Characterisation of soluble microbial products (SMPs) in anaerobic bioreactors

Le, Chencheng

2018

Le, C. (2018). Characterisation of soluble microbial products (SMPs) in anaerobic bioreactors. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/73164>

<https://doi.org/10.32657/10356/73164>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**CHARACTERISATION OF SOLUBLE MICROBIAL
PRODUCTS IN ANAEROBIC BIOREACTORS**

LE CHENCHENG

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2017

CHARACTERISATION OF SOLUBLE MICROBIAL PRODUCTS IN ANAEROBIC BIOREACTORS

LE CHENCHENG

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy

2017

ACKNOWLEDGEMENTS

It is always an honor to thank people who have helped me over the course of this work.

First of all, I would like to express my sincere gratitude and appreciation to my supervisors Professor David C. Stuckey and Professor Liu Yu, and thank them for accepting me as a Ph.D student and giving me the opportunity to do this work. In the course of this research project, David gave me invaluable supervision, advice, support and patient guidance. I am especially thankful for the sharing of his vast knowledge on various aspects of life, if not all. The knowledge, experience, teamwork and analytical thinking skills that I have gained during this period are the most precious assets I have gained from this work.

I am truly grateful for the support I received from the Nanyang Environment and Water Research Institute without which this experience would not have been possible.

It would also not have been possible to perform so many experiments without the expert assistance from Dr Chen Lin, Dr Roger Xiao Yeyuan, Dr Zhou Zhongbo and Dr Chinagarn Kunacheva. They have been my wonderful guardians, always there to clarify my doubts, share their experience, and help me out when I needed it the most. Special thanks to Assoc. Prof. Richard D. Webster and Dr Chen Jian Lin for invaluable discussion during this study.

Next I would like to extend my thanks to all of the group members in Advanced Environmental Biotechnology Centre (AEBC) for their tremendous help, and discussions throughout the period. Working with them has been the most enjoyable and enriching experience. Thanks to them for creating a helpful, friendly and accommodating research environment. Acknowledgements also go to my parents and friends for their love, understanding and constant encouragement throughout my study.

Lastly, but definitely not least, the assistance and contributions from the staff of AEBC on top of their selfless devotion in laboratory safety and facilitating in all forms are

greatly appreciated. The writer would also like to acknowledge the financial support from the Singapore National Research Foundation under its Environmental & Water Technologies Strategic Research Programme, and the research grant administered by the Environment & Water Industry (EWI) Programme Office of the Public Utilities Board (PUB), Singapore (PUB IDD 21100/36/6).

CONTENTS

TABLE OF CONTENTS	PAGE
Acknowledgement	i
Contents	iii
Abstract	viii
List of Publications	ix
List of Abbreviations	x
List of Figures	xiii
List of Tables	xiv
1 - Introduction	1
2 - Background and Literature Review	5
2.1 Introduction	5
2.2 Historical aspects of the anaerobic process	5
2.3 Fundamentals of anaerobic processes	7
2.4 Anaerobic treatment of wastewater	10
2.5 SMPs and their implications	11
2.5.1 Definitions of SMPs	11
2.5.2 SMPs and other biomass components	13
2.5.3 SMPs in anaerobic systems and their impact	14
2.6 Why are SMPs produced?	14
2.6.1 Feed strength, organic loading rate and HRT	15
2.6.2 Biomass concentration and solid retention time	15
2.6.3 Reactor configuration	16
2.6.4 Temperature	16
2.6.5 Substrate type	17
2.6.6 Other factors	17
2.7 Characterisation of SMPs	18
2.7.1 Characterisation of global parameters	18
2.7.1.1 MW distribution	19
2.7.1.2 Biodegradability assay	21
2.7.1.3 Toxicity	22

2.7.2	Chemical characterisation: individual compounds	23
2.7.2.1	Chemical characterisation: glycolysis	25
2.7.2.2	Chemical characterisation: proteolysis	26
2.8	Concluding remarks	27
2.9	Overview and research objectives	29
3	Materials and Methods	30
3.1	Introduction	30
3.2	Submerged anaerobic membrane bioreactor	30
3.3	General parameters	31
3.3.1	pH.....	31
3.3.2	Chemical Oxygen Demand	31
3.3.3	Volatile fatty acids	33
3.3.4	Suspended solids	33
3.3.5	Composition of gas	33
3.3.6	Calculation of SMP concentration	34
4	Characterisation of low MW SMPs	35
4.1	Introduction	35
4.2	Experimental and analytical details	37
4.2.1	Reagents and chemicals	37
4.2.2	Optimization of LLE for SMP analysis	37
4.2.3	Optimization of SPE for SMP analysis	39
4.2.4	Gas chromatography-mass spectrometry	41
4.2.5	Quantification	41
4.3	Results and discussion	43
4.3.1	Performance of SAMBR	43
4.3.2	Optimization of LLE for SMP analysis	44
4.3.2.1	Selection of solvents	44
4.3.2.2	Volume of extraction solvent	46
4.3.2.3	Effect of pH and additives	47
4.3.2.4	Selection of sample size	47
4.3.3	Optimization of SPE for SMP analysis.....	48
4.3.3.1	Comparison of Oasis®HLB and ENVI-Carb cartridge.....	48
4.3.3.2	Comparison of one cartridge with two cartridges	49
4.3.3.3	Effect of solvents for elution	50
4.3.4	Combination of LLE and SPE	51
4.4	Conclusions	59

5 - Characterisation of Polysaccharides	60
5.1 Introduction	60
5.2 Experimental and analytical details	63
5.2.1 Reagents and chemicals	63
5.2.2 Anthrone method	64
5.2.3 Phenol-sulfuric acid method	64
5.2.4 Periodic Acid-Schiff stain method	65
5.2.5 Metachromatic response on different saccharides	66
5.2.6 Colorimetric carbohydrate analysis with interfering solutes	66
5.2.7 Case study	67
5.2.8 Glycolysis protocol	67
5.2.9 Preparation of standards	70
5.3 Results and discussion	73
5.3.1 Performance of individual assays	73
5.3.2 Metachromatic response of different saccharides.....	74
5.3.3 Colorimetric carbohydrate analysis with interfering solutes	77
5.3.4 Case study	79
5.3.5 Exopolysaccharide versus exopolymeric substances	81
5.3.6 Glycolysis	82
5.3.6.1 Identification of monomeric structure	86
5.4 Conclusions	87
 6 - Characterisation of proteinaceous materials	 89
6.1 Introduction	89
6.2 Experimental and analytical details	91
6.2.1 Reagents and chemicals	91
6.2.2 Modified Lowry method	92
6.2.3 Bradford method - Coomassie	93
6.2.4 Bradford method - Coomassie Plus TM	93
6.2.5 Micro BCA TM method	93
6.2.6 Pierce [®] BCA method	94
6.2.7 Colorimetric protein analysis with interferences	94
6.2.8 Metachromatic response on different proteins and peptides	95
6.2.9 Case study	95
6.2.10 Proteolysis and amino acids analysis using HPLC	96
6.3 Results and discussion	98
6.3.1 Working range of individual assays	98
6.3.2 Colorimetric protein analysis with interferences	99
6.3.3 Metachromatic response of different proteins and peptides	102

6.3.4	Case study	104
6.3.5	Protein versus proteinaceous materials	105
6.3.6	Proteolysis and amino acids analysis using HPLC	108
6.3.7	FAAs in SAMBR	110
6.4	Conclusions	111
7	- Impact of nutrients on SMPs production	112
7.1	Introduction	112
7.2	Experimental and analytical details	115
7.2.1	Reagents and chemicals	115
7.2.2	Anaerobic sludge and medium	115
7.2.3	Sample preparation and fractionation	116
7.2.4	General parameters and analytical methods	117
7.3	Results and discussion	117
7.3.1	Performance of the reactors	117
7.3.2	MW distribution analysis of SMPs	120
7.3.3	Impact on low MW SMPs	122
7.3.4	Occurrence of FAAs	128
7.3.5	Polysaccharide composition	130
7.3.6	Physiological aspects of EPS production	133
7.3.7	Possible implications for the membrane	135
7.4	Conclusions	136
8	- Concluding discussion	138
8.1	Introduction	138
8.2	Final conclusions	138
8.3	Implications of this research	142
8.4	Recommendation for future research	145
	References	148

ABSTRACT

The primary aim of this research was to develop a variety of methods for the specific characterisation of soluble microbial products (SMPs) that are produced during substrate metabolism or cell lysis and degradation in anaerobic treatment. In biological wastewater treatment processes, the analysis of metabolic compounds that are produced during the process is critical to monitoring the performance of system; these soluble products directly affect process efficiency (removal of oxidizable compounds) and quality of the effluent after treatment, and are also one of the major causes of fouling in membrane bioreactors. However, analysing them is challenging due to their inherently dilute nature (sub ppm level) and heterogeneity. In this PhD project, the chemical composition of SMPs produced during the anaerobic condition will be identified and quantified using both sophisticated analytical instruments, e.g. GC-MS, and conventional chemical analyses. Firstly, an analytical methodology for low molecular weight SMPs (<580 Da) in wastewater treatment systems was established using a combination of solid phase extraction (SPE) and liquid-liquid extraction (LLE), which was optimized and enabled the maximum number of compounds to be identified. Next, conventional colorimetric measurements of carbohydrates and proteinaceous materials generated from anaerobic bioreactor were critically evaluated. These result highlighted the severe limitations of these dated assays, and the need for exercising care when interpreting results, particularly where complex heterogeneous wastewater is concerned. Then protocols for the characterisation of free amino acids using HPLC, and for elucidating the monomeric structure of loosely bound exopolysaccharides (EPS) using GC-MS were developed in order to gain greater insights into the biological transformations occurring in anaerobic digestion (AD), and the SMPs produced. Finally, all these optimized methods were employed to comprehensively assess the impact of different carbohydrate substrates (glucose, fructose and sucrose) and nitrogen sources (urea and NH_4Cl) on the production and composition of SMPs during AD. This study revealed that these macronutrients could strongly influenced many measures of

wastewater quality, and the results clearly demonstrated that well-characterized nutritional conditions ensure a cooperative bacterial symbiosis converting complex organic matter to biogas effectively. All the findings in this project provide fresh and useful insights into an area that is under researched, and often overlooked by engineers studying overall engineered wastewater treatment systems.

LIST OF PUBLICATIONS

1. Le, C.; Kunacheva, C.; Stuckey, D. C. (2016) “Protein” Measurement in Biological Wastewater Treatment Systems: A Critical Evaluation. *Environ. Sci. Technol.* 50, 3074-3081.
2. Le, C.; Stuckey, D. C. (2016) Colorimetric measurement of carbohydrates in biological wastewater treatment systems: A critical evaluation. *Water Res.* 94, 280-287.
3. Kunacheva, C.; Le, C.; Soh, Y. N. A.; Stuckey, D. C. (2017) Chemical characterization of low molecular weight soluble microbial products in anaerobic membrane bioreactors. *Environ. Sci. Technol.* 51, 2254-2261.
4. Le, C.; Stuckey, D. C. (2017) Impact of feed carbohydrates and nitrogen source on the production of soluble microbial products (SMPs) in anaerobic digestion. *Water Res.* 122, 10-16.
5. Le, C.; Stuckey, D. C. (2017) Influence of feed composition on the monomeric structure of free bacterial extracellular polysaccharides in anaerobic digestion. *Environ. Sci. Technol.* 51, 7009-7017.

LIST OF ABBREVIATIONS

2DE	Two Dimensional Gel Electrophoresis
2D-PAGE	Two Dimensional Polyacrylamide Gel Electrophoresis
AA	Amino Acids
AAA	Amino Acids Analysis
ABR	Anaerobic Baffled Reactor
AD	Anaerobic Digestion
AeTW	Aerobic Wastewater Treatment
AHS	Aquatic Humic Substance
AnMBR	Anaerobic Membrane Reactor
AnTW	Anaerobic Wastewater Treatment
APHA	American Public Health Association
BAP	Biomass Associated Product
BCA	Bicinchoninic Acid
BMP	Biochemical Methane Potential
BOD	Biochemical Oxygen Demand
BSA	Bovine Serum Albumin
BTSE	Biological Treated Sewage Effluent
COD	Chemical Oxygen Demand
CPS	Capsular Polysaccharide
CSTR	Continuous Stirred-Tank Reactor
CV	Coefficient of Variation
Da	Dalton
DAD	Diode Array Detector
DBP	Disinfection By-Products
DBPFP	Disinfection By-Products formation potential
DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EAA	Extended Amino Acids
ECP	Extracellular Polymers
ECPS	Extracellular Polysaccharides
EPG	Extracellular Proteoglycan
EPS	Extracellular Polymeric Substances

EPS	Exopolymeric substances
EPS	Exopolysaccharide
EPS	Extracellular Polysaccharides
EfOM	Effluent Organic Matter
FAAs	Free Amino Acids
FISH	Fluorescent <i>in situ</i> Hybridisation
FMOC	9-Fluorenylmethyl Chloroformate
FTIR	Fourier Transform Infrared Spectroscopy
GABA	Gamma-Amino-n-Butyric Acid
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GFC	Gel-Filtration-Chromatography
GPC	Gel-Permeation Chromatography
H-NMR	Hydrogen Nuclear Magnetic Resonance
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
IDL	Instrument Detection Limit
IQL	Instrument Quantification Limit
LC	Liquid Chromatography
LCFA	Long Chain Fatty Acids
LC-IT-TOF-MS	Liquid Chromatography Ion Trap Time-of-Flight Mass Spectrometry
LC-MS	Liquid Chromatography Mass Spectrometry
LLE	Liquid-Liquid Extraction
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry
MBR	Membrane Bioreactor
MES	2-(n-Morpholino)ethanesulfonic Acid
MLSS	Mixed Liquid Suspended Solids
MPA	3-Mercaptopropionic Acid
MS	Mass Spectrometry
MS-MS	Tandem Mass Spectroscopy
MW	Molecular Weight
MWCO	Molecular Weight Cut-Off
NMR	Nuclear Magnetic Resonance
OLR	Organic Loading Rate
OMP	Organic micropollutants
OPA	ortho-Phthalaldehyde
PAS	Periodic Acid-Schiff

PHP	Potassium Hydrogen Phthalate
PMAA	Partially Methylated Alditol Acetate
ppb	part per billion (10^{-9})
ppm	part per million (10^{-6})
PS	Polysaccharides
RPS	Released Polysaccharide
SAMBR	Submerged Anaerobic Membrane Reactor
SBR	Sequencing Batch Reactor
SCOD	Soluble Chemical Oxygen Demands
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size-Exclusion Chromatography
SMP	Soluble Microbial Product
SPE	Solid Phase Extraction
SRT	Sludge (Solid) Retention Time
SS	Suspended Solids
TCD	Thermal Conductivity Detector
TEP	Transparent Exopolymer Particles
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TOC	Total Organic Carbon
TSS	Total Suspended Solids
UAP	Utilisation Associated Products
USAB	Upflow Anaerobic Sludge Bed
UF	Ultrafiltration
VFA	Volatile Fatty Acid
VSS	Volatile Suspended Solid
UASB	Upflow Anaerobic Sludge Blanket
UF	Ultrafiltration
UNICEF	The United Nations Children's Fund
UV	Ultraviolet
UV/Vis	Ultraviolet-Visible
WHO	World Health Organization
XDLVO	Extended Derjaguin, Landau, Verwey, and Overbeek

LIST OF FIGURES

Figure 2.1 - Anaerobic process reaction	9
Figure 2.2 - Schematic of the metabolism of SMP and EPS formation	13
Figure 3.1 - Diagram of the SAMBR	32
Figure 4.1 - Particle size of well-known objects and particulates	36
Figure 4.2 - Chromatogram of alkanes standard at 1 mg/L (C10 – C40)	42
Figure 4.3 - Efficiency of different solvent volume evaluated for extraction	46
Figure 4.4 - Number of compounds detected using Oasis [®] HLB and ENVI-Carb cartridges	49
Figure 4.5 - Number of compound identified using 1 st and 2 nd Oasis [®] HLB cartridges	50
Figure 4.6 - Number of compounds eluted by different solvents	51
Figure 4.7 - Chromatograms of the sample 2 (SPE-LLE)	54
Figure 5.1 - An intergrated workflow for the MS-based glycomics analysis empolyed in this study	83
Figure 6.1 - Reaction of amino acids with OPA and FMOC-Cl, respectively	109
Figure 6.2 - HPLC chromatogram of amino acid derivatives	110
Figure 7.1 - Chromatograph of low MW SMPs in the 6 reactors	122
Figure 7.2 - Heat map of 15 FAA in 6 reactors supplemented with different substrates after 35 weeks of operations	129
Figure 7.3 - Schematic illustration of some membrane fouling mechanisms	136

LIST OF TABLES

Table 2.1 - Application of AnWT system in various industries	7
Table 3.1 - Composition of synthetic municipal wastewater used in this study...	31
Table 4.1 - Alkane standards for quantification	42
Table 4.2 - Parameters and performance of SAMBR	43
Table 4.3 - Efficiency of different organic solvents (20 mL) evaluated for extraction using 200 mL of sample	45
Table 4.4 - Efficiency of different additives evaluated for extraction	47
Table 4.5 - Efficiency of different sample sizes evaluated for extraction	48
Table 4.6 - Combination of SPE and LLE for extraction of low MW compounds in SMPs	52
Table 4.7 - List of 50 compounds that have the highest peak area (SPE as a Pretreatment)	57
Table 4.8 - List of 50 compounds that have the highest peak area (LLE as a pretreatment)	58
Table 4.9 - Comparison of the method of this study and literature results	59
Table 5.1 - Relative retention time of partially methylated alditol acetate derivatives	70
Table 5.2 - Performance of various carbohydrate assays	73
Table 5.3 - Effect of variations in different carbohydrates on metachromatic response	76
Table 5.4 - Effect of degree of glycosylation on metachromatic response	77
Table 5.5 - Solutes that can interfere with various carbohydrate assays	78
Table 5.6 - Average reading in the supernatant from each sample at various HRTs	79
Table 5.7 - Monosaccharide linkage composition presented in percentage mole .	86
Table 6.1 - Linearity range for 5 different assays	98
Table 6.2 - Substances that can interfere with various protein assays	100
Table 6.3 - Effect of composition on metachromatic response	103
Table 6.4 - Average reading in the effluent from each sample under various HRTs	104
Table 6.5 - Structures and abbreviations of the 20 standard amino acids	106
Table 6.6 - FAA concentration	111
Table 7.1 - Composition of feed	116
Table 7.2 - Performance of fill and draw reactors over 35 weeks	118
Table 7.3 - MW distribution of SMPs in 6 reactors after 35 weeks of operation ..	121
Table 7.4 - Molecular organic composition in the supernatant identified by GC/MS	122
Table 7.5 - Monosaccharide linkage composition with possible polysaccharide characteristics based on different feed compositions	132

CHAPTER 1

Introduction

Water is more precious than diamonds, and without it there would be no life on earth. Hence, it is axiomatic that water quality is essential for both aquatic ecosystem health and human health (Snow, 1855; Carr and Neary, 2008). Access to clean and safe water can be considered one of the basic human rights, and it can be said that this tenant is the key to the economic prosperity of a civilized society. On the other hand, water demand is increasing worldwide, but the capacity of water resources is not. In many areas of the world where *ab initio* freshwater is scarce, the problem of water management is becoming much larger and more complex.

According to the World Health Organization (WHO) and The United Nations Children's Fund (UNICEF) (2000), approximately 1 billion people are lacking access to an adequate supply of clean and safe water, despite its renewable character, and nearly 4 billion people are exposed to high levels of threat to water security (Vörösmarty *et al.*, 2010). In 2012, it is estimated that one-fifth of the world's population lives in parts of the planet where water scarcity directly threatens them (Hering and Ingold, 2012). The situation is alarming, and an intelligence assessment (Quinn, 2012) forecasts that water supplies are unlikely to keep up with global demand by 2040. A few years ago Faeth and his team (2014) predicted that by the 2020s about 35% of the world population will probably experience water scarcity. They state that anthropogenic climate change can further affect the situation, and by the year 2040 there will be water shortage worldwide. Sustainable management of water resources is hence a major challenge for this century. Meeting the challenge will require striking a balance between exploring new water resources, and using water more productively through conservation and wastewater reuse.

Wastewater reuse is one of the key responses to the challenge of water scarcity. Rapid urbanization and industrialization in the past two centuries have led to polluted water sources and a degraded environment. In response to these issues, research was undertaken that led to the growth of modern wastewater treatment systems; with adequate treatment high quality water can be regenerated from wastewater. Wastewater reuse has the potential to significantly increase water productivity because of additional goods and services produced. A prime example of regeneration is using anaerobic (without oxygen) biological processes to treat wastewater, and this has many inherent advantages over the more conventional aerobic treatment, such as:

- biogas generation (a gas rich in methane is produced biologically),
- considerably lower solids production (90% less than aerobic treatment) due to the different metabolic pathways involved,
- energy saving (a substantial reduction in energy required because of the methane produced, and no need to aerate the reactors),
- cost effective.

Because of these reasons, in addition to the technical advancements in recent years, this has resulted in the installation of thousands of full-scale anaerobic treatment facilities worldwide. The development of anaerobic treatment technology in the past century has been a major step towards sustainability in modern society, and it has a vast potential in the years ahead. However, one of the key limitations of anaerobic treatment is the high effluent chemical oxygen demand (COD), which usually exceeds the increasingly tight environmental legislation.

Effluents from biological wastewater treatment systems, either aerobic or anaerobic, contain a variety of organic matter. This is commonly known as effluent organic matter (EfOM), which consists mainly of non-degraded influent compounds, soluble microbial products (SMPs) and extracellular polymeric substances (EPS) produced during microbial metabolism, and trace levels of influent-derived organic micropollutants (OMPs) (Barker and Stuckey, 1999; Sharma *et al.*, 2011).

Generally, SMPs are recognized as organic compounds produced during substrate metabolism, or cell lysis or degradation. The term has been defined as the pool of organic compounds that are released into solution and do not contain known intermediates such as volatile fatty acids (VFA) or compounds in the influent (Aquino, 2004). EPS, which are the most important single microbial product group within SMPs, are a mixture of polymeric substances secreted by cells, and produced from cell lysis and hydrolysis.

There have been many studies showing that the majority of the EfOM were from SMPs and EPS (Stuckey, 2010b), and hence are of particular interest in minimizing their concentration in the effluent. This in turn leads to the achievement of consent levels for COD and Biochemical Oxygen Demand (BOD), and thus increases the overall performance of the biological treatment process (Barker and Stuckey, 1999; Ni *et al.*, 2009). Therefore, characterization of SMPs and EPS in the wastewater effluent is important because they not only satisfy the requirement of discharge quality but are also of great interest to the engineers and scientists.

The importance of SMPs and EPS has already been acknowledged in the literature. There is, however, a limited number of studies on the precise composition of SMPs and EPS, especially for anaerobic systems. Although colorimetric methods have been used to measure “protein” and carbohydrate in SMPs/EPS and their concentration for decades, a detailed evaluation of these assays with wastewater has received limited attention. In addition, very little is known about their chemical properties and what factors influence their production.

Moreover, identifying the major chemical components of SMPs and EPS is critical, and equally crucial is understanding the basic mechanisms of biological activity, which create these compounds, and how to reduce them in the effluent. Furthermore, it is not known whether the release of SMPs into the bulk solution is deliberate excretion, or as a result of a microbial strategy to survive in a hostile environment, or purely as a result of general cell lysis.

In order to understand the characteristics of SMPs and EPS in biological wastewater treatment systems, this research was planned to address such an issue. **Hence, the main aim of this project was to improve on the existing protocols for SMP and EPS analysis, and develop new methods that characterize, by means of chromatography and mass spectrometry techniques, chemical compounds in SMPs and EPS in the supernatant or effluent from anaerobic reactors operating under non-stressed conditions.**

Following this chapter, a literature review of the theory and previous work carried out is presented. Based on this review of the literature, detailed objectives and the scope of this work are set out in Chapter 2. Information regarding general experimental set-up and common analytical methods is presented in Chapter 3. In Chapter 4, development of a new analytical method for low molecular weight (MW) SMPs in wastewater treatment systems is presented. Critical evaluation of carbohydrate and proteinaceous materials analysis using colorimetric assays is discussed explicitly in Chapters 5 and 6, respectively, and improved analytical methods are proposed at the end of the respective chapters. Finally, Chapter 7 describes the impact of feed carbohydrate and nitrogen sources on the production of SMPs using the methods developed, while concluding remarks and recommendations for future research are presented in Chapter 8.

CHAPTER 2

Background and Literature Review

2.1 Introduction

The literature review presented here starts with a brief history of anaerobic processes, and its fundamentals and application in wastewater treatment. The definition of SMPs and their implications and global measurement are then discussed in detail. In addition, a review of past work on the characterization of SMPs is presented, with special attention focused on low MW SMPs, polysaccharides (PS) and proteinaceous materials. Each of the following Chaps 4-7 will address one of the specific topics that encompass the objectives of this work, which are defined in this chapter.

2.2 Historical aspects of the anaerobic process

The first recognition that anaerobic biological processes could be used as an effective method of converting waste organic material into methane can be attributed to Volta as early as 1776 (Barker, 1956). In the 1860s, a French engineer, Louis H. Mouras and a priest-cum-scientist, Abbé Moigno successfully conducted a full-scale application of the anaerobic digestion (AD) process for the treatment of domestic wastewater. The system, named "Mouras's Automatic Scavenger", was then patented on 2 September, 1881 (Moigno, 1881; 1882). This invention was solemnly defined at that time as "the most simple, the most beautiful, and perhaps, the grandest of modern inventions" (McCarty, 1985). The aerobic activated sludge process, at the heart of current sewage treatment technology, was invented almost 30 years later (Arden and Lockett, 1914).

In 1890-1891, W. D. Scott Moncrieff constructed the first biological wastewater treatment tank, in which the organic substances were first degraded anaerobically and the products were then further degraded and mineralized aerobically, producing an

effluent that is clear and gases that are inoffensive (McCarty, 2001). Shortly afterwards, the simplest and most widely used process was the septic tank (Jewell, 1987). Donald Cameron built the first patented septic tank in Exeter, England, successfully treating the entire city's wastewater (McCarty, 2001); thereafter, anaerobic processes have been continually improved and applied. Moreover, the value of methane gas collected was generally recognized for heating, lighting and power generation. Unfortunately, at that time anaerobic processes were treated as a “black box”, with very little or poor understanding of its microbiology and biochemistry, and consequently with little development in the process technology the application of anaerobic wastewater treatment (AnWT) did not take off. It was almost neglected around the 1930s in favour of the first-generation aerobic wastewater treatment (AeWT) systems, which were perceived by many as better understood and therefore considered as much more effective, feasible, and robust in operation.

30 years later, anaerobic technology was revitalized. Professor Perry McCarty and colleagues in the United States pioneered the development of high-rate AnWT system for the treatment of low and medium strength wastewater (McCarty, 1964; Young and McCarty, 1969). "Anaerobic wastewater treatment didn't enjoy the popularity it truly deserves" (McCarty, 1964) is his famous statement after recognition of his research on the anaerobic filter (AF). In April 1970, the great breakthrough came resulting from Lettinga's comprehensive investigations and his novel development of the upflow anaerobic sludge bed (USAB) reactor in the Netherlands (Lettinga, 1995). It was the first successful demonstration of a full-scale application of anaerobic technology for high-strength industrial wastewater treatment; since then the status of AnWT has changed considerably (Lettinga *et al.*, 1980). Because of all its advantages over aerobic treatment, a growing number of people are now calling for the substitution of AeWT as the core wastewater treatment method by AnWT.

Lettinga's environmentally-sustainable solution for the treatment of wastewater using AnWT processes have won him the Tyler Prize (2007) and the Lee Kuan Yew Water Prize (2009). USABs and their modifications have been successfully applied worldwide

for the treatment of high-strength industrial wastewaters. Not only used to treat industrial waste effluents such as food and beverages, AnWT process have also been used in the treatment of urban wastes, as shown in Table 2.1. It is estimated that thousands of AnWT systems have been built all over the world for those purposes.

Table 2.1 - Application of AnWT system in various industries

• Alcohol production	• Breweries
• Coffee processing	• Dairies
• Fish processing	• Fruit processing
• Leather production	• Palm oil production
• Potato processing	• Rubber production
• Soft drink production	• Starch production
• Sugar refineries	• Vegetable processing
• Wine production	• Yeast production

[Source: Chernicharo, 2007 (modified)]

In short, the current demand from an ever-increasing worldwide population, and the need for a more sustainable society are opening up new frontiers in the development of wastewater treatment processes. Nevertheless, the fundamentals of anaerobic processes, and their application in the treatment of wastewater should be understood in more depth.

2.3 Fundamentals of anaerobic processes

AeWT and AnWT processes use the same basic mechanism in that they use biological processes to oxidise organic compounds. The difference between the two types of treatment results from the different environmental conditions imposed on them. In AnWT, for example, the decomposition of organic and inorganic matter by micro-organisms occurs in the absence of oxygen. Under anaerobic conditions, there is a reducing situation and electron acceptors are needed since there is no molecular oxygen

present. Part of the influent organic carbon acts as an electron acceptor, and hence is reduced, while the rest of it is oxidised to carbon dioxide and water.

The breakdown of complex organic matter during anaerobic conditions requires the presence of a diverse and closely dependent group of bacteria (mainly fermentative, acetogenic and methanogenic) that coexist in an anaerobic ecosystem (McCarty and Smith, 1986). The whole degradation process, as well as the formation of the intermediate metabolites during degradation, can be described as a multiple process of series and parallel reactions (Gujer and Zehnder, 1983; Pavlostathis and Giraldo-Gomez, 1991b). According to van Haandel and Lettinga (1995), six distinct processes can be identified during AD:

1. Hydrolysis of biopolymers
2. Fermentation
3. Anaerobic oxidation of long chain fatty acids and alcohols
4. Anaerobic oxidation of intermediary products
5. Conversion of methanogenic substrates to methane
6. Conversion of hydrogen to methane

A simple schematic of the degradation is shown in Figure 2.1. The first step of the anaerobic process is hydrolyzing (breaking up) the big macromolecules into smaller pieces. Later, solubilisation of these particles and hydrolysis are aided by extracellular enzymes, including cellulases, hemicellulases, amylases, lipases and proteases, collectively known as hydrolases, which are produced and excreted by hydrolytic bacteria. Fermentation or acidogenesis is mediated by facultative and obligate bacteria, or specially known as acidogenic (organic acid forming) bacteria. The final fermentation is completed by hydrogen-producing (proton-reducing) acetogenic (acetate forming) bacteria. It is noteworthy that they can only function well under very lower partial pressures of hydrogen ($<10^{-4}$ atm). The net effect of these reactions is the conversion of complex polymers to acetate, hydrogen gas and carbon dioxide gas. The whole sequence is complete by the last group of bacteria, the methanogens, which

consume the end products from the previously mentioned bacteria and convert them to the final end-products - methane and carbon dioxide. In the last stage, hydrogen and carbon dioxide can also be converted to acetate by hydrogen-oxidising acetogens, while acetate is split into methane and carbon dioxide by aceticlastic methanogens. Generally, two-third or more of the methane produced under anaerobic condition is derived from the reduction of acetate, while the remainder is derived from the reduction of carbon dioxide with donation of electrons from formate or hydrogen (Speece, 1983). Production of methane by the oxidative or reductive dismutation of methanol or methylamines is a minor route compared to the other pathways (Pavlostathis and Giraldo-Gomez, 1991a; Ferry, 1992). Nevertheless, biogas production is the key advantage of anaerobic treatment of wastewater.

Figure 2.1 - Anaerobic process reaction [Sources: Gujer and Zehnder, 1983; van Haandel and Lettinga, 1995; Tchobanoglous *et al.*, 2003 (modified)]

2.4 Anaerobic treatment of wastewater

AeWT processes were very popular for the biological treatment of wastewater up until the 1960s, before the energy squeeze brought about a significant change in attitudes in the early 1970s (Lettinga, 2010). Energy conservation became a major concern, and AnWT processes rapidly emerged as an alternative to AeWT because they can produce biogas. In addition, an anaerobic system involves relatively low investments, with no aeration equipment, reduced sludge disposal facilities, and the advantage of natural gas recovery (Speece, 1996).

Despite its advantages AnWT processes have been, until lately, considered problematic and uneconomic. Among the factors that have contributed to its lack of popularity were the low growth rate of anaerobes (leading to long hydraulic retention times (HRT) in reactors), potential odour problems, high alkalinity requirements, greater sensitivity to toxins than aerobic systems, slow recovery from adverse conditions, and relatively lower efficiencies. Other advantages and disadvantages of AnWT processes have been well summarized, and are reported elsewhere (Seghezzo *et al.*, 1998). However, with the increase in research in the last 30 years, high rate AnWT systems have been developed where they can retain large quantities of biomass, even at short hydraulic retention times (HRT) (6-8 hr).

Today, AnWT technology continues to develop in many aspects, one of which is the development of new types of reactors. In the last decade, new anaerobic membrane reactors (AnMBRs) have been developed, with the membrane either external or submerged within the reactor; however, aerobic membrane bioreactors (MBRs) have been used in wastewater treatment for at least 40 years. There are over 500 full-scale commercial MBRs in operation globally, and more are under construction or being proposed (Stephenson *et al.*, 2000). In the 2000s, attention shifted to focus on the use of membranes in conjunction with anaerobic reactors over aerobic ones due to the increasing pressure on designing reactors that have lower energy consumption, less

sludge production, smaller footprints, and lower production of greenhouse gases. All these factors have led to the emergence of AnMBRs.

Since an AnMBR is a fully enclosed treatment tank, it incorporates solids removal and COD reduction in a single intensified unit operation. Hu and Stuckey (2006) constructed a novel submerged anaerobic membrane reactor (SAMBR), which operated down to as low as an HRT of 3 hours, producing a solids-free effluent with high COD removals (~98%). However, most of the soluble COD in the effluent (~30 mgCOD/L) was found to be SMPs (Hu and Stuckey, 2007). Other studies conducted by the same group have shown that the majority of soluble organics in the supernatant and effluent, when expressed in COD, are protein- or carbohydrate-like organics, and have MWs lower than 1kDa, and are difficult to remove even using different activated carbons (Barker *et al.*, 1999; Aquino *et al.*, 2006).

2.5 SMPs and their implications

It has been known that effluents from biological wastewater processes contain a variety of soluble organic compounds, and SMPs often form the majority of these in terms of effluent COD and BOD (Ni *et al.*, 2011b; Barker and Stuckey, 1999).

2.5.1 Definition of SMPs

SMPs result from processes common to most bacteria, and consist of a wide variety of soluble organic compounds with a range of MWs (~0.1-1500 kDa) which are released into solution from substrate metabolism and normal biomass decay in a mixed culture (Barker and Stuckey, 1999; Laspidou and Rittmann, 2002a). Boreo *et al.*, (1991) defined SMPs as intermediates or end products generated during substrate degradation and endogenous cell decomposition, whereas Noguera *et al.*, (1994) found otherwise. The definition of SMPs, according to Noguera *et al.*, (1994), is the pool of organic compounds that result from metabolism and decay during 'complete mineralization' of simple substrates. The end product of the incomplete degradation should not be included in the definition because the end product come directly from the substrate

instead of a microbial origin. In this case, many researchers excluded intermediate VFAs from the SMP pool thereafter (Kuo and Parkin, 1996; Kuo *et al.*, 1996; Nachaiyasit and Stuckey, 1997; Schiener *et al.*, 1998; Barker and Stuckey, 1999).

Barker (1999) further pointed out that eliminating intermediate products from the definition of an SMP does have a valid reason in practical terms as it permits the assessment of the degree of resistance of the effluent to biodegradation even though VFAs are of microbial origin. However, many publications only exclude acetic acid, propionic acid and butyric acid from the SMP pool (Kuo and Parkin, 1996; Barker, 1999), but it is well known that other VFAs such as formic acid, isobutyric acid, valeric acid and isovaleric acid are expected to be present, although only in low concentrations in well-operated systems (Parkin and McCarty, 1981a; Namkung and Rittmann, 1986; Rittmann *et al.*, 1987; Noguera *et al.*, 1994; Kuo *et al.*, 1996; Barker and Stuckey, 1999; Aquino, 2004). Nevertheless, a more realistic definition of SMPs is “soluble compounds that are released into the effluent from a biological system and not present in the influent”.

Nevertheless, the composition of SMPs is extremely complex (Barker *et al.* 1999), and it can be further classified into high MW and low MW fractions. The high MW fraction is generated as either SMPs from cell metabolism and lysis (Barker and Stuckey, 1999), or as EPS which constitutes around 20% of the SMPs (Aquino and Stuckey, 2004). EPS determine the structural and functional integrity of microorganisms' biofilms, and are considered the basic component that establishes the physiochemical properties of a biofilm (Flemming, 2000). EPS mostly consist of PS and proteins, but also include macro-molecules such as DNA, humic substances, and lipids (Jarusutthirak and Amy, 2006). Low MW SMPs are equally multifaceted, although very little is known about them; Aquino and Stuckey (2006) have analysed some of the low MW compounds and found substituted ring compounds, even in a reactor fed with simple sugars.

2.5.2 SMPs and other biomass components

SMPs can be classified into two groups based on the type of bacterial metabolism from which they are derived. Both utilisation associated products (UAP) and biomass associated products (BAP) are biodegradable components of SMPs. UAP are produced directly during substrate utilisation, while BAP are produced indirectly via the hydrolysis of dead biomass (cell lysis) (Laspidou and Rittmann, 2002a).

EPS, which are the most important microbial product group within SMPs, includes solid-phase products generated during metabolism (Laspidou and Rittmann, 2002a; Laspidou and Rittmann, 2002b; Aquino and Stuckey, 2008; Ni *et al.*, 2011b; Kunacheva and Stuckey, 2014). Later Aquino and Stuckey (2008) established a comprehensive mass balance with the intention that none of the components overlap or are left out. Figure 2.2 shows a diagram of the metabolism of SMP and EPS formation in a heterotrophic microbiological system of SMP and other biomass components.

Figure 2.2 - Schematic of the metabolism of SMP and EPS formation [Sources: Aquino and Stuckey, 2008; Ni *et al.*, 2011b (modified)]

Jarusutthirak and Amy (2006, 2007) found that the components making up BAP consist mainly of hydrophilic organic colloids and macromolecules such as PS, proteins-like material, and/or amino-sugar-like compounds, which have a MW greater than 10 kDa. Jiang *et al.* (2008, 2010) showed that UAP includes protein-like biopolymers and low-MW humic-like materials, and has a much lower MW than those of BAP (63% of BAP has a MW greater than 20 kDa). Ni *et al.* (2010) further confirmed that UAP is carbonaceous compounds with an MW less than 1 kDa, while BAP are primarily macromolecules with an MW greater than 10 kDa.

2.5.3 The impact of SMPs from anaerobic systems

Since most of the soluble COD in biological effluents (both aerobic and anaerobic) are SMPs, this has many implications. SMPs are ubiquitously present and important because they control the performance of the process, and effectively set the lower limit for treatment efficiency (Barker and Stuckey, 1999). Secondly, any post-treatment systems designed to remove the residual COD must aim at efficient SMP removal. Also, Lei *et al.* (2007) found that SMPs also influenced sludge settleability and dewaterability in activated sludge treatment systems. According to Chipasa and Medrzycka (2008), the presence of SMPs also has consequences for the composition of the microbial community in bioreactors. Moreover, some wastewater-based SMPs may become important precursor for disinfection by-products (DBP) in countries that chlorinate effluents (Liu and Li, 2010). Also of special importance is that membrane fouling in MBRs are caused primarily by SMPs (Judd, 2008; Meng *et al.*, 2009; Stuckey, 2012). Last but not least, the presence of SMPs may be a problem when recycling and reusing treated wastewater since many literature reviews suggest that secondary effluents may be more toxic than raw wastewater (Aquino, 2004).

2.6 Why are SMPs produced?

Pirt (1975) classified microbial products into three categories: growth-synonymous, growth-associated and growth-independent. A slightly different classification was

proposed by Parkin and McCarty (1981a, 1981b, 1981c), and developed by Chudoba (1985a, 1985b). Their classification is grouped in three categories:

1. compounds excreted by microorganism during their interaction with the environment;
2. compounds produced as a result of substrate metabolism and bacterial growth;
3. compounds released during lysis and degradation of microorganisms.

It is believed that many factors can affect the production of microbial products in pure or mixed cultures. The main operational parameters and environmental factors reported to influence SMP production in AnWT systems are presented, and briefly discussed below.

2.6.1 Feed strength, organic loading rate (OLR) and HRT

The work done by Kuo *et al.* (1996) using anaerobic chemostats was the first study showing the influence of OLR, HRT and initial substrate concentration on SMP production. In this study, SMP production increased as the retention time increased. Normalized production of SMP in glucose-fed chemostats followed a U-shaped pattern as a function of HRT, with a minimum near an HRT of 25 days. SMP production also increased as initial substrate concentration increased, agreeing with the findings of many studies (Chudoba, 1986; Germirli *et al.*, 1991; Kuo *et al.*, 1996).

2.6.2 Biomass concentration and solid retention time (SRT)

It was commonly believed that greater sludge ages would lead to lower effluent COD, but some studies showed that this is not necessarily true (Borero *et al.*, 1991; Kuo *et al.*, 1996). In contrast, it has been shown that accumulation of biomass may lead to higher SMP formation due to endogenous decay, and that there is an ideal retention time to minimize the residual COD. It was reported by Barker (1999) that the optimum SRT should be around 25 days for anaerobic systems.

Most of the SMPs could originate from cell decay at higher SRTs, and be comprised of BAPs, whereas most the SMPs should be UAPs at lower SRT. The reason is that

systems operated under lower SRT would lead to higher substrate utilization rates, and, therefore, generate higher concentration of UAP. As the SRT increases, more of the easily degradable substrate and intermediate (VFAs and more readily degradable SMPs) will be removed, leaving behind the more recalcitrant SMPs. Hence the balance between BAPs and UAPs is important as the former are usually more low MW and degradable, while the latter are higher MW and less biodegradable. Therefore, the effect of HRT and SRT shifts the balance between the more and less biodegradable SMPs. However, with a membrane present in AD, many of the higher MW compounds are rejected and are given the time to degrade, and hence the impact of these operating parameters is minimised. In the long run, operating at high SRTs does lead to lower sludge yields, and possibly optimum performance, however, this is still not totally clear.

2.6.3 Reactor configuration

Reactor configuration can affect SMP production; Barker (1999) found significant differences in SMP production between anaerobic baffled reactors (ABR) and anaerobic chemostats fed on synthetic wastewater. In addition, Pribyl *et al.*, (1997) found that a continuous flow system with a completely mixed tank produced consistently more SMP than a sequencing batch reactor (SBR). Later, Aquino and Stuckey (2006) presented that 2.1% of the substrate was channeled into the production of SMPs in an anaerobic continuous stirred-tank reactor, whilst in a SAMBR this was estimated to be 25%. In another study in which chromatography analysis showed that there was a decrease in aromatic compounds in SMPs compared with the components of raw wastewater in a full-scale upflow anaerobic sludge blanket (UASB) reactor (Wu and Zhou, 2010).

2.6.4 Temperature

Enhanced production of SMPs was observed at lower temperatures in ABRs fed on synthetic wastewater (Nachaiyasit and Stuckey, 1997; Schiener *et al.*, 1998). They believed that the accumulation of SMPs as the temperature decreased was due a combination of stress imposed on the microorganisms, and a decrease in the

biodegradation rate of SMP. Although higher temperatures may lead to higher hydrolysis and degradation rates, the accumulation of SMP may be enhanced as a result of stress and/or higher metabolic rates associated with thermophilic conditions.

2.6.5 Substrate type

Carbohydrates are known to be a primary source of nutrients for microbial growth in AD, and glycolysis plays a fundamental role in the degradation of fermentable carbon by microorganisms, providing the cell with both metabolic energy and essential intermediates for cell functions. Interestingly, most of the studies concerning SMPs in AD have been conducted with either glucose or acetate feeds (Barker and Stuckey, 2001), and there are only three comparative studies with different feed compositions in the literature (Noike *et al.*, 1985; O’Flaherty *et al.*, 1997; Barker and Stuckey, 2001). Barker and Stuckey (2001) noted that the normalized production of SMPs (SMP/influent COD) ranged from 12.0, 1.7 14.7 and 3.6% for the glucose-, glycerol-, lactic acid-, and starch-fed CSTRs, respectively. Furthermore, as far as we are aware, there are no studies on the effect of the nitrogen-source on the formation of SMPs, although it is known that high ammonia concentrations are of concern since they are inhibitory and result in unstable process performance, and increased risks of process failure (Westerholm *et al.*, 2012).

