Impact of iron oxide nanoparticles on the anaerobic digestion process

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**IMPACT OF IRON OXIDE NANOPARTICLES ON THE
ANAEROBIC DIGESTION PROCESS**

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IMPACT OF IRON OXIDE NANOPARTICLES ON THE ANAEROBIC DIGESTION PROCESS

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ABSTRACT

Anaerobic digestion (AD) is a practical option to address the energy depletion and pollution mitigation problems. The anaerobic digestion process is a multi-step reaction where complex organic substrates are converted to a mixture of gases such as methane and carbon dioxide. This conversion process is dependent on the concerted exchange of metabolites among five physiologically different microbial communities. Efforts to improve the anaerobic digestion process have included changes in reactor configurations as well as expensive physico-chemical interventions. Since the core of an AD process is its microcosm, changes in the microbial communities is thought to effect significant changes in the performance of anaerobic digestion.

Hydrolysis is frequently the rate - limiting step in the anaerobic digestion of organic substrates and cellulose is frequently used as the model substrate in the study of the hydrolysis reaction. The key biochemical activities involved in the conversion of cellulose to methane and carbon dioxide include: *hydrolysis*, *acidogenesis*, *acetogenesis* and *methanogenesis*, which also simulates the entire anaerobic digestion process.

The first phase of this study involved a preliminary investigation on the bio-augmentation effect of a pure culture of Dissimilatory Iron reducing Bacteria (DIRB) + iron oxide nanoparticles on the anaerobic digestion of feed sludge. Results obtained from this study led to the hypothesis that the hydrolysis event could be largely influenced by the addition of nanoparticles. This led to the next phase of the study which investigated the augmentation effects of specifically colloidal Iron Oxide Nanoparticles (IONp) on the anaerobic digestion of cellulose primarily to evaluate the hydrolysis potential as well as to study the effects of the IONp on the overall anaerobic digestion process.

Metaproteomic analysis revealed increased *Eubacteria* metabolic activity in response to the IONp augmentation, which resulted in increased enzyme/protein secretion into the mixed liquor. This biochemical event was accompanied by a large increase in soluble COD (837.5 mg/L in the IONp augmented test reactor and 358.4 mg/L within the control) in the early phase of the anaerobic digestion process. Relative and absolute quantification of the secreted proteins revealed a collection of hydrolytic enzymes such as *Endoglucanase*, *Hydrolases*, *Peptidases* and *Proteases* had been significantly expressed by microorganisms associated with hydrolytic activity such as

Kluyveromyces lactis, *Bacillus cereus*, *Pseudomonas sp.*, *Salmonella sp.*, *Yeasts* and *Fungi* in the IONp augmented system in comparison to the control.

Increased *acidogenic* activity could result in metabolic imbalances among the microbial communities which may further manifest as an accumulation of reduced fatty acids and well as hydrogen leading to process AD instability and failure. However, the results of this study indicated that in the presence of IONp, a well-balanced microbial syntrophy among the fatty acid oxidizing bacteria and the methanogens was promoted as evidenced by the comparatively reduced amounts of propionic acid (on day 7, 238.5mg/L in the IONp test reactor and 540mg/L in the control and on day 23, 14.8mg/L in the IONp test reactor and 579.7 mg/L in the control reactor) as well as negligible amount of hydrogen gas detected in the IONp augmented test reactors. Improved hydrolysis together with a well-balanced microbial syntrophy led to the overall improvement of methane production in the IONp augmented test reactors.

A significant increase in the *Eubacteria* 16S rRNA gene copy numbers ($p < 0.001$) had also been noted in the early phase of the anaerobic digestion process. Among the *Methanogen* communities, it was observed that *Methanomicrobiales* and *Methanosaetaeaceae* were the dominant *methanogens*. This indicated that both hydrogenotrophic and acetoclastic methanogenesis had been facilitated in the presence of the nanoparticles.

In this study, it was also noted that the addition of IONp led to the establishment of three distinct microbial phases: microbial stress and cell death of approximately one order of magnitude primarily due to oxidative stress and nanoparticle deposition of the cell surface, followed by microbial rewiring via the production of microbial nanowires and intercellular nanotubes, and recovery. This study presents evidence of intercellular nanotube communication within an environmental sample – i.e., anaerobic microbial consortium subjected to stress. This observation suggested a mode of microbial communication within the anaerobic process not previously explored and which could have implications on anaerobic bioprocess design. Taken together, these results suggest a promising potential for the use of iron oxide nanoparticles as a relatively simple and economically viable intervention to improve both the rate-limiting hydrolysis as well as microbial syntrophy in the anaerobic digestion process.

LIST OF PUBLICATIONS

Journal papers:

1. **John, M.**, A. P. Trzcinski, Y. Zhou and W. J. Ng (2017). “Microbial stress mediated intercellular nanotubes in an anaerobic microbial consortium digesting cellulose.” Scientific Reports **7**(1): 18006.
2. **John, M.**, Y. Zhou, A. P. Trzcinski and W. J. Ng (2017). “Metaproteomic evaluation of iron oxide nanoparticles augmentation on the anaerobic digestion of cellulose.” Nature Sustainability (*submitted*).

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ABBREVIATIONS

AD	Anaerobic Digestion
AFM	Atomic Force Microscopy
AMPTS II	Automatic Methane Potential Test System II
AS	Anaerobic Sludge
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BMP	Biochemical Methane Potential
BSI	Biomass Stress Index
COD	Chemical Oxygen Demand
cATP	Cellular Adenosine Triphosphate
dATP	Dissolved Adenosine Triphosphate
DGGE	Denaturant Gradient Gel Electrophoresis
DIET	Direct Inter species Electron Transfer
DIRB	Dissimilatory Iron Reducing Bacteria
DI	Deionized water
DNA	Deoxyribonucleic acid
FE-SEM	Field Emission Scanning Electron Microscopy
GC	Gas Chromatography
HAC	Acetic acid
HEP	Heptanoic acid
HPLC	High Performance Liquid Chromatography
HPR	Propionic acid
IONp	Iron Oxide Nanoparticles
IRB	Iron Reducing Bacteria
ISO-CAP	iso-caproic acid
ISO-HBU	iso-butyric acid
ISO-VAL	iso-valeric acid

iTRAQ	Iso-Baric Tags for Relative and Absolute Quantification
LC-MS/MS	Liquid Chromatography – tandem Mass Spectrometry
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MRB	Metal Reducing Bacteria
N-CAP	n–caproic acid
N-HBU	n–butyric acid
NTA	Trisodium Nitrilotriacetic Acid
ISO-VAL	iso–valeric acid
N-VAL	n-valeric acid
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
SRT	Solids Retention Time
tATP	Total Adenosine Triphosphate
VFA	Volatile Fatty Acid
WAS	Waste Activated Sludge
WWTP	Wastewater Treatment Plant

CHAPTER 1

INTRODUCTION

1.1 Background

Anaerobic digestion (AD) is regarded as a practical option to address the energy depletion and pollution mitigation problems. Anaerobic digestion technology is used to treat organic wastes/wastewaters because of its economic advantages in comparison to aerobic treatments (i.e., in anaerobic treatment - there is no need for a costly aeration process and a renewable fuel - biogas is also produced in the anaerobic digestion process) this advantage has been more pronounced in recent years because of the rapid increase in energy prices. The gross calorific value (at STP; 15 °C, 1atm) of typical biogas (60% methane content) is 23 MJ/m³ which is about 58% - 64% that of pipeline natural gas (generally 90% methane; 36 – 40 MJ/m³), indicating the economic value of biogas as a renewable source of energy.

One of the problematic, unavoidable and expensive by - product of anaerobic digestion treatment is sludge. Sludge is generally composed of undigested refractory organics and organic matter. In the European Union, annual sewage sludge production has increased by 51% in a 13 year period from 6.5 million dry tons in 1992 to 9.8 million dry tons in 2005 (Kelessidis and Stasinakis 2012). In China, in order to meet the increasing demand to treat the large amount of wastewater generated, over 600 wastewater treatment plants were constructed in the past decade till 2007, resulting in sludge management issues. Sludge production in China is on the rise; marked by an average increase of 5% every year (Tang, Yu et al. 2010).

Treatment of sludge through anaerobic digestion technology is gaining interest globally because of the process's relatively low energy requirement, efficiency, and production of a renewable fuel (Lettinga 1995, Appels, Baeyens et al. 2008, Appels, Lauwers et al. 2011). However, despite the popularity of conventional AD, one of the major drawbacks of this technology is the long sludge

retention time (SRT) required for efficient anaerobic digestion. The SRT is largely influenced by hydrolysis, which is frequently the rate-limiting step in sludge digestion (Keating, Cysneiros et al. 2012) as well as the slow growth rate of the anaerobic methanogens (Pind, Angelidaki et al. 2003). Although improvement of performance by altering process configurations have been successful (Lyberatos and Skiadas 1999, Burton, Stensel et al. 2002, Mosier, Wyman et al. 2005), there is still room for further process improvement.

Because the anaerobic digestion process involves a complex series of biological processes, i.e., *hydrolysis*, *acidogenesis*, *acetogenesis*, as well as *hydrogen- and acetate – utilizing methanogenesis*, and each activity is performed by diverse microbial groups, small changes in the microbial community composition has the potential to effect significant changes in the digester performance and vice versa. Therefore one of the fundamental interventions to improve anaerobic digestion treatment could be through bio-augmentation of the anaerobic digester with an exogenous microbial species to improve substrate utilization. Conversely, augmentation of the anaerobic digester with trace metals or micronutrients may also be considered.

Magnetite (micrometre – sized) has been reported to accelerate propionate degradation by enhancing syntrophy (Cruz Viggi, Rossetti et al. 2014), however its effects on the other trophic groups within the anaerobic digestion is not well understood. Because nanoparticles have been found to be more reactive due to their size and intrinsic properties, it was thought that augmentation of the anaerobic digestion process with iron oxide nanoparticles could produce better results and be an economically viable strategy to enhance the AD process even further.

Therefore, this study examined the effects of iron oxide nanoparticle augmentation on the overall anaerobic digestion bioprocess and evaluated its impact on the anaerobic sludge microbiota.

1.2 Organization of Thesis and Study Rationale

This thesis consists of seven chapters with the following organization:

Chapter 1 presents a brief introduction to the background, objectives and scope of this research.

Chapter 2 presents a comprehensive review of literature about the anaerobic digestion process, Dissimilatory Iron Reducing Bacteria and the use of iron oxide nanoparticles in wastewater treatment.

Chapter 3 elaborates the materials and methods used for the experiments in this study.

Chapter 4 presents an evaluation on the use of synthesized iron oxide nanoparticles on the anaerobic digestion process. Microcrystalline cellulose was used as the substrate to evaluate the hydrolysis potential of the nanoparticles. Metaproteomic analysis of the enzyme secretosome via Iso-baric Tags for Relative and Absolute Quantification (iTRAQ) and metagenomic analysis of the microbial communities through quantitative Polymerase Chain Reaction (RT-PCR) proved that hydrolysis and microbial syntrophy were the key biochemical events influenced by the nanoparticle addition which led to an overall improvement of the anaerobic digestion process and enhancement of methane production.

Chapter 5 extends the study of colloidal iron oxide nanoparticles on anaerobic digestion. Here the microbiological interaction of the nanoparticles and the anaerobic microbiota was studied through Field Emission Scanning Electron Microscopy (FE-SEM) and Atomic Force Microscopy (AFM) and second generation Adenosine triphosphate (ATP) analysis. It was found that microbial stress led to the establishment of intercellular nanotube mediating communication in the anaerobic microbiota. Furthermore, the addition of iron oxide nanoparticles resulted in three distinct microbial phases, which are, microbial stress and cell death followed by microbial rewiring and recovery.

Chapter 6 summarizes the main findings of this study and suggests recommendations for future studies.

The rationale and overall organization of this study is presented in the flow chart provided below;

Impact of Iron oxide Nanoparticles on the Anaerobic Digestion Process

CHAPTER 2

LITERATURE REVIEW

Overview of
Anaerobic Digestion
(AD)

INTRODUCTION

- Background
- Organization of Thesis

Dissimilatory Iron
Reducing Bacteria (DIRB)

Research Gaps

Objectives & Scope

The use of iron oxide in wastewater applications has been previously reported, however several knowledge gaps exist with respect to establishing the effect of these nanoparticles on the microbiota of the AD system as well as the impact on the metabolic and functional aspects of AD.

This research study aims to address this knowledge gap through a metaproteomic and metagenomic approach by identification of highly expressed proteins which provides direct evidence linking the metabolic activities of the microbiome to the functional aspects of AD, genomic study of shifts in the microbial communities as well as cellular ATP and microscopic evaluations on the effect of iron oxide nanoparticles on the AD system.

- I. To evaluate the impact of Iron oxide nanoparticles on the Anaerobic Digestion Process.
- II. To identify the key Biochemical event in the Anaerobic Digestion process most impacted by the Iron oxide nanoparticle augmentation.
- III. To examine the effects of the Iron oxide nanoparticles on the anaerobic sludge microbiota.

CHAPTER 3

MATERIALS & METHODS

CHAPTER 4

Metaproteomic & Metagenomic
analysis on iron oxide nanoparticles
augmentation on the anaerobic
digestion process

CHAPTER 5

Microbial Stress mediated
Inter cellular nanotubes in an
anaerobic microbial consortium
digesting cellulose –ATP, FESEM
& AFM- based evaluation

CHAPTER 6

**CONCLUSION
&
RECCOMENDATIONS**

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Anaerobic Digestion

Anaerobic digestion is a complex process dependent on a series of concerted and balanced metabolic interactions mediated by five physiologically different microbial communities including *hydrolytic bacteria*, *acidogenic (fermentative) bacteria*, *acetogenic bacteria*, *aceticlastic* and *hydrogenotrophic archaea* (Angenent, Karim et al. 2004). The metabolic interactions among the diverse trophic groups are carried out in serial as well as parallel biochemical reactions. Strict anaerobic conditions (oxidation reduction potential (ORP) < -200 mV) are required for the anaerobic digestion process (Appels, Baeyens et al. 2008).

In the anaerobic digestion process, large organic particulates are biologically hydrolysed to smaller soluble intermediates which are subsequently utilized by the acidogens as substrates for energy and growth. The acidogens produce fermentation products such as Volatile Fatty Acids (VFA's), NH₃, CO₂, H₂S and other by-products. The acetogens further digest the higher VFA's and organic acids produced during acidogenesis to primarily acetate as well as CO₂ and H₂. These metabolic intermediates are then utilized by the *methanogenic archaea* resulting in the production of methane (CH₄) and carbon dioxide (Appels, Baeyens et al. 2008).

Figure 2.1. Schematic representation of the decomposition pathways during the anaerobic digestion of activated sludge (and other organic material) (van Haandel and Van Der Lubbe 2007).

Figure 2.2. Composition of the anaerobic digestion gas (based on the assumption of total substrate mineralization) dependant on the mean oxidation state of the carbon in the substrate (Gujer and Zehnder 1983).

Hydrolysis

Hydrolysis is the first step in the anaerobic digestion process. In this process, the hydrolysis of particulate (complex) organic material (such as cellulose, lignin, proteins and lipids) into soluble monomers is achieved through the action of hydrolytic extracellular and cell – bound enzymes such as *cellulase*, *protease*, *lipase* Etc. The hydrolytic microorganisms may be either obligate or facultative anaerobes (Scharer and Moo-Young 1979). Hydrolysis is frequently the rate – limiting step in anaerobic digestion (Batstone, Keller et al. 2002). During the hydrolysis of biosolids, such as sludge, microbial cell walls are hydrolysed and extracellular polymeric substances (EPS) is released; which is further degraded by the acidogens (Appels, Baeyens et al. 2008) .The hydrolytic rate is a function of pH, temperature, type of particulate matter and concentration of hydrolysing biomass (Pavlostathis and Giraldo-Gomez 1991). Cellulose is the model substrate used in the study of hydrolysis and hydrolytic activity. (Angelidaki, Alves et al. 2007). The surface area of contact between the substrate and cellulolytic enzyme complex is a key factor in determining the rate of hydrolysis (Lynd, Weimer et al. 2002). The primary route of methane production from cellulose containing materials is as illustrated below:

Cellulose → Soluble sugars → Volatile Fatty Acids → CH₄, CO₂ (and other trace gases)

Examples of common hydrolytic microorganisms and their respective exo-enzymes and hydrolysis products are listed in Table 2.1 given below.

Table 2.1. **Hydrolysis products of biopolymers under anaerobic conditions** (Gujer and Zehnder 1983).

Biopolymer	Exo-enzymes	Hydrolysis Product	Microorganisms
Lipids	Lipase	Fatty acids Glycerol Alcohols	<i>Clostridia sp.</i>
Proteins	Protease Peptidase	Polypeptides Oligopeptides Amino acids	<i>Clostridium sp.</i> <i>Proteus vulgaris</i>
Cellulose	Cellulase	Polysaccharides Oligosaccharides Glucose	<i>Clostridium sp.</i> <i>Fungi</i> <i>Acetovibrio celluliticus</i> and other microorganisms
Hemi-cellulose (Xylans and Pentosans)	Hemicellulase Xylanase	Polysaccharides Oligosaccharides Hexoses Pentoses	<i>Clostridium sp.</i> <i>Fungi</i>

Acidogenesis

This is the second step of the anaerobic digestion process. The acidogenic or fermentative microorganisms convert the products of hydrolysis such as sugars, amino acids and fatty acids into acetate, propionate, butyrate, alcohols, ketones, CO₂ and H₂. Fermentation of glucose is

accomplished by the fermentative bacteria which are relatively fast – growing bacteria with an estimated doubling time of approximately 30 minutes (Mosey 1983). The group of microorganisms involved in these reactions include the major genera – *Clostridia*, *Acetobacter* and *Pseudomonas*. The fermentative microorganisms are less sensitive to changes in pH and are active at pH ranges between 4.0 – 8.5 (Hwang, Jang et al. 2004). The Embden - Meyerhoff pathway shown in the Fig.2.3 below is generally the main metabolic route in bacteria for the conversion of glucose to organic acids, where energy is primarily derived from the oxidation of glucose to acetate (Mosey 1983).

Figure 2.3. Metabolic pathways inside the acid – producing bacteria (Mosey 1983).

During acidogenesis, the H_2 produced is thought to influence the metabolic pathways for substrate degradation; an increase in H_2 concentration in the liquid phase or partial pressure in the gaseous phase is considered an early indicator of process instability (Rozzi and Remigi 2004)

Acetogenesis

This is the third phase in the anaerobic digestion process. Acetogenic bacteria (acetate and H_2 - producing) for example, *Syntrophomonas wolinii* and *Syntrophomonas wolfei* convert higher fatty acids (such as propionate and butyrate) produced during acidogenesis into acetate, H_2 and CO_2 (McInerney, Bryant et al. 1979). This conversion is influenced by the partial pressure of H_2 . Acetate is subsequently used by the acetotrophic methanogens and H_2 and CO_2 by the hydrogenotrophic methanogens. A syntrophic relationship between the fatty acid oxidizing bacteria and hydrogenotrophic methanogens helps in the maintenance of a low partial pressure of H_2 and formate thereby creating a favourable environment for anaerobic digestion (McInerney, Bryant et al. 1979). Syntrophic fatty acids degradation rate as well as specific growth rates have been shown to be influenced by the inter-microbial distances between the bacterial consortia and archaea (Stams, Sousa et al. 2012). The acetogens have a doubling time of 1.5 to 4.0 days (Mosey 1983). Examples of mesophilic syntrophic fatty acid oxidizers include mainly *Syntrophomonas sp.* and *Clostridium bryantii* (Stieb and Schink 1985).

The Table 2.2. given below illustrates the reactions and standard Gibbs free energy in the conversion of propionate and butyrate to acetate, formate and hydrogen.

Table. 2.2. **Syntrophic butyrate and propionate degradation with associated standard Gibbs free energy changes** (Thauer, Jungermann et al. 1977)

<i>Fatty – acid oxidation by acetogenic bacteria</i>	
$\text{Propionate}^- + 3\text{H}_2\text{O} \rightarrow \text{acetate}^- + \text{HCO}_3^- + \text{H}^+ + 3\text{H}_2$	$\Delta G^\circ = +76 \text{ kJ}$
$\text{Propionate}^- + 2\text{HCO}_3^- \rightarrow \text{acetate}^- + \text{H}^+ + 3\text{HCOO}^-$	$\Delta G^\circ = +72 \text{ kJ}$
$\text{Butyrate}^- + 2\text{H}_2\text{O} \rightarrow 2 \text{acetate}^- + 2\text{H}_2 + \text{H}^+$	$\Delta G^\circ = +48 \text{ kJ}$
$\text{Butyrate}^- + 2\text{HCO}_3^- \rightarrow 2 \text{acetate}^- + 2 \text{HCOO}^- + \text{H}^+ + 3\text{H}_2\text{O}$	$\Delta G^\circ = +46 \text{ kJ}$
<i>Hydrogen and formate utilization by the methanogenic archaea</i>	
$4\text{H}_2 + \text{HCO}_3^- + \text{H}^+ \rightarrow \text{CH}_4 + 3\text{H}_2\text{O}$	$\Delta G^\circ = -136 \text{ kJ}$
$4\text{HCOO}^- + \text{H}^+ \rightarrow 3\text{HCO}_3^- + 2\text{H}_2\text{O} + \text{CH}_4$	$\Delta G^\circ = -130 \text{ kJ}$
<i>Acetate utilization by the aceticlastic methanogens</i>	
$\text{Acetate}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^-$	$\Delta G^\circ = -31 \text{ kJ}$

Figure 2.4. **Microbial syntrophy** (a) Main Metabolic Pathways involved in the conversion of organic matter to methane (b) Redox reactions involved in syntrophic metabolism. At low formate and hydrogen concentrations, the redox potential for formate and hydrogen production is low as indicated by the blue highlighted regions. Unfavourable electron flow which requires reverse electron transfer is indicated in Orange and favourable electron flow is indicated in green colour. Adapted from (McInerney, Sieber et al. 2010).

