this study may trigger the enrichment of electro-active methanogens. Furthermore, both *Methanosarcina* and *Methanosaeta* are typical acetoclastic methanogens, which ruled out the possibility that the better hydrogen resistance capability in CM groups was due to the enrichment of hydrogentrophilic methanogens. It is highly possible that both DIET and hydrogen mediated interspecies electron transfer metabolisms co-existed in CM groups, and DIET may play a major role when H₂ partial pressure was high. It is proposed that the added CM may act as an electrical conduit, via which *Caloramator* metabolized fermentable substrates with electron transfer to *Methanosarcina* or *Methanosaeta* directly.

It should be noted that after 20 days mesophilic incubation, methanogens types and abundance were relatively similar between control and CM groups other than *Methanosaeta* which increased in GAC supplemented groups. The great distinction of methanogens between control and CM groups was also exhibited in thermophilic recovery phase. These facts indicate that the enrichment of electroactive bacteria and DIET pathway were likely triggered by temperature rise to some extent. In other words, the presence of CM or temperature shock alone may not be able to facilitate DIET efficiently. Thus, both conductive material and stress conditions would be required.

The above observations are considered to be the key solution to mitigate the negative impact of temperature shock on AD system. Firstly, DIET may enhance the substrate utilization due to faster interspecies electron transfer. Hence it can improve the

biomass growth rate of thermophilic exoelectrogens involved in DIET (Cruz Viggi et al. 2014). Secondly, DIET helped to balance the syntrophic metabolism rate under temperature shock. When molecular hydrogen is the electron transfer media, methanogenesis is often the rate-limiting step in the syntrophic metabolism process, as methanogens are more sensitive than other syntrophic partners (e.g. acidogens). The sudden increase in temperature would lead to accumulation of VFAs and hydrogen (> 500 ppm), in turn, resulting in further inhibition on the acidogenesis and acetogenesis (Gilroyed et al. 2008). The involvement of DIET may play a vital role in tackling the rate-limiting step since hydrogen will not affect the DIET pathway. Hence, a quicker activity recovery and more efficient co-metabolism during the thermophilic start-up period can be expected in CM groups. This improvement was evidenced with doubled maximum methane producing rate and lower accumulation of VFAs during thermophilic recovery period (Fig.4.2 and 4.4).

4.4 Conclusions

This study found that CM could promote the start-up of thermophilic process, as evidenced by low VFAs accumulation, high methane production rate and more robust responses against hydrogen inhibition. The facilitated phenomenon was related to the establishment of DIET. Exoelectrogens were highly enriched in CM reactors. DIET is likely to be a compensate electron transfer pathway at unstable AD state. Nevertheless, this study only monitored the thermophilic start-up process with glucose as feed. It is uncertain whether the promoted thermophilic methanogenesis will be reflected in the same way when the feed is changed to complex compounds i.e. sludge. Moreover, a scale-up study with various feed stock will be helpful to evaluate the feasibility of the full-scale application.

CHAPTER 5

Enhanced anaerobic phenol degradation by conductive materials via EPS and microbial community alteration

5.1. Introduction

Phenol is a common toxic compound in industry effluents. If it is discharged to open water bodies directly, serious ecological problems would be induced. Compared to physical, chemical and aerobic biodegradation treatment methods, anaerobic biological phenol degradation shows great advantages in terms of cost and efficiency (Rosenkranz et al. 2013). The complete anaerobic phenol degradation can be carried out through cooperation between phenol-degrading bacteria and methanogens (Li et al. 2013), and the syntrophic methanogenesis is performed via adopting molecular intermediates, typically hydrogen (McInerney et al. 2009). Recent studies show that direct extracellular electron transfer (EET) widely exists among syntrophic partners (Dube and Guiot 2015), and can be triggered by addition of CM (Kato et al. 2012a, Mu et al. 2012b). In addition, theoretical analysis reveals that electron transfer via CM could be around 10^6 times higher than that associated with interspecies hydrogen transfer, and certain organic compounds degradation could be stimulated via EET (Cruz Viggi et al. 2014, Kato et al. 2012b, Yin et al. 2018, Zhao et al. 2017b). The syntrophic phenol degradation and the faster electron transfer via DIET strongly suggest the feasibility of CM to stimulate anaerobic phenol degradation by triggering direct EET between syntrophic partners.

The enrichment of electroactive microorganism is required for conducting EET. Currently, CM, typically carbon-based and iron-based materials, are widely reported with the capability of altering anaerobic microbial community for promoting EET (Jing et al. 2016, Zhao et al. 2017b). Nevertheless, till now, limited studies compared

the effect of different CM on anaerobic microbes. In this study, single-walled carbon nanotube and magnetite nanoparticles are chosen as representative CM to compare their effect on phenol degradation efficiency and microbial community structure.

Extracellular polymeric substances (EPS) are ubiquitous heterogeneous mixtures of natural organic macromolecules. It can form a protective layer for microbial cells (Xiao and Zhao 2017b), with the thickness up to 1 μm (Flemming et al. 2007, Sheng et al. 2010). Humic substance and redox protein within EPS matrix are widely reported to be electrochemically active (Shi et al. 2016, Xiao and Zhao 2017b). Large number of functional groups in EPS, such as ketone, carboxylic acid, phenolic and alcoholic hydroxyls, can absorb/bind secreted redox protein such as flavoprotein to promote EET (Xiao and Zhao 2017b). CM promoted syntrophic electron transfer are based on applying CM as conduit, where direct contact between cells and CM are needed (Cruz Viggi et al. 2014, Liu et al. 2012a). Since CM likely interact with EPS layer first to establish contact with cells, it is important to explore the role of EPS in EET, which, however, has been ignored by most studies. To date, it is not clear if the EPS compositions would be influenced by the presence of CM, and if EPS acts as competitor or compensator of CM to facilitate EET. In this study, the influence of CM on EPS distribution, and the interaction between them as well as the role of EPS in EET were examined and discussed.

5.2 Experimental setup and operation

The seed sludge for anaerobic batch reactors was collected from a lab-scale mesophilic (35 °C) continuous stirred tank reactor (CSTR) fed with glucose as carbon source. All batch experiments were conducted using AMPTS II system (Bioprocess Control Company) to track the methane production automatically. Three groups, namely Control group (C), magnetite nanoparticles group (M) and single-walled carbon nanotube group (CNT), were setup with triplicates each. Each reactor had a

working volume of 360 mL with a final VSS concentration of 3.11 g/L. CNT (0.30 g CNT/g VSS) or magnetite particles (1.75 g magnetite/g VSS) was added into corresponding reactors. Reactors were purged with nitrogen gas for 10 mins to remove dissolved gas. All batch reactors were operated in semi-continuous mixing mode with 90 s on and 90 s off, and stirring speed was 80 rpm.

The phenol degradation experiment lasted for 165 days that can be divided into six phases. Detailed operation condition and sampling time can be found in Table 5.1. In the last feeding cycle of phase 3 (day 72), before feeding, all reactors were exposed to air for 60 mins to examine the influence of oxygen on the process. Phenol degradation performance were closely monitored at the first, middle and final feeding cycles in each FPC phase. The biomass for microbial community analysis was collected at the end of each FPC phase. Homogeneous sludge samples were taken out on day 160 for analyzing soluble iron ions (II and III) from all reactors.

Table 5.1 Operation conditions, phenol degradation performance monitoring and microbial community sampling time.

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6
Duration (Day)	1-15	15-45	45-75	75-105	105-135	135-165
First feeding cycle (Day)	1-4	15-17	45-47	75-81	105-112	135-144
Middle feeding cycle (Day)	7-10	31-33	61-63	87-93	120-127	144-154
Last feeding cycle (Day)	13-15	43-45	72-75	99-105	127-135	154-165
Glucose (mg/L)	138	\	\	\	\	\
Phenol (mg/L)	138	200	400	600	800	1000
Total COD (mg/L)	476	476	952	1428	1904	2380
DNA extraction (Day)	15	45	75	105	135	165

Magnetite nanoparticles were synthesized according to Kang et al. (1996). X-ray diffraction (XRD) and transmission electron microscope (TEM) were employed to confirm the product as magnetite nanoparticles (Fig. 5.1 and 5.2). In Figure. 5.1a, the pattern of synthesized product was similar to the standard pattern of Fe_3O_4 (75-0033). In Figure. 5.1b, the pattern of sludge sample from M group was similar to the standard pattern of FeCO_3 (29-0696), whereas little FeCO_3 was observed in Control group.

Figure 5.1. The X ray diffraction spectra of (a) synthesized magnetite nanoparticles (b) sludge of Control group and magnetite nanoparticles group

Figure 5.2. Images of contact between bacteria and (a) CNT (b) Magnetite

5.3 Results and discussion

5.3.1 Phenol degradation and methanogenic performance at different FPC

Fig. 5.3 exhibits the phenol degradation profile of final feeding cycle in each phase, and the corresponding methane production profile is shown in Fig. 5.4. Based on the performance, the influence of FPC and CM on methanogenic activity could be categorized into three stages. The first stage took place in phase 1, when co-substrate was used. Phenol was completely degraded in 60 hours (Fig. 5.3a). The degradation rates in all groups were similar (2.17 ± 0.01 mg/h). No significant difference was found for R_{max} in all groups (2.11 ± 0.10 mL/h, Fig. 5.5 P1). The second stage was from phase 2 to 3 (FPC from 200 – 400 mg/L). In general, phenol degradation and methane production rates increased with the acclimation time and FPC. In phase 2, phenol degradation rates in all groups increased to 10.57 ± 0.53 mg/h (Fig. 5.3b). It is noteworthy that R_{max} , on the other hand, exhibited a great difference among CM and Control group, e.g. CNT group (6.17 mL/h) vs. Control group (4.11 mL/h) (Figs. 5.4b and 5.5). From phase 3 onwards, the superiority of CM enlarged with the increased FPC. In particular, in the middle feeding cycle of phase 3, 2-fold R_{max} was

observed in CM group compared to Control group (8.36 mL/h in CNT and 7.06 mL/h in magnetite vs. 4.29 mL/h in control) (Fig. 5.5). Besides the improved R_{max} , faster phenol degradation rate was also observed in CM groups (Fig. 5.3c).

Figure 5.3. Phenol degradation profiles in each phase. (a): Phase 1; (b): Phase 2; (c): Phase 3; (d): Phase 4; (e): Phase 5; (f): Phase 6.

Figure 5.4. Methane production profiles in each phase. (a): Phase 1; (b): Phase 2; (c): Phase 3; (d): Phase 4; (e): Phase 5; (f): Phase 6.

Figure 5.5 Maximum CH₄ production rate (*R*_{max}) at beginning (B), middle (M) and final (F) feeding cycles of all phases.

Note: P1- P6 is the abbreviation of phase 1 to 6. The error bar is the *R*_{max} difference between the experimental and model fitting results.

The third stage was phase 4 - 6 (FPC 600 mg/L to 1000 mg/L). At stage, phenol degradation rate and *R*_{max} declined with FPC increase and could not reach the performance as before (Figs. 5.3, 5.5 and Table 5.2). As phenol is toxic, the unrecovered activity could be attributed to its inhibition. This was consistent with previous studies, which reported 400 - 600 mg/L phenol was the inhibitory threshold (Firozjaee et al. 2011, Rosenkranz et al. 2013). Nevertheless, CM groups still performed better. Specifically, a lag phase was observed in Control group at FPC > 600 mg/L, while a linear regression of phenol-time correlation was observed for CM groups (Figs. 5.3d, 5.3e, and 5.3f). Together with the methane production profile (Fig. 5.4), the results indicated high phenol concentration exert long-term inhibition on Control group, whereas the CM groups could reach a suboptimal condition after one-

month acclimation under each FPC condition.

