Applications of Nuclear Magnetic Resonance Spectroscopy to Food Wastewater Treatment

by

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ABSTRACT

APPLICATIONS OF NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY TO FOOD WASTEWATER TREATMENT

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This dissertation presents an investigation of the application of nuclear magnetic resonance (NMR) spectroscopy as an analytical tool for improved monitoring, diagnostics, and characterization of the wastewaters associated with food waste. NMR uses a non-targeted approach to gather high-resolution molecular-level data relating to the makeup of complex organic mixtures. One and two-dimensional experiments are used to generate an NMR fingerprint of anaerobic bioreactor samples after exposure to a known contaminant. The results determined by NMR are compared to biogas compositions measured using gas chromatography. In all bioreactor samples, key metabolites as well as the contaminant itself are clearly identified, demonstrating changes in the chemical profile in response to stress. For more simple mixtures of food waste, NMR has shown potential to be used as a tool to quickly quantify and predict biodegradability based on macromolecular structure of the organic material in the wastewater.

DEDICATION

I would like to dedicate this dissertation to my late grandfather, John Stocks. Although he passed away during my time as an undergraduate student, I know how proud he would be of me for pursuing and completing my masters.

I would also like to dedicate this dissertation to my mother, Julie Freemantle. I would not be where I am today without all of her love and support.

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LIST OF SYMBOLS, ABBREVIATIONS and NOMENCLATURE

AD	Anaerobic Digestion
Ala	Alanine
ATP	Adenosine Triphosphate
BAC	Benzalkonium Chloride
BOD	Biochemical Oxygen Demand
BOD_5	5-Day Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
$C_1 – C_4$	Carbone One – Carbon Four
$C_8 – C_{18}$	Carbone Eight – Carbon Eighteen
C/N	Carbon/Nitrogen Ratio
DBAS	Disulphine Blue Active Substance Test
DI	Deionized
DIPSI	Decoupling In the Presence of Scalar Interactions
D_2O	Deuterium Oxide
FID	Flame Ionization Detector
FOS	Total VFAs as Acetic Acid
FW	Food Waste
GC	Gas Chromatography
GC – MS	Gas Chromatography Mass Spectrometry
Glu	Glutamate

Gly	Glycine
HPLC	High Performance Liquid Chromatography
IHS	Interspecies Hydrogen Transfer
Ile	Isoleucine
Leu	Leucine
LOD	Limits of Detection
LC – MS	High Performance Liquid Chromatography Mass Spectrometry
HRT	Hydraulic Retention Time
Met	Methionine
METNOESY	Metabolomics Nuclear Overhauser Effect Spectroscopy
MHz	Megahertz
MIR	Mid Infrared Spectroscop
MS	Mass Spectrometry
MSW	Municipal Solid Waste
NIR	Near Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
OLR	Organic Loading Rate
ORP	Oxidation Reduction Potential
Phe	Phenylalanine
PPM	Parts Per Million
QAC	Quaternary Ammonium Compound

QAS	Quaternary Ammonium Salt
RPM	Revolutions Per Minute
SRT	Solid Retention Time
Ster-Bac (KQ-12)	Liquid Quaternary Ammonium Sanitizer
TAC	Total Alkalinity
Thr	Threonine
TLC	Thin Layer Chromatography
TMSP	3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid
TOC	Total Organic Carbon
TOCSY	Total Correlation Spectroscopy
Trp	Tryptophan
TS	Total Solids
Tyr	Tyrosine
U.S. EPA	United States Environmental Protection Agency
UV	Ultra-Violet
UV – Vis	Ultra-Violet Visible Spectroscopy
Val	Valine
VFA	Volatile Fatty Acid
VS	Total Volatile Solids
WWTP	Wastewater Treatment Plant
1D	One Dimensional
2D	Two Dimensional

$_{1}H$	Proton
$_{13}C$	Carbon

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Chapter 1 – Introduction and Literature Review

1.1 Introduction

Food waste (FW) is a global problem, with millions of tons generated from agricultural, industrial, commercial, and municipal sources daily. It is difficult to effectively and efficiently treat the waste streams associated with FW when the composition of the organic constituents is not fully identified. Many environmental systems contain unknown quantities of organic matter along with complex mixtures of contaminants that transform the chemical composition and pollutant strength. The chemical profile of these wastewaters needs to be better understood so that optimal treatment technologies can be used. The goal of this dissertation is to investigate the application of nuclear magnetic resonance (NMR) spectroscopy as a tool characterize the full organic composition of food waste effluents and process water. The overarching hypothesis is that a having a high-resolution molecular-level characterization of organics will improve our ability to treat wastewater and optimize bioreactor operation. The overall objective is to validate NMR spectroscopy as a FW characterization tool and demonstrate its effectiveness in bioprocess monitoring. Specific objectives relate to the development of NMR spectroscopy as a fingerprinting tool to quickly quantify and predict biodegradability as well as investigating the application of NMR methods to identify key changes in the chemical composition of a bioreactor.

This introductory chapter will outline the limitations associated with characterizing

organics in FW and the complications associated with monitoring anaerobic wastewater treatment. The limitations of conventional tools for the analysis of FW composition and quaternary ammonium compounds (QACs) will be discussed in relation to how NMR may help resolve these issues. The potential limitations of NMR analysis will also be addressed. This chapter will conclude with a brief review of current and past uses of NMR in the field of environmental science, specifically with reference to wastewater treatment systems and relating back to the overall objective of the thesis.

1.2 Food Waste and Wastewater

Whenever and wherever food is handled, processed, packaged and stored there will be an unpreventable generation of waste and wastewater. In the United States alone, approximately 40% of food is wasted during crop production, transportation, and final consumer use.[1] The rate of generation is now exceeding the rate of degradation under natural conditions,[2] and the amount of FW is expected to increase to meet economic and societal demands.[3] The United States Environmental Protection Agency (U.S. EPA) estimated that 251 million tons of municipal solid waste (MSW) was generated in 2012 and that FW made up 14.6 and 21.1% of the MSW generated and discarded, respectively.[4] The efficiency of treating the wastewaters associated with this FW depends on the quality of analytical data available during the treatment process, with the composition of the water being the most important parameter affecting treatment performance of biological wastewater treatment systems.[5] To accomplish this goal, there is a need to know the full organic composition of wastewater constituents before,

during, and after treatment. This is essential for proper management and treatment.

1.2.1 Composition and Source

The organic composition of FW varies immensely and is reliant on numerous dependent and independent variables. Eating habits, cultivation, and availability[2] all change according to regions, seasons, collection plans, and processing schemes.[6, 7] FW predominantly consists of three principal organic components: carbohydrates (sugars and fibers), lipids (fats), and proteins. These organic components will differ with the type of FW and its constituents.[8] The characteristics (i.e., pollutant strength, nature of constituents) and quantity of the associated wastewater will also vary with the product and production procedure.[9]

Generally, almost all manufactured products use water at some point during the production process. Water used in industry has a diverse range of applications, including fabricating, processing, washing, diluting, cooling, transporting a product, incorporating water into a product, or for sanitation purposes within the manufacturing facility.[10] The industries responsible for the highest consumption of water produce commodities such as food and beverage, paper, chemicals, refined petroleum, or primary metals.[11, 12] The research presented in chapter 2 of this dissertation deals with process water and effluents from a large-scale industrial food processing plant designed to manufacture potato products. Figure 1-1 outlines the use of water during the processing of potato chips. Process water can be described as water not suitable for

human consumption, which is used in connection with various technical processes and production procedures, requiring additional treatment for reuse or discharge.[13] Wastewater influent is water that will flow into a system in a raw and untreated state.[14] Wastewater effluent is water that will flow out of a system, having been treated and/or discharged to receiving surface waters.[14]

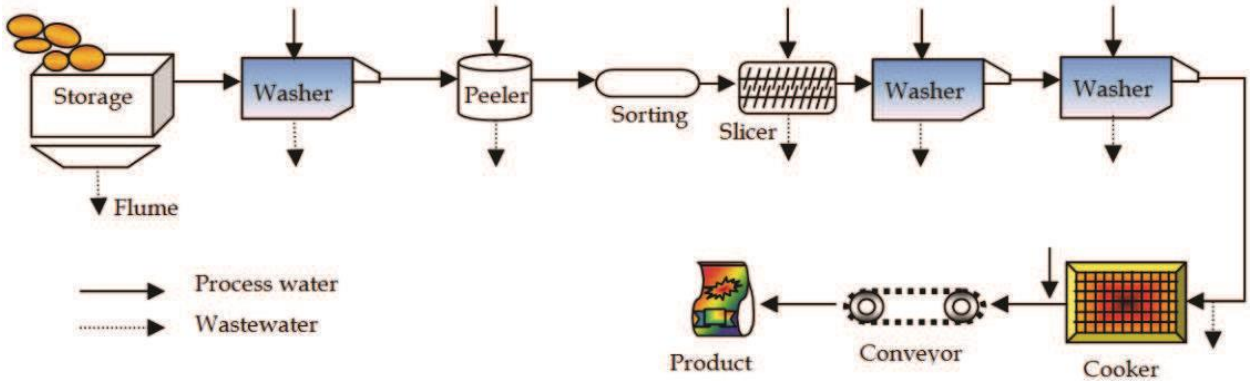

Figure 1-1: This illustration demonstrates a process flow diagram for a potato chip manufacturing plant.[15] It is important to note the high volume of water usage, both process water and influents, throughout the production procedure.

1.2.2 Conventional Analytical Techniques for the Characterization of Organics in Wastewater

The characterization of waste is a necessary step before it can be processed in a treatment facility. The quantities of different compounds (carbohydrates, proteins, and lipids) and anaerobic biodegradability (capacity to produce methane) are important parameters required to fully characterize FW and optimize treatment processes.[16] Modern advances in technology have, however, focused more on the chemical analysis

of specific substrate consumption or product formation rather than pre-treatment screening of organics, leaving a gap in knowledge between influents and treatment parameters. There are millions of known organic compounds in the environment, all of which cannot be individually identified in a short period of time, despite substantial analytical efforts. Therefore, analyses of non-specific, sum parameters are used to generalize overall estimates of biodegradability and pollutant strength.[17]

The most commonly used sum parameters in wastewater treatment are five-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD) and total organic carbon (TOC). BOD_5 is a measure of the amount of oxygen required for microorganisms to degrade the organic material present and is used to help quantify the concentration of organics.[17, 18] Since there are many organic compounds that are recalcitrant to degradation or even incapable of biological degradation, an analogous measurement was developed. COD is a measure of the amount of oxygen that would be needed to chemically oxidize both biodegradable and nonbiodegradable compounds, directly reflecting the amount of organics and inorganics present.[17, 18] Wastewaters containing high oxidative demands can lead to oxygen deficiencies in receiving water bodies, ultimately affecting aquatic organisms. TOC is a measure of the total amount of organic carbon that is found in both the dissolved and undissolved organic substances present in water, providing an estimate of the amount of organics present in the water source.[17, 18] Whereas BOD and COD reflect oxidative demands, TOC reflects total organic pollutants.

The analysis of sum parameters provides valuable information on the general characteristics of organic material present in wastewaters; however, there are two major drawbacks: time and accurate representation. The analysis of BOD_5 takes between 3-5 days and the estimations of COD and TOC takes between 3-4 hours.[19] This delay in response time does not provide sufficient insight, as it is merely a snap shot in time which does not accurately reflect the current status of all analytes present within a system. Furthermore, the analysis of each sum parameter is based on the macroscopic analysis of bulk properties, failing to represent the molecular-level heterogeneity that exists in the composition of the bulk organic constituents of wastewater. As a result of these limitations to conventional analytical approaches for the characterization of organics in wastewater, the full composition of most complex environmental systems remains unknown. The implementation of novel non-targeted analytical approaches that have the ability to differentiate between different classes of organics in a mixture as a whole, have the potential to improve the characterization of the organic fraction of wastewater from raw influent to treated effluent.

1.3 Anaerobic Digestion in Wastewater Treatment Systems

Plant operations of wastewater treatment plants (WWTPs) typically have high energy requirements, with regards to both pumping and treatment. The anaerobic digestion (AD) of organic material found in the waste from WWTPs can generate significant amounts of methane gas. The gas can be used to generate power to be used by the plant and heat the digester, as well as being sold to nearby industries as another source

of revenue. As energy costs continue to rise into the future, this should be the preferred direction of operation of WWTPs, evolving from energy consumers, into energy producers.[14]

The generation of biogas (methane and carbon dioxide) via AD has become a widely accepted and appropriate solution for FW management. Anaerobic treatment processes have a higher degree of waste stabilization, lower operational cost and lower residual waste production compared to aerobic systems[14, 20], while utilizing food waste as a renewable energy source.[9, 21] Bioreactors are the main apparatus used in the management of industrial waste streams and are the vessel from which AD is carried out, where microbial communities breakdown organic material from waste effluent, and in turn, create biogas.[22] Biological stability depends on the degradability of organic matter,[23, 24] therefore, optimal operating conditions and inhibition effects of AD processes will differ with diversity in the organic components of feedstock.[7] The AD of biomacromolecules in various microbial ecosystems is influenced by the variation in types, qualities, and quantities of chemical components,[25] with the characteristics of FW, particularly the chemical and physical composition, being the most important information for WWTP design and process stability.[1] Specific substrates (different types of FW) are utilized by specific types of bacteria, while exhibiting a symbiotic relationship with one another's activity, forming an anaerobic food chain.[26] AD is a complex biochemical and physiochemical process, as outlined in figure 1-2, that can be broken down into four distinct phases: hydrolysis, acidogenesis, acetogenesis, and methanogenesis.