Methanogenesis

Methanogenesis is the final stage of anaerobic digestion which is carried out by the methanogenic archaea. These methanogens are obligate anaerobes. They have an optimal pH range of 6.5 to 7.2 and are very sensitive to minor changes in pH (Turovskiy and Mathai 2006). Methanogens have a slow growth rate, with an estimated doubling time of 0.2 – 2 days when hydrogen, formate or high levels of acetate are used as substrates (Ghosh, Conrad et al. 1975, Jones, Nagle et al. 1987). Methanogenesis is carried out by two groups of methanogens: the aceticlastic methanogens which produce methane and carbon dioxide by the cleavage of acetate, and the hydrogenotrophic methanogens which use hydrogen as the electron donor and carbon dioxide as the electron acceptor to produce methane. Methane production through acetate oxidation is predominant and represents a minor pathway where the acetogens use carbon dioxide to oxidize hydrogen resulting in the formation of acetate, which is subsequently converted to methane via aceticlastic methanogenesis. In general, approximately 70% of methane is derived from the aceticlastic pathway and about 30% from the hydrogenotrophic pathway (Gujer and Zehnder 1983). It was noted that wherever the acetate concentration was equal to or higher than 350 mg/L, *Methanosarcina* sp. predominated, whereas when the acetate concentration fell below 350 mg/L, *Methanosaeta* sp. became more prevalent (Ehlinger, Audic et al. 1987). The major methanogen genera that have been identified under mesophilic conditions include *Methanobacterium*, *Methanococcus*, *Methanosaeta* and *Methanosarcina* (Demirel and Scherer 2008).

2.2 Dissimilatory Iron Reducing Bacteria (DIRB)

Iron (Fe) has long been recognized as a physiological requirement for life, however for many microorganisms found in soils, water and sediments, the role of Iron extends well beyond that of a nutritional necessity. Fe(II) can function as an electron source for iron-oxidizing microorganisms under both oxic and anoxic conditions and Fe(III) can function as a terminal electron acceptor under anaerobic conditions for iron-reducing microorganisms (Weber, Achenbach et al. 2006). Fe(III) is believed to be the most abundant potential electron acceptor for the anaerobic oxidation of organic contaminants in potential subsurface environments (Coates, Phillips et al. 1996). Previous studies had suggested that humic substances might be dynamically involved in carbon and electron flow in anaerobic environments (Coates, Phillips et al. 1996). The ability of Fe(III) - reducing bacteria to transfer electrons derived from the oxidation of organic matter and / or hydrogen to humic substances has been demonstrated (Lovley 2008). Under anaerobic conditions, this electron transfer yields energy to support growth and stimulate the mineralization of organic compounds (Cervantes, Dijkema et al. 2001). Under anaerobic conditions, in sedimentary environments where Fe(III) is the predominant electron acceptor, Dissimilatory Iron Reducing Bacteria (DIRB) have the ability to oxidize organic contaminants to carbon dioxide and a strong correlation between the activities of IRB, fermentative and respiratory organisms has been established (Lovley 2008).

The wide phylogenetic diversity of microorganisms capable of conserving energy coupled to growth by the dissimilatory reduction of Fe(III) has been identified throughout the *Archaea* and *Bacteria*. Their ubiquitous nature causes these microorganisms (IRB) to be found in a range of habitats and hence they include *hyperthermophiles*, *thermophiles*, *psychrophiles*, *acidophiles* and *alkalophiles* (Bowman, McCammon et al. 1997, Roh, Liu et al. 2002, Kashefi and Lovley 2003, Ye, Roh et al. 2004).

Dissimilatory Fe(III) reducing microorganisms can be broadly divided into two major groups - those that support growth by conserving energy from electron transfer to Fe(III) and those that do not. The dissimilatory Fe(III) reduction (DIRB) process involves the transfer of electrons by microorganisms to an external source of ferric iron which is subsequently reduced to ferrous iron but without the assimilation of iron.

In the terrestrial and subsurface environments, at circumneutral pH, microorganisms which belong to the family *Geobacteraceae* are among the most common and most comprehensively studied IRB (Bond and Lovley 2003). The *Geobacteraceae* are thought to have a significant role in dissimilatory Fe(III) reduction and the oxidation of organic matter in soils and sediments (Coates, Phillips et al. 1996). Apart from the δ -*proteobacteria*, the genus *Shewanella* in the γ -*proteobacteria* is another well characterized group of IRB, however, *Shewanella sp.* is reported to have only a minor role in the reduction of Fe(III) oxides in situ (Snoeyenbos-West, Nevin et al. 2000, Nealson, Belz et al. 2002). Recent evidence also indicates that other organisms belonging to the β -*proteobacteria* might also have a role in the reduction of Fe(III) in sediments, however the extent of this role remains yet to be quantified (Weber, Achenbach et al. 2006). Ferrihydrite reduction by several species of *Geobacter* is reported to have been stimulated by the presence of secondary bacteria including *Lactococcus lactis* (Straub and Schink 2004) indicative of microbial synergism for growth. The versatile behaviour of these facultative Fe(III) reducers suggests potential uses in bioremediation, degradation of recalcitrant organics and other environmental applications.

Iron is ubiquitous in most ecosystems, and the microbial reduction of Fe(III) oxyhydroxides plays a significant role in the biogeochemistry of these systems. Many microorganisms are capable of iron reduction e.g., fermentative bacteria and some sulfate reducers, however in contrast to dissimilatory iron-reducing bacteria (DIRB), they do not effectively couple the oxidation of organic matter to the reduction of Fe(III) (Lovley 1991).

DIRB are generally divided into groups depending on their substrate requirements and on whether they can completely oxidize organic matter to carbon dioxide. Fe(III) reducers which are capable of oxidizing products of fermentation such as acetate are thought to be responsible for the primary reduction of ferric iron in many ecosystems. Most of the DIRB that have been described are members of the delta subclass of the *Proteobacteria* that includes the genera *Geobacter*, *Desulfuromonas*, and *Pelobacter*. Other DIRB include *Shewanella spp.* and *Geovibrio*

ferrireducens, which are affiliated with the gamma subclass of the *Proteobacteria* and the *Flexstipes* lineage, respectively (Lovley 1991, Lloyd 2006).

At circumneutral pH, microbial iron redox cycling can significantly affect the geochemistry of hydromorphic soils and sediments, leading to the degradation of organic matter, mineral dissolution and weathering, and the mobilization or immobilization of various anions and cations, including contaminants (Lloyd 2006).

The redox transition between Fe(II) and Fe(III) valence states has a fundamental role in environmental geochemistry. Microorganisms from both the *Archaea* and *Bacteria* domains are capable of metabolically exploiting the favourable redox potential between the Fe(III)/Fe(II) couple and various electron donors or acceptors. In the anaerobic zone of neoteric environments at pH > 4.0, Fe(III) oxides are readily reduced and provide an important electron sink for both chemical and biological processes (Straub, Kappler et al. 2005). Direct enzymatic Fe(III) reduction by Fe(III)-reducing microorganisms has been proved to be rather substantial, and the activity of these organisms might account for as much as 90% of the oxidation of organic matter; they are further capable of using inorganic electron donors such as hydrogen as well (Bird, Bonnefoy et al. 2011).

Previous studies have also indicated that certain Fe(III)-reducing microorganisms are capable of utilizing structural or crystalline solid-phase Fe(III) as electron acceptors (Fredrickson and Gorby 1996, Kostka, Haefele et al. 1999). The insoluble nature of Fe(III) oxide minerals at pH values > 4.0 creates a metabolic dilemma for microorganisms utilizing Fe(III) oxides as a respiratory terminal electron acceptor. Various mechanisms have been proposed as possible strategies that microorganisms could use to transfer electrons to extracellular Fe (III) oxide minerals.

Direct contact between the microorganisms and the solid phase Fe(III) oxide minerals was shown to be a requirement for the reduction of insoluble Fe(III) oxides in *Geobacter spp.* (Snoeyenbos-West, Nevin et al. 2000). The formation of flagella and pili had been proposed as the mechanism by which *Geobacter spp.* directly attached to the Fe(III) oxide surface (Lovley 2011, Lovley and Malvankar 2015)

Recent evidence however, suggests that pili are not required for the attachment of *Geobacter spp.* to solid phase Fe(III) oxides. Instead, the pili function as an electrical conduit for the transfer of

electrons to insoluble Fe(III) oxides and, potentially other solid phase electron acceptors (Reguera, McCarthy et al. 2005, Lovley 2011). The formation of these conductive cellular 'nanowires' expands the accessible spatial area available beyond the cellular membrane, allowing the penetration of nanometre pore spaces in soils and sediments previously considered unavailable to the cell. The potential of these 'nanowires' to create a bridge between individual cells introduces the potential of cell - cell communication and also for the cell attached to the Fe(III) oxide or other electron acceptor to function as an electron shuttle (Reguera, McCarthy et al. 2005, Lovley and Malvankar 2015). These kind of conductive 'nanowires' are thought to be exclusive to *Geobacter spp.* as the pili produced by other MRB were non-conductive (Reguera, McCarthy et al. 2005). The direct involvement of these appendages in electron transport or Fe(III) reduction has been recently proved by (Reguera, McCarthy et al. 2005, Pirbadian, Barchinger et al. 2014, Lovley and Malvankar 2015).

Figure 2.6. Microbial strategies mediating electron transfer to insoluble Fe(III) oxides. *Three primary strategies have been proposed to facilitate the electron transfer between microorganisms and solid Fe(III) oxide surfaces. a) In Geobacter spp. direct contact with the oxide surface is required. The production of 'nanowires', conductive extracellular appendages, facilitates electron transfer by functioning as an electrical conduit to the Fe(III) oxide surface. b) An endogenously or exogenously produced electron shuttle mediates electron transfer to solid-phase Fe(III) oxides. c) The production of complexing ligands as in the case of Geothrix sp. aids in the dissolution of the solid-phase Fe(III) oxide providing a soluble Fe(III) form more readily available to the microorganism. These strategies have only been demonstrated for Fe(III)-reducing microorganisms (Weber, Achenbach et al. 2006)*

Fe(III) reducers might not have to establish direct contact with the solid-phase surface to bring about Fe(III) oxide reduction, as other alternative active mechanisms include the production of exogenous and endogenous soluble electron shuttles which mediate the transfer of electrons to the solid phase electron acceptors. These electron shuttles alleviate the need for the IRB to directly establish contact with the Fe(III) oxide and functions in a combination of a microbially catalysed and abiotic process; the IRB oxidizes an electron donor coupled to the reduction of the soluble electron shuttle and the reduced electron shuttle diffuses and subsequently donates electrons to the solid-phase Fe(III) oxide abiotically.

The abiotic regeneration of the oxidized electron acceptor restarts this cycle (Lonergan, Jenter et al. 1996). Redox - reactive organic compounds commonly found in soils and sediments, such as humic acids (Coates, Phillips et al. 1996), plant exudates (Snoeyenbos-West, Nevin et al. 2000) and antibiotics have been identified as electron shuttles (Stams, de Bok et al. 2006). Although the significance of these compounds as electron shuttles for microbially mediated Fe(III) reduction in eutrophic environments remains unknown, their utility in oligotrophic environments might be limited owing to the low availability of suitable redox - reactive refractory organic substrates.

Recent studies have suggested that the addition of humics or extracellular quinones could be an alternate strategy to stimulate dissimilatory Fe(III) reduction (Straub, Kappler et al. 2005, MacDonald, Moon et al. 2011). Fe(III) reducers can reduce the quinones in these organics to the hydroquinone state and the hydroquinones can then abiotically reduce Fe(III). The Fe(III) reduction step then oxidizes the hydroquinone to the quinone which can now undergo another cycle of reduction and oxidation. This alleviates the need for Fe(III) reducing microorganisms to directly come in physical contact with insoluble Fe(III) oxides in order to reduce them. Additionally, hydroquinones that are generated via quinone reduction by Fe(III) reducers could possibly enter pore spaces that are too small for Fe(III) reducers to enter and carry out Fe(III) reduction (Lovley 1991, Lovley 2008). These results are relevant to this study, when we consider that sludge is a complex mixture rich in humics and quinones and based on the results of the previous studies we may expect the growth and activity of *Geobacteriaceae* to be enhanced in the anaerobic sludge environment.

Iron reduction has also been demonstrated to occur in activated sludge and may possibly influence floc structure and the precipitation of phosphorus (Nielsen 1996). Acetate is generally the most

common substrate in most activated-sludge systems. Most known DIRB belong to the delta subclass of the *Proteobacteria*, and a few belong to the gamma subclass (Lonergan, Jenter et al. 1996). Beta *Proteobacteria* were the most abundant group found in activated sludge (Nielsen, Juretschko et al. 2002). However, despite this important implication, with the exception of a single isolate of the obligate iron reducer *Geobacter sulfurreducens*, not much information is available about the identity and diversity of iron-reducing bacteria in this ecosystem (Nielsen, Frølund et al. 1997).

Furthermore, the number of iron- reducing bacteria in activated sludge has primarily been determined by using a cultivation - dependant approach (Nielsen, Frølund et al. 1997). Cultivation based approaches tends to provide misleading information about the identity and abundance of DIRB because most of them greatly underestimate the actual number of DIRB since they are difficult to culture. Different techniques for the isolation and enumeration of DIRB have been developed (Lovley 1991, Lonergan, Jenter et al. 1996). The most commonly applied method for the enumeration of DIRB in different environments is the most-probable number (MPN) technique (Nielsen, Frølund et al. 1997). Fluorescence in situ hybridization (FISH) with rRNA - targeted oligonucleotide probes has also been used to detect and quantify bacterial species which are capable of catalysing dissimilatory iron reduction (DiChristina and DeLong 1993).

The importance of Fe(III) - reducing bacteria as an ecologically and environmentally important group of microorganisms has been widely recognized. The metabolic versatility of these microorganisms has resulted in their contribution to a range of biotechnological developments from remediating contaminated environments to harvesting energy (Bond, Holmes et al. 2002, Kim, Park et al. 2002, Bond and Lovley 2003). In this study, chapter 4 evaluated the effects and subsequent microbial transitions as a result of IRB-augmentation on an AD system digesting mixed sludge. In chapters 5 and 6, the effects of iron oxide nanoparticles on the anaerobic sludge microbiota and the anaerobic digestion process is evaluated in detail.

2.2.1 IRB – Bioaugmentation of the Anaerobic Digestion Process

Bio-augmentation is defined as “Introducing/ enriching a specific group of microorganisms into the treatment system which can induce positive effects” on the system/process. In general,

exogenous varieties are introduced into a system under study to broaden the metabolic diversity and versatility of the indigenous microbial community and thereby induce either new or enhanced functions. If the indigenous community contains a certain population of desired microorganisms, their active growth may be biochemically stimulated for enhanced performance (i.e., Bio-stimulation).

A unique group of bacteria called the metal – reducing bacteria (MRB's) have been discovered to gain energy for their growth by oxidizing a variety of organic compounds in conjunction with different metals (for example, Iron, Manganese, Uranium etc.) as the electron acceptor under anoxic conditions (Fredrickson and Gorby 1996, Fredrickson, Zachara et al. 2000).

Some of the major genera of MRB's which have been subject to recent attention are *Geobacter*, *Geothrix* and *Shewenella* which are frequently isolated from soils, freshwater and marine sediments (Coates, Phillips et al. 1996). The versatility in their use of difficult to degrade refractory organics as substrates and therefore the immense potential these MRB's have in bioremediation and bioenergy production has gained attention worldwide.

These MRBs have the ability to utilize recalcitrant organic compounds such as humic substances, phenols, polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) as the electron donor and Fe(III) as the electron acceptor in the dissimilatory iron reduction process (Zhang, Gannon et al. 2010); hence their unique ability to bio-remediate metal – or petroleum - contaminated sites and groundwater. In the past 10 years, the use of various MRBs in anaerobic digestion have been reported (Fig.2.3.2). However most of the reported research were carried out to study the bio-augmentation potential of MRBs with regard to outcomes such as hydrogen production, digester recovery after toxicant exposure etc.(Schauer-Gimenez, Zitomer et al. 2010, Huang, Li et al. 2014).

A strong potential for IRB – bioaugmentation with the objective of improving pollutant removal efficiency and hence resulting in enhanced methane production exists. Wastes/Wastewater is a complex mixture of organics; comprising to a large extent considerable amounts of refractory organic pollutants like industrial, animal wastes, sewage sludge etc. Therefore, within the AD systems, it could be hypothesized that on IRB-augmentation, a complete/incomplete oxidation of organic matter could take place - hence resulting in the release of subsequent substrates (e.g.

VFA's) to the methanogens. This in turn could result in enhanced methanogenesis. Studies have suggested that the enhanced methanogenesis could also be a result of the ambient neutral pH maintained with the AD system as a consequence of maintaining the delicate H_2 balance in the system - thus preventing over acidification and process failure.

It was recently observed that the inclusion of ATCC 55339 (MRB's derived from lake sediments) in conjunction with magnetite ore particles as the Fe^{3+} source, in an AD system was beneficial to the energy recovery efficiency (Hogen, Lighthart et al. 2010). These inventors have claimed, but not proved that even though the background is not very clear, they attribute the increase in biogas yield to the Fe^{3+} which when released from magnetite serves as a terminal electron acceptor for the added MRB's to stimulate their active growth. This resulted in enhanced degradation of organic compounds, thereby producing more substrate for the methanogens to in turn metabolize. However, this potential is still conceptual in nature and lacks fundamental information supporting practical use.

In the recent years, the concept of clean, green energy is gaining importance. Against this background, an important advantage of IRB-augmentation is the reduced formation of problematic sulfhydryl compounds ($R-SH$) as well as hydrogen sulphide (H_2S). This is a useful feature, in that, it will help mitigate odour issues as well as improve the quality of biogas generated. AD-generated biogas typically contains approximately 60 % methane, 30 % CO_2 and trace gases such as N_2 and H_2S . Even though H_2S is a trace gas (less than 1%), it is an important source of odour and corrosion (Truong and Abatzoglou 2005). Burning of biogas may therefore generate sulphur oxides (SO_x) in the exhaust gas, possible causing acid rain. The use of IRBs may involve the following sequence of reactions where, Fe^{3+} is reduced to Fe^{2+} , which in turn binds to the sulfhydryl groups to form an insoluble precipitate of ferrous sulphide (FeS).

A recent study on the factors affecting the anaerobic biodegradation kinetics of Canola oil in freshwater sediments revealed that an optimum dose of ferric hydroxide (10.5g $Fe(III)$ /kg sediment) was found to stimulate anaerobic biodegradation of Canola oil (Li, Wrenn et al. 2005)(18.6g oil/ kg). The effect of iron was shown to maximize methane yield and to minimize the lag time for initiation of active oil degradation (Li, Wrenn et al. 2005).

The versatility in the use of substrates by IRB is demonstrated by the following studies: 1,4 - dioxane is a xenobiotic which is generally recalcitrant to microbial degradation (Shen, Chen et al. 2008). It is used as a stabilizer for chlorinated solvents or is formed as an undesirable by product in many industrial processes. A study on sludge obtained from the Kangzhou municipal wastewater treatment plant and subsequently enriched with of Fe(III) - reducing bacteria demonstrated the ability of Fe(III) - reducing bacteria to anaerobically biodegrade 1,4 - dioxane by utilizing it as a sole carbon substrate for growth. Fe(III) oxides were found to stimulate the activity of Fe(III) - reducing bacteria in the sludge and the humic acids present in sludge were found to promote electron shuttling to Fe(III) in a catalytic manner. This resulted in the increased biodegradation of 1,4 - dioxane.

A study on the suitability of Fe(III) - reducing bacteria to control odour as well as aid in the efficient degradation of swine waste (Coates, Cole et al. 2005) revealed the following interesting outcomes - 1) Stimulated Fe(III) reduction in hog manure resulted in the rapid removal of malodorous compounds (volatile fatty acids) as well as 2) methane production was enhanced by 200 %. Several Fe(III) reducers including *Geobacter grificiae* were shown to readily digest some or all of the malodorous VFA's found in swine manure. This study concluded that Fe(III) supplementation combined with appropriate bioaugmentation provided a simple, cost - effective approach to deodorize and treat swine waste, thus removing a significant impediment to the expansion of pork production facilities. Fe(II) is the non - toxic end product of microbial Fe(III) reduction which can abiotically react with malodorous HS^- ions thus resulting in the formation of non - odorous metal sulphide precipitates. The study also revealed that IRB communities (predominantly *Geobacteriaceae*) were present in swine wastes but were limited by the supply of Fe(III) as the electron acceptor. However, further research is required to ascertain whether the enhancement of methane production was due to either the effective maintenance of a neutral pH in the wastes thereby providing an ambient growth environment for the methanogenic and syntrophic bacteria, or the rapid catabolism of complex organic matter through syntrophic cooperation.

A study by (Stabnikov and Ivanov 2006) on the effect of IRB isolated from anaerobic sludge and Fe^{3+} addition (as ferric hydroxide) for the anaerobic treatment of sulphate - containing wastewater resulted in the following conclusions - there was a significant diminishment in the reduction of

sulphate and sulphide concentration, the total organic carbon degradation was simultaneously increased and there was an increase in methane production. The results of this study is relevant to our proposed use of IRB's in the bio-augmentation of sludge because - in anaerobic wastewater reactors, the Sulphate-reducing bacteria (SRB's) can inhibit the growth and activity of methanogenic microorganisms by the following three major mechanisms 1) sulphide toxicity 2) competition for substrate between SRB's and methanogenic bacteria and 3) precipitation of trace elements by sulphide (Wiemann, Schenk et al. 1998). Therefore, it is important that future studies address efficient and inexpensive methods to inhibit the activity of sulphate - reducing bacteria and the prevention of sulphide production. This is an important aspect in the development of anaerobic technology for the purification of sulphate - containing wastewater.