Table 5.2 Parameters of methanogenic performance during each phase

		<i>R</i> _{max} (mL/h)	Root mean square	Lag phase (h)	Root mean square	Statistics R ²
Phase 1	Control	2.10	0.024	-1.01	0.437	0.992
	Magnetite	2.14	0.019	-2.79	0.410	0.993
	CNT	2.08	0.025	-1.67	0.439	0.991
Phase 2	Control	4.11	0.136	2.06	0.175	0.996
	Magnetite	4.98	0.087	0.17	0.167	0.996
	CNT	6.17	0.275	0.71	0.299	0.985
Phase 3	Control	2.33	0.041	14.39	0.443	0.993
	Magnetite	5.85	0.167	4.78	0.299	0.992
	CNT	5.89	0.104	5.96	0.273	0.994
Phase 4	Control	2.20	0.020	30.27	0.989	0.985
	Magnetite	3.57	0.059	12.73	0.384	0.994
	CNT	4.32	0.085	7.54	0.391	0.992
Phase 5	Control	1.16	0.02082	52.55	1.694	0.971
	Magnetite	3.45	0.053	21.25	0.531	0.993
	CNT	3.91	0.056	17.37	0.449	0.994
Phase 6	Control	1.56	0.024	66.84	1.410	0.979
	Magnetite	2.94	0.043	18.68	0.736	0.989
	CNT	2.84	0.039	12.03	0.716	0.989

It is noteworthy phenol degradation rate was much slower in phase 1 than in phase 2.

According to Movahedin et al. (2011), the influence of co-substrate on phenol degradation varied with acclimation methods and microbial community. Microorganism may choose to degrade glucose first, and then use phenol as alternative substrate, thus co-substrate may limit phenol degradation. The improvement of R_{max} in CM groups started from phase 2 (Fig. 5.4 and 5.5), whereas phenol degradation in CM groups was only accelerated from phase 3 (Fig. 5.3). It seemed phenol degradation and methane generation processes were decoupled and the activity of methanogens were enhanced earlier. CM, as electron shuttle to facilitate electron transfer among syntrophic partners, theoretically would not promote the metabolic activity of microbial electron donors directly. At low FPC (200 mg/L), the facilitated phenomenon was only observed in methanogenesis rather than in phenol degradation process. However, at FPC above 400 mg/L, the facilitated methanogenesis could speed up the consumption of upstream accumulated metabolites, thus resulting in enhancement of phenol degradation.

In the last feeding cycle of phase 3, all reactors were exposed to air for 60 mins to investigate the robustness of the systems. It has been reported that molecular oxygen once entered anaerobe cells can accept one electron from microbial electron respiratory chain and reduced to oxygen free radicals. As anaerobes are lack of superoxide dismutase, free radicals cannot be removed timely, which leads to inhibition on anaerobic metabolism and enzymatic systems (Jenney et al. 1999). The results showed the methane production rate declined by 46% in Control group, whereas the observed decline in magnetite and CNT groups were only 17% and 29%, respectively (Fig. 5.5). The existence of CNT can create a more negative redox potential in the anaerobic microcosms (Salvador et al. 2017), hence, such reduced environment may efficiently mitigate the impact from oxygen. As for Magnetite group, the presence of high concentration of Fe(II) ions can act as electron donor to remove dissolved oxygen (Liu et al. 2011). Therefore, the dosed CM to some extent

could resist oxygen triggered oxidative stress, and maintain a less affected environment for anaerobes.

5.3.2 Electron transport system (ETS) activities

ETS activity reflects the dehydrogenase activity of the microbial communities, which is associated with biological respiratory activity (Yin et al. 2018). The ETS activity in CM groups was much higher than that in Control group during 120 h anaerobic reaction (Fig. 5.6). In the first hour after feed, the ETS activity of CM groups was about 10 times higher than that in Control group (average 28.05 $\mu\text{g}/(\text{min} \cdot \text{mL})$ in CM VS 3.67 $\mu\text{g}/(\text{min} \cdot \text{mL})$). Although the difference narrowed at the end of the cycle when the substrate was fully consumed, the CM groups still showed about 2 times higher ETS. ETS activity results proved the facilitated electron transfer system with CM addition.

Figure 5.6 Electron transport system activity in phase 6

5.3.3 EPS alternation and its role in extracellular electron transfer

Thermodynamic analysis revealed the feasibility of DIET, when direct contact between cells and CM established (Liu et al. 2014b). TEM observation showed that microbes tend to contact with CNT, or their surface would be covered by dense magnetite nanoparticles (Fig. 5.2). In addition, the enrichment of electroactive genera was observed in CM groups, which will be discussed in the later part. These facts strongly indicate the successful establishment of EET condition.

As EPS act like a capsule of cells, CM would be in contact with EPS of the cells firstly. The electronactive moieties in EPS could play an important role in the EET process (Lovley 2017, Xiao and Zhao 2017a). In this study, the presence of CM clearly influenced all EPS compositions, especially protein and HS (Table 5.3).

Table 5.3 Quantification of sub-fractions of dissolved organic compounds in EPS extracted from end of phase 6. Unit: mg-C/L.

Sample	Hydrophobic	HMW	LMW	HMW	LMW	HS	Building	LMW	LMW
	DOC	PN	PS	PS	PS		blocks	Neutrals	Acids
SB-C	24.70	7.54	12.27	2.98	5.59	8.12	4.89	19.36	0.54
SB-M	43.58	4.67	234.82	7.25	1.16	4.94	3.65	4.28	0.00
SB-CNT	20.46	6.92	105.77	9.18	1.45	25.20	22.66	10.03	0.00
LB-C	2.15	1.27	7.12	0.60	0.95	1.10	1.16	4.86	0.35
LB-M	0.21	1.92	24.91	1.70	0.43	3.02	4.52	4.54	1.05
LB-CNT	0.15	1.64	18.85	0.58	1.19	1.39	2.19	5.16	1.22
TB-C	5.71	1.45	13.95	0.54	0.84	0.63	2.52	8.09	0.60
TB-M	0.82	2.41	24.25	2.13	1.35	1.71	3.56	8.45	0.46
TB-CNT	0.52	0.16	29.25	0.17	1.35	0.24	0.53	1.38	0.00

Protein profile was substantially different in CM and Control group. In particular, around 2.3-fold protein content was observed in bound EPS of CM groups. CM could facilitate the expression of redox active protein, such as cytochrome c oxidase which is an essential component of the electron transport chain (Jing et al. 2017). Ren et al. (2012) also reported that CNT could enhance the enzyme activity of horseradish peroxidase by binding to its activity center, with clear observation of electron transfer using near-infrared spectroscopy. Both magnetite and CNT would rely on direct contact with protein active center for accepting or donating electron (Lovley 2017). Given spatial distribution of those proteins, the higher quantity of protein that bound to cells may assist in better electron transfer between cells and CM. Therefore, the facilitated EET between syntrophic microbes was likely fulfilled by applying CM as mediator to accept and donate electrons from electro-active protein.

On the other hand, LMW protein from soluble EPS was 10 to 20 folds higher in CM (105.77 - 234.83 mg-C/L) groups than Control group (12.27 mg-C/L) (Table 5.3). It has been reported that some redox proteins (e.g. flavoprotein) involved in electron transfer during cell metabolism processes could be released into extracellular environment, and remain high electrochemical activity that can promote long distance electron transfer (Cao et al. 2011, Xiao and Zhao 2017b). It is reasonable to predict that greater amount of protein within soluble EPS in CM may also play a role in carrying out long distance interspecies electron transfer.

Building blocks are the break-down products or small fractions of humic substances. Building blocks and humic-like substance are combined called humic substance (HS) hereafter. The distribution of humic substance was very different in the three EPS layers. The highest humic substance concentration was observed in soluble EPS (47.86 mg-C/L) of CNT group, while the concentration of HS was much lower in magnetite and Control group regardless of EPS layers (Table 5.3). HS are widely reported as electron mediators facilitating long distance electron transfer between

syntrophic microbes via its redox moiety - quinone (Lovley et al. 1996, Roden et al. 2010). In this study, the significant high concentration of HS in soluble EPS of CNT group could promote long distance electron transfer, thus accelerated methanogenesis. In fact, the electron transfer by HS and CNT follows the similar multi-hopping mechanism, where electron transfer between syntrophic partners using these compounds as relays. This is because of the same functional moiety-quinone of HS and CNT (Lovley 2017). Therefore, CNT and HS may complement each other to support microbial cell and EET.

Lovley et al. (1996) reported that HS could stimulate iron-reducing bacteria for benzene-oxidizing, where soluble HS accelerated the reaction by alleviating direct contact as electron shuttles. Niedzwiecka et al. (2017) also reported both AQDS (a model HS compound) and ferric ions can act as electron shuttle during 2,4-Dinitroanisole reduction. These facts indicate soluble HS and/or ferric ions can go through reversible redox reaction for stimulating EET between syntrophic partners in anaerobic biological system. In this study, three factors, namely, abundant HS (Table 5.3), high concentration of ferric ions (Table 5.4), and highly enriched iron reducing bacteria (illustrated in microbial community part), were in favor of establishing EET between syntrophic microorganisms in Magnetite groups. It is hypothesized that the electron shuttles mediated EET performed in a two-step process in which iron reducing bacteria oxidized phenol with HS/ferric ions as electron acceptor, then the reduced shuttles transferred electron to terminal acceptors.

Therefore, other than the commonly reported EET pathway that CM conduit electrons transfer between syntrophic partners directly, CM also altered electron moieties compositions within EPS, i.e. HS and redox proteins, which could act as electron shuttles for long distance EET.

Table 5.4 Ferric iron concentration on Day 160 of different groups

Ferric ion	Control	Magnetite group	CNT group
Fe ²⁺ (mg/L)	17.1 ± 3.2	219.4 ± 5.6	15.8 ± 1.37
Fe ³⁺ (mg/L)	2.3 ± 0.3	5.4 ± 0.93	2.5 ± 0.34
Fe ²⁺ / Fe ³⁺ ratio	8.5 ± 1.4	31.2 ± 2.74	7.5 ± 0.87

5.3.4 Shifts of microbial community diversity and structure

The DNA samples were sequenced using the Illumina Miseq platform. High Good's coverage (0.9985 to 0.9993) was observed in all samples indicating sufficient sequencing depth. Generally, the index of OTUs and Chao1 declined with the increase of FPC (Table 5.5). The topology of UPGMA tree clearly exhibited the similarity of microbial communities among all samples. As shown in Fig. 5.7, microbial community derived significantly with the increase of FPC, which indicates that FPC was an important factor in determining microbial structure. In general, the microbial community, which was fed with phenol only, can be divided into three clusters, (1) cluster at 200 mg/L FPC, (2) cluster at 400 mg/L to 600 mg/L FPC, and (3) cluster at 800 to 1000 mg/L FPC. The first separation of clusters could be induced by the change of substrate components, changing from co-substrate (glucose and phenol) to phenol. The separation of second and third clusters was corresponding to the decreased methane production and phenol degradation rate. The synchronous thresholds suggest the close correlation of microbial community structure and system performance.

Table 5.5 OTUs and diversity index of microbial community

Sample Name	OTUs	Chao1	Shannon	Simpson	Good's Coverage
P2-C	346	388.775	3.890	0.829	0.9985
P2-M	363	394.189	4.105	0.810	0.9985
P2-CNT	357	390.478	3.827	0.817	0.9988
P3-C	302	325.033	3.819	0.821	0.9988
P3- M	310	310.115	3.889	0.849	0.9993
P3-CNT	318	322.800	3.872	0.829	0.9988
P4-C	267	295.333	3.684	0.792	0.9989
P4- M	309	338.400	3.825	0.837	0.9993
P4-CNT	272	305.781	4.089	0.841	0.9990
P5-C	236	274.077	4.368	0.873	0.9989
P5- M	293	327.902	4.759	0.933	0.9989
P5-CNT	277	317.320	4.715	0.931	0.9984
P6-C	221	266.000	4.206	0.865	0.9988
P6- M	276	318.029	4.750	0.925	0.9987
P6-CNT	244	310.120	4.640	0.915	0.9985

Note: P2- P6 are the abbreviation of phase 2 to phase 6.