2.6.6 Other factors

Cellular structures are constantly renewed, and the turnover of old structures may result in the excretion of SMPs into the medium (Hejzlar and Chudoba, 1996). Additionally, cell-to-cell communication has been discovered in some gram-negative bacteria that involves the release of quorum sensing chemicals into the media (Fuqua and Greenberg, 1998). Furthermore, during the normal growth phase biomass may be hydrolysed due to shear, releasing SMPs to the bulk solution (Chudoba, 1986). Lastly, SMPs may be produced as a direct result of cell lysis caused by the presence of toxic substances (Brown *et al.*, 1994, Chen *et al.*, 2014), and by means of efflux systems that involve the

excretion of metals bound with specific bimolecular (Kuo and Parkin, 1996; Mirimanoff and Wilkinson, 2000).

2.7 Characterisation of SMPs

Despite their significance in biological wastewater treatment systems, SMPs have proven to be a challenge to measure and characterise. Many attempts have been made to characterise them on three different levels such as; individual compounds, chemical groups, and collective parameters (Aquino, 2004, Kunacheva and Stuckey, 2014). Moreover, no comprehensive study has directly compared the production of SMPs in aerobic and anaerobic systems so far. There are, however, data that is available for indirect comparison and, interestingly, when comparing their results with the available literature reports, it was reported that production of SMPs in anaerobic systems were higher than that in aerobic system and more complex (Barker and Stuckey, 1998; Zhou *et al.*, 2009). Most importantly, the lack of information about anaerobic rather than aerobic systems is more acute.

2.7.1 Characterisation by global parameters

Chemical characterisation of SMPs is complex, and to date, it has not been possible to identify all the organic solutes in SMPs. The difficulty of this approach results from the great complexity of the organic matrix, constituted by a mixture of hundreds of simple molecules or hetero-polymers, and the very low concentration of these solutes. Barker (1999) highlighted the fact that most of the studies on SMP characterisation still employed “global parameters” such as MW, COD, carbohydrate and protein content, instead of identifying individual compounds or functional groups. The reasons for this were mainly due to the chemical complexity of the effluents, the requirements of time-consuming and tedious techniques, and the lack of a standard method which could accurately identify a majority of the compounds present. Moreover, other surrogate parameters such as total organic carbon (TOC) or dissolved organic carbon (DOC), although providing an indication of the total organic matter concentration, are non-specific testes. Measuring DOC/TOC will inform the user of the sum of all organic

carbon compounds, but not determine which particular SMP compounds are present, and hence are poorly indicative of SMPs. In addition, collective parameters such as biodegradability and toxicity are widely reported in the literature.

2.7.1.1 MW distribution

MW distribution analysis of SMPs has been extensively employed, and is suitable as a preliminary analysis (Logan and Qing, 1990; Amy *et al.*, 1992; Kuo and Parkin, 1996; Logan and Wagenseller, 2000), and Barker and Stuckey (1999) presented an extensive review on the MW distribution of aerobic systems. However, only a handful of studies have been carried out on the effluent from anaerobic systems (Aquino, 2004). Until now, there has been no standardized procedure to identify the MW distribution, although ultrafiltration (UF) and size-exclusion chromatography (SEC) have been widely used (Kunacheva and Stuckey, 2014).

For UF, the size of SMPs and extracted EPS were determined by their apparent MW using a known pore size. This technique is more suitable for the identification of compounds in SMPs and EPS since large amounts of the sample can be recovered for further characterisation. However, there are several drawbacks in using UF such as membrane pore size distribution, sample temperature, pressure of the stirred cell, pH, and membrane materials which can influence the transport of organics through the membranes (Logan and Qing, 1990).

A handful of researchers have conducted UF separation of SMPs since the 1980s (Barker and Stuckey, 1999); membranes with different MW cut-off (1 kDa, 3 kDa, 5 kDa, 10 kDa, 100 kDa, 300 kDa) are normally used in a stirred cell. Large volumes of sample can be separated according to MW (Kunacheva and Stuckey, 2014), and UF can be conducted in either parallel or serial modes, although Logan and Qing (1990) observed more errors in MW distribution using serial mode.

SEC, including gel-filtration-chromatography (GFC) and gel-permeation chromatography (GPC), is an old but versatile analytical technique useful in

determining the complete mass distribution of molecular sizes. The major advantages of SEC are (Kunacheva and Stuckey, 2014):

- easy and fast to obtain the MW distribution profile;
- good for comparing results between samples;
- useful for MW screening before using UF to obtain the separated sample for analysis;
- suitable for monitoring the reactor during transient loads under dynamic conditions.

However, there are a few disadvantages associated with using SEC (Barker and Stuckey, 1999):

- possible chemical interactions between the column materials, the solvent, and the organic compounds in SEC can overestimate or underestimate the MW profile;
- peaks from SEC can be difficult to differentiate between in some samples.

Recently and increasingly, high performance liquid chromatography (HPLC), in conjunction with an SEC column, has been used, and the SMP components are eluted according to the size of the molecules (Gorner *et al.*, 2003; Aquino, 2004; Jarusutthirak and Amy, 2006; Jarusutthirak and Amy, 2007; Tsai *et al.*, 2008; Wang *et al.*, 2009; Villain *et al.*, 2010; Guo *et al.*, 2011). There are many advantages and disadvantages using such technique:

Advantages

- short analysis time
- narrow bands
- no sample loss
- small amount of mobile phase required

Disadvantages

- limited number of peaks can be resolved within the short time scale
- the molecule masses of most of the chains will be too close for separation to show anything more than broad peaks

2.7.1.2 Biodegradability assay

Biodegradability is one of the most important collective parameters, and a useful way to evaluate methods to remove SMPs. One of the more noteworthy studies on biodegradability was carried out by Gaudy and Blachly (1985), who showed that the biodegradable SMPs can be degraded further by aerobic and anaerobic microorganisms (Schiener *et al.*, 1998). It is also known that the biodegradability assay (Owen *et al.*, 1979) can be carried out under either aerobic or anaerobic conditions. Aerobic biodegradability was determined based on the traditional BOD test in the American Public Health Association (APHA) Standard Methods (Rice, 2012). In contrast, anaerobic biodegradability was estimated by cumulative methane production (Owen *et al.*, 1979; Schiener *et al.*, 1998). Overall, it was suggested that SMPs produced by anaerobic systems are degradable, both aerobically and anaerobically, although with a slower rate than the original substrate (Aquino, 2004, Schiener *et al.*, 1998).

Schiener *et al.*, (1998) assessed the anaerobic biodegradability of the MW fraction of SMPs produced in different compartments of an ABR. It was expected that high MW compounds would be hydrolyzed to lower MW compounds, and then metabolized, as SMPs moved down the reactor. Analysis later revealed that the high MW fraction appeared to increase in the latter compartments of the ABR. These results suggested that the higher MW BAPs released during cell lysis remained relatively undergraded, and hence increased down the reactor. Biochemical methane potential (BMP) assays showed that all MW fractions were biodegradable in the range between 62-82%, and although the bulk of the effluent COD was anaerobically biodegradable, in some cases a residence time of 11 days was required.

In another study using BMP assays, Barker (1999) assessed the anaerobic biodegradability of return liquids, fermentation waste and ABR effluents. Biodegradability studies of individual MW fractions of the effluent showed that the low MW material was the most difficult to degrade anaerobically. Although SMPs produced by anaerobic systems are biodegradable, SMP biodegradability is slower

when compared to the original substrate. It was shown that the compounds that make up the residual COD in anaerobic systems cannot be considered “refractory *stricto sensu*”, as they just require relatively higher retention times to be completely biodegraded, and it is probable that many SMPs are similar, i.e. there are relatively few totally refractory compounds produced as SMPs.

2.7.1.3 Toxicity

Toxicity assessment of effluents becomes highly relevant since it is a parameter on virtually all discharge permits, and are also helpful in estimating the potential risk of introducing lethal compounds into the aquatic environment. Before Klinkow *et al.*, (1998) reported the first study on the toxicity of anaerobic effluents, all the other studies demonstrating the toxic properties of SMPs were carried out on aerobic effluents (Washington *et al.*, 1970; Chudoba, 1985a; Eckenfelder, 1988, 1995). Other studies have raised the question that some SMPs may also have genotoxicity, mutagenicity and/or carcinogenicity (Monarca *et al.*, 2000; Aquino and Stuckey, 2004). However, the complex wastewater samples treated in these toxicity studies make it impossible to ascertain which of the SMPs played a role in the observed properties. No information was given on the individual components or compounds in the SMPs making the secondary effluent more toxic than raw wastewater.

In the first study on the toxicity of anaerobic effluents, Klinkow *et al.* (1998) fractionated organic compounds according to their molecular size and polarity, and combined the fractionation with a luminescence inhibition test to measure the toxicity. It was found that the anaerobic effluent had less toxic fractions at lower MWs. Another study conducted by Monarca *et al.* (2000) reported on the influence of different disinfectants on mutagenicity and toxicity of urban wastewater. Samples of wastewater, untreated or subjected only to primary or secondary treatment, were collected before and after disinfection. Raw wastewater and treated wastewater collected before disinfection showed no mutagenicity using the Ames test (Mortelmans and Zeiger, 2000). However, both raw and treated wastewater showed bacterial toxicity, which

according to the authors, could have concealed the mutagenic activity. However, raw wastewater was positive for both the genotoxicity and toxicity tests, while the treated wastewater was positive only for the toxicity test.

2.7.2 Chemical characterisation: individual compounds

Even though attempts to chemically characterise SMPs started in the 1960s (Bunch *et al.*, 1961), most of the work was carried out on aerobic systems (Aquino, 2004), and there were only a handful of studies on the characterisation of anaerobic effluents. The first published work was by Reemtsma and Jekel (1997) who used gas chromatography mass spectrometry (GC-MS) to identify low MW components in tannery wastewaters, and their alterations through biological treatment systems. They classified the organic compounds present in the wastewater into 12 classes, and aromatic carboxylic acids, short chain carboxylic acids and dicarboxylic acids were dominant in the raw wastewater. A significant increase in aromatic compounds occurred during anaerobic treatment because of anaerobic cleavage of high molecular weight compounds. Cyclohexanes, fatty acids and a large variety of organic compounds were unable to be removed even after aerobic polishing.

Later, Klinkow *et al.*, (1998) used GPC to fractionate anaerobic effluents, and all five GPC fractions were subjected to a two-step SPE sequence. It was found that a MW of 600-2000 Da corresponded to the polar compounds, and most of them were hardly extractable. Their results also showed that the greatest amount of extracted dissolved organic carbon (DOC) had a MW of 30-100 Da, and seemed to contain many non-polar and acidic compounds, although none could be identified accurately. In the same year, Schiener *et al.* (1998) found that high MW SMPs were mainly comprised of monosaccharides and amino sugars in the effluent discharged from an ABR. Using probe-mass spectrometry and hydrogen nuclear magnetic resonance (H-NMR), Barker *et al.* (1999) found that 10% of the effluent COD was made up of carbohydrates and “proteins”. Other compounds existed in the anaerobic effluents released from either

laboratory scale reactors or treatment plants were alcohols, carboxylic acids and a considerable amount of aromatic compounds.

Galassi and Benfenati (2000) used SPE, HPLC, GC-MS to examine leachate from a textile effluent and an industrial landfill. They found 6 types of phthalates from the leachate in the concentration range of 9-6808 ppb. Imai *et al.* (2002) revealed that aquatic humic substances (AHS) and hydrophilic acids dominated in both sewage and human-waste treatment plants, accounting for more than 55% of the dissolved organic matter (DOM).

Fourier transform infrared spectroscopy (FTIR) has been utilized to characterise the functional groups of SMPs. The results obtained using FTIR indicated that the composition of SMPs was a mixture of PS, proteins, and/or amino-sugar like compounds due to the presence of hydroxyl and amino acid groups (Jarusutthirak and Amy, 2006; Wang *et al.*, 2009; Ni *et al.*, 2010; Mei *et al.*, 2014). These findings were closely related to the role of SMPs in membrane fouling and flux decline (Tian *et al.*, 2013).

SMPs have proven to be a challenge to characterise and identify, mainly because they are a mixture of unknown compounds that do not correspond to any well-defined groups, whether it is a substrate or product. For example, glucose is a well-defined substrate whereas acetate is a well-defined product. Recently, some researchers have tried to identify the chemical composition of SMPs using advanced instruments such as GC-MS (Aquino, 2004; Zhou, 2009; Trzcinski and Stuckey, 2009), high resolution liquid chromatography ion trap time-of-flight mass spectrometer (LC-IT-TOF-MS) (Mesquita *et al.*, 2010), and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Aquino, 2004; Mesquita *et al.*, 2010). Preliminary results show that long chain alkanes, alkenes, alcohols, acids, esters and amides, as well as aromatic compounds were present in anaerobic effluents.

Hence, it is critical to identify the individual components of SMPs and/or EPS in order to understand the fundamental biological pathways that create these compounds, and

hence how to minimize their production in the effluent. However, previous work on the characterization and measurement of SMPs used a wide range of different analytical techniques, making it a challenge to compare results in the literature. A methodology that is beneficial to compare the results of different researchers should be developed, which is one of the objectives of this work.

2.7.2.1 Chemical characterisation: glycolysis

Several studies have reported that the major organic constituents in SMPs and EPS are proteins-like compounds, PS/carbohydrates, humic substances, lipids, and other small molecules (Flemming and Wingender, 2001; Aquino, 2004; Jarusutthirak and Amy, 2006; Zhang *et al.*, 2008; Kunacheva and Stuckey, 2014). PS are of great importance, and have been extensively studied due to their effect in aerobic granules, biofouling in membrane-related processes, and bio-corrosion (Liu, 2007; Annuk and Moran, 2009; Stuckey, 2010).

Although the significant involvement of carbohydrates in these areas of interest has led to the development of various analytical techniques, the most rapid, simple and cheap approach for the micro-determination of total carbohydrate concentrations is probably the colorimetric methods. Most colorimetric methods involve reaction of the sample with sulfuric acid and addition of a reagent to develop the chromophore, and were initially developed many decades ago. Several colour developing reagents have been employed, but the most commonly used in wastewater treatment has been anthrone (Dreywood, 1946) and phenol (Du Bois *et al.*, 1951, 1956). Another colorimetric method is the periodic acid-Schiff (PAS) stain, which involves staining macromolecules such as the glycoproteins that contain a large amount of saccharide. This staining method is a common chemical test incorporating Schiff-type reagents that was developed by Hugo Schiff in 1866.

Despite their simplicity, there are two considerations which need to be noted; firstly, it must be emphasized that no single colorimetric method is absolutely specific (Mecozzi, 2005), and hence the results of these assays are often presented in terms of glucose-

equivalent concentrations. Such representation has a major drawback where the composition of the carbohydrates is not well known, and variable absorbance responses to different saccharides that are not glucose would very likely to be observed. Secondly, there is the problem with interferences (Ashwell, 1957); many of the organics present in wastewater may react with reagents in the presence of strong acids, especially concentrated sulfuric acid, or even simply react with H₂SO₄ alone producing a marked absorption in the ultraviolet region (Dische, 1955). Hence, the carbohydrates measured by these chromogenic methods could be incorrectly estimated.

Carbohydrates can also be detected by using titration, gravimetric, enzymatic, spectroscopy and chromatographic methods (Kunacheva and Stuckey, 2014). Among them, advanced instruments such as thin layer chromatography (TLC), GC, and HPLC, MALDI-TOF/MS based and Nuclear Magnetic Resonance (NMR) spectroscopy are less commonly used due to their high costs and time required. They also require highly skilled workers to interpret the spectra, and are not necessarily viable for routine measurements. Hence, new analyses need to be developed which must be simple enough to be used widely, and yet more accurate and informative than the existing assays.

2.7.2.2 Chemical characterisation: proteolysis

Many studies on the identification of SMPs show that most soluble organic compounds present in anaerobic biological treatment systems were proteins-like substances (Ni *et al.*, 2011b; Kunacheva and Stuckey, 2014). Rosenberger *et al.*, (2006) observed that macromolecules such as proteinaceous materials with a MW greater than 120 kDa contributed significantly to membrane fouling, while Liang *et al.*, (2007) postulated that these compounds appeared to be the major SMPs components that were liable to accumulate in the MBR, compared to aromatic compounds. There are many studies conducted to determine the amount of protein-like compounds present in SMPs, and numerous chemical analytical methods to do so (Kunacheva and Stuckey, 2014).

For proteolysis, use of the Lowry method, ion-exchange chromatography, resonance light-scattering, excitation emission matrix spectroscopy, and proteomics have been documented (Kunacheva and Stuckey, 2014); amongst these, the Lowry-Folin Assay is the most commonly used. Many researchers have used both the original Lowry method, and its modified version, to analyse the protein content in SMPs and EPS (Kunacheva and Stuckey, 2014) because of their convenience and speed. Similar to glycolysis, such colorimetric methods have been very popular for first stage screening of proteins, but are very likely to give an overestimation since the method only detects the peptide bond *per se* (Lowry *et al.*, 1951).

Recently, metaproteomic analysis has been applied to detect microbial functional proteins because this method can provide new insights into metabolic and physiological activities in mixed microbial cultures (e.g., sludge, biofilm, and soil bacteria) (Wilmes and Bond, 2006; Schneider and Riedel, 2010; Siggins *et al.*, 2012). Nonetheless, such applications are limited to microbial ecological functions in sample microbial communities in the laboratory (Schneider and Riedel, 2010, Wilmes *et al.*, 2015).

Overall, since most of the SMPs and EPS are carbonaceous materials that are made up of macromolecules such as proteinaceous materials, there is still much work needed to be done to fully analyze and understand their chemical characteristics. To this end, part of this work will address these aforementioned shortcomings.

2.8 Concluding remarks

The conclusions that can be drawn from the literature review are as follows:

- In recent years, the subject “anaerobic” has attracted much more attention than ten years ago, implying that an increasing number of studies are investigating the anaerobic process. However, a significant fraction of the soluble organic carbon content of final effluents is discharged (SMPs) that has partially refractory characteristics.

- The level of SMP in wastewater correspond directly to the levels of effluent COD, which in turn affects effluent discharge levels, treatment efficiency and energy use. Biodegradation of wastewater organics produces SMPs, which can be important DBP precursors. SMPs are also a major contributor to membrane fouling in MBRs, a technology of increasing interest for practitioners, and constrain the application of this promising technology. EPS, as part of the SMPs, are believed to play a crucial role in fouling due to their gelling properties. Thus, a better understanding of the composition and control of the SMPs/EPS is therefore necessary to meet industrial challenges, as well as the increasingly stringent limits applied to effluent quality.
- Generally, SMPs are known to result from biological processes common to most microorganisms, and consists of a wide variety of soluble organic compounds with a range of MWs. The composition of SMPs is extremely complex, although they can be classified into two general classes based on their source; UAPs and BAPs. On the other hand, there is no concrete answer to why are SMPs produced.
- Factors such as feed strength, HRT, OLR, SRT, temperature, toxins, reactor configuration and substrate type seem to affect SMPs production. It is found that SMPs production is enhanced at lower temperature, at relatively low or high HRT, at higher SRTs and with higher concentration of biomass. SMP production is believed to be directly proportional to feed strength, but there is lack of data on the production and characterisation of SMPs on different single carbohydrate substrates and different nitrogen sources.
- It also appears that the bulk of the residual COD from well-operated laboratory scale anaerobic systems is due to SMPs. However, most of the studies on the characterization of SMPs were carried out based mainly on collective parameters such as MW distribution, biodegradability and toxicity, and there is a lack of information on the chemical characterisation of SMPs produced in AD. A wide range of different analytical techniques have been used previously making it a challenge to compare results in the literature.

- SMPs and EPS are a mixture of a variety of unknown compounds that do not belong to a single well-defined group. The main components are believed to include protein-like compounds, carbohydrates, deoxyribonucleic acid (DNA), lipids, aquatic humic substances, and small molecules. However, measuring proteinaceous materials and carbohydrates in biological wastewater treatment systems on the basis of colorimetric assays can be very non-specific, and quantifying both substances is fraught with error. Developing suitable methods for isolating, charactering and quantify proteinaceous materials or exopolysaccharides is essential in order to understand the fundamental mechanism of their formation, as well as developing new approaches for minimizing membrane fouling in MBRs.

2.9 Overview and research objectives

Based on the literature findings above, the objectives of this project were to:

- methodologically optimize the pretreatment methods (LLE and SPE) to specifically identify the greatest number of low MW SMPs (<580 Da) in wastewater effluent, and to analyse them qualitatively and quantitatively using GC-MS so that a Standard Method can be developed which can be used to compare the results of different researchers;
- evaluate the different colorimetric measurements of carbohydrates presently used, and to optimize an analytical method using GC-MS that could determine the molecular structure of exopolysaccharides in anaerobic bioreactors;
- validate the different protein assay techniques currently used, and adopt an amino acid composition analysis that could be applied to a biological wastewater sample by using HPLC;
- comprehensively determine the impact of feed composition on the production of SMPs in AD by employing all the techniques developed above.

CHAPTER 3

Materials and Methods

3.1 Introduction

This chapter presents a description of a submerged anaerobic membrane bioreactor, experimental set ups, and feed material used for Chapter 4-6. It also documents general parameters used in this research. Other newly developed methodologies for specific experiments will be developed and discussed in their respective chapters.

3.2 Submerged Anaerobic Membrane Bioreactor

The experimental set-up for this work is shown in Figure 3.1. A 3-L working volume submerged anaerobic membrane bioreactor (SAMBR) was made from polymethyl methacrylate (Plexiglass®). A microfiltration flatsheet membrane (chlorinated polyethylene) from Kubota was used with a surface area of 0.116 m², and a nominal pore size of 0.2 µm, and the flux of the membrane was maintained at 15 LMH. The reactor was designed containing a standing baffle which directed the reactor liquid in the up and down direction. The reactor was placed inside a water bath to maintain its temperature at 35±1 °C.

Recycled biogas was pumped through a stainless steel tube diffuser with four holes generating coarse bubbles in order to mix the biomass in the reactor, and to scour the membrane surface (to minimize membrane fouling). The gas flow rate was controlled at 8 L.min⁻¹ using a gas flow meter, and the SAMBR was operated at a hydraulic retention time (HRT) of 6 h, and at 100 days SRT. The reactor was continuously fed with a synthetic feed (544 ± 22 mg COD.L⁻¹) comprised of glucose, peptone, meat extract, and essential nutrients (Table 3.1). Samples were collected from the effluent line of the SAMBR (Figure 3.1), as well as in the reactor. Feed samples were obtained

to identify the compounds present in the feed to act as a reference to differentiate between influent compounds in the system, and reactor/effluent SMPs.

Table 3.1 Composition of synthetic municipal wastewater used in this study [Sources: Owen *et al.*, 1979; Grobicki and Stuckey, 1991; Gerardi, 2003].

Feedwater	Ingredients	Concentration (mg.L ⁻¹)
Organics	Peptone	102.25
	Meat extract	34.25
	Glucose	341.75
	CoCl ₂ .6H ₂ O	0.305
Trace elements	FeCl ₂ .4H ₂ O	2.01
	MnCl ₂ .4H ₂ O	0.096
	Na ₂ MoO ₄ .2H ₂ O	0.048
	NiCl ₂ .6H ₂ O	0.058
Buffers	K ₂ HPO ₄	10.24
	NaHCO ₃	832

3.3 General parameters

3.3.1 pH

pH was measured using a regularly calibrated pH meter (Mettler-Toledo SevenCompactTM S220), and values obtained were accurate to within ± 0.01 . Otherwise, MColorpHastTM pH indicator strips (Merck) were used to estimate the pH. The pH of the SAMBR system was maintained in the range of 6.8 to 7.2 using 1M NaHCO₃ in the feed.

3.3.2 Chemical Oxygen Demand

COD measurement was based on the description in Section 5220-D of the American Public Health Association (APHA) Handbook (Rice, 2012) that requires the preparation of digestion solution and sulfuric acid reagent.

Figure 3.1 - Diagram of the SAMBR.

Digestion solution was first prepared by adding 10.216 g of potassium dichromate previously dried at 103 °C, 167 mL of concentrated sulfuric acid and 33.3 g of mercury sulfate to 500 mL of ultrapure water. The mixture was then left to cool to room temperature before diluting to 1000 mL using a volumetric flask. The sulfuric acid reagent was prepared by adding silver sulfate (technical grade) powder to concentrated sulfuric acid at the rate of 5.5 g $\text{Ag}_2\text{SO}_4/\text{kg H}_2\text{SO}_4$.

The COD procedure consisting of adding in sequence, 1 mL of sample, 0.6 mL of digestion solution and 1.4 mL of sulfuric acid (ISO grade, Merck) to a test tube with a screw cap, and sealed tightly before the mixture was mixed. The mixture was heated in a COD block heater at 150 °C for 2 hours, and after cooling they were analyzed in a

Shimadzu Ultraviolet-Visible (UV/Vis) spectrometer (UV-2600) at a wavelength of 600 nm. Potassium hydrogen phthalate (PHP) was used to prepare a standard solution in the range of 10-1000 mg/L. The detection limit was 5 mg/L, and the standard deviation (SD) was tabulated, and based on triplicates it was within $\pm 5\%$.

3.3.3 Volatile Fatty Acids

A total of seven VFAs, including formic, acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acid could be quantified with the following method. They were of analytical grade and purchased from Sigma-Aldrich. Measurement was carried out using a Shimadzu HPLC (SPD-20AD) with a UV diode array detector (DAD, SPD-M20A) at 210 nm with an Aminex[®] HPX-87H (300×7.8mm) column. Analysis time was 25 min for each sample using 0.005 M H₂SO₄ as a mobile phase at 55°C (Guerrant *et al.* 1982). The SD was within $\pm 8\%$.

3.3.4 Suspended Solids

Volatile suspended solids (VSS) was measured as described in APHA Standard Methods (Rice *et al.*, 2012) to estimate the biomass concentration. A 5 mL or 10 mL sample was added onto a pre-prepared Whatman glass fiber filter (pore size =1.58 μ m) in a pre-weighed foil tray and left overnight in a 105 °C oven. The dried sample was cooled in a desiccator before weighing to give a measure of total suspended solids (TSS); the tray was then placed in a furnace at 550 °C for 1 hour. After cooling in a desiccator, the tray was weighted again to obtain a measure of VSS. The SD for 3 identical sample was within $\pm 5\%$.

3.3.5 Composition of gas

The composition of the biogas produced (methane, oxygen, nitrogen, and carbon dioxide) from a SAMBR was determined using gas chromatography (GC) (Shimadzu) with a thermal conductivity detector (TCD), (GC-2010plus). A select permanent gases/CO₂ (CP7429) column from Agilent was used for gas separation, while gas

volume was measured from a gas-sampling bag using a gas pump with a gas flow meter. The SD was below $\pm 3\%$.

3.3.6 Calculation of SMP concentration

The amount of SMPs is typically estimated by subtracting COD due to intermediate VFAs and residual substrate, from the soluble effluent COD, as in the following equation (Equation 1) (Barker and Stuckey 1998).

$$\text{SMPs (as COD)} = \text{soluble effluent COD} - [\text{volatile fatty acids (as COD)} + \text{residual substrate (as COD)}] \quad \text{Equation 1}$$

where, VFA (as COD) = $0.35(\text{formate}) + 1.07(\text{acetate}) + 1.51(\text{propionate}) + 1.82(\text{butyrate} + \text{isobutyrate}) + 2.04(\text{valerate} + \text{isovalerate})$; residual substrate (as COD) = $1.07(\text{glucose})$; and

() = concentration in mg/L.

CHAPTER 4

Characterization of low MW SMPs¹

Effluents from wastewater treatment processes contain a variety of organic compounds, including end products from the degradation of influent substrates, non-biodegradable feed compounds, and SMPs produced by microbial metabolism. This chapter presents the development of an analytical methodology for low MW (<580 Da) SMPs in wastewater treatment systems. Work on analysing higher MW (500 – 1000 Da) SMPs was carried out by another group at NTU, and was published as Tiphara *et al.*, (2017). Pretreatment of samples using a combination of SPE (solid phase extraction) (Oasis[®] HLB) and LLE (liquid-liquid extraction) (n-hexane, chloroform and dichloromethane) was optimized, and enabled the extraction of a maximum number of compounds to be optimized. Methods for the identification and quantification of low MW SMPs using GC-MS were also established.

4.1 Introduction

SMPs, resulting from microorganism growth and decay, comprise a wide range of compounds with different MWs (Noguera *et al.*, 1994; Barker and Stuckey, 1999; Laspidou and Rittmann, 2002), and are considered as a major component of the soluble organic matter in effluents from biological wastewater treatment (Barker and Stuckey, 1999; Shon *et al.*, 2004; Jarusutthirak *et al.*, 2005; Rosenberger *et al.*, 2006; Aquino and Stuckey, 2008). SMPs have also been classified into two groups, the UAP and the BAP, based on the microorganism phase from which they are derived (Namkung and Rittmann, 1986; Grady *et al.*, 1999; Laspidou and Rittmann, 2002; Jarusutthirak and Amy, 2006; Aquino and Stuckey, 2008; Ni *et al.*, 2011b; Kunacheva and Stuckey,

¹ Published as Kunacheva, C.; Le, C.; Soh, Y. N. A.; Stuckey, D. C. (2017) Chemical characterization of low molecular weight soluble microbial products in anaerobic membrane bioreactors. *Environ. Sci. Technol.* 51, 2254-2261.

2014). Moreover, the MW distribution of SMPs has been extensively examined, and it was found that a majority of SMPs have a MW less than 1 kDa or greater than 10 kDa (Jarusutthirak and Amy, 2006). There is only a minority of SMPs that have a MW between 1 and 10 kDa. However, a bimodal distribution has also been proposed, with 30 % of MWs lower than 1 kDa, and 25 % with MWs greater than 100 kDa (Barker and Stuckey, 1999). Even though these findings are contradictory, they point out that the majority of SMP have a MW less than 1 kDa. In the later part of the chapter this issue will be addressed in more detail.

As shown in Chapter 2, chemical analysis has been carried out to identify the SMPs, but a majority of these studies have focused on global characterization. Furthermore, SMPs are a complex mixture of organic compounds with varying compositions and characteristics, so analysing them is a challenge due to their dilute nature (often in sub ppm or ppb level) and their heterogeneity. However, they occupy a continuous size range in molecular dimensions, as evident in Figure 4.1. For illustration purposes only - a glucose molecule has a MW of 180 Da.

Figure 4.1 - Particle size of well-known objects and particulates. (Illustrate the size in 1 angstrom units) [Source: Elimelech *et al.*, 1995; Ranville and Schmiermund, 1999 (modified)]

Although the actual mechanisms and factors that lead to the production of these organic compounds are still not clear, they are probably produced from bacterial activity. At

present, SMPs are measured using a wide range of pre-treatments and analytical methodologies, and hence the data produced is often difficult to compare. A standardised and detailed analytical method for SMPs in wastewater is required so that the data obtained by different researchers can be compared productively. The objective of this work was to optimize a pre-treatment method using both LLE and SPE to identify as many compounds in the effluent sample as possible, and to develop a simple and useful method for the quantification of low MW SMPs using GC-MS.

4.2 Experimental and analytical details

4.2.1 Reagents and chemicals

Solvents (acetone, chloroform, dichloromethane, and n-hexane) were of GC-MS grade or equivalent, and were purchased from Merck. Methanol (LC-MS grade) was purchased from Sigma-Aldrich. Other solvents used in this study were of chromatographic grade and were purchased from Fisher. Ultrapure water was obtained from a MilliQ water process (Millipore Advantage A10). The alkane standards mixture (C10 - C40, 50 mg/L each) was purchased from Sigma-Aldrich. Anhydrous sodium sulfate was of ACS grade and was purchased from Merck.

4.2.2 Optimization of LLE for SMP analysis

In this study, GC-MS was selected as a tool to identify the unknown low MW SMP compounds. Organic analytes present in environmental samples are in trace amounts (mg/L or less). Moreover, due to their different polarities and chemical properties, they cannot be analysed without sample preparation. Sample pre-treatment is thus an essential part of the chromatographic procedure, particularly for GCMS. LLE and SPE have been commonly used in sample preparation for the identification of organic compounds, and LLE has been used in several studies to identify low MW SMPs (Aquino, 2004; Zhou *et al.*, 2009; Wu and Zhou, 2010).

LLE is the first and most common technique used for the extraction of organic compounds from aqueous samples (Loconto, 2012). Historically, LLE has been used

for more than 60 years as a powerful sample preparation technique, and has since been continuously developed and applied widely in diverse fields despite its limitations. The conventional LLE approach is still in extensive use because of the availability of high quality solvents, simplicity of the extraction process and apparatus, and its widespread implementation in standardized methods. There are numerous official methods that involve LLE for the determination of organic compounds at very low concentrations, such as Part 6000 of Standard Methods for the Examination of Water and Wastewater (Rice, 2012).

LLE was also used in sample preparation for the identification of SMPs using GC-MS. Aquino (2004) once used four different organic solvents for LLE, while Zhou *et al.* (2009) and his team (Wu and Zhou, 2010) used dichloromethane, a commonly used solvent for LLE, to extract compounds from the effluent of various bioreactors. Despite the wide range of organic compounds identified using GC-MS, the data obtained by different researchers cannot be compared productively due to the variety of analytical methods used.

In this study, eight common laboratory solvents, namely n-hexane ($\log K_{ow} = 4.00$), n-heptane ($\log K_{ow} = 4.50$), toluene ($\log K_{ow} = 2.73$), chloroform ($\log K_{ow} = 1.97$), diethyl ether ($\log K_{ow} = 0.89$), methyl tert-butyl ether ($\log K_{ow} = 0.94$), dichloromethane ($\log K_{ow} = 1.25$) and ethyl acetate ($\log K_{ow} = 0.73$) were selected for solvent screening so as to determine the solvent that can extract the most compounds from the sample. N-Hexane, chloroform and dichloromethane were found to be able to extract most of compounds using a minimum amount of solvent as presented in Section 4.3.2.1. A mixture of these three solvents was later demonstrated to be able to extract even more organic compounds. Further optimization of LLE was performed by adjusting the solvent to sample volume ratio, pH of the sample, and the solubility of the analyte through the addition of a neutral salt to “salt out” the solutes in order to establish a “capture all” pre-treatment procedure.

LLE was carried out at room temperature; the sample was placed into a 1-L separating funnel and the pH was adjusted when necessary to 2 or 11. The funnel was sealed after addition of the solvent mixture, and then agitated vigorously for one minute with periodic venting to release the pressure. The funnel was left to stand for at least 1 min; after the organic and aqueous layers had separated completely, the organic layer was recovered as completely as possible into a 500 mL conical flask. The extraction was repeated twice and the organic extracts were combined. Any moisture content in the combined organic layer was removed using anhydrous sodium sulfate, through which it would be filtered. After filtration, the filtrate was collected in a dry Florentine flask, and the solvent removed using a rotary evaporator. Possible losses of highly volatile compounds could be expected due to evaporation during the resulting solvent extraction. A micro-syringe (Hamilton) was used to measure precisely 1.0 mL of dichloromethane to dissolve the extract for chromatographic analysis. Finally, a 2 μ L aliquot was injected into the GC-MS system directly. To avoid contamination, all glassware used in this study was washed with tap water, cleaned with ultrapure water and rinsed with acetone. During the procedure, all apparatus was made of glass to avoid contamination.

4.2.3 Optimization of SPE for SMP analysis

Another pre-treatment option is SPE, and there are many types of adsorption cartridges available from various companies such as Waters, Sigma-Aldrich, and Biotage. These cartridges are typically designed for the identification of specific compounds or classes of compounds. Since the aim of this study was to obtain the maximum number of unknown compounds in the sample, a “capture all” cartridge should be suitable. There were two groups of researchers who used SPE followed by GC-MS to identify compounds in SMPs (Kunacheva and Stuckey, 2014). Aquino (2004) used a sorbent ENV+ (Biotage) and further analysed using GC-MS to identify specific SMP compounds which surprisingly revealed the presence of alkenes and alkanes as well as phthalates. Both Trzcinski and Stuckey (2010) and Vyrides *et al.*, (2010) used an Oasis[®]HLB cartridge to capture the compounds in the effluent from the SAMBR, and

analysed them using GC-MS. Alkanes, alkenes, phthalates, as well as esters, phenols, and amides were found in significant amounts. In this study, two “capture it all” reversed phase cartridges was selected, Oasis[®]HLB (Waters) and Supelclean ENVI-Carb (Sigma-Aldridge). Oasis[®]WAX and Oasis[®]WCX cartridges were also used to obtain strong acids and strong bases, respectively. However, no compounds were found from the eluents of both Oasis[®]WAX and Oasis[®]WCX. Therefore, Oasis[®]HLB and ENVI-Carb were selected for further optimization to obtain most of the compounds in the sample. Four solvents (methanol, acetone, dichloromethane, n-hexane) were selected based on their dielectric constants to elute the retained compounds from non-polar to polar ranges. The sequence of solvents for elution was methanol, acetone, dichloromethane, and hexane because the polarity of methanol is most comparable to that of water. Furthermore, the filtrate from the first cartridge was passed through a second one, which was connected inline to evaluate the capacity of the first cartridge. The elution and GC-MS analyses were performed separately for each cartridge.

Collected samples were filtered through a 0.45 µm glass fibre filter to remove suspended solids (SS). The filtrate (1 L) was passed through a cartridge (Oasis[®]HLB (225mg) or ENVI-Carb (250mg), which was preconditioned by 10 mL of methanol followed by 20 mL of ultrapure water. A flow rate of 10 mL/min was maintained using a peristaltic pump purchased from Watson-Marlow (120U). The tubing of the pump was rinsed with methanol (5 min) followed by ultrapure water (10 min) prior to sample loading. Then, the sample bottle was rinsed using ultrapure water to ensure the entire sample was loaded. Finally, the compounds were eluted with 2 mL of the individual solvent selected (methanol, acetone, dichloromethane, n-hexane) into the glass tubes (one for each solvent). Plastic material was avoided since solvent contacted with plastic can leach contaminants into the sample. SPE using ultrapure water blanks was also conducted to identify contamination during pre-treatment.

There are various advantages and disadvantages of LLE and SPE, and different kinds of compounds were obtained in different ways. To achieve the maximum recovery of most SMP compounds, a combination of LLE and SPE was also studied. In order to

avoid co-elution and/or saturation, the sequences of pre-treatment using LLE and SPE as well as the practicality of the procedure were evaluated.

4.2.4 Gas chromatography-mass spectrometry

Samples obtained from both LLE and SPE were then separately analyzed using a GC-MS system (GCMS-QP2010ULTRA, Shimadzu). 2-3 μL of sample (LLE: 2 μL ; SPE: 3 μL for acetone, dichloromethane, and n-hexane samples; 2 μL for methanol sample) was injected onto an RTX[®]-5MS (30m $\times$ 0.25mmID, Restek) column for identification of low to mid polarity compounds. A ZB-WAX*plus* (Phenomenex) column was also employed to identify high polarity compounds, but a much lower response in peak numbers and area were obtained, so the RTX[®]-5MS column was selected. Splitless injection was used with the temperature controlled at 280°C, and helium was used as the carrier gas at a column flow rate of 1 mL/min. The temperature programme was: 50°C, hold 7 min, rate 7°C/min to 325°C, and hold for 14 min; total run time for one sample was 60 min. This temperature programme was modified based on a study of volatile organics by Ansorena *et al.* (2001). The mass spectrometer was operated in the electron ionisation (EI) mode with the ion source temperature maintained at 230 °C. Mass spectra were acquired from an m/z of 30 to 580 after a 12 min solvent cut time. The chromatographic peaks were identified using the NIST11 library (*National Institute of Standards and Technology, Gaithersburg, MD, USA*, <http://www.nist.gov/srd/index.cfm>), and a compound with a match greater than 80% was considered as identified. Similarity index, mass spectrum and retention index were also used as selection criteria for compound identification. Compounds with a match lower than 80% were mentioned as unknown peaks.

4.2.5 Quantification

Alkanes were selected as representative compounds for quantification of the SMPs based on a literature review, their availability, and cost. The most important factor is that alkanes have a wide range of chain lengths (C10 – C40) covering most of the volatility range of the RTX[®]-5MS column. A 5-point calibration curve with

concentrations of 0.1, 0.25, 0.5, 1 and 2 mg/L was used for quantification. The coefficients of multiple correlation (R^2) were above 0.99 for all compounds except C38 and C40, which had lower intensities (Table 4.1).

Table 4.1 - Alkane standards for quantification.

Compound name	Formula	Molecular weight	Retention time	R^2	IDL (mg/L)	IQL (mg/L)	Retention Index
Decane	C ₁₀ H ₂₂	142	12.15	0.999	0.026	0.087	1015
Dodecane	C ₁₂ H ₂₆	170	17.16	0.999	0.017	0.056	1214
Tetradecane	C ₁₄ H ₃₀	198	21.96	0.999	0.019	0.064	1413
Hexadecane	C ₁₆ H ₃₄	226	25.70	0.997	0.020	0.066	1612
Octadecane	C ₁₈ H ₃₈	254	29.03	0.997	0.015	0.050	1810
Eicosane	C ₂₀ H ₄₂	282	32.04	0.996	0.020	0.068	2009
Docosane	C ₂₂ H ₄₆	310	34.78	0.997	0.022	0.073	2208
Tetracosane	C ₂₄ H ₅₀	338	37.30	0.991	0.017	0.055	2407
Hexacosane	C ₂₆ H ₅₄	366	39.62	0.995	0.013	0.042	2606
Octacosane	C ₂₈ H ₅₈	394	41.77	0.993	0.014	0.046	2804
Triacontane	C ₃₀ H ₆₂	422	43.78	0.994	0.016	0.054	3003
Dotriacontane	C ₃₂ H ₆₆	450	45.66	0.990	0.015	0.051	3202
Tetratriacontane	C ₃₄ H ₇₀	478	47.51	0.991	0.010	0.033	3401
Hexatriacontane	C ₃₆ H ₇₄	506	49.72	0.990	0.014	0.046	3600
Octatriacontane	C ₃₈ H ₇₈	534	52.59	0.981	0.011	0.038	3800
Tetracontane	C ₄₀ H ₈₂	562	56.45	0.984	0.054	0.182	3997

Note: IDL= Instrumental Detection Limit, IQL= Instrumental Quantification Limit

Figure 4.2 - Chromatogram of alkane standards at 1 mg/L (C10 – C40).

Figure 4.2 shows the chromatogram of alkane standards (C10 – C40) used in this study. Alkane standards were used as a reference to quantify the unknown compounds based on their retention time, and quantification was done separately for each unknown compound with reference to the alkane with the closest retention time. A set of standard was run in every analysis batch to minimise instrumentation error, and blank solvents (methanol, acetone, dichloromethane, n-hexane) were also run in every batch. The blank was subtracted from the concentration of individual compound in the sample. The instrument detection limit (*IDL*) of the alkane standards was evaluated for each compound based on the maximum blank concentration, and a signal-to-noise ratio of 3:1, whereas the signal-to-noise for the instrument quantification limit (*IQL*) was 10:1

4.3 Results and discussion

4.3.1 Performance of SAMBR

Table 4.2 - Parameters and performance of SAMBR.

Parameters	SAMBR
Membrane	Flatsheet (Kubota)
Membrane type	Chlorinated Polyethylene 0.4µm (max) 0.2µm (ave)
Surface area (sq.m.)	0.116
Membrane flux (LMH)	15
Reactor volumn (L)	3
HRT (hr)	6
VSS (mg/L)	6000
Feed type	Glucose + nutrients
COD _{in} (mg/L)	492±18
COD _{out} (mg/L)	13±1
Efficiency	98% COD removal
Methane production (L/d)	2.77±0.29
VFAs in effluent (as mgCOD/L)	ND
SMP production (as mgCOD/L)	13±1

Note: ND = No Detection

The SAMBR was fed with a low strength synthetic wastewater (492±18 mgCOD.L⁻¹) under stable conditions (HRT = 6 h) for 30 days; Table 4.2 shows the parameters and performance of the SAMBR during this period. The performance of the SAMBR was excellent, with 98% COD removal and a soluble chemical oxygen demand (SCOD) in

the effluent as low as $13 \pm 1 \text{ mg.L}^{-1}$; VFAs were not found in any samples in this experiment. According to Equation 1, 100 % of the SCOD accounted for SMPs in effluent. Biogas contained an average of 74 % methane, 11 % carbon dioxide, and 15 % nitrogen, with no oxygen detected. Biogas production was an average of 2.77 L.day^{-1} , which accounted for $323 \text{ mL.CH}_4(\text{g.COD})^{-1}$ (92 % of the theoretical value).