Another example of the IRB's ability to degrade recalcitrant organics is provided by the following study by (Ivanov, Lim et al. 2010) who investigated the effect of Fe(III) and a facultatively anaerobic strain of IRB on the anaerobic biodegradation of estrogens in reject water. The team concluded that the strain of IRB used was able to degrade 92% of the natural estrogen - 17 β estradiol after 15 days of batch incubation with IRB in reject water.

Fe(III) is generally the most abundant possible electron acceptor for dissimilatory metal - reducing microorganisms in soils and sediments (Bird, Bonnefoy et al. 2011). Microorganisms that conserve energy to support growth via metal reduction directly use electron donors such as acetate, short chain fatty acids, long chain fatty acids, monoaromatic compounds and hydrogen and interestingly, many studies have reported that the addition of electron donors effectively stimulated Fe(III) reduction in batch cultures in both laboratory and field experiments (Fahrbach, Kuever et al. 2006).

In general, Fe(III) reducers may be expected to utilize the fermentation products produced by fermentative microorganisms as evidenced by the increased number of *Geobacter* in glucose amended soils (Coates, Phillips et al. 1996, Snoeyenbos-West, Nevin et al. 2000). However, in contrast to fermentable sugars, acetate, lactate and monoaromatic compounds can be directly oxidized to carbon dioxide by Fe(III) - reducing microorganisms (Ohnishi, Yoshimoto et al. 2004, Fahrbach, Kuever et al. 2006). It has been subsequently established that some Fe(III) reducers, including *Geobacter* species can completely oxidize lactate to carbon dioxide, furthermore, acetate was remarkably effective in stimulating Fe(III) reduction by members of the *Geobacter* family (Coates, Phillips et al. 1996).

Conventionally, it has been accepted that within an AD system, bacterial “cross - talk” with the methanogenic archaea is generally via quorum sensing and the exchange of diffusive carriers such as hydrogen and formate (Batstone, Keller et al. 2002, Appels, Baeyens et al. 2008, Ali Shah, Mahmood et al. 2014); in the presence of (semi)conductive nanoparticles (Kato, Hashimoto et al. 2012), however, a recently discovered phenomenon, Direct Interspecies Electron Transfer (DIET) has been shown to facilitate the direct exchange of electrons among bacteria as well as between bacteria and archaea via microbial nanowires in defined co-cultures and wastewater digester aggregates (Lovley 2011, Morita, Malvankar et al. 2011, Kato, Hashimoto et al. 2012, Pirbadian, Barchinger et al. 2014, Lovley and Malvankar 2015).

The evidence for DIET associated with hydrogenotrophic methanogens frequently detected in anaerobic digesters is mixed. Electrons from insoluble iron oxides and electrodes have supported the growth of hydrogenotrophic methanogens such as *Methanobacterium* (Dinh, Kuever et al. 2004, Karri, Sierra-Alvarez et al. 2005).

Furthermore, growth via DIET mediated by syntrophic interactions is thought to be highly dependent on the organisms present (Rotaru, Shrestha et al. 2014). For example, attempts to promote DIET in CH₄-generating co-cultures and anaerobic digesters have focused primarily on addition of electrically conductive materials such as magnetite and carbon (Rotaru, Shrestha et al. 2014) and it was found that the main biochemical changes in AD affected by the addition of conductive particles included, a reduction in the lag of propionate degradation (Cruz Viggi, Rossetti et al. 2014).

IET (Inter-species Electron Transfer) between syntrophic bacteria and methanogenic archaea is known to occur through the diffusion of electron carriers such as formate and hydrogen in the aqueous phases which may be rate limited by the low concentration of carriers in an AD system (Stams, Sousa et al. 2012). Recent advances in microbial communication have clarified that in the presence of iron oxides, DIRB produce extra-cellular appendages such as conductive pili and microbial nanowires. Microbial nanowires have been determined to be extensions of the outer membrane and periplasm in *Shewenella oneidensis* MR 1 having a unique *beaded* appearance in Atomic Force Microscopy (Pirbadian, Barchinger et al. 2014) (Fig. 6) and *Type IV pili* in *Geobacter sulfurreducens* (Lovley and Malvankar 2015), while *flagella* have also been implicated in iron reduction (Childers, Ciufo et al. 2002).

In the case of extracellular electron transfer mediated by *Shewanella oneidensis* MR 1, it was found that magnetite can compensate for the lack of pilin associated c - type cytochrome in extracellular electron transfer (Liu, Rotaru et al. 2015). Furthermore, methane production via DIET would require ability of the methanogens to directly accept electrons during methane production in conjunction with the DIRB as seen in *Methanobacterium*, *Methanosaeta* and *Methanococcus* (Smith and Ingram-Smith , Dinh, Kuever et al. 2004, Uchiyama, Ito et al. 2010).

The use of nanoparticles for various applications in the electronics, materials engineering, food, cosmetics, energy, pharmaceutical, biomedical, consumer goods as well as environmental industries have seen a significant rise in recent years (Alvarez and Cervantes 2011). However most of these nanoparticles have been engineered or coated with different biomaterials (such as biotin) to enhance their reactivity, stability and function, this has increased the cost of specialized nanomaterial production and made their overall application more expensive (Casals, Barrena et al. 2014). The usefulness of nanoparticles in various applications lie in their small size, quick reactivity, surface area, sequestration capability, Sub-surface transport, physical and chemical properties, effective clean-up of wastes compared to conventional materials, bioremediation Etc. Some nanoparticles (such as – Titanium dioxide, Silicon dioxide, carbon nanotubes, uncoated fullerenes, C60 fullerene, Alumina nanoparticles Etc.) have been found to be toxic to the environment and nanomaterial toxicity to biological systems – with effects such as membrane damage, disrupted cellular functions, infertility, loss of cell structure integrity, anti-microbial activity, abnormal growth function, lipid peroxidation Etc. has been documented (Brar, Verma et al. 2010, García, Espinosa et al. 2011). Therefore, there is widespread debate about the usefulness of *engineered nanoparticles* against their potentially hazardous effects to biological systems.

However, iron oxide nanoparticles are generally regarded as less toxic and can be easily eliminated from a system by high gradient magnetic separation. The use of different iron oxides, iron ores and zero valent iron in wastewater treatments have been reported (Diao and Yao 2009, Liu, Zhang et al. 2012, Huang, Li et al. 2014). However, most of these studies have focused on the use of micrometer size iron oxide particles for the bioprocesses. (Cruz Viggi, Rossetti et al. 2014) reported that micrometer sized magnetite enhanced and accelerated syntrophy during propionate degradation. This group computed theoretical syntrophic calculations to compare the kinetic efficiency of diffusive H₂ exchange and electric conduction via an iron oxide (magnetite) based

conduit by (Cruz Viggi, Rossetti et al. 2014), the difference between these 2 fluxes was found to be very large (i.e., a 10^6 factor) clearly indicative of the kinetic advantage of direct interspecies electron transfer via conduction. The calculations are given below:

Theoretical calculations of Interspecies Electron Transfer via H_2 diffusion and Electron conduction

Figure 2.7. **Proposed electron transfer mechanisms between an Acetogen and a Methanogen in iron oxide supplemented cultures:** (a) interspecies H_2 transfer and (b) electronic conduction through magnetite particles. For each of the two mechanisms, a simplified model to compute the electric current associated with the flux of electron carriers (electrons or H_2) is also depicted. Adapted from (Cruz Viggi, Rossetti et al. 2014)

The electron carrier flux mediated by Interspecies H₂ transfer was computed as presented in studies by (Dong and Stams 1995, De Bok, Plugge et al. 2004, Felchner-Zwirello, Winter et al. 2013) where Fick's law was used to calculate the rate of H₂ diffusion from acetogens to methanogens.

Here, the maximum driving force for H₂ diffusion is given by the difference between the highest concentration of H₂ which can be generated by the acetogen during propionate oxidation (approximately, 180nM, corresponding to $\Delta G = 0$) and the lowest H₂ concentration which can be attained by the methanogens (approximately 0.5 nM, corresponding to $\Delta G = 0$) during bicarbonate reduction. For thermodynamic computation, the following values of substrates and products were used: propionate 5×10^{-3} mol / L, acetate 5×10^{-4} mol / L, bicarbonate 3×10^{-2} mol / L and methane 5×10^{-4} atm. The inter-microbial distance was assumed to be 0.5 μ m between arbitrarily spherical cells with average diameters of 2 μ m and diffusion constant of H₂ in water of 4.5×10 cm²/s (Dong and Stams 1995, Stams, De Bok et al. 2006). Under these conditions the maximum H₂ flux was calculated to be approximately 2×10^{-8} nmol / s, theoretically corresponding to an equivalent electric current of 4×10^{-12} A. Similarly, the maximal electron carrier flux for electronic conduction-based mechanisms via aligned magnetite particles of 100nm (electrical conductivity of magnetite assumed to be 2.5×10^2 Ω cm⁻² (Verwey 1939) conveyed to a methanogen, upon propionate oxidation (using the stoichiometry of the half-life reaction: $4\text{Propionate}^- + 12 \text{H}_2\text{O} \rightarrow 4\text{HCO}_3^- + 28\text{H}^+ + 24\text{e}^-$) with the microorganism carrying out reduction of bicarbonate to methane gas (using the stoichiometry of the half-life reaction: $3\text{HCO}_3^- + 27\text{H}^+ + 24\text{e}^- \rightarrow 3\text{CH}_4 + 9\text{H}_2\text{O}$) with the maximum electron transfer was given by the redox potential (ΔE) of the overall reaction ($4\text{Propionate}^- + 3\text{H}_2\text{O} \rightarrow 4\text{Acetate}^- + \text{HCO}_3^- + \text{H}^+ + 3\text{CH}_4$) which accounted for approximately 0.070V was found to be 3×10^{-5} A. Thus indicating that the maximum electron conduction via magnetite particles was 10⁶ times higher than interspecies H₂ transfer. This theoretical computation by (Cruz Viggi, Rossetti et al. 2014) therefore, could account for the improved methanogenesis via improved syntrophy in the presence of the magnetite nanoparticles seen in the test system.

Studies exploring the use of iron oxide nanoparticles in anaerobic digestion are not well documented with the exception of a study by (Casals, Barrena et al. 2014) who reported the use of engineered serum coated iron oxide nanoparticles to boost biogas production, it was thought the programmed release of Fe²⁺ by the surface coated nanoparticles enhanced biogas production. Given that a significant portion of the nanoparticles used in the various industries would eventually make their way to the wastewater treatment plants, it is imperative to assess the impact of these

nanoparticles on the sludge microbiota. An urgent knowledge gap exists in the study of the effects of *non-engineered* iron oxide nanoparticles on the anaerobic digestion process and specifically the effects of these nanoparticles on the anaerobic sludge microbiota.

2.3 Research Gaps

The use of iron oxide microparticles in wastewater applications have been reported and the results were largely contradictory and inconclusive. Nanoparticles have been shown to be more reactive due to their size and intrinsic physico – chemical properties. However, conclusive knowledge on the effects of iron oxide nanoparticles in the anaerobic digestion process is lacking. This study addresses this knowledge gap by investigating the impact of iron oxide nanoparticles on the anaerobic digestion process and the anaerobic sludge microbiota through metaproteomic and metagenomic analyses. In addition, this study seeks to identify the anaerobic metabolic process/ processes most influenced by the nanoparticles amendment. This would then aid in the development of effective pre-treatment interventions to enhance the anaerobic digestion process.

2.4 Objectives and Scope

The main objectives of this study are:

- 1) To evaluate the impact of iron oxide nanoparticles on the anaerobic digestion process.
- 2) To identify the key biochemical event in anaerobic digestion process most impacted by the addition of iron oxide nanoparticles.
- 3) To examine the effects of the iron oxide nanoparticles on the anaerobic sludge microbiota.

CHAPTER 3

MATERIALS AND METHODS

3.1 Biochemical Methane Potential Test (Automatic Methane Potential Test System (AMPTS II) Bioprocess Control, Sweden)

The design and construction of full-scale anaerobic digesters based on substrates from single or multiple sources requires a good understanding of the physical and biochemical characteristics of each individual substrate. Conventional BMP tests provide a preliminary indication of the biodegradability of a substrate as well as its potential to produce methane via anaerobic digestion.

The AMPTS II (Bioprocess Control, Sweden) had been developed for the on-line measurements of ultra-low biogas and bio-methane flows produced from the anaerobic digestion of biodegradable substrates. The AMPTS II (Bioprocess Control, Sweden) system includes - an automated analytical procedure, real-time data display and analysis overview, real-time data logging of accumulated bio-methane volume and flow rate and an algorithm to avoid overestimation of gas volume and flow.

The standard protocols for the AMPTS II designed by Bioprocess Control, Sweden (BCS) laboratory was used in the experiments.

Instrumentation

In this study, the batch digestion tests for estimating methane potential were carried out using Bioprocess Control's AMPTS equipment. The instrumentation was as shown in (Fig. 3.1 and Fig. 3.2). Unit A was a thermostatic water bath used for reactor incubation. In each reactor a small amount of substrate and inoculum were incubated at the desired temperature and mixed by a slow rotating agitator. Biogas produced in each reactor was passed on to the next unit. Unit B was a CO₂ -fixing unit, the biogas produced in each reactor went through an individual CO₂-trap bottle filled with a pH indicator-alkali solution (3 M NaOH admixed with 0.4% Thymolphthalein

indicator). Gases such as CO_2 and H_2S were removed by chemical reactions with the alkali solution, this ensured that CH_4 was the only gas that passed through the CO_2 -fixing unit. Carbon dioxide fixing bottles were prepared for gases such as CO_2 and H_2S to chemically react with an alkali solution and be separated from the remaining methane. Unit C was the gas volume measuring device where the volume of CH_4 gas released from unit B was measured using a wet gas flow measuring device with a multi-flow cell arrangement (15 cells). This measuring device works according to the principle of liquid displacement & buoyancy and can monitor ultra-low gas flows; a digital pulse is generated when a defined volume of gas flows through the device. An integrated embedded data acquisition system was used to record, display and analyse the results.

Figure 3.1. Experimental set – up for anaerobic batch digestion using the AMPTS II (Bioprocess Control, Sweden).

Figure 3.2. **Schematic representation of the AMPTS system.**

The biochemical methane potential was computed as the accumulated methane produced per gram of VS added in each reactor, as determined by the equation -

$$BMP = \frac{V_S - V_B \frac{m_{IS}}{m_{IB}}}{m_{VS,SS}}$$

where,

BMP - is the normalised volume of methane produced per gram VS of substrate added (NL/gVS)

V_S - is the accumulated volume of methane produced from the reactor with sample (i.e., inoculum and substrate)

V_B - is the mean value of the accumulated volume of methane produced by the 2 blanks (i.e., inoculum)

m_{IS} - is the total amount of inoculum in the sample

m_{IB} - is the total amount of inoculum in the blank

m_{VS,SS} - is the amount of organic material (i.e., volatile solids) of substrate contained in the sample bottle.

The amount of methane produced per gram of VS added and the degradation profile versus time was measured.

3.2 Analytical Methods

3.2.1 pH

pH was measured using a digital pH meter. Values obtained are accurate to within ± 0.02 units.

3.2.2 Total COD and Soluble COD

The standard protocols as described in Standard Methods (APHA 2007) for the measurement of total COD and soluble COD were used in this study.

3.2.3 Total Solids (TS), Volatile Solids (VS), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

The biomass was characterized with respect to the total (TS), Volatiles solids (VS), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS). The standard protocol for the measurement of TS, VS, TSS and VSS as described in Standard Methods (APHA 2007) was utilized. Briefly, empty aluminium dishes (TS and VS), empty aluminium dishes with a filter paper (TSS and VSS) were placed in a furnace at 550 °C for 1 hour. After cooling in a desiccator, the sample was loaded into the pre-weighted dish (TS and VS) for (TSS and VSS) 2 mL was filtered through the filter paper, and the liquid fraction evaporated overnight in an oven at 103 - 105 °C until the weight stabilised. The resulting weight was recorded for the measurement of TS and TSS. The same dish was then placed in a furnace at 550 °C for 2 hours. For VS and VSS measurement, the final weight was then recorded on removal from the furnace and subsequent cooling in a desiccator. Calculations were then performed as given in Standard Methods (APHA 2007).

3.2.4 Volatile Fatty Acids (VFAs)

Analysis of acetic, propionic, n-butyric, iso-butyric, n-valeric, iso-valeric and n-caproic acids was performed using an Agilent gas chromatograph system (6890N) equipped with a flame ionization detector (FID) according to (Zhou, Lim et al. 2012). The different VFA's were separated with a 30 m $\times$ 0.32 mm $\times$ 0.50 μ m DB-FFAP fused silica capillary column. The injector and detector temperatures were set at 260 °C and 300 °C respectively. The oven temperature was initially 60 °C, ramped up to 120 °C at a rate of 20 °C/min and held for 1 min. The temperature was then raised

to 240 °C at 20 °C/min and held for 3 min. Helium was the carrier gas at a constant pressure of 103.0 kPa. The injector was in split mode with split ratio of 50:1. Prior to VFA analysis, test samples were withdrawn from the mixed liquor and centrifuged at 10,000 rpm for 10 minutes followed by filtration of the supernatant through a 0.2 micron syringe filter. The samples were then diluted at 100 µL of 10% formic acid to 900 µL of the sample.

3.2.5 Biogas Composition

The composition of biogas was determined using a Shimadzu GC-TCD fitted with a Porapak N column (1500 × 6.35 mm). The carrier gas was Helium set at a flow rate of 50 ml/min. The column, detector and injector temperatures were 28, 38 and 128 °C, respectively. The peak areas were calculated with a Shimadzu Chromatopac C-R6A integrator. 5 mL samples were withdrawn from the gas sampling ports in the reactors using a gas- tight Agilent glass syringe and the sample was injected into the instrument for analysis.

3.2.6 Preparation of the Iron Calibration Curve – Modified Phenanthroline Assay

Hydroxylamine solution: 10 g of $\text{NH}_2\text{OH} \cdot \text{HCl}$ was dissolved in 100 mL of deionised water.

Ammonium acetate buffer solution: 250 g of Ammonium acetate was dissolved in 150 mL distilled water and 700 mL concentrated Acetic acid was added.

Phenanthroline solution: 100 of mg 1, 10 – Phenanthroline monohydrate ($\text{C}_{12}\text{H}_8\text{N}_2 \cdot \text{H}_2\text{O}$) was dissolved in distilled water. The solution was stirred continuously. 2 drops of concentrated HCl (1 mL is sufficient < 100 µg Fe) was added to the solution.

Stock Iron solution (200mg/L): 20 mL concentrated sulphuric acid was added to 50 mL deionised water. Into this solution 1.404 g of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ was dissolved and 0.1 N Potassium permanganate (KMnO_4) was added dropwise until a faint pink colour persisted, this solution was then diluted to 1 L with deionised water.

Total Iron measurement procedure: 2.5 mL of the sample was pipetted out into a 50 mL volumetric flask and 2 mL concentrated hydrochloric acid, 1 mL $\text{NH}_2\text{OH} \cdot \text{HCl}$ solution, 20 mL of deionised water were added. 2 (diameter 5mm, volume about 0.5 mL) glass beads were introduced into the

mixture and heated to boil for about 10 – 15 minutes. The mixture was cooled to room temperature and 10 mL Phenanthroline solution, 10 mL ammonium acetate buffer solution were added, and the volume made up to 50 mL. Then 0.8 mL deionised water was added to compensate for the volume occupied by the glass beads. The solution was mixed well and incubated for 10 – 15 minutes, absorbance was then measured at 510 nm.

Total Ferrous Iron measurement procedure: The test sample was acidified with 2 mL concentrated HCl / 100 mL sample. 2.5 mL of the test sample was pipetted into a 50 mL volumetric flask and 10 mL Phenanthroline solution, 10 mL ammonium acetate buffer solution was added, the final volume was made up to 50 mL with deionised water. The solution was mixed well and incubated for 10 – 15 minutes. Absorbance was measured at 510 nm.

Total Ferric Iron = Total Iron – Total Ferrous Iron.

Total Dissolved Iron: Iron was filtered through a 0.45 µm membrane filter. 1 mL concentrated HCl / 100 mL sample was added. The Total iron procedure was subsequently followed.

Calibration Procedure:

Solution A (10 mg/L iron): 5ml standard solution was diluted to 100 ml with deionised water.

Solution B (200 mg/ L iron): 5ml standard solution was diluted to 50 ml with deionised water.

Table 3.1. Iron Measurement Table (for standard curve)

Concentration (mg/ L)	0	1	5	10	20	40	60	80	100	120	160	200
Add (mL)	0	0.25	0.625	2.5	2.5	0.5	0.75	1.0	1.25	1.5	2.0	2.5
		A	B	A	B							

Procedure: The required volume of solution was added into the volumetric flask. To this solution, 2 mL concentrated HCl, 1 mL NH₂OH.HCl, add 20 ml deionised water were added; 2 (diameter 5 mm, volume about 0.5 mL) glass beads were introduced into the mixture and heated for about 10

– 15 minutes. The mixture was cooled to room temperature and 10 ml Phenanthroline solution, 10 ml ammonium acetate buffer solution were added, and the final volume was made up to 50 mL. Subsequently, 0.8 mL deionised water was added to compensate for the volume occupied by the glass beads. The solution was mixed well and incubated for 10 – 15 minutes. Absorbance was measured at 510 nm.