Figure 5.7 UPGMA cluster based-on 16s RNA of community samples

Note: The scale bar refers to evolutionary distances in substitutions per site.

The results also suggest that CM were another important factor affecting microbial community evolution. Different materials showed diverged influence. The microbial community of CNT always clustered with Control group in all phenol fed only phases, whereas Magnetite groups appeared to separate from them at all phases (Fig. 5.7). The results imply CNT imposed little influence on community structure, whereas magnetite nanoparticles could alter the community structure greatly. The great divergence between Magnetite groups and CNT/Control group likely because magnetite can enrich iron reducing bacteria (Yang et al. 2016), which may induce extracellular iron redox electron transfer pathway. The results of microbial phylotype

at phylum level also confirm this point (Fig. 5.8).

5.3.5 Microbial phylotypes alternation and correlation with CM

5.3.5.1 Microbial community at phylum level

Fig. 5.8 shows the phylogenetic levels of phylum obtained from microbial community analysis. Similar to UPGMA tree result, all phylum of CNT and Control group show similar trend with increased FPC. At FPC 200 mg/L, the most abundant phylum was *Firmicutes* (control 50.3%, CNT 53.7%). And the highest abundant population shifted to *Proteobacteria* at FPC 400 mg/L (control 36.7 %, CNT 44.7%), and further to *Aminicenantes* and *Proteobacteria* beyond FPC 600 mg/L. *Firmicutes* are able to form spores to resist environmental stress, and quickly respond to substrate availability, thus they are able to outgrow other organisms (Schaechter 2009). *Proteobacteria* are gram-negative bacteria, in which many phenol degradation strains belong to (Jiang et al. 2016b). The high abundance of *Proteobacteria* in this study suggests its important role in phenol degradation. *Aminicenantes* are commonly found in extreme environments (Farag et al. 2014), which were also found in this study when FPC was higher than 600 mg/L.

However, the most predominate phylum in M group at FPC of 200 mg/L, 400 mg/L, and > 600 mg/L were *Bacteroidetes* (48.5%), *Actinobacteria* (48.8%), and *Proteobacteria/Actinobacteria* (around 30%), respectively. The abundance of *Bacteroidetes* could be triggered by ferric iron. According to Pan et al. (2017), *Bacteroidetes* were enriched, while *Firmicutes* were weakened in an anaerobic dye degradation reactor after dosing zero valent iron. Therefore, it is possible that the enrichment of *Bacteroidetes* were related with iron reducing activities. *Actinobacteria* were commonly found in active metal-reducing communities (Lin et al. 2007, Wang et al. 2009), and its enrichment only happened in high concentration of carbon source impacted environment (Akob et al. 2011). Therefore, *Actinobacteria*,

selected by high FPC, may be responsible for iron reducing as well. The enrichment of *Proteobacteria* was probably responsible for phenol degradation. However, the abundance of this phylum was remarkably different between magnetite and control/CNT group. Compared with CNT group, the existence of magnetite nanoparticle had significantly altered the microbial structure. Except for *Proteobacteria*, the main enriched phylum in Magnetite groups was predicted to be related to iron reducing bacteria, which could interact with methanogens via direct EET for stimulating electron transfer (Kato et al. 2012a, Morita et al. 2011a).

Figure 5.8 Relative abundances of phylogenetic level in source sludge, Control group, CNT group and M group. Microbial communities are presented in taxonomic phylum. Phylogenetic groups accounting for ≤ 2.0 % of all classified sequences are summarized in the artificial group “others”.

5.3.5.2 Functional genera identification

The addition of CM certainly enhanced the enrichment of phenol degradation bacteria (Fig. 5.9). Specifically, the total percentage of identified phenol degradation bacteria in CNT and Magnetite groups were 24.4% and 33.8%, while only 13.9% was observed in control (Fig. 5.9). The functional genera were also quite different between the two CM groups. The predominate functional genus was *Syntrophus* (12.3%) in CNT group, whereas *Syntrophorhabdus* (14.1%) was the most abundant genus in Magnetite group. In CNT group, *Brooklawnia* and *Treponema* constituted 3.5% and 3.4%, whereas their corresponding abundance were 9.3% and 0.9% in Magnetite group. It is noteworthy that the exoelectrogen- *Syntrophus* (Khilyas et al. 2017, Reimers et al. 2006, Toczyłowska-Mamińska et al. 2018), exhibited around 6 times higher abundance in CM groups compared to Control group.

Figure 5.9 Relative abundance of identified phenol degradation bacteria in phase 6.

Four genera of methanogens, i.e. *Methanosaeta*, *Methanolinea*, *Methanobacterium*, and *Methanoregula*, were observed. Fig. 5.10 shows the archaea community from

genus level, which also exhibited great difference among control and CM groups. The predominate archaea in CM were *Methanosaeta* (around 40%), whereas *Methanobacterium* was the most abundant in Control group (56.3%). Among the methanogens, only *Methanosaeta* and *Methanosarcina* were reported with the capability of accepting electron directly (Rotaru et al. 2014c, Yin et al. 2018). Similar to former magnetite amended anaerobic digestion (Lei et al. 2018), the high abundance of *Methanosaeta* in CM groups was likely induced by the presence of CM. *Methanobacterium* is a typical hydrogenotrophic methanogens that live exclusively through the reduction of carbon dioxide by hydrogen. The different methanogens in CM and Control group indicates the change of methanogenesis from hydrogenotrophic to direct EET pathway. Thus, it is reasonable that both *Syntrophus* and *Methanosaeta* were essential in anaerobic phenol degradation via direct EET pathway.

Figure 5.10 Relative abundance of archaea in phase 6

5.4 Conclusions

The study demonstrated that the addition of CM could improve anaerobic microbial

activity in phenol degradation and enhance microbial resilience to adverse environmental impact. The observed phenomenon may be attributed to three factors. (1) CM can act as electron conduit to facilitate direct EET; (2) CM altered the EPS compositions, which were in favor of EET in both liquid phase and cell surface; (3) CM enhanced the growth of functional genera in phenol degradation and direct electron transfer. It was found out that CNT and magnetite nanoparticles can trigger different microbial community to stimulate EET. Interestingly, the microbial community in Control group was not significantly different compared to carbon nanotube supplemented groups during the whole operation period, whereas the Magnetite groups diverged largely from the initial stage.

CHAPTER 6

The influence of iron-based materials on organic compounds transformation

6.1 Introduction

Anaerobic treatment has gained increasing attention within recent decades with less energy consumption and potential energy recovery. Currently, the most important application of anaerobic digestion lies in waste activated sludge, animal and food waste digestion, as it can greatly reduce the volume of solids, generate bioenergy, and reduce the cost. However, its application was greatly impeded by system instability as well as low transformation efficiency.

Anaerobic digestion is carried out by cooperation between several groups of microorganisms. The intermediates, typically hydrogen, often act as the electron or energy carrier between each community, and their transport efficiency determines the system stability as well as AD speed. Recent studies demonstrated the syntrophic metabolism could be promoted by supplementing CM, such as magnetite (De Vrieze et al. 2016, Yamada et al. 2015). The stimulated methane production could be attributed to the promotion of direct interspecies electron transfer (DIET) (Liu et al. 2012a, Rotaru et al. 2014b, Rotaru et al. 2014c). The mechanism is that the non-biological magnetite could act as conductive pili or c-type cytochromes for interspecies electron transfer, thus electron transfer limiting factor – hydrogen- could be removed (Zhao et al. 2015). Another possibility is that iron ions are important minerals for microbes, thus it is also possible to be the facilitating factor by supplying nutrients for microbial growth and metabolism activity (Symeonidis and Marangos 2012). To make this clear, it is important to compare the role of magnetite nanoparticles and iron ions in one system. However, this point was rarely paid

attention by researchers, as most studies only focus on either promoting role of nutrient or DIET.

Till now, many studies demonstrated CM could promote DIET and stimulate various organic matters degradation, such as WAS and VFA. However, no study reported the effects of CM on the transformation of organic matters in different molecular range. In fact, the enhancement varied with specific compounds. For example, our previous studies indicated CM can greatly promote the degradation of phenol, whereas it did not work in the case of glucose. Therefore, it is important to study the effects of CM on different organic matters for better understanding and application in industry.

Based on the above rationale, this study compared the role of Fe^{2+} and magnetite nanoparticles in AD. The WAS fermented liquor, which is rich of various types of organic matter, was chosen as substrate. The methane generation profile was monitored to compare the methanogenic performance. The degradation process of dissolved organic matters (DOM) was closely monitored to figure out the transformation of each type of organics with different effects of iron-based materials. The alteration of syntrophic bacteria and methanogens was explored to reveal the mechanism of the influence from magnetite nanoparticles and Fe^{2+} .

6.2 Substrates and Experimental set-up

6.2.1 Fermented liquor

Waste activated sludge (WAS) was collected from a local water reclamation plant. The fermentation process was conducted in a fermenter at $\text{pH } 10.0 \pm 0.1$ for 8 days according to previous study (Yuan et al. 2006). After centrifuged at 3500 g for 10 mins, the supernatant was collected as the substrate for AD reactors. Total COD was then adjusted to $7120 \pm 33 \text{ mg COD/L}$. The characteristics of fermented liquor can be found in Table 6.1.

Table 6.1 Fermented liquor composition. Unit: mg/L-C						
Cycle	Total TOC	Hydrophobic	Hydrophilic	Biopolymer	HMW PN	HS
1 st & 2 nd	2254 ± 54.6	140 ± 17.8	2114 ± 41.5	156 ± 18.4	39.4 ± 5.8	394 ± 47.5
3 rd	2047 ± 56.1	116 ± 14.6	1932 ± 54.2	218 ± 13.1	55 ± 9.1	742 ± 50.2
Cycle	HMW	LMW	LMW	LMW	LMW	
	PS	Neutrals	Acids	PN	PS	
1 st & 2 nd	715 ± 48.7	224 ± 30.1	304 ± 44.4	164 ± 23.3	117 ± 13.6	
3 rd	163 ± 12.1	226 ± 22.5	746 ± 36.6	95 ± 5.7	72 ± 4.5	

6.2 Experimental set-up

The inoculum was collected from a local wastewater treatment plant. All batch studies were conducted by AMPTS II system to record methane production automatically. In this study, three batch groups were designed, namely control (Control), magnetite nanoparticles supplemented groups (M), and FeCl₂ supplemented group (FeCl₂). Each reactor was added with 200 mL fermented liquor and 150 mL inoculum with final VSS of 5.5 g/L. Magnetite nanoparticles and FeCl₂ was added into reactor to reach final concentration of 10 mmol/L-Fe and 5 mmol/L-Fe. Then, each reactor was purged with nitrogen for 10 mins to remove dissolved gas and sealed immediately. The reactors were stirred at a speed of 80 rpm and operated for 3 feeding cycles (1month/cycle). Before 2nd and 3rd feeding cycle, reactors were settled for 24 hours and 200 mL supernatant was replaced with fermented liquor. During each cycle, supernatant was sampled for organic matters analysis at the exponential gas production stage, the stationary gas generation stage, and end of gas generation stage. Detailed sampling schedule can be found in Table 6.2.