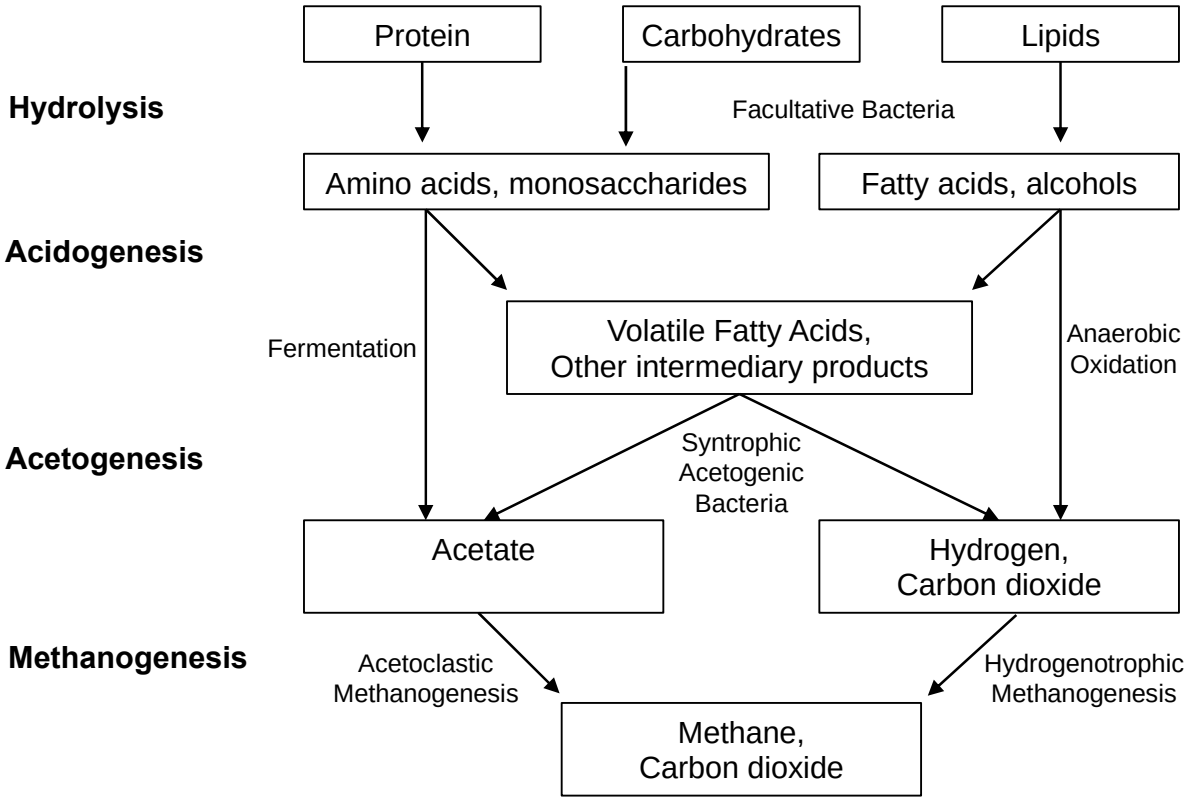

Figure 1-2: Flow diagram of the anaerobic digestion process.

Hydrolysis is the first phase in the AD process. In this phase, polymers that cannot be transported into cell membranes are broken down into simple and soluble monomers.[8, 27] Complex organic molecules such as proteins, carbohydrates and lipids are converted into amino acids, monosaccharides, glycerol and fatty acids, respectively.[2, 8] This enzyme-mediated transformation[28] is facilitated by hydrolases secreted by facultative bacteria, which can thrive with or without the presence of oxygen.[8, 28] No waste stabilization occurs during this phase, but rather the organic material is converted to a form that can be more readily taken up by microorganism in the next phases.[14]

Acidogenesis is the second phase in the AD process. In this phase, the monomers generated from hydrolysis will become substrates for microbes to further degrade into simpler, and smaller molecules.[8, 28] The products of hydrolysis are predominantly fermented into volatile fatty acids (VFAs), acetate, hydrogen (H_2), and carbon dioxide (CO_2), along with a variety of other soluble C_1 – C_4 end products, such as organic acids, alcohols, and ketones.[8, 29] Organic substrates serve as both electron donors and electron acceptors,[27] with the presence of oxygen-removing bacteria being crucial to facilitate anaerobic conditions.[2] During this acidification, facultative anaerobes utilize oxygen and carbon to create and maintain favourable conditions for the subsequent development of obligatory anaerobes.[2, 30]

During AD, VFAs are formed from larger molecules, such as carbohydrates, proteins and lipids. These large molecules are hydrolyzed by fermentative bacteria into simple soluble compounds, such as sugars, amino acids and fatty acids. Various VFAs exist in AD systems, all of which have different and cooperative effects on the bacteria and archaea present within.[31] The characteristics of wastewater effluents in regard to certain VFA concentrations have critical impacts on the overall performance of an anaerobic digester, as an excess of one type of acid may favour the predominance of specific kinds of microbial consortia.[32, 33] Acetic acid, propionic acid, butyric acid, and valeric acid are formed directly from the fermentation of carbohydrates and proteins, as well as during the anaerobic oxidation of lipids.[29, 34, 35] Obligate H_2-producing acetogenic bacteria are responsible for oxidizing VFAs to acetate, CO_2 and H_2. The acetate, H_2, and CO_2 generated from acidogenesis can be utilized directly for methane production,[28]

however, propionate, isobutyrate, isovalerate and other VFAs require further degradation via syntrophic acetogenic bacteria to form acetate and hydrogen.[36, 37, 38] Acetogenesis is the third phase in the AD process. In this phase, the products of acidogenesis are converted into acetate, H_2, and CO_2 as the precursors for methane production.[1, 2, 8] All VFAs and other short chain organic products are metabolized by syntrophic acetogenic bacteria,[27, 28] which convert the acid-phase products to acetate, H_2, and CO_2.[8] Syntrophic acetogenic bacteria belong to a group of strictly anaerobic organisms known as acetogens.[28] The organic end products of anaerobic metabolism would accumulate in the environment if it were not for acetogenic bacteria and methanogenic archaea.

Methanogenesis is the fourth phase in the AD process. In this final phase, the products of acetogenesis are used to generate methane (CH_4) gas by a group of organisms known collectively as methanogens.[39] Specifically, there are two groups of microbes responsible for methane production: Acetoclastic methanogens and hydrogen-utilizing methanogens.[2, 8] Acetoclastic methanogens split acetate into CH_4 and CO_2, while hydrogen-utilizing methanogens uses H_2 as the electron donor and CO_2 as the electron acceptor to produce CH_4.[8, 27] Approximately 72% of the methane generated can be attributed to the cleavage of acetate, whereas the remaining 28% can be attributed to the reduction of CO_2.[14] Overall, the process generates biogas in an approximate ratio of 60% CH_4 and 40% CO_2,[40] with a strong relationship existing between the quantity of methane produced and the organic matter used.[16]

1.3.1 Factors Affecting Optimal Performance

Complex metabolic interactions exist between the different microbial groups present within a bioreactor system and an imbalance in microbial activities can spawn the accumulation of metabolic intermediates, and in turn seriously inhibit the AD efficiency.[41] The accumulation of VFA concentrations in AD is a well-studied phenomenon with their effects on methanization being widely publicized.[29, 31, 42] Amino acids are another important intermediate that generally degrade into VFAs via the Strickland reaction. A key product of this amino acid breakdown is the production of ammonia, originating from deamination, which is known to be an inhibitor of AD.[24, 43] Conditions and variables influencing AD must be considered in order to obtain a proper breakdown of the organic compounds.

The operating parameters of a digester must also be controlled so as to optimize the microbial activity and thus increase the AD efficiency. For anaerobes to work with high metabolic activity, it is imperative to have controlled environmental conditions.[40] Digestor upsets can be costly, resulting in heavy losses of biogas and subsequent profit depletion.[44] A digestor upset can occur from a multitude of problems, ranging from internal environmental factors within the system to the addition of inhibitory or toxic substances, and can play a major role in modifying reaction rates of individual sub-processes.[45, 46] Anaerobic processes may take place within a wide range of temperatures, influencing the dynamics and kinetics of the reactions.[47] Each group of bacteria are vulnerable at different pH levels and have unique thresholds for optimum

performance.[48] All microorganisms require essential elements for growth, with carbon, nitrogen, and phosphorus ratios playing significant roles in high-methane yield.[48] Moisture content in feedstock and particle size, as well as organic loading rate (OLR) and solid retention time (SRT) are also factors that affect the optimal performance of anaerobic systems.[48]

Reactor design, mixing, temperature, pH and buffering capacity, feedstocks, co-digestion, as well as pre-treatments and additives are often parameters that can be manipulated by the operator.[40] The presence of toxic or inhibitory substances, on the other hand, is often a factor that the operator has no control over. With the nature of our industrialized world, chemicals are readily used in almost all consumer products. The fate and effect that these chemicals have on environmental system varies immensely and has become a recent topic of discussion amongst the scientific community in regard to their effects on wastewater treatment process.

1.3.1.1 Exposure to Quaternary Ammonium Compounds

The term xenobiotic encompasses a large group of man-made organic compounds that are not found naturally in the environment.[49] Quaternary ammonium compounds (QACs) are a class of xenobiotic that are extensively used in agricultural, industrial, domestic, and healthcare applications as disinfectants, surfactants, detergents, emulsifiers, pesticides, and personal care products.[50, 51] QACs are amphiphilic compounds which act as disinfectants through their interaction with the bipolar cell

membrane of bacteria[52] and are extremely versatile organic chemicals, having both hydrophobic and hydrophilic parts.[53] Xenobiotics, along with ammonia, sulphide and metal ions are the most common toxicants inhibiting the AD process.[54] Food processing facilities often use antimicrobial QAC sanitizers to maintain cleanliness,[55] leading to QAC accumulation in wastewaters used as feedstock for anaerobic digestion. Approximately 75% of all QACs consumed during domestic and industrial application are released into wastewater treatment systems annually.[56]

Xenobiotics, and in particular QACs, can enter a waste stream at numerous points. The compound can either be found already within incoming influents or can be introduced to the system at any point along a food processing line from improper cleaning or accidentally cross contamination. The overall effect that QACs have on process performance of anaerobic digestors is relatively well understood, with the introduction of QACs decreasing methane production efficiency and consequently economic revenue.[57] However, there are few studies that go beyond the scope of monitoring the fate and effect of only specific analytes and metabolites involved in the transformation of QACs in anaerobic systems. This leaves an opportunity to explore the entirety of the metabolic profile created from the overall digestion process with the hope of better understanding how QACs interact and inhibit AD.

1.3.2 Conventional Analytical Techniques for Bioreactor Monitoring

A complex, difficult and multivariate process, the monitoring of AD has few reliable in-

line (i.e. those that monitor the process constantly) sensors for the monitoring of operational parameters.[40] Only a small number of process variables are commonly measured in-line in a bioreactor. These are pressure, temperature, pH dissolved oxygen, gas and liquid flow rates, and stirring speed.[58] Additional probes are used for fermentation processes to measure oxidation reduction potential (ORP), dissolved carbon dioxide, turbidity, and optical density.[60] In anaerobic systems, there are several parameters that can be used as indicators of process imbalance. These are carbon-nitrogen (C/N) ratio, total solids (TS), total volatile solids (VS), alkalinity, VFAs, organic loading rate (OLR), hydraulic retention time (HRT), and nutrient concentrations.[1, 8] No single operational parameter can provide a complete assessment of a treatment system, as the majority of parameters are interrelated. Although the aforementioned parameters are more expensive and time consuming to quantify, they provide a more comprehensive evaluation of the current status of the system compared to the easy to measure variables: biogas composition, production, and reactor temperature.

One of the most common methods used for operational process control is FOS/TAC, an endpoint titration that gives a measure of total VFAs as acetic acid (FOS) and compares it to the total alkalinity (TAC) in the system.[61, 62, 63] If the system is disrupted, often a spike in VFA production will be observed, causing the FOS/TAC ratio to jump.[63-65] The main failure of this method is that it cannot differentiate VFA types, so total VFA concentration could remain constant even if a shift from acetic acid production (“good”) to propionic and butyric acid production (“bad”) is observed.[66-68] This flaw exposes the inability to quickly differentiate between the different fatty acids that coexist within a

bioreactor system. The FOS/TAC method is sufficient to ensure optimal performance provided conditions within the reactor are normal, however, problems arise when conditions stray from the norm as this method often fails to diagnose or predict these changes.[63, 65, 66] Most traditional methodologies used for monitoring are costly, tedious, time-consuming and only provide a snapshot of the process.[69] Furthermore, these methodologies provide limited information on the chemical composition of the organic matter present,[70] thus, there is a need to improve the analytical techniques used in order to give an up to date, precise analysis of the key chemical parameters governing the system.

Chromatography involves the separation of a mixture in order to derive its chemical constituents, and it have been deployed as a method to observe product development within bioreactor systems. Chromatographic techniques such as gas chromatography (GC),[71] high performance liquid chromatography (HPLC),[72] GC mass spectrometry (GC-MS)[73] and HPLC mass spectrometry (LC-MS)[74] have all been successful in determining specific targeted products in anaerobic biodegradation pathways,[75] however they lack variation in the diversity and complexity of analytes studied. These chromatographic techniques are able to make quantitative determinations for specific compounds, but in order to do so, the user must first know which specific compounds to look for.[76] The technological advancements in chromatography have focused more on monitoring individual biochemical processes and products with a targeted analysis rather than observing the overall metabolic profile.