Figure 3.3. **Phenanthroline Assay.** *In this colorimetric assay, the intensity of the colour change is proportional to the concentration of iron in the sample.*

3.2.7 Extraction and Quantification of Fe^{2+} ions and Total Fe^{3+} ions

Briefly, 2 mL of test sludge was mixed with 8 mL of 0.5 N hydrochloric acid taken in a capped test tube, 2 glass beads were added into the mixture to enhance mixing. The test tube was capped and incubated at room temperature on an orbital shaker for 30 minutes to facilitate the extraction of Fe^{3+} and Fe^{2+} iron from the sludge sample. The samples were then centrifuged at 10000 r.p.m for 10 minutes and the supernatant was filtered through a 0.2 μm syringe filter. This filtrate was used for quantification of Fe^{3+} and Fe^{2+} iron.

3.2.8 Preparation and Characterization of colloidal Iron oxide nanoparticles

A homogenous, aqueous suspension of magnetite (Fe_3O_4) nanoparticles without the use of any surfactant was synthesized according to the methodology described (Kang, Risbud et al. 1996).

Briefly, the synthesis was performed in an aqueous solution maintaining a molar ratio of Fe (II)/Fe (III) = 0.5 and pH = 11- 12. The requisite volume of Milli-Q water was deoxygenated by Nitrogen gas purging prior to the synthesis reaction. 3.4 ml of 12.1 N HCl was slowly added to 100 mL of deoxygenated water, followed by the subsequent dissolution of 20.8 grams FeCl₃ and 8 grams of FeCl₂. This solution was then added drop-wise to 1000 mL of 1.5 M NaOH solution with constant stirring. A black precipitate of Fe₃O₄ was generated. The paramagneticity was checked by placing a super - magnet near the black precipitate. This precipitate was isolated in a magnetic field and the supernatant was decanted. The precipitate was then suspended in deoxygenated Milli - Q water and centrifuged at 4000 rpm. The supernatant was discarded. This centrifugation step was repeated three times. This was followed by neutralization of the anionic charges on the nanoparticles by the addition of 2000 mL of 0.01 M HCl to the black iron oxide precipitate with constant stirring. Finally, the suspension was again centrifuged at 4000 rpm and the cationic colloidal nanoparticles were separated and peptized by the addition of deoxygenated water. A colloidal suspension of iron oxide nanoparticles was generated.

Nanoparticle characterization (size and morphology) was carried out on vacuum freeze - dried samples by Field Emission Scanning Electron Microscopy (FESEM) JEOL JSM-7600F. The nanoparticles were uniform in shape distribution and almost spherical with an average diameter of 8.7 ± 2 nm. The nanoparticle structure was determined via the Bruker D8 Advance X-ray Diffractometer (XRD) with monochromatic high intensity Cu K α radiation ($\lambda = 1.5418$ Å).

Calculation of Molarity (M) of Iron oxide nanoparticle suspension was performed using the formulae given below,

$$M = \frac{g \text{ Magnetite}}{mL \text{ of solution}}$$

$$M = \frac{g \text{ Magnetite}}{mL \text{ of solution}} \times \frac{1 \text{ mol magnetite}}{\text{molecular wt. of magnetite}}$$

Figure 3.4. **Scanning Electron Micrograph of the synthesized Iron oxide nanoparticles.** *Image magnification is 100000X. The scale bar is 100nm.*

Figure 3.5. Scanning Electron Micrograph of the synthesized Iron oxide nanoparticles and particle size measurements.

The iron oxide nanoparticles were found to be roughly spherical with relatively uniform particle diameters. The nanoparticle size measurements are shown in the electron micrographs. Magnification is 400000X. Scale bar is 10nm.

Element	Weight %	Atomic %
C K	18.91	45.62
O K	9.44	17.10
Al K	0.05	0.05
Si K	0.14	0.14
Fe K	71.46	37.08
Totals	100.00	

Figure 3.6. **FE-SEM Energy Dispersive X ray Spectroscopy (EDX) analysis of the synthesized nanoparticles.** *The elemental composition of the iron oxide nanoparticle mixture is presented in the spectrum analysis and table. Scale bar is 10nm.*

Figure 3.7. **Laboratory synthesis of iron oxide nanoparticles.** a) *This image represents the nanoparticle synthesis process by Co-precipitation.* b) *The paramagneticity test. In this image, the synthesized (freeze-dried) iron oxide nanoparticles (indicated by the pink arrow) are seen attracted to a magnet.*

3.3 DNA Sample Preparation and Extraction

Sludge samples were initially diluted with DI water. The diluted sludge samples were centrifuged at 10,000 rpm for 5 minutes and the pellet washed with phosphate buffered saline (PBS), this washing cycle was repeated three times to remove any impurities and inhibitory substances in the sample before DNA extraction. The washed pellet was then re-suspended in PBS and the final volume made up to 1mL. 100µL of this processed sludge sample was then loaded into an automated nucleic acids extractor (MagNa Pure Nucleic Acids Extractor, Roche, Germany). A standard pre-loaded nucleic acid extraction protocol was initiated. The final DNA elution volume was set at 100µL. The extracted DNA was stored at -20°C before further analyses.

3.4 Real-time Quantitative Polymerase Chain Reaction

The abundance of the 16S rRNA gene of each of the microbial communities was quantified by RT-PCR on a Roche LightCycler 480 II system (Germany). DNA samples from each reactor was analysed in duplicate and the average of four replicates from each reactor test set are reported in this study. Shifts in 16S rRNA gene copy numbers of the *Eubacteria*, two hydrogenotrophic orders – *Methanobacteriales* and *Methanomicrobiales* as well as an acetoclastic order comprising two families – *Methanosarcinaeaceae* and *Methanosaetaceae* during AD was monitored. The primer/probe sequences of the target microbial communities were adapted from (Yu, Lee et al. 2005) and are listed in (Supplementary table 1). The probes as well as the forward/reverse primers were synthesized by TIB MolBio (Berlin, Germany). Each well of the qPCR LightCycler microplate contained a reaction mixture of 2µL DNA template, 1 µL forward primer, 1 µL reverse primer, 2 µL TaqMan probe (community – specific), PCR – grade water as well as 10 µL of LightCycler mastermix (Roche, Germany). The final reaction volume in each well was 20 µL. All samples were analyzed in duplicates. The microplates were sealed and loaded into a Roche LightCycler 480 – II (Roche Diagnostics, Mannheim, Germany) for the qPCR reaction. The construction of the standard curves are as described by (Bialek, Kim et al. 2011).

A two-step amplification of the target DNA, combining the annealing and the extension steps, as described in a previous study (Yu, Kim et al. 2006) with slight modifications was followed.

Briefly, the 2 step protocol included an initial incubation step for the activation of Taq DNA polymerase at 95° C for 10 minutes followed by thermal denaturation at 95° C for 10 minutes (55 cycles) and simultaneous primer annealing and extension at 60° C for 30 seconds. The emitted fluorescence signal data was recorded in the “single mode” and the results were processed with the Roche LightCycler Software version 4.0.

Table 3.2. Composition of Taq man probe and primer sets (Yu, Lee et al. 2005)

Set name	Target group	Oligonucleotide sequence (5' → 3') ^b
ARC787F	<i>Archaea</i>	F: ATTAG ATACC CSBGT AGTCC
ARC915F		T: AGGAA TTGGC GGGGG AGCAC
ARC1059R		R: GCCAT GCACC WCCTC T
BAC338F	<i>Eubacteria</i>	F: ACTCC TACGG GAGGC AG
BAC516F		T: TGCCA GCAGC CGCGG TAATA C
BAC805R		R: GACTA CCAGG GTATC TAATC C
MBT857F	<i>Methanobacteriales</i>	F: CGWAG GGAAG CTGTT AAGT
MBT929F		T: AGCAC CACAA CGCGT GGA
MBT1196R		R: TACCG TCGTC CACTC CTT
MMB282F	<i>Methanomicrobiales</i>	F: ATCGR TACGG GTTGT GGG
MMB749F		T: TYCGA CAGTG AGGRA CGAAA GCTG
MMB832R		R: CACCT AACGC RCATH GTTTA C
Msc380F	<i>Methanosarcinaceae</i>	F: GAAAC CGYGA TAAGG GGA
Msc492F		T: TTAGC AAGGG CCGGG CAA
Msc828R		R: TAGCG ARCAT CGTTT ACG
Mst702F	<i>Methanosaetaceae</i>	F: TAATC CTYGA RGGAC CACCA
Mst753F		T: ACGGC AAGGG ACGAA AGCTA GG
Mst862R		R: CCTAC GGCAC CRACM AC

Adapted from Yu et al. (2005). F, T, and R indicate forward primer, TaqMan probe, and reverse primer, respectively.

3.5 iTRAQ – based Proteomic Analysis

Identification of proteins in the secretosome and their relative quantification is a key facet in elucidating the key microbial players and fundamental metabolic processes that are either enhanced or suppressed on the augmentation of iron oxide nanoparticles. Meta – proteomic analysis via the Iso – Baric tags for Relative and Absolute Quantification (iTRAQ) coupled to a 2 – dimensional LC-MS/MS was conducted according to protocols described by (Zhou and Chen 2011) with minor modifications as given below.

Protein Extraction, Digestion and Labelling

3 mL of mixed liquor was withdrawn through the sampling port from each of the reactors (C1, C2, C3, T1, T2 and T3) at selected time points of the anaerobic digestion reaction. Samples C1, C2 and C3 were combined together to form one control set (C); similarly, samples T1, T2 and T3 were combined together to form a test set (T) for that time point. In this study, relative quantification of the secretosome proteins expressed in the control (C0 and C7) and test set (T0 and T7) on day zero and day seven was studied. The samples were centrifuged at 10000 rpm at 4° C for 5 minutes, the supernatant (containing the secreted proteins) was transferred to a new test tube and was subsequently used for the proteomic analysis. The proteins in the secretosome were quantified via the Bradford assay. Sample volumes were then adjusted to generate a final concentration of 40 µg protein in each of the samples (Control and Test) before commencing the iTRAQ protocol. The proteins were precipitated by the addition of 4 volumes of cold Acetone to each of the samples followed by overnight incubation at – 20° C. After incubation, the samples were centrifuged at 12000 rpm for 5 minutes and the acetone was gently decanted. The pellet was air dried and then subjected to protein reduction and cysteine blocking. The standard protocol as provided by the iTRAQ Reagents Multiplex Kit (Applied Biosystems, Inc., Foster city, CA) was followed. Briefly, samples were reduced by the addition of 20 µL Dissolution buffer and 1 µL Denaturant. This was followed by the addition of 2 µL Reducing reagent and incubation at 60 ° C for 1 hour. 1 µL of Cysteine Blocking reagent was then added to each of the tubes followed by incubation for 10 minutes at room temperature. The proteins were then digested by the addition of 10 µL trypsin solution to each of the tubes and subsequent overnight incubation at 37° C. Post incubation, the samples C0, T0, C7 and T7 were labelled with iTRAQ reagent- labelled tags 114, 115, 116 and

117 respectively and incubated at room temperature for 1 hour. The labelled samples were then pooled together and condensed to an approximate final concentration of 1 µg/µL.

On-Line 2D Nano LC/MS/MS Analysis

An online 2D Nano- LC-MS/MS (Agilent 1200 Series LC system, interfaced with a 6530 Q-TOF mass spectrometer) was performed on the iTRAQ reagent – labelled samples (Tang, Feng et al. 2013). An online 2D Nano- LC-MS/MS (Agilent 1200 Series LC system, interfaced with a 6530 Q-TOF mass spectrometer) was performed on the iTRAQ reagent – labelled samples. Briefly, 4 µg of the pooled iTRAQ reagent - labeled peptides was introduced into the system (first dimension) and then separated by a strong cation exchange (SCX) nano-column (0.32×50 mm, 5 µm) (Agilent Technologies, CA). Stepwise elution of the labelled peptides was achieved by the injection of a gradient (concentrations were 20, 40, 60, 80, 100, 200, 500 and 1000 mM) ammonium formate solution.

In the second dimension, the sequentially eluted peptides from the SCX column were subsequently trapped in a Zorbax C18 enrichment column (0.3×5 mm, 5 µm). They were subject to an isocratic wash with 97% solvent A (water with 0.1% formic acid) and 3 % solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 4 µl/min. After the elution of the peptides and subsequent binding on to a C18 reverse phase column integrated with a nano – LC Chip, the peptides were eluted out with 95% solvent A (water with 0.1 % formic acid) and 5% solvent B (acetonitrile with 0.1% formic acid) at a flow rate of 3 µl/min over 45 minutes. The flow was maintained at 90% B for 5 minutes before being set back to the initial state for 10 minutes to recondition the column. Each round consisted of 9 runs. Electrospray analysis was conducted through a 6530 Q-TOF mass spectrometer, interfaced with a Nano-LC-Chip Cube nano - spray source. Mass spectrometry data was obtained using a The Agilent Masshunter Workstation Q-TOF. Survey scans were acquired from m/z 450-1500 at 3 Hz, with up to two precursor's selected for MS/MS from m/z 50-2000 at 2 Hz.

LC/MS/MS Data Analysis

Peptides in each of the samples were identified by the Spectrum Mill system (Agilent Technologist, CA). The Uni Prot KB / Swiss-Prot Protein database (Geneva, Switzerland) was

used for peptide comparison and analysis using each of the LC/MS/MS spectrums obtained. The high confidence for searched proteins was carried out by auto-validation. The relative abundances of the identified peptides were considered for further analysis if the peptide expressed was greater than 0.5 fold.

3.6 Adenosine triphosphate (ATP) Analysis

QuenchGone21™ Wastewater Test Kit (LuminUltra Technologies Ltd. New Brunswick, Canada) which utilizes second generation ATP technology was used to monitor Total and Dissolved ATP with the experimental test sets. This test kit is known to provide real-time measurement of the active biomass population, biomass stress level as well as solids viability in a biological wastewater treatment system.

The QuenchGone21™ Wastewater Test Kit (LuminUltra Technologies Ltd. New Brunswick, Canada) is based on the principle of measurement of ATP (which is a direct, interference-free, microbiologically non-specific indicator of the total living biomass. The ATP is measured using the luciferase assay, wherein, a study sample containing ATP is introduced into a solution containing the Luciferase enzyme to produce a quantifiable light reaction. The light produced is detected in a luminometer as Relative Light Units (RLU).

Some definitions relevant to this study and evaluation are as follows:

Total ATP (tATP)

Is a measurement of all the ATP within a sample, including ATP from living cells in addition to ATP that has been released from dead cells.

$$tATP \text{ (ng ATP / mL)} = \frac{RLU_{tATP}}{RLU_{ATP1}} \times 11 \text{ (ng ATP / mL)}$$

Dissolved ATP (dATP)

It is defined as a measurement of only the dead biomass. It represents the ATP present within a sample that has been released from dead cells.

$$dATP \text{ (ng ATP / mL)} = \frac{RLU_{dATP}}{RLU_{ATP1}} \times 101 \text{ (ng ATP / mL)}$$

Cellular ATP (cATP)

Represents ATP from living microorganisms. Hence, cATP is a direct indicator of the living microbial population (quantity) and represents the ATP contained within the living cells.

$$cATP \text{ (ng ATP / mL)} = tATP \text{ (ng ATP / mL)} - dATP \text{ (ng ATP / mL)}$$

Biomass Stress Index (BSI)

Represents the stress level experienced by the microbial population present within the test system. It is representative of the microbiological quality of the biomass in terms of stress experienced. It is an indicative parameter to monitor the toxicity within a bioreactor.

$$\text{BSI (\%)} = \frac{\text{dATP (ng ATP / mL)}}{\text{tATP (ng ATP / mL)}} \times 100\%$$

ATP extraction and analyses was carried out according to standard kit instructions. Briefly, the assay steps are as follows;

1) ATP Standard Calibration

The ATP Standard Calibration (ATP1) converts luminometer RLU values into real ATP concentration. One calibration was carried out per test set. Briefly, using clean pipet tips, two drops of (100 μ L) of Ultracheck 1(standardized ATP solution) was added to 300 μ L of Luminase taken in a 12 $\times$ 55 mm assay tube. The mixture was gently mixed. 100 μ L of the sample was loaded onto a microplate well and promptly inserted into a TECAN infinite 200 PRO microplate reader and luminescence was measured. Each of the measurements were carried out in duplicates. The measured value was designated as RLU_{ATP1} .

2) Total ATP (tATP) Analysis

Total ATP analysis of individual samples withdrawn from each of the triplicate reactors at different sampling time points was performed as follows;

2.1) Extraction

The test sample was mixed well. Using a wide-mouth pipette tip, 1mL of the sludge sample was added to 2 mL UltraLyse 30 (Extraction Tube). The tube was capped and inverted three times to ensure thorough mixing. This tube was then incubated for one minute to facilitate extraction.

2.2) Dilution

The contents of the UltraLyse 30 (Extraction Tube) post incubation was poured into a new eight mL UltraLute/Resin (Dilution) tube. The mixture was transferred back and forth between the two

tubes several times to ensure thorough mixing. The Ultra Lute/ Resin (Dilution) tube beads were then allowed to settle.

2.3) Assay

Using a clean pipette tip, 100 μ L of the Ultra Lute/ Resin (Dilution) tube content was added to 300 μ L of Luminase taken in a 12 $\times$ 55mm test tube. The mixture was gently swirled, 100 μ L was sample was withdrawn and loaded onto the prepared microplate, which was then inserted into the TECAN infinite 200 PRO microplate reader and luminescence was measured.

3) Dissolved ATP (dATP) Analysis

Dissolved ATP analysis of individual samples withdrawn from each of the triplicate reactors at different sampling time points was performed as follows;

3.1) Dilution

Using a clean pipette tip, 100 μ L of well-mixed anaerobic sludge reactor sample was added to 10 mL LumiSolve (Stabilizer) Tube. The tube was capped and inverted three times to allow through mixing. The sample was then incubated for one minute.

3.2) Assay

Using a clean pipette tip, 100 μ L of the LumiSolve (Stabilizer) Tube mixture containing the test sample was added to 300 μ L of Luminase taken in a clean 12 $\times$ 55mm assay test tube. The mixture in the test tube was gently swirled, 100 μ L of the sample was extracted and loaded into a prepared microplate which was promptly inserted into the TECAN infinite 200 PRO microplate reader and luminescence was measured.

Equations provided by the kit manufacturer, which are provided in section 3.6. was used to determine Biomass Stress Index and concentrations of cellular ATP.

3.7 Field Emission Scanning Electron Microscopy (FE-SEM)

Samples extracted from the bioreactors were immediately frozen at -80°C for 30 minutes followed by freeze-drying under vacuum (Labconco 73820 FreezeZone Plus™ 4.5 Liter Cascade Freeze Dry System, Kansas City, MO) at 0.021 mBar and -70°C for 18 hrs - 20 hrs.

The samples were mounted on a carbon tape and sputter-coated with Platinum (JEOL JCF-1600) (Fig.3.8) prior to examination by Field-Emission Scanning Electron Microscopy (FE-SEM, JEOL JSM – 7600F) at 5kV acceleration voltage.

Elemental mapping was performed using an Energy Dispersive X-ray spectrometer (EDX) (Oxford Instrument, X-max, 80 mm²) attached to the FE-SEM.

Figure 3.8. **Preparation of samples for FE-SEM Energy Dispersive X ray Spectroscopy (EDX) analysis.** *The freeze-dried samples for analysis were mounted on a carbon tape and sputter coated with Platinum at 5kV acceleration voltage using JEOL JCF-1600 coater.*

3.8 Cell imaging by Atomic Force Microscopy (AFM)

At selected data points, samples were extracted from the reactors and 0.5 mL of the sample was placed on a clean glass slide and gently smeared. The smear was air-dried and immediately

observed using an Atomic Force Microscope (XE-100 AFM, Park Systems Corp., Suwon, Korea)
Images were collected in ambient air in both contact and non-contact mode.

CHAPTER 4

METAPROTEOMIC AND METAGENOMIC ANALYSIS OF IRON OXIDE NANOPARTICLES AUGMENTATION ON THE ANAEROBIC DIGESTION PROCESS

4.1 Abstract

This study investigated the impact of iron oxide nanoparticles (IONp) on the Anaerobic Digestion (AD) process. Quantitative PCR results revealed an initial loss of microbial numbers followed by recovery. Increased *Eubacteria* activity in response to the iron oxide nanoparticles was indicated by a significant increase in the 16S rRNA gene copy numbers from 2.73×10^{10} to 1.15×10^{11} ($p < 0.001$) accompanied by an increase in the relative abundances of secreted proteins on day 7 of the anaerobic process. Furthermore, comparatively higher methane production was noted in the nanoparticle augmented reactors. *Methanomicrobiales* and *Methanosaetaeaceae* were the dominant methanogens. Analysis of the protein secretosome in response to IONp revealed increased relative abundances of proteins relating to the Inosine monophosphate (IMP) and glycolysis pathways in addition to ribosome biogenesis and stress-related proteins. IONp augmentation enhanced hydrolysis and facilitated inter – species syntrophic association which resulted in enhanced methane production.

4.2 Introduction

Anaerobic Digestion (AD) is regarded a practical option to reducing energy consumption and environmental pollution problems (Lee, Kim et al. 2008, Shin, Han et al. 2010). Anaerobic digestion is dependent on a series of concerted and balanced metabolic interactions mediated by five physiologically different microbial communities including hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria, acetoclastic and hydrogenotrophic archaea (Angenent, Karim et al. 2004). Hydrolysis is the first step and is frequently the rate – limiting step in the degradation of complex organic substrates (Appels, Baeyens et al. 2008). Hydrolysis is mediated through the extracellular secretion of hydrolytic enzymes primarily by members of the *Eubacteria*. An increased surface area of contact between the hydrolytic enzyme complex and substrate has been shown to be important for efficient hydrolysis (Adney, Rivard et al. 1991, Lynd, Weimer et al. 2002, Zhang and Lynd 2004). Metabolic imbalances in syntrophic association between the fatty acid oxidizing bacteria and hydrogenotrophic methanogens can also result in impaired anaerobic digestion. The catabolic interactions mediated by syntrophic bacteria are in many cases thermodynamically unfavorable, however, interactions are thermodynamically feasible when reducing equivalents, such as H_2 are efficiently scavenged by their syntrophic partners, such as methanogenic archaea (Hansen, Schmidt et al. 2004, Stams, Sousa et al. 2012) (Sieber, McInerney et al. 2012, Stams, Sousa et al. 2012). Electric syntrophy via Direct Inter species electron transfer (DIET) is a recently established phenomenon that has been shown to occur primarily in the presence of (semi) conductive iron oxide nanoparticles. DIRB such as *Geobacter sp.* use conductive minerals as electron conduits and establish an electric syntrophy via DIET with the methanogenic *Archaea* (Lovley 2011, Kato, Hashimoto et al. 2012).