Table 6.2 Sampling time of organic compounds

Cycle	Time (Day)	Sampling Time			
1 st	1-30	68h	117h	350h	720h
2 nd	31-60	39h	112h	284h	720h
3 rd	61-90	100h	250h	720h	/

6.3 Results and conclusion

6.3.1 Methane generation profile

As shown in Fig. 6.1, methane generation profiles mainly composed of two stages: exponential gas production stage and slow stationary gas production stage. Minor difference was shown between control and treated groups during the rapid gas producing period. Then, gas production in Control group stopped with negligible stabilization time. On the contrary, a slow stationary stage appeared in Fe groups, which continued three times longer than gas generation time of Control group. The slow generation phase in Fe groups were mainly because of the promoted hydrolysis and recalcitrant compounds degradation by iron, which will be explained in section 6.3.2. As a result, 35% (1st and 2nd cycles) to 86% (3rd cycle) more methane was generated in Fe groups compared to Control group. Detailed information about the enlarged difference at 3rd cycle would be illustrated in the later section.

Since similar methane production trend appeared among each cycle, the 3rd cycle was chosen as a representative to describe the methanogenic performance. At the fast gas production period, the specific methane production rate was 0.28 ± 0.01 mL/g VSS/h in all groups, which is because a large quantity of easily biodegradable compounds existed in the feed (Table 6.1). Control group stopped gas production at 139 h with 158 mL accumulative methane production. Then, a slow stationary stage continued in Fe groups with average gas production rate at 0.035 mL/g VSS/h (M group) and

0.022 mL/g VSS/h (FeCl_2) and last for more than 500 h. Consequently, the accumulative methane production in M and FeCl_2 reached 294 mL and 254 mL, exhibiting 86% and 61% higher methane production than that in Control group.

Figure 6.1 Methane generation profile of three cycles

6.3.2 DOM transformation

Clearly, DOM fractions were degraded in sequence during the digestion (Fig. 6.2). In general, LMW acids was the most preferable compounds for degradation, followed by LMW neutral, LMW PS/PN, biopolymer and lastly humic substances like compounds (HS). This was evidenced by significant degradation of LMW acids during the first period, and the consumption of HS only started after other compounds degraded to very low concentration. Compared to methane generation profile, similar DOM degradation trend was observed among all cycles, thus the DOM transformation of 3rd cycle was described as an example to show the whole process (Fig. 6.3). LMW acid was the major organic component of the feed accounting for more than 30% of TOC. As an easily biodegradable compound, more than 96% was consumed during the first 100 h degradation. From 250 h onwards, its concentration remained below 1 mg/L-C in all groups. As LMW acid is a typical indicator of AD performance, the minor accumulation suggested stable AD process.

Figure 6.2 DOM compositions at 720h of 1st cycle (a) and 2nd cycle (b)

Hydrophobic compounds, LMW neutral, biopolymer including HMW PN/PS and LMW PN/PS could be compounds existed in feed or hydrolyzed products from macromolecules or particles organics (Lu et al. 2018b). All of them were degraded rapidly in the first 100 h. Specifically, the average decline ratio of HMW PN was 82%, followed by LMW neutral (47%) and LMW PS (44%), LMW neutral (34%) and biopolymers (23%). Although DOM exhibited similar degrading trend during the first 100 h, further analysis revealed that different degradation performance also exhibited among each group. Typically, the hydrophobic compounds in Control group remained as high as 54.1 mg/L-C, whereas the corresponding concentration in M and FeCl₂ group were only 32.8 mg/L-C and 11.8 mg/L-C. During 100 h to 250 h, the quantity of these DOM varied greatly among three treated groups due to the varied extracellular hydrolytic and intracellular metabolic activities. During this period, hydrolysis seemed to be the rate-limiting step evidenced by little LMW acids accumulation, balanced quantity of HMW PN/PS, and increased quantity of hydrophobic compounds. It is worth pointing the microbial metabolism activity associated increase of hydrophobic compounds only appeared in M and FeCl₂ groups, indicating the metabolism activity during this period was more active. On the contrary, 90% of hydrophobic compounds degraded in Control group. From 250 h to 720 h, all of these compounds were further degraded in Fe groups. As a result, the residue concentration of LMW PN and LMW were below 10 mg/L-C among all groups. Biopolymers and LMW neutral exhibited a much higher concentration in Control group, while hydrophobic compounds were at much lower concentration.

Due to the high molecular weight and the aromatic structures, HS was the least favorable compounds. The consumption of HS only started when the availability of other substrates was limited. The concentration of HS was 318 mg/L-C accounting for more than 30% of the TOC in fermented liquor. At the first 100 h, minor reduction was observed for them. From 100 h to 250 h, only 14.5% and 25.7% degradation was

observed for M and FeCl₂ groups, and none degradation was observed in Control group. At the end of this period, the remaining HS were 278 mg/L-C, 217 mg/L-C and 315 mg/L-C in M, FeCl₂ and Control group, respectively. The greatest degradation of HS took place from 250 h to 720 h, when the concentration of other substrates was limited. However, the capability to degrade HS of Control group could not compete with the Fe groups. As a result, the remaining HS in control (221 mg/L-C) exhibited 3.5 times higher than the other two groups (around 63 mg/L).

The remained dissolved TOC in Control group (346.3 mg/L-C) was much higher than treated groups (both around 172 ± 5 mg/L). This fact indicates iron-based material could greatly promoted the degradation of recalcitrant compound. It is noteworthy the final COD difference could only convert into 75 mL methane calculated based on the calculation of COD=49.2+3.00*TOC (Dubber and Gray 2010), and 1g COD can generate 361 mL CH₄ at 35 °C, 1atm. Therefore, the greater methane production of Fe groups also partially came from organic solids hydrolysis.

It is noteworthy the remaining DOM of 1st and 2nd cycles did not exhibit statistical difference between control and Fe groups (Fig. 6.2). In addition, only 35% greater methane was generated in Fe groups in the 1st/2nd cycles, which is remarkably less compared to 3rd cycle. The larger gap of methane production between control and Fe groups of 3rd cycle was because of the composition difference of the fermented liquor, in the quantity of HS (Table 6.1). The fermented liquor for 1st/2nd came from fresh WAS, whereas the WAS for 3rd cycle had preserved at 4 °C for 6 months. The long storage caused the consumption of easily biodegradable compounds and the production of recalcitrant compounds. Therefore, one-folder higher HS existed in fermented liquor of 3rd cycle than 1st/ 2nd cycle. However, greater degradation of recalcitrant compounds occurred in Fe groups than that in Control group (Fig. 6.3). As a result, greater discrepancy of methane production between control and Fe groups was observed in 3rd cycle than 1st/ 2nd cycle.

Figure 6.3 DOM Compositions of 3rd cycle at different time

6.3.3 Microbial community analysis

As shown in Table 6.3, the values of goods coverages were higher than 0.99 for all samples indicating that most operational taxonomic units (OTUs) were identified. The supplement of magnetite nanoparticles and Fe^{2+} did result in reduction of species richness of microbial communities reflected by the values of observed species, Chao 1 richness estimator and abundance-based coverage estimator (ACE). Shannon index reflects alpha diversity, and takes into consideration of both species richness and the evenness in the distribution of individual species. The lower Shannon index in Fe groups suggests the magnetite nanoparticles and Fe^{2+} were in favor the growth of certain species.

Table 6.3 Microbial community analysis results

Sample name	Observed species	Shannon	Chao1	ACE	Goods_coverage
Inoculum	1171	7.483	1184.505	1201.239	0.999
M	995	5.511	1089.033	1123.633	0.997
FeCl_2	1070	5.669	1176.265	1177.698	0.997
C	1128	6.092	1247.715	1248.751	0.996

As shown in Fig. 6.4, great difference was exhibited between inoculum sludge and cultured sludge, which was attributed to operational condition and substrate difference. Among all phylum, *Euryarchaeota* exhibited the greatest difference. After operation for 90 days, its relative abundance increased more than two times suggesting well-established anaerobic condition and methanogenic performance. The microbial community variety also existed among cultured groups. Typically, the abundance of *Euryarchaeota* was 36.9% in Control group, whereas the value reached

46.2% and 47.1% in M and FeCl₂ group, respectively. *Euryarchaeota* can only use limited types of substrate, such as H₂/CO₂, acetate, formate, methanol and methylamines, which are the metabolic products from its syntrophic partners (Blaut 1994). The greater abundance of *Euryarchaeota* suggested more metabolites from its upstream bacteria and greater methane generation, which has been observed in Fig. 6.1. The second varied phylum was *Actinobacteria*, which belong to gram-positive bacteria and are commonly found in soil. In natural environment, they act as fungi in decomposing complex organics from dead organisms and humus. As natural waste enriched in fallen leaves and biological remains, therefore its abundance decreased more than 10% after changed into fermented liquid (Fig. 6.4). Interestingly, its abundance was higher in M (9.3%) and FeCl₂ (9.4%) groups than Control group (7.9%). This was consistent with the greater degradation of HS in Fe group.

Figure 6.4 Microbial community at phylum level

The results from genus level could further illustrate the functions of the bacterial communities. For better understanding the influence of magnetite nanoparticles and Fe^{2+} on microbial community, only genus with abundance more than 0.5% and the difference between control and Fe groups greater than 20% was considered (Fig. 6.5). The growth of *Aneurinibacillus* was closely related with available iron ion. Under iron limited circumstance, it could release siderophores, high-affinity iron-chelating compounds, for serving to iron transport through cell membrane under low iron stress (Kumar et al. 2018, Neilands 1995). In this study, great quantity of iron ion existed in Fe^{2+} supplemented group. Consistent to the characteristics of this bacteria, less than 50% of *Aneurinibacillus* was observed in FeCl_2 group compared with the others. Zhao et al. (2017a) reported functional gene involved in degrading complex organics were increased in presence of magnetite nanoparticles. Here, this superiority was observed in both M and FeCl_2 groups. A typical example was genera *Alkaliphilus* and *Syntrophorhabdus*, both of which are capable of degrading aromatic compounds, and exhibited less relative abundance in Control group. Genus *Alkaliphilus* obtained the whole gene for degrading polycyclic aromatic hydrocarbon, and its sub-genus - *Alkaliphilus oremlandii* *OhILAs* was often adopted as the model species for interpreting the metabolic pathway of polycyclic aromatic hydrocarbon (Wrzodek et al. 2011). Similarly, *Syntrophorhabdus* was the first cultured anaerobe capable of degrading aromatic compounds (Qiu et al. 2008). Besides the previous two genera, species under *Syntrophus* are also widely reported with the capability of degrading benzoate and fatty acids, and its abundance increased by 50% in Fe groups. The recalcitrant characteristics of HS were mainly attributed to the polycyclic or heterocyclic aromatic structure. Furthermore, the abundance of *VadinBC27*, which was predicted to function in degrading recalcitrant organic, also increased greatly in Fe groups. Therefore, it is concluded that the greater methane generation and less remaining TOC, especially HS, was because of enriching functional genera in

degrading refractory compounds in Fe groups.

Figure 6.5 Bacteria abundance compare from genus level

Note: Only relative abundance is more than 0.5%, and the difference between control and M/ FeCl₂ is greater than 20% was exhibited in this figure.

Magnetite nanoparticle is widely reported of triggering direct electron transfer between syntrophic partners. The presence of electroactive strains is the necessary condition for completing this process. *Shewanella* and *Trichococcus* are typical electroactive bacteria, capable of reducing ferric ion as well as contact with electrode for electricity production. Specifically, the relative abundance of *Shewanella* was around 0.6% in M group, but it was not found in the other two groups. *Trichococcus*, functioned in utilizing various polysaccharides for electron generation, also exhibited 2 to 6 times higher abundance than the other two groups. Study of Baek et al. (2015) reported its potential ability of forming electric syntrophy with *Methanosaeta* in

enhancing methanogenic performance. It is noteworthy both *Trichococcus* and *Shewanella* were capable of reducing ferric oxides to ferrous ion under anaerobic condition. However, the release of ferrous iron from magnetite nanoparticles are not significant indicating inactivate iron reducing activity in M group (Table 6.4). The enrichment of *Methanosaeta* and *Methanosarcina*, both of which are capable of accepting extracellular electron directly from magnetite nanoparticles, and the capability of conducting electron to magnetite nanoparticles by the two genera. These facts evidenced the existence of direct electron transfer via magnetite nanoparticles between the syntrophic partners.