4.3.2 Optimization of LLE for SMP analysis

4.3.2.1 Selection of solvents

The development of this technique required a selection of suitable solvents. Although water is highly polar and immiscible with most organic solvents, polar organic solvents like alcohol and acetone will not separate well with water using LLE techniques because of their high miscibility with water. The appropriate solvent for aqueous extraction should also fulfil several other criteria: (a) high volatility of the solvent to make concentration of the sample easier and faster, (b) strong capability to extract analytes, (c) impurities should preferably be insoluble in the extracting solvent, (d) low cost, and (e) preferably non-toxic and non-flammable. From the 30 common solvents that can be found in the laboratory, eight inexpensive and easily accessible solvents were chosen for this study. Additionally, they exhibited good solubility for a range of organic compounds including other organic solvents.

First, the extraction efficiency of different solvents was studied; an average number of peaks and their standard deviations (SD) were obtained from a quadruplicate analysis ($n = 4$). The results, as shown in Table 4.3, reveal that n-hexane, chloroform and dichloromethane have good extraction capability, while no peaks were found in the extract using n-heptane and toluene due their poor extraction ability. Other solvents like diethyl ether, methyl tert-butyl ether and ethyl acetate showed higher degrees of miscibility with water resulting in poor extraction outcomes.

After GC-MS analysis 146 organic compounds with identical structures could be identified in either n-hexane, chloroform or dichloromethane. Some of the compounds

detected such as alkanes, alkenes, fatty acids, phthalates (in particular dibutyl phthalate and bis(2-ethylhexyl)phthalate) have also been reported previously (Aquino, 2004; Zhou *et al.*, 2009; Trzcinski and Stuckey, 2010b; Wu and Zhou 2010). There were 13, 33, and 11 organic compounds that could only be extracted by n-hexane, chloroform and dichloromethane independently, respectively, and this led to an investigation of solvent extraction using a mixture of solvents. The average number of organic compounds extracted was higher when a (1:1) mixture of chloroform and dichloromethane as well as a (1:1:1) mixture of all three solvents were used, compared to using three individual solvents. When employing a similar protocol, a mixture of n-hexane and chloroform and a mixture of n-hexane and dichloromethane were less effective in extracting analytes, producing an inferior result. This is because both mixtures had a high miscibility in water, and thus no analytes were extracted. Therefore, a mixture of n-hexane, chloroform and dichloromethane was selected as the extraction solvent for further study.

Table 4.3 - Efficiency of different organic solvents (20 mL) evaluated for extraction using 200 mL of sample.

Solvents (ratio)	Total number of peaks (Mean $\pm$ SD)
n-Hexane	196 $\pm$ 2
n-Heptane	0
Toluene	0
Chloroform	215 $\pm$ 3
Diethyl ether	0
Methyl tert-butyl ether	0
Dichloromethane	232 $\pm$ 2
Ethyl acetate	0
n-Hexane + Chloroform (1:1)	0
n-Hexane + Dichloromethane (1:1)	0
Chloroform + Dichloromethane (1:1)	260 $\pm$ 3
n-Hexane + Chloroform + Dichloromethane (1:1:1)	274 $\pm$ 1

Note: SD = standard deviation

4.3.2.2 Volume of extraction solvent

After identifying the optimal extraction solvent mix, another set of experiments was carried out in an attempt to enhance the extraction concentration by optimizing the solvent volume. The purpose of doing this was to determine the minimum amount of solvent required to perform LLE, and hence minimise the generation of waste since the extraction solvents are either flammable or potentially toxic.

In order to evaluate the effect of solvent volume, different volumes of solvent mixture containing n-hexane, chloroform and dichloromethane were subjected to the same extraction procedure. The volume of organic solvent mixture was investigated in the range of 20 mL to 100 mL, with all the other experimental conditions fixed. Based on the results shown in Figure 4.3, the degree of extraction efficiency was influenced by the volume of extraction solvent used. Both the number of peaks, and the total peak area obtained increased with an increase in the volume of extraction solvent below 70 mL. However, a plateau in both was observed after the solvent volume exceeded 80 mL, although this decrease in peak number was not statistically significant. Hence, 70 mL of solvent mixture was chosen as the optimum volume for subsequent experiments.

Figure 4.3 - Efficiency of different solvent volume evaluated for extraction

4.3.2.3 Effect of pH and additives

It is well known that pH plays a significant role in the extraction of organic analytes in environmental water samples, because the pH of solution determines the molecular form of the organic compounds, and thus affects their extraction. The “salting out” effect is another method commonly used to enhance LLE performance by decreasing the solubility of the analyte in aqueous samples, and increasing the partition coefficient with the addition of neutral salts (Dean, 2003). In this work, the effect of pH was evaluated in either acidic ($\text{pH} < 2$) or basic ($\text{pH} > 11$) solutions, while different neutral additives such as NaCl and MgSO_4 were added to the solution. From Table 4.4, the experimental results demonstrated that both the performance indicators showed no obvious improvement, and they may even decrease the efficiency as compared to the results obtained at neutral pH with no addition of salts. As a result, the future procedures were conducted at $\text{pH} = 7$ with no additives.

Table 4.4 - Efficiency of different additives evaluated for extraction.

Additives	pH	Total peak area (Mean ($\times 10^9$) $\pm$ SD)	Total number of peak (Mean $\pm$ SD)
Nil	7	2.99 ± 0.06	395 ± 1
HCl	< 2	2.24 ± 0.05	388 ± 1
NaOH	> 11	2.91 ± 0.36	372 ± 1
NaCl	7	2.98 ± 0.22	393 ± 1
MgSO_4	7	2.78 ± 0.25	383 ± 1

Note: SD = standard deviation

4.3.2.4 Selection of sample size

To study the influence of sample size on extraction performance, experiments were performed using varying volumes of effluent sample while keeping all other experimental conditions constant. When the volume of sample increased from 200 mL to 400 mL, the number of compounds detected did not increase significantly, although the total peak area almost doubled, as shown in Table 4.5. On the other hand, as the volume of sample decreased from 200 mL to 100 mL and 50 mL respectively, a decline

in the number of peaks, and their peak area, was observed. Since the total mass of analytes will decrease with a reduction in effluent sample volume, some of the analytes' concentration in the solvent extract could fall below the detection limits of the GC-MS. Hence, a minimum sample volume of 200 mL was deemed as optimal to maximise the extraction of low MW SMPs.

Table 4.5 - Efficiency of different sample sizes evaluated for extraction.

Sample size (mL)	Total peak area (Mean ($\times 10^9$) $\pm$ SD)	Total number of peaks (Mean $\pm$ SD)
400	5.82 $\pm$ 0.02	396 $\pm$ 3
200	2.99 $\pm$ 0.06	395 $\pm$ 1
100	0.55 $\pm$ 0.33	326 $\pm$ 1
50	0.46 $\pm$ 0.39	290 $\pm$ 1

Note: SD = standard deviation

4.3.3 Optimization of solid phase extraction for SMP analysis

4.3.3.1 Comparison of Oasis[®]HLB and ENVI-Carb cartridge

Two cartridges, Oasis[®]HLB and ENVI-Carb, were tested to compare their performance in capturing compounds from the effluent. Effluent from the SAMBR was collected and loaded onto the cartridges on the same day. The compounds identified were categorized as alkanes, alkenes, esters, alcohols, nitrogen compounds (N-compounds), phenols, and others according to previous studies (Trzcinski and Stuckey, 2010b; Wu and Zhou, 2010). The reactor feed was also analysed to give a background against which SMPs could be assessed. Both cartridges showed similar results in terms of the number of compounds retained and subsequently eluted (Oasis[®]HLB: 73 and ENVI-Carb: 70) (Figure 4.4), however, the compounds that both cartridges captured were noticeably different, with only about a 50% similarity. Figure 4.4 also shows the number of compounds that were retained in each cartridge under different categories. Oasis[®]HLB retained more of the higher volatility compounds than ENVI-Carb, with average retention indices (retention time normalized to the retention times of adjacently

eluting n-alkanes: lower retention index equals higher volatility) of 1820 and 1942, respectively. Both cartridges showed very good performance in retaining low MW compounds from the effluent of the SAMBR. However, Oasis®HLB was selected because it retained compounds with a wider range of volatility.

Figure 4.4 - Number of compounds detected using Oasis®HLB and ENVI-Carb cartridges.

4.3.3.2 Comparison of one cartridge with two cartridges

The capacity of the Oasis®HLB cartridge was evaluated by varying the sample volume (50 mL, 100 mL, 500 mL, and 1000 mL) loaded onto it. The concentrations of the compounds detected varied over a wide range, and hence low concentration compounds were not detected using small sample volumes. Since our objective was to capture as many compounds as possible, a 1000 mL sample size was selected for extraction. Nevertheless, some compounds were found at very high concentrations, and as a result the cartridge was saturated. Consequently, a second cartridge was connected in-line with the first cartridge to capture the compounds that could “overflow” from the first cartridge. Figure 4.5 shows the number of compounds identified using the first and second Oasis®HLB cartridges. The number of compounds identified using the first and

second cartridges were 50 and 33, respectively, and the second cartridge captured 14 more compounds than the first one was unable to adsorb. Another option was to use a bigger capacity cartridge, however, the benefit of using two cartridges was that the eluted sample could be analysed separately to avoid overlapping peaks.

Figure 4.5 - Number of compound identified using 1st and 2nd Oasis® HLB cartridges.

4.3.3.3 Effect of solvents for elution

A single solvent cannot elute all compounds of different polarities, and hence four (methanol, acetone, dichloromethane, n-hexane) were selected based on their dielectric constants to elute as many of the compounds as possible, from non-polar to polar. The elution sequence was methanol, acetone, dichloromethane, and hexane due to their polarities. Figure 4.6 shows number of compounds eluted by different solvents. Methanol and acetone eluted more than dichloromethane and hexane as expected; non-polar compounds such as alkanes and alkenes were more likely to elute with the later since the percentage of alkanes and alkenes increased with reducing dielectric constants of the solvents. Some compounds, mostly long chain alkanes and alkenes, were not eluted completely using more polar solvents such as methanol and acetone. As a result,

these compounds were also found in dichloromethane and hexane samples. The GC-MS analysis was done separately for each solvent to avoid co-elution, overlapping peaks, dilution of compounds, and higher backgrounds (reduced signal to noise ratio). Ultimately, the final optimized SPE procedure was as follows: 1 L of effluent sample was loaded onto two in-line Oasis[®]HLB cartridges and eluted with four solvents in sequence (methanol, acetone, dichloromethane and hexane) separately for each cartridge, and all eluted samples were then analysed using GC-MS.

Figure 4.6 - Number of compounds eluted by different solvents.

4.3.4 Combination of LLE and SPE

Compared to SPE, LLE captured more compounds from the effluent, although most of the compounds were non-polar. SPE, on the other hand, could identify a wider range of compounds, and hence combinations of SPE and LLE were evaluated as a pre-treatment.

Both the SPE→LLE and LLE→SPE sequence were analysed. It was found that there were several practical disadvantages with using LLE→SPE; the first was that the LLE chromatogram had a higher baseline due to the matrices present (higher MW organics extracted). In contrast, with SPE→LLE, SPE captured some matrices which resulted in a lower baseline in the LLE chromatogram, and better resolution. The second was that dichloromethane was present in the sample after LLE, and this affects adsorption in SPE. The last issue was sample volume; for LLE→SPE, five extracts (200 mL each) with LLE were needed for further extraction by SPE (1 L). For SPE→LLE, 1 L of sample was loaded onto the Oasis®HLB cartridges and then 200 mL that had passed through the cartridges was extracted using LLE. Hence, SPE→LLE was selected as an optimal pre-treatment for low MW SMPs. An effluent sample was collected and split into 3 samples (Sample 1, 2, 3) for statistical validation of method reproducibility. Table 4.6 shows the number of peaks (with minimum peak area/peak height > 3000) identified using a combination of SPE and LLE for extraction, and demonstrates good reproducibility across the three samples. The chromatograms of sample 2, blanks and alkanes are shown in Figure 4.7, while the list of compounds identified is shown in Tables 4.7 and 4.8.

Table 4.6 - Combination of SPE and LLE for the extraction of low MW compounds in SMPs.

Sample	Number of peaks (SPE)			Number of peaks (LLE)			Total number of peaks
	Identified	Unidentified	Total	Identified	Unidentified	Total	
1	56	22	78	67	113	180	258
2	52	25	77	68	109	177	254
3	53	24	77	68	110	178	255

Note: SPE = Solid phase extraction, LLE = liquid-liquid extraction,
Identified = mass spectral similarity > 90%, Unidentified = mass spectral similarity < 90%

Table 4.9 compares our method to other methods in the literature, and LLE was found to be the most popular pre-treatment in previous research. Aquino (2004) was the first to try to analyse low MW SMPs, and he used four solvents in LLE extraction. 18 compounds and 9 compounds were found in a sugar feed CSTR and a SAMBR,

respectively. Zhou *et al.* (2009) used LLE with dichloromethane for analysing SMPs in the effluent of UASBs treating industrial wastewater, and more than 27 compounds were identified. The same group of researchers found less low MW SMPs in a reactor fed with glucose using a similar LLE method (Wu and Zhou, 2010). SPE was also applied previously to separate compounds in the effluent of a SAMBR, and 4 compounds were identified using the sorbent ENV+ (Aquino 2004). Another study was carried out using Oasis®HLB to identify compounds in the effluent of a SAMBR treating solid waste leachate, and 11 compounds were identified (Trzcinski and Stuckey, 2009). Until now there has been no information in the literature on pre-treatment optimization; in this study pre-treatment was optimized, and an average of 77 and 178 compounds were identified using SPE and LLE techniques, respectively, an order of magnitude more compounds identified than in past work.

Finally, approximate quantification was done separately for each compound using the alkanes with the closest retention time. A set of standards were run in and between every batch of samples analysed to minimise discrepancy, and blank solvents (methanol, acetone, dichloromethane, n-hexane) were also run concurrently for background subtraction. Combined concentrations of the low MW compounds using SPE and LLE were 0.009 mg.L⁻¹ and 0.598 mg.L⁻¹, respectively, and the compounds identified from both methods accounted for 2.1 mgCOD.L⁻¹ (16% of the total SMP as COD calculated from 3.46 gO₂/g docosane). While the concentration estimation using the alkane standards will not be the most accurate way to quantify SMP, the quantification developed here is currently the best estimate in the literature, and the most practical for unknown samples. The comparison of compounds identified as SMPs from different type of wastewater effluents were presented in Kunacheva *et al.* (2017).

Figure 4.7. Chromatograms of the sample 2 (SPE-LLE).

Figure 4.7. Chromatograms of the sample 2 (SPE-LLE) (*cont.*).

Figure 4.7. Chromatograms of the sample 2 (SPE-LLE) (*cont.*).

Table 4.7 - List of 50 compounds that have the highest peak area (SPE as a pretreatment).

RT	Compound name	Chemical formula	Mass	Retention Index	Compound Group	Peak Area	Similarity Index
24.38	Phenol, 3,5-bis(1,1-dimethylethyl)-	C14H22O	206	1555	Phenol	4805680	92
22.27	Tetradecane	C14H30	198	1413	Alkane	1893471	96
17.77	Dodecane	C12H26	170	1214	Alkane	1749187	96
26.04	Hexadecane	C16H34	226	1612	Alkane	1542929	96
35.82	Tributyl acetyl citrate	C20H34O8	402	2594	Ester	1371280	94
29.18	Octadecane	C18H38	254	1810	Alkane	1172653	93
12.19	Decane	C10H22	142	1015	Alkane	1075648	91
32.18	Eicosane	C20H42	282	2009	Alkane	816872	95
32.01	9H-Pyrido[3,4-b]indole, 1-methyl-	C12H10N2	182	1746	N-compound	669681	84
16.52	5-Undecene, (E)-	C11H22	154	1123	Alkene	636746	83
25.91	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	C16H30O4	286	1605	Ester	448224	87
34.49	1-Propene-1,2,3-tricarboxylic acid, tributyl ester	C18H30O6	342	2297	Ester	448127	88
35.05	Docosane	C22H46	310	2208	Alkane	446279	91
37.52	Tetracosane	C24H50	338	2407	Alkane	409996	88
39.79	Hexacosane	C26H54	366	2606	Alkane	376296	84
38.64	Eicosane, 7-hexyl-	C26H54	366	2542	Alkane	343980	94
28.78	Heptadecane, 3-methyl-	C18H38	254	1746	Alkane	295922	91
41.72	Octacosane	C28H58	394	2804	Alkane	264170	85
24.64	Benzoic acid, 4-ethoxy-, ethyl ester	C11H14O3	194	1448	Ester	239171	81
16.51	Camphor	C10H16O	152	1121	Others	226671	92
25.49	Pentadecane, 3-methyl-	C16H34	226	1548	Alkane	226363	94
29.06	1-Octadecene	C18H36	252	1801	Alkene	195662	89
25.76	Cetene	C16H32	224	1602	Alkene	190939	96
31.36	1H-Pyrido[3,4-b]indole, 2,3,4,9-tetrahydro-1-methyl-	C12H14N2	186	1820	N-compound	188408	83
33.82	Eicosane, 10-methyl-	C21H44	296	2045	Alkane	183125	83
20.14	3-Methyl-2,3-dihydro-benzofuran	C9H10O	134	1097	Others	172360	84
42.93	2-methyloctacosane	C29H60	408	2840	Alkane	160679	91
28.89	Tryptophol	C10H11NO	161	1606	Alcohol	155851	80
31.82	Dibutyl phthalate	C16H22O4	278	2037	Ester	148063	87
36.32	Heptadecane, 9-octyl-	C25H52	352	2442	Alkane	147424	86
38.57	Pyrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	C14H16N2O	228	2138	N-compound	145538	91
31.70	Nonadecane, 2-methyl-	C20H42	282	1945	Alkane	145244	92
26.65	Benzophenone	C13H10O	182	1603	Others	133971	80
31.91	2-(Hexyloxy carbonyl)benzoic acid	C14H18O4	250	2027	Ester	132798	80
29.13	Benzenesulfonamide, N-butyl-	C10H15NO2 S	213	1797	N-compound	130013	84
31.22	Pentadecanoic acid, 14-methyl-, methyl ester	C17H34O2	270	1814	Ester	121465	86
31.37	1-Naphthalenemethanamine	C11H11N	157	1588	N-compound	111091	80
23.26	4-Hydroxyphenylacetamide	C8H9NO2	151	1523	N-compound	103739	84
34.58	Heneicosane, 3-methyl-	C22H46	310	2144	Alkane	99344	87
37.85	Triphenyl phosphate	C18H15O4P	326	-	Others	95462	84
33.70	9-Octadecenoic acid (Z)-, methyl ester	C19H36O2	296	2085	Ester	87321	83
31.19	Hexadecanoic acid, 15-methyl-, methyl ester	C18H36O2	284	1914	Ester	75783	80
23.03	Ethanone, 1-(4-ethoxyphenyl)-	C10H12O2	164	1318	Others	74947	83
28.72	Pyrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	C7H10N2O2	154	1404	N-compound	74614	94
43.93	Triacontane	C30H62	422	3003	Alkane	66715	87
29.26	Octadecane, 2-methyl-	C19H40	268	1846	Alkane	64001	84
27.13	[1,1'-Biphenyl]-2,2'-diol	C12H10O2	186	1808	Others	61775	84
30.84	9-Hexadecenoic acid, methyl ester, (Z)-	C17H32O2	268	1886	Ester	60770	87
26.21	Benzothiazole, 2-(methylthio)-	C8H7NS2	181	1548	Others	43624	82
40.88	2-methylhexacosane	C27H56	380	2641	Alkane	40676	92
28.69	Heptadecane, 2,6-dimethyl-	C19H40	268	1782	Alkane	36068	92
40.87	Docosane, 7-hexyl-	C28H58	394	2740	Alkane	30692	87

Table 4.8 - List of 50 compounds that have the highest peak area (LLE as a pretreatment).

RT	Compound name	Chemical formula	Mass	Retention Index	Compound Group	Peak Area ($\times 10^5$)	Similarity Index
11.20	2,3-Dimethylpentanol	C7H16O	116	832	Alcohol	11961	82
36.24	13-methylpentacosane	C26H54	366	2534	Alkane	5523	93
30.48	Palmitic acid	C16H32O2	256	1968	Others	4890	92
29.91	n-Nonadecane	C19H40	268	1910	Alkane	3805	93
36.15	11-pentyl-heneicosane	C26H54	366	2453	Alkane	3510	89
33.46	10-Methylhenicosane	C22H46	310	2144	Alkane	3183	90
29.16	Di-iso-Butyl phthalate	C16H22O4	278	1908	Ester	2950	90
33.26	Stearic acid	C18H36O2	284	2167	Others	2787	95
32.91	n-Octadecanol	C18H38O	270	2053	Alcohol	2746	92
38.08	Diisooctyl phthalate	C24H38O4	390	2704	Ester	2535	89
18.35	Allyl 4-oxopentanoate	C8H12O3	156	1110	Ester	2309	88
26.57	2-Methylheptadecane	C18H38	254	1746	Alkane	2309	89
30.56	5-methylhex-2-yl butyl phthalate	C19H28O4	320	2207	Ester	2300	95
27.28	8-Methylheptadecane	C18H39	254	1746	Alkane	2108	91
35.66	2-methyltetracosane	C25H52	352	2442	Alkane	2065	90
40.60	(Z)-13-Docosenamide	C22H43NO	337	2625	N-compound	2061	90
19.05	(E)-2-Dodecene	C12H24	168	1222	Alkene	1838	92
38.61	9-methylheptacosane	C28H58	394	2735	Alkane	1779	92
11.43	(E)-Oct-2-enal	C8H14O	126	1013	Others	1616	81
41.07	Squalene	C30H50	410	2914	Alkene	1559	95
15.02	5-Chloro-1-pentyl acetate	C7H13ClO2	164	1110	Others	1526	91
33.53	(Z)-9-Octadecenamide	C18H35NO	281	2228	N-compound	1509	80
35.86	Nonadecanamide	C19H39NO	297	2319	N-compound	1467	91
24.72	2,2,4-Trimethylpentanediol-1,3-diisobutyrate	C16H30O4	286	1605	Others	1344	92
28.14	Tris(2-chloro-1-methylethyl) phosphate	C9H18Cl3O4P	326	-	Others	1282	95
18.48	5-Sec-butylnonane	C13H28	184	1185	Alkane	1130	91
40.89	n-Octacosane	C28H58	394	2804	Alkane	1106	91
23.83	7,9-Dimethylhexadecane	C18H38	254	1682	Alkane	1061	80
38.16	2-methylhexacosane	C27H56	380	2641	Alkane	1061	91
22.83	n-Tetradecane	C14H30	198	1413	Alkane	966	93
19.47	2-Methyldodecane	C13H28	184	1249	Alkane	956	93
19.53	4,6-Dimethyldodecane	C14H30	198	1285	Alkane	918	95
33.35	2-Methyl-1-hexadecanol	C17H36O	256	1890	Alcohol	837	94
12.54	4-Methyl-2-heptanone	C8H16O	128	888	Others	759	90
38.68	2-methyloctacosane	C29H60	408	2840	Alkane	741	89
30.76	8-Hexylpentadecane	C21H44	296	2045	Alkane	717	92
30.89	10-Methylnonadecane	C20H42	282	1945	Alkane	717	96
33.70	5-methyl-henicosane	C22H46	310	2151	Alkane	716	92
12.76	4,4-Dimethylheptane	C9H20	128	831	Alkane	581	93
12.80	3,7-Dimethyldecane	C12H26	170	1086	Alkane	567	94
43.01	11-Decyltetracosane	C34H70	478	3337	Alkane	560	92
14.12	2,6-Dimethyldecane	C12H26	170	1086	Alkane	555	92
26.38	n-Hexadecane	C16H34	226	1612	Alkane	493	91
40.67	2-Octyl-1-dodecanol	C20H42O	298	2188	Alcohol	490	92
30.64	5-methylnonadecane	C20H42	282	1953	Alkane	478	91
30.52	5,5,7,7-Tetraethylundecane	C19H40	268	1741	Alkane	464	88
27.42	2,6,10,15-Tetramethylheptadecane	C21H44	296	1852	Alkane	432	96
33.94	8-butyl-9-ethyloctadecane	C24H50	338	2140	Alkane	428	89
12.18	4-Ethyl-4-methyl-1-hexene	C9H18	126	821	Alkene	418	82
29.98	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	C17H24O3	276	2081	Others	416	87

Table 4.9 - Comparison of the method of this study and literature results.

Sample	Type of Biological treatment	Pretreatment	Analysis	Number of identified compounds	References
Mixed liquor, Effluent	CSTR, SAMBR	LLE (hexane, monochloro benzene, dichloromethane, ethyl acetate), for GC-MS SPE (sorbent ENV+), for GC-MS	GC-MS [Column: SGE Phase BPX5, Identification of m/z 40 to 600]	CSTR: 18 compounds (LLE), 9 compounds (SPE) SAMBR: 9 compounds (LLE), 4 compounds (SPE)	Aquino (2004)
Effluent	UASB (full scale 3,100m ³)	LLE (dichloromethane)	GC-MS [Column: DB 5MS, Identification of m/z 33 to 500, NIST98 and WILEY Registry 7.0]	27 compounds (major)	Zhou <i>et al.</i> (2009)
Effluent	SAMBR	SPE (Oasis®HLB), Elution solvent: 10% methanol/90% MTBE	GC-MS [Column: SGE HT5, Identification of m/z 33 to 500, NIST05 library]	11 compounds	Trzcinski and Stuckey (2009)
Effluent	activated sludge	Lyophilized	LC-IT-TOF-MS [Identification of MW 100 to 4,000 Da]	Not mentioned	Mesquita <i>et al.</i> (2010)
Effluent	UASB	LLE (dichloromethane)	GC-MS [NIST98 and WILEY Registry 7.0]	13 compounds (major)	Wu and Zhou (2010)
Effluent	SAMBR	SPE (Oasis®HLB), Elution solvent: methanol, acetone, dichloromethane, hexane LLE: mixture of n-hexane, chloroform or dichloromethane	GC-MS [Column: RTX®-5MS, Identification of m/z 33 to 580, NIST11]	SPE: 63 compounds LLE: 227 compounds	This study

Note: CSTR = continuous stirred tank reactor, LC-IT-TOF-MS = High resolution liquid chromatography ion trap time-of-flight mass spectrometry, LLE = liquid-liquid extraction, SAMBR = submerged anaerobic membrane bioreactor, SPE = solid phase extraction, UASB = upflow anaerobic sludge blanket

[Source: Kunacheva and Stuckey, 2014, (modified)]

4.4 Conclusions

Based on the results of this study, the combination of SPE→LLE coupled with the GC-MS was shown to be “optimum” for separating and identifying low MW SMPs. This proposed new method is a good step towards further characterizing and understanding SMPs produced from bioreactors. Combining SPE and LLE in the pre-treatment of effluent samples before GC-MS resulted in the best performance, and the most practical method using alkanes as a reference standard was a way of quantifying the compounds present in the SAMBR effluent; the total compounds identified accounted for 2.1 mg COD.L⁻¹ or 16% of the total SMP as COD. The origin of these SMPs is of great importance, however, it is beyond this study, and this will be done in Chapter 7.

CHAPTER 5

Characterization of Polysaccharides²

While Chapter 4 deals with methodology development for the characterization of low MW SMPs, this chapter examines how to measure carbohydrates in SMPs generated from anaerobic wastewater bioreactors. This information is important to obtain in the water research field since these colorimetric methods, despite widespread use, are not being used correctly. In addition, a method for the elucidation of the glycan structure and composition in biological wastewater treatment systems using GC-MS is also developed. This new method will be more accurate and informative than the existing assays, and its application is covered in detail, including qualification and quantification.

5.1 Introduction

It has been well-documented that the ability to synthesize and secrete PS is widespread among microorganisms under virtually all physiological conditions (Sutherland *et al.*, 2011), and various secreted extracellular compounds are often found to be glycosylated even though such compounds are only produced in eukaryotic cells (Upreti *et al.*, 2003). It is also known that these extracellular PS are heavily involved in the formation of three-dimensional structures, and improve the long term stability of aerobic granules (Liu, 2007). Cho and Fane (2002) found that increasing membrane resistance was linked to the increasing production of EPS, and Okamura *et al.* (2009) suggested that this resistance directly correlates with PS, which undergo intermolecular or intramolecular ionic cross-linking thus clogging the membrane pores and leading to

² Published as Le, C.; Stuckey, D. C. (2016) Colorimetric measurement of carbohydrates in biological wastewater treatment systems: A critical evaluation. *Water Res.* 94, 280-287. and Le, C.; Stuckey, D. C. (2017) Influence of feed composition on the monomeric structure of free bacterial extracellular polysaccharides in anaerobic digestion. *Environ Sci. Technol.* 122, 10-16.

further fouling of the membrane. Furthermore, PS-related biofilms of both aerobic and anaerobic microorganisms have been recognised as the most significant contributor to microbially influenced corrosion, which is a major issue for material used in industrial wastewater treatment plants (Beech and Gaylarde, 1999; Pitonzo *et al.*, 2004; Coester and Cloete, 2005).

Numerous analytical methods have been developed to measure carbohydrate in SMPs and their concentration, and colorimetric methods are the most commonly used. As mentioned in Chapter 2, these assays are simple, requiring only chemicals, tubes and a spectrophotometer, and are universally accepted. The reagents can be economically prepared in a laboratory, and a number of assays are available from commercial suppliers such as Sigma-Aldrich, Thermo Scientific, and Abnova. Recently, scaled-down methods have been developed and 96-well microplates have been adapted for higher throughput, and to further economize on reagents (Laurentin and Edwards, 2003; Masuko *et al.*, 2005).

In addition, a detailed evaluation of these colorimetric saccharide quantification methods is often time consuming, and thus has received limited attention (Raunkjaer *et al.*, 1994; Frøhlund *et al.*, 1996; Jimenez *et al.*, 2013). When using these methods with wastewater it is important to recognize whether or not the methods have a high sensitivity to the different saccharides measured, and/or a low sensitivity to interfering compounds, because wastewater is a relatively complex mixture of different components at different concentrations. Thus there is an urgent need to evaluate these existing assay techniques in some depth, with and without interfering solutes, to see whether they can accurately measure carbohydrates in wastewater. By comparing the performance of these carbohydrate assays simultaneously and comprehensively with a large number of (poly)saccharides, this study will highlight the need to understand the chemistry and limitations of the colorimetric assays, particularly when studying the complex environments in which wastewater is present. It is important to understand that the results are values relative to the amount of glucose, and are not an absolute value. Due to the issues surrounding carbohydrate assays, as we will detail in the first

part of this chapter, we will conclude with an establishment of a new methodology to measure free extracellular polysaccharides and their composition analysis using GC-MS.

Free extracellular polysaccharides are an intricate assortment of extracellular polymeric substances secreted by a broad range of bacterial species (Dong *et al.*, 2006). The free exopolysaccharide can be found on the outermost surface of a wide range of bacteria, but in its unbound form it maintains only a limited association with the surface of the bacterial cells (Whitefield and Valvano, 1993; Deng *et al.*, 2000). Moreover, certain free exopolysaccharides used to be part of the capsular PS that may themselves be released into the growth medium (i.e. become free) as consequence of their weak adhesion properties (Roberts, 1996; Dong *et al.*, 2006). The commonly described biological functions of bacterial exopolysaccharides includes; resistance to desiccation (Roberson 1992), protection against harmful substances, and acting as a permeability barrier that facilitates selective transportation of nutrients (Dong *et al.*, 2006). More significantly, exopolysaccharides play a major role in mediating the bacterial colonization of surfaces by enabling cell adhesion and co-aggregation via dipole interactions, covalent or ionic bonding, steric interactions, and hydrophobic association (Beveridge and Graham, 1991; Fletcher, 1996; Bruno and Yves, 2002; Li *et al.*, 2014; Flemming *et al.*, 2106). Of contemporary concern, membrane autopsies revealed significant fouling resistance contributed by an uneven distribution of exopolysaccharides (Cho and Fane, 2002), and this resistance was directly correlated with exopolysaccharides which undergo intermolecular or intramolecular ionic cross-linking, and subsequently clog the membrane pores and lead to further fouling (Okamura *et al.*, 2009; Lin *et al.*, 2014).

Unsurprisingly then, exopolysaccharides are as functionally and structurally diverse as the bacteria that synthesize them. Since they can be present in many forms, and seeking the chemical basis for such a chief physical distinction is of considerable importance, considerable effort has been made to catalogue the enormous structural complexity of the exopolysaccharides made possible by the wide assortment of available

monosaccharide combinations, and linkage types, and to elucidate their biosynthesis and export. Analyses of the PS structure was coined “glycomics”, which has received considerable interest in recent years (Paulson *et al.*, 2006), but its progress is far behind proteomics and genomics partly because of analytical and preparative difficulties. Since carbohydrates or PS cannot be “amplified” like proteins and nucleic acids, there is no template that encodes their sequence. Beside technical challenges associated with unique chemical properties, another significant challenge in analyzing them derives from their vast structural complexity (Bertozzi and Kiessling, 2001). Even though there are only nine monosaccharides commonly found in biological systems, with at least three different ways of forming linkages between each monomers, a short chain of four monosaccharides results in 15 million possible combinations (Bertozzi and Saisiekharan, 2009). Understandably, this complexity makes glycomics analysis very challenging.

Conversely, no systems-level analysis of a biological process is complete without interrogating the glycomics studies. Understanding how a collection of glycans is related to a particular biological event is of great significance in academic research. Hence this work accepted the challenge of trying to analyse these compounds, and shedding more light on this area.

5.2 Experimental and analytical details

5.2.1 Reagents and chemicals

All analytical grade chemicals were purchased from Sigma-Aldrich, while solvents of GC-MS grade or equivalent were purchased from Merck. Sulfuric acid was of ISO grade and purchased from Merck, while ultrapure water was obtained from a MilliQ water process (Millipore Advantage A10). A 2 mg.mL⁻¹ stock solution of glucose was prepared and stored at 4 °C before use.

5.2.2 Anthrone method

The reaction of anthrone with saccharides to form a blue-green colored complex was first reported by Dreywood (1946). Heat and a strongly acidic environment induce both hydrolysis of the glycosidic bonds of PS, and dehydration of monosaccharides to produce furfural derivatives. These furfuraldehyde compounds react with anthrone producing a coloured product, which is then measured spectrophotometrically. Since Dreywood's work a number of modifications have been reported to optimise the experimental conditions and improve agreement between the experiential evidence obtained from different carbohydrates (Morris, 1948; Loewus, 1952; Scott and Melvin, 1953; Brooks *et al.*, 1986; Raunkjaer *et al.*, 1994; Frohland *et al.*, 1996; Laurentin and Edwards, 2003; Rondel *et al.*, 2013).

Since it is photosensitive, and its absorption decreases over time, the anthrone-sulfuric acid reagent is prepared freshly on the day of analysis. One hundred micro liters of a glucose standard, or the sample, is added to separate wells of the same 96-well microplate (Corning), and then 200 μL of 0.1% or 0.2% anthrone in concentrated H_2SO_4 was added to each well. The solution was well mixed using a horizontal shaker for 1 min at room temperature, and the reaction incubated for 30 min at 80°C in an oven; the microplate was covered and protected from light during the incubation. The plate was then cooled to room temperature before the absorbance was read at 625 nm using a microplate reader (TECAN, Infinite 200 PRO).

5.2.3 Phenol-sulfuric acid method

Possibly the most reliable and well-known colorimetric assay for carbohydrates is that devised by Du Bois *et al.* (1951, 1956) utilizing phenol and H_2SO_4 . The assay involves mixing carbohydrates and phenol in water, then adding H_2SO_4 , and allowing the heat of reaction to drive the dehydration and formation of furfural derivatives that condense with phenols to form orange-yellow complexes. The original method was modified several times, and eventually adapted for 96-well microplates for greater simplicity and speed (Rao and Pattabiraman, 1989; Taylor, 1995; Masuko *et al.*, 2005).

To 50 μL of a glucose standard (0, 4, 8, 12, 16 and 20 $\mu\text{g}/\text{well}$), or the sample, in the well of a 96-well Corning microplate was added 30 μL of freshly prepared 5% or 8% phenol solution (purified by redistillation). The mixture was shaken for 10 min before 150 μL of concentrated H_2SO_4 was added rapidly; the mixture was then shaken for another 5 min and allowed to stand at room temperature for 25 min. The plate was covered and protected from light during incubation; after incubation the absorbance was read at 490nm by an Infinite 200 PRO microplate reader (TECAN) with TECAN i-control software.

Two commercially available phenol-sulfuric acid assay kits were purchased from Sigma-Aldrich (MAK104) and Abnova (KA3756). A glucose standard, or a sample, was first added to a series of wells in a 96-well microtiter plate (Corning), and the final volume of each well then adjusted to 30 μL with ultra-pure water. One hundred and fifty micro liters of concentrated H_2SO_4 was added to each well and the solution mixed for 1 min on a shaker. The plate was then covered and incubated at 90°C for 15 min in an oven protected from light; after 15 min, 30 μL of a developer was then added. The plate was shaken at room temperature for another 5 min and the $\text{OD}_{490\text{nm}}$ measured by a microplate reader (TECAN, Infinite 200 PRO).

5.2.4 Periodic Acid-Schiff (PAS) stain method

The PAS reaction estimates the carbohydrate content in glycoproteins (Schiff, 1866), and is where the carbohydrate portion of a glycoprotein is first oxidized with sodium meta-periodate to form an aldehyde, which is critical for the staining. Treatment with Schiff's reagent gives a distinct, but not necessarily intense, colour staining of a reddish purple, and a high quality reagent is essential in order not to compromise the staining abilities of the reagent (Kiernan 1999). Hence, a commercially available glycoprotein carbohydrate estimation kit was purchased (Thermo Scientific 23260).

Fifty micro liters of a standard, or the sample, was pipetted into a 96-well microplate (Corning). For the blank, 50 μL of a Glycoprotein Assay Buffer was used, and 25 μL of sodium meta-periodate solution was added to each well using a multi-channel pipette.

The plate was shaken for 1 min on a horizontal shaker, and then covered and incubated at room temperature for 10 min. One hundred and fifty micro liters of the Glycoprotein Detection Reagent was then added to each well before another 1 min of shaking; the plate was covered, protected from light and incubated at room temperature for 1 hour. The absorbance of the resulting solution was measured at 550 nm in a microplate reader (TECAN, Infinite 200 PRO).

5.2.5 Metachromatic response to different saccharides

The results of these colorimetric assays are often presented in terms of glucose-equivalent concentrations, and this may lead to misrepresentation. In this part of the work, an attempt was made to determine the variations in response for various classes of saccharides with all the different carbohydrate assays mentioned. The metachromatic response of different assays towards a range of monosaccharides (ribose, arabinose, xylose, mannose, fructose, and galactose), sugar alcohols (mannitol, sorbitol, and myo-inositol), sugar acids (galacturonic acid and glucuronic acid), deoxy sugars (fucose and rhamnose), monosaccharide derivatives (N-acetyl-D-glucosamine and N-acetylneuraminic acid), disaccharides (maltose, lactose and sucrose), PS (xanthan gum, laminarin, dextran, pullulan, phytigel, sodium alginic acid, carrageenan and fucoidan) and various proteins (lysozyme, bovine serum albumin, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum, α -acid glycoprotein) were investigated using glucose as the reference standard. All these samples were analysed at a fixed concentration of 10 μ g/well using the assays mentioned above, while a glucose standard was assayed in tandem for each batch and calibration simultaneously.

5.2.6 Colorimetric carbohydrate analysis with interfering solutes

Wastewater contains a great variety of solutes and some of them, if not all, might affect the colour development of the chromophore (Dische, 1955; Ashwell, 1957). A total of 14 compounds that had been previously identified, or known to be present in wastewater samples (Box, 1983; Raunkjaer *et al.*, 1994; Aquino *et al.*, 2006; Zhou *et al.*, 2009; Trzcinski and Stuckey, 2010a; Wu and Zhou 2010), representing different

classes of chemicals, were chosen as interfering substances. Each of them was deliberately added to known amounts (10 µg/well) of a glucose standard at 1 mol/L, and the usual colorimetric carbohydrate procedure was followed for all the assays; a blank containing only glucose (10 µg/well) was run concurrently with every batch. A new standard curve was also prepared each time the assay was run, and statistical analyses for quadruplicates were performed using the Student's *t*-test in Excel.

5.2.7 Case study

Validation of these methods for wastewater samples was performed using an added spike of glucose to test for possible interferences from solutes that absorb in the UV range. The effluent was initially collected at the outlet of a laboratory scale SAMBR (Hu and Stuckey, 2006). However, it was found that the concentration of carbohydrates fell below the limit of detection for most of these assays, so reactor supernatant (centrifuged reactor contents) was collected instead. The SAMBR, as mentioned in Chapter 3, was operated at 35±1°C and an HRT of 6, 4, and 2 h, and infinite SRT. Samples were either measured on the day of collection, or refrigerated at 4°C, and glucose at 10 µg/well was spiked into the filtered (0.45 µm) supernatant samples. The centrifugation and filtration steps prevent the analysis of internal stored or insoluble PS (such as ECPs) that are associated with the sludge, and only the freely available soluble PS in the supernatant were measured. The precision of the assays was investigated by analysing several replicates (*n* = 4) with all assays, and a glucose standard was assayed simultaneously as a reference for every batch.

5.2.8 Glycolysis protocol

The supernatant obtained from respective reactors were centrifuged at 3600 *g* at 4 °C for 15 min, and then filtered with 10 µm filter paper (Sartorius) to remove sludge flocs and obtain the supernatant, which was lyophilized. It is believed that the main components of the supernatants consist of “protein-like” compounds, carbohydrates, lipids, genetic materials, humic substances and small molecules (Aquino *et al.*, 2006; Jarusutthirak and Amy, 2006; Ni *et al.*, 2011b). In order to eliminate contaminating

proteinaceous materials and nucleic acids, treatment with proteinase K, DNase, and RNase was added prior to the extraction step (Whister and Wolfrom, 1965; Sekine *et al.*, 1985). For this purpose, proteinase K (100 µg/mL) was added to the re-solubilized supernatant and the tubes were kept at 65 °C for one hour. The mixture was subsequently treated with DNase (50 µg/mL) and RNase (50 µg/mL) in the presence of 10 mM MgCl₂ and 4 µL/mL chloroform, and incubated at 37 °C overnight. The enzymatic-digested residue was separated by centrifugation at 20,000 g at 4 °C for 40 min and delipided by successive washing with methanol, methanol:chloroform (1:1, v/v) and chloroform. The supernatant was finally dialyzed against water (molecular weight cutoff 1000 Da) for 1 day at 4 °C and lyophilized before derivatization.

The fluffy white lyophilized sample (5 mg) was dissolved in 1 mL of ultrapure water before 200 µL of 0.2 M 2-(n-morpholino)ethanesulfonic acid (MES) and 400 µL of 500 mg/mL carbodiimide reagent were added. The solution was vortexed and incubated for 3 h at 30 °C. Four milliliter of ice-cold 4 M imidazole-HCl was added, and 1 mL of freshly prepared 30 mg/mL NaBD₄ was added to the solution on ice for the first two additions at 5 min intervals. The third addition was performed after 2h. The excess reductant was destroyed by slowly adding 500 µL of glacial acetic acid until fizzing ceases. The solution was dialyzed against water overnight at 4 °C and lyophilized, and the sample can be stored frozen until monosaccharide linkage composition analysis is carried out.

The first step of glycosylic linkage analysis is methylation; each carboxyl-reduced sample was dissolved in 400 µL dimethyl sulfoxide (DMSO), and 300 µL of DMSO-NaOH suspension (120 mg/mL NaOH) was added to the sample solution. After the sample was stirred at room temperature for 10 min, 130 µL of methyl iodide was added slowly with a syringe, and the mixture then stirred vigorously for 10 min; methylation was terminated by adding 1 mL of water. The sample was extracted by adding an equal volume of DCM to the reaction mixture. The aqueous layer was removed and discarded, and the organic layer washed with 3 mL of water three times. The DCM phase was then dried under a stream of nitrogen.