In the study of microbial interactions within natural and engineered ecosystems, high throughput metaproteomic and metagenomic analysis has proved indispensable and more representative of the microbial communities over the conventional culture – based methods (Desai, Pathak et al. 2010, Armengaud, Hartmann et al. 2013, Chourey, Nissen et al. 2013). This study evaluated the impact of iron oxide nanoparticles on the anaerobic digestion of cellulose through quantitative polymerase chain reaction (where changes in 16S rRNA gene copy abundances was used to study the microbial community shifts during anaerobic digestion) and Iso – Baric tags for Relative and Absolute Quantification (iTRAQ) coupled to a 2 – dimensional LC-MS/MS to detect and quantify the

relative abundance of secretosome proteins. This study had aimed to determine if iron oxide augmentation enhanced hydrolysis and improved syntrophic associations among the microbial communities which resulted in improved methane production.

4.3 Materials and Methods

4.3.1 Inoculum and Substrate

The inoculum used in this study was anaerobic sludge obtained from a mesophilic anaerobic digester treating municipal sludge at a reclamation plant in Singapore. The inoculum was stored in a cold room for 5 days and subsequently degassed as reported by (Angelidaki, Alves et al. 2009) before use in the Automatic Methane Potential Test System II. The sludge characteristics were as follows: Total Solids = 12.86 g/L, Volatile Solids = 9.59 g/L, Total Suspended Solids = 12.4 g/L, Volatile Suspended Solids = 9.7 g/L and pH = 6.8. The substrate was microcrystalline cellulose (Sigma Aldrich) and used at 3.4g cellulose/L water. Theoretically, methane and carbon dioxide would be produced from cellulose in the molar ratio of 1:1 as follows:

Cellulose has often been accepted as the model substrate to study the hydrolysis reaction (Zhang and Lynd 2004, Keating, Cysneiros et al. 2012). The key biochemical activities involved in the conversion of cellulose to methane and carbon dioxide involve microbial communities that include: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Scharer and Moo-Young 1979) which closely simulates the whole anaerobic digestion process.

4.3.2 Biochemical Methane Potential Tests

The Automatic Methane Potential Test System II (AMPTS II) (Bioprocess Control AB, Sweden) was used to evaluate the effects of iron oxide nanoparticle augmentation on the anaerobic process. The AMPTS II (Bioprocess Control AB, Sweden) parallel batch reactor system was used for the detection and estimation of methane from each of the reactors in real-time. The standard protocols for the AMPTS II (Bioprocess Control AB, Sweden) were used in this study. Three reactors were used for each set of tests. These were: (i) Control (C1 – C3) set which contained 140 ml of anaerobic inoculum sludge, 1.53 grams cellulose powder mixed in 210 ml of deoxygenated water and 100 ml of deoxygenated water to obtain a final working volume of 450 ml, (ii) Test reactors (T1-T3) which contained 140 ml anaerobic inoculum sludge, 1.53 grams cellulose powder mixed in 210 ml of deoxygenated water and 100 ml of synthesized colloidal nano iron oxide suspension and, (iii) Blank reactors (B1-B3) which comprised 140 ml anaerobic sludge and 310 ml

deoxygenated. The total volume of each AMPTS batch reactor is 600 ml and the reaction volume 450 ml. Each of the reactors was connected to the carbon dioxide absorption unit and flushed with nitrogen gas for 2 minutes each to maintain an anaerobic environment. The batch reactors were subsequently connected to the on-line gas monitoring system.

The AMPTS batch tests were conducted over a period of 40 days. The incubation temperature was maintained at $35 \pm 1^\circ\text{C}$. The initial pH across all the reactors was 7.4. The background methane production from the seed (blank) as determined with the blank assays with inoculum and no substrate, was subtracted from the methane produced in the substrate assays (Angelidaki, Alves et al. 2009).

4.3.3 Analytical methods

4.3.3.1 Wastewater Physico-chemical Analysis

TS, VS, TSS, VSS, TCOD and SCOD measurements were conducted in accordance with the standard methods (APHA 2007). Analysis of acetic, propionic, n-butyric, iso-butyric, n-valeric, iso-valeric and n-caproic acids was performed as detailed in section 3.2. Extraction and Quantification of Fe^{2+} ions and Total Fe^{3+} ions is as described in section 3.2.6 and 3.2.7.

4.3.3.2 Preparation and Characterization of colloidal Iron oxide nanoparticles

The synthesis is as described in section 3.2.8

4.3.3.3 DNA Sample Preparation and Extraction

The experimental protocols are as detailed in section 3.3.

4.3.3.4 Real-time Quantitative Polymerase Chain Reaction

The experimental protocol followed is as detailed in section 3.4.

4.3.4 iTRAQ - based Proteomic Analysis

Meta – proteomic analysis of the secretosome via Iso – Baric tags for Relative and Absolute Quantification (iTRAQ) coupled to a 2 – dimensional LC/MS/MS as described by (Zhou and Chen 2011) with minor modifications was conducted according to protocols detailed in Chapter 3 (section 3.5) .

Protein Extraction, Digestion and Labelling

The tests were evaluated in triplicate. 3 mL of mixed liquor was withdrawn through the sampling port from each of the reactors – Control (C1, C2, and C3) and the iron oxide nanoparticle – augmented Test reactors (T1, T2 and T3) at selected time points of the anaerobic digestion reaction. Control samples C1, C2 and C3 were pooled together to form one control set (C); similarly, test samples T1, T2 and T3 were combined together to form a test set (T) for each individual sampling time point.

In this study, relative and absolute quantification of the secretosome proteins expressed in the control (C0 and C7) and test set (T0 and T7) on day zero and day seven respectively was further evaluated. Protein secretosome quantification was conducted via the Bradford assay. Sample volumes were then adjusted to generate a final concentration of 40 µg protein in each of the samples (Control and Test) before commencing the iTRAQ protocol. Detailed description of the iTRAQ protocol is provided in chapter 3 (Materials and methods) under section 3.5.

On-Line 2D Nano LC-MS/MS Analysis

An online 2D Nano- LC-MS/MS (Agilent 1200 Series LC system, interfaced with a 6530 Q-TOF mass spectrometer) was performed on the iTRAQ reagent – labelled samples (Tang, Feng et al. 2013). Online 2D Nano- LC-MS/MS (Agilent 1200 Series LC system, interfaced with a 6530 Q-TOF mass spectrometer) was performed on the iTRAQ reagent – labelled samples.

Electrospray analysis was conducted through a 6530 Q-TOF mass spectrometer, interfaced with a Nano-LC-Chip Cube nano - spray source. Mass spectrometry data was obtained using a The Agilent Masshunter Workstation Q-TOF. Survey scans were acquired from m/z 450-1500 at 3 Hz, with up to two precursor's selected for MS/MS from m/z 50-2000 at 2 Hz. Detailed experimental protocols are provided in Chapter 3 (Materials and Methods) section 3.5.

LC/MS/MS Data Analysis

Peptides in each of the samples were identified by the Spectrum Mill system (Agilent Technologist, CA). The Uni Prot KB / Swiss-Prot Protein database (Geneva, Switzerland) was used for peptide comparison and analysis using each of the LC/MS/MS spectrums obtained.

The high confidence for searched proteins was carried out by auto-validation.

The relative abundances of the identified peptides were considered for further analysis if the peptide expressed was greater than 0.5 fold.

4.3.5 Statistical Analysis

Two tailed Student *t*-test with equal variance was used to determine the statistical significances in the anaerobic digestion process between the Control and Iron oxide nanoparticles augmented Test reactors as well as to compare the statistical differences in the microbial populations between the Control reactors and the iron oxide nanoparticles augmented Test reactors throughout anaerobic digestion. In the formation of the null hypothesis, α was defined as 0.05 and the confidence intervals on the difference between the mean was adjusted to 95%. Results were considered statistically significant when $p \leq 0.05$.

4.4 Results and Discussion

4.4.1 Bioprocess Performance

Significant increases in cumulative methane production from the substrate was noted between day 5 and day 8 in the test reactors (152.3 NmL) when compared to the control (3.4 NmL) (Fig.4.1).

Figure 4.1. **Accumulated methane production as a function of time** from (1) Blank containing only inoculum, (2) Control containing inoculum and microcrystalline cellulose, (3) Test containing inoculum, microcrystalline cellulose and iron oxide nanoparticles. Background methane is not subtracted from any of the reactors. Each data point represents the average values of triplicate sets. The error bars represent the standard deviation among the replicates.

Similarly, on day 11, comparison of the differences in methane produced from the substrate between the control (149.53 NmL) and test sets augmented with iron oxide nanoparticles (412.3 NmL) revealed statistically significant ($p < 0.002$) volumes of methane were produced in the test reactors relative to the controls.

Evaluation of volatile fatty acids produced in the control revealed the presence of reduced fatty acids such as propionate and butyrate (Fig. 5.2) together with an accumulation of soluble COD from the start of anaerobic digestion till day 7 which was found to be statistically significant ($p < 0.0005$), (Fig. 4.3). The accumulation of Propionate control reactors from the start of anaerobic digestion (11.8 mg/L) till day 7 (540.1 mg/L) was found to be statistically significant ($p < 0.01$). This suggested an unsuccessful syntrophic association between the fatty acid oxidizing bacteria and hydrogenotrophic archaea. In contrast, samples analyzed from the test reactors showed negligible concentrations of reduced volatile fatty acids (Fig. 4.2) together with low amounts of soluble COD (Fig. 4.3) this suggested a balanced syntrophic partnership between the fatty acid oxidizing bacteria and the hydrogen scavenging methanogens.

Figure 4.2. **Changes in volatile fatty acids composition** as a function of time in the control and test reactors. Volatile fatty acids abbreviations are as follows; *HEP* (Heptanoic acid), *N-CAP* (n-caproic acid), *ISO-CAP* (iso-caproic acid), *N-VAL* (n-valeric acid), *N-HBU* (n-butyric acid), *ISO-HBU* (iso-butyric acid), *HPR* (Propionic acid), *HAC* (Acetic acid)

Figure 4.3. **Changes in soluble COD as a function of time.** *Each of the data points represents the average values of triplicate sample sets. The error bars represents the standard deviation among the replicate sample sets.*

Mutualistic syntrophic interactions among the fatty acid oxidizing microorganisms and the hydrogenotrophic methanogens has been thought to occur via the diffusion and consumption of hydrogen/formate. Metabolic imbalances among these syntrophic partners could result in a digester imbalance and eventual process failure. (Sieber, McInerney et al. 2012). Accumulation of large amounts of propionic acid is indicative of process failure. Propionate was estimated to be 14.8 mg/L on day 23 in the test reactors and 579.7 mg/L in the control reactors.

The addition of iron oxide nanoparticles promoted the establishment of Dissimilatory Iron reducing bacteria (DIRB) indicated by the increase in Fe^{2+} iron (Fig. 4.4). Direct Inter-species Electron Transfer (DIET) has been proved to occur via (semi) conductive iron oxide in DIRB - enriched pure cultures from acetate which resulted in accelerated methanogenesis (Kato, Nakamura et al. 2010, Kato, Hashimoto et al. 2012). The low amounts of reduced fatty acids detected throughout the experiment in the presence of iron oxide nanoparticles (Fig. 4.2) as well as the improved methane production (Fig. 4.1) suggested the possible establishment of DIET among the microbial communities.

Given that the only difference between the control and test reactors was the presence of iron oxide nanoparticles in the test reactors, the establishment of a balanced syntrophic association in the test reactors could largely be attributed to the presence of iron oxide nanoparticles and the DIRB community - which helped in the creation of a favorable environment for the anaerobic digestion process primarily in terms of pH conditions (Table 4.1) as well as prevention of accumulation of reduced volatile products and hydrogen within the test system (Fig. 4.2).

Table 4.1 Changes in pH during anaerobic digestion

TIME (days)	Blank 1	Blank2	Control 1	Control 2	Control 3	Test 1	Test 2	Test 3
0	6.8	6.8	7.4	7.4	7.4	7.5	7.5	7.5
7	7.3	7.3	5.6	5.6	5.5	6.6	6.4	6.6
23	7.4	7.2	6.1	6.3	6.3	7	6.9	7
40	7.2	7	6.7	6.7	6.8	6.9	6.8	6.9

Figure 4.4. Production of Fe (II) as a function of time. *Each data point represents average values of triplicate reactor sets. The error bars represent the standard deviation values of the replicate reactor sets.*

4.4.2 Microbial Response to Iron oxide Nanoparticle Augmentation

Quantitative PCR analysis of the 16S rRNA gene copy numbers revealed three distinct microbial phases as a result of iron oxide nanoparticle addition: initial “microbial shock” or cellular stress followed by acclimatization/adaptation phase with a rapid increase in both the total *Eubacteria* ($p < 0.001$) and *Methanogen* 16S rRNA gene copy numbers and finally the establishment of a syntrophically balanced high – performance anaerobic digestion system.

Initial contact between the microorganisms embedded in the sludge flocs and the nanoparticles resulted in a net decline in 16S rRNA gene copy numbers across all microbial communities (Fig.4.5. (a–f)). *Eubacteria* showed the largest decline in gene copy numbers (9.74×10^{10} to 2.73×10^{10}) followed by the *Methanogens* (6.63×10^8 to 2.89×10^8). Nanoparticle toxicity has been largely attributed to release of free ions due to instability and agglomeration (Codina, Ascensión Muñoz et al. 1998, Brar, Verma et al. 2010, García, Espinosa et al. 2011). In this experiment, the colloidal iron oxide nanoparticles remained largely stable throughout anaerobic digestion, therefore it is possible that deposition of the nanoparticles on the microbial cell surface may have resulted in the initial cytotoxic response as indicated by the increased expression of oxidative stress response enzymes such as *protein P52677 (Probable hydrogen peroxide-inducible genes activator)*, *Chaperone protein DnaK* as well as DNA damage repair enzymes such as *DNA helicase*, *DNA Polymerase IV* and *DNA ligase* detected in the iron oxide nanoparticles augmented reactors (Table 4.2 and Table 4.3).

However, on day 7, significant increases in 16S rRNA gene copy numbers in the *Eubacteria* ($p < 0.001$) (Fig. 4.5a) as well as *methanogens* (Fig. 4.5b) indicated microbial acclimation. The largest microbial response to the addition of iron oxide nanoparticles, both in terms of decline as well as enhancement of cell numbers was recorded by the *Eubacteria* (Fig.4.5a). According to literature, the doubling time for hydrolytic and acidogenic bacteria was determined to be about 1.0 to 1.5 days, while it was found to be 1 to 4 days for acetogenic bacteria and approximately 5 to 15 days for methanogens (Gerardi 2003).

In the test reactors, *Methanomicrobiales* was the dominant methanogen community throughout anaerobic digestion. This suggested that hydrogenotrophic methanogenesis was an important route in methanogenesis (Fig.4.5d). Within the test reactors, the highest increase in *Methanomicrobiales*

16S rRNA gene copy numbers occurred between day 0 and day 7 (9.4×10^8 to 2.39×10^9) of anaerobic digestion. A similar trend was noted in *Methanobacteriales* with the largest increase between day 0 and day 7 (3.94×10^7 to 6.48×10^7) of anaerobic digestion. Quantification of the acetoclastic families revealed the clear dominance of *Methanosaetaceae* over *Methanosarcinaceae*. Continuous increase in *Methanosaetaceae* 16S rRNA gene copy numbers was observed in the test reactors throughout anaerobic digestion (Fig. 4.5e); the largest increase occurred between day 7 and day 23 (4.10×10^7 to 7.85×10^7). Similarly, quantification of the *Methanosarcinaceae* 16S rRNA gene copy numbers also indicated a continuous increase till day 23 (7.03×10^5 to 1.84×10^6). This suggested acetoclastic methanogenesis was favored in the test reactors between day 7 and day 23 corroborated by the decline in acetate detected in the test reactors (151mg/L to 0.4 mg/L). In the control reactors, however, a net decrease in the *Methanosaetaceae* gene copy numbers from day 0 to day 23 (8.29×10^7 to 2.4×10^7) was noted (Fig.4.5e) together with a similar decline in *Methanosarcinaceae* gene concentration upto day 7 was noted (Fig.4.5f). The loss of *Methanosaetaceae* and *Methanosarcinaceae* gene copy numbers correlates with the significant accumulation of volatile fatty acids, mainly acetic and predominantly propionic acid (Fig.4.2) indicated impaired syntrophy.

Figure 4.5.a. **Changes in the 16S rRNA gene copy numbers of *Eubacteria* in the control and test reactors.** The error bars represent the standard deviation values of the replicate reactor sets.

Figure 4.5.b. **Changes in the 16S rRNA gene copy numbers of *Methanogens* in the control and test reactors.** The error bars represent the standard deviation values of the replicate reactor sets.

Figure 4.5.c. **Changes in the 16S rRNA gene copy numbers of *Methanobacteriales* in the control and test reactors.** The error bars represent the standard deviation values of the replicate reactor sets.

Figure 4.5.d. **Changes in the 16S rRNA gene copy numbers of *Methanomicrobiales* in the control and test reactors. The error bars represent the standard deviation values of the replicate reactor sets.**

Figure 4.5.e. **Changes in the 16S rRNA gene copy numbers of *Methanosaetaceae* in the control and test reactors. The error bars represent the standard deviation values of the replicate reactor sets.**

Figure 4.5.f. **Changes in the 16S rRNA gene copy numbers of *Methanosarcinaceae* in the control and test reactors. The error bars represent the standard deviation values of the replicate reactor sets.**

4.4.3 Isobaric Tags for Relative and Absolute Quantification Proteomic Analysis (iTRAQ)

Iso-Baric tags for Relative and Absolute Quantification (iTRAQ) coupled to a 2 – dimensional LC-MS/MS was used to evaluate the impact of iron oxide nanoparticles on the enzyme secretomics of the anaerobic sludge microbiota. Significant differences in cumulative methane production between the control and test reactors was noted on day 7 of anaerobic digestion (Fig.4.2). Evaluation of the differences in protein secretion in the control and test reactors revealed increased secreted protein concentration in the mixed liquor of the test reactors ($41 \mu\text{g/mL}$) compared to the control ($31 \mu\text{g/mL}$). Relative quantification of the day 7 proteins expressed (> 0.70 fold) in the test reactors compared to the control is presented in (Table 4.2).

The highest relative protein expression was depicted by *Bacillus cereus* which expressed *Phosphoribosylformylglycinamide cyclo-ligase*, a key enzyme in the IMP pathway 35.75 fold greater than the control. This was followed by *Pseudomonas syringae* which expressed *Phosphoribosylamine-glycine ligase* (IMP biosynthesis) 25.54 fold more than the control. *Salmonella Heidelberg* expressed *Glucokinase* (a key enzyme in the glycolytic pathway) 8.82 fold higher than the control, *Phosphoglycerate kinase* another key enzyme associated with Glycolysis was also detected in the test reactors. Pyruvate is the metabolic end product of glycolysis which can be converted to metabolic intermediates such as ethanol and lactate.

The results of this study indicate the up regulation of relative protein expression by yeasts in the test reactors such as: *Debaryomyces hansenii*, (13.6), *Kluyveromyces lactis* (7.7 fold) and *Saccharomyces cerevisiae* (5.07 fold). *Bacillus cereus*, *Pseudomonas sp.*, *Salmonella sp.*, yeasts and *fungi* are the major classes of microorganisms generally associated with hydrolytic activity. *Endoglucanase* (responsible for the endohydrolysis of (1->4)-beta-D-glucosidic linkages in cellulose), *hydrolases*, *peptidases* and *proteases* are classes of hydrolytic enzymes which were also expressed in significantly greater relative abundances in the iron oxide nanoparticles augmented reactors in comparison to the control (Table 4.2 and Table 4.3) thus accounting for the increased hydrolytic activity. The metabolic end products of anaerobic fermentation by members of *Saccaromycetaeaceae* is ethanol and carbon dioxide (Breuer and Harms 2006) which is further oxidized to acetate and formate. *Prochlorococcus marinus* expressed the protein *1-deoxy-D-xylulose-5-phosphate synthase* (5.36 fold more than the control). This is a key enzyme in the xylose fermentation pathway in which the substrate is generally *hemicellulose*, and suggests the involvement of this microorganism in the enhanced hydrolysis observed.

Methanoculleus marisnigri, which belongs to the order *Methanomicrobiales* is frequently associated with acetate oxidation (Mayumi, Mochimaru et al. 2011) and is unique in its ability to utilize hydrogen, ethanol, secondary alcohols, formate and butyrate as electron donors for methanogenesis (Hattori 2008) showed protein expression to be 6.38 fold higher than the control. This is consistent with qPCR findings which revealed increases in the hydrogenotrophic *Methanomicrobiales* 16S rRNA gene abundances during anaerobic digestion.