Spectroscopy is the science that studies the interaction between photon energy and matter. Various spectral ranges (UV-Vis, NIR and MIR) have been used to study the organic matter in waste in order to predict chemical composition and directly monitor bioreactor operation. Ultra-violet visible (UV-Vis),[77, 78] near infrared (NIR),[79, 80] and mid infrared (MIR)[16] spectroscopic techniques have previously been utilized for monitoring numerous AD parameters such as VFAs, alkalinity, COD, TOC, TS, and VS. These techniques are fairly low maintenance and generate relatively reproducible results for multiple parameters; however in order for their application to be practical, the spectral data must be coupled with multivariate statistical analysis (chemometrics) to derive relevant information.[60, 69] As a result of these limitations to conventional analytical techniques for the monitoring of bioreactors, the operation of these systems remains at less than optimal performance. The implementation of novel non-targeted analytical approaches that reveal the entire chemical profile in one analysis, while enabling specific parameters to be highlighted and interpreted efficiently without excessive processing has the potential to improve the monitoring of industrial bioreactors.

1.3.2.1 Identifying Quaternary Ammonium Compounds

The widespread use of QACs has led to its inevitable release into wastewater treatment systems as well as in some cases, directly into the environment.[81] Conventional WWTPs are not fully equipped to process QAC contaminated wastewaters, therefore having the ability to monitor these contaminants as they move through the treatment process is vital as it can reveal key information on the fate and effect it has within the

system. At present, it is challenging to develop analytical techniques that can guarantee the detection, identification, and quantitative determination of the broad spectrum of QACs that exist within the various compartments of the environment. Most current monitoring techniques for QACs in bioreactor systems involve the use of spectrophotometry, GC or HPLC. Spectrophotometry can be utilized for both ionic and non-ionic surfactants, but its application is hindered by the influence of organic interferents and high limits of detection (LOD).[82] GC is mainly applied in combination with single or tandem mass spectrometry (MS). This approach is satisfactory for regulating highly volatile surfactants, however less-volatile compounds require a derivatization step.[83] HPLC is also capable of measuring both ionic and non-ionic surfactants, separating homologues, oligomers, and isomers of mixture of complex surfactants. Although QAC levels can be measured in a short period of time with this method, tedious sample preparation, numerous extraction techniques and a high operation cost are involved.[84] Disulfine blue ion-pair extraction[81] and colorimetric methods[85, 86] have also been reported for monitoring QACs.

Often times, QACs themselves are not monitored as they move through the system, rather their effects on certain operational parameters are quantified instead. Measurements of methane, COD, VFAs, nitrogen, phosphorus, and ammonia are used to characterize the extent to which QACs effects a systems performance and can also be used to determine recovery time.[87] Observing the toxic effects QACs have on a wastewater system by monitoring percent COD reduction, percent ammonia reduction, levels of free bacteria, total adenosine triphosphate (ATP), optical density, and biogas

can help model a systems disruption and progression towards recovery.[88] The implementation of a novel non-targeted approach capable of monitoring selected operational parameters, as well as detecting, identifying and quantifying QACs in a single measurement has the potential to optimize wastewater treatment processes and limit environmental impacts.

1.4 Fundamentals of Nuclear Magnetic Resonance Spectroscopy

The work presented in this thesis makes extensive use of Nuclear Magnetic Resonance (NMR) spectroscopy to monitor and characterize the organic constituents present in food wastewaters. As the focus of this thesis is on environmental chemistry rather than NMR spectroscopy specifically, an in-depth discussion of the theory of NMR is beyond the scope of this work. For a thorough discussion, textbooks by Keeler and Gunther are recommended.[89, 90] This section will cover some basic concepts in the theory of NMR spectroscopy that touch on the underlying principles and explain how it is applicable to this work. Figure 1-3 presents the photo of the NMR spectrometer used throughout the research presented in chapter 2 of this dissertation for illustration.

NMR has quickly become an indispensable analytical tool within all reaches of the scientific community and is primarily used in environmental chemistry to characterize compounds and elucidate molecular structure.[91] NMR utilizes the magnetic properties of NMR active nuclei in a non-targeted, non-biased, and non-destructive manner.[91, 92] It is used extensively in both industry and research settings as a method of quality control,

drug screening, chemical identification and conformation analysis, however, its applications in environmental science is relatively underdeveloped.[91, 92] Unlocking the ability to use NMR to study sensitive environmental systems would provide numerous benefits that may help to improve our understanding of the complex physical and chemical interactions that exist within wastewater treatment processes. Some benefits include minimal sample preparation, rapid and comprehensive analysis, reproducible results, and the ability to elucidate the chemical structure of unknown compounds.[91, 93]

Figure 1-3: Two of the six NMR spectrometers from the University of Guelph NMR Centre are displayed in this photo. In the foreground on the right-hand side is a 600 MHz Bruker Avance III NMR spectrometer equipped with a TCI cryoprobe. In the background on the left-hand side is an 800 MHz Bruker Avance III NMR spectrometer.

In general, NMR is based on the excitation of atomic nuclei, typically $_1$H, using a combination of radiofrequency radiation and external magnetic fields. In an NMR measurement, signals are observed from all $_1$H nuclei present, with the position of a signal in the NMR spectrum being controlled by molecular structure. Generally speaking, the NMR spectrum of a compound is intrinsic to the structure of the substance being analyzed and can be used as a fingerprint to both identify constituents present and to compare differences between samples acquired under different conditions or at different times. In addition, the measurement of an NMR signal is fully quantitative and non-biased towards different types of molecular structures.

1.4.1 Nuclear Spin, Magnetic Moments and Resonance

The basic principle behind NMR is nuclear spin. Any atomic nucleus containing either odd mass, odd atomic number, or both, possess a quantized spin angular momentum and a magnetic moment.[94] The most commonly studied NMR active nuclei are protons (hydrogen nuclei, $_1$H). $_1$H have a nuclear spin state of ½ meaning that there are two allowed spin states for its nucleus: -½ and +½.[95] When no external magnetic field is applied (under normal conditions) the spin states are equal in energy (degenerate) with each spin state being equally populated throughout the collection of atoms, resulting in the cancellation of the magnetic moment.[94] When an external magnetic field is applied, however, spin states are not equal in energy; A nucleus is a charged particle in motion and as such generates a magnetic field of its own, therefore, the nucleus will have a magnetic moment generated by its charge and spin.[94]

With no external magnetic field being applied, a proton may spin either clockwise (+½) or counterclockwise (-½) allowing the magnetic moment to be pointed in opposite directions.[94] On the other hand, with an external magnetic field being applied, protons will have a preference for their magnetic moments aligned either with the field or opposed to it.[94] Hence, when a proton is placed into a magnetic field the spin states split into two states of unequal energy.[94] Now the magnetic moment may align with (low energy) or against (high energy) the external magnetic field, creating two energy levels.[96-98]

When NMR active nuclei are aligned with an external magnetic field and are induced to absorb energy, changing their spin orientation (with respect to the applied field), the NMR phenomenon occurs.[94] If the nuclei are subjected to radiofrequency radiation at the proper frequency, they will absorb the energy and transfer spins from the lower to the higher energy level. This absorbed energy is a quantized process and the response of the system as it returns to equilibrium is known as resonance. An NMR spectrum is produced by measuring and processing the signal that matches this transfer in energy.[95] The frequency of absorption is characteristic to the type of nuclei, dependent the applied static magnetic field and subject to the chemical environment surrounding the nuclei. The difference between resonance frequencies of nuclei in a molecule are very small and are expressed as the chemical shift, in ppm, compared to a reference compound.[96-98] It is this chemical shift that allows for the discrimination between molecules in the same compound, and between compounds in the same mixture as illustrated in figure 1-4.

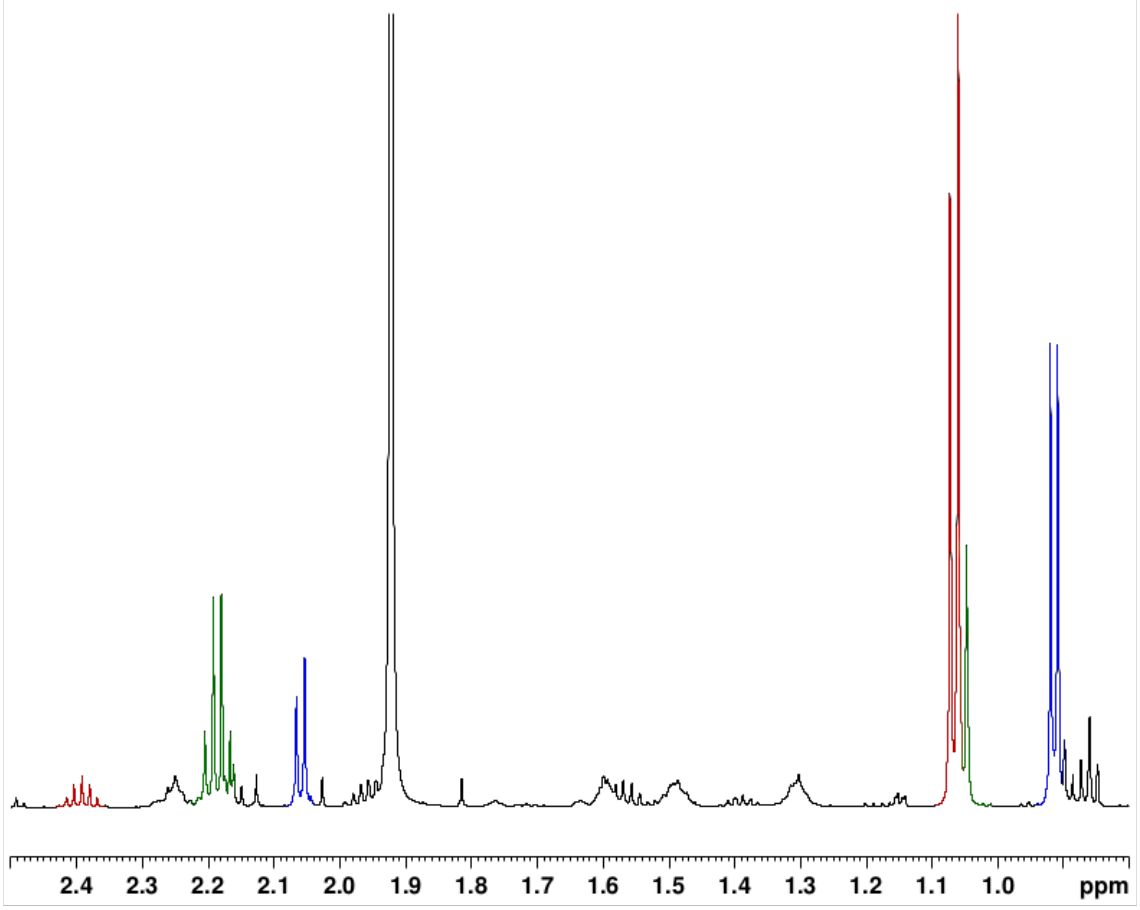

Figure 1-4: A one-dimensional $_1$H NMR spectrum of a bioreactor sample is shown above. The spectrum is zoomed between 0.8 – 2.5 ppm and colour coded to highlight selected key metabolites identified. Propionic acid (green), Isobutyric acid (red), and Isovaleric acid (blue).

1.4.2 Chemical Shift and Shielding

The term given to the observed resonance frequency of any given nucleus is chemical shift. Not all protons in a molecule have resonance signals at the same frequency because the protons in a molecule are surrounded by electrons and are in different electronic environments from one another. Each proton in a molecule also exists in a different chemical environment, and as such experience different amounts of shielding,

resulting in different resonance frequencies. As a result of this chemical shielding, different types of molecular structures exhibit different measured chemical shifts, and it is this variability that gives NMR tremendous utility. Chemical shift is generally defined as the resonance frequency of an NMR active nuclei relative to a standard known to have a chemical shift of 0. It is the most common feature of NMR spectroscopy used for chemical analysis and structural elucidation, and is expressed in parts per million (ppm).

1.4.3 Two-Dimensional NMR

Two-dimensional (2D) NMR experiments are often required to verify and elucidate the chemical structure of compounds present in a mixture. One-dimensional (1D) experiments provide an initial investigation from which preliminary information can be gathered, but they can be difficult to interpret due to the overlapping of signals. If signals cannot be clearly resolved in a 1D spectrum, or are not matched via a spectral database, 2D experiments can be utilized to further investigate and gain more information on the sample. 1D experiments have a single frequency dimension whereas 2D NMR experiments have two.[99] Having signals dispersed over a second dimension greatly improves the resolution of 2D experiments compared to 1D,[100] and enabling magnetization transfer allows the operator to see which signals are coupled to one another. Both of these features help in verifying and elucidating the chemical structure of complex molecules. Total correlation spectroscopy (TOCSY) is a 2D NMR experiment that correlates all the protons present in an uninterrupted spin system. $^{1}H - {}^{1}H$ TOCSY NMR experiments are used as a confirmation tool to verify the

structures of compounds identified in 1D spectra, as well as a tool to further elucidate the structure of unknown compounds. As illustrated in figure 1-5, TOCSY experiments show the hydrogen coupling network between molecules of the same compound. This network acts as a visual guide to confirm the presence of compounds found in 1D ^{1}H NMR spectra as well as tool to piece together the structure of unidentified compounds.