Next, the acid hydrolysis step was performed; to each sample was added 1 mL of 2.0 M trifluoroacetic acid (TFA) containing 0.1 mg/mL of *myo*-inositol as an internal standard. The tubes were capped, vortexed, and incubated at 120 °C for 1 h; the sample was later dried under nitrogen at 30 °C. After TFA hydrolysis, the residue was re-suspended in 10 mg/mL solution of NaBD₄ in 2 M NH₄OH (500 µL). The sample was mixed and left at room temperature for 90 min. Excess NaBD₄ was decomposed by the addition of 250 µL acetone, and the solvent was evaporated under a flow of dry nitrogen.

The last step of the derivatization is acetylation to form alditol acetates; each sample was dissolved in 100 µL of glacial acetic acid, and 500 µL of ethyl acetate and 1.5 mL of acetic anhydride were added before mixing. Acetylation was catalyzed by the addition of 58 µL of perchloric acid (60%). After 5 min at room temperature, 5 mL of water and 100 µL 1-methylimidazole were added to each partially methylated alditol acetate (PMAA) sample, and then mixed to decompose any excess acetic anhydride. Once the tubes were cooled to room temperature, each was extracted with 2 × 1 mL DCM. The combined DCM was washed with 4 mL of water thrice, and following the last extraction, DCM was carefully transferred and injected into the GC-MS for analysis.

A QP2010ULTRA GCMS (Shimadzu) was used, with high purity helium as the carrier gas at a constant flow rate of 1 mL/min; all chromatographic separations were performed on a BPX70 column. The injector and detector temperature were maintained at 240 °C, while split injection (1/10) was used with 1 µL. A five times solvent wash was performed between samples. The oven temperature was programmed from 140 °C, held for 2 min after sample injection; increased to 170 °C at 10 °C/min, held for 2 min; and then ramped to 320 °C at 6 °C/min at which it was held for 10 min. The total run time was less than 45 min. Temperature of the MS source was maintained at 230 °C. The mass spectra were recorded in the positive-ion EI mode with an acquisition range of *m/z* 100 to 350 at 2.14 scans per 1 second, and with a solvent delay of 3 min. The chromatographic peaks were identified using the NIST11 library as previously mentioned.

5.2.9 Preparation of Standards

Seven commonly available monosaccharides (each 0.15 mol of glucose, mannose, galactose, xylose, arabinose, fucose and rhamnose) were weighed out into separate Teflon-lined screw-capped tubes. Two milliliters of 1 M methanol-HCl was added to each sample, and the tubes were capped tightly and heated for 90 min at 80 °C. The samples were cooled to room temperature followed by the addition of 200 µL of anhydrous 2-methyl-2-propanol to each tube; they were then heated at 40 °C under a stream of nitrogen to concentrate. Each sample was then dissolved in 1 mL of methanol and re-concentrated. Without pre-reduction, all the 7 methyl glycoside samples were subjected to partial methylation separately. They then underwent partial acetylation, hydrolysis, reduction, acetylation, and GC-MS analysis identical to those samples described above. Relative retention time (R_t) was assigned to these partial methylated, partially acetylated alditol acetate standards (Table 5.1). The R_t of known standards was used a guide to deduce the identity of the monosaccharide in the actual sample. The area of the peaks was used to yield a relative molar value of the individual monosaccharide portion.

Table 5.1. Relative retention time of partially methylated alditol acetate derivatives.

Deduced Linkage	Derivatives	MW	R_t	R_f
Pyranose	Rhamnose			
T-Rha(p)	1,5-di- <i>O</i> -acetyl-6-deoxy-2,3,4-tri- <i>O</i> -methyl rhamnitol	293	6.189	0.204
2-Rha(p)	1,2,5-tri- <i>O</i> -acetyl-6-deoxy-3,4-di- <i>O</i> -methyl rhamnitol	321	9.860	0.325
3-Rha(p)	1,3,5-tri- <i>O</i> -acetyl-6-deoxy-2,4-di- <i>O</i> -methyl rhamnitol	321	10.103	0.333
4-Rha(p)	1,4,5-tri- <i>O</i> -acetyl-6-deoxy-2,3-di- <i>O</i> -methyl rhamnitol	321	10.163	0.335
2,3-Rha(p)	1,2,3,5-tetra- <i>O</i> -acetyl-6-deoxy-4- <i>O</i> -methyl rhamnitol	349	13.470	0.444
2,4-Rha(p)	1,2,4,5-tetra- <i>O</i> -acetyl-6-deoxy-3- <i>O</i> -methyl rhamnitol	349	14.259	0.470
3,4-Rha(p)	1,3,4,5-tetra- <i>O</i> -acetyl-6-deoxy-2- <i>O</i> -methyl rhamnitol	349	12.772	0.421
Rhamnitol	1,2,3,4,5-penta- <i>O</i> -acetyl-6-deoxy rhamnitol	377	14.562	0.480
Pyranose	Fucose			
T-Fuc(p)	1,5-di- <i>O</i> -acetyl-6-deoxy-2,3,4-tri- <i>O</i> -methyl fucitol	293	7.645	0.252
2-Fuc(p)	1,2,5-tri- <i>O</i> -acetyl-6-deoxy-3,4-di- <i>O</i> -methyl fucitol	321	11.771	0.388
3-Fuc(p)	1,3,5-tri- <i>O</i> -acetyl-6-deoxy-2,4-di- <i>O</i> -methyl fucitol	321	10.770	0.355

4-Fuc(p)	1,4,5-tri- <i>O</i> -acetyl-6-deoxy-2,3-di- <i>O</i> -methyl fucitol	321	11.437	0.377
2,3-Fuc(p)	1,2,3,5-tetra- <i>O</i> -acetyl-6-deoxy-4- <i>O</i> -methyl fucitol	349	14.896	0.491
2,4-Fuc(p)	1,2,4,5-tetra- <i>O</i> -acetyl-6-deoxy-3- <i>O</i> -methyl fucitol	349	15.139	0.499
3,4-Fuc(p)	1,3,4,5-tetra- <i>O</i> -acetyl-6-deoxy-2- <i>O</i> -methyl fucitol	349	13.288	0.438
Fucitol	1,2,3,4,5-penta- <i>O</i> -acetyl-6-deoxy fucitol	377	15.230	0.502
Furanose Arabinose				
T-Ara(f)	1,4-di- <i>O</i> -acetyl-2,3,5-tri- <i>O</i> -methyl arabinitol	293	6.462	0.213
2-Ara(f)	1,2,4-tri- <i>O</i> -acetyl-3,5-di- <i>O</i> -methyl arabinitol	307	9.647	0.318
3-Ara(f)	1,3,4-tri- <i>O</i> -acetyl-2,5-di- <i>O</i> -methyl arabinitol	307	10.618	0.350
5-Ara(f)	1,4,5-tri- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl arabinitol	307	12.105	0.399
2,3-Ara(f)	1,2,3,5-tetra- <i>O</i> -acetyl-5- <i>O</i> -methyl arabinitol	335	13.197	0.435
2,5-Ara(f)	1,2,4,5-tetra- <i>O</i> -acetyl-3- <i>O</i> -methyl arabinitol	335	16.686	0.550
3,5-Ara(f)	1,3,4,5-tetra- <i>O</i> -acetyl-2- <i>O</i> -methyl arabinitol	335	15.927	0.525
Pyranose Arabinose				
T-Ara(p)	1,5-di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl arabinitol	293	7.827	0.258
2-Ara(p)	1,2,5-tri- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl arabinitol	307	12.408	0.409
3-Ara(p)	1,3,5-tri- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl arabinitol	307	12.166	0.401
2,3-Ara(p)	1,2,3,5-tetra- <i>O</i> -acetyl-4- <i>O</i> -methyl arabinitol	335	16.504	0.544
Arabinitol	1,2,3,4,5-penta- <i>O</i> -acetyl arabinitol	363	18.840	0.621
Pyranose Xylose				
T-Xyl(p)	1,5-di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl xylitol	279	8.040	0.265
2-Xyl(p)	1,2,5-tri- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl xylitol	307	13.076	0.431
3-Xyl(p)	1,3,5-tri- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl xylitol	307	11.984	0.395
4-Xyl(p)	1,4,5-tri- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl xylitol	307	13.076	0.431
2,3-Xyl(p)	1,2,3,5-tetra- <i>O</i> -acetyl-4- <i>O</i> -methyl xylitol	335	17.839	0.588
2,4-Xyl(p)	1,2,4,5-tetra- <i>O</i> -acetyl-3- <i>O</i> -methyl xylitol	335	17.596	0.580
3,4-Xyl(p)	1,3,4,5-tetra- <i>O</i> -acetyl-2- <i>O</i> -methyl xylitol	335	17.748	0.585
Xylitol	1,2,3,4,5-penta- <i>O</i> -acetyl xylitol	363	22.056	0.727
Pyranose Galactose				
T-Gal(p)	1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl galactitol	323	11.225	0.370
2-Gal(p)	1,2,5-tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methyl galactitol	351	15.776	0.520
3-Gal(p)	1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methyl galactitol	351	14.987	0.494
4-Gal(p)	1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl galactitol	351	16.018	0.528
6-Gal(p)	1,5,6-tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl galactitol	351	18.263	0.602
2,3-Gal(p)	1,2,3,5-tetra- <i>O</i> -acetyl-4,6-di- <i>O</i> -methyl galactitol	379	18.688	0.616
2,4-Gal(p)	1,2,4,5-tetra- <i>O</i> -acetyl-3,6-di- <i>O</i> -methyl galactitol	379	20.175	0.665
2,6-Gal(p)	1,2,5,6-tetra- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl galactitol	379	23.573	0.777
3,4-Gal(p)	1,3,4,5-tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methyl galactitol	379	18.567	0.612

3,6-Gal(p)	1,3,5,6-tetra- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl galactitol	379	22.693	0.748
4,6-Gal(p)	1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl galactitol	379	22.389	0.738
2,3,4-Gal(p)	1,2,3,4,5-penta- <i>O</i> -acetyl-6- <i>O</i> -methyl galactitol	407	20.933	0.690
2,3,6-Gal(p)	1,2,3,5,6-penta- <i>O</i> -acetyl-4- <i>O</i> -methyl galactitol	407	27.092	0.893
2,4,6-Gal(p)	1,2,4,5,6-penta- <i>O</i> -acetyl-3- <i>O</i> -methyl galactitol	407	27.122	0.894
3,4,6-Gal(p)	1,3,4,5,6-penta- <i>O</i> -acetyl-2- <i>O</i> -methyl galactitol	407	24.513	0.808
Galactitol	1,2,3,4,5,6-hexa- <i>O</i> -acetyl galactitol	435	27.274	0.899

Pyranose

Glucose

T-Glc(p)	1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl glucitol	323	10.103	0.333
2-Glc(p)	1,2,5-tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methyl glucitol	351	14.471	0.477
3-Glc(p)	1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methyl glucitol	351	14.138	0.466
4-Glc(p)	1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl glucitol	351	16.534	0.545
6-Glc(p)	1,5,6-tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl glucitol	351	16.018	0.528
2,3-Glc(p)	1,2,3,5-tetra- <i>O</i> -acetyl-4,6-di- <i>O</i> -methyl glucitol	379	19.568	0.645
2,4-Glc(p)	1,2,4,5-tetra- <i>O</i> -acetyl-3,6-di- <i>O</i> -methyl glucitol	379	20.205	0.666
2,6-Glc(p)	1,2,5,6-tetra- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl glucitol	379	21.995	0.725
3,4-Glc(p)	1,3,4,5-tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methyl glucitol	379	19.204	0.633
3,6-Glc(p)	1,3,5,6-tetra- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl glucitol	379	21.176	0.698
4,6-Glc(p)	1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl glucitol	379	21.904	0.722
2,3,4-Glc(p)	1,2,3,4,5-penta- <i>O</i> -acetyl-6- <i>O</i> -methyl glucitol	407	22.389	0.738
2,3,6-Glc(p)	1,2,3,5,6-penta- <i>O</i> -acetyl-4- <i>O</i> -methyl glucitol	407	27.486	0.906
2,4,6-Glc(p)	1,2,4,5,6-penta- <i>O</i> -acetyl-3- <i>O</i> -methyl glucitol	407	26.151	0.862
3,4,6-Glc(p)	1,3,4,5,6-penta- <i>O</i> -acetyl-2- <i>O</i> -methyl glucitol	407	25.029	0.825
Glucitol	1,2,3,4,5,6-hexa- <i>O</i> -acetyl glucitol	435	28.639	0.944

Pyranose

Mannose

T-Man(p)	1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methyl mannitol	323	9.951	0.328
2-Man(p)	1,2,5-tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methyl mannitol	351	14.320	0.472
3-Man(p)	1,3,5-tri- <i>O</i> -acetyl-2,4,6-tri- <i>O</i> -methyl mannitol	351	14.259	0.470
4-Man(p)	1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methyl mannitol	351	15.048	0.496
6-Man(p)	1,5,6-tri- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methyl mannitol	351	15.927	0.525
2,3-Man(p)	1,2,3,5-tetra- <i>O</i> -acetyl-4,6-di- <i>O</i> -methyl mannitol	379	17.717	0.584
2,4-Man(p)	1,2,4,5-tetra- <i>O</i> -acetyl-3,6-di- <i>O</i> -methyl mannitol	379	19.720	0.650
2,6-Man(p)	1,2,5,6-tetra- <i>O</i> -acetyl-3,4-di- <i>O</i> -methyl mannitol	379	21.752	0.717
3,4-Man(p)	1,3,4,5-tetra- <i>O</i> -acetyl-2,6-di- <i>O</i> -methyl mannitol	379	17.960	0.592
3,6-Man(p)	1,3,5,6-tetra- <i>O</i> -acetyl-2,4-di- <i>O</i> -methyl mannitol	379	21.388	0.705
4,6-Man(p)	1,4,5,6-tetra- <i>O</i> -acetyl-2,3-di- <i>O</i> -methyl mannitol	379	20.508	0.676
2,3,4-Man(p)	1,2,3,4,5-penta- <i>O</i> -acetyl-6- <i>O</i> -methyl mannitol	407	20.023	0.660
2,3,6-Man(p)	1,2,3,5,6-penta- <i>O</i> -acetyl-4- <i>O</i> -methyl mannitol	407	25.818	0.851
2,4,6-Man(p)	1,2,4,5,6-penta- <i>O</i> -acetyl-3- <i>O</i> -methyl mannitol	407	25.848	0.852
3,4,6-Man(p)	1,3,4,5,6-penta- <i>O</i> -acetyl-2- <i>O</i> -methyl mannitol	407	23.512	0.775

Mannitol	1,2,3,4,5,6-hexa- <i>O</i> -acetyl mannitol	435	26.151	0.862
----------	---	-----	--------	-------

INT-STD *myo*-inositol

<i>myo</i> -inositol	<i>myo</i> -inositol hexaacetate	432	30.338	1.000
----------------------	----------------------------------	-----	--------	-------

Deduced linkages based on *O*-acetyl and *O*-methyl position, molecular weight (MW) and a retention time (R_f) relative to *myo*-inositol hexacetate as internal standard (INS-STD, R_f) under the conditions described in the text.

5.3 Results and discussion

5.3.1 Performance of individual assays

Commercial reagents were purchased in an attempt to compare them with the laboratory preparations; the performance of individual assays were first validated according to the respective procedures mentioned above, and are detailed in Table 5.2.

Table 5.2. Performance of various carbohydrate assays

Assays	Coefficients of multiple correlation (R^2)	Linearity range (ug/well)	Variation (% CV)		
			Inter-plate	Intra-plate	Day-to-day
Anthrone (0.1%)	0.9977	4-90	4.03	3.18	6.63
Anthrone (0.2%)	0.9935	4-60	2.12	3.02	7.09
Phenol-sulfuric acid (5%)	0.9982	4-50	1.12	1.78	5.35
Phenol-sulfuric acid (8%)	0.9959	4-40	2.64	1.61	5.89
Sigma-Aldrich assay kit	0.9942	4-35	4.78	2.88	3.22
Abnova assay kit	0.9935	4-35	2.93	2.22	3.51
Glycoprotein assay kit	0.9972	4-30	3.83	2.06	4.08

Typically, data from concentration series were compiled from 4 independent experiments, each performed 4 times per plate. Each plate contained glucose concentrations of 0, 4, 8, 12, 16, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 $\mu\text{g}/\text{well}$, allowing for a calibration curve to be determined, and points that displayed signal saturation were excluded from the curve fitting procedure. Good linearity was generally observed for all the assays since the coefficients of multiple correlation, R^2 , were greater than 0.99. The linearity of the 5% phenol-sulfuric acid was the highest ($R^2 = 0.9982$) among them, although the linearity range reached its limit at 50 $\mu\text{g}/\text{well}$. A calibration curve up to 90 $\mu\text{g}/\text{well}$ was only achieved for the 0.1% Anthrone, the highest among all the assays. It is important to note that samples with a higher

concentration than the linearity range usually require dilution, which can introduce errors; hence a compromise in accuracy has to be made.

The sources of variation associated with glucose concentration measurements were analysed and shown in Table 5.2. The % CV value represents the average coefficient of variance generated using assays for glucose at a concentration of 10 µg/well. Inter-plate variance is the error associated with the average value from 4 separate microtiter plates; while intra-plate variance is the error obtained from quadruplicate separate reactions in a single plate. The variation of inter-plate was typically 1-4% CV, indicating a high reproducibility (repeat assays were performed using the same operator on a single day). The % CV of intra-plate measurements was observed to be below 3.5; this means that there was low human error in liquid handling, specific errors related to the plate (such as an "edge effect"), and a detection error attributed to the microplate reader. Assay robustness was also examined by comparing the variation in glucose quantitation when the individual assay was repeated over an extended time-scale. Day-to-day variation was generated from 4 glucose samples having the same concentration (10 µg/well), each repeated on 4 different days performed by the same operator. Table 5.2 also suggests that when the amount of glucose was fixed, day-to-day variations could possibly introduce errors since the variation was typically 3-8%, with the greater variation displayed by the assays that were freshly prepared in the laboratory. This suggests that artifacts such as fluctuations in the reagent makeup play an importance role in quantifying carbohydrates when using the selected assays. On the other hand, the use of a commercial reagent probably results in an improvement in the long-term repeatability and performance of the assay.

5.3.2 Metachromatic response to different saccharides

Bacteria contain a great variety of heteropolysaccharides and produce diverse PS extracellularly, and some of these PS consist of many different types of monosaccharides which can be present in solution in very small amounts. Hence biologically treated wastewater may contain diverse classes of carbohydrates. Since the

word *carbohydrate* applies to a class of organic compounds, monomeric, oligomeric and polymeric in nature, which do not necessarily have the general formula of glucose, $C_6H_{12}O_6$, and their oxygen and hydrogen atoms in the molecular ratio of 1:2, the specificity of using glucose as a reference standard to quantify the total sugar content is debatable. Another objective of this work was to determine the variation in response for a range of carbohydrates with all these commonly used assays.

The metachromatic response of 7 assays with a large number of carbohydrates including monosaccharides, sugar alcohols, sugar acids, deoxy sugars, monosaccharide derivatives, disaccharides, PS and glycoproteins was investigated when glucose was used as the reference carbohydrate. All the samples were analysed several times ($n = 4$) at a fixed concentration of 10 $\mu\text{g}/\text{well}$, which is within the linearity range of each assay. Variations in the signal observed (as glucose equivalents) for each assay are summarised in Table 5.3.

It is well accepted that saccharides with free aldehyde or keto groups can undergo dehydration to form furfural and its derivatives on heating with concentrated sulfuric acid (Dische, 1955; Du Bois *et al.*, 1956; Ashwell, 1957). Any saccharides (sugar alcohols, sugar acids, monosaccharide derivatives) that lack this property will show a large deviation from its original concentration of 10 μg (Table 5.3). The above observation is consistent with the fact that any saccharide (such as maltose, lactose, sucrose, laminarin, and dextran) that contains a high proportion of glucose units will give a reading closer to 10 μg . Noticeable differences in readings among neutral PS were also observed; laminarin and dextran, which consist solely of glucose, gave higher readings than those of xanthan gum, which consists of a mixture of glucose, mannose and glucuronic acid in a 2:2:1 ratio. This suggests that the lack of reaction or slower rate of conversion to form furfural derivatives is reflected when the carbohydrate is not comprised solely of glucose. Readings amongst different classes of commercially available bacterial exopolysaccharides such as neutral (xanthan gum, laminarin, dextran, pullulan), anionic (phytagel, sodium alginic acid) and sulfated ones (κ -carrageenan, ι -carrageenan fucoidan) were also not the same. Thus assays that use a glucose solution

as a calibration standard may not be directly applicable for different classes of carbohydrates.

Table 5.3. Effect of variations in different carbohydrates on metachromatic response.

Carbohydrate sample		Glucose-equivalent concentration (µg/well)						Glycoprotein kit
		(% CV)						
Reference standard	D-(+)-Glucose	10.1 (2.0)	10.3 (2.0)	10.3 (1.1)	10.2 (2.7)	9.7 (1.6)	10.4 (1.3)	10.1 (0.6)
Monosaccharide	D-(-)-Ribose	0.9 (4.4)	9.9 (1.8)	22.6 (0.5)	17.6 (0.4)	4.6 (2.2)	3.3 (2.2)	13.4 (1.6)
	D-(-)-Arabinose	0.6 (4.3)	1.1 (4.2)	12.4 (0.9)	0.7 (4.3)	4.2 (2.7)	4.3 (1.9)	- (-)
	L-(+)-Arabinose	2.1 (3.8)	0.7 (4.4)	11.4 (0.8)	0.1 (4.2)	4.0 (2.5)	4.2 (1.9)	- (-)
	D-(+)-Xylose	2.3 (3.9)	1.6 (5.2)	20.8 (0.5)	13.8 (0.6)	8.1 (1.5)	8.2 (1.0)	15.2 (2.7)
	D-(+)-Mannose	7.8 (2.1)	6.1 (2.4)	18.0 (0.5)	13.7 (0.7)	4.5 (2.5)	3.7 (2.1)	11.2 (1.1)
	D-(-)-Fructose	12.5 (2.1)	13.7 (2.0)	12.2 (1.2)	11.8 (1.1)	7.4 (1.9)	7.0 (1.3)	2.0 (0.7)
	D-(+)-Galactose	5.6 (2.8)	6.3 (2.3)	7.5 (1.0)	0.5 (4.1)	5.5 (2.5)	4.7 (2.1)	8.7 (2.1)
	D-Mannitol	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	- (-)
Sugar alcohol	D-Sorbitol	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	- (-)
	Myo-inositol	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.4 (2.3)
Sugar acid	D-(+)-Galacturonic acid	0.9 (5.6)	1.1 (5.7)	4.1 (1.7)	0.2 (3.7)	2.3 (3.4)	1.7 (2.6)	0.6 (2.1)
	D-Glucuronic acid	2.1 (4.0)	0.4 (5.9)	2.3 (2.1)	2.4 (2.1)	0.7 (5.5)	0.6 (3.9)	0.4 (2.7)
Deoxy sugar	L-(-)-Fucose	10.8 (1.4)	8.9 (1.5)	8.3 (1.3)	0.8 (3.5)	1.9 (3.8)	2.5 (2.9)	2.0 (0.6)
	L-(+)-Rhamnose	12.1 (2.1)	7.9 (2.4)	15.4 (0.6)	10.4 (0.8)	2.1 (4.1)	1.5 (3.1)	2.1 (1.0)
Monosaccharide derivatives	N-Acetyl-D-glucosamine	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	9.4 (1.2)	4.1 (0.7)
	N-Acetylneuraminic acid	4.1 (2.1)	0 (0)	0 (0)	0 (0)	0 (0)	2.5 (2.9)	13.8 (0.6)
Disaccharide	D-(+)-Maltose	9.5 (1.5)	11.4 (1.6)	6.6 (1.5)	2.9 (1.7)	9.7 (1.6)	9.4 (1.2)	0.9 (1.4)
	α-Lactose	7.6 (2.4)	7.6 (2.4)	11.0 (0.6)	4.8 (1.2)	8.0 (1.8)	7.2 (1.3)	0.9 (1.2)
	Sucrose	10.6 (1.8)	12.3 (1.6)	14.5 (0.8)	10.0 (0.8)	11.0 (1.4)	9.1 (1.1)	0.5 (2.2)
Polysaccharide	Xanthan Gum	1.1 (3.0)	2.2 (2.6)	2.4 (2.4)	0.1 (4.3)	2.7 (2.8)	4.2 (1.6)	0.3 (0.6)
	Laminarin	8.7 (1.9)	9.9 (1.8)	6.9 (1.0)	9.8 (1.0)	8.1 (1.7)	7.0 (1.3)	0.5 (2.8)
	Dextran	7.7 (1.7)	8.8 (1.8)	9.4 (0.8)	6.1 (1.0)	9.4 (1.5)	8.3 (1.2)	0.1 (3.1)
	Pullulan	7.5 (1.7)	9.4 (1.8)	8.2 (1.1)	0.9 (2.8)	9.4 (1.5)	8.2 (1.2)	0.2 (2.8)
	Phytigel	5.0 (1.7)	5.0 (1.9)	7.0 (1.3)	1.2 (2.8)	4.5 (2.2)	3.8 (1.6)	0.1 (3.1)
	Sodium alginic acid	0 (0)	0.7 (3.9)	2.9 (1.8)	1.1 (3.1)	0.1 (5.6)	0 (0)	0.1 (3.3)
	κ-Carrageenan	4.1 (2.5)	5.3 (2.7)	5.4 (1.5)	3.9 (1.6)	2.9 (2.6)	1.9 (2.3)	0.1 (3.3)
	ι-Carrageenan	0.4 (4.4)	1.4 (3.7)	2.0 (2.6)	0 (0)	2.7 (2.8)	1.8 (2.3)	0.1 (3.2)
	Fucoidan	4.7 (3.0)	3.8 (3.3)	3.8 (2.1)	3.2 (2.5)	0.4 (4.7)	0 (0)	0.1 (3.6)

In the case of PAS, the reagent will oxidise carbohydrates to form aldehydes exclusively, and if there is an insufficient concentration of aldehyde, a lack of colour development is obvious, as shown in Table 5.3. Since the most likely application of the PAS assay is for the quantification of carbohydrates in glycoproteins found in bacterial cell walls, which are conjugate PS (oligomers or polymers of monosaccharides linked to a peptide), the degree of glycosylation was found to be directly proportional to the absorbance reading in which non-glycosylated glycoproteins, such as lysozyme and BSA, produce a lower value (Table 5.4). Inferior performance was observed for assays using anthrone, while inconsistent results were observed for other assays. The PAS assay, although generating results in good agreement with the carbohydrate content, still results in a large difference with the original concentration. Hence, no single colorimetric assay is absolutely specific, and total sugars are inaccurately determined when a single sugar - glucose - was used as the reference standard representing many other individual carbohydrates in complex mixtures such as wastewater.

Table 5.4. Effect of degree of glycosylation on metachromatic response

Protein/Glycoprotein	Carbohydrate content (%)	Glucose-equivalent concentration (µg/well)						
		Anthrone (0.1%)	Anthrone (0.2%)	Phenol (5%)	Phenol (8%)	S-A kit	Abnova kit	Glycoprotein kit
Lysozyme	-	0	0.2	0.2	0.6	0.5	1.4	0.1
		(0)	(2.6)	(4.1)	(3.5)	(4.1)	(3.9)	(2.9)
Bovine serum albumin	Trace	0	0	1.2	0.1	0	0	0.1
		(0)	(0)	(3.8)	(5.2)	(0)	(0)	(2.9)
Ovalbumin	3.2	0	0.2	0.4	0.2	0.6	1.2	0.3
		(0)	(3.1)	(3.7)	(3.9)	(4.0)	(3.8)	(2.8)
Apo-transferrin	5.8	0	0	0.1	0.2	1.3	1.5	0.3
		(0)	(0)	(3.4)	(3.9)	(3.5)	(3.4)	(2.6)
Fetuin	22.9	0	0	0.4	0.4	0.8	1.0	0.9
		(0)	(0)	(3.6)	(3.7)	(3.4)	(3.2)	(2.1)
α-acid glycoprotein	41.4	0	0.2	1.1	1.1	1.3	1.2	1.5
		(0)	(2.7)	(3.0)	(2.7)	(3.5)	(3.1)	(1.9)
D-(+)-Glucose	100	10.1 (2.0)	10.3 (2.0)	10.3 (1.1)	10.2 (2.7)	9.7 (1.6)	10.4 (1.3)	10.1 (0.6)

5.3.3 Colorimetric carbohydrate analysis with interfering solutes

Besides the problem of non-specificity, certain solutes found in wastewater samples can strongly interfere with the quantitative determination of carbohydrates. Some solutes react with the strong acid alone, but not with the developer, whereas other impurities react with the developer but not the acid. A few solutes even produce coloured compounds with a marked absorption in the UV region. Since the risk of interference

must be considered with typical carbohydrates analyses, 14 different chemicals were selected to represent those commonly found in wastewater samples, and were deliberately added at a concentration of 1 mmol.L⁻¹ to glucose standards as interfering reagents; these result are shown in Table 5.5.

Table 5.5. Solutes that can interfere with various carbohydrate assays

Interfering solutes (1mmol/dm ³)	Anthrone (0.1%)	Anthrone (0.2%)	Phenol (5%)	Phenol (8%)	S-A kit	Abnova kit	Glycoprotein kit
Hexadecane	-	○	-	●	○	○	●
Octadecene	○	○	-	●	○	○	●
n-Hexadecanol	○	-	○	●	●	○	●
Sorbitol	●	●	-	●	○	○	●
Urea	-	○	-	●	○	●	●
Uric acid	-	-	-	○	●	●	●
Butyric acid	●	-	-	●	●	●	○
Hexanoic acid	-	-	-	●	●	●	○
Palmitic acid	-	-	○	○	○	○	●
Ascorbic Acid	○	-	-	●	●	●	○
Humic acid	●	○	●	○	●	●	●
Squalane	○	-	-	○	●	●	●
Dibutyl phthalate	○	-	-	●	●	●	●
2,6-Di-tert-butylphenol	-	-	-	●	●	○	●
All	●	●	●	●	●	●	●

●: there is a statistically highly significant difference in measurement between standard w/o interfering solutes ($p \leq 0.001$)
 ○: there is a statistically significant difference in measurement between standard w/o interfering solutes ($p \leq 0.05$)
 -: there is no statistically significant difference in measurement between standard w/o interfering solutes

Many colorimetric carbohydrate analysis methods have been proposed in an effort to; decrease the assay time, provide a linear standard curve, and produce more stable reagents. However, none can effectively remove the interfering properties of certain compounds. As can be seen in Table 5.5, at the 95% confidence level ($n = 4$, $p = 0.05$) many substances interfere with carbohydrate assays and give false readings. In general, the findings confirm that the interfering properties of many compounds in a 5% phenol-sulfuric acid assay are slight. However, the 8% phenol-sulfuric acid procedure, and three commercial assays, appear to be sensitive to many (at 99.9% confidence level), if not all (at 95% confidence level) compounds giving erroneous values. A few compounds such as uric acid, short chain fatty acids and 2,6-di-tert-butylphenol do not interfere with the anthrone methods. Of particular note is the presence of humic acid,

which is commonly present in wastewater samples, is particularly troublesome when attempting to quantify glucose content using colorimetric assays.

Although the actual mechanism of interference is unknown, Table 5.5 indicates that the presence of these solutes, even at low concentrations, is likely to compromise the accuracy of carbohydrate determinations. Unless quantitative carbohydrate determinations are carried out under rigorously controlled conditions where the possibility of interfering substances has been carefully eliminated, the assays will not produce reasonably accurate results; needless to say, pretreatment to remove all interfering solutes is infeasible and impractical for samples obtained from wastewater treatment systems.

5.3.4 Case study

The quantification of carbohydrates in a complex medium was validated by assaying various actual wastewater samples; reactor supernatants at 3 different HRTs were characterised by measuring their COD and carbohydrate content. Table 5.6 shows the results from all the different assay methods.

Table 5.6. Average reading in the supernatant from each sample at various HRTs

HRT (h)	COD (mg/L)	Glucose-equivalent concentration ($\mu\text{g}/\text{well}$) $\pm$ SD						
		Anthrone (0.1%)	Anthrone (0.2%)	Phenol (5%)	Phenol (8%)	S-A kit	Abnova kit	Glycoprotein kit
6	60.36	1.5 ± 0.4	0.7 ± 0.1	0.9 ± 0.4	1.4 ± 0.4	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
4	73.12	1.8 ± 0.1	0.8 ± 0.2	0.9 ± 0.6	1.3 ± 0.4	0.8 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
2	91.49	2.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
6 (SA*)	-	10.2 ± 1.3	9.4 ± 0.5	9.7 ± 0.1	4.3 ± 1.3	9.9 ± 0.3	10.3 ± 0.3	16.4 ± 0.1
4 (SA*)	-	10.5 ± 0.1	10.3 ± 0.6	10.5 ± 1.1	4.1 ± 1.4	10.1 ± 0.2	12.1 ± 0.2	16.3 ± 0.1
2 (SA*)	-	10.9 ± 2.4	10.6 ± 0.4	10.1 ± 2.6	6.1 ± 1.7	10.2 ± 0.7	11.6 ± 0.9	17.8 ± 0.7

*: Standard Addition of glucose at 10 $\mu\text{g}/\text{well}$.

As a controllable reactor parameter, HRT is important and controls treatment performance and biomass characteristics, which in turn affect membrane fouling in the SAMBR. From Table 5.6, it is obvious that, as expected, the supernatant COD increases with decreasing HRT. However, no significant differences were observed with all the assays in terms of carbohydrate concentration when the HRT changes,

except with the anthrone methods, which show a slight increase in concentration. It is probable that a reduced HRT resulted in an increased release of EPS, which in turn was responsible for the rise in SMPs leading to membrane fouling (Meng *et al.*, 2007). In some cases, using the Du Bois phenol-sulfuric acid method (Du Bois *et al.*, 1956) and glucose as the standard reference, Huang *et al.* (2011) found similar trends to this in that the carbohydrate concentration was not affected by a change in HRT. Fundamentally, however, these seven assay methods gave widely varying readings; at 6 h HRT, the carbohydrate concentration varied by a factor of 4 (0.4 - 1.5), while this ratio was even greater at 7 (0.3 - 2.1) when the HRT was 2 h. In addition, there was little or no agreement between them in terms of absolute concentrations, which echoes the difference in their metachromatic response towards different carbohydrates. It is known that prokaryotic exopolysaccharides are a major fraction of the EPS matrix (Frøhlund *et al.*, 1996), and some are homopolysaccharides such as sucrose-derived glucans, whereas most bacterial exopolysaccharides are heteropolysaccharides that consist of a variety of sugar residues (Sutherland, 2007). Owing to the heterogeneous structure and composition of various kinds of PS, a diverse response to these analytical assays was observed, which is shown in the previous section.

Finally, the values shown in Table 5.6 for carbohydrate content after a glucose spike of 10 µg/well are generally different from expected. This clearly shows that all the samples must contain solutes, and this was also reported in Chapter 3, and that they interfere with the carbohydrate determination to varying degrees. Moreover, the use of glucose as a reference standard could possibly result in misleading concentrations for all the assays used. Careful interpretation of these results is required, and the unit should be analogized as glucose equivalents whenever glucose was selected as the reference standard to assess the concentration of carbohydrate. These methods, although useful for determining glucose content, are completely non-specific and rarely appropriate for the investigation of complex wastewater samples.

5.3.5 Exopolysaccharide versus Exopolymeric substances

The status of cell-bound PS is often unclear in the literature, and sometimes they are called; extracellular polymeric substances (EPS), exopolymeric substances (EPS), exopolysaccharides (EPS), extracellular polysaccharides (EPS or ECPS), extracellular proteoglycan (EPG), released polysaccharide (RPS), capsular polysaccharides (CPS), transparent exopolymer particles (TEP) or polysaccharides (PS). Although the abbreviation of “EPS” is the same as exopolysaccharides or extracellular polysaccharides, extracellular polymeric substances and exopolymeric substances include any substance of microbial origin, and have been shown to be a rich matrix of polymers, including PS, protein, glycoproteins, nucleic acid, phospholipids, and humic substances (Geesey, 1982). The lack of a strict definition in the use of these terms, and their interchangeability in the published paper makes their use confusing.

On the other hand, cell-bound exopolysaccharides are defined as two groups of exopolysaccharides: tightly bound exopolysaccharides and free exopolysaccharides (Whitefield and Valvano 1993; Deng *et al.*, 2000). The free exopolysaccharides can be found on the outermost surface of a wide range of bacteria, but in its unbound form it only maintains a limited association with the surface of bacterial cells (Whitefield and Valvano 1993; Deng *et al.*, 2000). Moreover, certain free exopolysaccharides used to be part of the CPS that may themselves be released into the growth medium (i.e. become free) as consequence of their weak adhesion properties (Roberts, 1996; Dong *et al.*, 2006). CPS in this case can be used interchangeably as tightly bound exopolysaccharides, and associated with the cell surface.

In the many previous studies, measurement of PS was used to represent the amount of total cell-bound PS, but was actually free exopolysaccharides. In fact, little information is available that distinguishes between the proportions of the two types of EPS, and their possibly different effects on the properties of a bacterial surface. Exopolysaccharides are believed to be essential to the formation of sludge floc, but excessive exopolysaccharides in the form of free exopolysaccharides may reduce cell

attachment and weaken the floc structure, which results in poor-sludge-water separation (Li and Yang, 2007). Therefore, based on the results obtained in this study, and the information discussed earlier, it is understandable that if any carbohydrates were measured with the assays tested above it is very likely to be misleading in published papers, and the data lacks credibility.

5.3.6 Glycolysis

Numerous techniques have been developed for interrogating the glycome at various levels (Shriver *et al.*, 2004; Raman *et al.*, 2005; Novotny, *et al.*, 2008; Ruhaak *et al.*, 2010), although no single technique can define all aspects of the glycome (Pilobello and Mahal, 2007). In terms of environmental studies, advanced spectroscopic analyses such as matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS), as well as 1D and 2D nuclear magnetic resonance (NMR) spectroscopy have been applied to reveal important structural features of the exopolysaccharides (EPS) in wastewater treatment systems such as aerobic granular sludge (Lin *et al.*, 2010; Seviour *et al.*, 2010), anaerobic granular sludge (Gonzalez-Gil *et al.*, 2015), and membrane bioreactors (MBRs) (Kimura *et al.*, 2012). However, MALDI-TOF/MS-based and NMR spectroscopy are expensive both in terms of investment, and running costs. High resolution GC-MS is perhaps one of the more popular tools (Zaia, 2004) for analysing a wide range of compounds, and a de facto primary technique for characterizing the structures of individual glycans (Dell and Morris, 2001) when only small quantities are available, as is the case in environmental samples. In addition, another great advantage of GC-MS glycan profiling is that multiple glycans of any given subtype can be characterized at once, increasing their throughput. Surprisingly, although this technique is comparatively powerful and relatively economical, it has not been applied to analyze free EPS.

It is known that several bacterial EPS are homopolysaccharides, but most are heteropolysaccharides that consist of a mixture of neutral and charged sugar residues (Sutherland, 2007). There seems to be general agreement that the exopolysaccharides

found in wastewater treatment systems contain acidic moieties (Lin *et al.*, 2010; Seviour *et al.*, 2010; Seviour *et al.*, 2012; Gonzalez-Gil *et al.*, 2015). We conclude this chapter by providing a detailed protocol for an analytical procedure that can be used routinely in a reasonably equipped laboratory to simultaneously determine both the neutral and acidic monosaccharides, how each residue is linked, and the possible PS structures in the free EPS produced from a mixed culture during AD.

Figure 5.1: An integrated workflow for the MS-based glycomics analysis employed in this study **(A)**: The specific strategies employed in every stage of sample preparation and derivatization. **(B)**: Methods for the conversion of uronic acids containing PS to their corresponding PMAAs. Also shown in red is the simultaneous labelling of the reduced uronic acid with deuterium. The hydroxyl groups not involved in the glycosyl-linkage are methylated with blue. After TFA hydrolysis, the partially methylated saccharides are released and reduced with NaBD₄, which concurrently opens the cyclic ring to form the alditol and tags the C1 atom with a red deuterium atom. To increase the

volatility of the derivatives, C atoms involved in the glycosidic linkage and the ring are acetylated (purple in colour) with acetic anhydride.

The general workflow in a typical MS-based glycomics analysis may be viewed as comprising three stages, from sample preparation to data acquisition and then data analysis, preceded first by strategic planning. The strategic planning involves a thoughtful consideration of the sample sources and therefore what kind of glycans are to be expected, since these will ultimately affect the sample preparation steps. The strategy described in this chapter can be found in Figure 5.1.

Although uronic acids can be detected by colorimetric methods such as the carbazole method (Galambos, 1967), or the hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973; Filisetti-Cozzi and Carpita, 1991), they often give a global estimate and do not differentiate between individual moieties, as mentioned early in the chapter. The acidic moieties, on the other hand, are not detectable through acetylation and require pre-reduction to their neutral monosaccharide counterparts (Bemiller, 1967). Reduction of the carboxyl group usually requires activation and subsequent reduction. The free acidic group has to be activated first by carbodiimide and reduced with sodium borodeuteride (NaBD_4) to generate 6,6'-dideuterio-saccharides (Taylor and Conrad, 1972), which is well suited to distinguish it from neutral glycans using GC-MS by the presence of fragment ions with increased mass ($M^+ + 2$). Besides, providing a link between mass and composition, other advantages of using GC-MS for PS compositional analysis are its robustness and high resolution. Since various derivatization reagents have been reported in the literature (Ruiz-Matute *et al.*, 2011), linkage analysis is also possible if the glycan is derivatized by pre-*O*-methylation before hydrolysis.

Methylation is an important tool for the elucidation of PS structure (Jay, 1996). In our procedure, all the free hydroxyl groups of the glycans are methylated and, following TFA hydrolysis, the partially methylated saccharides released are further reduced and acetylated to yield PMAAs, which can be easily separated, identified and quantified by GC-MS. There are many ways to perform methylation with methyl iodide (Hakomori,

1964; Ciucanu, 2006); we used a revised Ciucanu and Kerek (Ciucanu and Kerek, 1984; Ciucanu *et al.*, 2003; Ciucanu and Caprita, 2007) method as it generates cleaner chromatograms, and does not require the bulk generation of unstable and potentially explosive methylsulfinyl carbanion. NaOH-DMSO slurry is a powerful base and must be prepared freshly, and will deprotonate the high- pK_a hydroxyl groups on the monosaccharide residues. Once ionized, the saccharides are treated with CH_3I , which results in the complete methylation of the hydroxyl groups not involved in the glycoside linkage. Notwithstanding, methylation analysis is more qualitative than quantitative since it is difficult to obtain standards for each individual monosaccharide derivative. Nonetheless, standards are extremely important and provide both retention time and mass spectral data that are necessary for derivative identification. For more complex and unknown samples, such as in the case of environmental analysis, it is advised to generate a series of partially methylated, partially acetylated monosaccharide standards from their methyl-glycosides.

Both quantitative and qualitative analysis of monosaccharide composition are possible by derivatization to alditol acetates after pre-*O*-methylation. The partially methylated saccharides will first be subject to acid hydrolysis, which is crucial and can vary depending on the nature of the sample and its component glycans. Two most common reagents for hydrolysis are TFA and sulfuric acid. Sulfuric acid is a harsher acid and better suited than TFA for complete hydrolysis. However, the presence of sulfuric acid is troublesome since the entire mixture is used in the subsequent steps. It can be removed, for example, by precipitation with barium hydroxide, but it is not practical when the sample is in micrograms. TFA, on the other hand, is volatile and therefore can be easily removed by evaporation. After acid hydrolysis, the monosaccharide residues are released, but they need to be reduced with either $NaBH_4$ (Abdek-Akher *et al.*, 1951) or $NaBD_4$ before acetylation. We chose $NaBD_4$ because it simultaneously opens the cyclic ring to form the alditol, and tags the anomeric carbon (C1) atom with another deuterium isotope. Glycomic analysis using isotopic labeling highlights the potential advantages of incorporating the mass labels into samples during derivatization, and

relating spectral intensities to the relative quantities of the carbohydrates under investigation.