The addition of iron oxide nanoparticles elicited an oxidative stress response, as indicated by the significantly increased expression of cellular oxidative stress proteins such as *protein P52677*

(*Probable hydrogen peroxide-inducible genes activator*, expressed 5.57 fold greater than the control), *Chaperone protein DnaK* (0.91 fold greater than control) and DNA damage repair enzymes such as *DNA helicase* (2.32 fold greater than control), *DNA Polymerase IV* (1.77 fold greater than the control) and *DNA ligase* (1.21 fold greater than the control) (Table 5.2, Table 5.3). This is in tandem with the decline in 16S rRNA gene copy numbers across the microbial communities noted after nanoparticle addition (Fig. 4.5 (a – f)), which corroborated the cytotoxicity of the nanoparticles on the anaerobic sludge biomass.

However, despite the initial cytotoxic response of the anaerobic sludge microbiota to the iron oxide nanoparticles, a significant recovery of the *Eubacteria* 16s rRNA gene copy numbers was noted on day 7 (Fig. 4.5a) together with significantly increased expression of key enzymes such as *Phosphoribosylformylglycinamide cyclo-ligase* (35.75 fold) *Phosphoribosylamine--glycine ligase* (25.54) associated with the synthesis of nucleotides via the IMP pathway as well as proteins involved in ribosome biogenesis such as the *Pre-rRNA-processing protein ESF2* (13.6 fold) and various enzymes associated with the protein biosynthesis machinery including *tRNA pseudouridine synthase 1* (7.7 fold), *50S ribosomal protein L22* (6.38 fold), *Eukaryotic translation initiation factor 3 subunit C* (5.1 fold), *Isoleucine--tRNA ligase* (1.14 fold greater than the control) clearly indicative of enhanced *Eubacteria* growth and synthesis activity.

Taken together the findings presented in this study indicate that despite the initial cytotoxicity of the iron oxide nanoparticles which resulted in a loss of microbial numbers across all the communities, a significant recovery of *Eubacteria* microbial numbers and the increased expression of key enzymes involved in cellular recovery, metabolic activity and hydrolytic activity facilitated the enhanced hydrolysis of cellulose which increased the carbon flow and made available substrates for the acidogen, acetogen and methanogen communities and eventually resulted in the enhanced methane production noted (Fig. 4.1).

In the next Chapter 6 of this thesis, a deeper microscopic insight using FESEM and Atomic Force Microscopy image analysis as well as second generation ATP analysis provides a more detailed insight about the effect of the iron oxide nanoparticles on the anaerobic sludge microbiota as well as provides answers on the survival and growth mechanisms of the microbial communities which aided in cell proliferation and recovery despite the initial cytotoxicity.

Table 4.2. **Relative quantification of increases in protein expression (greater than 0.70 fold) in the test enzyme secretosome compared to the control.**

Relative abundances of proteins expressed > 0.7 fold (test samples to control)	Protein Database Accession No.	Protein Name	Microorganism
35.75	A7GKI0	Phosphoribosylformylglycinamidine cyclo-ligase	<i>Bacillus cereus</i> subsp. <i>cytotoxis</i> (strain NVH 391-98)
25.54	Q87VR8	Phosphoribosylamine--glycine ligase	<i>Pseudomonas syringae</i> pv. <i>tomato</i> (strain DC3000)
13.6	Q6BSS5	Pre-rRNA-processing protein ESF2	<i>Debaryomyces hansenii</i> (strain ATCC 36239)
8.82	B4TCD7	Glucokinase	<i>Salmonella heidelberg</i> (strain SL476)
7.7	Q6CWQ8	tRNA pseudouridine synthase 1	<i>Kluyveromyces lactis</i> (strain ATCC 8585)
7.21	Q6BV76	Serine/threonine-protein kinase TEL1	<i>Debaryomyces hansenii</i> (strain ATCC 36239)
6.38	A3CT02	50S ribosomal protein L22	<i>Methanoculleus marisnigri</i> (strain ATCC 35101 / DSM 1498)
5.57	P52677	Probable hydrogen peroxide-inducible genes activator	<i>Mycobacterium avium</i>
5.36	A2C220	1-deoxy-D-xylulose-5-phosphate synthase	<i>Prochlorococcus marinus</i> (strain NATL1A)
5.07	A6ZN26	Eukaryotic translation initiation factor 3 subunit C	<i>Saccharomyces cerevisiae</i> (strain YJM789) (Baker's yeast)

3.69	A0B6K7	3-dehydroquinate dehydratase	<i>Methanosaeta thermophila</i> (strain DSM 6194 / PT)
3.68	P17051	HTH-type transcriptional regulator AlkS	<i>Pseudomonas oleovorans</i>
2.32	Q2SS60	Holliday junction ATP-dependent DNA helicase RuvA	<i>Mycoplasma capricolum</i> subsp. <i>capricolum</i> (ATCC 27343)
2.31	P14010	4-aminobutyrate aminotransferase	<i>Emericella nidulans</i> ATCC 38163
1.77	Q974T8	DNA polymerase IV	<i>Sulfolobus tokodaii</i> (strain DSM 16993)
1.29	O60183	Protein sat1	<i>Schizosaccharomyces pombe</i> (strain 972 / ATCC 24843)
1.21	C0QVE5	DNA ligase	<i>Brachyspira hyodysenteriae</i> (strain ATCC 49526 / WA1)
1.14	Q7V352	Isoleucine--tRNA ligase	<i>Prochlorococcus marinus</i> subsp. <i>pastoris</i> (strain CCMP1986)
0.97	P36634	Peptide transport periplasmic protein SapA	<i>Salmonella typhimurium</i> (ATCC 700720)
0.91	Q8YE76	Chaperone protein DnaK	<i>Brucella melitensis</i> biotype 1 (strain 16M / ATCC 23456)
0.72	Q8K9L0	Flagella basal body P-ring formation protein FlgA	<i>Buchnera aphidicola</i> subsp. <i>Schizaphis graminum</i> (strain Sg)

Table 4.3. **Proteins detected in the test enzyme secretosome but not expressed/ detectable in the control.**

Protein Database Accession No.	Protein Name	Microorganism
Q1WU83	Elongation factor Tu	<i>Lactobacillus salivarius</i> (strain UCC118)
P06540	ATP synthase subunit beta	<i>Nostoc sp.</i> (strain PCC 7120 / UTEX 2576)
A0LUH1	Chorismate synthase	<i>Acidothermus cellulolyticus</i> (strain ATCC 43068 / 11B)
O00852	Phosphoglycerate kinase	<i>Glaucoma chattoni</i>
Q98QG1	Elongation factor Tu	<i>Mycoplasma pulmonis</i> (strain UAB CTIP)
B8G990	50S ribosomal protein L1	<i>Chloroflexus aggregans</i> (strain MD-66 / DSM 9485)
Q06836	Arf guanine nucleotide exchange factor SYT1	<i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) (Baker's yeast)
C3L3G5	Ribosomal protein L11 methyltransferase	<i>Clostridium botulinum</i> (strain 657 / Type Ba4)
P76505	Uncharacterized protein YfdF	<i>Escherichia coli</i> (strain K12)
B0B8H3	Protein TolB	<i>Chlamydia trachomatis</i> serovar L2 (strain 434/Bu / ATCC VR-902B)
Q5HPJ0	Aconitate hydratase	<i>Staphylococcus epidermidis</i> (strain ATCC 35984 / RP62A)
A6H1D1	NAD kinase	<i>Flavobacterium psychrophilum</i> (strain JIP02/86 / ATCC 49511)
Q6CHE5	DNA mismatch repair protein MSH3	<i>Yarrowia lipolytica</i> (strain CLIB 122 / E 150) (Yeast) (<i>Candida lipolytica</i>)
A4XL45	tRNA dimethylallyltransferase	<i>Caldicellulosiruptor saccharolyticus</i> (strain ATCC 43494 / DSM 8903)

Q3IJ53	Translation initiation factor IF-2	<i>Pseudoalteromonas haloplanktis</i> (strain TAC 125)
C4LFH3	Threonine--tRNA ligase	<i>Tolumonas auensis</i> (strain DSM 9187 / TA4)
B2FLM5	8-amino-7-oxononanoate synthase	<i>Stenotrophomonas maltophilia</i> (strain K279a)
P31002	Inosine-5'-monophosphate dehydrogenase	<i>Acinetobacter calcoaceticus</i>
Q88U41	ATP-dependent helicase/nuclease subunit A	<i>Lactobacillus plantarum</i> (strain ATCC BAA-793 / NCIMB 8826 / WCFS1)
A1RRV9	50S ribosomal protein L14	<i>Pyrobaculum islandicum</i> (strain DSM 4184 / JCM 9189)
P42480	Elongation factor Tu	<i>Hymenobacter ocellatus</i> (Taxeobacter ocellatus)
P49378	NAD(P)H-dependent D-xylose reductase	<i>Kluyveromyces lactis</i> (strain ATCC 8585)
A1SL79	UDP-N-acetylmuramate--L-alanine ligase	<i>Nocardioides</i> sp. (strain BAA-499 / JS614)
Q8ST66	ABC transporter G family member 18	<i>Dictyostelium discoideum</i> (Slime mold)
Q58428	Archaeosine synthase	<i>Methanocaldococcus jannaschii</i> (strain ATCC 43067)
Q9K492	HTH-type transcriptional repressor DasR	<i>Streptomyces coelicolor</i> (strain ATCC BAA-471 / A3(2) / M145)
Q2FJK3	Uncharacterized lipoprotein SAUSA300_0411	<i>Staphylococcus aureus</i> (strain USA300)
O26786	50S ribosomal protein L15e	<i>Methanothermobacter thermautotrophicus</i> (strain ATCC 29096)
C6A2A3	Geranylgeranylglyceryl phosphate synthase	<i>Thermococcus sibiricus</i> (strain MM 739 / DSM 12597)

Q9P7M9	Probable GTP cyclohydrolase-2	<i>Schizosaccharomyces pombe</i> (strain 972 / ATCC 24843) (Fission yeast)
Q47ZE0	Bifunctional protein FolD 1	<i>Colwellia psychrerythraea</i> (strain 34H / ATCC BAA-681)
Q759B6	Pre-mRNA-processing protein 45	<i>Ashbya gossypii</i> (strain ATCC 10895)
P33306	Protein BCK2	<i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) (Baker's yeast)
Q0SPE9	Uridine kinase	<i>Borrelia afzelii</i> (strain PKo)
P38181	Nucleoporin NUP170	<i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) (Baker's yeast)
Q30Z38	Elongation factor G	<i>Desulfovibrio alaskensis</i> (strain G20) (<i>Desulfovibrio desulfuricans</i> (strain G20))
Q57989	Probable porphobilinogen deaminase	<i>Methanocaldococcus jannaschii</i> (strain ATCC 43067)
P22446	DNA gyrase subunit A	<i>Mycoplasma pneumoniae</i> (strain ATCC 29342 / M129)
P15917	Lethal factor	<i>Bacillus anthracis</i>
Q82WX6	Phosphoglucosamine mutase	<i>Nitrosomonas europaea</i> (strain ATCC 19718 / NBRC 14298)
Q4JQU7	DNA polymerase catalytic subunit	<i>Varicella-zoster virus</i> (strain Oka vaccine) (HHV-3) (Human herpesvirus 3)
O68897	Glutaminase-asparaginase	<i>Pseudomonas fluorescens</i> biotype A
A6TLM6	Deoxyuridine 5'-triphosphate nucleotidohydrolase	<i>Alkaliphilus metalliredigens</i> (strain QYMF)
A0R4Q0	Putative succinate-semialdehyde dehydrogenase [NADP(+)]	<i>Mycobacterium smegmatis</i> (strain ATCC 700084 / mc(2)155)
P54472	Putative GTP cyclohydrolase 1 type 2	<i>Bacillus subtilis</i> (strain 168)

P41450	Uncharacterized 22.7 kDa protein in GTA-P79 intergenic region	<i>Autographa californica nuclear polyhedrosis virus</i> (AcMNPV)
A3LZ57	COPII coat assembly protein SEC16	<i>Scheffersomyces stipitis</i> (strain ATCC 58785 / CBS 6054 / NBRC 10063 / NRRL Y-11545) (Yeast) (<i>Pichia stipitis</i>)
Q11YP2	ATP synthase subunit delta	<i>Cytophaga hutchinsonii</i> (strain ATCC 33406 / NCIMB 9469)
Q72RP6	Tyrosine--tRNA ligase	<i>Leptospira interrogans</i> serogroup Icterohaemorrhagiae serovar copenhageni (strain Fiocruz L1-130)
Q6G5P7	Phosphoglucosamine mutase	<i>Bartonella henselae</i> (strain ATCC 49882 / Houston 1) (<i>Rochalimaea henselae</i>)
Q8EQV3	30S ribosomal protein S2	<i>Oceanobacillus iheyensis</i> (strain DSM 14371 / JCM 11309 / KCTC 3954 / HTE831)
P38771	Ribosome-recycling factor, mitochondrial	<i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) (Baker's yeast)
Q1GWT5	Adenosylhomocysteinase	<i>Sphingopyxis alaskensis</i> (strain DSM 13593 / LMG 18877 / RB2256) (<i>Sphingomonas alaskensis</i>)
A4TSI1	Aspartate--ammonia ligase	<i>Yersinia pestis</i> (strain Pestoides F)
B5XL08	Phosphopentomutase	<i>Streptococcus pyogenes</i> serotype M49 (strain NZ131)
Q0H8Y3	Probable intron-encoded endonuclease aI8	<i>Ustilago maydis</i> (strain 521 / FGSC 9021) (Corn smut fungus)
Q6CG46	Nucleolar protein 58	<i>Yarrowia lipolytica</i> (strain CLIB 122 / E 150) (Yeast) (<i>Candida lipolytica</i>)
O32236	HTH-type transcriptional repressor RghR	<i>Bacillus subtilis</i> (strain 168)

Q55F68	Adenylate cyclase, terminal-differentiation specific	<i>Dictyostelium discoideum</i> (Slime mold)
B2KAW2	GTPase Obg.	<i>Elusimicrobium minutum</i> (strain Pei191)
Q8EQL0	Putative membrane protein insertion efficiency factor	<i>Oceanobacillus iheyensis</i> (strain DSM 14371 / JCM 11309 / KCTC 3954 / HTE831)
C3K1H6	Phosphoribosylglycinamide formyltransferase 2	<i>Pseudomonas fluorescens</i> (strain SBW25)

4.5 Conclusion

This study investigated the impact of iron oxide nanoparticles on the anaerobic digestion process. The evaluation was primarily conducted through metaproteomic analysis of the protein secretosome in the presence and absence of the iron oxide nanoparticles as well as 16S rRNA gene quantification of the anaerobic digestion-associated microbial communities.

It was seen that the relative abundances of proteins expression systems related to hydrolysis, IMP and glycolysis pathway as well as cellular stress and repair proteins was increased in the presence of IONp. This study showed that iron oxide nanoparticle augmentation initially exerted significant microbiological stress on the anaerobic sludge microbiota which resulted in the increased expression of *cellular stress* and *repair enzymes* together with an initial loss of microbial numbers across all the microbial communities. This phenomenon improved as the anaerobic digestion reaction progressed. Increased expression of extracellular *hydrolytic* and *acidogenesis – related enzymes* in the secretosome in the presence of IONp in tandem with an increase in the soluble COD established improved substrate hydrolysis. Acidogenesis and improved microbial syntrophy among the microbial communities in the presence of IONp resulted in better overall AD performance in the presence of IONp. This study concluded that IONp augmentation significantly improved both hydrolysis and acidogenesis as well as facilitated the establishment of a well - balanced syntrophic association which resulted in enhanced methane production. These results suggested a promising potential for the use of IONp as a pre-treatment strategy in anaerobic digestion systems to improve the rate – limiting hydrolysis.

CHAPTER 5

MICROBIAL STRESS MEDIATED INTERCELLULAR NANOTUBES IN AN ANAEROBIC MICROBIAL CONSORTIUM DIGESTING CELLULOSE

5.1 Abstract

The anaerobic process is a multi-step reaction dependent on concerted activities such as exchange of metabolites among physiologically different microbial communities. This study investigated the impact of iron oxide nanoparticles on the anaerobic sludge microbiota. It was shown there were three distinct microbial phases following addition of the nanoparticles: microbial stress and cell death of approximately one order of magnitude, followed by microbial rewiring, and recovery. Furthermore, it was noted that cellular stress led to the establishment of intercellular nanotubes within the microbial biomass. Intercellular nanotube mediated communication among genetically engineered microorganisms and “ad hoc” assembled co-cultures has been reported. This study presents evidence of intercellular nanotube communication within an environmental sample – i.e., anaerobic microbial consortium subjected to stress. The observation suggested a mode of microbial communication in the anaerobic process not previously explored and which may have implications on process design.

5.2 Introduction

Treatment of sludge through anaerobic digestion (AD) is gaining interest because of the process's relatively low energy requirement, efficiency, and production of a renewable fuel in comparison with aerobic digestion (Lettinga 1995, Appels, Baeyens et al. 2008, Appels, Lauwers et al. 2011). Despite the popularity of conventional AD, one of the major drawbacks of this technology is the comparatively long sludge retention time, attributable to the slow growth rate of the anaerobic methanogens (Pind, Angelidaki et al. 2003) and to hydrolysis. The latter is frequently the rate-limiting step in sludge digestion (Keating, Cysneiros et al. 2012). Improvement of performance by altering process configurations have been successful (Lyberatos and Skiadas 1999, Mosier, Wyman et al. 2005). The microbial consortium is the “core” of an anaerobic digester, and broadly encompasses four metabolically diverse microbial communities involved in the degradation of complex organic substrates to methane and carbon dioxide; they include, the hydrolytic bacteria, acidogenic bacteria, acetogenic bacteria and the methanogenic archaea (Narihiro and Sekiguchi 2007, Appels, Baeyens et al. 2008). Each of these microbial communities share a cooperative relationship and are inter-dependent for growth, and therefore interspecies microbial communication plays an indispensable role. Conventionally, it has been accepted that within an AD system, bacterial “cross - talk” with the methanogenic archaea is generally via the exchange of diffusive carriers such as hydrogen and formate (Batstone, Keller et al. 2002, Appels, Baeyens et al. 2008, Ali Shah, Mahmood et al. 2014); in the presence of (semi)conductive nanoparticles (Kato, Hashimoto et al. 2012) however, a recently discovered phenomenon, Direct Interspecies Electron Transfer (DIET) has been shown to facilitate the direct exchange of electrons among bacteria as well as between bacteria and archaea via microbial nanowires in defined co-cultures and wastewater digester aggregates (Lovley 2011, Morita, Malvankar et al. 2011, Kato, Hashimoto et al. 2012, Pirbadian, Barchinger et al. 2014, Lovley and Malvankar 2015). These microbial nanowires have been shown to be mainly conductive pili as well as conductive filaments (MallaaShrestha 2014, Pirbadian, Barchinger et al. 2014).

Among diverse but interdependent microbial communities, intra-and-inter-species microbial communication has been thought to occur primarily through quorum sensing, production of membrane vesicles, pili and nanotube mediated communication (Bassler, Bassler and Losick 2006, West, Griffin et al. 2006, Dubey and Ben-Yehuda 2011). Parasitism, mutualism or

cooperative microbial interactions largely depend on the production and detection of signaling molecules by the interacting microorganisms (West, Griffin et al. 2006, van Vliet and Ackermann 2015). In a hostile environment, the loss of signaling molecules will likely prove detrimental to the microbial communities present. In such a scenario, the advantages of direct cell-cell contact confers a more positive fitness to the microorganisms and results in a more robust response to the environmental conditions. Among microorganisms, direct cell-to-cell interactions have been shown to occur via pili (Lovley and Malvankar 2015) and more recently interconnecting nanotubes (Dubey and Ben-Yehuda 2011). Bacteria have also been shown to exchange nutrients and distribute metabolic functions among interconnected cells and auxotrophic mutants through nanotubes (Pande, Shitut et al. 2015). Although the cytoplasmic exchange of materials through intercellular nanotubes has been proved in “ad hoc” co-cultures, evidence of the same in an un-engineered and/or “real” environmental sample is lacking.

This study reports evidence of microbial communication mediated by intercellular nanotubes among microorganisms in an environmental sample. This would suggest nanotube-mediated communication in the environment may be more prevalent than previously suspected and hence indicates a microbial shift toward multi-cellularity under certain conditions. This study has shown environmental and consequent microbial stress can induce the production of intercellular nanotubes in a medium such as anaerobic sludge. The discovery of intercellular nanotubes within anaerobic sludge furthers understanding of microbial communication in anaerobic digestion with implications on process design, monitoring and operations.

This study would indicate that use of Iron Oxide Nanoparticles (IONp) could significantly improve hydrolysis while simultaneously enhancing syntrophy among the microbial communities and so resulting in improved methane yield.

5.3 Materials and Methods

5.3.1 Inoculum and Substrate

The anaerobic sludge inoculum used in this experiment was collected from a mesophilic anaerobic digester treating primary and secondary municipal sludge. The anaerobic seed was stored in a cold room for no more than 5 days and degassed (Angelidaki, Alves et al. 2009) prior to inoculation into the anaerobic digestion batch reactors.

The substrate was insoluble microcrystalline cellulose (Sigma Aldrich). Cellulose is the substrate of choice used in the study of hydrolysis (Angelidaki, Alves et al. 2007) (Scharer and Moo-Young 1979); since the complete anaerobic degradation of cellulose closely simulates a conventional AD bioprocess (O’Flaherty, Collins et al. 2006) involving hydrolysis, acidogenesis, acetogenesis and methanogenesis.

5.3.2 Process Operation

The effect of iron oxide NP on the anaerobic digestion of microcrystalline cellulose was evaluated through the BMP test using the Automatic Methane Potential Test System (AMPTS II) (Bioprocess Control AB, Sweden), designed for the automatic, real-time monitoring of methane production during anaerobic digestion. Batch AD of cellulose was carried out at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under standard pressure for 20 days after which methane production stopped. Reactor performance was analyzed simultaneously. Details of the reactor system is as shown in Table 5.1. No pH amendment or nutrient solutions were added to mitigate possible interactions with the iron oxide suspension. Each sample set was tested in duplicate. The average values of results from each of the reactor sets are reported in this study.