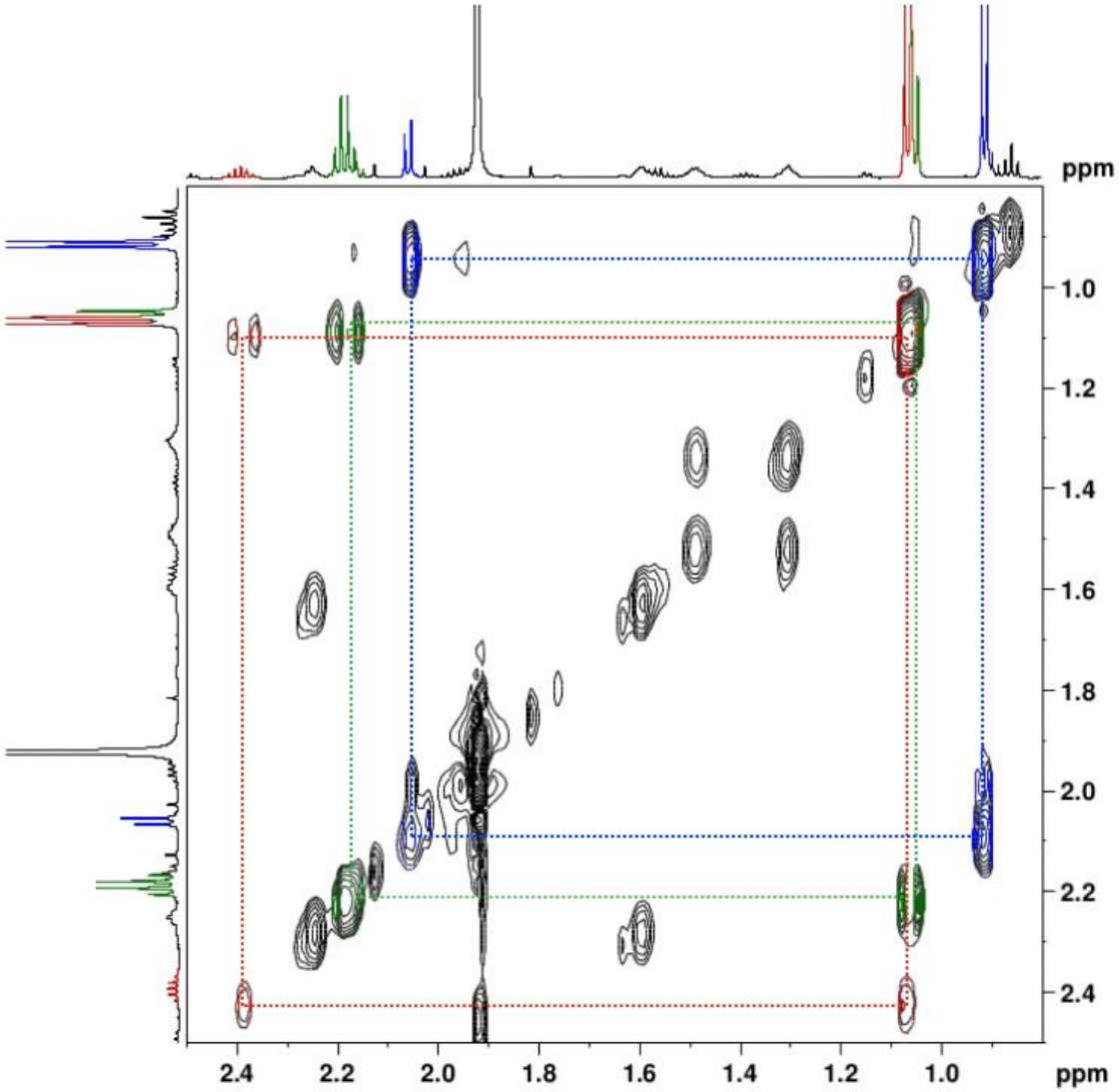

Figure 1-5: A two-dimensional $_1$H – $_1$H TOCSY NMR spectrum of Figure 4. is shown above. This image highlights the connection between resonances of each compound, with a characteristic 'box' forming between signals of the same compound. This 'box' is a total correlation of all protons of a chain with each other.

A unique feature of NMR is that even though the identity of a compound can remain unknown, it still has the ability to be monitored. There can be unknown compounds present in a sample that are not positively identified after comparison to standards or

NMR databases. In a 1D spectrum, these compounds appear as individual peaks with no correlation to one another, however, when pulled apart in a 2D spectrum, those unidentified peaks can be correlated to one another with the coupling network between hydrogen molecules of the same compound being visible. A TOCSY spectrum gives a total correlation of all protons of a chain with each other, which enables this elucidation of peaks that correlate to one other. This versatility is exceptionally useful for monitoring wastewater treatment systems as unknown chemicals are often introduced to waste streams. Having the ability to monitor and elucidate the structure of unknown compounds as they evolve over time within a treatment system would be an asset.

1.4.4 NMR Spectroscopy as an Analytical Tool

The dynamic and robust nature of NMR spectroscopy generates the largest non-biased dataset available amongst all spectroscopic techniques. Having a direct probe into the local molecular environment at a subatomic level allows for a detailed investigation into molecular structure and the complex physio-biochemical process that take place within sensitive environmental systems that are often unattainable using other analytical techniques. Despite intrinsically low sensitivity, NMR offers great utility because the response of magnetically active nuclei in a spectrum is nearly independent of the physical and chemical properties of the analyte.[101] The low sensitivity is also offset by the non-destructive nature of this technique. Measurements for the same experiment, as well as numerous different experiments can be conducted and repeated on the same sample without having to change out or modify the sample in any way.

As the most effective analytical tool available for determining the structure of organic compounds, NMR is used to study both physically and chemically complex systems.[102] Environmental samples are most commonly characterized with reference to the targeted analysis of only specific chemical parameters. This makes it a difficult task to derive relevant information on the otherwise “known unknowns and unknown unknowns” that are ubiquitous in environmental systems.[92] NMR is an extremely useful tool as it allows for simultaneous targeted and untargeted analysis of multiple chemical parameters. NMR can be used to screen for multiple known and unknown substances.[92] From a single data set, specific metabolites, by-products, inhibitors and/or regulatory compounds can all be detected, identified, and quantified. Despite this utility, the use of NMR in environmental science is underdeveloped relative to other scientific disciplines.

1.5 Applications of NMR spectroscopy in the Characterization of Wastewater

Globally, wastewater quality is generally characterized using physical, chemical, and microbiological tests. However, these parameters depend on expensive, labor intensive and/or time-consuming methods, offering only snapshots of moments in time, which makes them unsuitable for real time monitoring.[60, 69, 103] Recent technological advances have pushed the practical applications of NMR spectroscopy in environmental sciences to the forefront. One of the most common applications of NMR is in the characterization of waste streams.

NMR spectroscopy has been used to characterize and quantify the substituents in

wastewater influents and effluents, as well as the resulting sludge that is produced during the treatment process. However, due to the complex make-up of wastewater, with varying amounts of organic, inorganic, and toxic constituents, the level of detail is often limited.[104] In a ^{1}H NMR spectrum of a complex environmental mixture, the signals from varying constituents are highly overlapped, mainly limiting the characterization to specific functional groups rather than individual compounds.[105, 106] This makes it difficult to identify the macromolecular level heterogeneities that exist within different waste streams.

Efimova et al. (2013) used NMR in combination with thin-layer chromatography (TLC) to study the lipid profile of various wastewaters originating different sources in order to estimate their potential for biofuel production. This study was only able to utilize NMR to determine functional groups from the lipid extracts, rather than the specific compounds due to the complex multicomponent mixtures.[107] In a similar study conducted by Dignac et al. (2001), changes in the organic composition of wastewaters were observed using ^{1}H NMR as a tool for the direct chemical analysis of structural information. Changes in the organic matter identified during biological wastewater treatment were monitored through the transformation of functional groups.[108] Both studies were limited by their inability to differentiate between individual components present within specific groups. In another study conducted in Brazil, ^{1}H NMR was utilized in combination with multivariate statistical analysis (chemometrics) to determine the concentration of the major components of wastewaters before and after flowing through a sewage treatment plant.[102] However, due to the complexity of the NMR data sets and similarities amongst

samples, chemometric methods were required to complement the spectroscopic data. The U.S. EPA also used ^{1}H NMR in a 2018 study to assess the impacts of WWTP effluent on downstream drinking water quality. Cell-based metabolomics coupled with spectroscopy showed usefulness as a tool for assessing the biological effect of complex pollutant mixtures.[109] By using the NMR data in tandem with multivariate statistics, both the research group in Brazil as well as the U.S. EPA were able extract pertinent information.

1.5.1 Bioprocess Monitoring

NMR spectroscopy provides the highest density of non-biased data wherefrom relevant information can be quickly and easily extracted.[110] NMR spectra act as chemical fingerprints that contain vast amounts of information relating to chemical structure, concentration, biodegradation products, as well as operational parameters for wastewater treatment. These spectral fingerprints captured during bioprocess monitoring can subsequently be used for system diagnostics and management.[60, 69] However, the majority of studies utilizing NMR for bioprocess monitoring focus solely on the targeted quantitative monitoring of specific chemical compounds rather than employing a non-targeted approach for quantitative and qualitative monitoring and diagnostics.[60, 69]

Yamazawa et al. (2013) used NMR spectroscopy to characterize the microbial degradation of complex substrates as well as the metabolic dynamics of large

macromolecules present in the AD process. NMR measurements were used to demonstrate that cellulose was anaerobically degraded, fermented, and converted into biogas by targeting key chemical markers that would be present throughout each step of the process.[111] A study conducted in Germany evaluated the effectiveness of using non-invasive low-field NMR for on-line monitoring of the fermentation process.[112] The results highlighted NMR to be highly robust and chemically specific for reaction monitoring in opaque media. Once again specific by-products were quantified and used to monitor the respective process.[112] Xue et al. (2014) explored coupling a novel in-situ NMR bioreactor with ex-situ high-resolution NMR spectroscopy to determine the metabolic profile of an advantageous bacteria. This study successfully demonstrated NMR as a tool to monitor the fermentation process, specifically identifying intermediate and endpoint metabolites.[113] Jawien et al. (2016) applied ^{1}H NMR combined with chemometrics to generate the metabolic fingerprint as a bioprocess control tool for monitoring the progression in the brewing process of oil seeds. Changes in concentrations of chemical biomarkers were explained relative to the biochemical processes and external conditions, which could ultimately be utilized for quality control in the food industry.[114]

The real-time monitoring of bioreactors is paramount for effective bioprocess control.[60] The combination of at-line (either on-line or in-line) NMR process data with on-line process variable data has the potential to greatly improve the ability to fully characterize the bioprocesses.[60] Chemical constituents of bioreactor media (substrates, products and intermediates) are predominantly monitored off-line by methods requiring multiple

stages of sample preparation,[69, 115] compared to NMR, which could offer an at-line analysis of those key chemical components in a single measurement, with minimal sample preparation. Successful bioprocess monitoring in real-time can lead to increased efficiency and reproducibility, as well as improving quality control and profit maximization.[69, 116]

1.5.2 Quaternary Ammonium Compounds

QACs are widely applied in domestic and industrial applications. Therefore, QACs will inevitably get into different compartments of ecosystems. Their toxicity and persistence are such that an accurate and sensitive analytical method must be developed to better understand the occurrence, distribution and fate of QACs in various environmental samples.[56] NMR has shown great versatility in the types of compounds that it can analyze, with QACs emerging as a trending topic in new research. Targeted compound analysis for QACs is difficult because standard and reference compounds are not always available. Different QAC homologues are found within environmental systems and are hard to positively identify because often times QACs are not expected to be present within certain waste streams. NMR spectroscopy provides a unique non-targeted approach to detect QACs in wastewater.

Alves Filho et al. (2014) utilized both 1D and 2D NMR experiments to gather a non-targeted analysis of organics, and specifically recalcitrant compounds found in urban wastewaters. This combination of spectroscopic techniques allowed for the detection

and elucidation of a non-specific contaminant that was present in the mixture at a low concentration without the use of standard compounds.[117] An experiment conducted in Poland examined the genotoxicity and biodegradation of quaternary ammonium salts (QAS) using both ^{1}H NMR and ^{13}C NMR.[118] The hypothetical mechanism for QAS biodegradation were proposed by correlating degradation to decomposition of specific molecular positions and measuring intermediates and by-products. NMR was utilized to monitor different stages of the biodegradation process but, the concentrations of QAS, however, were measured using disulphine blue active substance test (DBAS).[118] A study conducted by the Department of Chemical and Biomolecular Engineering at Ohio University used ^{1}H NMR to characterize quaternary ammonium corrosion inhibitors having different alkyl tail length.[119] ^{1}H NMR analysis confirmed the desired structure and purity of the compounds.

Other analytical instruments have been used to complement one and two-dimensional NMR experiments in order to better examine complicated mixtures.[120, 121, 122] Al-Tamimi et al. (2019) linked HPLC-MS with ^{1}H NMR to quantify the main QACs and metabolites present in the roots and leaves of a medicinally important plant in Saudi Arabia and Italy. Luo et al. (1992) also linked HPLC with ^{1}H NMR to aid in the identification and characterization of metabolites found during the synthesis of quaternary ammonium-linked glucuronide metabolites of drugs containing an aliphatic tertiary amine group.