In the next step, acetylation not only increase the volatility of the derivatives for GC separation, the C atoms that were involved in the glycosidic linkage and the ring also carry the acetyl group (Figure 6.1). There are a variety of acetylation methods (Ruiz-Matute *et al.*, 2011); the simplest is with addition of acetic anhydride and catalysis by either 1-methylimidazole (Blakeney *et al.*, 1983) or 60% perchloric acid (Fritz and Schenk, 1959). Typically, *myo*-inositol is used as the internal standard, and the acetates can be identified by their retention time relative to the internal standard. Finally, the PMAA derivatives can be injected into a GC-MS system directly.

5.3.6.1 Identification of monomeric structure

Most microbial exopolysaccharides are highly soluble and are attached to the microbial cells through covalent bonds to other surface polymers (Sutherland, 2007). On the other hand, some are detached from cell walls or form very rigid gels (Meng *et al.*, 2017). Structurally, these exopolysaccharides usually contain a backbone in which there is predominance of either 1,3 or 1,4 linkages (Sutherland, 2007). The monosaccharide linkage associated to the possible free exopolysaccharides present in the anaerobic bioreactors is shown in Table 5.7.

Table 5.7. Monosaccharide linkage composition presented in percentage mole

Monosaccharide
(Mol % $\pm$ SD)	23.3 $\pm$ 0.3%	22.0 $\pm$ 0.6%	18.6 $\pm$ 0.5%	14.1 $\pm$ 0.1%	7.2 $\pm$ 0.3%

At 6 h HRT, the total concentration of free exopolysaccharides found in our reactor was 11.8 mg/L, which is clearly different from the results obtained from the aforementioned colorimetric carbohydrates analysis as shown in Table 5.6. This further reinforce the idea that the carbohydrates present in wastewater measured using colorimetric assays could be wrongly estimated. The concentration is also in close agreement with other

workers, who characterized PS composition in a SAMBR using simple monosaccharide analysis (Zhou *et al.*, 2016).

Furthermore, Table 5.7 shows the descending ranking of various monomeric saccharide presented as a percentage mole, and most of the monosaccharide are either neutral (e.g. glucose) or polyanionic (e.g. glucuronic acid) residues with various linkages. It is not uncommon that 15-20% of the linkages are not assigned because of the complexity and dynamic nature of the PS structures. Despite this, this protocol is an effective measure of free exopolysaccharides both, qualitatively and quantitatively, and can also be used as the basis for further in-depth analysis.

Moreover, it is known that the presence of a large amount of polyuronic acid moieties in the exopolysaccharide structure enable the formation of the cross-links in the presence of divalent cations such as Ca^{2+} (Xin *et al.*, 2016). These cations can act as bridges for the carboxyl groups in the PS (Meng *et al.*, 2017), which will become much more crystalline and form less soluble gels. As shown in our findings, the concentration of uronic moieties in our system is comparatively lower than the other moieties, indicating a lower possibility of gelation behavior.

However, exopolysaccharides are considered to be the major components of key fouling-causing constituents in MBRs (Rosenberger *et al.*, 2006). It is also believed that PS have higher membrane rejection degrees than proteinaceous materials and humic substances because they can form large crosslinked structures (Wang and Waite, 2009) as well as having low biodegradation rates (Meng *et al.*, 2017), which might contribute to the accumulation of exopolysaccharides in both the reactor liquor and bio-cake significantly (Meng *et al.*, 2011).

5.4 Conclusions

This chapter highlights the serious problems associated with carbohydrate analysis in biological wastewater systems; considerable fluctuations in sample quantification, lack of a suitable standard for analysis, and frequent interference with coexisting compounds

found in wastewater. Assessing carbohydrate concentrations on the basis of colorimetric assays that reference glucose leads to a misrepresentation of the PS content, especially for SMPs/EPS found in wastewater samples. Because of these issues surrounding carbohydrate analysis in wastewater, a new and detailed protocol for the analysis of carbohydrates was developed which can be used routinely in a reasonably equipped laboratory to determine simultaneously the both neutral and acidic monosaccharides, and how each residue is linked within the free exopolysaccharides. A further application of this method will be presented in Chapter 7, and the link between exopolysaccharides' physiochemical properties and the underlying biofouling mechanisms under the influence of different macronutrients will be discussed in more depth.

CHAPTER 6

Characterization of Proteinaceous Materials³

In Chapter 5, we concluded that the characterisation of carbohydrates in wastewater samples using colorimetric methods had serious drawbacks. In this companion Chapter, when we evaluated the colorimetric measurement of proteinaceous materials, this also delivered a clear message that these assays are not specific; using Bovine Serum Albumin (BSA) as a standard we quantified a variety of other proteins, and found that the assays resulted in considerable under- or over-estimation. It also addressed the accuracy and consistency of current protein quantitation methods on samples from wastewater treatment systems, and highlighted the need for truly understanding the chemistry and limitation of these assays, and in using care in interpreting results, particularly with wastewater. Next, we attempted to do a full scale MS-based metaproteomic analysis using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The attempt was failed, however, due to insufficient amount of proteinaceous material being extracted from the wastewater effluent for 2D-PAGE analysis. Therefore, a method for characterizing amino acids (AA) in biological treatment systems using HPLC was adopted and optimized. This method will enable us to gain some insights into the composition of the proteinaceous materials in SMPs.

³ Published as Le, C.; Kunacheva, C.; Stuckey, D. C. (2016) “Protein” Measurement in Biological Wastewater Treatment Systems: A Critical Evaluation. *Environ. Sci. Technol.* 50, 3074-3081. and Le, C.; Stuckey, D. C. (2017) Impact of feed carbohydrates and nitrogen source on the production of soluble microbial products (SMPs) in anaerobic digestion. *Water Res.* 122, 10-16.

6.1 Introduction

In this chapter, we use the term “proteins” because despite the fact that many old and dated assays are still being used to evaluate “protein” concentrations, they are not designed for use in wastewater systems, and do not measure true protein concentration. In the last few decades, several studies have pointed out that "proteins" are the main compounds present in various wastewater systems (Raunjker *et al.*, 1994, Comte *et al.*, 2007), and that they seem to be positively correlated with the aggregation process and flocculation ability (Urbain *et al.*, 1993; Lazarova and Manem, 1995; Frohnlund *et al.*, 1996; Liu and Fang, 2003; Wilen *et al.*, 2003; Nielsen *et al.*, 2004). Nevertheless, quantification of these "proteins" sometimes shows great variability, and such variations were attributed to sludge origin and process treatments, with direct measurement of "protein" content in activated sludge varying between 224 and 462 mg protein.(g VSS sludge)⁻¹.

Understanding the composition of proteins in SMPs and EPS in wastewater treatment systems is increasingly important. Several colorimetric methods have been used to quantify proteins (Noble and Bailey, 2009; Kunacheva and Stuckey, 2014), namely; Kjeldahl (Kjedahl, 1883), Biuret (Gornall *et al.*, 1949), Lowry (Lowry *et al.*, 1951), Bicinchoninic acid (BCA) (Smith *et al.*, 1985) and Bradford (Bradford, 1976). While time- and sample-consuming, organic nitrogen compounds other than proteins can also be measured using the Kjeldahl method, and hence this procedure is not used often. The Biuret method is somewhat insensitive compared to the others, and therefore is not used for analyzing wastewater samples. Owing to their simplicity and precision, the BCA, Lowry or Bradford methods have been used extensively in the analysis of proteins found in biological treatment effluents (Aquino, 2004; Adav and Lee, 2008; Zhang *et al.*, 2008; Wang *et al.*, 2009). Such chromogenic methods are used to analyze global “protein” content, and are very popular for first measuring proteins in SMPs and EPS (Kunacheva and Stuckey, 2014).

Although these assays offer possible insights into protein concentrations, they are likely to overestimate protein quantities where matrices are present. In one study, Ikawa and co-workers (2003) performed an exhaustive study on the Lowry reagent; they tested over 60 nitrogen-containing compounds and declared that many classes showed reactivity toward the Folin-Ciocalteu reagent. However, Everette *et al.* (2010) later revealed that over 80 non-nitrogenous chemicals, representing several chemical classes, also react with Lowry's reagent leading to an erroneous estimation of protein content. Moreover, many colorimetric methods detect only specific peptide bonds *per se*, and this detection could include oligopeptides, polypeptides and/or other biological polymers in SMPs and EPS (Kunacheva and Stuckey, 2014). In addition, not only are these protein assays markedly influenced by protein composition, but also a wide range of solutes can interfere with them (Raunjker *et al.*, 1994; Price, 1996; Frohlund *et al.*, 1996; Ras *et al.*, 2008). As a result, the "proteins" measured by these chromogenic methods could be wrongly estimated; in addition, a detailed evaluation of these colorimetric protein quantification methods is often time-consuming and has thus received limited attention in the literature (Jimenez *et al.*, 2013) Lastly, none of these assays successfully provided qualitative identification of the proteinaceous materials.

Therefore, the objectives of this study were to evaluate all the different existing assay techniques, with and without these interfering solutes, to see whether they can accurately measure "proteins" in wastewater solutions. We compared the performance of these protein assays simultaneously and comprehensively with a large variety of proteins and peptides, and through a case study by adding a 'spike' of BSA to effluent samples. Finally, it aims to establish an amino acid composition analysis using HPLC to measure proteinaceous materials found in biological wastewater treatment systems.

6.2 Experimental and analytical details

6.2.1 Reagents and chemicals

GC-MS grade n-hexane was purchased from Merck, while Sodium acetate and hydrochloric acid were of ACS grade and purchased from Merck. Sodium hydroxide,

boric acid, disodium hydrogen phosphate and sodium azide were supplied by Merck, Singapore. Acetonitrile (LC-MS grade) was purchased from Fisher. The high purity mixed AA standard (17 AAs dissolved in 0.1 M HCl at 2.5 mM except L-cystine at 1.25 mM), additional HPLC grade AA and the internal standards (ISTD) DL-norvaline, sarcosine and gamma-amino-n-butyric acid (GABA) were all purchased from Sigma-Aldrich, Singapore. All derivatization reagents were of spectrophotometric grade and supplied by Sigma-Aldrich, Singapore. All other chemicals and supplementary solvents exhibited HPLC or higher grade were obtained from Sigma-Aldrich, Singapore. Ultrapure water was prepared from a MilliQ water process (Millipore Advantage A10).

6.2.2 Modified Lowry method

The Lowry method (and its modifications) is based on a two-step procedure; initially, Cu^{2+} ions are reduced to Cu^+ by protein in alkaline medium, followed by the amplification stage, the reduction of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate) (Peterson, 1979) producing a characteristic blue. Factors that play a role in the development of colour are due, not only to the reduced copper-amide bond complex, but also to the interactions with specific amino acids such as tyrosine, tryptophan, and to a lesser extent cystine, cysteine, and histidine residues (Peterson, 1977; Wu *et al.*, 1978).

The Thermo Scientific modified Lowry protein assay kit (23240) combines a stabilized formulation of the original Lowry reagent and the Folin-Ciocalteu Reagent. BSA (1-1,500 mg/L) from Thermo Scientific (23209) was used as the standard for calibration curve preparation. Two hundred micro liters of standard or sample were added to 1.0 mL of the Modified Lowry reagent at 15-second intervals, and after 10 min of incubation at room temperature, 0.1 mL of Folin-Ciocalteu Phenol reagent was added at 15-second intervals. The samples were then incubated at room temperature for 30 min, and the absorbance of all the samples was measured at 750 nm (Shimadzu UV-2600 UV/Vis double-beam spectrometer).

6.2.3 Bradford method - Coomassie

The ease and high sensitivity of the Bradford assay has driven its extensive use for the quantitation of protein in a wide variety of protein samples. The assay, first described by Bradford (1976), is based on the binding of the dye Coomassie Brilliant Blue G-250 at acidic pH to arginine, histidine, phenylalanine, tryptophan and tyrosine residue (de Moreno *et al.*, 1986) and hydrophobic interactions (Fountoulakis *et al.*, 1992), which results in a dye-protein complex with a metachromatic shift. The exact mechanism is still not fully understood, but the majority of the observed signal is due to the interaction with lysine and arginine residues (Sapan *et al.*, 1999).

The Thermo Scientific Coomassie protein assay kit (23200) is a quick Bradford method for the quantification of proteins that have a molecular weight greater than 3,000 Da. Because the colour response with Coomassie is non-linear with increasing protein concentration, a standard curve (100-1,500 mg/L) was prepared with each assay using a BSA standard (Thermo Scientific 23209). Thirty micro liters of standard or sample was added to 1.5 mL of the Coomassie Reagent; after incubation for 10 min at room temperature the absorbance was measured at 595 nm.

6.2.4 Bradford method - Coomassie Plus™

The Coomassie Plus assay kit (Thermo Scientific 23236) improves the linearity of the colour response, and results in less protein-to-protein variations than other Bradford formulations. A standard curve with a dynamic range of 100-1,500 mg/L was prepared using the BSA standard (Thermo Scientific 23209). Fifty micro liters of standard or sample was combined with 1.5 mL of the Coomassie Plus Reagent; for the most consistent results, samples were incubated for 10 min at room temperature before the absorbance measurement was carried out at 595 nm.

6.2.5 Micro BCA™ method

This method replaces the Folin-Ciocalteu reagent, as described in the Lowry method, with BCA which was developed by Smith *et al.* (1985). This results in a protein assay with improved sensitivity and uses BCA to detect the Cu⁺ ions, generated from Cu²⁺

ions, by reaction with protein in an alkaline pH (Sapan *et al.*, 1999). The residues that reduce cupric ion include the cysteine, cystine, tryptophan, tyrosine, and the peptide bonds (Smith *et al.*, 1985; Box, 1983). It is noteworthy that most of the commercial sources are formulated close to the original preparation to improve the sensitivity of the assay.

A variation of the original BCA method is the Micro BCA protein assay (Thermo Scientific), which is useful for dilute protein samples (0.5-20 mg/L); a BSA standard (Thermo Scientific 23209) was used for preparing the calibration curve. One milliliter of standard or sample was added to 1.0 mL of the Micro BCA working reagent, and mixed thoroughly before incubation at 60 °C for 1 hour. After cooling to room temperature, the samples were measured spectrophotometrically at 562nm within 10 minutes.

6.2.6 Pierce® BCA Method

The Pierce BCA protein assay (Thermo Scientific 23250), a variation of the original preparation, enables the quantitation of total protein in samples while minimizing interference from reducing agents, and enhancing sensitivity. A standard curve was generated in the range of 125 to 2,000 mg/L using BSA (Thermo Scientific 23209). Twenty-five micro liters of standard or sample was added to an equal volume of Reducing Agent-Compatibility Reagent solution, mixed thoroughly, and incubated at 37 °C for 15 min. Subsequently, 1.0 mL of BCA working reagent was added to the samples which were incubated at 37 °C for 30 min. After cooling to room temperature, the samples were measured spectrophotometrically at 562 nm within 10 minutes.

6.2.7 Colorimetric protein analysis with interferences

While employing protein assays to determine the concentration of protein, it is noted that certain compounds that are present in wastewater samples might affect colour development of the chromophore (Bensadoun and Weinstein, 1976; Raunjker *et al.*, 1994). Seventeen compounds that were previously identified, or known to be in the wastewater sample (Aquino, 2004; Zhou *et al.*, 2009; Trzcinski and Stuckey, 2010b;

Wu and Zhou 2010), representing different class of chemicals, were chosen as interfering substances. Each of them was deliberately added at concentrations of 1 mM to known amounts (either 10 mg/L or 300 mg/L) of BSA, which is used as the standard protein. The usual colorimetric protein analysis procedure was followed for all the five commercial test kits. A reference BSA standard containing no interfering substances was run concurrently for every batch. Calibration was also carried out using varying concentrations of standard stock protein solution (2 mg.mL⁻¹, Thermo Scientific 23209). A statistical analysis for quadruplicate samples was performed with the Student's *t*-test using Excel.

6.2.8 Metachromatic response for different proteins and peptides

An attempt was made to find any variation in the metachromatic response of different proteinaceous materials with five different protein assays. Eight different polyamino acid standard (poly-L-lysine, poly-L-proline, poly-L-arginine, poly-DL-aspartic acid, poly-(glu,ala,tyr) 1:1:1, Poly-(glu,ala,tyr) 6:3:1, Poly-(arg-pro,thr) 1:1:1, Poly-(arg-pro,thr) 6:3:1); a mixture of short chain peptide standards (gly-tyr, val-tyr-val, tyr-gly-gly-phe-met, tyr-gly-gly-phe-leu, asp-arg-val-tyr-ile-his-pro-phe); and a protein standard mixture (ribonuclease, cytochrome C, holo-transferrin, apomyoglobin) and various individual proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum, α -acid glycoprotein) were purchased from Sigma-Aldrich. A known amount (either 10 mg/L or 300 mg/L) of the 16 standard samples was measured using all the five commercial test kits. A BSA standard was assayed concurrently as reference for every batch, and calibration was also carried out simultaneously.

6.2.9 Case study

In order to evaluate the applicability of these methods to wastewater samples, a test was performed using an added spike of BSA; this will reveal any possible interferences by adding known amounts of the standard protein to wastewater effluent. The effluent was collected at the outlet of a laboratory scale SAMBR (Hu and Stuckey, 2006) mentioned in Chapter 3. Samples measured on the day of collection were not preserved, while

other samples were refrigerated at 4 °C. The protein spiked into the effluent samples was BSA at either 10 mg/L or 300 mg/L. The precision of the assays was investigated by analyzing several replicates ($n = 4$) with all five commercial test kits. A BSA standard was assayed simultaneously as a reference for every batch, and calibration was also carried out concurrently.

6.2.10 Proteolysis and amino acids analysis (AAA) using HPLC

Amino acid analysis (AAA) is a typical analytical technique that characterize proteins, peptides and free amino acids (FAA). It is essential for qualification and quantitation of proteinaceous materials and widely used in research, clinical and industrial facilities. AAA usually involves four steps: hydrolysis, separation, derivatization, and detection (Halpine, 2005). Because of the sample's dilute nature, lyophilization is performed before hydrolysis, in which a dehydration process typically is employed to reduce sample volumes, and concentrate the sample without destroying its physical structure. Hydrolysis breaks the peptide bonds and releases their constituent amino acids, while separation by column chromatography is not carried out due to sample dilution; direct pre-column derivatization with a chromogenic reagent is used instead. Lastly, the resulting HPLC chromatogram is compared with a calibrated standard based on their peak area.

In this study, FAAs were analyzed by simple, direct and rapid pre-column derivatization and an HPLC method which was adopted and modified based on previous studies (Jones *et al.*, 1981; Einarsson *et al.*, 1986; Schuster, 1988). The method does not differentiate between D and L forms of amino acids (AA), but was valid for the determination of 23 different primary and secondary AAs with high sensitivity. FAAs in the sample were analyzed directly after lyophilization of the filtered supernatant, and a calibration with both internal and external standard samples of AAs was used to approximate concentration.

For the AA standard solution, 100 μ L of the 17 AA standard solution was transferred into a conical vial at varying concentrations of 1.0, 0.25, 0.1, 0.025, and 0.010

$\mu\text{mol/mL}$. For the ISTD AA solution, sarcosine and norvaline were transferred into a 50 mL volumetric flask. The flask was firstly filled with 0.1 N HCl to halfway to dissolve the AA; water was then added for a final concentration of 10 nmol/ μL solution. For the extended AA (EAA) solution, asparagine, hydroxyproline, glutamine and tryptophan were transferred into a 25 mL volumetric flask. The flask was firstly filled with 0.1 N HCl to halfway to dissolve the AA; water was then added for a final concentration of 18 nmol/ μL . For the borate buffer, 2.48 g of boric acid was dissolved in a 50 mL volumetric flask; to another 50 mL volumetric flask, 1.6 g NaOH was added and dissolved. Two solutions were mixed under stirring and the pH was adjusted to 9.5 with 5M HCl. For mobile phase A solution, 10 mM Na_2HPO_4 , 10 mM $\text{Na}_2\text{B}_4\text{O}_7$ and 5 mM NaN_3 were added and dissolved in a 2L flask, and the pH adjusted to 8.2. For mobile phase B solution, acetonitrile, methanol and water were mixed at 45:45:10, v:v:v ratio in a 1L bottle. For the diluent, 0.4 mL of concentrated H_3PO_4 was added to 100 mL of mobile phase A solution. All the solutions were stored at 4°C before using.

For the ortho-phthalaldehyde (OPA) derivatization reagent, 10.1 mg of OPA was dissolved in 92 μL of methanol before 0.4 M borate buffer and 8.2 μL 3-mercaptopropionic acid (MPA) were added and mixed. For the 9-fluorenylmethylchloroformate (FMOC) derivatization reagent, the concentration of the solution was 2.5 mg. mL^{-1} FMOC in acetonitrile. These two reagents have to be prepared freshly before use.

In the first step, 25 μL of borate buffer and 10 μL of sample were mixed. Next, 5 μL of OPA reagent was mixed into solution to derivatize the primary AAs. To this mixture, 4 μL FMOC reagent was added to derivatize the free secondary AAs. Later, 320 μL injection diluent was added to the mixture before injection; all buffer solutions were filtered through 0.45 μm porosity mixed cellulose ester filters (Advantec).

The resulting mixture of primary AAs as isoindole (OPA) derivatives, and secondary AAs as FMOC derivatives was injected into an HPLC (Prominence UFLC, Shimadzu, Japan) installed with a UV diode array detector (DAD, SPD-M20A) at a wavelength of

338 nm for primary AAs, and 266 nm for secondary AAs, and an Agilent Zorbax Eclipse Plus C18 column. The flow rate was adjusted to 1.5 mL/min at 40 °C, and the gradient programs applied were set as follow: 0-2% B (0-0.84 min), 2-57% B (0.84-33.4 min), 57-100% B (33.4-33.5 min), 100% B (33.5-39.3 min), 100-2% B (39.3-39.4 min), and 2% B (39.4-45 min).

6.3 Results and discussion

6.3.1 Working range of individual assays

Colorimetric protein assays are methods that use UV-Vis spectroscopy to determine the concentration of protein, relative to a standard. An increase in the number of these assays has been observed over the last few decades, however, in the field of wastewater the BCA, Lowry and Bradford methods are still the most commonly used. These assays can be run at a high throughput using inexpensive reagents with equipment found in most laboratories. The reagents can either be economically prepared in bulk and stored for prolonged periods, or purchased from commercial sources such as Bio-Rad, Novagen, Roche, Sigma-Aldrich, and Thermo Scientific. It should be noted that different preparations of the same method may not give equal responses when using an identical protein (Stoscheck, 1990). The main advantage of using commercial sources is the improvement in long-term repeatability and performance. However, each assay has its own advantages and disadvantages relative to sensitivity, ease of performance, linearity and accuracy. A comparison of the use of 5 commercial assays with the same protein sample is presented here; the linearity range of the 5 methods was tested and the comparative results are given in Table 6.1.

Table 6.1. Linearity range for 5 different assays

Assays	Duration of reaction (min)	Dynamic range (mg BSA/L)	Linearity range (mg BSA/L)	Regression coefficient	Coefficients of multiple correlation (R^2)
Modified Lowry	40	1-1500	1-125	$y = 0.0423x + 0.1197$	0.99946
Micro BCA	60	0.5-20	0.5-20	$y = 0.00324x + 0.03193$	0.99980
Pierce BCA	10	125-2000	125-500	$y = 0.0007x + 0.08959$	0.99966
Bradford (Coomassie)	10	100-1500	125-750	$y = 0.00111x + 0.51937$	0.99932
Bradford (Coomassie Plus)	10	100-1500	125-500	$y = 0.00131x + 0.458$	0.99943

As can be seen in Table 6.1, it is often necessary to use more than one type of protein assay to cover a wide concentration range. The dynamic range of the assays was obtained based on the manufacturers' claims of a commercial product, and is not a rigorous measure of the accuracy range of the assay. Although the signal is adequately determined by the spectrophotometer, the accuracy and precision can vary beyond what is acceptable to report as a true measure of the concentration. A linearity study was performed in order to determine the linear reportable range. A single run testing of at least five concentrations was carried out in quadruplicate, and a linear regression equation was obtained. The result should not have an intercept significantly different from zero, and no value should deviate greatly from the others after the result is graphically and statistically analyzed. Hence the linearity range was obtained, where a linear response over a wide concentration range is produced, and the analyte concentration can be quantified with acceptable reliability and precision. Since it is a stricter measure and requires both sensitivity and accuracy, the linearity range is narrower than the dynamic range, and hence a more reliable measure of the accurate range of the concentration being quantified.

Superior linearity was observed for the Micro BCA method compared to the other 4 methods, and its dynamic range was similar to its linearity range indicating high sensitivity with the BSA protein sample. The protein-dye binding methods such as the Bradford assay give sensitivities generally in the same linear range as the Pierce BCA method. Despite shorter preparation and reaction times than that of the Micro BCA, the other methods do not generate a linear response with BSA; although shorter segments of their standard curve approximate linearity, a quadratic curve must be used to model the data over a wider range of concentrations for a more reliable and reportable result. A fourth order or more polynomial equation is necessary in order to provide a better fit than that of a second-degree polynomial.

6.3.2 Colorimetric protein analysis with interferences

Despite their advantages, and the substantial use of these techniques for the determination of "protein" concentration in wastewater sample, many solutes present in

wastewater have been found to interfere with the colour development of the chromophore. Seventeen chemicals were selected to represent those most commonly found in wastewater samples, and were deliberately added at a concentration of 1 mmol/L to the BSA standard as interfering reagents. Table 6.2 provides a broad list of compounds that were studied.

Table 6.2 - Substances that can interfere with various protein assays.

Interfering solutes (1mmol/L)	Modified Lowry	Micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
Hexadecane	-	-	-	○	-
Octadecene	○	-	-	●	○
n-Hexadecanol	●	●	●	●	●
Glucose	○	●	●	○	-
Sucrose	-	-	-	○	-
Sorbitol	-	-	-	-	-
Urea	-	-	-	○	-
Uric acid	●	●	○	○	-
Acetic acid	-	-	-	○	○
Butyric acid	-	-	-	-	-
Hexanoic acid	-	-	-	-	●
Palmitic acid	○	●	●	●	○
Ascorbic Acid	●	●	○	-	○
Humic acid	●	●	●	○	●
Squalane	-	-	●	●	●
Dibutyl phthalate	-	-	-	-	-
2,6-Di-tert-butylphenol	●	●	○	○	○
All	●	●	●	●	●

●: there is a statistically highly significant difference in measurement between standard w/o interfering solutes ($p = 0.001$)

○: there is a statistically significant difference in measurement between standard w/o interfering solutes ($p = 0.05$)

- : there is no statistically significant difference in measurement between standard w/o interfering solutes

Standard curves were first obtained with BSA concentrations varying from 0.5 mg/L to 500 mg/L; BSA was then assayed in parallel with or without the individual interfering substance. At the 95% confidence level ($n = 4$, $p = 0.05$), several compounds that interfered with the Bradford assay did not interfere with the others, causing an increase in the absorbance. Antioxidants such as ascorbic acid, uric acid, and substituted phenols can act as reducing agents and result in artificially high values for the protein concentration. At the 99.9% confidence level ($n = 4$, $p = 0.001$) fatty alcohols such as hexadecanol appeared to result in an erroneous reading that almost certainly reflects on its interactions with the reagents. Similarly, it is noteworthy that lipid-rich compounds such as palmitic acid interfere with protein assays, giving a false protein concentration. Box (1983), and Randtke and Larson (1983) reported that humic substances interfere

with the Lowry procedure, and it seems like humic acids can be expected to interfere with all the colorimetric protein measurements due to their ability to complex metal ions (Ghabbour and Davies, 2001), and their heteropolycondensate structure that absorbs in the ultraviolet region (Kumada, 1965). Although the Coomassie and Coomassie Plus assays require little sample preparation time, they have little tolerance to most of the interfering substances, and result in significantly different "protein" concentrations. Interestingly, a plasticizer such as dibutyl phthalate, a common laboratory contaminant, shows no sign of interference.

Although the mechanism of interference varied with the solute, they all resulted in strongly erroneous absorbance values. The easiest method for coping with interfering compounds is to add them into the blank sample, and prepare a standard curve in their presence; however, this requires knowing the identity and amount of the interfering solute. Moreover, the presence of interfering solutes is difficult to adequately control for during colorimetric protein analysis. Considerable study of the interference by a compound over a range of appropriate concentrations for its effects on several different proteins is usually required. This is clearly infeasible and impractical due to the diverse range of chemical compounds, SMPs, and EPS found in biologically treated wastewater samples. The most common strategy for coping with interfering compounds is to remove them by selective isolation techniques (Peterson, 1979), and most low MW interfering substances can often be removed by dialysis. Another method is to precipitate the protein in acid and collect the precipitate by membrane filtration, although recovery of precipitated protein is not quantitative at low concentrations. Even though specific interfering substances can sometimes be removed prior to concentration determination, this adds additional steps to the overall procedure and can consequently result in dilution, or incomplete recovery of the original sample leading to errors. Hence, interfering substances are particularly troublesome when attempting to quantify protein content directly and reliably.

6.3.3 Metachromatic response to different proteins and peptides

Many of the traditional colorimetric protein assays depend on both protein quantity and composition, while another influential property is MW. In this work, we critically evaluated the variation in metachromatic response with five commercially available protein assays for a variety of synthetic polyamino acids, a mixture of short chain polypeptides, a mixture of protein standards and several protein samples using BSA as the reference protein. All the samples were analysed at a fixed concentration within the quantitation range of each assay. Proteinaceous compounds with different MWs were also assayed to determine the sensitivity of the methods towards MW, and the results are summarised in Table 6.3.

From Table 6.3, it is clear that there is a noticeable difference between all the samples, even when their concentration was identical to that of the reference protein, BSA, and each of the assays tested exhibited some degree of varying response towards different proteins. Some of the factors that could possibly contribute to this difference are; the amino acid sequence, isoelectronic point (pI), its three-dimensional structure, and the presence of certain side chains or prosthetic groups. These results highlight the fact that the metachromatic response is predominantly dependent on amino acid content. In particular, the presence of a few specific residues enhances colour development in the colorimetric protein assays, and hence the dissimilar metachromatic response. For example, colour formation is highly dependent on the arginine amino residues in the binding of Coomassie Blue to the protein. Furthermore, residues like tryptophan and tyrosine in all of the di-, tri-, or polypeptides are capable of reducing cupric ions to cuprous ions in the BCA reaction, and consequently gave a stronger response than other residues. Moreover, it was demonstrated that peptides with a molecular mass of less than 3,000 Da did not form a complex in the Bradford assay, and this could result in very serious errors of omission; low MW SMPs and EPS such as short chain peptides would fall into this category.

Table 6.3 - Effect of composition on metachromatic response

	Protein or polypeptide sample	Molecular weight (Da)	Reading (mg BSA /L) $\pm$ SD				
			Modified Lowry	Micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
Reference protein	Bovine serum albumin	~66500	10 $\pm$ 1.4	10 $\pm$ 0.9	300 $\pm$ 11	299 $\pm$ 8.3	300 $\pm$ 1.3
	Poly-L-lysine	1000-5000	6.4 $\pm$ 1.4	9.1 $\pm$ 0.8	303 $\pm$ 25	22.5 $\pm$ 6.6	19.3 $\pm$ 5.7
	Poly-L-proline	1000-10000	0.1 $\pm$ 1.2	0.2 $\pm$ 0.2	1.00 $\pm$ 2.1	21.1 $\pm$ 5.4	17.9 $\pm$ 7.9
	Poly-L-arginine	5000-15000	3.3 $\pm$ 1.3	7.5 $\pm$ 0.2	129 $\pm$ 20	995 $\pm$ 8.6	912 $\pm$ 10
Polymeric amino acids	Poly-DL-aspartic acid	2000-11000	0.7 $\pm$ 1.0	0.2 $\pm$ 0.2	0.40 $\pm$ 2.1	20.9 $\pm$ 6.1	17.3 $\pm$ 7.3
	Poly-(glu,ala,tyr) 1:1:1 *	20000-50000	10 $\pm$ 2.1	14 $\pm$ 0.5	675 $\pm$ 41	109 $\pm$ 10	183 $\pm$ 11
	Poly-(glu,ala,tyr) 6:3:1	20000-50000	3.6 $\pm$ 1.0	10 $\pm$ 0.5	340 $\pm$ 48	0.6 $\pm$ 0.8	46.8 $\pm$ 5.1
	Poly-(arg-pro,thr) 1:1:1	5000-20000	7.7 $\pm$ 2.1	13 $\pm$ 0.1	175 $\pm$ 26	619 $\pm$ 26	594 $\pm$ 15
	Poly-(arg-pro,thr) 6:3:1	10000-30000	2.3 $\pm$ 1.4	6.4 $\pm$ 0.2	120 $\pm$ 19	981 $\pm$ 20	922 $\pm$ 21
	Gly-tyr	238.2					
	Val-tyr-val	379.5					
	Tyr-gly-gly-phe-met	573.7	46 $\pm$ 1.0	19 $\pm$ 0.4	412 $\pm$ 14	13.1 $\pm$ 2.9	12.5 $\pm$ 7.9
Short chain peptide standard mixture	Tyr-gly-gly-phe-leu	555.6					
	Asp-arg-val-tyr-ile-his-pro-phe	1046.2					
	Ribonuclease A	~13700					
	Cytochrome C	~12000	27 $\pm$ 0.6	18 $\pm$ 0.6	432 $\pm$ 10	362.1 $\pm$ 14	360 $\pm$ 17
	Holo-transferrin	76000-81000					
Protein standard mixture	Apomyoglobin	~16900					
	Lysozyme	14388	18.3 $\pm$ 0.3	15 $\pm$ 0.3	567 $\pm$ 15	186 $\pm$ 8.6	410 $\pm$ 6.3
	Ovalbumin	45000	11.6 $\pm$ 0.4	12 $\pm$ 0.4	410 $\pm$ 5.8	148 $\pm$ 5.5	307 $\pm$ 5.7
	α -Acid glycoprotein	41000-43000	9.0 $\pm$ 1.2	6.9 $\pm$ 0.1	255 $\pm$ 12	132 $\pm$ 6.7	172 $\pm$ 1.9
	Apo-transferrin bovine	76000-81000	9.5 $\pm$ 1.1	11 $\pm$ 0.2	415 $\pm$ 17	346 $\pm$ 6.5	389 $\pm$ 2.4
	Fetuin from fetal bovine serum	48400	6.7 $\pm$ 0.8	7.0 $\pm$ 0.2	255 $\pm$ 6.8	191 $\pm$ 2.9	210 $\pm$ 2.0

*: Molar ratio of random co-polymers of amino acid

It is also remarkable that a large variability was measured between the BSA standard and the 5 proteins (lysozyme, ovalbumin, apo-transferrin bovine, fetuin from fetal bovine serum, α -acid glycoprotein). However, the standard deviation (SD) suggests that this variation in concentration between the different proteins was not due to repeatability, but rather to the individual colorimetric assay responses being dependent on the amino acid content of the protein. Moreover, the protein concentration measured was highly sensitive to the degree of glycosylation of the protein analysed, and the specific sugars present in the protein (Wu *et al.*, 1978). Comparison of an equal amount of lysozyme (0% carbohydrate content), fetuin (22.9% carbohydrate content) and acid

glycoprotein (41.4% carbohydrate content) suggests that the non-glycosylated forms of the protein generally gave higher responses. This difference was observed for all of the assays analysed, which suggests this was not an erroneous result.

Taken together, since the assay responses are in fact dependent on the amino acid content of the protein, extreme caution should be exercised in using BSA to estimate the concentration of unknown "proteins" in a wastewater sample due to the large variability observed. It has been documented that the colorimetric assays require an appropriate protein standard to obtain a good estimate of the concentration present, and the ideal protein standard to use would be the same protein being assayed (Peterson, 1979). In practice, we often do not know what protein or polypeptide we are looking for, and there is not always a matched protein standard available, especially in wastewater analysis.

6.3.4 Case study

A study was carried out in order to evaluate the applicability of these methods to actual wastewater samples; samples at 3 different HRTs were characterised by measuring their COD and "protein" content. The effluent samples were taken under the conditions shown in Table 6.4, and analysed with various colorimetric protein assay methods.

Table 6.4 – Average reading in the effluent from each sample under various HRTs

HRT (h)	COD (mg/L)	Reading (mg BSA/L) $\pm$ SD				
		Modified Lowry	Micro BCA	Pierce BCA	Bradford (Coomassie)	Bradford (Coomassie Plus)
6	19.12	5.6 $\pm$ 0.8	6.8 $\pm$ 0.1	6.5 $\pm$ 1.8	1.9 $\pm$ 2.0	15.2 $\pm$ 1.8
4	31.16	8.3 $\pm$ 0.5	10.3 $\pm$ 0.3	16.5 $\pm$ 2.1	3.0 $\pm$ 1.2	16.6 $\pm$ 0.8
2	40.65	9.5 $\pm$ 0.5	23.7 $\pm$ 0.8	31.5 $\pm$ 1.4	3.5 $\pm$ 0.7	18.7 $\pm$ 2.4
6 (SA*)	-	24.6 $\pm$ 1.4	19.2 $\pm$ 0.1	369 $\pm$ 22	379 $\pm$ 15	373 $\pm$ 5.4
4 (SA*)	-	32.9 $\pm$ 1.1	20.4 $\pm$ 0.2	373 $\pm$ 24	384 $\pm$ 9.9	375 $\pm$ 4.4
2 (SA*)	-	33.1 $\pm$ 2.8	25.9 $\pm$ 0.5	388 $\pm$ 20	386 $\pm$ 11	380 $\pm$ 5.2

*: Standard Addition of BSA at 10 mg/L for Modified Lowry and Micro BCA, and 300 mg/L for the others.

As mentioned previously, HRT is an important operational parameter that impacts on treatment performance, and affects SMP production and hence membrane fouling in a SAMBR. From Table 6.4 it is clear that, as expected, the effluent COD increased with

decreasing HRT, and an increase in "protein" concentration with all assays was also observed. While a low operating HRT is desirable for anaerobic reactors in order to reduce their overall footprint, it enhances biomass growth and the accumulation of SMPs leading to membrane fouling (Smith *et al.*, 2012). Using a modified Lowry method, Chae *et al.* (2006) also observed a similar trend with the "protein" concentration increasing with decreasing HRT. However, these five methods gave widely varying readings; at 6 h HRT the "protein" concentration varied by a factor of 8 (1.9-15.2), the highest measured with the Bradford (Coomassie Plus) assay. At 2 h HRT the highest was obtained using the Pierce BCA kit, and the ratio was even greater at 9. It is true that all assays showed an increase in "protein" with decreasing HRT, but each ratio varied markedly, with a small increase of 23% from 6 to 2 h with Bradford (Coomassie Plus), while the largest increase was 485% with the Pierce BCA assay. Hence, while all these assays predicted a general trend, there was little or no agreement between them in terms of absolute concentrations, or relative increases.

In addition, the values shown in Table 6.4 for protein content after the addition of a BSA "spike" are generally higher than expected (a spike of 10 mg/L was added to the Modified Lowry and Micro BCA, and 300 mg/L to the Pierce BCA and Bradford methods). This clearly shows that all the samples must contain solutes that interfere with the protein determination for all the five assays to varying degrees. Moreover, the use of BSA as a reference standard could also possibly introduce errors since it is clearly not an appropriate protein standard for estimating proteinaceous material present in the wastewater sample.

6.3.5 Proteins versus proteinaceous materials

Proteins are large biopolymers that are comprised of the 20 standard amino acids shown in Table 6.5. These amino acids are usually classified according to the polarities of their side chain, and amino acids polymerize, at least conceptually, through condensation to form peptide bonds. Polymers composed of two, three, a few (3-10) and many amino acids residues are known as dipeptides, tripeptides, oligopeptides, and

polypeptides, respectively. Proteins are macromolecules that consist of one or more polypeptide chains ranging in length from ~40 to 34,000 amino acids residues (even though few have more than 1500 residues) (Voet and Voet, 2011). Since the average mass of an amino acid residue is ~110 Da, proteins usually have molecular masses that range from ~40 to over 3700 kDa. Moreover, proteins are constantly being degraded by a variety of catabolic pathways during biological treatment processes, adding a dynamic component to the system, and therefore, based on the results obtained in this study, and information discussed earlier, it is understandable that if BSA was chosen as the reference standard, the result should be analogized and reported as mg BSA/L despite the fact that they are not the actual protein mass concentrations in the samples. Hence it is clear that enumerating changes in “proteins” measured with the assays tested above, with varying operational parameters is very likely to be misleading in published papers, and the data lacks credibility. Increasingly, many authors are starting to use the term "protein-like materials" (Kunacheva and Stuckey, 2014), although a more suitable term could be "proteinaceous" material, whose definition includes any materials relating to, resembling, or being proteins that are synthesised or decomposed by bacteria or eukaryotic organisms, and describes all forms of polypeptides/proteins (Fahnestock and Steinbüchel, 2003; Metcalf & Eddy *et al.*, 2003).

Table 6.5 - Structures and abbreviations of the 20 standard amino acids

Name, Three-Letter Symbol, and One-letter symbol	Structural formula	Name, Three-Letter Symbol, and One-letter symbol	Structural formula
<i>Nonpolar, hydrophobic side chain</i>			
Glycine Gly G	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{H} \end{array}$	Alanine Ala A	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_3 \end{array}$
Valine Val V	$\begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CHCH}_3 \\ \\ \text{CH}_3 \end{array}$	Proline Pro P

Leucine Leu L	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CHCH}_3 \\ \\ \text{CH}_3 \end{array} $	Isoleucine Ile I	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CHCH}_3 \\ \\ \text{CH}_2 \\ \\ \text{CH}_3 \end{array} $
Phenylalanine Phe F	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{C}_6\text{H}_5 \end{array} $	Tryptophan Trp W	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{Indole} \end{array} $
Methionine Met M	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{S} \\ \\ \text{CH}_3 \end{array} $		
<i>Polar, uncharged side chain</i>			
Serine Ser S	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{OH} \end{array} $	Threonine Thr T	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CHOH} \\ \\ \text{CH}_3 \end{array} $
Cysteine Cys C	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{SH} \end{array} $	Tyrosine Tyr Y	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{C}_6\text{H}_4 \\ \\ \text{OH} \end{array} $
Asparagine Asn N	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{C=O} \\ \\ \text{NH}_2 \end{array} $	Glutamine Gln Q	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{C=O} \\ \\ \text{NH}_2 \end{array} $
<i>Polar, charged side chain (Acidic)</i>			
Aspartic acid Asp D	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{C=O} \\ \\ \text{OH} \end{array} $	Glutamine acid Glu E	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{C=O} \\ \\ \text{OH} \end{array} $

<i>Polar, charged side chain (Basic)</i>			
Lysine Lys K	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{NH}_2 \end{array} $	Arginine Arg R	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{CH}_2 \\ \\ \text{NH} \\ \\ \text{C}=\text{NH} \\ \\ \text{NH}_2 \end{array} $
Histidine His H	$ \begin{array}{c} \text{O} \\ \parallel \\ \text{H}_2\text{N}-\text{CH}-\text{C}-\text{OH} \\ \\ \text{CH}_2 \\ \\ \text{N} \quad \text{C} \\ \diagup \quad \diagdown \\ \text{C} \quad \text{NH} \end{array} $		

[Source: Voet and Voet, 2011 (modified)]

Based on the results of this study, we have shown that a wide variety of solutes can interfere with colorimetric protein assays. In addition, the metachromatic response of these assays is clearly influenced by sample composition, and hence the proteinaceous material measured by these methods could be wrongly estimated. Given the complexities, vast dynamic range of proteinaceous material concentrations, and analytical limitations associated with traditional colorimetric assays, these five colorimetric methods for protein determination are, therefore, not recommended for the measurement of "proteins" in wastewater samples.

6.3.6 Proteolysis and amino acids analysis using HPLC

AAA was used as the "gold standard" technique to determine the absolute protein amount due to its reproducibility and robustness (Noble and Bailey, 2009; Voet and Voet, 2011). It is also a fundamental technique to determine the protein composition, and offers accurate qualitative identification of proteinaceous materials. The analysis of proteins using AAA methods generally involves hydrolysis, separation, derivatization, and detection. However, the analysis that developed for wastewater treatment process involves four steps: lyophilisation, derivatization and detection.