Table 5.1. **Reactor composition**

Content/Reactor	Blank (Bl)		Fe-Blank (Fe-Bl)		Control (CC)		Test (CT)	
	Bl 1	Bl 2	Fe-Bl1	Fe-Bl 2	CC 1	CC 2	CT 1	CT2
Substrate – Microcrystalline Cellulose (Grams)	0	0	0	0	1.53	1.53	1.53	1.53
Inoculum (mL)	140	140	140	140	140	140	140	140
Deoxygenated water (mL)	310	310	210	210	310	310	210	210
Iron oxide nanoparticle suspension (mL)	0	0	100	100	0	0	100	100
Total volume (mL)	450	450	450	450	450	450	450	450

5.3.3 Analytical Methods

Characterization of the sludge samples was conducted in accordance with the standard methods(American Public Health Association 1998). VFA analysis of acetic, propionic, n-butyric, iso-butyric, n-valeric, iso-valeric and n-caproic acids is as given in section 3.2.4

Composition of the biogas generated was determined as detailed in section 3.2.5.

5.3.3.1 Extraction and Quantification of Fe^{2+} ions and Total Fe^{3+} ions

The analysis is as detailed in section 3.2.6 and 3.2.7.

5.3.3.2 Preparation and Characterization of Colloidal Iron oxide Nanoparticle Suspension

Synthesis and characterization of iron oxide nanoparticles is as given in section 3.2.8.

5.3.3.3 DNA Sample Preparation and Extraction

Experimental protocol is as detailed in section 3.3.

5.3.3.4 Real-time Quantitative Polymerase Chain Reaction

Experimental protocol is as detailed in section 3.4.

5.3.3.5 Adenosine triphosphate (ATP) analysis

QuenchGone21TM Wastewater kits (LuminUltra Technologies Ltd. New Brunswick, Canada) which utilizes second generation ATP technology was used to monitor total and dissolved ATP. ATP extraction and analyses was carried out according to standard kit instructions. TECAN infinite 200 PRO microplate reader was used to detect and quantify luminescence. Detailed experimental protocols used for evaluation are provided in Chapter 3 (Materials and Methods) under section 3.6.

Equations provided by the kit manufacturer was used to determine Biomass Stress Index and concentration of cellular ATP.

5.3.3.6 Field Emission Scanning Electron Microscopy (FE-SEM)

Samples extracted from the bioreactors were immediately frozen at – 80 °C for 30 minutes followed by freeze-drying under vacuum (Labconco 73820 FreezeZone PlusTM 4.5 Liter Cascade Freeze Dry System, Kansas City, MO) at 0.021 mBar and – 70 °C for 18 hrs - 20 hrs.

The samples were mounted on a carbon tape and sputter-coated with Platinum (JEOL JCF-1600) prior to examination by Field-Emission Scanning Electron Microscopy (FE-SEM, JEOL JSM – 7600F) at 5kV acceleration voltage. Elemental mapping was performed using an Energy

Dispersive X-ray spectrometer (EDX) (Oxford Instrument, X-max, 80 mm²) attached to the FE-SEM.

5.3.3.7 Cell imaging by Atomic Force Microscopy (AFM)

At selected data points, representative samples were extracted from the reactors and 0.5 mL of the sample was placed on a clean glass slide and gently smeared. The smear was air-dried and immediately observed using an Atomic Force Microscope (XE-100 AFM, Park Systems Corp., Suwon, Korea). Images were collected in ambient air in both contact and non-contact mode.

5.3.3.8 Statistical Analysis

Two tailed Student *t*-test with equal variance was used to determine the statistical significances in the bioprocess results between the control and test systems, as well as differences in the 16s rRNA gene copy numbers in the microbial populations between the control and iron oxide nanoparticles - augmented test reactors. α was defined as 0.05 in the formation of the null hypothesis. The confidence intervals on the difference between the mean was adjusted to 95%. Results were considered statistically significant when $p \leq 0.05$.

5.4 Results and Discussion

5.4.1 Evaluation of Anaerobic Digestion Performance

Supplementation of the batch anaerobic reactors digesting cellulose with iron oxide nanoparticles (IONp) initially resulted in cytotoxicity and loss of microbial numbers (Fig. 5.1 (*a – f*) and Fig. 5.5).

Figure 5.1.a. **Changes in the 16S rRNA gene copy numbers of *Eubacteria* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

Figure 5.1.b. **Changes in the 16S rRNA gene copy numbers of *Methanogens* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

Figure 5.1.c. **Changes in the 16S rRNA gene copy numbers of *Methanosaetaceae* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

Figure 5.1.d. **Changes in the 16S rRNA gene copy numbers of *Methanosarcinaceae* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

Figure 5.1.e. **Changes in the 16S rRNA gene copy numbers of *Methanobacteriales* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

Figure 5.1.f. **Changes in the 16S rRNA gene copy numbers of *Methanomicrobiales* in the control and test reactors.** The error bars represent the standard deviation values among each of the reactor sets.

The control reactors not supplemented with nanoparticles had better initial performance in comparison to the test reactors. The IONp inhibited methane production following supplementation, this is most likely because of the high biomass stress and cytotoxicity due to oxidative stress exerted on the microbial biomass which resulted in a loss of microbial numbers by approximately one order of magnitude across all the microbial communities.

However, on day five, there was improvement in methane production in the test reactors which was slightly better compared to the control (Fig. 5.2). This was accompanied by a large increase in soluble COD ($p < 0.05$) (Fig. 5.3a) and acetate (Fig. 5.3b) concentration. A drop in pH from 8.1 to 7.1 was recorded indicating hydrolytic and increased acidogenic activity (Table 5.3).

Figure 5.2. Differences in methane yields per gram volatile substance destroyed is presented. The volume of methane from each reactor was subtracted from the blank. The error bars are defined as the standard deviation between each duplicate reactor set.

Table 5.2. Methane and carbon dioxide composition of the biogas from each of the reactor sets during AD.

Day		BL 1	BL 2	FEBL 1	FEBL 2	CC 1	CC 2	CT 1	CT2
0	CO ₂	0	0	0	0	0	0	0	0
	CH ₄	0	0	0	0	0	0	0	0
5	CO ₂	5.2	1.8	2.9	3.7	39.9	28.6	50.4	47.1
	CH ₄	18.3	8.6	11.6	11.5	58.9	53.5	46.6	51.4
12	CO ₂	3.7	4.1	3.8	2.4	42.6	39.4	30.6	29.6
	CH ₄	13.3	20.5	18.9	14.5	61.9	64.5	74.3	71.5
21	CO ₂	6.3	6.6	6.8	4.7	33.4	23.6	22.4	11.2
	CH ₄	20.1	20.2	29.7	22.5	60	44	66.3	41

Table. 5.3. pH changes in each of the reactor sets during Anaerobic digestion

Day	Blank	Fe Blank	Control	Test
0	7.8	8	7.9	8.1
5	8.0	8	7.5	7.1
12	7.4	7.7	6.8	7.0
20	7.4	7.6	6.9	7.0

Figure 5.3. Changes in soluble COD and composition of Volatile Fatty Acids (VFA) detected

Average values of each reactor set is presented. The error bars are defined as the standard deviation between each duplicate reactor set (a) Changes in soluble COD during AD.

(b) Differences in volatile fatty acids composition on day five of AD between the control and test reactors. Volatile fatty acids abbreviations are as follows; N-HBU (n-butyric acid), ISO-HBU (Iso-butyric acid), HPR (Propionic acid), HAC (Acetic acid).

Increased acidogenic activity results in an increase in hydrogen production and this could disrupt the balance among the microbial communities (Sieber, McInerney et al. 2012). Hence the scavenging of hydrogen by hydrogenotrophic methanogens via microbial syntrophy would be important for process stability. Microbial syntrophy was by then enhanced in the presence of IONp

as suggested by the negligible amount of propionate (Fig. 5.3b) and hydrogen gas detected in the test reactors. Quantification of the 16S rRNA gene copy numbers revealed a significant increase in *Eubacteria* (Fig. 5.1a) followed by the *Methanogens* (Fig.5.1b). In the *Methanogen* community, the largest increase in 16S rRNA gene copy numbers was noted in *Methanomicrobiales*, closely followed by *Methanosarcinaceae*, *Methanosaetaceae* and a minor increase in *Methanobacteriales* (Fig.5.1 (c-f)). With the two acetoclastic families, *Methanosarcinaceae* and *Methanosaetaceae*, the growth rate of *Methanosarcinaceae* is known to exceed *Methanosaetaceae* at higher acetate concentrations (Yu, Kim et al. 2006), as was observed in the test reactors.

These results showed addition of IONp did, after the initial lag, enhance cellulose hydrolysis which made available substrates for the acidogen, acetogen and methanogen communities (indicated by the increase in cellular ATP as well as 16S rRNA microbial gene copy numbers). Within the control reactors, however, metabolic activity was limited and quantitative PCR analysis revealed a decline in 16S rRNA gene copy numbers across all communities from day zero till day five suggestive of a lack of microbial adaptation to and impaired hydrolysis of the cellulose substrate which in turn restricted carbon flow to the acidogen, acetogen and methanogen communities. A drop in pH from 7.5 to 6.8 on day 12 within the control reactors signaled the establishment of acidogenesis accompanied by a net increase in 16S rRNA gene copy numbers across all the microbial communities (Fig. 5.1 (a – f)). This suggested a slower acclimation process (more than twice the time required by the iron supplemented reactors). A decline in 16S rRNA gene copy numbers was noted within the test reactors on day 12, possibly due to substrate depletion.

5.4.2 Impact of Iron oxide Nanoparticles (IONp) on the Microbial Community

Results from earlier experiments (authors' unpublished data) had suggested iron oxide nanoparticles could be cytotoxic to microbial cells within the anaerobic consortium. A combination of second generation ATP test to monitor the cellular ATP (cATP) (indicative of the living and “healthy” microbes (Fig.5.4)) and the Biomass Stress Index (BSI %) ((Fig.5.5) to evaluate the fraction of dying or dead biomass within the microbial consortium) was used with quantitative PCR to evaluate the effect of IONp on the microbial communities (*Methanogen*, *Eubacteria*, *Methanobacteriales*, *Methanomicrobiales*, *Methanosaetaceae* and

Methanosarcinaeaceae). Field Emission Scanning Electron Microscopy and Atomic Force Microscopy were then used to observe the interaction between the nanoparticles and microbial cells.

Figure 5.4. Estimation of cellular ATP during anaerobic digestion.

The cellular ATP (ng ATP/mL) estimated in each of the reactor set. The cellular ATP represents the amount of ATP contained within the living cells and is a direct measure of the total living biomass within the reactor. The ATP results represent the averaged values of each duplicate reactor set. The error bars are defined as the standard deviation between each duplicate reactor set.

Figure 5.5. Estimation of the Biomass Stress index

The ATP results represent the averaged values of each duplicate reactor set. The error bars are defined as the standard deviation between each duplicate reactor set. The Biomass Stress Index (BSI %) estimated within each sample set. The BSI indicates the microbial stress level (quality) of the microbiota. A BSI % above 50 % indicates severe microbiological stress.

In the test reactors, quantitative PCR analysis on day zero showed a marked decline of approximately one order of magnitude in 16S rRNA gene copy numbers across all microbial communities upon introduction of the IONp (Fig. 5.1 (a-f)). The loss in 16S rRNA gene copy numbers was supported by data from evaluation of cATP which demonstrated a similar decline in concentration from 81.6 ng ATP/mL on day zero before nanoparticle addition to 32.3 ng ATP/mL after nanoparticle addition (Fig. 5.4). This was indicative of a decline in living/active biomass. The Biomass Stress Index (BSI) estimated at these time points further corroborated the finding. It

was observed the BSI had increased from 30.9% to 53.2% shortly after the addition of IONp on day zero (Fig. 5.5). Scanning electron microscopy of the sludge aggregates revealed large numbers of nanoparticle-coated microbial cells with loss of both structural as well as cellular integrity (Fig. 5.10 (a-e)). This likely resulted in cytotoxicity and cell death. However, it was also noted there had seemed to be “selectivity” in the deposition of iron oxide nanoparticles on the surface of the microbial cells, where a small proportion of the microbial biomass seemed “resistant” to IONp deposition (Fig. 5.10 d). It was not determined if this was due to the differences in cell surface properties such as difference in surface charges.

On day five of the anaerobic digestion process, quantitative PCR analysis of samples from the control reactors had shown a decline in 16S rRNA gene copy numbers across all the microbial communities (Fig. 5.1 (a-f)) but the test reactors revealed significant increase in the *Eubacteria* 16S rRNA gene copy numbers (1.25×10^{10} to 1.71×10^{11}) ($p < 0.0002$) as well as Methanogen 16S rRNA gene copy numbers (3.18×10^8 to 1.14×10^9) ($p < 0.0003$). Within the methanogenic community, *Methanomicrobiales* recorded the largest increase in gene copy numbers from 6.80×10^8 to 2.74×10^9 ($p < 0.001$) followed by *Methanosarcinaceae* (Fig. 5.1 (c-f)). The significant increases observed in *Methanomicrobiales* in the early phase of AD, suggests that hydrogenotrophic methanogenesis was also an important route of methane production which is in contrast to conventional methanogenesis.

Consistent with this data, test reactors cellular ATP also showed a 324.8 % increase (Fig. 5.4). Fe^{2+} ions in the test reactors increased by 128% (Fig. 5.6) indicating the presence of a dissimilatory iron reducing community. Fe^{2+} ions increase in the control reactors was negligible (Fig. 5.6).

Figure 5.6. **Estimation of Ferrous iron during anaerobic digestion.**

The average values of Fe^{2+} estimated in each duplicate reactor set during AD is presented in this graph. The error bars are defined as the standard deviation between each duplicate reactor set.

Scanning electron micrographs and Atomic Force microscopy revealed the presence of structures resembling microbial nanowires as well as intercellular nanotubes in samples taken from the test reactors. These microbial nanowires were, however, not detected in samples from the control reactors (Fig. 5.7a, Fig. 5.7b). Previous studies have shown Dissimilatory Iron Reducing Bacteria (DIRB) produce pili-like appendages which function as microbial nanowires in the presence of solid iron oxide aiding iron reduction (Lovley 2011, Lovley and Malvankar 2015). It was also observed the structural integrity of the microbial nanowire producing cells appeared largely uncompromised by the nanoparticles. The microbial nanowires were predominantly embedded among the iron oxide nanoparticles (Fig. 5.7b, 5.12 (a-d)). This suggested these cellular structures could have played an important role in dissimilatory iron reduction and DIET within the test system contributing to the enhanced AD performance despite the initial loss of microbial cells.

Figure 5.7.a. **Comparison of Control and Test samples using Atomic Force Microscopy.** *In the first image which is the Control only microcrystalline cellulose and microbial cells are seen with no nanowires. Scale bar is 5 μm . In the second AFM image of test sample presence of iron oxide nanoparticles and microbial nanowires are indicated by pink arrows. Scale bar is 3 μm .*

Figure 5.7.b. *Comparison of day 8 AFM images of Control and Test samples. In the first image which is the Control (a.) only microcrystalline cellulose and microbial cells are seen with no nanowires. Scale bar is 3 μm . In the second AFM image of test sample (b) presence of iron oxide nanoparticles and microbial nanowire is indicated by pink arrows. Scale bar is 3 μm .*

Figure 5.8.a. **Formation of Intercellular nanotubes between cells**

A coccus and bacillus interconnected via interspecies nanotube in a sample taken from the test reactor on day 12. Scale bar is 1µm.

Figure 5.8.b. **Formation of Intercellular nanotubes between cells**

Intercellular nanotubes visualized by FE-SEM in both the control and test reactors during AD. Pink dashed circles and arrows indicate the nanotubular connections between cells. A coccus and bacillus interconnected via interspecies nanotube in a sample taken from the test reactor on day 12. Scale bar is 1 μm.

Figure 5.8 (c) & (d). **Formation of Intercellular nanotubes between cells (c).** *Coccoid cell in a day five sample from the control reactor with protruding intercellular nanotubes. Scale bar is 100nm. (d) Coccoid cell embedded in iron oxide nanoparticles in a day five sample from the test reactor with protruding intercellular nanotubes. Scale bar is 100nm.*

Figure 5.8.e. **Formation of Intercellular nanotubes between cells.** *Interspecies nanotubes detected in a control sample on day 5. Scale bar is 1μm.*

Figure 5.8.f. **Formation of Intercellular nanotubes between cells.** *Microbial cell extruding interspecies nanotubes in a sample from the test reactor on day 20. Figure (i) Magnification is 30000X. Figure (ii) Magnification is 65000X. Intercellular nanotubes are highlighted in pink. The cellular integrity appears compromised but undistorted to microbial stress. Scale bar is 100nm.*

5.6.3 Microbial Stress and Intercellular Nanotubes

Continuous monitoring of methane production during the initial phase of anaerobic digestion revealed a slow start in methane production in the control as well as the test reactors. While initial methane production inhibition was expected due to IONp cytotoxicity within the test reactors, both the test and control reactors could also have suffered from stress associated with introduction of the substrate, cellulose. Evaluation of the BSI on day zero in the control and test reactors (after IONp addition) indicated relatively high BSI values at 31.8% and 53.2% respectively (Fig.5.5). By day five, however, while BSI in the control reactors had remained largely unchanged at 31.1% indicative of microbial stress, the BSI had declined in the test reactors (44.3%) indicating microbial adaptation and cell recovery (Fig.5.5). These results were supported by the cATP results where the cATP in the test reactors had initially declined from 81.6 ng ATP/mL to 32.3 ng ATP/mL. Thereafter cATP had increased 324.8% by day five (137.2 ng ATP/mL). These mirrored increase in the 16s rRNA gene copy numbers in both the *Eubacteria* (1268 %) as well as the *Methanogen* community (258.5 %) (Fig. 5.1 (a –b)). The anaerobic process performance had also improved in tandem (Fig.5.2).

Nanotube-mediated bacterial communication had been thought to represent a significant form of direct cell-cell communication in microbial communities although evidence to support this had been lacking. In this study, improved performance despite the relatively high BSI, have led to the hypothesis that intercellular nanotube mediated communication may have played a significant role. To test this hypothesis, a combination of FE-SEM and EDX was used to examine the control and the test microbial aggregates at various time points during the anaerobic process. Scanning electron micrographs revealed extensive presence of interspecies nanotubes interconnecting the microorganisms at all the sampling time points. Interspecies nanotubes were, however, also noted in the control samples (Fig. 5.8.c, Fig. 5.8.d). The diameters of the nanotubes were between 0.13 μ m and 0.47 μ m. Elemental mapping through Energy Dispersive X-ray Spectrometer (EDX) showed that the elemental compositions of the bacterial cells and the interconnecting nanotubes were similar (Fig. 5.11a, 5.11b, 5.11c, and 5.11d). This indicated the tubular structures were cell-derived as previously reported (Dubey and Ben-Yehuda 2011). Given the relatively high BSI in the control as well as test reactors, this study would suggest an environment leading to microbial stress was an inducer for the production of intercellular nanotubes. It has been reported that

microbial phenomena such as development of bacterial competence (Hotchkiss 1954), quorum sensing (Oleskin, Botvinko et al. 2000), bioluminescence (Antunes and Ferreira 2009), and rapid increase in cell density is preceded by an initial lag phase, and this was similarly observed in this study - where an initial lag phase preceded rapid increase in microbial numbers. This study would propose this phenomenon could also be a characteristic feature of intercellular nanotube formation. Intercellular nanotube formation between microorganisms has been thought to occur only on solid surfaces or in liquid cultures, this study showed that intercellular nanotubes between microorganisms could also be formed in anaerobic sludge which was subjected to shear forces arising from intermittent stirring at 50 rpm (Fig.5.8a). This suggested the production of interspecies nanotubes connecting microorganisms may be independent of the nature of the culture medium.

Figure 5.8.g. **Formation of Intercellular nanotubes between cells.** *Microbial cells interconnected by intercellular nanotubes in a sample taken from the test reactor on day 20. Scale bar is 1µm.*

Figure 5.8.h. **Formation of Intercellular nanotubes between cells.** *Test reactor day 20. Scale bar is 1µm.*

Figure 5.9. **FE-SEM micrographs of the Blank and Iron oxide nanoparticle augmented Blank reactor.** Intercellular nanotubes are not visible in the anaerobic sludge inoculum. *Figure (i) Blank reactor Day 0. Magnification is 10000X. Figure (ii) Blank reactor Day 0. Magnification is 20000X. Test reactor (day 24). Here healthy microbial cells are seen with surface deposition of the iron oxide nanoparticles. Magnification is 20000X. Scale bar is 1μm.*

Figure 5.10.a. **Cytotoxicity caused by Iron oxide nanoparticle deposition.** *Iron oxide nanoparticle deposition on the microbial cells visualized within a day 20 sample from the test reactor. The cellular surfaces appear damaged. Scale bar is 1μm.*

Figure 5.10.b. **Cytotoxicity caused by Iron oxide nanoparticle deposition.** *Iron oxide nanoparticle deposition on the microbial cells visualized within a day 20 sample from the test reactor. The cellular surfaces appear damaged. Scale bar is 1μm.*

Figure 5.10.c. **Cytotoxicity caused by Iron oxide nanoparticle deposition.** *Despite the toxicity of the IONp, this microbial cell appears healthy. The nanoparticles are present on cellular appendages resembling nanowires. Scale bar is 100nm.*

Figure 5.10.d. **Cytotoxicity caused by Iron oxide nanoparticle deposition.** *Some microbial cells appear less susceptible to nanoparticle surface deposition indicated by a pink arrow. An adjacent microorganism, however is heavily coated with the nanoparticles, indicated by a blue arrow. Scale bar is 1µm.*

Figure 5.10.e. **Cytotoxicity caused by Iron oxide nanoparticle deposition and intercellular nanotubular connections.** *FE-SEM microstructure of sludge sample taken from a test reactor on day 20. In these images, microbial cells appear distorted, however the cellular integrity appears uncompromised in some cells. Figure (i) magnification is 12000X. Scale bar is 1µm. Figure (ii) magnification is 30000X. Scale bar is 100nm.*

Element	Weight%	Atomic%
C K	85.40	89.59
O K	12.54	9.88
Al K	0.16	0.07
Si K	0.28	0.12
S K	0.19	0.07
Cl K	0.09	0.03
Ca K	0.17	0.05
Fe K	0.14	0.03
Zr L	1.09	0.15
Totals	100.00	

Figure 5.11.a. **Elemental composition of intercellular appendages.** *Points at which EDX spectra were obtained and the respective elemental composition are shown (Scale bars: 9µm).*

Element	Weight%	Atomic %
C K	66.35	71.14
N K	22.86	21.02
O K	9.35	7.52
Al K	0.07	0.03
Si K	0.16	0.07
S K	0.12	0.05
Cl K	0.10	0.04
Ca K	0.07	0.02
Zr L	0.67	0.09
Pt M	0.25	0.02
Totals	100.00	

Figure 5.11.b. **Elemental composition of intercellular appendages.** Points at which EDX spectra were obtained and the respective elemental composition are shown (Scale bars: 9 μm).