1.6 Outline of Thesis

Despite the versatility offered by NMR spectroscopy, it can be argued that it is currently underutilized in the field of wastewater treatment. Practical applications of NMR used in this niche sector are limited to screening for functional groups or identifying specific metabolites and by-products found in influents and effluents. The use of NMR as a tool for the targeted analysis of compounds has been demonstrated with great success, however there are limited examples for the use of NMR as a tool for the non-targeted analysis of complex environmental mixtures. The powerful and diverse nature of NMR spectroscopy will improve the non-targeted analysis of waste streams, which will complement traditional analytical techniques. Combining methods will provide a more comprehensive assessment of the chemical composition of waste streams, leading to improved characterization and optimization of the wastewater treatment process. This dissertation will investigate and validate the novel application of NMR spectroscopy as a tool to characterize the full organic composition of food waste effluents and process water.

Chapter 2 discusses the non-targeted characterization of bioreactor samples after exposure to a QAC using a combination of one and two-dimensional NMR experiments. The compounds present are identified and confirmed by comparing the results to an NMR spectral database. The results from the NMR analysis are compared to the results obtained for biogas quality and quantity.

Chapter 3 explores the use of NMR spectroscopy as a unique way to investigate the correlation that exists between food waste composition and oxidative demand.

Chapter 4 will conclude the results obtained from the chapters 2 and 3, drawing connections between gaps in the research and connecting them to future works. The expansion of NMR in the field of environmental science will also be discussed.

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Chapter 2 – Non-targeted Characterization of the Metabolic Profile of Quaternary Ammonium Affected Wastewater Bioreactors using Nuclear Magnetic Resonance.

2.1 Abstract

Quaternary ammonium compounds (QACs) are disinfection agents used in industrial cleaning processes. QACs are known to interfere with the proper functioning of anaerobic waste digestion and impact the quality and quantity of the biogas produced (i.e. CO_2 and CH_4). While the impact of these contaminants on waste digestors are well known, the impact these compounds have on the metabolic profile of an anaerobic digestor is less understood. This paper describes the use of nuclear magnetic resonance (NMR) spectroscopy as a non-targeted and non-biased tool to monitor variations in the metabolic profile of an anaerobic bioreactor used to treat industrial wastewater that has been exposed to Benzalkonium chloride, a key QAC. Using NMR, the variation in the metabolic profile of microcosms is compared to variations in the quality and quantity of the biogas produced. A clear development of propionic, isobutyric, isovaleric, and other volatile fatty acids (VFAs) is observed indicating a disruption to the overall ability of the system to convert fatty acids to methane. The ability to successfully identify both the overall metabolic profile and the occurrence of the individual VFAs in one analysis helps to provide valuable information on the metabolic pathways involved in the bioreactor disruption.

2.2 Introduction

Anaerobic digestion (AD) is a biological process in which microorganisms biodegrade and stabilize complex organic matter in the absence of oxygen, yielding treated effluent and methane for energy recovery.[1, 2] AD has historically been used by humans for waste management and wastewater treatment and has recently emerged as a promising solution for food waste reduction, energy recycling, and nutrient recovery.[2, 3] The process of AD consists of four phases. First, hydrolysis depolymerizes complex biomolecules (proteins, carbohydrates, and fats) into soluble organic monomers (amino acids, sugars, and fatty acids). Second, acidogenesis converts these products into alcohols, carbonic acid, and volatile fatty acids. Third, acetogenesis generates acetic acid, carbon dioxide, and hydrogen. Finally, methanogenesis occurs, producing biogas (typically 60% methane, 40% CO_2).[1-5]

Anaerobic bioreactors are complex, multi-variable systems, as during their operation, substrates are consumed and products and intermediate metabolites are formed.[6] The development of volatile fatty acids (VFAs) are a key intermediate in the process of AD, however, the production and accumulation of specific VFA concentrations have shown the ability to be inhibitory, ultimately limiting biogas generation.[3, 4, 7] A study by Murto et al. (2004) suggests that only VFAs can be considered reliable for process monitoring, therefore successful identification of the individual VFAs formed is vital,[4] as it can supply valuable information on the different metabolic pathways involved in the process. The typical operation of bioreactors is based on the routine monitoring of several key

parameters including: biogas production, 5-day biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total organic carbon (TOC), pH, and measurements of specific nutrients and by-products, such as carbohydrates, VFAs, amino acids, and ammonium.[9, 10] These traditional methodologies used for monitoring are costly, time-consuming and only provide a narrow snapshot of the process, providing limited information on the chemical composition of the organic matter present.[9, 11] As such, there are benefits to exploring new analytical approaches to characterize bioreactor operation in order to give an improved analysis of the key chemical parameters governing the system.

Microorganisms are the driving force behind the complex biological process of AD,[3] with the microbiome being extremely sensitive and heavily dependent upon the environmental conditions present within the bioreactor.[12] The disruption of a digester can be costly and result in a substantial reduction of biogas production. Quaternary ammonium compounds (QACs) are commonly used industrial disinfectants and are known to interfere with the proper functioning of the AD process, but their impact on the microbiome, and in particular their metabolic pathways, are not yet fully understood.[13, 14] Benzalkonium chloride (BAC), which is a mixture of alkyl benzyl dimethyl ammonium chlorides with chain lengths C_8 – C_{18},[15, 16] is the most frequently found QAC in municipal wastewaters worldwide and is perceived to be recalcitrant under anaerobic conditions.[15, 17-20] Studies exploring the impact of BAC on anaerobic digestors show this impact primarily through the measured reduction in biogas production and do not explore the metabolomic profile of the anaerobic digestion process.[18-21] This limited knowledge of

how QACs impact anaerobic digestor systems limits the ability to determine the extent to which QACs have impacted the digestion processes.

The measurement of QACs in wastewater is a key step for monitoring potential impacts on AD and have been performed in the past using various technologies, but each with their own limitations. QACs can be measured spectrophotometrically using anionic dyes or chromogenic reagents.[22, 23] While quick and simple, this technique can be influenced by the presence of anionic surfactants as QACs have a higher affinity for them than the dyes.[24] Moreover, this technique is unable to identify individual QAC structures, limiting its widespread use.[14] High-performance liquid chromatography (HPLC) is promising for screening BAC, but this technique is limited by its inability to analyze non-chromatic surfactants, as they are unable to absorb ultra-violet (UV).[14, 25] Gas chromatography/mass spectrometry (GC/MS) has been used for qualitative determination of BAC in river water and sewage effluent,[24, 26] however, a complex pre-treatment is required.[14, 26]

Nuclear magnetic resonance (NMR) spectroscopy is a powerful non-targeted analytical tool used to obtain high-resolution molecular-level data relating to the makeup of complex mixtures of organic compounds with minimal sample preparation.[27-29] This approach provides a non-biased, non-targeted and fully quantitative approach that has the potential to simultaneously monitor VFA development, the chemical stresses imposed from BAC, and the BAC itself. Considerable efforts are being undertaken to develop spectroscopic methods for monitoring and quantifying key parameters involved

in wastewater treatment processes,[9] however, the use of NMR, specifically in the field of bioprocess control, is relatively underdeveloped in comparison to other spectroscopic techniques.[6]

The overall objective of this study is to determine the effects QACs have on bioreactor systems by monitoring the evolution of the composition of anaerobic digesters in response to QAC exposure and relate those variations to the quality and quantity of the biogas produced (i.e. CO_2 and CH_4). The goal of this research is to explore the use of NMR spectroscopy as a tool to provide both a qualitative and quantitative analysis of the metabolic footprint generated from the interaction between BAC and the bioreactor samples and by doing so be able to recognize deviations away from normal operating procedures.

2.3 Material and Methods

2.3.1 Anaerobic Digester Pilot Study

An anaerobic digester system was constructed using mixed sludge from an anaerobic potato digestor. This reactor was fed potato wash-water and monitored over a 6-month period. Once the reactor was determined to be stable, approximately 20 µL of a QAC-based disinfection agent (Ster-Bac KQ-12) was injected into the system and monitoring continued. Figure 2-1 presents the photo of the anaerobic digestor used throughout the research presented in chapter 2 of this dissertation for illustration; A single stage 10-liter

New Brunswick Bioflo 3000 bioreactor, operating with ~ pH 7, a mesophilic temperature of ~ 31 ° C, and an HRT of 10 days.

Figure 2-1: The lab scale anaerobic digestor owned and operated by Geosyntec Consultants is displayed in this photo.

2.3.2 Microcosm Tests

Microcosms were prepared using mixed sludge from the anaerobic digestor inside a LABstar Glove Box Workstation (MBraun) under a 100% argon atmosphere to prevent the mixed sludge from oxygen exposure. Fifteen (15) mL of mixed sludge was placed into 24 individual 40 mL glass vials with 24 mm mininert valves (Supelco). The vials were then divided into 4 series for tests of exposure to 0, 16.7, 33.3, and 66.7 mg/L benzalkonium chloride (BAC, Sigma Aldrich). BAC was added to each vial using 15 ml of a 0.01 M phosphate buffer (Sodium phosphate monobasic dihydrate / Sodium phosphate dibasic, Sigma Aldrich), pH 6.92, containing the appropriate amount of BAC to achieve the desired concentrations.

Samples were placed into an Innova 3100 water bath shaker (New Brunswick Scientific) and left to incubate at a temperature of 35ºC and a shake rate of 75 rpm until being tested on days 1, 2, 5, 9, 12, and 18. Samples were vented daily to alleviate built up biogas.

2.3.3 NMR Sample Preparation

To prepare the samples for NMR analysis, 10 mL was removed from the vial being tested and placed into a 15 mL centrifuge tube. Samples were then placed in a Sorvall Legend X1R Centrifuge (Thermo Scientific) and spun at 5000 rpm for 5 mins. 600 µL of the supernatant was passed through a 0.45 µm syringe filter to remove the fine

particulates and combined with 60 µL of deuterium oxide (D_2O, Sigma Aldrich) that included 0.05 (w/v) 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt (TMSP, Sigma Aldrich) as an internal standard and chemical shift reference. Samples were transferred into a 5 mm diameter glass NMR tube (Wilmad) for the analysis to be carried out.

2.3.4 NMR Analysis

All NMR experiments were carried out on a Bruker Avance III 600MHz NMR spectrometer equipped with a Bruker TCI cryoprobe. 1D $_1$H METNOESY (Metabolomics Nuclear Overhauser Effect Spectroscopy) experiments were acquired using a 90º excitation pulse, 256 transients, and a 2 s delay with pre-saturation. 2D $_1$H-$_1$H TOCSY experiments with excitation sculpting water suppression were acquired using 32 transients and 256 increments, 1.5 s recycle delay, and DIPSI mixing time of 80 ms. TOCSY spectra were processed with 1024 points in the F2 dimension, 1024 in the F1 dimension and phase corrected manually, then readjusted with an automatic phase correction.

2.3.5 NMR Identification and Quantitation

Chenomx NMR Suite 8.3 professional (Chenomx Inc.), was used to identify and quantify compounds in each spectrum using reference spectra. 2D $_1$H-$_1$H TOCSY measurements were used to verify the structure of identified metabolites.

2.3.6 Gas Chromatography Analysis

The biogas (CO_2 and CH_4) content in each sample vials headspace was measured at the various testing points using an SRI 8610C Gas Chromatograph (SRI Instruments) equipped with a 6 ft Haysep D packed column and a flame ionization detector (FID) methanizer, utilizing nitrogen as the carrier gas, and a sample loop of 20 µL. Peak Simple chromatography acquisition and integration software (SRI Instruments) was used to process the data, with peaks being measured by area.