Due to the analytes' dilute nature, lyophilisation is necessary where a dehydration process is typically employed to reduce sample volume, and at the same time concentrate the sample without destroying its physical structure. Next, pre-column derivatization with a chromogenic reagent such as OPA and FMOC-Cl enhances the chromatographic separation, and increases detection sensitivity. Although numerous pre-column derivatization reagents could be used for the analysis of amino acids, all of them show various inadequacies. An ideal reagent should react rapidly and quantitatively, under mild conditions, with both primary and secondary amino acids, without being disturbed by sample matrix components. Additionally, reagents should give rise to a single, stable amino acid derivative. Lastly, the derivative should be detectable with high sensitivity and separated by available chromatographic methods.

Both OPA and FMOC-Cl meet most of these criteria; they react rapidly and quantitatively under mild conditions, and the resulting amino acid derivatives (Figure 6.1) exhibit high chromogenic responses (detection at the low picomole level) (Schuster, 1988). These derivatives are stable at room temperature for a number of days, and show excellent chromatographic behavior on reversed-phase columns (Jones *et al.*, 1981; Einarsson *et al.*, 1986).

Figure 6.1 - Reaction of amino acids with OPA and FMOC-Cl, respectively

Figure 6.2 - HPLC chromatogram of amino acid derivatives

The elution profiles of the 23 amino acid standard derivatized by reaction with both OPA and FMOC-Cl are shown in Figure 6.2. Each amino acid represents 50 pmol, except for the internal standard cystine (25 pmol). The resulting chromatogram, based on the peaks, can be used to compare them to a calibrated standard. The final results, often expressed as micro-mole of residue per sample, determine the percentage composition of each amino acid in the sample.

6.3.7 FAAs in SAMBR

Few studies have looked into the FAAs that result from the proteolysis of proteinaceous materials in the feed, and most of these studies only focused on how ammonia from protein degradation inhibits the activity of AD (Liu and Sung, 2002). The analytical results of FAA content in the SAMBR are shown in Table 6.6, where these values are the mean of four determinations. As shown in Table 6.6, there were 15 kinds of natural AAs emerging in the anaerobic bioreactors when it's HRT was = 6h. Although a small number of anaerobic bacteria are known to ferment single AAs, the highest concentration found in the reactors was isoleucine at 10.64 mmol/L. Isoleucine and other AAs with relatively high concentrations were identified to undergo oxidatively deamination (Barker, 1981), and seem to last longer than the other AAs did. Their accumulation means possible inhibition of methanogenesis (Orlygsson *et al.*, 1994) and lack of hydrogen transfer from methanogenic bacteria (Nagase and Matsuo, 1982),

although the accumulated concentration is way below inhibition levels (Nagase and Matsuo, 1982; Orlygsson *et al.*, 1994). The result further suggested that the quantitative contents of FAAs could be an indicator of the efficiency of AD since the AA are the main intermediates produced from proteins, and which will ultimately degrade to ammonia. Nevertheless, the analysis of FAAs using pre-column derivatization and reversed-phase HPLC is attractive as it can quantify both primary and secondary AA with low detection limits and high accuracy. In addition, this method sheds fresh insights into the analysis of proteinaceous materials in wastewater, and gives us a better understanding of the biological transformations occurring in AD.

Table 6.6 – FAA concentration

FAA	mmol/L $\pm$ SD	FAA	mmol/L $\pm$ SD	FAA	mmol/L $\pm$ SD
Aspartic acid	2.10 $\pm$ 0.12	Threonine	1.44 $\pm$ 0.07	Methionine	1.77 $\pm$ 0.27
Glutamic acid	1.62 $\pm$ 0.14	Arginine	0.94 $\pm$ 0.08	Phenylalanine	10.50 $\pm$ 0.50
Serine	1.05 $\pm$ 0.09	Alanine	9.63 $\pm$ 0.18	Isoleucine	10.64 $\pm$ 0.52
Histidine	1.13 $\pm$ 0.10	Tyrosine	7.10 $\pm$ 0.27	Leucine	4.42 $\pm$ 0.09
Glycine	6.93 $\pm$ 0.24	Valine	9.85 $\pm$ 0.50	Lysine	6.75 $\pm$ 0.06

The number in parenthesis refers to the average value (n = 4) and the number after the ($\pm$) symbol refers to SD

6.4 Conclusions

Based on the results of this study, we have shown that the metachromatic response of these colorimetric assays is clearly influenced by sample composition, and hence the proteinaceous material measured by them could be incorrectly estimated, at times by up to an order of magnitude. Moreover, a wide variety of solutes can strongly interfere with colorimetric protein assays. In summary then, these five colorimetric methods for protein determination are not recommended for accurate measurement of “protein” in wastewater samples, and an AA analysis method was adopted to more accurately identify and quantify the proteinaceous material found in wastewater. The application of this method will be further discussed in Chapter 7, where new insights into the production of SMPs under varying nutritional conditions will be presented.

CHAPTER 7

Impact of Nutrients on SMP Production⁴

In the previous Chapters several analytical methods for the characterization of SMPs were developed and documented. In Chapter 7, we are going to employ these optimized methods to comprehensively and critically assess the impact of feed composition, in particular carbohydrate substrates and nitrogen sources, on the production of SMPs in AD to demonstrate the use of these analytical techniques, and gain insights into the SMPs produced. This study revealed that the type of SMPs that dominate in a particular system depend strongly on the chemical characteristics of the feed. Therefore, it is evident that further fundamental research on the biochemistry of the process is of prime importance, and a much better understanding of the biochemical mechanisms is imperative for the efficient control of an essentially biological process such as AD if it is to be utilized to its full capacity.

7.1 Introduction

It was noted in Chapter 2 that AD is a sensitive multi-stage process that is comprised of hydrolysis, acidogenesis, acetogenesis and methanogenesis, and depends on a wide variety of microorganisms to carry out the task in a coordinated manner (Batstone and Jensen, 2011). Nutrients that microorganisms take up are one of the most influential factors in supporting and regulating their metabolism to maintain an effective digestion process. It is also understood that nutrients supply the basic cellular building blocks for microbial growth, and ensure that the cell can synthesize the enzymes and cofactors that drive the biochemical and metabolic reactions.

⁴ Published as Le, C.; Stuckey, D. C. (2017) Impact of feed carbohydrates and nitrogen source on the production of soluble microbial products (SMPs) in anaerobic digestion. *Water Res.* 122, 10-16 as well as Le, C.; Stuckey, D. C. (2017) Influence of feed composition on the monomeric structure of free bacterial extracellular polysaccharides in anaerobic digestion. *Environ. Sci. Technol.* 51, 7009-7017.

In proportion to their required quantities, nutrients can be divided into two groups: macro- and micro-nutrients. Macronutrients such as carbon, nitrogen, phosphorus, are required in large quantities because most cell mass is made up of carbon and nitrogen along with hydrogen and oxygen. Micronutrients that are required at relatively lower concentrations include magnesium, sulfur, potassium, calcium, and trace elements such as manganese, iron, cobalt, nickel, copper, zinc, and molybdenum. Both macro- and micro-nutrients must be present in an available form in the growth environment to allow effective uptake. The effect of trace nutrient supplements, particularly with regard to transition metals, has been studied extensively in the literature and was summarized by several authors (Jarrell and Kalmkoff, 1988; Oleszkiewicz and Sharma, 1990; Schattauer *et al.*, 2011; Thanh *et al.*, 2016). However, these publications paid very little attention to the influence macronutrients may have on the production of SMPs.

Carbohydrates are known to be a primary source of macronutrients for microbial growth in AD, and the glycolysis pathway plays a fundamental role in the degradation of fermentable carbon by microorganisms, providing the cell with both metabolic energy and essential intermediates for cell functions. As discussed in Chapter 2, most of the studies concerning SMPs in AD have been conducted with either glucose or acetate feeds (Barker and Stuckey, 2001), and there have only been three comparative studies with different feed compositions in the literature (Barker and Stuckey, 2001; O’Flaherty *et al.*, 1997; Noike *et al.*, 1985). Furthermore, as far as we are aware, there are no studies on the effect of the nitrogen-source on the formation of SMPs, although it is known that high ammonia concentrations are of concern since they are inhibitory and result in unstable process performance, and increased risks of process failure (Westerholm *et al.*, 2012). Moreover, only a few studies have examined the effect of feed composition on SMP MW distribution, and used crude parameters such as soluble COD and colorimetric measurement of proteins and carbohydrates, to analyse the compounds produced. Hence more fundamental information is required for a better

understanding of the accumulation and global composition of SMPs, particularly in response to different carbon (carbohydrate) and nitrogen sources in the feed.

Furthermore, it also seems as if a change in nutrients can have a significant effect on extracellular polysaccharide (EPS) production and composition (Sheng *et al.*, 2010). Nevertheless, past research in this field has mainly been concerned with manipulating external parameters such as nutrient levels and imbalances in the C:N:P ratio on the global production of EPS (Sheng *et al.*, 2010; Flemming *et al.*, 2016) in a “black-box” approach. It is striking that this kind of research is still in its infancy, and systematic research on this topic is missing in spite of its obvious importance.

As highlighted in Chapter 2, understanding what SMPs and EPS are, and why they are produced, is important because they are usually the main constituent of effluent chemical oxygen demand (COD) (Ni *et al.*, 2011a; Ni *et al.*, 2011b; Jarusutthirak and Amy, 2007; Barker and Stuckey, 1999; Barker *et al.*, 1999). Hence, they dictate process performance, since effluent COD is set by discharge consents. In addition, some low MW SMPs are known to be recalcitrant (Barker *et al.*, 1999) and are precursors for DBP (Liu and Li, 2010).

Also of contemporary importance is the role of SMP/EPS in membrane fouling of MBR technologies, which are becoming more prevalent globally nowadays (Roseenberger *et al.*, 2006; Jarusutthirak and Amy, 2006; Meng *et al.*, 2009; Wang and Waite, 2009; Jiang *et al.*, 2010; Stuckey, 2010a). Membrane autopsies reveal significant fouling resistance is contributed by an uneven distribution of SMP/EPS (Cho and Fane, 2002), and this resistance is directly correlated with SMP/EPS, which undergo intermolecular or intramolecular ionic cross-linking, which subsequently clogs the membrane pores and leads to further fouling (Lin *et al.*, 2014; Okamura *et al.*, 2009). Therefore, more work is needed to understand the characteristics of SMP/EPS, and whether nutrients affect their formation in anaerobic systems.

In the present study we examined if the type of nutrient (both carbon and nitrogen) had any effect on the formation of SMPs and EPS. Since the production of SMPs is closely

related to the mechanisms of microbial defense, acclimation and survival (Wang and Zhang, 2010), the purpose of this study was to investigate the effect on AD performance of different substrates. The low MW SMPs generated under different conditions were collected and analyzed using GC-MS, while FAAs and other organics were determined using HPLC. Besides deducing the fine structure of EPS, we also provide insights into the changes in glycan composition with changes in feed composition. Identification of the composition and characteristics of these free EPS could be useful to clarify various contradictions about EPS in previous studies (Seviour *et al.*, 2012). After the composition of EPS is analyzed in detail, understanding the factors influencing its production would be useful to manipulate the EPS content in microbial activities, and thus improve the performance of anaerobic membrane reactors.

7.2 Experimental and analytical details

7.2.1 Reagents and chemicals

All analytical grade chemicals were purchased from Sigma-Aldrich, and solvents were of GC-MS grade, or equivalent, and purchased from Merck. Sulfuric acid was of ISO grade and was purchased from Merck, while ultrapure water was obtained from a MilliQ water process (Millipore Advantage A10). A 2 mg.mL⁻¹ stock solution of glucose was prepared and stored at 4 °C before use.

Given the variety of basic carbohydrates potentially available to feed the reactors, we decided to use the ones commonly used in synthetic feeds such as glucose, and sucrose, which is a disaccharide of both glucose and fructose. In addition, to challenge the anaerobic culture we also chose fructose which is not commonly used, but constitutes half of the sucrose, and represents the other half of the carbohydrate spectrum.

7.2.2 Anaerobic Sludge and Medium

AD seed sludge was obtained from municipal wastewater treatment plant digesters (Ulu Pandan, Singapore), and identical cultures were started and then grown in six 5.0 L mixed laboratory batch reactors operating at mesophilic conditions (30 ± 1.0 °C) with

an agitation rate of 300 rpm, and the mixed liquid suspended solids (MLSS) were kept at ~3000 mg/L. The reactors were designed to provide strictly anaerobic conditions with constant feeding, and sampling of gas and liquid contents. The reactors were fed at a 7-day retention time over a period of 35 weeks using fill-and-draw mode with a synthetic medium (OECD). The composition of the feed media (Table 7.1) was a mixture of a simple soluble substrate and trace metals, which has been well characterized for work with anaerobic mixed culture (Speece and McCarty, 1964; Owen *et al.*, 1979; Grobicki and Stuckey, 1991; Noguera *et al.*, 1994; Gerardi, 2003). To minimize the possibility of inorganic salts precipitating out, the procedure for preparing 2 L of concentrated feed media was adopted and modified from previous work (Owen *et al.*, 1979). Phosphate salts, salts of magnesium and calcium, and transitional metal salts except iron (II) chloride were added to 1.8 L of ultrapure water. The feed media was autoclaved for 15 min at 121 °C and cooled to room temperature; the remaining feed ingredients and sodium bicarbonate (5 g/L) were then added to the solution while flushing with N₂ gas at approximately 1 L/min. The final pH of the feed media was adjusted to 7.2 using trace metal free grade HCl. The concentrated feed stock produced was stored under 4 °C. Ultrapure water was used to make up the feed to the correct dilution, and to give a final COD of about 2 g/L. Only ultrapure water and 5 g of sodium bicarbonate were added to reactor 6.

Table 7.1. Composition of feed

Reactor	Major constituent	C/N ratio (mg/L)	Micronutrient (mg/L)			
1	Glucose	2000:0	K ₂ HPO ₄	28	MgSO ₄ ·7H ₂ O	2
2	Glucose, urea	2000:200	KH ₂ PO ₄	28	MnCl ₂ ·4H ₂ O	3
3	Glucose, NH ₄ Cl	2000:200	CaCl ₂ ·2H ₂ O	4	Na ₂ MoO ₄ ·2H ₂ O	1
4	Fructose	2000:0	CoCl ₂ ·6H ₂ O	1	Na ₂ S·9H ₂ O	20
5	Sucrose	2000:0	FeCl ₂ ·4H ₂ O	10	NiCl ₂ ·6H ₂ O	1
6	Nil	Nil	NaHCO ₃ 1000			

7.2.3 Sample Preparation and Fractionation

Sample preparation is a critical step, and constrains most analytical processes. The supernatant obtained from the six bioreactors was centrifuged at 1450 *g* at 4 °C for 15

min, and then filtered through 10 µm filter paper to remove any flocs. Soluble samples in the supernatant were fractionated using a Slide-A-Lyzer Dialysis Cassette (Thermo Scientific, Singapore) (Yang *et al.*, 2005). Four dialysis cassettes with molecular weight cut-off (MWCO) membranes of 2 k, 3.5 k, 7k and 10 kDa were used; hence the MW distribution was categorized as follows: <2 kDa, 2-3.5 kDa, 3.5-7 kDa, 7-10 kDa and >10 kDa. The liquor was then lyophilized, and the carbohydrate content was estimated as a glucose-equivalent concentration using a phenol-sulfuric acid assay kit (Sigma-Aldrich, Singapore), while the proteinaceous material was estimated as Bovin Serum Albumin-equivalent concentration using a Micro BCA Kit (Thermo Scientific, Singapore). Although these kits were found to be inaccurate in quantification, they are simple, cheap, rapid, widely accepted and function as a relatively good estimator for global profiling.

7.2.4 General parameters and analytical methods

Methods to determine general parameters at 7-day intervals can be found in Chapter 3. GC-MS as used to identify and quantify the unknown low MW SMPs compound. The method procedure can be found in Chapter 4. The protocol for elucidation of the glycan structure and composition in biological wastewater treatment systems using GC-MS is detailed in Chapter 5. In addition, analytical procedures that characterized the amino acids profile using HPLC are documented in Chapter 6.

7.3 Results and discussion

7.3.1 Performance of the Reactors

Methane production is a key indicator when monitoring the performance of AD systems (Owen *et al.*, 1979) because it is produced from the final stage of methanogenesis, which is the most biologically sensitive (Khan *et al.*, 2016). Over more than 35 weeks of operation, good and steady performance was achieved for reactor 1, which was the control. Methane conversion was found to be near 100% (99%

COD removal), and its production rate was 1.05 L/d. No VFAs were detected, and the supernatant COD was found to be 45.5 ± 2.8 mg/L, as shown in Table 7.2.

Table 7.2. Performance of fill and draw reactors over 35 weeks

Reactor	Major constituent	Influent COD (mg/L)	Supernatant COD (mg/L)	pH	% CH ₄ conversion ^a	Gas production rate (L/d)	VFA (mg/L)	SMP (mg/L)
1	Glucose	2075 ± 22	42.9 ± 2.7	7.12 ± 0.12	99 ± 0.42	1.07 ± 0.06	ND*	42.9 ± 2.7
2	Glucose, urea	2063 ± 21	64.5 ± 2.4	7.30 ± 0.08	96 ± 0.21	1.07 ± 0.06	ND	64.5 ± 2.5
3	Glucose, NH ₄ Cl	2002 ± 4.5	926 ± 2.4	7.00 ± 0.29	54 ± 0.05	0.94 ± 0.13	534 ± 56	435 ± 47
4	Fructose	2033 ± 23	419 ± 35	7.15 ± 0.23	79 ± 0.34	1.03 ± 0.07	319 ± 45	116 ± 25
5	Sucrose	2080 ± 21	546 ± 65	7.07 ± 0.20	74 ± 0.24	1.05 ± 0.14	335 ± 49	192 ± 34
6	Nil	0	68.5 ± 10.7	6.81 ± 0.09	ND	ND	ND	68.5 ± 10.7

The number in parenthesis refers to the average value (n = 35) and the number after the (±) symbol refers to standard deviation (SD)

*ND: Not detected

^a % based on theoretical 350 mL methane per gm COD removed at STP.

Given the variety of basic carbohydrates potentially available to feed the reactors, we decided to use the ones commonly used in synthetic feeds such as glucose, and sucrose, which is a disaccharide of both glucose and fructose. In addition, to challenge the anaerobic culture we also chose fructose which is not commonly used, but when bonded to glucose forms sucrose, and represents the other half of the carbohydrate spectrum. The performance of the AD system supplemented with medium containing different substrates is presented in Table 7.2. It is evident that after more than 35 weeks the reactor system had a high capacity to degrade glucose (reactor 1), but a lower degradation rate for both fructose (reactor 4) and sucrose (reactor 5) was observed. Fructose and sucrose, although having the same chemical moiety as glucose, but different structures, did not cause complete inhibition of the sludge syntrophic and archaeal populations. It is also evident that large amounts of VFAs were left undegraded (Table 7.2) in reactors 4 and 5, even after 35 weeks of constant feeding. Glucose, a simple monosaccharide, would likely bypass hydrolysis and undergo acidogenesis converting it into VFAs. Even though glucose acidifies quickly, a high methane conversion rate suggests that the methanogens were utilizing the feed well in

the presence of a high buffer capacity and sufficient micronutrients. Sucrose, on the other hand, undergoes hydrolysis to give glucose and fructose. All cells contain hexokinase that phosphorylates glucose and fructose, but when the glucose level is high, metabolism of glucose via glycolysis is preferred over fructose. In the early stages of the experiment, there is a possibility that catabolite repression takes place in the sucrose-fed reactor (reactor 5) since the system of gene control in some bacterial operons dictates glucose to be used preferential, and the metabolism of fructose is repressed in the presence of glucose. This, however, is unlikely to be observed in reactor 1 and 4 where glucose and fructose are the only dominant carbon sources, respectively. Such a preference is due to glucose undergoing glycolysis without further modification, and requires less energy to metabolize than fructose. However, after 35 weeks, catabolite repression should not have been the main reason for lower methane production and higher VFA concentrations in reactor 5, and hence more experiments are required to understand this phenomenon.

Moreover, in the 28th week, one day after the introduction of the feed into the reactors, the VFAs in the supernatant were analyzed, and acetate production in the fructose supplemented reactor (reactor 4) was 1.5-fold higher than that in the glucose supplemented reactor (reactor 1). In this case, there are two possible explanations; firstly, the increased production of acetate may be due to lactate oxidation to acetate. Secondly, this could be due to the ATP-producing acetate reaction being promoted at the expense of the NADH-dependent production of lactate. Nevertheless, when the rate of acetate production is faster than that of acetate-utilizing methanogenesis, an unstable process results. Overall, these data suggested that the efficiency of biogas generation was strongly associated with the metabolic pathway of various carbon sources, indicating that the risk of system imbalance depends partially upon the prevailing nutritional environment as the study was carried out for more than 35 weeks.

In this study, the effect of adding 200 mg/L of ammonium-N or urea-N on the performance of AD was also investigated. It is generally believed that this concentration is below the threshold concentration of ammonia inhibition, and

beneficial to AD (Liu *et al.*, 2015). A stable AD process was achieved with the addition of urea (reactor 2), but surprisingly with NH_4Cl (reactor 3), the COD increased sharply from 42.9 to 926 mg/L, both biogas and CO_2 production decreased slightly, and the percentage of methane production halved, all of which indicated instability. In addition, the pH decreased marginally, although due to the large buffer capacity of the media this drop was small, but the VFAs were relatively high illustrating the accumulation of acids, and unstable performance. Interestingly, the degradation of urea to ammonia affected the performance to a lesser extent, and propionic acid accumulation could explain this observation. The concentration of propionic acid after the first 24 hours was 101 ± 3.7 mg/L in reactor 2, while in reactor 3 was 352 ± 2.1 mg/L; after 7 days, the propionic acid concentration in reactor 2 still showed no tendency to decrease, and this was true even after more than 35 weeks of operation. Such interactions between FAs and VFAs may lead to an “inhibited steady state”, a condition where the process runs stably, but with a lower methane yield (Angelidaki and Ahring, 1993).

7.3.2 MW distribution analysis on SMPs

Besides methane production, the level of SMPs corresponds directly to the effluent COD, which in turn indicates treatment efficiency. As shown in Table 7.2, SMPs was estimated by subtracting the COD due to intermediate VFAs and residual substrate from supernatant COD, as in the following equation, which was described in Chapter 2 (Aquino 2004):

$$\text{SMP (as COD)} = \text{Supernatant} - [\text{VFA (as COD)}] + [\text{residual substrate (as COD)}] \quad (1)$$

Since glucose was the macronutrient in the feed to the first 3 reactors, and readily biodegradable, there were no intermediate VFAs detected, and the presence of organics (SMPs) (45.47 mg/L COD) in the supernatant indicates the strong likelihood that they were produced by microorganisms.

SMPs have a broad spectrum of MW distributions. In the present study, MW distribution was identified using 2 kDa, 3.5 kDa, 7 kDa and 10 kDa MW cut-off

dialysis membranes in cross-flow membrane modules, and the low MW SMPs were determined using GC-MS. Table 7.3 summaries the global distribution profile of MW, proteinaceous material and carbohydrates found in the different reactors after 35 weeks of operation. We found that both protein-like compounds and carbohydrates less than 2 kDa had the highest concentration, while low MW SMPs contribute considerably to the concentration of total SMPs (~16% of the total SMP as COD, Kunacheva *et al.*, 2017). Among the six reactors, large amounts of low MW solutes, and low levels of high MW solutes indicate that decay associated with the products of cell lysis was steady, but some SMPs with lower MWs resist further biodegradation which resulted in their accumulation. Interestingly, under controlled conditions such as in reactor 1, the concentration of low MW SMPs was found to be even lower than in reactor 6 (the background). On the other hand, addition of NH_4Cl reduces the production of low MW (<2 kDa) proteinaceous material and carbohydrates, while inducing the production of higher MW (2-3.5 kDa) and low MW SMPs. When sucrose (reactor 5) was used as the main carbon source, both proteinaceous material and carbohydrates decreased, but higher concentrations of low MW SMPs were observed, even though all six reactors received the same amount of substrate C. These data from 35 weeks of operation, support the hypothesis that the type of substrate was a significant factor affecting the relative quantities of various SMPs, particularly the low MW SMPs.

Table 7.3. MW distribution of SMPs in 6 reactors after 35 weeks of operation

Reading $\pm$ SD		Reactors					
		1	2	3	4	5	6
MW distribution ($\mu\text{g/L}$)	<500 Da	7466 $\pm$ 40	9758 $\pm$ 40	67407 $\pm$ 75	18816 $\pm$ 40	28271 $\pm$ 5.4	10098 $\pm$ 17
	2-3.5k Da	233 $\pm$ 5.5	1423 $\pm$ 2.0	1843 $\pm$ 1.9	50 $\pm$ 5.7	212 $\pm$ 7.9	103 $\pm$ 1.1
	3.5-7k Da	117 $\pm$ 1.5	113 $\pm$ 0.5	17 $\pm$ 0.7	108 $\pm$ 0.5	35 $\pm$ 1.2	33 $\pm$ 1.3
	7-10k Da	17 $\pm$ 0.8	10 $\pm$ 0.5	23 $\pm$ 0.4	27 $\pm$ 0.5	17 $\pm$ 0.9	20 $\pm$ 0.6
	>10k Da	17 $\pm$ 1.2	13 $\pm$ 0.1	13 $\pm$ 0.1	23 $\pm$ 0.2	17 $\pm$ 0.6	13 $\pm$ 0.7
Proteinaceous material distribution ($\mu\text{g/L}$)	<2k Da	1311 $\pm$ 21	1163 $\pm$ 18	841 $\pm$ 27	1134 $\pm$ 29	942 $\pm$ 21	531 $\pm$ 21
	2-3.5k Da	62 $\pm$ 3.1	252 $\pm$ 3.1	306 $\pm$ 3.7	34 $\pm$ 1.3	45 $\pm$ 7.9	27 $\pm$ 0.8
	3.5-7k Da	24 $\pm$ 0.5	22 $\pm$ 0.6	9 $\pm$ 0.7	26 $\pm$ 0.1	11 $\pm$ 1.2	11 $\pm$ 0.6
	7-10k Da	17 $\pm$ 0.8	10 $\pm$ 0.5	23 $\pm$ 0.4	27 $\pm$ 0.5	17 $\pm$ 0.9	20 $\pm$ 0.6
	>10k Da	17 $\pm$ 1.2	13 $\pm$ 0.1	13 $\pm$ 0.1	23 $\pm$ 0.2	17 $\pm$ 0.6	13 $\pm$ 0.7
Carbohydrates distribution ($\mu\text{g/L}$)	<2k Da	6822 $\pm$ 45	6053 $\pm$ 18	4374 $\pm$ 26	5899 $\pm$ 29	4900 $\pm$ 46	2765 $\pm$ 49
	2-3.5k Da	322 $\pm$ 4.7	1308 $\pm$ 14	1594 $\pm$ 16	175 $\pm$ 3.1	235 $\pm$ 2.9	143 $\pm$ 2.0
	3.5-7k Da	126 $\pm$ 1.8	115 $\pm$ 1.6	48 $\pm$ 0.8	133 $\pm$ 2.2	57 $\pm$ 1.1	56 $\pm$ 0.4
	7-10k Da	ND	ND	ND	ND	ND	ND
	>10k Da	ND	ND	ND	ND	ND	ND

The number in parenthesis refers to the average value ($n = 35$) and the number after the ($\pm$) symbol refers to standard deviation (SD)

*ND: Not detected.

7.3.3 Impact on Low MW SMPs

Characterization of SMPs at a structural level is important for expanding knowledge about AD; Figure 7.1 shows a typical chromatography of low MW SMPs in the six differently fed reactors. In Table 7.4 we summarize 20 of the most concentrated organics with a MS library match similarity greater than 90%. Large amounts of olefins (e.g. tridecane, icosane), long chain fatty acids (LCFA) and their derivatives (e.g. stearic acid, methyl stearate), long chain alcohols (e.g. 2-octyldecan-1-ol, n-hexadecan-1-ol) contributed 50% of the total concentration of low MW SMPs, while even small amounts of a plasticizer (e.g. bis(2-methylpropyl) phthalate, bis(2-ethylhexyl) adipate) were observed.

Figure 7.1. Chromatograph of low MW SMPs in the 6 reactors.

Table 7.4. Molecular organic composition in the supernatant identified by GC/MS.

Reactor 1					
Compound name		Similarity	CAS#	MW (Da)	Molecular formula
1	2-Ethylhexanol	94	104-76-7	130	C8H18O
2	Tridecane	91	629-50-5	184	C13H28
3	2,3,5,8-Tetramethyldecane	90	192823-15-7	198	C14H30
4	cis-3-Tetradecene	90	41446-67-7	196	C14H28
5	2,6,11-Trimethyldodecane	92	31295-56-4	212	C15H32
6	cis-3-Hexadecene	95	34303-81-6	224	C16H32

7	Heptadecane	92	629-78-7	240	C17H36
8	Croctane	92	504-44-9	282	C20H42
9	Octadecane	95	593-45-3	254	C18H38
10	Icosane	92	112-95-8	282	C20H42
11	Bis(2-methylpropyl) phthalate	91	84-69-5	278	C16H22O4
12	Heneicosane	90	629-94-7	296	C21H44
13	Methyl elaidate	94	1937-62-8	296	C19H36O2
14	2-methyltetracosane	91	1560-95-8	352	C25H52
15	2-methylhexacosane	90	1561-02-0	380	C27H56
16	Benzyl butyl phthalate	91	85-68-7	312	C19H20O4
17	Bis(2-ethylhexyl) adipate	95	103-23-1	370	C22H42O4
18	Bis(2-ethylhexyl) phthalate	96	117-81-7	390	C24H38O4
19	2-Octyldecan-1-ol	90	45235-48-1	270	C18H38O
20	Stigmasterol	91	83-48-7	412	C29H48O
Reactor 2					
	Compound name	Similarity	CAS#	MW (Da)	Molecular formula
1	2-Ethylhexanol	94	104-76-7	130	C8H18O
2	p-Cresol	94	108-39-4	108	C7H8O
3	Tridecane	91	629-50-5	184	C13H28
4	2,3,5,8-Tetramethyldecane	90	192823-15-7	198	C14H30
5	Tetradec-1-ene	94	1120-36-1	196	C14H28
6	3,5-Di-tert-butylphenol	94	1138-52-9	206	C14H22O
7	Trans-9-Icosene	93	74685-29-3	280	C20H40
8	n-Hexadecan-1-ol	90	36653-82-4	242	C16H34O
9	N-Butylbenzenesulfonamide	96	3622-84-2	213	C10H15NO2S
10	Diisobutyl phthalate	91	84-69-5	278	C16H22O4
11	Methyl palmitate	96	112-39-0	270	C17H34O2
12	1-Heneicosanol	92	15594-90-8	312	C21H44O
13	Methyl elaidate	95	1937-62-8	296	C19H36O2
14	Methyl stearate	95	112-61-8	298	C19H38O2
15	Benzyl butyl phthalate	96	85-68-7	312	C19H20O4
16	Bis(2-ethylhexyl) adipate	95	103-23-1	370	C22H42O4
17	Bis(2-ethylhexyl) phthalate	97	117-81-7	390	C24H38O4
18	2-methyltetracosane	91	1560-95-8	352	C25H52
19	2-methylhexacosane	90	1561-02-0	380	C27H56
20	trans-Geranylgeraniol	90	24034-73-9	290	C20H34O
Reactor 3					
	Compound name	Similarity	CAS#	MW (Da)	Molecular formula

1	p-Cresol	97	106-44-5	108	C7H8O
2	3,5-Di-tert-butylphenol	92	1138-52-9	206	C14H22O
3	Lauric acid	93	143-07-7	200	C12H24O2
4	1-Pentadecanal	92	2765-11-9	226	C15H30O
5	Methyl palmitate	92	112-39-0	270	C17H34O2
6	Pentadecanoic acid	91	1002-84-2	242	C15H30O2
7	Icosane	92	112-95-8	282	C20H42
8	Methyl oleate	91	112-62-9	296	C19H36O2
9	Stearic acid	90	57-11-4	284	C18H36O2
10	Heneicosane	94	629-94-7	296	C21H44
11	Octadecanol acetate	95	822-23-1	312	C20H40O2
12	8-Hexylpentadecane	91	13475-75-7	296	C21H44
13	Bis(2-ethylhexyl) adipate	91	103-23-1	370	C22H42O4
14	Docosyl acetate	90	822-26-4	368	C24H48O2
15	Bis(2-ethylhexyl) phthalate	97	117-81-7	390	C24H38O4
16	2-methyltetracosane	91	1560-95-8	352	C25H52
17	2-methylhexacosane	90	1561-02-0	380	C27H56
18	Pentatriacontane	93	630-07-9	492	C35H72
19	Hexatriacontane	92	630-06-8	506	C36H74
20	Stigmasterol	90	83-48-7	412	C29H48O
Reactor 4					
	Compound name	Similarity	CAS#	MW (Da)	Molecular formula
1	2-Ethylhexanol	94	104-76-7	130	C8H18O
2	3-Ethyl-3-methylheptane	92	17302-01-1	142	C10H22
3	p-Cresol	97	106-44-5	108	C7H8O
4	1-Tridecene	95	2437-56-1	182	C13H26
5	cis-3-Tetradecene	90	41446-67-7	196	C14H28
6	3,5-Di-tert-butylphenol	92	1138-52-9	206	C14H22O
7	Lauric acid	90	143-07-7	200	C12H24O2
8	1-Heptadecene	94	6765-39-5	238	C17H34
9	Icosane	93	112-95-8	282	C20H42
10	Methyl palmitate	95	112-39-0	270	C17H34O2
11	Methyl elaidate	95	1937-62-8	296	C19H36O2
12	Methyl stearate	94	112-61-8	298	C19H38O2
13	Benzyl butyl phthalate	94	85-68-7	312	C19H20O4
14	Bis(2-ethylhexyl) adipate	96	103-23-1	370	C22H42O4
15	Bis(2-ethylhexyl) phthalate	97	117-81-7	390	C24H38O4
16	2-methyltetracosane	91	1560-95-8	352	C25H52
17	2-methylhexacosane	91	1561-02-0	380	C27H56
18	trans-Geranylgeraniol	90	24034-73-9	290	C20H34O
19	1-Octacosanol	91	557-61-9	410	C28H58O

20	17-Pentatriacontene	91	6971-40-0	490	C35H70
Reactor 5					
	Compound name	Similarity	CAS#	MW (Da)	Molecular formula
1	2-Ethylhexanol	94	104-76-7	130	C8H18O
2	3,7-Dimethyldecane	91	17312-54-8	170	C12H26
3	p-Cresol	98	106-44-5	108	C7H8O
4	1-Tridecene	94	2437-56-1	182	C13H26
5	Tridecane	91	629-50-5	184	C13H28
6	2,3,5,8-Tetramethyldecane	90	192823-15-7	198	C14H30
7	Lauric acid	93	143-07-7	200	C12H24O2
8	Pentadecanoic acid	90	1002-84-2	242	C15H30O2
9	1-Pentadecanal	90	2765-11-9	226	C15H30O
10	Nonadecane	90	629-92-5	268	C19H40
11	1-Nonadecene	92	18435-45-5	266	C19H38
12	Methyl palmitate	96	112-39-0	270	C17H34O2
13	Methyl elaidate	95	1937-62-8	296	C19H36O2
14	Methyl stearate	93	112-61-8	298	C19H38O2
15	Benzyl butyl phthalate	94	85-68-7	312	C19H20O4
16	Bis(2-ethylhexyl) adipate	97	103-23-1	370	C22H42O4
17	Bis(2-ethylhexyl) phthalate	97	117-81-7	390	C24H38O4
18	2-methyltetracosane	91	1560-95-8	352	C25H52
19	2-methylhexacosane	91	1561-02-0	380	C27H56
20	Pentatriacontane	90	630-07-9	492	C35H72
Reactor 6					
	Compound name	Similarity	CAS#	MW (Da)	Molecular formula
1	2-Ethylhexanol	93	104-76-7	130	C8H18O
2	p-Cresol	95	106-44-5	108	C7H8O
3	1-(2-Butoxyethoxy)ethanol	93	54446-78-5	162	C8H18O3
4	1-Pentadecene	95	13360-61-7	210	C15H30
5	Pentadecylic acid	90	1002-84-2	242	C15H30O2
6	1-Heptadecene	95	6765-39-5	238	C17H34
7	Nonadecane	91	629-92-5	268	C19H40
8	Icosane	90	112-95-8	282	C20H42
9	2-Hexyldecanol	90	2425-77-6	242	C16H34O
10	2-Octyldecan-1-ol	90	45235-48-1	270	C18H38O
11	1-Nonadecene	93	18435-45-5	266	C19H38
12	Diisobutyl phthalate	93	84-69-5	278	C16H22O4
13	Methyl palmitate	96	112-39-0	270	C17H34O2
14	Palmitic acid	90	57-10-3	256	C16H32O2

15	Methyl elaidate	95	1937-62-8	296	C19H36O2
16	Methyl stearate	93	112-61-8	298	C19H38O2
17	2-Octyldecanol	90	5333-42-6	298	C20H42O
18	Benzyl butyl phthalate	94	85-68-7	312	C19H20O4
19	Bis(2-ethylhexyl) adipate	97	103-23-1	370	C22H42O4
20	Bis(2-ethylhexyl) phthalate	97	117-81-7	390	C24H38O4

The presence of olefins (medium: C18-C27 or long: C25-C35) can be explained by the decarboxylation of unsaturated fatty acids, or the decarbonylation of aldehydes as the primary mechanism (Dennis and Kolattukudy, 1992; Schirmer *et al.*, 2010). Fatty aldehydes such as 1-pentadecanal (Table 7.4) and fatty alcohols are likely the biosynthetic intermediates in this process. These longer-chain olefinic hydrocarbons (>C35) such as pentatriacontane (Table 7.4) are thought to derive from a head-to-head fatty acid condensation mechanism (Albro and Dittmer *et al.*, 1969). The role of these hydrocarbons in microorganisms is still not completely understood, but it is suggested that extracellular saturated hydrocarbons are involved in the autoregulation of cell adhesion to surfaces, and promote cell aggregation (Nikolaev *et al.*, 2001). In the case of reactor 3, when large amounts of VFAs were produced, higher level aliphatic hydrocarbons were also observed. It is believed that they are associated with the formation of capsules on the cell surface, and protect the microorganism from high concentrations of excreted acids (Bagaeva and Zinurova, 2004).

Bacterial survival also depends on membrane lipid homeostasis, and on the ability to adjust lipid composition so that the cell can acclimatize to different environments. The adjustments in fatty acid composition that maintain the biophysical properties of cell membranes are referred to as homoviscous adaptation, and are interpreted as a mechanism that modifies the permeability of the lipid bilayer to minimize energy expenditure and optimize growth (Cronan and Gelmann, 1975). When bacteria are subjected to abrupt changes in their extracellular environment, the existing LCFAs rapidly adapt to the new conditions, and through this process, the LCFAs are either eliminated, or recycled to maintain membrane homeostasis. Huge amounts of LCFAs

and their derivatives were found in reactors 2-6, and it is suggested that bacteria in these reactors were possibly modifying their existing membrane lipids to decrease the membrane permeability of ammonia (reactor 2 and 3), or to increase their stability due to acid stress (reactor 3-5), or to conserve energy by recycling the fatty acids due to starvation (reactor 6).

Although no plastic materials were used in the extraction procedure, a few phthalates were found at concentrations greater than detection limits in the SMPs analysed. Other workers in our laboratory have carried out control studies by soaking tubing and reactor parts in ultrapure water for 30 days, and found that most of the phthalates were probably biologically produced (Kunacheva *et al.*, 2017).

Remarkably, we found that biodegradation preferentially mineralized oxidized hydrocarbons, whereas reduced aliphatic compounds were generally at a higher concentration, and thus plausibly persist in the aquatic systems. This in turn has prompted a new view on the dynamics of this type of organic carbon – whether the persistence of some SMPs is due to their intrinsic molecular properties/structures, or because of extrinsic factors such as physiochemical and biological influences from the surrounding environment that controls the probability and therefore rate of decay, thereby allowing the organic matter to persist. Accordingly, the molecular structure of biomass and organic matter has long been thought to determine their long-term degradation rate in natural aquatic systems (Hedges *et al.*, 1997). For example, the change of Gibbs free energy for the uptake of LCFAs is positive;

Long chain olefins often persist longer than solutes such as long chain alkanolic acids in anaerobic environments. Nevertheless, the persistence of organics may be due to the

complex interactions between the organic and its environment, such as the interdependence of compound chemistry, acidity, redox potential, and the presence of potential degraders in the immediate microenvironment. In other words, compound chemistry has a very important effect on biological degradation rates, but its influence is also dependent on the physical environment. The concentration of low MW SMPs in reactor 1 was noticeably lower than in reactor 6 (Table 7.4), and this may be due to the lower production of SMPs, although there is no clear reason for this, and hence these are probably the refractory end-products of the mineralization of more complex organics. Nevertheless, it can be seen from Table 7.4 that other potentially degradable compounds, such as PS or proteinaceous material, cannot be degraded completely within this suitable environment in the short retention time of 7 days.

7.3.4 Occurrence of FAAs

FAAs are one source of the endogenous or exogenous AAs in biological systems, and provide an elementary connection between metabolism and the proteome, since the FAAs and those incorporated into peptides and proteins are essentially the same. In addition, certain amino acids at nanomolar concentrations may be a widespread signal for biofilm disassembly (Kolodkin-Gal *et al.*, 2010). On the other hand, FAAs cannot be removed by coagulation (Chinn and Barrett, 2000), and their chlorination can lead to the formation of haloacetamides (Shah and Mitch, 2012), an emerging class of nitrogenous DBP which are of environmental concern. However, in the present study our analysis of FAAs does not enable us to identify specific proteins/peptides since they were probably degraded due to enzymatic hydrolysis in the reactor.

Figure 7.2. Heat map of 15 FAA in 6 reactors supplemented with different substrate after 35 weeks of operation (n = 1). Value represents the concentration in mmol/L.

The occurrence of FAAs during AD is presented as a heat map (Figure 7.2), and are exclusively of microbial origin, since neither proteins nor peptides were present in the feed. Generally, the abundance of 15 AAs in each reactor was found to be diverse, but typically less than 2 mmol/L; the exception was in the NH_4Cl -fed reactor where ammonia-induced inhibition of proteinaceous uptake was likely. One explanation for this observation could be that the peptidases expressed by microorganisms have been inhibited due to the high amounts of ammonium (200 ± 15 mg) since deamination of proteins will produce addition NH_4^+ (Barker, 1981). High concentrations of VFAs may inhibit FAA uptake, as deamination also generates VFAs, as shown below;

Proteinaceous materials must be degraded to FAAs before they are absorbed by cells. AAs can be utilized in different ways by different microorganisms (Greenberg 1961), and each has characteristic pathways by which they are degraded. Phenylalanine, for example, can be degraded by some bacteria via homogenetic acid and by others via β -oxaloadipic acid (Barker, 1961), while P-cresol (Table 7.4, reactor 2-6) is known to be one of the metabolic end-products of phenylalanine. However, some the end-products

of AAs are very similar to those of the degraded carbohydrates, and not all favorably degrade to small organic compounds such as aspartic acid, which has a large positive Gibbs free energy under standard conditions.

Overall, these data suggested that the presence of different carbon substrates or nitrogen sources possible induce readjustments in AA fermentative metabolism. It is evident that there is a paucity of knowledge on the biochemistry of these processes, and that a much better understanding of the processes is imperative for the efficient characterization and control of AD, and formation of SMPs.

7.3.5 Polysaccharide composition

One advantage of obtaining monosaccharide linkage composition is the potential to estimate the relative proportion of different saccharides from a single analysis. The accuracy of this estimation depends on prior knowledge of existing classes of bacterial PS that are likely to be present. It will also depend, amongst others things, on the taxonomic origin of the bacteria and sample treatment. In the case of biological treatment systems, it is impossible to identify the origin of the microorganisms in a mixed culture. Nevertheless, information obtained through the protocol discussed above can still be used to assign monosaccharide linkages, and thus associate them with the possible free EPS present in various reactors, as shown in Table 7.5.

Although the theoretical number of structures that can be derived from combining a certain number of monosaccharides is high (Bertozzi and Sasisekhran, 2009), the actual number of structures naturally occurring is fortunately significantly smaller, and there are often nested structures, reflecting the limited number of monosaccharides and specific biosynthetic machinery present in a given biological context. Table 7.5 shown the descending ranking of various monosaccharide linkages presented in percentage mole, and most of the saccharide monomers are neutral glucose (Glc_p), galactose

(Galp), mannose (Manp), and rhamnose (Rhap) residues, and a polyanionic glucuronic acid (Glc pA) residue with various linkages.