Table 6.4 The quantity of Fe^{2+} during 1st and 3rd cycle

Cycle	Group name	Sampling time					
	Sampling time	Feed	21h	68h	117h	350h	720h
1 st cycle	M (mg/L-Fe)	0.9 ± 0.24	2.2 ± 0.35	1.8 ± 0.26	1.8 ± 0.39	1.3 ± 0.15	1.2 ± 0.16
	FeCl_2 (mg/L-Fe)	0.9 ± 0.24	9.4 ± 1.32	60 ± 4.8	50 ± 2.6	30 ± 3.6	30 ± 0.35
	C (mg/L-Fe)	0.9 ± 0.24	1.1 ± 0.33	1.1 ± 0.25	1.2 ± 0.17	0.9 ± 0.21	0.9 ± 0.15
	Sampling time	Feed	100h	250h	720h		
3 rd cycle	M (mg/L-Fe)	0.8 ± 0.16	0.9 ± 0.27	1 ± 0.16	1.2 ± 0.24		
	FeCl_2 (mg/L-Fe)	0.8 ± 0.16	5.3 ± 0.67	5 ± 0.38	4.7 ± 0.67		
	C (mg/L-Fe)	0.8 ± 0.16	0.8 ± 0.43	0.9 ± 0.21	0.8 ± 0.24		

Taxonomic classification showed that the most abundant archaea was assigned to *Methanosaeta*, followed by *Methanosarcina* and *Methanobacterium* (Fig. 6.6). Consistent with the phylum result, the relative abundance of methanogens increased greatly as a result of optimized condition and substrate difference. The dosing of iron-based materials increased the related abundance of methanogens population greatly.

Specifically, the abundance of *Methanosaeta* was 25.8% in Control group, while the corresponding value for M and FeCl₂ groups were 32.1% and 31.1%, respectively. Similarly, greater abundance of *Methanosarcina* and *Methanobacterium* were observed in M and FeCl₂.

Figure 6.6 Archaea abundance compare from genus level

The greater quantity of methanogens could be caused by two factors. One was the addition nutrient iron in stimulating the growth of methanogens, and the other could be more substrates provided (Glass and Orphan 2012). Iron requirement for methanogenesis is vast. Almost every metalloenzyme involved in the methanogenic pathway contains iron, such as the first enzyme in the CO₂ reduction pathway, Ferredoxins that transfer electrons from H₂ to other enzymes, and the Ni-Fe hydrogenase which involved in intermediates electron transfer in methanogenesis (Glass and Orphan 2012). This was true for FeCl₂ group with greater quantity of ferrous ion observed, but did not work for M group as very limited iron was released from magnetite nanoparticles. Furthermore, more pronounced methanogenic

performance was observed in M group compared with FeCl₂. Therefore, another factor, i.e. magnetite nanoparticles triggered DIET, definitely played an important role in promoting the whole process. This was confirmed by enriched electroactive microbes in M group.

6.4 Conclusions

The study demonstrated that addition of magnetite nanoparticles and Fe²⁺ significantly enhanced AD performance in the terms of methane production and organic conversion. Such bio-stimulation with magnetite nanoparticles and Fe²⁺ could promote anaerobic hydrolysis as well as recalcitrant compounds degradation, in particular, aromatic hydrocarbon. This was proved by analyzing the transformation of DOM fractions during the whole AD process. Study on microbial community revealed both magnetite nanoparticles and Fe²⁺ enriched the abundance of bacteria that are capable of degrading complex compounds. An interesting point is that the enhancement of performance was more pronounced in M group than FeCl₂ group. Evidenced by limited releasing of Fe²⁺ and greater abundance of electroactive bacteria, it is concluded the better performance was attributed to magnetite nanoparticles conducted DIET between syntrophic anaerobic partners.

CHAPTER 7

The interactive effects of ammonia and CNT on anaerobic digestion

7.1 Introduction

Carbon-based materials have been widely applied to environmental bioremediation technologies (Hadi et al. 2015, Mauter and Elimelech 2008), such as wastewater treatment (Rivera-Utrilla et al. 2011), pollutant adsorbent (De Celis et al. 2009) and support for biofilm formation (Liu et al. 2010b) in activate sludge processes. Recently, in anaerobic digestion (AD) systems, they were employed for stabilizing and promoting the conversion of organics to methane via conducting direct interspecies electron transfer (DIET) between syntrophic partners (Fagbohunge et al. 2016, Liu et al. 2012a, Zhao et al. 2017a). Besides stimulating organic compound degradation, carbon-based materials also exhibited positive effect on stability of AD (Pereira et al. 2016a), and mitigating toxicity of substrates (Qu et al. 2015). Such technologies were also applied in the engineering AD systems. For instance, Zhao et al. (2017a) reported the potential application of carbon-based material in promoting complex organic compound degradation. In another study, the methane yield of an activated carbon supplemented pilot-scale AD system (1000 L) was increased by 41% compared with none materials supplemented system at same organic loading rate(Zhang et al. 2018). Given the improved performance and shortened retention time, the reactor size can be reduced, or the loading of the systems can be increased significantly.

It is known that the performance of AD is closely related to organic loading rate and the solids concentration in the feed (Mata-Alvarez et al. 2000, Rincón et al. 2008). To improve the loading and solids content, many solids pretreatment methods have been developed, e.g. ultrasound-alkaline (Liu et al. 2008, Tyagi and Lo 2011), thermal hydrolysis process (THP) (Xue et al. 2015) etc. Most pretreatment methods, THP in

particular, would yield high ammonia concentration, typically ranging from 1000 mg-N/L to 4000 mg-N/L (Abu-Orf and Goss 2012, Oosterhuis et al. 2014). Moreover, anaerobic digestion of livestock and food waste often encounter great accumulation of ammonia. The high ammonia condition could greatly disturb the AD performance and inhibit microbial activity (Zhang et al. 2011). The inhibition was often characterized with declined methane production rates and intermediates, typically VFA accumulation (Liu and Sung 2002). Toxicity is usually manifested by a total cessation of methanogenic activity (Calli et al. 2005, Sung and Liu 2003).

It was hypothesized that the strategy to stimulate DIET may help to reduce the accumulation of intermediate products in AD and mitigate the inhibition from toxic organic compounds in the feed. This promoted mechanism was mainly based on the following advantages. Firstly, electron can be directly delivered to syntrophic partner without the involvement of hydrogen. Secondly, the electron transfer speed via DIET is 10^6 times faster than interspecies hydrogen transfer (Cruz Viggi et al. 2014), which allows much more rapid substrate conversion. As the disruption of AD was mainly attributed to the decoupled metabolic activities, the advantage of DIET could greatly alleviate the unsynchronized metabolism between syntrophic partners. This was in fact proven in many studies (Niedzwiecka et al. 2017, Zhuang et al. 2015). For example, Li et al. (2015) reported 1 g/L carbon nanotube (CNT) induced faster substrate consumption and methane production rate via accelerating syntrophic electron transfer (Ambuchi et al. 2017). Another study on pure culture of methanogens showed 5 g/L CNT could improve methanogenesis via reducing redox potential of the environment conditions. DIET is especially helpful in case of complex or toxic organics compounds as feed by promoting faster syntrophic metabolism. However, to date, it is not clear how the carbon-based material would interact with the inhibition from inorganic compounds, typically ammonia. If the

above DIET mechanisms work universally, the inhibition from inorganic compounds should be also mitigated efficiently.

In this study, CNT was chosen as the representative carbon-based materials to explore its role in anaerobic system with the presence of ammonia. During the operation, the methanogenic activity was closely monitored to reveal the performance difference at the coexistence of CNT and ammonia. Intracellular ATP level and potassium transport activity were also investigated to understand the interactive mechanism.

7.2 Experimental set-up

7.2.1 Long-term anaerobic CNT reactor operation under different ammonia concentration

The experiment was conducted in an automatic methane potential test system (AMPTSII, Bioprocess Control Company, Sweden). Control and CNT group (1 g/L CNT) were setup with working volume of 360 mL. Reactors were purged with nitrogen for 10 mins, then operated with continuous stirring at a speed of 80 rpm. The reactors were fed with 1.38 g/L COD, trace elements, and ammonia at various pre-set concentration (Table 7.1 and 7.2), conducting batch feeding mode. The operation can be divided into 5 phases according to ammonia concentration. Ammonia was added into reactors from phase 1500(a) with initial concentration of 1500 mg/L-N, adjusted the pH to 7 ± 0.1 at phase 1500 (b), then additional 1000 mg/L-N was added step-wisely during the following phases. The reason to adjust pH was to remove the inhibitory factor (low pH) on microbial activity. NH_4Cl was used as ammonia source for phase 1500(a). Detailed operating information can be found in Table 7.1 and 7.2. Since the adsorbed ammonia by CNT was less than 2% at all tested ammonia concentration (1500 mg/L-N to 3500 mg/L-N), the interference of adsorption was ignored in this study.

Table 7.1 Operational process of long-term study of CNT group

Phase	None Ammonia	1500 (a)	1500 (b)	2500	3500
NH_4^{+}N (mg/L-N)	0	1500	1500	2500	3500
Duration (h)	1-48	48-545	545-778	778-1084	1084-1504
1 st cycle (h)	48	186	85	108	89
2 rd cycle (h)	\	124	52	70	98
3 rd cycle (h)	\	187	53	71	109
4 th cycle (h)	\	\	43	57	124
Ammonia type	\	NH_4Cl	NH_4Cl	NH_4Cl	NH_4Cl
			NaHCO_3	NaHCO_3	NaHCO_3

Note: Under 1500 mg/L-N condition, the pre-pH adjustment phase named 1500 (a), and post pH adjustment phase named 1500 (b).

Table 7.2 Operational process of long-term study of Control group

Phase	None Ammonia	1500 (a)	1500 (b)	2500	3500
NH_4^{+}N (mg/L-N)	0	1500 (a)	1500 (b)	2500	3500
Duration (h)	1-48	48-434	434-667	667-948	948-1356
1 st cycle (h)	48	94	85	83	89
2 rd cycle (h)	\	100	52	70	98
3 rd cycle (h)	\	195	53	71	109
4 th cycle (h)	\	\	43	57	112
Ammonia type	\	NH_4Cl	NH_4Cl ,	NH_4Cl ,	NH_4Cl ,
			NaHCO_3	NaHCO_3	NaHCO_3

7.2.2 Short-term influence of CNT with the coexistence of ammonia

This short-term study aimed to investigate different concentration level of CNT on

methanogenic performance and intracellular ATP level. Sludge source and operating conditions were similar with the setup described in section 7.2.1. CNT was added to the reactors at a final CNT concentration of 0, 1, 3 or 5 g/L, respectively. Each CNT concentration group was performed in triplicates. Three cycles were operated before ammonia was added in to stabilize the microbial activity. Ammonia (NH_4Cl at 1500 mg/L-N) was then added together with COD substrate into the reactors during the following cycles. The reason to choose NH_4Cl at 1500 mg/L-N was due to its moderate inhibition effect (Fig. 7.1). The methane production profile was monitored by AMPTS system automatically.