2.4 Results

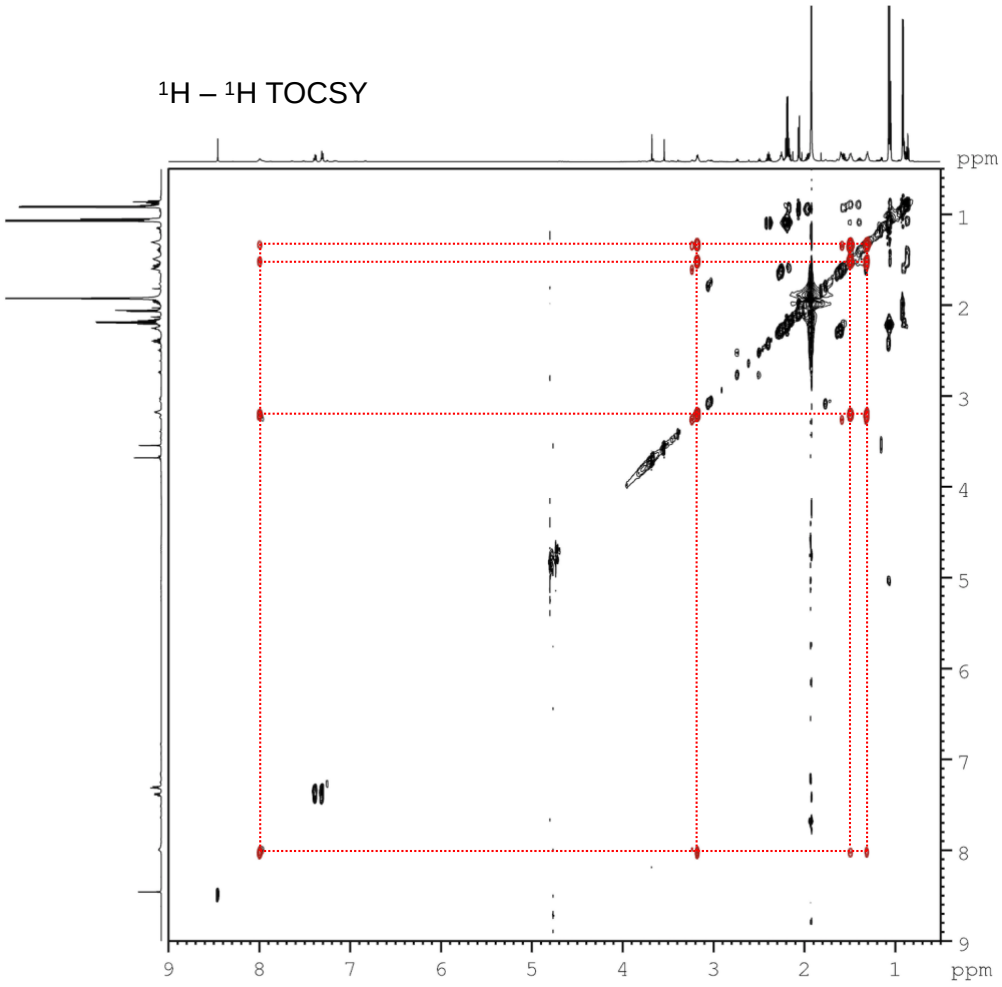
2.4.1 Operation of Anaerobic Digester Pilot Study

The performance of the digestor became unstable after introduction of the QAC as measured in variations in the biogas composition, however the overall metabolic response to this disturbance could not be fully observed and quantified using NMR due to the low concentration of metabolites present. The biogas composition baseline before injection was 35% CO_2 and 65% CH_4. After injection, the biogas ratio proceeded to shift between a range of 30-50% CO_2 and 50-70% CH_4, however there was never any significantly observable difference in the NMR spectra. It was determined that a greater concentration of QAC was needed in order to observe a more significant metabolic response using NMR, however instead of injecting a greater amount of Ster-Bac (KQ-12) into the system and risk the potential of a digester crash, it was elected instead to

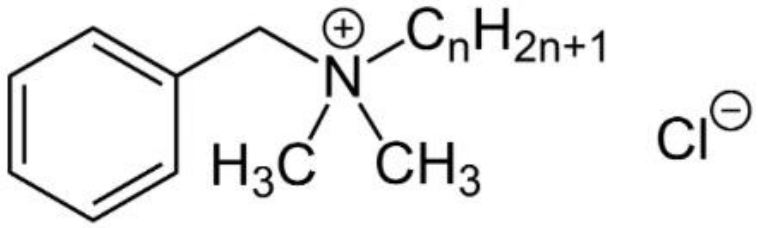
remove mixed sludge from the system and conduct microcosm tests outside of the digester.

2.4.2 Measurement of Benzalkonium Chloride

Figure 2-2 shows the structure of BAC and the ^{1}H-^{1}H TOCSY NMR spectrum of the microcosm with 33.3 mg/L after 18 days. The signals corresponding to BAC are highlighted in red in the TOCSY NMR spectrum. The TOCSY spectrum shows clear correlations between the alkyl signals at around 1.5 ppm, the signals from CH_2 groups between aromatic ring and the amine functional group at around 3 ppm, and the aromatic signals at 8 ppm.[30] This signal pattern, which is observed in all microcosm samples, including the control, increases with increasing dosage of BAC and is consistent with both the general structure of BAC and the presence of multiple similar BAC structures, confirming BAC being a technical mixture.



Benzalkonium Chloride



n = 8, 10, 12, 14, 16, 18

Figure 2-2: The chemical structure of benzalkonium chloride (BAC) shown alongside the ^{1}H – ^{1}H TOCSY NMR spectrum of a microcosm sample after 18 days of exposure to 16.7 mg/L BAC. The resonances from BAC are highlighted in red.

2.4.3 Characterization of Microcosms

Figures 2-3, 2-4, and 2-5 show the alkyl (0 – 2.5 ppm), functionalized alkyl (2.5 – 4 ppm), and aromatic regions (6.5-9 ppm) of the $_1$H NMR spectra of the microcosm samples after 18 days of exposure to BAC, respectively. Key fatty acids, amino acids, and related compounds are identified and listed in table 2-1. The labelled structures were initially identified by comparison with standard spectra in the CHENOMX metabolite database and confirmed using 2D $_1$H-$_1$H TOCSY NMR, which are shown in figure 2-6 for microcosm samples after 18 days of exposure to BAC. Figure 2-6 shows the occurrence of different structural classes (linear fatty acids, branched fatty acids, amino acids, and alcohols) in each microcosm treatment as different colours.

Table 2-1: Metabolites identified in microcosm studies using $_1$H NMR spectroscopy.

Reference #	Compound	Reference #	Compound	Reference #	Compound	Reference #	Compound
	Fatty Acids		*Amino Acids*		*Alcohols*		*Auxins*
1	Formic Acid	10	Glycine	20	Ethanol	26	Indole-3-Acetate
2	Acetic Acid	11	Alanine	21	Ethylene Glycol		*Disinfecting Agents*
3	Propionic Acid	12	Threonine	22	Propylene Glycol	27	Benzalkonium Chloride
4	Butyric Acid	13	Glutamine		*Amines*		
5	Isobutyric Acid	14	Methionine	23	Methyl Amine		
6	Valeric Acid	15	Valine		*Amides*		
7	Isovaleric Acid	16	Isoleucine	24	Acetamide		
8	Succinic Acid	17	Tyrosine		*Pyrimidines*		
	Fatty Acid Metabolites	18	Phenylalanine	25	Uracil		
9	Phenylacetate	19	Tryptophan				

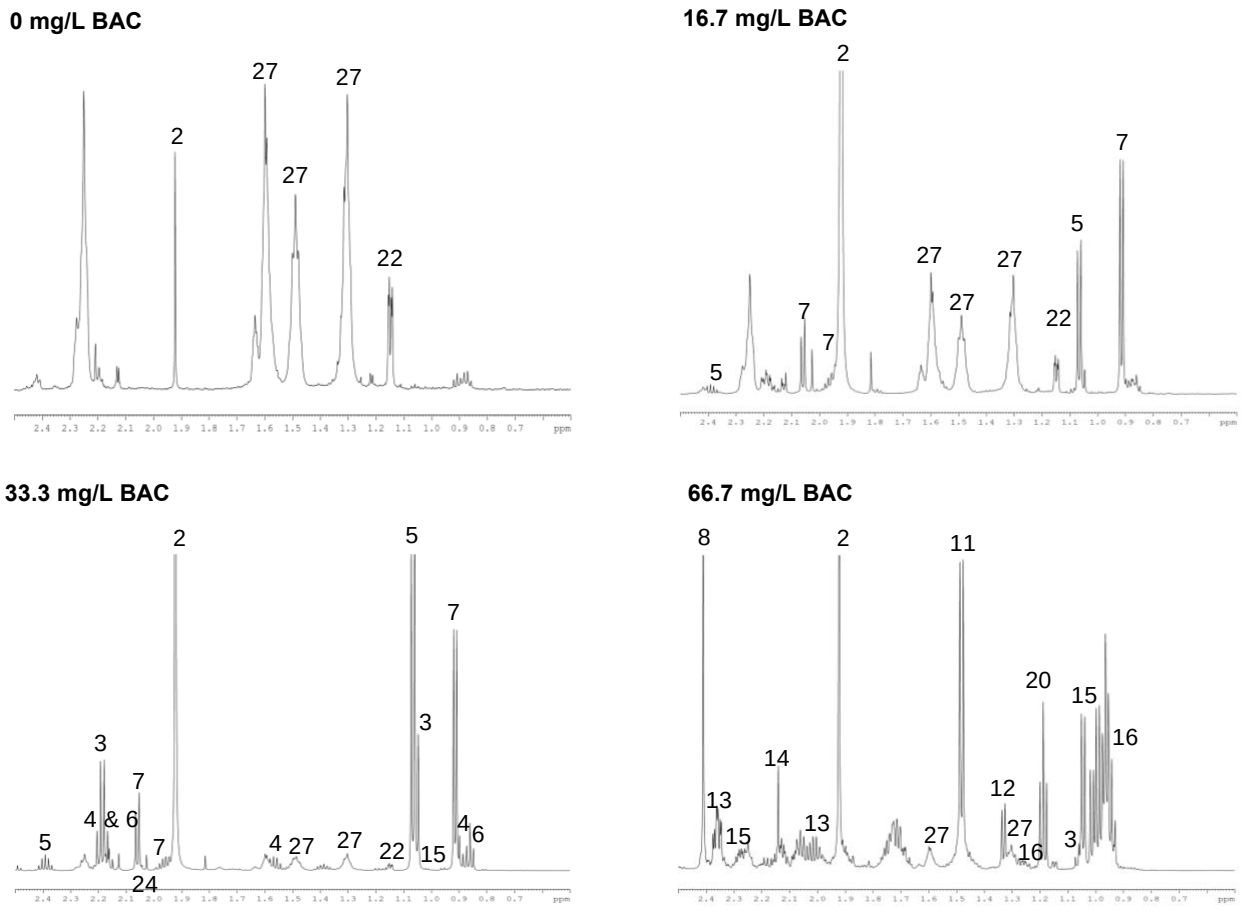


Figure 2-3: The alkyl region of the $_1$H NMR spectra microcosm samples after 18 days.

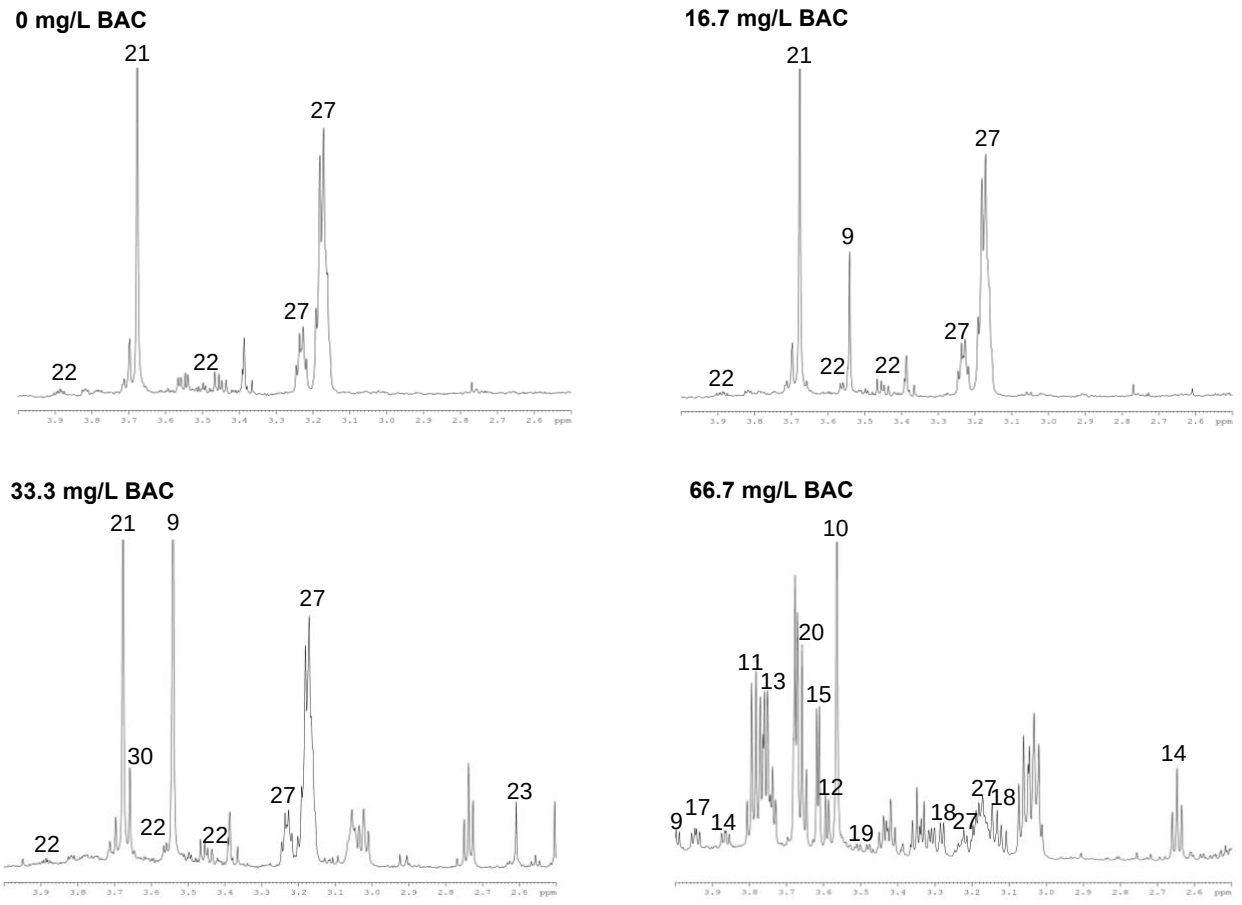


Figure 2-4: The functionalized alkyl region of the $_1$H NMR spectra microcosm samples after 18 days.