Due to the diversity of linkage types, bacterial EPS presents a wide range of physical properties. It is these linkages in PS, coupled with variations in monomer sequence, that produce diverse possible structures (Atkins, 1985). The effects which certain monosaccharide residues confer on their physical properties can be seen in Table 7.5. It is clear that these physical attributes could be determined based on the chemical composition and structural niceties of the PS. For example, a composition of $\beta 1 \rightarrow 4$ or $\beta 1 \rightarrow 3$ linkages may confer considerable rigidity, while $\alpha 1 \rightarrow 2$ linkages may yield more flexible structures (Sutherland, 2007). Furthermore, it has also been suggested that the presence of a significant amount of linear neutral monosaccharides with $\beta 1 \rightarrow 4$ or $\beta 1 \rightarrow 3$ linkages tends to decrease the solubility of the PS, and with the presence the right amount of uronic acid residues, they yield highly viscous aqueous solutions (Geremia and Rinaudo, 2004; Sutherland, 2007), as in the case of reactor 3. The uronic acid residues modify the original stereoregularity and helical conformations in solution; their altered semi rigid character is further affirmed in the presence of several monovalent or divalent cations (Geremia and Rinaudo, 2004). As the molar ratio of ionic substituents increases, the solubility of the PS is amplified because of the strong ionic and electrostatic interactions.

Nevertheless, it is also not uncommon that 15-20% of the linkages are not assigned because of the varied and dynamic nature of their polysaccharide structures, and experimental analytical errors, and this can be seen in Table 7.5 where the total top five monomeric structures only sum to 80-90%. It is also notable that the information generated from this type of analysis is indicative only, and should not be used to assign absolute composition. Despite this, it is a relatively simple and effective measure of free EPS composition that can be used as the basis for further in-depth analysis.

Table 7.5. Monosaccharide linkage composition with possible polysaccharide characteristics based on different feed compositions.

Reactor	1	2	3	4	5	6
Major Feed Constituent	Glucose	Glucose, Urea	Glucose, NH_4Cl	Fructose	Sucrose	None
C/N ratio (mg/L)	2000:0	2000:200	2000:200	2000:0	2000:0	None
Total free EPS concentration (mg/L $\pm$ SD)	11.0 $\pm$ 0.35	12.0 $\pm$ 0.35	18.2 $\pm$ 0.15	9.45 $\pm$ 0.15	10.7 $\pm$ 0.25	1.4 $\pm$ 0.15
Statistically significant difference in total against reactor 1 (p = 0.05)	-	No	Yes	Yes	No	Yes
Monosaccharide (Mol % $\pm$ SD)	(25.6 $\pm$ 0.3%)	(22.4 $\pm$ 1.1%)	(28.8 $\pm$ 0.5%)	(24.4 $\pm$ 0.2%)	(26.3 $\pm$ 0.1%)	(23.6 $\pm$ 0.1%)
	(24.3 $\pm$ 0.6%)	(19.6 $\pm$ 0.4%)	(27.6 $\pm$ 0.4%)	(22.8 $\pm$ 0.2%)	(24.7 $\pm$ 0.2%)	(21.9 $\pm$ 0.2%)
	(22.4 $\pm$ 0.6%)	(18.5 $\pm$ 0.3%)	(13.0 $\pm$ 0.3%)	(21.4 $\pm$ 0.1%)	(22.8 $\pm$ 0.3%)	(20.4 $\pm$ 0.2%)
	(9.7 $\pm$ 0.4%)	(14.0 $\pm$ 0.2%)	(10.5 $\pm$ 0.3%)	(12.5 $\pm$ 0.3%)	(8.4 $\pm$ 0.2%)	(18.5 $\pm$ 0.5%)
	(7.9 $\pm$ 0.1%)	(7.8 $\pm$ 0.2%)	(6.5 $\pm$ 0.5%)	(9.7 $\pm$ 0.1%)	(5.8 $\pm$ 0.1%)	(2.2 $\pm$ 0.1%)
Total Monosaccharide (Mol % $\pm$ SD)	89.9 $\pm$ 0.8%	82.3 $\pm$ 0.6%	86.4 $\pm$ 1.0%	90.8 $\pm$ 0.6%	88.0 $\pm$ 0.9%	86.6 $\pm$ 0.6%
Physical properties	soluble	soluble	Viscous solution with a loose gel-like behaviour	soluble	slightly viscous	soluble

Note:

The number in parenthesis refers to the average value (n = 2) and the number after the ($\pm$) symbol refers to the standard deviation (SD)

7.3.6 Physiological aspects of EPS production

The formation and excretion of EPS is a process requiring considerable amounts of energy, and is favored by growth under conditions of a plentiful and readily utilizable carbon source. The extent of EPS production could depend on the types of precursors, and also on other physiological conditions employed. Since the biomass concentration in each reactor over the 35 weeks was relative constant, the amount of free EPS should not change based on biomass variations. However, when there is no freely available energy source, microorganisms stop producing EPS, as can be seen in reactor 6 where there is no carbon source. This presumably can be attributed to the conservation of energy in preference to EPS synthesis.

Carbohydrates are widespread in nature and serve as initial substrates for bacteria in AD. On a molecular basis, the initial steps in the EPS biosynthesis pathway essentially follow Enter-Doudoroff (Enter and Doudoroff, 1952), and the carbonaceous substrates are metabolized intracellularly to intermediate compounds - “sugar nucleotides” that serve as precursors and, in turn, form energy-rich monosaccharide donors for EPS synthesis and provide a means for interconversion (Geremia and Rinaudo, 2004; Novotny *et al.*, 2008). It was found that the amount of total free EPS produced in the fructose-fed reactor (reactor 4) was statistically lower (95% confidence level ($n=2$, $p = 0.05$) than in the glucose-grown cultures (reactor 1), while the sucrose-fed (reactor 5) appeared lower, but was not statistically significant. Given that sucrose is a disaccharide composed of fructose and glucose, this means during its hydrolysis and metabolism half the feed is actually glucose, and hence it is perhaps not surprising that the total free EPS produced from sucrose was not very different from the pure glucose feed. In addition, only one of the constituent monosaccharides was statistically different from those produced by glucose.

With fructose the total free EPS was significantly different from glucose, and had a different monomeric sugar composition, as observed in Table 1, and 3 out of the 5 major monomers produced from fructose were statistically different from glucose. It is

noteworthy that these EPS are free and soluble, and are not cell bound; there is evidence that most of the EPS is distributed on the outer layer of the cell (bound EPS), and one study even reported that the EPS content on these outer layers was about four times greater than free EPS (Wang *et al.*, 2005). This further suggests that the regulation of the biosynthetic pathway of EPS, or free EPS, and that of the monomeric saccharide composition, both depend on the nature of the monosaccharide in the feed. It was proposed that a regulatory enzyme, fructose 1,6-bisphosphatase had an activity much lower than that of 6-phosphofructokinase, which is in the “backward” direction in the synthesis of “sugar nucleotides” (Reizer and Peterkofsky, 1987); and this leads to a lower production of EPS in a fructose-fed medium (Table 7.5).

The C source is not the only parameter affecting EPS synthesis, although it was reported that an adjustment in the C/N ratio could potentially improve the characteristics of the feed and produce less EPS (Lin *et al.*, 2014). While we did not change the C/N ratio in the feed due to the work involved, our work suggested that the use of a different nitrogen feed (at a C/N ratio of 10) promotes the production of EPS, although the addition of urea did not lead to a significant increase in total EPS ($p>0.05$) in contrast to ammonia addition. It can also change the monomeric sugar composition noticeably, and 3 out of the 5 monomeric sugars with both N sources were significantly different from glucose alone. As shown in Table 7.5, reactors 2 and 3 had a higher estimated free EPS concentration (based on duplicates) than those reactors that had no N in the feed, while the ammonium-fed reactor (reactor 3) had the highest amount among all the reactors. In addition, comparison between the two N sources with glucose revealed that they produced significantly different amounts of free EPS. Even though the theoretical total ammonium nitrogen (TAN) was 200 mg/L for both reactors 2 and 3, reactor 3 had a net 200 mg/L free ammonia nitrogen (FAN). It is this FAN that diffuses into microbial cells and apparently alters the balance of intracellular activities and normal material transport (Sheng *et al.*, 2010). This in turn reinforces the idea that changes in EPS composition will bring about different interactions between bacteria and their extracellular environment by modifying the normally negatively charged

surface to repel NH_4^+ cations (Lin *et al.*, 2014; Sheng *et al.*, 2010). Such action is an inherent mechanism apparently that protects the cell from fluctuations in environmental conditions (Beveridge and Graham, 1991; Sutherland, 2007; Lin *et al.*, 2014).

7.3.7 Possible implications for membrane fouling

EPS are one of the most important classes of membrane foulants, and may affect membrane performance through pore clogging, and adhesion to the membrane surface, while leads to biofouling. This enhances cake layer consolidation and results in profound impacts on membrane fouling (Sheng *et al.*, 2010; Lin *et al.*, 2014) leading to a significant rise in the overall cost and energy use.

The correlation between free extracellular polysaccharides and membrane fouling is illustrated in Figure 7.3. Based on our results, it is known that most of the free EPS consist of varying amounts of the negatively charged uronic acid moiety (Table 7.5), and monomer composition changes with feed composition, and from a structural point of view, the uronic acid moiety is a unique component of EPS. Although present at relatively low concentrations, the adsorption of free EPS will be bolstered by the ubiquitous divalent cations on the membrane surface through the formation of metal-bridges with the negatively charged membrane surface (Herzberg *et al.*, 2009; Mo *et al.*, 2012). In addition, blocks of uronic acid moieties are able to yield an array of coordination sites that assist divalent cations in their cavities, and provide a gel-forming capacity (Figure 7.3-D). In contrast, other blocks of the free EPS that are neutral will provide the chain with a flexibility and network structure during gelation. After adsorption more free EPS and other extracellular polymeric substances form a gel layer which radically alters the characteristics of the membrane surface (Tang *et al.*, 2011), and hence may change the membrane fouling propensity. Concurrently or subsequently, free EPS will further mediate in bacterial attachment and reinforces the biofilm structure which is likely to be the same as those resulting from EPS self-assembly (Bar-Zeev *et al.*, 2012). Again, being negatively charged these organic films will significantly increase bacterial attachment by hydrophobic interactions and hydrogen

bonding with overlying cells (Hwang *et al.*, 2013). Such phenomenon of bacterial-surface, particle-particle and particle-surface interactions have been described by the extended Derjaguin, Landau, Verwey, and Overbeek (XDLVO) (Hermansson, 1999) theory in conjunction with electrokinetic theory (Ohshima, 1994).

Figure 7.3. Schematic illustration of some membrane fouling mechanisms: (A) Cell biomass and EPS. The inner layer consists of tightly bound EPS (TB-EPS), which is bound tightly to the cell surface. The outer layer is loose and dispersible free extracellular polymeric substances, which arises from cell growth, cell lysis, or from attachment. (B) Pore clogging. Most organics, including SMPs and colloids, could enter the membrane pores and then partially accumulate due to their “sticky nature” (i.e., characterized by adhesive force up to 8.5 nN determined by atomic force microscopy) (Bar-Zeev *et al.* 2012). (C) Biofilm. Development of floc adhesion and gel layer formation emphasizing the contribution of the extracellular polymeric substances species. This will subsequently lead to the formation of a second “dynamic” membrane – cake layer. (D) Ion bridging. Extracellular polysaccharides contain an uronic acid moiety, which is negatively charged at near-neutral pHs, and will react with divalent cations such as calcium and magnesium ions to form complexes. This ion bridging mechanism can also enhance polymer entanglement, facilitating the adhesion of foulants, and strengthening the cake layer structure.

7.4Conclusions

In this present study, the impact that certain feed carbohydrates and sources of nitrogen have on the production of SMPs in anaerobic digestion was examined. Our data suggested that the efficiency of biogas generation (COD removal) was noticeably

associated with the metabolic pathway of various carbon/nitrogen sources, indicating that the risk of system imbalance to some extent depends on the prevailing nutritional environment as the study was carried out for more than 35 weeks, and hence was not a measure of transient behavior. Moreover, it is believed that the composition of substrate also alters the physicochemical characteristics of the proteinaceous material and free EPS. Our findings also highlight the fact that previous studies on the C/N ratio in the feed tended to overestimate EPS production, while variations in the chemical nature of the nitrogen source were overlooked. Therefore, a good knowledge of the composition of the substrate to be treated anaerobically is necessary in the interpretation of the behavior of a reactor, particularly in the co-digestion of other wastes. Furthermore, well-characterized nutritional conditions ensure a cooperative bacterial symbiosis involved in the conversion of complex organic matter to methane. Finally, a deeper understanding of the pathways which form SMPs and EPS, and their dynamic physiochemical nature, could result in new pretreatment methods for the efficient removal of them from the supernatant, and novel cleaning strategies targeting the membrane surface.

CHAPTER 8

Concluding Discussion

8.1 Introduction

The extent to which this work has fulfilled the initial objectives is concluded at the beginning of this chapter. The importance of the findings and their implication are then discussed. Finally, the areas of research that could be further explored, and the knowledge gaps that need to be further investigated are highlighted in the last section.

8.2 Final conclusions

The main objectives of this work were to improve the existing protocols and develop new methods that characterize, by means of chromatography and mass spectrometry techniques, chemical compounds in SMPs and EPS in the supernatant or effluent from anaerobic bioreactors. We also intended to investigate whether macronutrients such different carbohydrate and/or nitrogen sources played any role in influence SMPs production. Based on these objectives (Section 2.9), the following conclusion can be drawn:

(I) Chemical characterization of low MW SMPs in a SAMBR

- In Chapter 2, it is known that effluents from wastewater treatment systems contain a variety of organic compounds, including end-products from the degradation of influent substrate, non-biodegradable feed compounds, and, in particular, SMPs produced by microbial metabolism. The importance of clearly identifying these SMPs to understand the fundamental mechanisms of biological activity that create these compounds was emphasized. Section 2.7.1 summarized various analytical methods that have been used to date to analyze individual compounds in SMPs, and highlighted their shortcomings.

- In Chapter 4, we presented an optimized physical pre-pretreatment of wastewater samples to extract and concentrate low MW SMPs from the effluent of a SAMBR. A combination of SPE (Oasis HLB) followed by LLE (n-hexane, chloroform, dichloromethane) was shown to maximize compound identification and quantitation.
- An approximate quantification was done and the combined concentration of the low MW compounds accounted for 2.1 mg COD/L (16% of the total SMP as COD calculated from 3.46 gO₂/g docosane). While concentration estimation using an alkane standard may not be the most accurate way to quantify SMPs, this is currently the best estimate and most practical for unknown samples so that it is also beneficial to compare the results of different researchers.
- In Chapter 7, the optimized pretreatment methods successfully and specifically identify the greatest number of low MW SMPs in different AD reactors. This proposed new method is further demonstrated that it is a good step towards further characterizing and understanding SMPs produced from bioreactors at molecular level.

(II) Measurement of carbohydrates in biological wastewater treatment systems

- As discussed in Section 2.7.2.1, many studies on the identification of effluent SMPs show that most of these compounds were not present in the influent. Among them, PS are of great importance and have been extensively studied. Despite the development of various analytical techniques, the most rapid, simple and cheap approach for measuring the total carbohydrate concentration are the colorimetric methods.
- However, despite their simplicity, there are major drawbacks when using these assays. In Chapter 5, we presented a study where four laboratory preparations and three commercially available assay kits were tested on the same carbohydrates samples with the addition of 14 different possible interfering solutes typically found in wastewater treatment plants. This work

shows that a wide variety of solutes can interfere with these assays. In addition, a comparative study on the use of these colorimetric methods with different carbohydrates was also investigated, and the metachromatic response was clearly influenced by variation in sample composition.

- Nonetheless, assessing carbohydrate concentration and characteristics on the basis of colorimetric assays that reference glucose leads to a misrepresentation of PS content, especially when studying a complex environment of which wastewater constitutes. Hence, a new analytical method using GC-MS was developed to measure the monomeric structure of exopolysaccharides in anaerobic bioreactors.
- This newly improved method can be used routinely in a reasonably equipped laboratory to determine simultaneously the both neutral and acidic monosaccharides, and how each residue is linked within the free exopolysaccharides. In Chapter 7, this method sheds some light on the diversity of exopolysaccharides present in wastewater, and the link between exopolysaccharides' physiochemical properties.

(III) “Protein” measurement in biological wastewater treatment systems

- We understood from Chapter 2 that proteins are one of the main compounds present in various wastewater systems, and that they seem to be strongly correlated with the aggregation process and flocculation ability. However, there is a lack of thorough validation study on different protein assays currently used.
- In this companion study unveiled in Chapter 6, five commercially available protein assay kits were tested on the same BSA molecule with the addition of 17 different types of interfering substances, and comparison of the use of these colorimetric methods with 22 different protein and peptide samples was also presented. Similar to carbohydrate measurements, it was shown that a wide variety of substances can interfere dramatically with these assays; the metachromatic response was clearly influenced by different

proteinaceous materials. Quantitative results of the “protein” concentration in the different effluent samples, with or without spiked addition of BSA, showed considerable disagreement.

- Nevertheless, given the complexities, the vast dynamic range of proteinaceous material concentrations, and analytical limitations associated with traditional colorimetric methods, using these assays for an accurate measurement of “protein” in wastewater samples was not recommended. Thus, in the later part of Chapter 6, a method analyzing 23 primary and secondary FAA by simple, direct and rapid pre-column derivatization and HPLC was adopted.
- This FAA analysis method effectively identified and quantified the proteinaceous material found in wastewater. Its application was presented in Chapter 7, where new insight into production of FAA in a particular nutritional condition was revealed.

(IV) Impacts of feed carbohydrates and nitrogen source on the production of SMPs in AD

- Key nutrients are required to supplement basic cellular metabolism for microbial growth, and ensure the cell synthesizes the enzymes and cofactors that are needed to drive the biochemical and metabolic reactions. However, both carbon and nitrogen are known to be a primary source of macronutrients for microbial growth, but there is little or no studies on their effect on the formation of SMPs.
- In this comprehensive study presented in Chapter 7, six stirred fill-and-draw batch reactors with a range of carbohydrate feeds (glucose, fructose and sucrose), and nitrogen sources (ammonium salt and urea) at various concentration were used to investigate the effect of feed composition on the production of SMPs during AD. To gain greater insights into the SMPs formed, the composition of various fraction was analyzed by employing all the newly developed techniques. Note, however, this level of chemical

analysis has never been carried out in the past because of analytical limitations.

- It was found that the presence of FAN at 200 mg/L stimulated the production of not only VFAs, but also SMPs of different MW fractions, and reduced the production of biogas noticeably. Moreover, the study revealed that the type of SMP that dominates in a particular system depends on the chemical characteristic of the feed, and this insight has profound implications on the composition of the effluent from AD reactors (and their potential chlorination by-products), and membrane fouling in membrane bioreactors.
- Such insights demonstrate that past studies on C/N ratios tended to overestimate SMPs production, while variations in the chemical nature of the nitrogen source were overlooked. The discoveries provide fresh and valuable insights into an area that is plainly under researched, and often disregarded by specialists studying overall engineered wastewater treatment systems.

8.3 Implications of this research

Anaerobic treatment has proven to be an effective way of degrading organic matter in sewage, sludges and various wastewaters. Anaerobic technology is cheaper than aerobic, and is unique because of its potential for the production of renewable biogas. Such features unquestionably contribute to the wider application of this technology, however, one key aspect which mitigates against these benefits is the comparatively low treatment efficiency compared to aerobic systems. AD needs to achieve lower effluent COD levels in order to compete against aerobic systems, and convince sceptics. Considerable research has been carried out on reactor configuration, and various technical parameters and microbial ecology in reactors, however, only limited research has looked more deeply into the characterization of SMPs, which are microbial products. This research has gone one more step further by improving the

characterisation methodology employed to investigate the characteristics and origins of effluent COD, and exploring the factors that affect the production of SMPs in AD. We proposed a new method that is a good first step toward understanding chemical characteristic of low MW SMPs. By combining SPE and LLE in the pretreatment of effluent samples before GC-MS resulted in an order of magnitude increase in the SMP compounds capable of being identified, and a total of 255 compounds were found in our study.

The findings in Chapter 5 reinforce one of the central premises that measuring carbohydrates in biological wastewater treatments on the basis of colorimetric assays is not specific, and quantifying exopolysaccharides based on glucose content is fraught with error. Even though various distinctively different PS were characterized in wastewater treatment systems, reliable quantification of carbohydrates has proven to be a real challenge. For decades most of the EPS that has been isolated is from either aerobic or facultative anaerobic conditions, and there has been very little work on the products from strict anaerobes. Furthermore, being heterogeneous in nature, accurately characterizing EPS composition, and quantifying their concentration in different wastewater systems is not straightforward. Their chemical composition and structure has remained uncertain for a long time, and much of the knowledge of their nature has been largely been based on indirect evidence. We successfully developed a suitable method for isolating, charactering and quantifying extracellular polysaccharide in anaerobic bioreactor, and further pushed the boundaries of our understanding of their structures and properties.

It is known that a detailed evaluation of colorimetric protein quantification methods is often time-consuming and hence has received very little attention. In Chapter 6, we critically evaluate the existing assay techniques to see they can accurately measure “protein” in the wastewater samples. By comparing the performance of these assays simultaneously and comprehensively with a large variety of proteinaceous materials, the data generated clearly shows that all the assays wrongly estimated by varying degrees. Moreover, the use of BSA as a reference standard could possible introduce

errors since it is not an appropriate protein standard for estimating various proteinaceous materials present in biological wastewater treatment systems. Later, a method that can analyze 23 primary and secondary AAs with low detection limits and high accuracy was adopted. These results suggested that the quantitative contents of certain FAAs could be a good indicator of the efficiency of anaerobic processes.

Last but not least, the composition of the substrate is clearly a major factor that determines the characteristics of an ecosystem in AD, and its efficiency. Our results suggest that the physiological differences associated with the digestion of various carbon and/or nitrogen substrates clearly alters metabolic pathways, indicating that the risk of system failure to some extent depends on the prevailing nutritional environment. Knowledge of the composition of the substrate to be treated anaerobically is important in the interpretation of the behavior of a reactor, particularly in co-digestion with other wastes. Well-characterized nutritional conditions ensure a cooperative bacterial symbiosis converting complex organic matter to methane.

As already indicated in Chapter 7, a wide variety of organic compounds can be produced in an AD system. Heterogeneous microbial communities, such as those in AD, will effect sequential waste removal when complex substrates are biodegraded because certain substances are more easily degraded than others. The composition of a waste fed to a symbiotic relationship determines the eventual combination of solutes such as low MW SMPs, FAAs, proteinaceous material and carbohydrates remaining in the system, as well as the corresponding biological activities of such a system. This work not only analyzed the monomeric components, but also their specific linkage patterns, and their modification associated with changes in carbon substrates or nitrogen sources, which can have profound biological consequences, is revealed. We expect our methodologies will help in laying the groundwork necessary for probing the structural details of various SMPs/EPS, and represents a step towards understanding the role they play in anaerobic processes and membrane fouling.

8.4 Recommendation for future research

Based on the findings of this research, and on the difficulties faced throughout the study, the following recommendations for future research are made:

- Current developments in water science and technology are often dependent on what has been evolving in other sectors, such as advanced analytical instruments, biotechnology and biochemical knowledge. These emerging technologies are often disruptive, but require water professionals to be aware of the advances taking place, and to learn from other disparate sectors. Collaboration is critical to finding solutions in an increasingly interconnected world.
- Analysis of high MW SMPs and EPS in the supernatant can be achieved, however, only up to a certain molecular weight (~ 2 kDa). Chapter 4 showed chemical characterisation of low MW SMPs of mostly non-polar molecules (< 580 Da) using GC-MS. Recently, Tiphara *et al.* (2017) identified a range of polar and non-polar metabolites and lipid SMPs (100 to 1000 Da MW) using UPLC-ESI-Q-ToF. Moreover, Zhou *et al.*, 2017 investigated the sub-visible particles and colloids (0.45 -10 μm) using photometric analysis. Nevertheless, there is still a profound lack of understanding of SMP macromolecules in the range of 1000 Da up to 0.45 μm (Figure 4.1). Possible techniques that seem to hold promise for the detection of high MW SMPs are MALDI-ToF-MS, and tandem mass spectrometry (MALDI-ToF-MS/MS) since they are diagnostic tool for microbial identification and characterization based on the detection of the mass of molecules.
- It is known that SMPs are the final products of the natural biodegradation process, but little is known about whether they are important DBP precursors in wastewater treatment, and worsen the DBP problem in water supplies. Since large numbers of solutes have been identified in our study, as well as in previous work, these compounds should be evaluated for their DBP formation potential (DBFPF). In addition, the effectiveness of other SMP removal methods (e.g. carbon adsorption, precipitation) as well as the related change in DBFPF of the water after the treatments should also be evaluated.

- Clearly, our direct knowledge of the role of exopolysaccharides is still rather limited. It is possible that each mixture of microbial species could be regarded as distinct, with its own unique microenvironments. The variance between exopolysaccharides of different structures and different physical properties in complex mixtures provides an enormous number of possible microenvironments, and requires much more detailed study. Hopefully, with a combination of molecular biology techniques of isolation and pure culturing, with chemical characterisation, this may allow for the determination of the species and whether they are responsible for the production of certain EPS that are of particular interest in fouling and act as DBP precursors. This may provide a clearer picture of the exact function(s) of these vital constituents of SMPs, as well as developing new approaches for minimizing membrane fouling in MBRs.
- Improvements in analysing proteinaceous materials should be continued. Chapter 5 shows that colorimetric techniques for protein analysis for wastewater sample are totally non-specific, and often lead to large errors. Even though an HPLC method was established, proteinaceous materials are still considered to be a very difficult challenge to identify specifically. Although not reported in this thesis, we attempted to extract and concentrate proteins from effluent or supernatant samples. The results we obtained were poor due to the dilute nature of proteins in the effluent, and the complexity of the matrix present in the supernatant. Nevertheless, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separation enabled the identification of a single protein (Higgins and Novaks, 1997). Interference with humic substances, however, can hinder protein visualisation in SDS-PAGE (Denecke, 2006), and this could explain the small number of research reports in the wastewater treatment literature. Recently, two dimensional gel electrophoresis (2DE), which enables the concurrent visualisation of multiple proteins/polypeptides in a single analysis was used to provide a metaproteomic analysis of biocake protein in order to understand membrane fouling in a SAMBR (Zhou *et al.*, 2015). This could be employed with the purpose of overcoming shortcomings such as MW distribution encountered in earlier studies for proteinaceous material identification.

- The impact of co-digestion of different carbohydrate substrates and proteinaceous materials in AnMBRs could be explored. Systematic research on the impact of influent carbon and nitrogen sources on membrane fouling is still lacking. The influence of influent characteristics on membrane fouling, with particular emphasis on the chemical characterization of the main foulants, should be further explored. Furthermore, the microbial mechanisms of co-digestion are still largely unknown, and thus it would also be important to gain a deeper understanding of the biochemistry behind why such organics are synthesized, their biological, physical or chemical functions, and eventually find ways to minimize their production.
- Lastly, more advanced proteomic techniques involving isobaric or fluorescence labeling and LC separation along with size exclusion and other high-resolution chromatographic techniques with border proteome coverage will enable more information to be obtained on proteomic responses to nutrients. Consequently, deeper enzymatic and metatranscriptomics studies are needed for a better understanding of SMPs production at a biochemical level. The most exciting prospect of glycomic research is its potential to be used in combination with prevailing genetic and biochemical tools and, when possible, for integrating other data sets. A deeper understanding of glycan formation, and their dynamic physiochemical nature, could result in new pretreatment methods for the efficient removal of EPS from the supernatant, and enable novel cleaning strategies to be developed which target the membrane surface.

REFERENCES

- Abdek-Akher, M., Hamilton, J. K., Smith, F. (1951) The reduction of sugars with sodium borohydride. *J. Am. Chem. Soc.*, 73, 4691-4692.
- Adav, S.S., Lee, D.J. (2008) Extraction of extracellular polymeric substances from aerobic granule with compact interior structure. *J. Hazard Mater.*, 154(1-3), 1120-1126.
- Albro, P. W., Dittmer, J. C. (1969) The biochemistry of long-chain nonisoprenoid hydrocarbons. III. The metabolic relationship of long chain fatty acids and hydrocarbons and other aspects of hydrocarbon metabolism in *Sarcina lutea*. *Biochemistry*, 8, 3317-3324.
- Amy, G.L., Sierka, R.A., Bedessem, J., Price, D., Tan, L. (1992) Molecular-size distributions of dissolved organic-matter. *J. Am. Water Works Assoc.*, 84(6), 67-75.
- Angelidaki, I., Ahring, B. K. (1993) Thermophilic digestion of livestock waste: the effect of ammonia. *Appl. Microbiol. Biotechnol.*, 38, 560-564.
- Annuk, H. Moran, A.P. (2010) Microbial biofilm-related polysaccharides in biofouling and corrosion. in *Microbial Glycobiology*; Holst, O., Brennan, P.J., Itzstein, M.V., Ed.; Academic Press, USA, pp. 781-801.
- Ansorena, D., Gimeno, O., Astiasaran, I. and Bello, J. (2001) Analysis of volatile compounds by GC-MS of a dry fermented sausage: chorizo de Pamplona. *Food Research International*, 34(1), 67-75.
- Aquino, S.F. (2004) Formation of soluble microbial products (SMP) in anaerobic reactors during stress conditions, *Department of Chemical Engineering and Chemical Technology*. London, Imperial College of Science Technology and Medicine.
- Aquino, S.F., Hu, A.Y., Akram, A., Stuckey, D.C. (2006) Characterization of dissolved compounds in submerged anaerobic membrane bioreactors (SAMBRs). *Journal of Chemical Technology and Biotechnology*, 81, 1894-1903.
- Aquino, S.F., Stuckey, D.C. (2004) Soluble microbial products formation in anaerobic chemostats in the presence of toxic compounds. *Water Res.*, 38(2), 255-266.
- Aquino, S.F., Stuckey, D.C. (2006) Chromatographic characterisation of dissolved organics in effluents from two anaerobic reactors treating synthetic wastewater. *Wat. Sci. Technol.*, 54(2), 193-198.

- Aquino, S.F., Stuckey, D.C. (2008) Integrated model of the production of soluble microbial products (SMP) and extracellular polymeric substance (EPS) in anaerobic chemostats during transient conditions. *Biochem, Eng. J.*, 38, 138-146.
- Arden, E., and Lockett, W.T. (1914) Experiments on the oxidation of sewage without the aid of filters. *Journal of the Society of Chemical Industry*, 33(10), 523-539.
- Ashwell, G. (1957) Colorimetric analysis of sugars, in *Methods in Enzymology*, Vol. 3. Colowick, S.P. and Kaplan, N.O. Ed., Academic Press, New York, pp. 73-105.
- Bagaeva, T.V., Zinurova, E.E. (2004) Comparative characterisation of extracellular and intracellular hydrocarbons of *Clostridium pasteurianum*. *Biochemistry*, 69, 427-428.
- Barker, D.J. (1999) Characterisation, production and removal of residual soluble chemical oxygen demand (COD) from anaerobic treatment effluents, *Department of Chemical Engineering and Chemical Technology*. London, Imperial College of Science Technology and Medicine.
- Barker, D.J., Mannucchi, G.A., Salvi, S.M.L., Stuckey, D.C. (1999) Characterization of soluble residual chemical oxygen demand (COD) in anaerobic wastewater treatment effluents. *Water Res.*, 33, 2499-2510.
- Barker, D.J., Stuckey, D.C. (1998) A review of soluble microbial products (SMPs) in wastewater treatment systems. *Water Res.*, 33, 3063-3082.
- Barker, D. J.; Stuckey, D. C. (2001) Modeling of soluble microbial products (SMP) in anaerobic wastewater treatment systems: the effect of feed strength and composition. *Water and Environ Res.* 73, 173-185.
- Barker, H.A. (1956) Biological formation of methane, in *Bacterial Fermentations*, John Wiley and Sons, Inc., New York, pp 1.
- Barker, H. A. (1961) Fermentation of nitrogenous organic compounds. In *The Bacteria*. Vol II, Gunsales, I. C.; Stanier, R. Y. Eds.; Academic Press: New York; pp 151-207.
- Barker, H. A. (1981) Amino acid degradation by anaerobic bacteria. *Annual Review of Biochemistry*, 50(7), 23-40.
- Batstone, D. J., Jensen, P. D. (2011) Anaerobic process. In *Treatise water science*; Wilderer, P., Eds.; Elsevier: Oxford; pp 615-639.
- Beech, I.B., Gargyarde, C.C. (1999) Recent advances in the study of biocorrosion - an overview. *Rev. Microbiol.* 30, 177-190.
- Bemiller, J. N. (1967) Acid-catalyzed hydrolysis of glycosides. *Advan. Carbohydr. Chem. Biochem.*, 22, 25-108.
- Bensadoun, A. Weinstein, D. (1976) Assay of proteins in the presence of interfering materials. *Anal. Biochem.*, 70, 241-250.
- Bertozzi, C.R., Kiessling, L.L. (2001) Chemical glycobiology. *Science*, 291, 2357-2364.

- Bertozzi, C.R., Sasisekharan, R. Glycomics. (2009) In *Essentials of Glycobiology*. 2nd Eds. Varki, A., Cummings, R. D., Esko J. D., Freeze, H. H., Stanley, P., Bertozzi, C. R., Hart, G. W., Etzler, M. Eds.; Cold Spring Harbor Laboratory Press: New York, Chapter 48.
- Beveridge, T.J., Graham, L.L. (1991) Surface layers of bacteria. *Microbiol. Mol. Biol. Rev.*, 55, 684-705.
- Blakeney, A. B., Harris, P. J., Henry, R. J., Stone, B. A. (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.*, 113, 291-299.
- Blumenkrantz, N., Asboe-Hansen, G. (1973) New method for quantitative determination of uroinc acid. *Anal. Biochem.*, 54, 484-489.
- Boero, V.J., Eckenfelder, W.W.J., Bowers, A.R. (1991) Soluble microbial product formation in biological systems. *Wat. Sci. Technol.*, 23, 1067-1076.
- Box, J.D. (1983) Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. *Water Res.*, 17, 511-525.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the prinicple of protein-dye binding. *Anal. Biochem.*, 72, 248-254.
- Brooks, J.R., Griffin, V.K., Kattan, M.W. (1986) A modified method for total carbohydrate analysis of glucose syrups, maltodextrins, and other starch hydrolysis products. *Cereal Chemistry* 63, 465-466.
- Brown, N.L., Lee, B. T. O., Silver, S. (1994) *Metal ions in Biological Systems*. New York, Dekker.
- Bruno, C., Yves, F. (2002) In situ characteriation of bacterial extracellular polymeric substances by AFM. *Colloids Surf. B: Biointerfaces*, 23, 173-182.
- Bunch, R.L., Brath, E.F., Ettinger, M.B. (1961) Organic materials in secondary effluents. *Journal WPCF*, 33(2), 122-126.
- Carlson, K. H., Amy, G. L. (2000) The importance of soluble microbial product (SMPs) in biological drinking water treatment. *Water Res.*, 34, 1386-1396.
- Carr, G.M. and Neary, J.P. *Water Quality for Ecosystem and Human Health*, 2nd Ed. United Nations Environment Progamme Global Environment Monitoring System/Water Programme, Canada. 2008.
- Chae, S-. Y., Ahn, Y-. T., Kang, S-. T., Shin, H-.S. (2006) Mitigated membrane fouling in a vertical submerged membrane bioreactor (VSMBR). *J. Membr. Sci.*, 280, 572-581.
- Chen, J. L., Ortiz, R., Terry, W. J. S., Stuckey, D. C. (2014) Toxicants inhibiting anaerobic digestion: A review. *Biotechnol. Adv.*, 32, 1523-1534.

- Chernicharo, C.A.L. (2007) *Anaerobic Reactors*. Volume 4. Biological Wastewater Treatment Series. IWA Publishing, UK.
- Chinn, R., Barrett, S. (2000) Occurrence of amino acids in two drinking water sources. In *Natural Organic Matter and Disfection By-Products*, Barret, S.; Krasner, S.; Amy, G. Eds.; Oxford University Press: New York.
- Chipasa, S., Medrzycka, K. (2008) The influence of soluble microbial products on microbial community composition: hypothesis of microbial community succession. *Pol. J. Microbiol.*, 57(1), 59-70.
- Cho, B.D., Fane, A.G. (2002) Fouling transients in nominally sub-critical flux operation of a membrane bioreactor. *J. Membr. Sci.*, 209, 391-403.
- Chudoba, J. (1985a) Inhibitory effect of refractory organic compounds produced by activated sludge micro-organisms on microbial activity and flocculation. *Water Res.*, 19(2), 197-200.
- Chudoba, J. (1985b) Quantitative estimation in COD units of refractory organic compounds produced by activated sludge microorganism. *Water Res.*, 19(1), 37-43.
- Chudoba, J. (1986) Microbial polymers in the aquatic environment – II. Isolation from biological nonpurified and purified municipal wastewater and analysis. *Water Res.*, 20(10), 1217-1221.
- Ciucanu, I. (2006) Per-O-methylation reaction for structural analysis of carbohydrates by mass spectrometry. *Anal. Chim. Acta.*, 147-155.
- Ciucanu, I., Caprita, R. (2007) Per-O-methylation of neutral carbohydrates directly from aqueous samples for gas chromatography and mass spectrometry analysis. *Anal. Chim. Acta.*, 81-85.
- Ciucanu, I., Costello, C. E. (2003) Elimination of oxidative degradation during the per-O-methylation of carbohydrates. *J. Am. Chem. Soc.*, 125, 16213-16219.
- Ciucanu, I., Kerek, F. (1984) A simple and rapid method for the permethylation of carbohydrates. *Carbohydr. Res.*, 131, 209-217.
- Coester, S.E., Cloete, T.E. (2005) Biofouling and biocorrosion in industrial water system. *Crit. Rev. Microbiol.* 31, 213-232.
- Comte, S., Guibaud, G., Baudu, M. (2006) Biosorption properties of extracellular polymeric substances (EPSs) resulting from activated sludge according to their type: soluble or bound. *Process Biochem.*, 41, 815-823.
- Comte, S., Guibaud, G., Baudu, M. (2007) Effects of extraction methods on EPS from activated sludge: an HPSEC investigation, *J. Hazard. Mater.*, 140 (1-2), 235-247.
- Cronan, J. E., Gelmann, E. P. (1975) Physical properties of membrane lipids: biological relevance and regulation. *Bacteriol. Rev.*, 39, 232-256.

- de Moreno, M.R., Smith, J.F., Smith, R.V. (1986) Mechanism studies of coomassie blue and silver staining of proteins. *J. Pharm. Sci.*, 75, 907-911.
- Dean, J.R. (2003) *Methods for environmental trace analysis*, John Wiley & Sons.
- Dell, A., Morris, H. M. (2001) Glycoprotein structure determination by mass spectrometry. *Science*, 291, 2351-2356.
- Denecke, M (2006) Protein extraction from activated sludge. *Water Sci. Technol.*, 54(1), 175-181.
- Deng, L., Kasper, D.L., Krick, T.P., Wessels, M.R. (2000) Characterisation of the linkage between the type III capsular polysaccharide and the bacterial cell wall of group B *serptococcus*. *J. Biol. Chem.*, 275, 7497-7504.
- Dennis, M., Kolattukudy, P. E. (1992) A cobalt-porphyrin enzyme converts a fatty aldehydes to a hydrocarbon and CO. *Proc. Natl. Acad. Sci. USA*, 89, 5306-5689.
- Dische, Z. (1955) New color reactions for determination of sugars in polysaccharides, in *Methods of Biochemical Analysis*, Vol. 2. Glick, D. Ed., John Wiley & Sons, Inc. USA. pp. 313-358.
- Dong, C., Beis, K., Nesper, J., Brunkan-LaMontagne, A.L., Clarke, B.R., Whitfield, C., Naismith, J.H. (2006) Wza the translocon for *E. coli* capsular polysaccharides defines a new class of membrane protein. *Nature*, 444, 226-229.
- Dove, A. (2001) The bittersweet promise of glycobiology. *Nat. Biotechnol.*, 19, 913-917.
- Drews, A. (2010) Membrane fouling in membrane bioreactors - characterisation, contradictions, cause and cures, *J. Membr. Sci.*, 363, 1-28.
- Drewes, J.E., Reinhard, M., Fox, P. (2003) Comparing microfiltration-reverse osmosis and soil-aquifer treatment for indirect potable reuse of water. *Water Res.*, 37(15), 3612-3621.
- Dreywood, R. (1946) Qualitative test for carbohydrate material. *Industrial and Engineering Chemistry-Analytical Edition*, 18 (8), 499.
- Du Bois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F. (1951) A colorimetric method for determination of sugars. *Nature*, 168, 167.
- Du Bois, M., Gilles, K., Hamilton, J., Rebers, P., Smith, F. (1956) Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, 28 (3), 350-356.
- Eckenfelder, W.W.J (1988) *Point toxics control for industrial wastewater*, Civil Engineering Practice.
- Eckenfelder, W.W.J (1995) Biological treatability studies: new regulations require a new approach. *Environmental Progress*, 14(3), 172-175.

- Einarsson, S., Folestad, S., Josefsson, B., Lagerkvist, S. (1986) High-resolution reversed-phase liquid chromatography system for the analysis of complex solutions of primary and secondary amino acids. *Anal. Chem.*, 58, 1638-1643.
- Elimelech, M. G. J., Jia, X. and Williams, R. A. (1995) *Particle Deposition and Aggregation*. Butterworth-Heinemann, Oxford.
- Everette, J.D., Bryant, Q.M., Green, A.M., Abbey, Y.A., Wangila, G.W., Walker, R.B. (2010) Thorough study of reactivity of various compounds classes toward the Folin-Ciocalteu reagent. *J. Agric. Food. Chem.*, 58(14), 8139-8144.
- Faeth, P., Sovacool, B. K., Thorkildsen, Z., Rao, A., Purcell, D., Eidsness, J., Johnson, K., Thompson, B., Imperiale, S., Gilbert, A. (2014) A clash of competing necessities, in *Center for Naval Analyses Research Memorandum*, Washington, United State, pp. 6-80.
- Fahnestock, S.R., Steinbüchel, A. (2003) Polyamide and complex proteinaceous materials II, in *Biopolymers*, (Vol. 8) Wiley-Blackwell.
- Ferry, J.G. (1992) Biochemistry of methanogenesis, *Critical Review in Biochemistry and Molecular biology*, 27(6), 473-503.
- Filisetti-Cozzi, T. M. C. C., Carpita, N. C. (1991) Measurement of uronic acids without interference from neutral sugars. *Anal. Biochem.*, 197, 157-162.
- Flemming, H., Wingender, J. (2001) Relevance of microbial extracellular polymeric substances (EPSs) - Part 1: Structural and ecological aspects. *Water Sci. Technol.* 43, 1-8.
- Flemming, H.C., Wingender, J., Griebe, T., Mayer, C. (2000) Physico-Chemical Properties of Biofilm, in *Biofilms: Recent Advances in their study and Control*. Evan, L.V. Ed., CRC press, p. 20.
- Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A., Kjelleberg, S. (2016) Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.*, 14, 563-575.
- Fletcher, M. (1996) Bacterial attachment in aquatic environments: a diversity of surfaces and adhesion strategies. In *Bacterial Adhesion: molecular and ecological diversity*. Fletcher, M. Eds.; Wiley-Liss: New York, pp 1-24.
- Fountoulakis, M., Juranville, J.F., Manneberg, M. (1992) Comparison of the Coomassie brilliant blue, bicinchoninic acid and Lowry quantitation assays, using non glycosylated and glycosylated proteins. *J. Biochem. Biophys. Methods*, 24, 265-274.
- Fritz, J. S., Schenk, G. H. (1959) Acid-catalyzed acetylation of organic hydroxyl groups. *Anal. Chem.* **1959**, 31, 1808-1812.
- Frohlund, B., Palmgren, R., Keiding, K., Nielsen, P.H. (1996) Extraction of extracellular polymers from activated sludge using cation exchange resin. *Water Res.*, 30 (8), 1749-1758.