Figure 7.1. Methane generation profile at different ammonia concentration

ATP is the product of oxidative phosphorylation, in which electrons are transferred from electron donor (substrate) to electron acceptor. Its production is coupled with catabolism of microbe, and it is a reliable and fast indicator of microbial metabolism activity (Kennicutt 1980). The antagonistic action towards ammonia is an ATP

consumption process (Rajagopal et al. 2013). To reveal the effects from ammonia and CNT on microbial activity, VFA and ATP samples were taken at the beginning of the cycle, the exponential gas production stage, the stationary gas production stage, and the end of gas production stage. Specifically, samples were taken at 7 h, 22 h and 42 h after feeding in no ammonia/control cycle. For ammonia supplemented cycle, the samples taken time were 7 h, 22 h, 46 h, 80 h and 180 h after substrate and ammonia addition.

7.2.3 Batch study on the impact of ammonia and CNT on K⁺ transport

The batch study was conducted in 55 mL serum bottles with 35 mL working volume. CNT concentrations of 0, 1, 3 or 5 g/L were applied in 4 groups of reactors, respectively. Each CNT group consisted of nine identical reactors with the same COD feed and seed sludge. After acclimation for 10 days, samples were taken from each reactor for membrane integrity analysis and background K⁺ analysis. Then, the nine replicate reactors of each CNT group were further divided into three sub-groups, of which three ammonia concentration levels, i.e. 0, 800, 1500 mg/L-N, were achieved by adding NH₄Cl together with the COD substrate. For all the reactors, samples were then taken for potassium measurement at two time points, i.e. before substrate (containing COD and different ammonia concentration) addition, and 8 min after substrate addition.

EPS samples were also taken from each reactor to reveal the potential different impact of ammonia and CNT on cell stress level. Baseline EPS content was identified before NH₄Cl addition, and another batch of EPS samples was taken at 200 h after NH₄Cl supplemented. The net change of EPS, including increase and decrease, was imputed for Spearman correlation analysis.

7.3 Results and discussion

7.3.1 Long-term anaerobic CNT reactor operation under different ammonia concentration

Maximum methane production rate was closely monitored in all the phases. In the first phase - none ammonia phase, a slightly better methanogenic performance was observed in CNT group (2.35 mL/g VSS/h) compared to Control group (2.23 mL/g VSS/h) (Fig. 7.2). In phase 1500(a), with 1500mg/L-N addition, dramatic inhibition on methane production was observed in both groups and a greater inhibition occurred in CNT group at the 1st cycle. Specifically, the specific methane production rate declined to 0.41 mL/g VSS/h in CNT (82.6% decrease) and 1.11 mL/g VSS/h in Control (50% decrease) (Fig. 7.2). In the second and third feeding cycle, the methanogenic activity was gradually recovered in CNT group, whereas the corresponding activity continued to decrease in Control group. Consequently, better performance was shown in CNT group (0.67 mL/g VSS/h) than Control group (0.41 mL/g VSS/h) at the last feeding cycle of phase 1500(a). The existence of carbon-based materials could trigger the DIET process and enhance substrate and fatty acids degradation (Li et al. 2015, Pereira et al. 2016b, Zhao et al. 2017b).

Figure 7.2 Specific methane production rate of five phases

Note: The number in the X- axis means the corresponding ammonia concentration in each phase.

According to Procházka et al. (2012), low buffer capacity could greatly reduce bacterial capability to cope with ammonia. In phase 1500 (b), the pH was adjusted from 6.4 to 7 and maintained at 7 with pH buffer to avoid the low pH impact on methanogens. Methanogenesis activity recovered immediately after the pH adjustment, and CNT group resumed the activity to the none ammonia level at the end of phase 1500(b). However, Control group still had 27% lower activity compared to that of CNT group. Similar results were observed in other studies, where CNT promoted VFA degradation (Li et al. 2015, Pereira et al. 2016b) and AD recovery.

Ammonia concentration increased to 2500 mg/L-N in phase 2500. Greater inhibition

was also observed in CNT group, with 31.7% activity reduction, whereas the corresponding value was 19.2% in Control group. Although the activity of both groups could partially recover from the second feeding cycle, the recovery speed was slower than previous phase. In particular, CNT group was much slower than Control group, indicating CNT may affect AD performance adversely at 2500 mg/L-N condition. When ammonia concentration further increased to 3500 mg/L-N, the performance of both groups was deteriorated. During the four feeding cycles in phase 3500, the specific methane production rate in Control group fluctuated around 1.0 ± 0.08 mL/g VSS/h, whereas the corresponding value continuously decreased from 0.9 mL/g VSS/h to 0.49 mL/g VSS/h in CNT group. It seems methanogenic communities showed the capability to adapt to ammonia inhibition in Control group. Nevertheless, this adaptation may not always result in a complete recovery of AD performance as well as methane yield. Here, the methanogenic activity of Control group, despite decreased in the phase 3500, was not further reduced significantly in the following 3 cycles. However, the CNT group may potentially have earlier system failure with continuous reduction of methanogenic activity.

Although CNT could promote the recovery at lower ammonia concentration (< 2500 mg/L-N), the sudden increase in ammonia concentration always posed greater inhibition on CNT group. Moreover, beyond 2500 mg/L-N, the methanogenic activity of CNT group was always lower than Control group, which was opposite to the expectation that carbon-based materials would enhance AD performance. The mechanism behind this phenomenon was further explored in the following sections.

7.3.2 Short-term influence of CNT with the coexistence of ammonia

7.3.2.1 Methane generation profile

Before ammonia addition, methane generation profile was similar in all groups (Fig. 7.3a). After 1500 mg/L-N was added, inhibition was observed in all groups with greater impact on CNT group. Specifically, the specific methane production rate (R) decreased 52.5% in 0 g/L CNT group, whereas the corresponding reduction were 61.2%, 72.5%, 82.2% in 1 g/L, 3 g/L and 5 g/L CNT group, respectively (Table 7.3). Spearman rank correlation revealed a strong negative correlation between R and CNT concentration ($R_s = -0.972$, $p < 0.01$), indicating that AD performance deteriorated with the increasing CNT concentration at the presence of ammonia. Besides, the lag time was significantly longer in CNT group, and around 30% less methane was observed in 3 or 5 g/L CNT group (Fig. 7.3, Table 7.3). Hence, CNT could further worsen the shock inhibition of ammonia on AD system, which confirmed the results from long-term study (Fig. 7.2).

Figure 7.3 Methane production profile before (a) and after (b) ammonia supplement

Table 7.3 Methane production parameters before and after ammonia addition

	CNT Conc.	R (mL/g VSS/h)	Potential CH ₄ yield (mL)	Lag time (h)	R^2
Before ammonia addition	0 g/L	2.76 ± 0.09	148.89 ± 1.78	2.37 ± 0.45	0.990
	1 g/L	2.78 ± 0.12	144.78 ± 1.59	1.89 ± 0.58	0.982
	3 g/L	2.39 ± 0.07	147.97 ± 2.14	2.96 ± 0.48	0.991
	5 g/L	2.12 ± 0.07	152.10 ± 3.27	3.93 ± 0.62	0.987
After ammonia addition	0 g/L	1.31 ± 0.02	143.80 ± 0.89	3.66 ± 0.45	0.995
	1 g/L	1.08 ± 0.02	127.27 ± 0.83	4.35 ± 0.46	0.995
	3 g/L	0.66 ± 0.01	101.51 ± 0.32	9.38 ± 0.66	0.991
	5 g/L	0.38 ± 0.01	98.78 ± 0.80	19.79 ± 1.12	0.989

7.3.2.2 VFA profile

The accumulation of VFA reflects a kinetic uncoupling between acids producers and consumers and is considered as typical indicator of AD reaction imbalance (Ahring et al. 1995). In this short-term study, VFA profile was closely monitored. Before ammonia supplement, the VFA concentration peaked at 4 h after the pulse feeding with glucose (Figure 7.4 a). At 50 h, VFA concentration was below detectable limitation in all groups (Figure 7.4 a). With the addition of 1500 mg/L-N ammonia, the VFA peaked time delayed by 5 h - 15h depending on the CNT concentration (Figure 7.4 b). Comparing to none ammonia case, the accumulated VFA concentration increased by 31.2%, 51.6%, 36.5%, and 49.2% in 0, 1, 3, and 5 g/L CNT group, respectively. The delayed peak time and higher VFA accumulation indicated both acidogenic and methanogenic microflora was inhibited, while methanogenic archaea suffered more from high ammonia inhibition. After 90 h, limited VFA was detected in 0 or 1g/L CNT group, whereas there were still about 245

mg/L COD and 315 mg/L COD remained in 3 g/L and 5 g/L CNT group (Figure 7.4b). From 180 h onwards, the methane producing rate in 3 g/L and 5 g/L groups was negligible (Figure 7.3), while the VFA in the two groups still remained as high as 184 and 235 mg/L COD till the end of monitoring time (Figure 7.4b). In consistent with methane production profile (Figure 7.3), the accumulation of VFA further confirmed the negative impact of CNT on the AD system.

Figure 7.4 VFA profile before (a) and after (b) ammonia addition

7.3.2.3 Intracellular ATP quantification

In CNT group without ammonia, the intracellular ATP level was similar in all groups at the sampling time points, with a slightly lower level in 3 g/L and 5 g/L CNT group (Figure 7.5a). After 1500 mg/L-N of ammonia addition, 25% to 55% less ATP was observed within all experimental group, suggesting the metabolism was repressed (Figure 7.5b). Specifically, 3 or 5 g/L CNT group had 25% lower ATP compared to 0 g/L CNT group at 22 h (Figure 7.5 b). At 46 h the difference enlarged to 35%, which indicates severer inhibition occurred in high CNT concentration groups. Afterwards, the difference between control and CNT groups was narrowed down due to limited substrate available in control and 1 g/L CNT group. The results demonstrated microbial activity was repressed much more seriously in CNT group.

Figure 7.5 ATP profile before (a) and after (b) ammonia addition

Bacterial antagonistic action towards ammonia is an ATP consumption process (Rajagopal et al. 2013). Studies demonstrated it was free ammonia rather than total ammonia that directly related to AD inhibition. This is because hydrophobic ammonia molecular can diffuse passively into bacterial cell and further convert into ammonia by absorbing proton and result in intracellular pH disturbance. Then, bacteria and methanogens will activate an energy driven detoxicate effect, during which a K^+/H^+ antiporter like process is stimulated to maintain intracellular pH via pumping out K^+ for exchanging proton. Considering the reduced ATP level in CNT group, it can be concluded that the adverse effects was associated with the interruption of ATP generation and/or consumption. In addition, studies evidenced CNT could form potassium porin like channel for K^+ transport through cell membrane (Geng et al. 2014, Lacerda et al. 2013, Lee et al. 2010). Therefore, it is important to track the change of K^+ for better understanding the effects from CNT coupled ammonia inhibition.

7.3.3 Potential synergistic effects of ammonia and CNT on AD

It has been reported that the physical perturbation could be the cause of CNT

cytotoxicity (Liu et al. 2012b). In this study, there was no statistical difference between control and CNT group in terms of crystal violet uptake capability (Table 7.4). This suggests the existence of CNT did not affect bacterial membrane or cell wall integrity, which was also proved by SEM images (Fig. 7.6 b), in which no cell lysis was observed in CNT group. Previous studies also reported that cell membrane remained intact even with CNT insertion (Lacerda et al. 2013, Liu et al. 2013, Qu et al. 2015). On the other hand, both modeling and experimental studies evidenced CNTs could be an ideal transmembrane channel as they obtained graphitic characterization, could insert into the hydrophilic lipid membrane bilayer (Hinds et al. 2004, Lee et al. 2010, Liu et al. 2010a), and could function like biological channels for ion, typically proton and potassium, transport (Geng et al. 2014, Sui et al. 2001). As shown in Fig. 7.6 a, CNT exhibited nanofibers structure and well wrapped the cell surface. Since CNT obtained graphene shell, its rigid characterization made it possible to insert into cell membrane when cells were encapsulated by CNT (Serpell et al. 2016). In addition, many cells exhibited depression deformation with hollow like area on their membrane indicating the successful formation of CNT channel (Geng et al. 2014, Lacerda et al. 2013). Thus, the presence of CNT may affect ion transport that further influence AD performance.