Element	Weight%	Atomic%
C K	52.88	65.25
N K	18.05	19.09
O K	14.05	13.02
Al K	0.16	0.09
Si K	0.23	0.12
S K	0.22	0.10
Ca K	0.65	0.24
Fe K	5.46	1.45
Pt M	8.83	0.67
Bi M	-0.53	-0.04
Totals	100.00	

Figure 5.11.c. **Elemental composition of intercellular appendages.** Points at which EDX spectra were obtained and the respective elemental composition are shown (Scale bars: 5µm).

C Ka1_2

O Ka1

S Ka1

Ca Ka1

Fe Ka1

Zr La1

Figure 5.11.c. **Elemental composition of intercellular appendages.** *Points at which EDX spectra were obtained and the respective elemental composition are shown (Scale bars: 5µm).*

Figure 5.11.d. **Elemental composition of intercellular appendages.** *Points at which EDX spectra were obtained and the respective elemental composition are shown (Scale bars: 2μm).*

Figure 5.12.a. **Microbial nanowires.** *AFM image of iron oxide nanoparticles and microbial nanowires indicated by pink arrows. Scale bar is 850 nm.*

Figure 5.12.b. **Microbial nanowires.** *FE-SEM image of the iron oxide nanoparticles with cellular appendages, thought to be microbial nanowires indicated by pink arrows. The scale bar is 100nm.*

Figure 5.12.c. **Microbial nanowires.** AFM image of IONp and microbial nanowires highlighted by pink dashed circles. Scale bar is $3\mu\text{m}$.

Figure 5.12.d. **Microbial nanowires.** Microbial appendages such as flagella have been implicated in iron reduction activities. Test reactor (day 20). Intercellular nanotubes are highlighted in Pink. Figure (i) magnification is 5000X. Scale bar is 1µm. Figure (ii) magnification is 10000X. Scale bar is 1µm.

Figure 5.13. Atomic Force Microscopic images of day 5 test samples showing healthy microbial cells. Scale bar is 5 μm.

5.5 Conclusion

This study investigated the impact of iron oxide nanoparticles on an anaerobic microbial consortium digesting cellulose. Cytotoxic stress and loss in microbial numbers as a result of iron oxide nanoparticle addition was reversed as anaerobic digestion progressed. Establishment of a dissimilatory iron reducing bacterial community aided the recovery of *Eubacteria* and *Methanogens*. This was largely through the supply of Fe^{2+} which resulted in improved enzymatic activity (Casals, Barrena et al. 2014). Additionally, it was possible the IONp increased the surface area of contact between the hydrolytic enzyme complex and substrate which resulted in enhanced hydrolysis of the insoluble cellulose (Lynd, Weimer et al. 2002). This increased bioavailability of substrates to the acidogen, acetogen and methanogen communities and enabled cell number recovery. Microbial syntrophy was also enhanced in the presence of IONp which created a conducive environment for the anaerobic process.

In this study, unfavorable environmental conditions led to biomass stress which resulted in the establishment of intercellular nanotubes within the microbial communities present in both the Control as well as Test reactors. Nanotube-mediated bacterial communication was first demonstrated by Dubey and Ben-Yehuda in 2011, it was shown to facilitate exchange of cytoplasmic contents between identical species as well as different species of bacteria (Dubey and Ben-Yehuda 2011). Subsequent studies have reported that metabolic cross-feeding (Pande, Shitut et al. 2015) is facilitated between bacteria interconnected by intercellular nanotubes and nutritional stress has been reported to induce nanotube formation to facilitate cytoplasmic exchange and energetic coupling between interconnected cells (Benomar, Ranava et al. 2015). Under adverse environmental conditions, direct cell-cell contact ensures the targeted exchange of metabolic products between cells mitigating loss of metabolic product to the environment as well as ensuring synergistic growth and activity (Pande, Shitut et al. 2015). Evaluation of this study's results would suggest microorganisms preferentially established communication through intercellular nanotubes under influence of unfavorable environmental conditions. Given the improved performance of the test reactors despite the toxicity, it was likely that intercellular nanotube – mediated communication played an important role. This study has shown evidence of the spontaneous establishment of such intercellular nanotubes within an anaerobic microbial community. The

microbial transition from uni-cellularity to multi-cellularity under unfavorable conditions could have significant implications both in anaerobic digestion as well other microbial processes.

CHAPTER 6

STUDY CONCLUSIONS AND RECOMMENDATIONS

This thesis investigated the impact of iron oxide nanoparticles augmentation on the anaerobic sludge microbiota as well as the overall anaerobic digestion process. The system used in this study was an Automatic Methane Potential Test System II (AMPTS II) (Bioprocess Control AB, Sweden) which operated 15 stirred batch reactors anaerobically digesting microcrystalline cellulose with anaerobic sludge from a local municipal wastewater treatment plant used as the inoculum. The test reactors were augmented with synthesized iron oxide nanoparticles (IONp) and the overall impact of the nanoparticles augmentation on the test systems with respect to methane production activity was monitored. The main motivation for this study was to enhance the rate-limiting hydrolysis step in the anaerobic digestion process and thereby improve methane production. This would then enable a reduction in the carbon footprint and simultaneously allow for the production of a renewable fuel – methane from waste substrates through anaerobic digestion. The following conclusions may be drawn from the study:

Objective 1: To evaluate the impact of iron oxide nanoparticles on the anaerobic digestion process

In this thesis work, microcrystalline cellulose was used as the substrate in the anaerobic digestion study because the key biochemical events in the anaerobic conversion of cellulose to methane and carbon dioxide simulates the entire anaerobic digestion process, therefore each stage of the AD process may be examined in detail.

Results indicated that the IONp largely improved the overall anaerobic digestion process. The enhancement of cellulose substrate hydrolysis within the IONp augmented reactors was indicated by a large increase in soluble COD (837.5 mg/L in the test reactor and 358.4 mg/L in the control) in the early phase of the anaerobic digestion process. Furthermore, metaproteomic analysis also revealed a relative increase in the concentration of secreted proteins into the mixed liquor in the IONp augmented reactors in comparison to the control system (the protein concentration was

determined to be 41µg/mL in the test and 31 µg/mL in the control). Relative and absolute quantification of the secreted proteins revealed a collection of hydrolytic enzymes such as *Endoglucanase*, *Hydrolases*, *Peptidases* and *Proteases* were significantly expressed by microorganisms associated with hydrolytic activity such as *Kluyveromyces lactis*, *Bacillus cereus*, *Pseudomonas sp.*, *Salmonella sp.*, yeasts and fungi in the IONp augmented system in comparison to the control.

Increased acidogenic activity generally results in an increase in hydrogen production and this could in turn disrupt the delicate syntrophic balance among the microbial communities (Sieber, McInerney et al. 2012). Hence the scavenging of hydrogen by hydrogenotrophic methanogens via microbial syntrophy would be important for process stability.

In this study, it was observed, that the presence of iron oxide nanoparticles within the anaerobic reactors also facilitated the establishment of a well - balanced syntrophic association among the microbial communities. This was primarily evidenced by the comparatively reduced/negligible amounts of propionic acid in the nanoparticles – augmented anaerobic digestion reactors as well as the negligible amount of hydrogen gas detected. Conversely, large amounts of propionate and reduced volatile fatty acids were detected in the control reactors not amended with the iron oxide nanoparticles on day 7 (238.5 mg/L in the test and 540 mg/L in the control (Chapter 4) and on day 23, 14.8 mg/L in the test reactor and 579.7 mg/L in the control (Chapter 4) indicative of impaired microbial syntrophy in the Control while establishing that syntrophy between the fatty acids oxidizing bacteria and methanogens was enhanced in the presence of iron oxide nanoparticles.

The efficient scavenging of hydrogen by the hydrogenotrophic methanogens and the enhanced syntrophy within the Iron oxide nanoparticles augmented anaerobic digestion system therefore prevented the accumulation of toxic reduced volatile fatty acids and helped in the maintenance of optimal pH conditions which in turn facilitated an enhancement of methane production. Therefore, this study would conclude that the addition of iron oxide nanoparticles to the anaerobic digestion of cellulose had an overall ameliorative effect and improved both hydrolysis (and acidogenesis) and simultaneously enhanced microbial syntrophy.

Objective 2: To identify the key biochemical event in anaerobic digestion process most impacted by the addition of iron oxide nanoparticles

Our preliminary experiments indicated that the Iron oxide nanoparticles (IONp) could potentially influence the hydrolysis event in the anaerobic digestion process. Therefore, in order to investigate and evaluate this potential, insoluble microcrystalline cellulose (which is the standard substrate of choice in the study of hydrolysis (Angelidaki, Alves et al. 2007)) was used in this thesis work (Chapter 4 and Chapter 5). The results of this thesis study showed that augmentation of the anaerobic digestion system with the Iron oxide nanoparticles did indeed primarily enhance hydrolysis and also had an ameliorative effect on the overall anaerobic digestion process.

Augmentation of the anaerobic digestion test reactors with IONp resulted in an initial net decline (approximately one order of magnitude) of 16S rRNA gene copy numbers across all the microbial communities largely due to oxidative stress. However, as the anaerobic digestion progressed, an increase in the concentration of Fe^{2+} ions was indicative of Dissimilatory Iron Reducing bacterial activity. This supply of Fe^{2+} (important for microbial biosynthesis pathways and enzyme repair functions) by the DIRB activity as well as, the establishment of microbial nanowires in the IONp augmented reactors (as proved by AFM analysis) facilitated Interspecies Electron Transfer (IET) among the microbial communities and thereby aided in microbial energy conservation and enhanced metabolism which allowed for the initial recovery of microbial numbers.

Enhanced cellulose hydrolysis in response to IONp augmentation was indicated by a significant increase in the *Eubacteria* community from 2.73×10^{10} to 1.15×10^{11} ($p < 0.001$) on day 7 of the anaerobic digestion together with a large increase in soluble COD (in the test reactor 837.5 mg/L and in the control 358.4 mg/L). Absolute and relative quantification of the IONp augmented protein secretosome revealed increased relative abundances of enzymes such as *Glucokinase* (expressed 8.82 fold higher than the control) and *Phosphoglycerate kinase* involved in Glycolysis which was indicative of the efficient solubilization of cellulose to glucose (the metabolic substrate for Glycolysis)

Furthermore, increased hydrolytic activity in the IONp augmented reactors was supported by an increased production of secreted enzymes (the secreted protein concentration was found to be 41 $\mu\text{g/mL}$ in the test and 31 $\mu\text{g/mL}$ in the control) into the mixed liquor of the anaerobic digestion

system. Relative and absolute quantification of the test protein secretosome revealed increased expression of hydrolytic enzymes such as *Endoglucanase*, *Hydrolases*, *Proteases*, *Peptidases* and *Xylanases* from microorganisms frequently associated with hydrolytic activity such as *Bacillus sp.*, *Schizosaccharomyces sp.*, *Saccharomyces sp.*, *Kluyveromyces lactis*, *Lactobacillus sp.*, *Clostridium sp.* and *fungi* (Chapter 5).

An improved surface area of contact between the hydrolytic enzyme complex and the cellulose substrate mediated by the IONp which allowed for sustained contact between the extracellular hydrolytic enzyme complex and the particulate microcrystalline cellulose is implicated in the enhanced hydrolysis reaction.

Improved cellulose hydrolysis increased the carbon flow to the acidogen, acetogen and methanogen communities indicated by the volatile fatty acids production and consumption (Chapter 4 and Chapter 5). Increased acidogenic activity could result in a digester imbalance due to an increase in hydrogen production as well as accumulation of reduced volatile fatty acids which could then disrupt the balance among the microbial communities and lead to process failure. However, the results of this thesis study revealed that IONp augmentation led to the establishment of a well – balanced syntrophic association between the fatty acids oxidizing bacteria and the hydrogenotrophic methanogens.

This is indicated by the comparatively low to negligible levels of propionate and hydrogen gas detected in the IONp augmented reactors, which was determined to be 238.5 mg/L in the test and 540mg/L in the control on day 7 (Chapter 4) and 14.8mg/L in the test reactor and 579.7 mg/L in the control on day 23 (Chapter 4) clearly indicative of impaired microbial syntrophy in the Control and enhanced microbial syntrophy in the presence of iron oxide nanoparticles. Given that the only difference between the control and test reactors was the presence of iron oxide nanoparticles in the test reactors, the establishment of a balanced syntrophic association in the test reactors could largely be attributed to the presence of iron oxide nanoparticles and the DIRB community - which helped in the creation of a favorable environment for the anaerobic digestion process primarily in terms of pH conditions (Chapter 4) as well as prevention of accumulation of reduced volatile products and hydrogen within the test system.

The results of this thesis study therefore conclude that the key biochemical event largely influenced by the IONp augmentation was primarily hydrolysis with an improved overall anaerobic digestion reaction due to enhanced microbial syntrophy among the microbial communities.

Objective 3: To examine the effects of the iron oxide nanoparticles on the anaerobic sludge microbiota

Augmentation of the anaerobic digestion of cellulose with IONp resulted in cellular stress, largely due to the cell surface deposition of nanoparticles with loss of both structural as well as cellular integrity in a portion of the microbial consortium, as indicated by the Field emission scanning electron microscopy and Atomic Force microscopic analysis. ATP analysis of the microbial biomass also indicated increased biomass stress from 30.9% to 53.2% and a decline in cellular ATP from 81.6 ng ATP/mL on day zero before nanoparticle addition to 32.3 ng ATP/mL shortly after nanoparticle addition on day zero (Chapter 5). Similarly, quantitative PCR analysis of the 16S rRNA gene revealed a net decline of approximately one order of magnitude across all the microbial communities. Relative and absolute quantification of the IONp augmented protein secretosome further corroborated the initial cytotoxicity of the nanoparticles on the anaerobic sludge microbiota was largely due to oxidative stress; increased relative abundances of proteins such as *protein P52677 (Probable hydrogen peroxide-inducible genes activator)*, *Chaperone protein DnaK* as well as DNA damage repair enzymes such as *DNA helicase*, *DNA Polymerase IV* and *DNA ligase* were expressed in the iron oxide nanoparticles augmented reactors in comparison to the control (Table 4.2 and Table 4.3).

This cytotoxic event was followed by microbial rewiring (through intercellular nanotubes and microbial nanowires) and recovery. The anaerobic sludge microbiota in our experiments had produced intercellular nanotubes which inter-connected microorganisms across different species in response to microbial and nutritional stress (found in both the IONp augmented and control system without the nanoparticles). Nanotube-mediated bacterial communication had been thought to represent a significant form of direct cell-cell communication in microbial communities which facilitates the exchange of cytoplasmic contents and cell factors between interconnected microbial cells.

Because these intercellular nanotubes are produced in an inter-species manner, this would then suggest the potential exchange of metabolic intermediates between microbial species with metabolically distinct capabilities in a coordinated and cooperative manner to efficiently share metabolites and cell factors thus simulating a transition from uni-cellularity to multi-cellularity, especially to survive hostile environmental conditions. In this study, improved performance despite the relatively high Biomass stress in the IONp augmented reactors as well as visual evidence of intercellular nanotubes (Chapter 5) throughout the anaerobic digestion process suggested that intercellular nanotube-mediated communication may have played a significant role in the recovery and enhancement of microbial numbers possibly by mitigating loss of metabolic intermediates and cellular factors to the environment.

Given that despite, the initial loss of microbial numbers and increased biomass stress in the IONp augmented reactors, better performance was noted (Chapter 4 and Chapter 5), the significant recovery of *Eubacteria* microbial numbers is also attributed to the production of microbial nanowires by the Dissimilatory Iron Reducing Bacteria (DIRB). Microbial nanowires have been found to be produced in response to iron oxide materials mainly by the DIRB. Microbial nanowires are cellular extrusions mainly composed of the outer membrane and are periplasmic cell extensions which allow for extracellular electron transport with potential function as an outer surface-type cytochrome analogue (Pirbadian, Barchinger et al. 2014, Liu, Rotaru et al. 2015).

The presence of microbial nanowires was similarly noted only within the test reactor augmented with iron oxide nanoparticles through Atomic force microscopic analysis, evidence of the same is presented in Chapter 6. Interspecies electron transfer within the anaerobic digestion system is primarily thought to occur via the exchange of diffusive metabolites such as H_2 and formate. However, Direct Interspecies Electron Transfer (DIET) has been shown to be more efficient and to occur at faster rates (Kato, Hashimoto et al. 2012, Cruz Viggi, Rossetti et al. 2014, Pirbadian, Barchinger et al. 2014, Liu, Rotaru et al. 2015, Lovley and Malvankar 2015). Theoretical syntrophic calculations to compare the kinetic efficiency of diffusive H_2 exchange and electric conduction via an iron oxide (magnetite) based conduit by (Cruz Viggi, Rossetti et al. 2014) have computed the difference between these 2 fluxes to be very large (i.e., a 10^6 factor) clearly indicative of the kinetic advantage of direct interspecies electron transfer via conduction. The main function of the nanowires is to facilitate the long - range extracellular electron transport in an interspecies

manner linking the electron donors and acceptor. This in turn is thought to help in energy conservation and an efficient, directed transfer of electrons.

Our experiments indicated the minor presence of a native, uncharacterized DIRB community present in the anaerobic seed sludge before inoculation into the reactors. Augmentation of the test reactor with iron oxide nanoparticles resulted in an increase in Fe^{2+} iron within the IONp augmented test reactor (indicative of DIRB activity) but not in the control reactor (Chapter 4 and Chapter 5). This, together with the presence of microbial nanowires in the test system indicates that despite the initial loss of microbial numbers due to the addition of iron oxide nanoparticles across the microbial communities, DIRB growth was promoted which aided in the supply of Fe^{2+} to the microbial communities (important for microbial biosynthesis pathways and enzyme repair functions) which facilitated the recovery of microbial numbers (Chapter 4 and Chapter 5).

Therefore, as the anaerobic digestion progressed, a decline in the Biomass Stress Index (from 53.2% to 44.3%) and a simultaneous 324.8 % increase in cellular ATP which is indicative of living biomass (32.3 ng ATP/mL to 137.2 ng ATP/mL) as well as 16S rRNA gene copy numbers across all the microbial communities (from 1.25×10^{10} to 1.71×10^{11} in *Eubacteria* and from 3.14×10^8 to 1.14×10^9 in *Methanogens* on day 5 of anaerobic digestion (Chapter 4 and Chapter 5) indicated microbial adaptation and recovery in the nanoparticle - augmented reactors, while BSI in the control reactors had remained largely unchanged (from 31.8% on day zero to 31.1% on day 5) indicative of microbial stress due to impaired substrate utilization. Furthermore, optimal pH conditions regulated by the microbiota, enhanced microbial syntrophy and the efficient scavenging of H_2 by the hydrogenotrophic methanogens ensured no accumulation of reduced metabolic products which in turn provided an optimal condition for the anaerobic digestion process.

Recommendations

Based on the findings of this thesis, some research questions arise which may suggest perspectives for future research direction, they are:

In the study of hydrolysis in anaerobic digestion, current technology used to quantify the rate of hydrolysis primarily includes spectrophotometric quantification of the reducing sugars released from the hydrolysis of cellulose. Although widely used, because of its simplistic nature, this method has many demerits which include a lack of reproducibility and inaccurate results. Therefore, in this study the hydrolysis potential was evaluated through the use of a model hydrolytic substrate (Microcrystalline cellulose), changes in soluble COD together with a high throughput metaproteomic analysis of the secreted proteins via Iso-baric Tags for relative and Absolute Quantification to relatively quantify and identify the secreted proteins. It is suggested that future studies may consider establishing a similar, but more economical high throughput automated technique which would minimize human errors and produce accurate and reproducible results.

Chapter 5 showed that microbial stress resulted in the formation of intercellular nanotubes among the anaerobic microbial consortium. Thus far, accepted knowledge of microbial cross – talk within wastewater and anaerobic digestion system have been largely restricted to microbial communication via pili, biofilms, quorum sensing and release and detection of extracellular molecules. This thesis work provided evidence of intercellular nanotube mediated microbial communication among the anaerobic sludge microbiota. This is a novel mode of microbial communication, especially in biological wastewater treatment. Therefore, future research should be directed toward gaining a deeper insight into the study of intercellular nanotubes, how they may influence quorum sensing among the anaerobic sludge microbiota and their influence on biological wastewater treatment efficiency and process design.

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