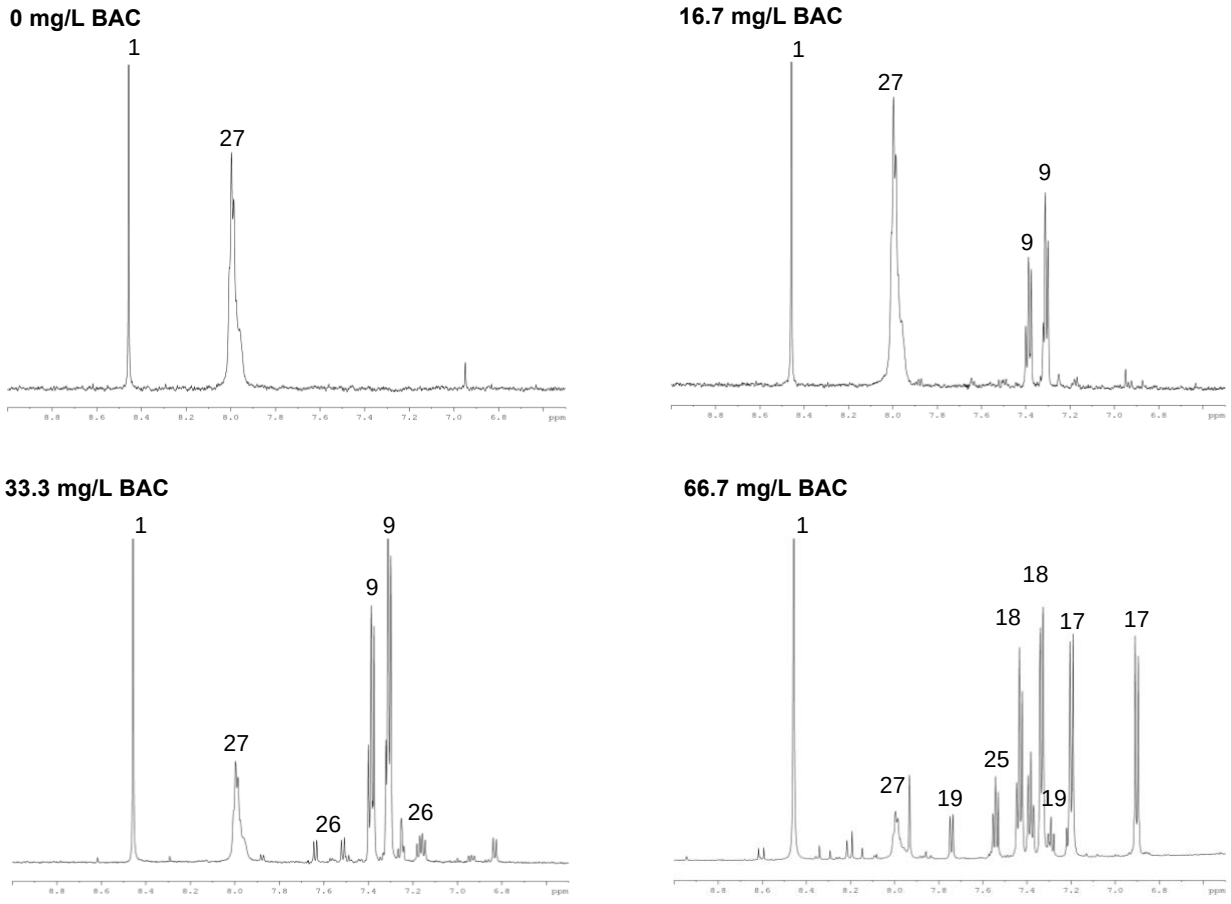


Figure 2-5: The aromatic region of the $_1$H NMR spectra microcosm samples after 18 days.

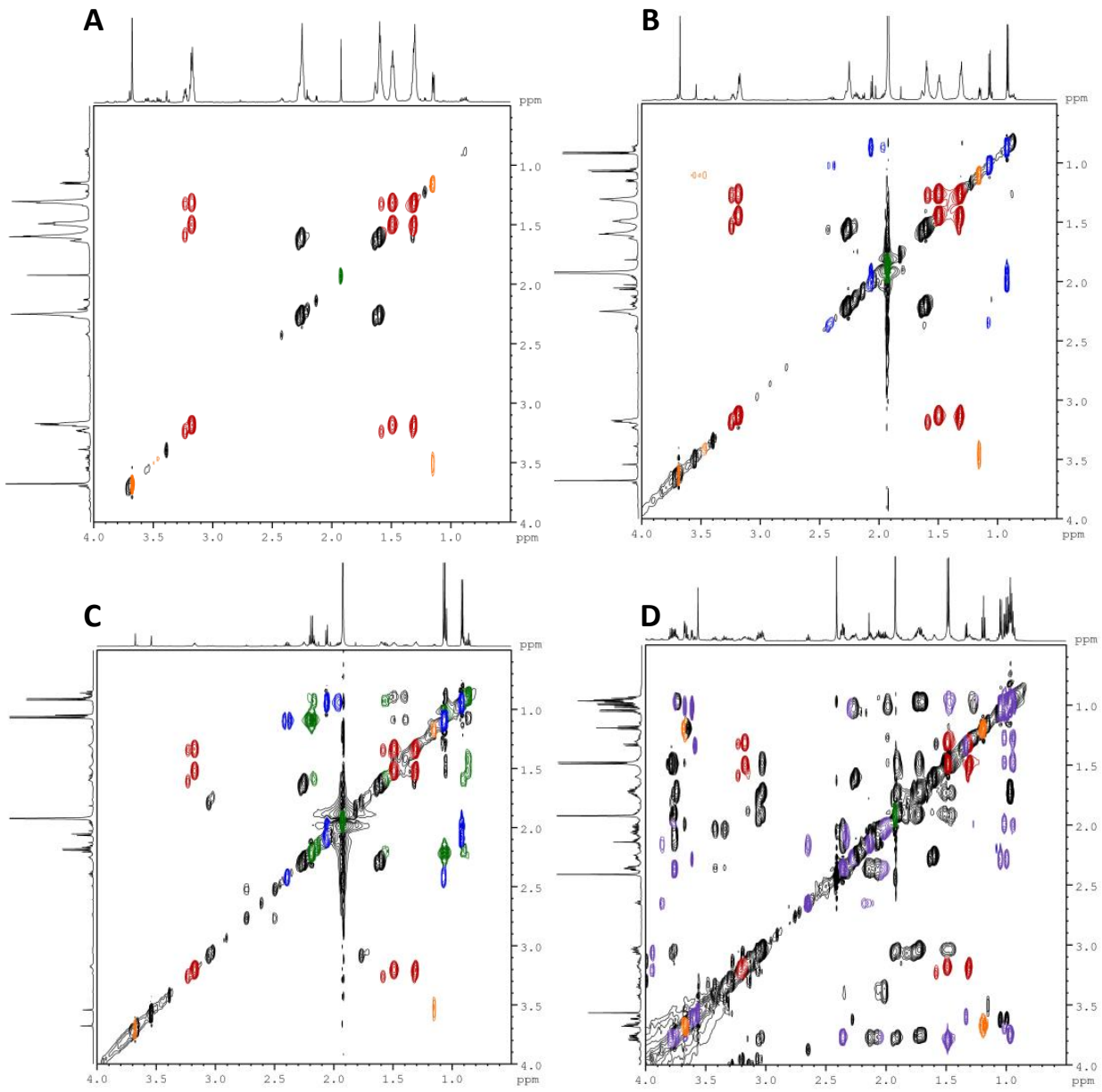


Figure 2-6: $_1$H – $_1$H TOCSY NMR spectra of the functionalized alkyl and alkyl regions of microcosm samples after 18 days of BAC exposure. A: 0 mg/L BAC; B: 16.7 mg/L BAC; C: 33.3 mg/L; D: 66.7 mg/L BAC. Colours denote different classes of compounds; BAC (red), linear fatty acids (green); branched chain fatty acids (blue); amino acids (purple); alcohols (orange).

Once signals were identified, CHENOMX was used to quantify selected fatty acids and amino acids by spectral deconvolution and comparison to an internal standard. Figure 2-7 compares the progression of fatty acid concentrations for each microcosm treatment over time. Figure 2-8 compares the progression of amino acid concentrations over time for the 33.33 mg/L and 66.7 mg/L microcosm treatments. No amino acid signals were measured in the 0 or 16.7 mg/L microcosm treatments, indicating no significant accumulation of metabolic intermediates.

Measurements of biogases (CO_2 and CH_4) in the microcosm headspace was performed by GC-FID before NMR measurements were performed. Figure 2-9 compares the % contribution of CH_4 to the biogas for each microcosm treatment over the duration of the experiment. Figure 2-10 compares the change in the total biogas (CO_2 and CH_4) relative to the total biogas in the day 2, 0 mg/L BAC control microcosm.

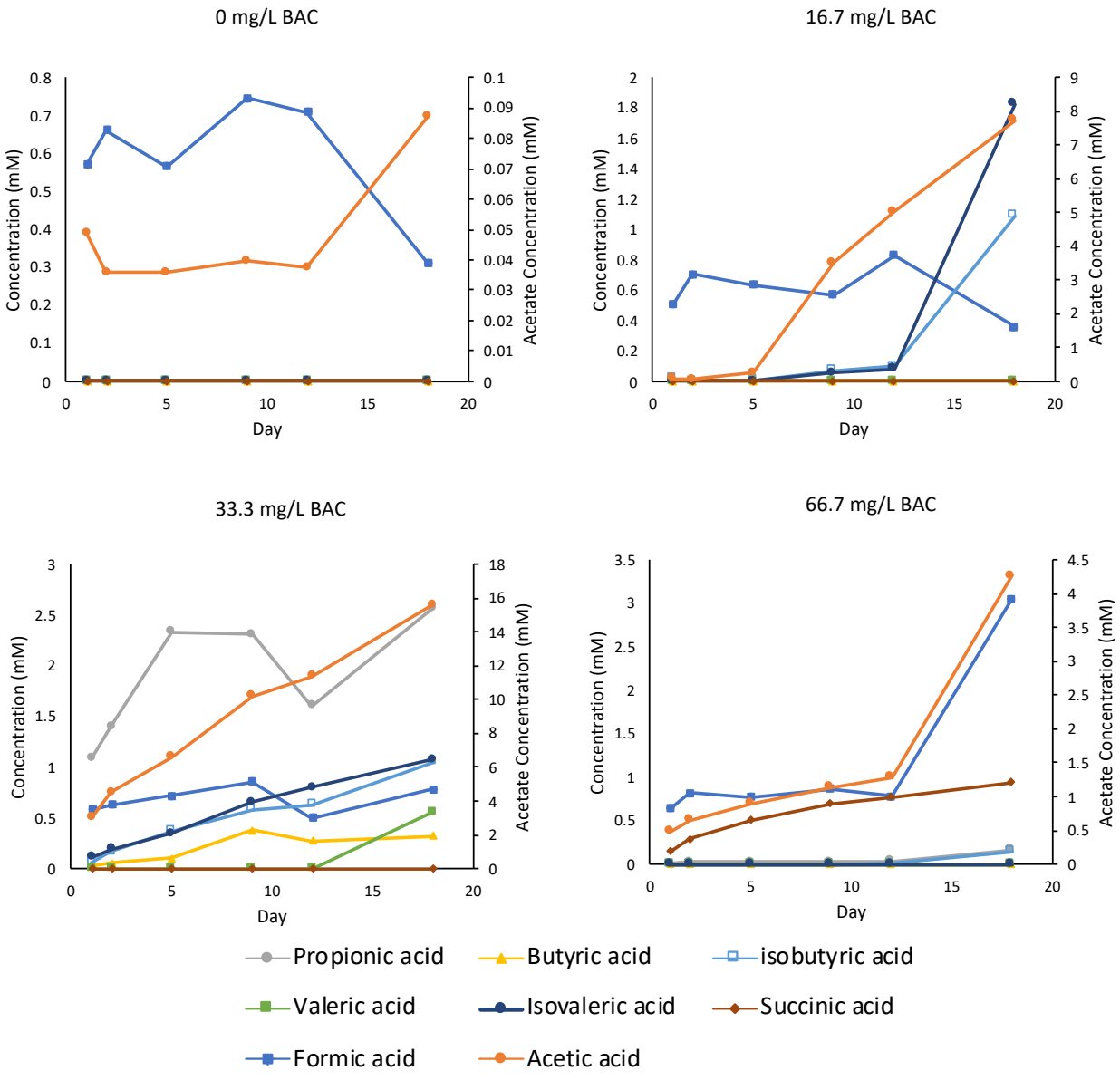


Figure 2-7: Selected fatty acid concentrations as measured using $_1$H NMR spectroscopy.