- Fuqua, C., Greenberg, E. P. (1998) Cell-to-cell communication in *Escherichia Coli* and *Samonella Trphumurium*: they may bet talking, but who's listening? *Proc, Natl, Acad, Sci.*, 95, 6571-6572.
- Galambos, J. T. (1967) The reaction of carbazole with carbohydrates: I. Effects of borate and sulfamte on the carbazole colour of sugars. *Anal. Biochem.*, 19, 119-132.
- Galassi, S., Benfenati, E. (2000) Fractionation and toxicity evaluation of wastewaters. *Journal of Chromatography, A* 889, 149-154.
- Gaudy Jr., A.F., Blachly, T.R. (1985) A study of the biodegradability of residual COD. *Journal WPCF*, 57(4), 332-338.
- Geesey, G. G. (1982) Micorbial exopolymer: ecological and economic consideration. *ASM News* 48, 9-14.
- Gerardi, M. H. (2003) *The microbiology of anaerobic digesters*, New Jersey: John Wiely & Sons, Inc..
- Germirli, F., Orhon, D., Artan, N. (1991) Assessment of the initial inert soluble COD in industrial wastewaters. *Wat. Sci. Tech.* 23, 1077-1086.
- Ghabbour, E.A., Davies, G. (2001) *Humic Substances: Structure, Models and Functions*. RSC publishing: Cambridge, U.K.
- Gonzalez-Gil, G., Thomas, L., Emwas, A.-H., Lens, P. N. L., Saikaly, P. E. (2015) NMR and MALDI-TOF MS based characterization of exopolysaccharides in anaerobic micorbial aggregates from full-scale reactors. *Sci. Rep.*, 5, 14316.
- Gornall, A.G., Bardawill, C.J., David, M.N. (1949) Determination of serum protein by means of the Biuret reaction. *J. Biol. Chem.*, 177, 751-766.
- Gorner, T., de Donato, P., Ameil, M.H., Montarges-Pelletier, E., Lartiges, B.S. (2003) Activated sludges exopolymers: separation and identification using size-exclusion chromatography and infrared micro-spectroscopy *Water Res.*, 37(10), 2388-2393.
- Grady Jr, C.P.L., Daigger, G.T., Lim, H.C. (1999) *Biological wastewater treatment* 2nd Ed., Marcel Dekker, New York, pp. 282-284.
- Greenberg, D. M. (1961) Carbon catabolism of amino acids. In *Metabolic Pathways*. Vol II Greenberg, D. M. Eds.; Academic Press: New York; pp 79-121.
- Grobicki, A., Stuckey, D. C. (1991) Performance of the anaerobic baffled reactor under steady and shock loading conditions. *Biotechnology and Bioenginerring*, 37, 344-355.
- Guerrant, G.O., Lambert, M.A. Moss, C.W. (1982) Analysis of Short-Chain Acids from Anaerobic-Bacteria by High-Performance Liquid-Chromatography. *Journal of Clinical Microbiology*, 16(2), 355-360.
- Gujer, W., Zehnder, A.J.B. (1983) Conversion process in anaerobic digestion. *Wat. Sci. Tech.*, 15, 127-167.

- Guo, J., Peng, Y., Guo, J., Ma, J., Wang, W., Wang, B. (2011) Dissolved organic matter in biological treated sewage effluent (BTSE): characteristics and comparison. *Desalination*, 278, 365-372.
- Guo, R., Sim, W.-J., Lee, E.-S., Lee, J.-H., Oh, J.-E. (2010) Evaluation of the fate of perfluoroalkyl compounds in wastewater treatment plants. *Water Res.*, 44(11), 3476-3486.
- Haberkamp, J., Ernst, M., Böckemlmann, U., Szewzyk, U., Jekel, M., (2008) Complexity of ultrafiltration membrane fouling caused by macromolecular dissolved organic compounds in secondary effluents. *Water Res.*, 42(12), 3153-3161.
- Hakomori, S.-I. (1964) A rapid permethylation of glycolipid, and polysaccharide catalysed by methylsulfinyl carbanion in dimethyl sulfoxide. *J. Biochem.*, 55, 205-208.
- Halpine, S.M. (2005) Amino acid analysis by HPLC, in *The Encyclopedia of Chromatography*, 2nd Ed., Cazes, J. Pub Ed., Marcel Dekker, Inc., New York.
- Hedges, J. L., Keil, R. G., Benner, R. (1997) What happens to terrestrial organic matter in the ocean? *Org. Geochem.*, 27, 195-212.
- Hejzlar, J., Chudoba, J. (1996) Microbial polymers in the aquatic environment – I. Production by activated sludge microorganism under different conditions. *Water Res.* 20(10), 1209-1216.
- Hering, J. G. and Ingold, K. M. (2012) Water resources management: What should be integrated? *Science*, 336, 1234-1235.
- Higgins, M.J., Novak, J.T. (1997) Characterisation of exocellular protein and its role in bioflocculation. *J. Environ. Eng. ASCE*, 123(5), 479-485.
- Hu, A.Y., Stuckey, D.C. (2006) Treatment of dilute wastewater using a novel submerged anaerobic membrane bioreactor. *J. Environ. Eng. ASCE*, 132(2), 190-198.
- Hu, A.Y., Stuckey, D.C. (2007) Activated carbon addition to a submerged anaerobic membrane bioreactor: effect on performance, transmembrane pressure, and flux. *J. Environ. Eng. ASCE*, 133(1), 73-80.
- Huang, Z., Ong, S.L., Ng, H.Y. (2011) Submerged anaerobic membrane bioreactor for low-strength wastewater treatment: effects of HRT and SRT on treatment performance and membrane fouling. *Water Res.* 45 (2), 705-713.
- Imai, A., Fukushima, T., Matsushige, K., Kim, Y.-H., Choi, K. (2002) Characterisation of dissolved organic matter in effluents from wastewater treatment plants. *Water Res.*, 39, 859-870.
- Jang, N., Ren, X., Kim, G., Ahn, C., Cho, J., Kim, I.S. (2007) Characteristics of soluble microbial products and extracellular polymeric substances in the membrane bioreactor for water reuse. *Desalination*, 202(1-3), 90-98.

- Jarrell, K. F., Kalmkoff, M. L. (1988) Nutritional requirements of the methanogenic archaeobacteria, *Canadian Journal of Microbiology*, 34(5), 557-576.
- Jarusutthirak, C., Amy, G. (2006) Role of soluble microbial products (SMP) in membrane fouling and flux decline. *Environ. Sci. Technol.*, 40, 969-974.
- Jarusutthirak, C., Amy, G. (2007) Understanding soluble microbial products (SMP) as a component of effluent organic matter (EfOM). *Water Res.*, 41(12), 2787-2793.
- Jarusutthirak, C., Amy, G., Croue, J.P. (2002) Fouling characteristics of wastewater effluent organic matter (EfOM) isolates on NF and UF membranes. *Desalination*, 145, 247-255.
- Jay, A. (1996) The methylation reaction in carbohydrate analysis. *J. Carbohydr. Chem.*, 15, 897-923.
- Jewell, W.J. (1987) Anaerobic sewage treatment. *Environmental Science and Technology*, 21(1), 14-21.
- Jiang, T., Myngheer, S., De Pauw, D.J.W., Spanjers, H., Nopens, I., Kennedy, M.D., Amy, G., Vanrolleghem, P.A. (2008) Modeling the production and degradation of soluble microbial products (SMP) in membrane bioreactors (MBR). *Water Res.*, 42, 4955-4964.
- Jiang, T., Kennedy, M.D., Schepper, V.D., Nam, S.-N., Nopens, I., Vanrolleghem, P.A., Amy, G. (2010) Characterisation of soluble microbial products and their fouling impacts in membrane bioreactors. *Environ. Sci. Technol.*, 44, 6642-6648.
- Jimenez, J., Vedrenne, F., Denis, C., Mottet, A., Déléris, S., Steyer, J.-P., Rivero, J.A.C. (2013) A statistical comparison of protein and carbohydrate characterisation methodology applied on sewage sludge samples. *Water Res.*, 47 (6), 1751-1762.
- Jones, B. N., Paabo, S., Stein, S. (1981) Amino acid analysis and enzymatic sequence determination of peptides by an improved o-phthaldialdehyde precolumn labeling procedure. *J. Liquid Chromatogr.*, 4, 565-586.
- Judd, S. (2008) The status of membrane bioreactor technology. *Trends Biotechnol.*, 26, 109-116.
- Khan, M. A.; Ngo, H. H.; Guo, W. S.; Liu, Y. W.; Zhou, J. L.; Liang, S.; Ni, B. J.; Zhang, X. B.; Wnag, J. (2016) Comparing the value of bioproducts from different stages of anaerobic membrane bioreactors. *Bioresour. Technol.*, 214, 816-825.
- Kiernan, J.A. (1999) Histological and histochemical methods: Theory and practices, Ed. 3 Butterworth Heinemann, Oxford, UK.
- Kim, M., Ahn, Y.-H., Speece, R. E. (2002) Comparative process stability and efficiency of anaerobic digestion: mesophilic vs. thermophilic. *Water Res.*, 36, 4369-4385.

- Kimura, K., Tanaka, I., Nishimura, S. -I., Miyoshi, R., Miyoshi, T., Watanabe, Y. (2012) Further examination of polysaccharides causing membrane fouling in membrane bioreactors (MBRs): Application of lectin affinity chromatography and MALDI-TOF/MS. *Water Res.*, 46, 5725-5734.
- Kjeldahl, J. (1883) New method for determination of nitrogen in organic substances, *Zeitschrift fur analytische Chemie*, 22 (1), 366-383.
- Klinkow, N., Oleksy-Frenzel, J., Jekel, M. (1998) Toxicity-directed fractionation of organic compounds in tannery wastewater with regard to their molecular weight and polarity. *Water Res.*, 32(9), 2583-2592.
- Kolodkin-Gal, I.; Romero, D.; Cao, S.; Clardy, J.; Kolter, R.; Losick, R. (2010) D-amino acids trigger biofilm disassembly. *Science* 328, 627-629.
- Koster, I. W., Lettinga, G. (1985) The influence of ammonium-nitrogen on the specific activity of pelletized methanogenic sludge. *Agric. Wastes*, 9, 205-216.
- Kumada, K. (1965) Studies on the colour of humic acids. *Soil Science and Plant Nutrition*, 11 (4), 151-156.
- Kunacheva, C., Stuckey, D.C. (2014) Analytical methods for soluble microbial products (SMP) and extracellular polymers (ECP) in wastewater treatment systems: A review. *Water Res.*, 61, 1-18.
- Kunacheva, C.; Le, C.; Soh, Y. N. A.; Stuckey, D. C. (2017) Chemical characterization of low molecular weight soluble microbial products in an anaerobic membrane bioreactor. *Environ. Sci. Technol.* 51, 2254-2261.
- Kuo, W.C., Parkin, G.P. (1996) Characterisation of soluble microbial products from anaerobic treatment by molecular weight distributions and nickel-chelating properties. *Water Res.*, 30(4), 915-922.
- Kuo, W.C., Sneve, M.A., Parkin, G.P. (1996) Formation of soluble microbial products during anaerobic treatment. *Water Environmental Research*, 68, 279-285.
- Laspidou, C.D., Rittmann, B.E. (2002a) A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Res.*, 36, 2711-2720.
- Laspidou, C.D., Rittmann, B.E. (2002b) Non-steady state modeling of extracellular polymeric substances, soluble microbial product, and active and inert biomass. *Water Res.*, 36, 1983-1992.
- Laurentin, A., Edwards, C.A. (2003) A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. *Anal. Biochem.* 315, 143-145.
- Lazarova, V., Manem, J. (1995) Biofilm characterization and activity analysis in water and wastewater treatment. *Water Res.*, 29 (10), 2227-2245.

- Lei, Z., Luo, X., Zhang, Z., Sugiura, N., (2007) Effects of variations of extracellular polymeric substances and soluble microbial products on activated sludge properties during anaerobic storage. *Environ. Technol.*, 28(5), 529-544.
- Lettinga, G., van Velsen, A.F.M., Hobma, S.W., de Zeeuw, W.J., Klapwijk, A. (1980) Use of the upflow sludge blanket (USB) reactor. *Biotechnol. Bioeng.*, 22, 699-734.
- Lettinga, G. (1995) Anaerobic digestion and wastewater treatment systems. *Anthony van Leeuwenhoek*, 67, 328.
- Lettinga, G. (2010) The route of anaerobic wastewater Treatment towards global acceptance, in *Environmental Anaerobic Technology: Applications and New developments*; Fang H.H.P., Ed.; Imperial College Press, 2010; pp 1-15.
- Li, X. Y., Yang, S. F. (2007) Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. *Water Res.*, 41, 1022-1030.
- Li, W. -W.; Yu, H. -Q. (2014) Insight into the roles of microbial extracellular polymer substances in metal biosorption. *Bioresour. Technol.*, 160, 15-23.
- Liang, S., Liu, C., Song, L. (2007) Soluble microbial products in membrane bioreactor operation: behaviors, characteristics, and fouling potential. *Water Res.*, 41(1), 95-101.
- Lin, H., Zhang, M., Wang, F., Meng, F., Liao, B.-Q., Hong, H., Chen, J., Gao, W. (2014) A critical review of extracellular polymeric substances (EPSs) in membrane bioreactors: Characteristics, roles in membrane fouling and control strategies. *J. Membr. Sci.*, 460, 110-125.
- Lin, Q., De Vrieze, J., He, G., Li, X., Li, J. (2016) Temperature regulates methane production through the function centralization of microbial community in anaerobic digestion. *Bioresour. Technol.*, 216, 150-158.
- Lin, Y., de Kreuk, M. C. M., van Loosdrecht, M. C. M., Adin, A. (2010) Characterization of alginate-like exopolysaccharides isolated from aerobic granular sludge in a pilot-plant. *Water Res.*, 44, 3355-3364.
- Liu, H., Wang, J., Liu, X., Fu, B., Chen, J., Yu, H. Q. (2012) Acidogenic fermentation of proteinaceous sewage sludge: effect of pH. *Water Res.*, 46, 799-807.
- Liu, J.-L.; Li, X.-Y. (2010) Biodegradation and biotransformation of wastewater organics as precursors of disinfection byproducts in water. *Chemosphere*, 81, 1075-1083.
- Liu, S., Ge, X., Liew, L.N., Liu, Z., Li, Y. (2015) Effect of urea addition on giant reed ensilage and subsequent methane production by anaerobic digestion. *Biotechnol. Bioeng.*, 192, 682-688.
- Liu, T.; Sung, S. (2002) Ammonia inhibition on thermophilic aceticlastic methanogens. *Water Sci. Technol.*, 45, 113-120.

- Liu, Y. (2007) Wastewater purification: Aerobic granulation in sequencing batch reactors. CRC Press, USA.
- Liu, Y., Fang, H. (2003) Influence of extracellular polymeric substances (EPS) on flocculation, settling and dewatering of activated sludge. *Crit. Rev. Environ. Sci. Technol.*, 33 (3), 237-273.
- Loewus, F.A. (1952) Improvement in anthrone method for determination of carbohydrates. *Anal. Chem.* 24 (1), 219.
- Loconto, P.R. (2012) *Trace environmental quantitative analysis: Principles, techniques and applications*, CRC Press.
- Logan, B.E., Qing, J. (1990) Molecular-size distributions of dissolved organic-matter. *J. Environ. Eng. ASCE*, 116(6), 1046-1062.
- Logan, B.E., Wagenseller, G.A. (2000) Molecular size distributions of dissolved organic matter in wastewater transformed by treatment in a full-scale trickling filter. *Water Environ. Res.*, 72(3), 277-281.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randhall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193(1), 265-275.
- Mao, C., Feng, Y., Wang, X., Ren, G. (2015) Review on research achievements of biogas from anaerobic digestion. *Renewable Sustainable Energy Rev.*, 45, 540-555.
- Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S.-I., Lee, Y.C. (2005) Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Anal. Biochem.* 339, 69-72.
- McCarty, P.L. (1964) *Anaerobic Waste Treatment Fundamentals*. Public Works, September, 107-112, October, 123-126, November 91-94, December, 95-99.
- McCarty, P.L. (1985) Historical trends in the treatment of dilute wastewater, in *Proceeding of the Seminar-Workshop on Anaerobic Treatment of Sewage*, Switzenbaum, M.S., Ed., Amherst, USA, pp 3-16.
- McCarty, P.L., Smith, D.P (1986) Anaerobic wastewater treatment. *Environmental Science Technology*, 20, 1200-1206.
- McCarty, P.L. (2001) The development of anaerobic treatment and its future. *Wat. Sci. Technol.*, 44(8), 157-176.
- Mecozzi, M. (2005) Estimation of total carbohydrate amount in environmental samples by the phenol-sulfuric acid method assisted by multivariate calibration. *Chemometrics and Intelligent Laboratory Systems*, 79 (1-2), 84-90.
- Mei, X.J., Wang, Z.W. Zheng, X., Huang, F., Ma, J.X., Tang, J.X., Wu, Z.C. (2014) Soluble microbial product in membrane bioreactors in the presence of ZnO nanoparticles. *J. Memb. Sci.*, 451, 169-176.

- Meng, F., Chae, S.-O., Drews, A., Kraume, M., Shin, H.-S., Yang, F. (2009) Recent advances in membrane bioreactors (MBRs): Membrane fouling and membrane material. *Water Res.*, 43, 1489-1512.
- Meng, F., Shi, B., Yang, F., Zhang, H. (2007) Effect of hydraulic retention time on membrane fouling and biomass characteristics in submerged membrane bioreactors. *Bioprocess Biosyst. Eng.* 30, 359-367.
- Meng, F., Zhang, S., Oh, Y., Zhou, Z., Shin, H.-S., Chae, S.-R. (2017) Fouling in membrane bioreactors: An updated review. *Water Res.*, 114, 151-180.
- Meng, F., Zhou, Z., Ni, B.-J., Zheng, X., Huang, G., Jia, X., Li, S., Xiong, Y., Kraume, M., (2011) Characterisation of the size-fractionated biomacromolecules: Tracking their role and fate in a membrane bioreactor. *Water Res.*, 45, 4611-4671.
- Mesquita, P.L., Aquino, S.F., Xavier, A.L.P., da Silva, J.C.C., Afonso, R.C.F., Silva, S.Q. (2010) Soluble Microbial Product (SMP) Characterization in Bench-Scale Aerobic and Anaerobic Cstrs under Different Operational Conditions. *Brazilian Journal of Chemical Engineering*, 27(1), 101-111.
- Metcalf & Eddy., Tchobanoglous, G., Burton, F.L., Stensel, H.D. (2003) *Wastewater engineering: treatment and reuse*, 4th ed., Boston: McGraw Hill.
- Mirimanoff, N., Wilkinson, K. J. (2000) Regulation of Zn accumulation by a freshwater gram-positive bacterium (*Rhodococcus opacus*) *Environ. Sci. Technol.*, 34, 616-622.
- Moigno, A.F. (1881) Mouras' Automatic Scavenger. *Cosmos*, p. 622.
- Moigno, A.F. (1882) Mouras' Automatic Scavenger. *Cosmos*, p. 97.
- Monarca, S., Feretti, D., Collivignarelli, C. Guzzella, L., Zerbini, I. Bertanza, G. Pedrazzani, R. (2000) The influence of different disinfectants on mutagenicity and toxicity of urban wastewater. *Water Res.*, 34(17) 4261-4269.
- Morris, D.L. (1948) Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science* 107, 254.
- Mortelmans, K., Zeiger, E. (2000) The Ames Salmonella/microsome mutagenicity assay. *Mutat. Res.* 455, 29-60.
- Nachayasit, S., Stuckey, D.C. (1997) The effect of shock loads on the performance of anaerobic baffled reactor (ABR). *Water Res.*, 31(11) 2737-2746.
- Nagase, M., Matsuo, T. (1982) Interactions between amino-acid –degrading bacteria and methanogenic bacteria in anaerobic digestion. *Biotech. Bioeng.* 24, 2227-2239.
- Namkung, E., Rittmann, B.E. (1986) Implications of soluble microbial products (SMPs) formation on biofilm reactor performance, in *ASCE 1986 Specialty Conference on Environmental Engineering*, Cincinnati, USA.

- Ni, B.-J., Fang, F., Xie, W.-M., Xu, J., Yu, H.-Q. (2011a) Formation of distinct soluble microbial products by activated sludge: kinetic analysis and quantitative determination. *Environ. Sci. Technol.*, 46, 1667-1674.
- Ni, B.-J., Fang, F., Xie, W.-M., Sun, M., Sheng, G.-P., Li, W.-H., Yu, H.-Q. (2009) Characterization of extracellular polymeric substances produced by mixed microorganisms in activated sludge with gel-permeating chromatography, excitation–emission matrix fluorescence spectroscopy measurement and kinetic modeling. *Water Res.*, 43(5), 1350-1358.
- Ni, B.-J., Rittmann, B.E., Yu, H.-Q. (2011b) Soluble microbial products and their implications in mixed culture biotechnology. *Trend in Biotechnology*, 29(9), 454-463.
- Ni, B.-J., Zeng, R.J., Fang, F., Xie, W.-M., Sheng, G. -P., Yu, H.-Q. (2010) Fractionating soluble microbial products in the activated sludge process. *Water Res.*, 44, 2292-2302.
- Nielsen, P.H., Thomsen, T.R., Nielsen, J.L. (2004) Bacterial composition of activated sludge - importance for floc and sludge properties. *Wat. Sci. Technol.*, 49 (10), 51-58.
- Nikolaev, Y., Panikov, N.S., Lukin, S.M., Osipov, G.A. (2001) Saturated C21-C33 hydrocarbons are involved in the self-regulation of *Pseudomonas fluorescens* adhesion to a glass surface. *Microbiol.*, 70, 174-181.
- Noble, J.E., Bailey, M.J.A. (2009) Quantitation of Protein. *Methods Enzymol.*, 463(Chapter 8), 73-95.
- Noike, T.; Endo, G.; Chang, J. –E.; Yaguchi, J. –I.; Matsumoto, J. –I. (1985) Characteristics of carbohydrate degradation and the rate-limiting step in anaerobic digestion. *Biotechnol. Bioeng.* 27, 1482-1489.
- Noguera, D.R., Araki, N., Rittmann, B.E. (1994) Soluble microbial products (SMPs) in anaerobic chemostats. *Biotechnology and Bioengineering*, 44, 1040-1047.
- Novotny, M. V., Soini, H. A., Mechref, Y. (2008) Biochemical individually reflected in chromatographic, electrophoretic and mass-spectrometric profiles. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 866, 26-47.
- OECD. *Test No. 303: Simulation Test – Aerobic Sewage Treatment – A: Activated Sludge Unit; B: Biofilms*; OECD Publishing.
- O’Flaherty, V.; lens, P. N. L.; de Beer, D.; Colleran, E. (1997) Effect of feed composition and upflow velocity on aggregate characteristics in anaerobic upflow reactors. *Appl. Microbiol. Biotechnol.* 47, 102-107.
- Okamura, D., Mori, Y., Hashimoto, T., Hori, K. (2009) Identification of biofoulant of membrane bioreactors in soluble microbial products. *Water Res.*, 43, 134-144.
- Oleszkiewicz, J. A., Sharma, V. K. (1990) Stimulation and inhibition of anaerobic processes by heavy metals – a review. *Biol. Wastes*, 31, 45-67.

- Orlygsson, J., Houwen, F.P., Swensson, B. H. (1994) Influence of hydrogenotrophic methane formation on the thermophilic anaerobic degradation of protein and amino acids. *FEMS Microbiol. Ecol.* 13, 327-334.
- Owen, W.F., Stuckey, D.C., Healy, J.B., Young, L.Y. McCarty, P.L. (1979) Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Res.*, 13(6), 485-492.
- Parkin, G.P., McCarty, P.L. (1981a) Production of soluble organic nitrogen during activated sludge treatment. *Journal WPCF*, 53(1), 99-112.
- Parkin, G.P., McCarty, P.L. (1981b) Sources of soluble organic nitrogen in activated sludge effluents. *Journal WPCF*, 53(1), 89-98.
- Parkin, G.P., McCarty, P.L. (1981c) A comparison of the characteristics of soluble organic nitrogen in untreated and activated sludge treated wastewater. *Water Res.*, 15, 139-149.
- Paulson, J. C., Blixt, O., Collins, B. E. (2006) Sweet spots in functional glycomics. *Nat. Chem. Biol.*, 2, 238-248.
- Pavlostathis, S.G., Giraldo-Gomez, E. (1991a) Kinetics of anaerobic treatment, *Wat. Sci. Tech.*, 24(8), 35-39.
- Pavlostathis, S.G., Giraldo-Gomez, E. (1991b) Kinetics of anaerobic treatment: a critical review, *Critical Review in Environmental Control*, 21, 411-490.
- Pernet-Coudrier, B., Croue, J.P., Varrauly, G., Dignac, M.F., Saad, M., Mouchel, J.M. (2011) Characterization of dissolved organic matter in Parisian urban aquatic systems: predominance of hydrophilic and proteinaceous structures. *Biogeochemistry*, 106, 89-106.
- Peterson, G.L. (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.*, 83, 346-356.
- Peterson, G.L. (1979) Review of the Folin phenol quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.*, 100, 201-220.
- Pilobello, K. T., Mahal, L. K. (2007) Deciphering the glycode: the complexity and analytical challenge of glycomics. *Curr. Opin. Chem. Biol.*, 11, 300-305.
- Pitonzo, B.J., Castro, P., Amy, P.S., Southam, G., Jones, D.A., Ringelberg, D. (2004) Microbiologically influenced corrosion capability of bacterial isolated from Yucca Mountain. *Corrosion* 60, 64-74.
- Pirt, S.J. (1975) Product formation in microbial cultures. *Principal of microbe and cell cultivation*. Oxford, Blackwell scientific.
- Pribyl, M., Tucek, F., Wilderer, P. A., Wanner, J. (1997) Amount and nature of soluble refractory organics produced by activated sludge microorganisms in sequencing batch and continuous flow reactors. *Wat. Sci. Technol.*, 35, 27-34.
- Price, N.C. (1996) in *Enzyme LabFax* Engel, P.C., Ed., BIOS Scientific Publishers, Oxford, pp. 34-41.

- Quinn, A. (March 22, 2012) *U.S. Intelligence Sees Global Water Conflict Risks Rising*, Reuters.
- Raman, R., Raguram, S., Venkataraman, G., Paulson J. C., Sasisekharan, R. (2005) Glycomics: an integrated systems approach to structure-function relationship of glycans. *Nat Method.*, 2, 817-824.
- Ramesh, A., Lee, D.J., Hong, S.G. (2006) Soluble microbial products (SMP) and soluble extracellular polymeric substances (EPS) from wastewater sludge. *Appl. Microbiol. Biotechnol.*, 73(1), 219-225.
- Randtke, S. J., Larson, R.A. (1983) Comment. *Water Res.*, 18, 1597-1599.
- Ranville, J.F., Schmiermund, R.L. (1999) General Aspects of Aquatic Colloids in Environmental Geochemistry, in *The Environmental Geochemistry of Mineral Deposits: Part A: Processes, techniques, and health issues*. Reviews in Economic Geology, Vol 6A., Society of Economic Geologists, p 183-199.
- Rao, P., Pattabiraman, T.N. (1989) Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. *Anal. Biochem.* 181 (1), 18-22.
- Ras, M., Gribal-Neuhauser, E., Paul, E., Sperandio, M., Lefebvre, D. (2008) Protein extraction from activated sludge: an analytical approach. *Water Res.*, 42 (8-9), 1867-1878.
- Raunjker, K., Hvittved-Jacobsen, T., Nielsen, P.H. (1994) Measurement of pools of protein, carbohydrates and lipid in domestic wastewater. *Water Res.* 28 (2), 251-262.
- Reemtsma, T., Jekel, M. (1997) Dissolved organic in tannery wastewater and their alternation by a combined anaerobic and aerobic treatment. *Water Res.*, 31(5), 1035-1046.
- Reizer, J.; Peterkofsky, A. (1987) Sugar transport and metabolism in gram-positive bacteria. Ellis Horwood, Chichester.
- Rice, E.W.; Baird, R.B. Eaton, A. D. Clesceri, L. S. (2012) *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association. Washington D.C.
- Rittmann, B.E., Bae, W., Namkung, E., Lu, C.-J. (1987) A critical evaluation of microbial product formation in biological processes. *Wat. Sci. Technol.*, 19, 517-528.
- Roberson, E. B., Firestone, M. K. (1992) Relationship between desiccation and exopolysaccharide production in a soil *Pseudomonas* sp. *Appl. Environ Microbiol.*, 58, 1284-1291.
- Roberts, I. S. (1996) The biochemistry and genetics of capsular polysaccharide production in bacteria. *Ann. Rev. Microbiol.*, 50, 285-315.

- Rondel, C., Marcato-Romain, C.-E., Girbal-Neuhauser, E. (2013) Development and validation of a colorimetric assay for simultaneous quantification of neutral and uronic sugars. *Water Res.* 47, 2901-2908.
- Rosenberger, S., Laabs, C., Lesjean, B., Gnirss, R., Amy, G., Jekel, M., Schrotter, J.-C. (2006) Impact of colloidal and soluble organic material on membrane performance in membrane bioreactors for municipal wastewater treatment. *Water Res.*, 40, 710-719.
- Ruhaak, L. R., Zauner, G., Huhn, C., Bruggink, C., Deelder, A. M., Wuhrer, M. (2010) Glycan labeling strategies and their use in identification and quantification. *Anal. Bioanal. Chem.*, 397, 3457-3481.
- Ruiz-Matute, A. I., Hernandez-Hernandez, O., Rodriguez-Sanchez, S., Sanz, M. L., Martinez-Castro, I. (2011) Derivatization of carbohydrates for GC and GC-MS analyses. *J. Chromatogr. B*, 879, 1226-1240.
- Sapan, C.V. Lundblad, R.L., Price, N.C. (1999) Colorimetric protein assay techniques. *Biotechnol. Appl. Biochem.*, 100, 201-220.
- Schattauer, A., Abdoun, E., Weiland, P., Plochl, M., Heiermann, M. (2011) Abundance of trace elements in demonstration biogas plant. *Biosyst. Eng.*, 108, 57-65.
- Schiener, P., Nachayasit, S., Stuckey, D.C. (1998) Production of soluble microbial products (SMPs) in an anaerobic baffled reactor: composition, biodegradability, and the effect of process parameters. *Environmental Technology*, 19, 391-400.
- Schiff, H. (1866) Eine neue Reihe organischer Diaminen. *Justus. Liebigs. Ann. Chemie*. 140, 92-137.
- Schirmer, A., Rude, M.A., Li, X., Popova, E., del Cardayre, S.B. (2010) Microbial biosynthesis of alkanes. *Science*, 329, 559-562.
- Schneider, T., Riedel, K. (2010) Environmental proteomics: analysis of structure and function of microbial communities. *Proteomics*, 10(4), 785-798.
- Schuster, R. (1988) Determination of amino acid in biological pharmaceutical, plant and food samples by automated precolumn derivatization and high-performance liquid chromatography. *J. Chromatogr.*, 431, 271-284.
- Scott, T.A., Melvin, E.H. (1953) Determination of dextran with anthrone. *Anal. Chem.* 25, 1656-1661.
- Sedmak, J.J., Grossberg, S.E. (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.*, 79, 544-552.
- Sekine, K., Toida, T., Saito, M., Kuboyama, M., Kawashima, T., Hasimoto, Y. (1985) A new morphologically characterized cell wall preparation (Whole peptidoglycan) from *Bifidobacterium infantis* with a higher efficacy on the regression of an established tumor in mice. *Cancer Res.*, 45, 1300-1307.

- Seghezzo, L., Zeeman, G., van Lier, J.B., Hamelers, H.V.M., Lettinga, G. (1998) A review: the anaerobic treatment of sewage in UASB and EGSB reactors. *Bioresource Technology*, 65, 175-190.
- Seviour, T., Lambert, L. K., Pijuan, M., Yuan, Z. (2010) Structural determination of a key exopolysaccharide in mixed culture aerobic sludge granules using NMR spectroscopy, *Environ. Sci. Technol.*, 44, 8964-8970.
- Seviour, T., Yuan, Z., van Loosdrecht, M. C. M., Lin, Y. (2012) Aerobic sludge granulation: A tale of two polysaccharides? *Water Res.*, 46, 4803-4813.
- Shah, A. D., Mitch, W.A. (2012) Halonitroalkanes, halonitriles, haloamides, and N-nitrosamines: A critical review of nitrogenous disinfection by-product formation pathways. *Environ. Sci. Technol.*, 46, 119-131.
- Sharma, S.K., Maeng, S.K., Nam, S-N., Amy, G. (2011) Characterization Tools for Differentiating Natural Organic Matter from Effluent Organic Matter. In *Treatise on Water Science*; Wilderer, P., Ed.; Elsevier B. V.; pp 417-427.
- Sheng, G.-P., Yu, H.-Q., Li, X.-Y. (2010) Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnology Advance*, 28, 882-894.
- Shon, H.K., Vigneswaran, S., Kim, I.S., Cho, J., Ngo, H.H. (2004) The effect of pretreatment to ultrafiltration of biological treated sewage effluent: a detailed effluent organic matter (EfOM) characterization. *Water Res.*, 38, 1933-1939.
- Shon, H.K., Vigneswaran, S., Kim, I.S., Cho, J., Ngo, H.H. (2006) Fouling of ultrafiltration membrane by effluent organic matter: a detailed characterization using different organic fractions in wastewater. *J. Membr. Sci.*, 278(1-2), 232-238.
- Shriver, Z., Raguram, S., Sasisekharan, R. (2004) Glycomics: a pathway to a class of new and improved therapeutics. *Nat. Rev. Drug Discov.*, 3, 863-873.
- Siggins, A., Gunnigle, E., Abram, F. (2012) Exploring mixed microbial community functioning: recent advances in metaproteomics. *FEMS Microbiol. Ecol.* 80, 265-280.
- Smith, A.L., Stadler, L.B., Love, N.G., Skerlos, S.J., Raskin, L. (2012) Perspectives on anaerobic membrane bioreactor treatment of domestic wastewater: a critical review. *Bioresource Technology*, 122, 149-159.
- Smith, P.K., Krohn, R.I. Hermansen, G.T. Malia, A.K., Gartner, F.H., Provenzano, M.D. Fuiomoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, 150, 76-85.
- Snow, J (1855) *On the Mode of Communication of Cholera*, John Churchill, London.
- Speece, R.E. (1983) Anaerobic biotechnology for industrial wastewater treatment. *Environ. Sci. Technol.*, 17(9), 416A-427A.

- Speece, R.E. (1996) *Anaerobic biotechnology for industrial wastewaters*, Archae Press, Tennessee, USA.
- Speece, R.E., McCarty, P.L. (1964) Nutrient requirements and biological solids accumulation in anaerobic digestion. In *Advances in Water Pollution Research*, Vol 2. Eckenfelder, W. W. Eds.; Oxford: Pergamon Press; pp305-322.
- Stephenson, T., Judd, S., Jefferson, B., Brindle, K. (2000) *Membrane bioreactors for wastewater treatment*, International Water Association, London.
- Stoscheck, C.M. (1990) Quantitation of protein. *Methods Enzymol.*, 182, 50-68.
- Stuckey, D.C. (2010a) Anaerobic Membrane Reactor, in *Environmental Anaerobic Technology: Applications and New developments*; Fang H.H.P., Ed.; Imperial College Press, London; pp 137-161.
- Stuckey, D.C. (2010b) Anaerobic Baffled Reactor (ABR) for Wastewater Treatment, in *Environmental Anaerobic Technology: Applications and New Developments*; Fang H.H.P., Ed.; Imperial College Press, London; pp 163-184.
- Stuckey, D.C. (2012) Recent developments in anaerobic membrane bioreactors. *Bioresource Technology*, 122, 137-148.
- Sutherland, I. W. (2007) Bacterial Exopolysaccharides in *Comprehensive Glycoscience Vol. 2*; Kamerling, J.P., Ed.; Elsevier, Dordrecht; pp 521-558.
- Taylor, K.A.C.C. (1955) A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbances. *Applied Biochemistry and Biotechnology* 53, 207-214.
- Taylor R. L., Conrad, H. E. (1972) Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbimide-activated carboxyl groups. *Biochemistry*, 11, 1383-1388.
- Tchobanoglous, G., Burton, F.L., Stensel, H.D. (2003). *Wastewater Engineering: Treatment and Reuse*. Metcalf & Eddy, Inc. 4th Ed., McGraw-Hill, Boston.
- Tian, Y., Li, Z.P., Ding, Y., Lu, Y.B. (2013) Identification of the change in fouling potential of soluble microbial products (SMP) in membrane bioreactor coupled with worm reactor. *Water Res.*, 47(6), 2015-2024.
- Tipthara, P., Kunacheva, C., Soh, Y.N.A., Wong, S.C.C., Pin, N.G., Stuckey, D.C., Boehm, B. O., (2017) Global profiling of metabolite and lipid soluble microbial products in anaerobic wastewater reactor supernatant using UPLC-MS. *J. Proteome Res.*, 16, 559-570.
- Thanh, P.M., Kethessan, B., Zhou, Y., Stuckey, D. C. (2016) Trace metal speciation and bioavailability in anaerobic digestion: A review. *Biotechnology Advance*, 34, 122-136.
- Trzcinski, A.P. and Stuckey, D.C. (2009) Continuous treatment of the organic fraction of municipal solid waste in an anaerobic two-stage membrane process with liquid recycle. *Water Res.*, 43(9), 2449-2462.

- Trzcinski, A.P., Stuckey, D.C. (2010) Treatment of municipal solid waste leachate using a submerged anaerobic membrane bioreactor at mesophilic and psychrophilic temperatures: Analysis of recalcitrants in the permeate using GC-MS. *Water Res.*, 44, 671-680.
- Tsai, B.N., Chang, C.H., Lee, D.J. (2008) Fractionation of soluble microbial products (SMP) and soluble extracellular polymeric substances (EPS) from wastewater sludge. *Environ. Technol.*, 21(10), 1127-1138.
- Upreti R.K., Kumar, M., Shankar, V (2003) Bacterial glycoproteins: functions, biosynthesis and applications. *Proteomics* 3 (4), 363-379.
- Urbain, V., Block, J.C., Manem, J. Biofloculation in activated sludge: an analytical approach. *Water Res.* **1993**, 27 (5), 829-838.
- van Haandel, A.C. and Lettinga, G. (1995) *Anaerobic Sewage Treatment - A Practical Guide for Regions with a Hot Climate*. Wiley, New York.
- Villain, M., Simon, S., Bourven, I., Guibaud, G. (2010) The use of a new mobile phase, with no multivalent cation binding properties, to differentiate extracellular polymeric substances (EPS), by size exclusion chromatography (SEC) from biomass used for wastewater treatment. *Process Biochem.*, 45(8), 1415-1421.
- Voet, D., Voet, J.D. (2011) *Biochemistry*, 4th Ed., John Wiley & Sons, Inc. USA.
- Vörösmarty, C.J., McIntyre, P.B., Gessner, M.O., Dudgeon, D., Prusevich, A., Green, P., Bunn, S.E., Sullivan, C.A., Liermann, C.R., and Davies, P.M. (2010) Global threats to human water security and river biodiversity, *Nature*, 467, 555-561.
- Vyrides, I., Conteras, P.A., Stuckey, D.C. (2010) Post-treatment of a submerged anaerobic membrane bioreactor (SAMBR) saline effluent using powdered activated carbon (PAC) *Journal of Hazardous Materials*, 177, 836-841.
- Wang, X.-M., Waite, T.D. (2009) Role of gelling soluble and colloidal microbial products in membrane fouling. *Environ. Sci. Technol.*, 43(24), 9341-9347.
- Wang, Z.-P., Zhang, T. (2010) Characterization of soluble microbial products (SMP) under stressful conditions. *Water Res.*, 44, 5499-5509.
- Wang, Z.-W., Liu, Y., Tay, J.-H. (2005) Distribution of EPS and cell surface hydrophobicity in aerobic granules. *Appl. Microbiol. Biotechnol.* 69, 469-473.
- Wang, Z.W., Wu, Z.C., Tang, S.J. (2009) Extracellular polymeric substances (EPS) properties and their effect on membrane fouling in a submerged membrane bioreactor. *Water Res.*, 43, 2504-2512.
- Washington, D.R, Clesceri, L.S., Young, L.C., Hardt, F.W. (1970) Influence of microbial waste products on metabolic activity of high solids activated sludge, in *Industrial Wastewater*, Purdue, USA.
- Watson, S.D. Akhurst, T., Whitely, C.G., Rose, P.D. Pletschke, B.I. (2004) Primary floc degradation is accelerated under biosulphidogenic conditions: enzymological aspects. *Enzyme Micro. Technol.*, 34 (6), 595-602.

- Westerholm, M., Leven, L., Schnuer, A. (2012) Bioaugmentation of syntrophilic acetate-oxidising culture in biogas reactors exposed to increasing level of ammonia. *Appl. Environ. Microbio.*, 78, 7619-7625.
- Westgate, P.J., Pack, C. (2010) Evaluation of proteins and organic nitrogen in wastewater treatment effluents. *Environ. Sci. Technol.*, 44, 5352-5357.
- Whister, R. L., Wolfrom, M. L. (1965) *Method in Carbohydrate Chemistry*. Vol V; Academic Press: New York.
- Whitefield, C., Valvano, M.A. (1993) Biosynthesis and expression of cell-surface polysaccharides in Gram-negative bacteria. *Adv. Microb. Physiol.*, 35, 135-246.
- Wiechelman, K. J., Braun, R.D., Fitzpatrick, J.D. (1988) Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal. Biochem.*, 175, 231-237.
- Wilén, B.M., Jin, B., Lant, P., (2003) The influence of key chemical constituent in activated sludge on surface and flocculating properties. *Water Res.*, 37 (9), 2127-2139.
- Wilmes, P., Bond, P.L. (2006) Metaproteomics: studying functional gene expression in microbial ecosystems. *Trends Microbiol.*, 14, 92-97.
- Wilmes, P., Heintz-Buschart, A., Bond, P.L. (2015) A decade of metaproteomics: where we stand and what the future holds. *Proteomics*, 15, 3409-3417.
- World Health Organization, The United Nations Children's Fund. (2000) *Global water supply and sanitation assessment 2000 report*. Retrieved from http://www.who.int/water_sanitation_health/monitoring/jmp2000.pdf
- Wu, A.M., Wu, J.C., Herp, A. (1978) Polypeptide linkages and resulting structural features as powerful chromogenic factors in the Lowry phenol reaction. Studies on a glycoprotein containing no Lowry phenol-reactive amino acids and on its desialylated and deglycosylated products. *Biochem. J.*, 175, 47-51.
- Wu, B., Zhou, W. (2010) Investigation of soluble microbial products in anerobic wastewater treatment effluents. *J. Chem. Technol. Biotechnol.*, 85, 1597-1603.
- Xin, Y.J., Bligh, M. W., Kinsela, A.S., Waite, T. D. (2016) Effect of iron on membrane fouling by alginate in the absence and presence of calcium. *J. Membr. Sci.* 497, 289-299.
- Young, J.C., McCarty, P.L. (1969) The anaerobic filter for wastewater treatment. *J. Wat. Pollut. Control Fed.*, 41(5), R160-173.
- Zaia, J. (2004) Mass spectrometry of oligosaccharides. *Mass Spectrom. Rev.*, 23, 161-227.
- Zamanzadeh, M., Hagen, L.H., Svensson, K., Linjordet, R., Horn, S.J. (2016) Anaerobic digestion of food waste – effect of recirculation and temperature on performance and microbiology. *Water Res.*, 96, 246-254.

- Zhang, M.L., Sheng, G.P., Yu, H.Q. (2008) Determination of proteins and carbohydrates in the effluents from wastewater treatment bioreactors using resonance light-scattering method. *Water Res.*, 42(13), 3464-3472.
- Zhou, Z., Meng, F., He, X., Chae, S.-R., An, Y., Jia, X. (2015) Metaproteomic analysis of biocake proteins to understand membrane fouling in a submerged membrane bioreactor. *Environ. Sci. Technol.*, 49, 1068-1077.
- Zhou, Z., Tan, Y., Xiao, Y., Stuckey, D. C. (2016) Characterisation and significance of sub-visible particles and colloids in a submerged anaerobic membrane bioreactor (SAnMBR). *Environ. Sci. Technol.*, 50(23), 12750-12758.
- Zhou, W.L., Wu, B.T., She, Q.H., Chi, L., Zhang, Z.J. (2009) Investigation of soluble microbial products in a full-scale UASB reactor running at low organic loading rate. *Bioresource Technology*, 100(14), 3471-3476.