Table 7.4 Crystal violet uptake by anaerobic bacteria

CNT Conc.	0 g/L	1 g/L	3 g/L	5 g/L
X	28.9 ± 3.91%	32.9 ± 4.97 %	30.6 ± 3.45%	32.6 ± 1.74%

Where X is the membrane integrity index.

Figure 7.6 Bacterial were encapsulated by CNT (Fig. 7.6a) and hollow like area onsite bacterial surface (Fig. 7.6b)

Note: The arrow pointed to the hollow like area onsite bacterial surface.

Potassium is the major intracellular cation in bacteria as well as archaea. The uptake and release of K^+ is the most universal process at the change of external environment (Epstein 2003). In this study, the concentration of extracellular K^+ can be affected by three factors in CNT group: the osmotic driven potassium uptake, the efflux of K^+ for anti- ammonia toxicity effect, and the CNT channel conducted potassium transport. In order to reveal the mechanism behind the severer inhibition in CNT group, the uptake of potassium under different ammonia/CNT concentration was closely monitored. As shown in Fig.7.7, extracellular K^+ did not exhibit significant difference among all treated groups ($p > 0.05$, 94.4 mg/L in average) at the end of none ammonia addition cycle. However, the dose of ammonia diverged the quantity of extracellular K^+ among all groups immediately.

Figure 7.7 Extracellular potassium concentration

Note: 0 (a) means the sample taken before substrate addition cycle. 0 (b), 800 (b) and 1500 (b) denote the sample taken at substrate/ammonia added cycle and the ammonia concentration was 0, 800, 1500 mg/L-N, respectively.

Among 0 g/L CNT sub-groups, the extracellular K^+ concentration declined in 0 mg/L-N (new feeding cycles) and 800 mg/L-N groups, whereas the corresponding quantity increased remarkably in 1500 mg/L-N group (Fig. 7.7). This could be because the sudden dose of ammonia increased the medium osmolarity, which is the general driven force for K^+ uptake, thus resulted in less extracellular K^+ . On the other hand, when extracellular ammonia reached inhibition level of 1500 mg/L-N, the process of K^+ efflux for exchanging proton would be activated to maintain the intracellular pH (Martinelle and Häggström 1993, Rajagopal et al. 2013, Sprott et al. 1984, Sprott and Patel 1986). Therefore, it is likely that the elevated extracellular K^+ in 1500 mg/L-N

group was because the potassium efflux caused by antagonistic effects which surpassed its uptake activity driven by osmotic pressure.

Contrary to the effects of ammonia, the existence of CNT may cause the decrease of extracellular potassium. Among 0 mg/L-N groups, less K^+ was observed in CNT groups once COD substrate was added (Fig. 7.7 – 0 (b)). Typically, extracellular K^+ decreased 28.8 % in 5 g/L CNT group (from 94.9 to 64.1 mg/L), whereas only 3.4 % decline was shown in 0 g/L CNT group (from 94.3 to 91.3 mg/L). Moreover, 5 g/L CNT group always exhibited 20 % to 30 % less extracellular potassium than that in 0 g/L CNT group regardless of the ammonia concentration level. These facts evidenced that the existence of CNT could greatly stimulate the uptake of K^+ .

Spearman rank correlation analysis revealed strong negative correlation between CNT and extracellular K^+ ($R_s = -0.807$, $p < 0.01$), while a positive correlation was shown for ammonia and extracellular K^+ ($R_s = 0.398$, $p < 0.05$). In addition, two-way ANOVA analysis concluded that a strong interaction existed within the two factors, namely, CNT and ammonia concentration, which both affected extracellular K^+ concentration ($p = 0.017$). These facts further confirmed CNT acted as channel for potassium influx, bacterial detoxicate activity led to potassium efflux, and the two factors had interactive effects on K^+ transport.

In fact, the influx of K^+ was only observed after the COD feed indicating that K^+ transportation through cell membrane or CNT channel is an energy consuming process (Epstein 2003, Geng et al. 2014). This is reasonable as the diffusion of ion across CNT channel depends on a combination of ion concentration gradient and the electrochemical gradient (Geng et al. 2014, Hinds et al. 2004). The metabolism of substrate would result in activate efflux of proton as well as the generation of electrochemical gradient, thus driven the passive influx of proton and potassium via

CNT channel. Similarly, previous studies also observed ion including proton and K^+ passing through CNT channel to neutralize the osmotic gradient (Fornasiero et al. 2008, Geng et al. 2014). The generated electrochemical gradient, also called proton motive force, was the driven force for ATP generation (Mitchell 1961). The influx of potassium and proton through CNT channel would gradually dispatch the proton motive force, consequently less ATP could be generated. This hypothesis was also evidenced by lower ATP level in the culture of CNT group (Fig. 7.5). As bacterial detoxicate effect is an energy consumption activity, which may further reduce the intracellular ATP level, leading to worsened AD performance in CNT group. It is worth pointing out that NH_4^+ is small ion, which may also pass through the CNT channel and affect bacterial activity. However, as the concentration of extracellular NH_4^+ was too high, its variation was failed to be identified in this study.

7.3.4 EPS fractions influenced by coexistence of ammonia and CNT

The change of EPS constitution is usually considered as the response of microorganism to harsh environmental conditions (Henriques and Love 2007). Confocal Laser Scanning Microscope images showed the uniformly distributed fractions of bound EPS could form into meshwork-like structure as an adaption to exotic materials (Li et al. 2015, Ye et al. 2018). As shown in Table 7.5 and 7.6, bound EPS, especially TB-EPS, showed a stronger correlation with the concentration of ammonia and CNT than SB-EPS ($p > 0.05$) in term of dissolved organic matters as well as other sub-fractions (Table 7.6). This is because bound EPS directly contact with the cell surface and may respond to external circumstance changes first (More et al. 2014, Mu et al. 2012a). Therefore, analysis the change of bound EPS can aid in understanding the response of microorganisms to ammonia and CNT exposure.

Table 7.5 TB-EPS fraction change after ammonia addition (unit: ug/L- C) (Standard deviation range - 1% to 8%)

CNT (g/L))	NH ₄ ⁺ - N (mg/L)	T-DOC	Hydrophobic DOC	Hydrophilic DOC	Biopolymer	HMW PN	HMW PS	HS	LMW Neutral	LMW Acids
0	0	-2499	182	-2735	-1542	969	-2471	-837	-881	504
0	800	21304	4769	16886	1125	4431	-3038	2623	10897	1038
0	1500	6603	874	6056	-442	2512	-2950	563	5374	859
1	0	-7986	-4511	-3334	-1199	-273	-1002	-1436	-1044	239
1	800	1565	-2500	4115	-487	533	-1057	535	3300	786
1	1500	5860	-2315	8822	1492	1218	288	853	5343	761
3	0	-8040	-11203	3483	120	105	10	739	1358	1279
3	800	-4800	-11274	6337	360	457	-100	1108	3586	1206
3	1500	-1153	-10714	9054	1494	860	617	1690	4442	1306
5	0	700	237	510	-590	-439	-138	-5	372	704
5	800	4232	484	3720	1259	716	520	813	1131	587
5	1500	6269	574	5343	1046	724	327	1299	2415	676

A strong positive correlation was observed between the T-DOC from TB-EPS and the ammonia concentration ($R_s = 0.648$, $p < 0.01$) (Table 7.6). This correlation was mainly attributed to the hydrophilic ($R_s = 0.773$, $p < 0.01$) rather than hydrophobic compounds ($R_s = 0.226$, $p = 0.185$) (Table 7.6). Specifically, HMW protein ($R_s = 0.622$, $p < 0.01$), HS ($R_s = 0.616$, $p < 0.01$), LMW neutral ($R_s = 0.737$, $p < 0.01$) were positively correlated to ammonia concentration, while none statistical correlation was observed for HMW polysaccharides and LMW acids ($p > 0.05$) (Table 7.6). The positive correlation of HMW protein with ammonia concentration was consistent with previous study that bacterial detoxicate activity can enhance protein or enzyme secretion (Cao et al. 2017, Zhang et al. 2016a, Zheng et al. 2013).

Humic substances of TB-EPS were mainly derived from the resynthesis of macromolecular organics. Under highly stressed condition, e.g. high ammonia concentration in this study, the inhibition state could induce the production of secondary metabolite, thus promote the production of HS (Zeng et al. 2016). Similar to HS, LMW neutrals were the metabolic by-products under stressful condition (Wang and Zhang 2010, Zhang et al. 2016a). On the contrary, none significant correlation was observed between T-DOC of LB-EPS and ammonia concentration. As for the subfractions of LB-EPS, only LMW neutral and LMW acids showed moderate positive correlation with ammonia concentration ($p < 0.05$). Therefore, bacteria response to ammonia inhibition was more related with TB - EPS fractions.

Table 7.6 Spearman Correlation Coefficients (Rs) between ammonia/CNT and EPS fractions

EPS layer	SB - EPS		LB - EPS		TB - EPS	
Items	Ammonia	CNT	Ammonia	CNT	Ammonia	CNT
T-DOC	0.056	-0.189	0.296	0.107	0.648**	-0.167
Hydrophobic DOC	0.115	0.301	-0.042	0.271	0.226	-0.194
Hydrophilic DOC	-0.065	-0.057	0.547*	-0.176	0.773**	-0.151
Biopolymers	-0.386*	-0.024	-0.145	0.206	0.698**	0.299
HMW Protein	-0.364*	0.218	-0.37	0.677**	0.662**	-0.586**
HMW Polysaccharide	-0.39*	-0.163	0.067	-0.171	0.321	0.773**
HS	-0.239	-0.237	0.319	0.463*	0.616**	0.189
LMW Neutrals	0.409*	-0.024	0.533*	-0.228	0.737**	-0.332*
LMW Acids	-0.285	0.729**	0.384*	-0.191	0.288	-0.103

**Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Compared to ammonia, the correlation between CNT and TB-EPS ($p > 0.05$) or LB-EPS ($p > 0.05$) was minor (Table 7.5 and 7.6). Among T-DOC subfractions from TB-EPS, the significant correlation was only observed with HMW polysaccharide ($R_s = 0.773$, $p < 0.01$) and HMW protein ($R_s = -0.586$, $P < 0.01$) (Table 7.6). HMW polysaccharide has been visualized as fine strands attached on the bacterial cell surface and forming networks for protecting the cells (Sutherland 2001). CNT are graphic nanoparticles and can physically interact with cell surface. As reported by Li et al. (2015) that the excessive production of polysaccharide could be a result of defense against the intrusion of CNT. This could explain the positive correlation between CNT and polysaccharide. As for HMW protein, its correlation with CNT depended on its location. Contrary to the negative correlation between CNT and HMW protein from TB-EPS, the corresponding value of HMW protein from LB-EPS was $R_s = 0.677$ ($p < 0.01$) (Table 7.6). This is probably attributed to the electrostatic attraction between the oppositely charged amino group of proteins and CNT surface (Ye et al. 2018), which led to the outward movement of HMW protein from TB-EPS to LB-EPS.

This study clearly demonstrated EPS, especially TB-EPS, was more sensitive to high ammonia concentration than CNT. The production of HMW protein from TB-EPS could be stimulated under high ammonia as a response to its inhibition. In addition, the secondary metabolism related products, i.e. HS and LMW neutrals, also exhibited strong positive correlation with ammonia rather than CNT. This phenomenon indicated high ammonia was the predominate inhibitor, which was corresponding to the observed methanogenic performance, where the adverse effects of CNT only observed after ammonia addition. The HMW polysaccharide could function in physical protection of bacterial surface. As a response to CNT exposure, its quantity

increased greatly to resist CNT physical interruption on cell membrane (Table 7.6). However, more study is needed to figure out the interaction mechanism between EPS fractions and ammonia/CNT. Isolating individual compounds should be a solution to this question, which, however, is not easy to be operated because of the complex compositions of EPS.

7.4 Conclusions

The effects of CNT on AD performance varied with ammonia concentration. Specifically, CNT could only accelerate the recovery of AD from ammonia inhibition when the ammonia concentration was in the range of 1500 to 2500 mg/L-N, beyond which it could affect AD performance negatively. CNT group always exhibited worse inhibition upon the shock addition of ammonia, characterized by decreased methane production rate, extended lag time, less gas production, as well as higher VFA accumulation. The phenomenon was likely because CNT could form transportation channel for proton and potassium influx thus reduced the proton motive force, which is important for ATP generation. As a result, less ATP could be available for microbial detoxification in CNT group compared to Control group. The change of TB-EPS fractions revealed that the stimulated production of HMW protein was associated with high ammonia concentration and might attribute to microbial anti-toxicity activity. On the other hand, the production of polysaccharide was promoted by the existence of CNT and this could be related with physical protection of cell membrane from CNT intrusion.

CHAPTER 8

Conclusions and Recommendations

8.1 Major findings

Anaerobic degradation has attracted great attention in recent decades. However, its application has greatly been impeded by the low organics converting efficiency as well as its sensitivity to environment such as temperature change and toxic compounds. This thesis focuses on introducing DIET into the AD system by supplementing CM for solving AD application obstacles, namely thermophilic AD start-up, toxic compounds degradation, and low DOM converting efficiency. For better understanding the effects of conductivity on AD system, the interaction effects of CM and inorganic inhibitor-ammonia were also explored. The findings are concluded as follows.

8.1.1 Effects of CM on thermophilic AD reactor start-up

- Methane production rate in CM supplemented reactors was almost two times higher than Control reactors. CM can greatly shorten the start-up period of thermophilic anaerobic system.
- Acetate and propionate were the major VFAs accumulated during the thermophilic recovery stages. The results showed that faster VFAs degradation was observed in CM groups.
- The supplement of carbon-based CM could greatly mitigate inhibition from high hydrogen partial pressure, which indicated CM introduced DIET pathway. Sludge morphology and conductivity further evidenced the existence of DIET after dosing CM.
- Microbial community analysis revealed the enrichment of exoelectrogens in CNT

group. Specifically, *Caloramator sp.*, a candidate of electroactive bacteria, was significantly enriched in the CNT groups (12.89%) as compared to Control group (1.26% only). Together with the doubled abundance of *Methanosaeta* and *Methanosarcina* methanogens, which are capable of accepting extracellular electron directly. It was predicted *Caloramator sp.* and *Methanosaeta/Methanosarcina* established syntrophic DIET in AD system, where CM acted as electron conduit.

8.1.2 Effects of CM on anaerobic phenol degradation

- CM could stimulate phenol degradation up to one-fold higher than that in Control group, which was also confirmed by methane generation profile.
- EPS compounds that functioned as electron shuttles, i.e. protein and humic substances, were greatly enriched at the existence of CM. In particular, CM groups contained 2.3 and 10 to 20 folds higher low molecular weight protein in bound and soluble EPS compared with Control group. In addition, CNT group exhibited 3-fold higher HS in soluble EPS.
- The overall electron transport system activity in CM groups was much higher than that Control group. The addition of CM enriched phenol degradation bacteria—*Syntrophorhabdus*, *Brooklawnia*, *Treponema* and *Syntrophus*, as well as electroactive methanogens—*Methanosaeta*. It was proposed that *Syntrophus* and *Methanosaeta* were the functional genera in methanogenic phenol degradation via extracellular electron transfer.

8.1.3 Effects of CM on organic matters transformation

- The dosing of iron-based materials resulted in 35% to 86% more methane generation in Fe groups compared with Control group. The greater generation of methane was attributed to organic particles hydrolysis and HS degradation.
- Obvious degradation sequence was observed among DOM fractions. In general,

LMW acids were the most preferable compounds for degradation, followed by LMW neutral, LMW polysaccharides/protein, biopolymer and lastly HS. This was evidenced by greatest degradation of LMW acids during the first period, and the consumption of HS was only started after other compounds degraded to limited concentration.

- The supplement of iron-based materials can greatly enrich methanogens, and bacteria that are capable of degrading complex compounds.
- The enhancement of AD performance was more pronounced in magnetite nanoparticle group than Fe^{2+} group. The mechanisms of bio-stimulation by Fe^{2+} and magnetite nanoparticles are different. Evidenced by limited releasing of Fe^{2+} and greater abundance of electroactive bacteria in magnetite nanoparticles group. It is concluded that the better performance was associated with magnetite nanoparticles stimulated DIET.

8.1.4 The synergistic effects of ammonia and CM on AD performance

- The presence of CNT worsened the ammonia inhibition, Typically, with 1500mg/L-N addition, the specific methane production rate declined 82.6% in CNT group, while only 50% decrease was observed in Control group. Spearman rank correlation revealed a strong negative correlation between specific methane production rate and CNT concentration ($R_s = -0.972, p < 0.01$), indicating, with the presence of ammonia, AD performance could be deteriorated with the increasing CNT concentration.
- CNT could shorten the performance recovery period, when ammonia concentration was below 2500 mg/L-N.
- The existence of CNT greatly reduced ATP level in CNT group. Specifically, 3 or 5 g/L CNT group had 25% lower ATP compared to 0 g/L CNT group after 22h of dosing ammonia. Considering bacteria anti-ammonia inhibition activity was an energy driven process, it was concluded that the adverse effects of CNT was associated with

the interruption of ATP generation and/or consumption.

- CNT could potentially interrupt potassium transport, whose activity associated with detoxicate efficiency. Spearman rank correlation analysis revealed strong negative correlation between CNT and extracellular K^+ ($R_s = -0.807$, $p < 0.01$), while a positive correlation was shown for ammonia and extracellular K^+ ($R_s=0.398$, $p < 0.05$). In addition, two-way ANOVA analysis concluded that a strong interaction existed within the two factors (CNT and ammonia concentration) in affecting extracellular K^+ concentration ($p=0.017$). These facts confirmed CNT and ammonia had interactive effects on K^+ transport.
- Study on EPS demonstrated TB-EPS was more sensitive to high ammonia concentration but less sensitive to CNT. Specifically, the production of HS and LMW neutral, which were closely related with secondary metabolism process, exhibited strong positive correlation with ammonia rather than CNT. The secretion of HMW protein was stimulated under high ammonia as a response to its inhibition.
- The generation of polysaccharide was positively related with CNT concentration, which may function in resisting CNT physical interruption on cell membrane.

8.2 Implications of this research

Anaerobic digestion is an economical and sustainable technology in treating various organic wastes with the production bioenergy. Such feature unquestionably attracted its application. However, one key factor that limits its popularity is the comparatively system instability and low treatment efficiency compared to aerobic digestion. Conclusively, the drawback of AD was primarily attributed to uncoupled or slow syntrophic electron transfer. This study demonstrated that the dosing of CM could greatly promote methanogenic performance in different situations, such as short-cut thermophilic AD reactor start-up time, stimulating toxic compounds degradation rate, and improving DOM converting efficiency. Currently, such strategy has already been

applied in full-scale AD systems. For instance, the methane yield of an pilot-scale AD system (1000L) increased by 41% after supplementing activated carbon at the same organic loading rate (Zhang et al. 2018). However, the benefits of CM are not universal. Under ammonia inhibition state, CNT could bring about negative effects on anaerobic process. This fact suggests that engineering-DIET to improve AD performance should be studied in specific case, and prior study is needed for better application.

8.3 Recommendation for future study

- The quantity of CM in AD reactor will be inevitably decreased after a long-term operation because of the discharge with effluent or sludge. For industry application, it is important to know whether the positive effect still exists after removing the CM out of the AD system. Therefore, the role of CM needs to be explored in long-term.
- In studies performed with CM, changes of microbial community were often observed as an evidence to justify the shift of interspecies hydrogen transfer to DIET. This conclusion is achieved from detecting the enrichment of *Geobacter* species, or other bacteria that are capable of conducting electron to electrode or reducing extracellular chemicals. In fact, DIET was only proved in cocultures of *Geobacter metallireducens* with *Methanosarcina barkeri* and *Methanosaeta harundinacea*. However, frequently *Geobacter. sp.* was not detected in the promoted methanogenic system. Therefore, pure culture studies are needed to further understand the collaboration between the enriched exoelectrogenic bacteria and its methanogenic partner.
- It is predicted that exoelectrogens widely exist in natural environment. Currently, that the most commonly adopted technology to identify exoelectrogens was to isolate the strains, and then detect their capability of producing electricity in microbial fuel cell system. However, this procedure is time consuming with huge amount of workload, especially for the complex strains existed AD system. Thus, faster and effective

techniques are urged to be developed to detect and quantify the abundance of exoelectrogens.

- EPS are ubiquitous heterogeneous mixtures of natural organic macromolecules. It can form a protective layer for microbial cells with the thickness up to 1 μm (Flemming et al. 2007, Sheng et al. 2010, Xiao and Zhao 2017b). HS and redox proteins within EPS matrix are widely reported to be electrochemically active (Shi et al. 2016, Xiao and Zhao 2017b). Since CM likely interact with EPS layer first to establish contact with cells, it is important to explore the role of EPS in DIET. In chapter 5, the results of EPS fractions showed greater quantity of HS and protein in CM groups, which were predicted of associated with the extracellular electron transfer. Further study is needed to confirm the hypothesis by identify the protein and HS. In addition, it is interesting to know whether these extracellular shuttles act as competitor or compensator of CM in facilitating syntrophic electron transfer.
- It is unquestionable CM promoted organic compounds degradation. However, studies in chapter 6 observed the superiority of CM was not obvious in converting easily biodegradable compounds. Moreover, promoted glucose degradation was only observed after AD performance became unstable (chapter 4). Study on the metabolic proteins could potentially reveal how CM influence the degradation of different organic compounds, and when the influence of CM could be more effective.
- The positive effects of CM have been proved in AD system operated under different situation. Some wastewater treatment plants have already started its full-scale application. On the contrary, the result of chapter 7 demonstrated the co-existence of high ammonia and CM could introduce further adverse effect on AD performance. Chapter 7 explored the effects from the perspective of anti-toxicity, ATP and potassium transport. In fact, the alteration on microbial community by CM could also attribute to the negative interactive effects. Till now, only two types of acetolactic methanogens are reported to be capable of accepting extracellular electron. Greater abundance of

these two genera were also detected in study of chapter 4, 5 and 7. However, acetolactic methanogens are more sensitive to high ammonia concentration than hydrogenotrophic methanogens. Therefore, the conflict interests of methanogens could also be the cause of deteriorated effects after dosing CM. Therefore, the interactive effects of CM and high ammonia on AD system need to be further explored from the perspective of microbial community.

- The specific surface area determined the conductivity and cell-bacteria contact efficiency, thus it was hypothesized to be closely related with the DIET performance. For example, Dang et al. (2017) reported GAC amended digesters performed better than carbon cloth amend digester, possibly due to the larger specific surface area provided by GAC. However, the surface area of these conductive materials in accelerating DIET kinetics is not yet conclusive. Hence, more studies would be required to optimize the specific surface area for efficiently stimulating DIET in AD system.

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