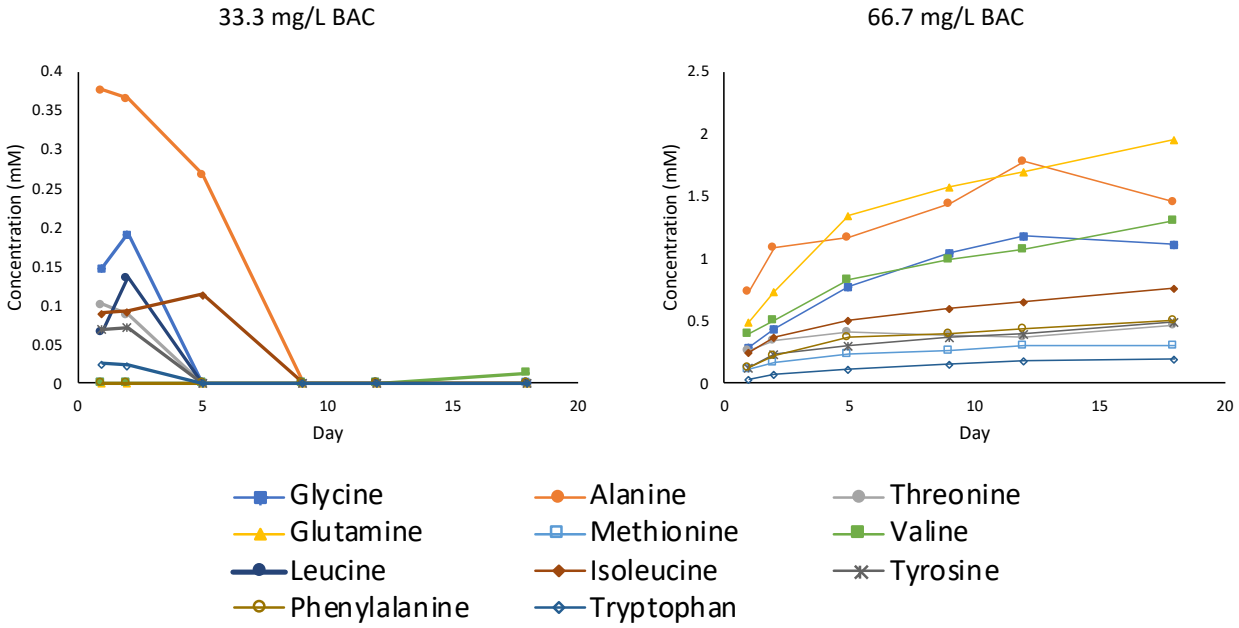


Figure 2-8: Selected amino acid concentrations in the 33.3 mg/L and 66.7 mg/L BAC microcosms as measured using $_1$H NMR spectroscopy.

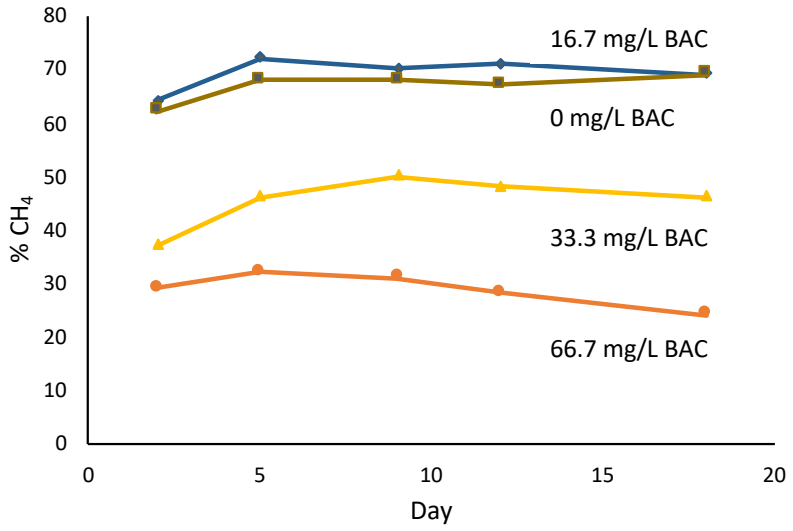


Figure 2-9: Percent CH_4 contribution to biogas after exposure to BAC.

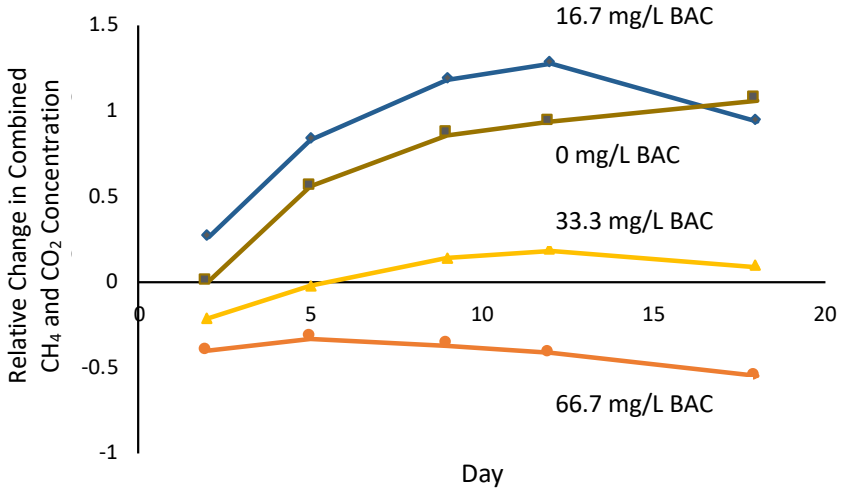


Figure 2-10: The change in combined CH_4 and CO_2 concentration after exposure to BAC relative to the 0 mg/L microcosm at day 2.

2.5 Discussion

The presence of BAC signals in the control samples indicate that the sludge provided from the bioreactor for the construction of the microcosms was indeed impacted by BAC. In the microcosms prepared with 0 mg/L additional BAC, the formation and persistence of additional fatty acids, aside from acetic acid and formic acid, were not observed throughout the study, even though a low-level of BAC was observed throughout. For the 0 mg/L added BAC microcosms, the initial composition of CH_4 in the biogas is ~65 %, which increases to ~70% after 5 days. This level remains constant for the duration of the study. The total combined CH_4 and CO_2 concentration also increases significantly in these microcosms over the duration of the study.

The addition of 16.7 mg/L BAC does not change the biogas composition of the microcosms, including the total combined CH_4 and CO_2 production, as the levels of CH_4 in the 0 mg/L and 16.7 mg/L added BAC are similar throughout the experiment. Nevertheless, the fatty acid profile of these two microcosms treatments do vary from each other. While the formic acid levels in the 0 and 16.7 mg/L added BAC microcosms are similar, the acetic acid levels in the 16.7 mg/L treatments increase after 5 days by levels that are 100 times those observed in the 0 mg/L BAC microcosms. Additionally, isovalerate and isobutyrate are observed in the 16.7 mg/L added BAC microcosms but not in the 0 mg/L treatment. This change indicates that fatty acid metabolism is being affected by the presence of increased levels of BAC even though the amount and

quality of the biogas production remains unchanged. As the measurement of biogas composition does not show a significant difference between the 0 and 16.7 mg/L BAC reactors, this suggests that the NMR measurements are able to detect and diagnose metabolic stress due to BAC exposure, as well as detecting BAC itself, without observable changes in biogas production.

Significant deviations from the control are observed for the 33.3 and 66.7 mg/L added BAC microcosm treatments. In the 33.3 mg/L microcosm treatments, the total acetic acid concentration was 2 times that of the 16.7 mg/L microcosms, while propionic acid, butyric acid, valeric acid, isobutyric acid, and isovalerate are also measured at significant levels. Obligate H_2-producing acetogenic bacteria are responsible for oxidizing VFAs to acetate, CO_2 and H_2.[32, 33] The buildup of VFA concentrations indicates that these syntrophic acetogenic bacteria are unable to facilitate further degradation in order to form acetate and hydrogen.[3, 34-36] Methanogens occupy the terminal position in the anaerobic food chain and are dependent on the metabolic activities of other organisms to provide their growth substrate.[37, 38] Interspecies hydrogen transfer (IHT) is well studied phenomenon that describes the flow of electrons from which CO_2 is finally reduced by H_2 to produce CH_4.[39, 40] The accumulation of intermediates could be an indication of a disruption to IHT;[41] The methanogens are being impacted by BAC and we are seeing the effects upstream (accumulating intermediates) in the digestion process.[13, 16] In the 66.7 mg/L added BAC microcosms the fatty acid concentrations are lower than in the 33.3 mg/L microcosms, with only acetic acid and formic acid being observed at significant concentrations. Succinic acid is

also observed in the 66.7 mg/L microcosms indicating a significant disturbance in the metabolism of the microbial communities. Succinic acid is a key intermediate in the citric acid cycle and an integral part of the electron transport chain, from which oxidative phosphorylation produces ATP.[42, 43] The connection between succinic acid metabolism and ATP synthase indicates that the higher BAC concentration directly inhibits regular cellular activity.[42, 43] Amino acids are measured in the 33.3 mg/L added BAC treatments up until 10 days, after which these metabolites are no longer observed. In the 66.7 mg/L added BAC treatments, the concentration of amino acids increases over the course of the experiment. The lack of fatty acids and the presence of amino acids in the 66.7 mg/L added BAC treatments indicate a serious disturbance of the metabolisms of the anaerobic bacteria. The biogas production in the 33.3 and 66.7 mg/L added BAC microcosms is also significantly affected compared to the 0 and 16.7 mg/L treatment microcosms. Both treatment series shows less total biogas (see figure 2-10), of which a significantly smaller contribution is due to CH_4 (see figure 2-9). The changes in biogas production due to exposure to higher BAC concentrations is consistent with the disturbance in the microbial metabolism as measured using NMR.

In general, the concentration of BAC used in this study is directly proportional with the magnitude of toxicity presented. This is observed by a greater reduction in CH_4 production following exposure to higher concentrations of BAC, which is in agreement with previous studies conducted by Garcia et al., 1999, Durham and Young, 2009, and Flores et al, 2015. BAC is known to be inhibitory to fermentation and methanogenesis.[16, 18, 44, 46] The accumulated levels of VFAs in the 16.7 and 33.3 mg/L

treatments indicates that BAC seems to affect the acetate consumers, which have been proven to be inhibited under a variety of toxic conditions.[16, 47] This means that BAC toxicity and resistance to biodegradation in anaerobic biological systems will ultimately result in its persistence.[13]

Marchaim and Krause (1993) suggested that common indicators, such as VFAs, gas composition and pH were useful for monitoring gradual changes but did not directly reflect the current metabolic status of the active organisms in the system. Our findings show that NMR data coupled with biogas measurements gave a sufficient reflection of the current metabolic state enabling the identification of a disruption and allowing for a possible response.

2.6 Conclusion

This study has shown that NMR spectroscopy provides an improved approach for the monitoring and diagnostics of an anaerobic digester after exposure to the disinfection agent, benzalkonium chloride. The NMR spectra of bioreactor microcosms provide a rapid and high-resolution molecular characterization of the full organic composition of wastewater samples, including fatty acids, amino acids, other metabolites, and the BAC itself in a single analysis. This approach allows for a detailed investigation of the ways in which BAC affects the microbial metabolism that results in less than optimal biogas production, both in terms of total biogas and biogas composition. Improved

understanding of the metabolic impacts of stressors on anaerobic digestion, as well as a rapid tool for monitoring and diagnostics, will lead to the future development of more efficient and effective operation of anaerobic digesters by giving us the ability to better identify and respond to potential fouling events.

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Chapter 3 – An NMR Study of the Correlation Between Food Waste Composition and Oxidative Demand

3.1 Abstract

The current methods for characterizing Food Waste (FW) require hours or even days. As such there is a need to refine the approaches used in order to give a more precise analysis of the organic constituents entering wastewater treatment systems. The development of novel analytical techniques that provide improved chemical information from complex environmental systems will help to improve the overall treatment and management of FW. Nuclear Magnetic Resonance (NMR) Spectroscopy is a robust analytical tool that shows the chemical differences between mixtures of complex organic substances in only minutes and with minimal sample preparation. The goal of this study is to apply NMR as a tool to characterize simulated food waste biodegradability by exploring the correlation between NMR spectra and measurements of biological and chemical oxygen demands (BOD_5/COD). Using different compositions of principal organic components (sugars, fats, proteins, and fibers), measurements of oxidative demand were taken and compared to the corresponding NMR spectra of these same mixtures. Similarities and differences between the distinct spectral regions and the associated chemical parameters were compared. In this sense, NMR acts much as a nutritional label, allowing for a rapid and comprehensive identification of the organic constituents present. Overall, we observed correlations between BOD_5/COD and the signals observed in the NMR spectrum for individual food classes, excluding protein.

These differences are due to the different degradability profiles of these foods based on different macromolecular compositions, which are observed in the NMR spectrum. This research will help to develop the use of NMR spectroscopy as a fingerprinting tool to quickly quantify and predict biodegradability based on macromolecular structure of the organic material in the wastewater.

3.2 Introduction

Food waste (FW) poses threats to the environment and human health in both industrialized and developing countries, yet the standard methods of FW characterization have remained the same for decades,[1] requiring hours or even days. This approach needs to be refined in order to give a more rapid, up to date, and precise analysis of the organic constituents governing the system. The overall degradability of FW as a substrate for biological wastewater treatment is mainly dependent on its chemical composition, however the deconvolution of that chemical composition is rather challenging. Sugars, proteins and lipids influence anaerobic digestion performance differently and it is difficult to determine the exact percentage of each chemical constituent responsible for the make-up of a complex substrate because of its heterogeneous nature.[2]

Effluents from the food industry are characterized by high oxidative demands, along with large quantities of organics and recoverable nutrients.[3] The 5-day biochemical oxygen demand (BOD_5) test is the base standard for quantifying biodegradable organic

matter. Like the name states, the test takes 5 days, therefore additional rapid tests are frequently conducted for operational process control of the biodegradation treatment process.[4] Chemical oxygen demand (COD) and total organic carbon (TOC) are alternate tests based on the macroscopic analysis of bulk properties, and these tests take hours instead of days. However, these tests do not represent the molecular-level heterogeneity in the composition of the bulk organic constituents of wastewater, which varies significantly.[5, 6, 7] These heterogeneities include degradable and non-degradable organics,[6] carbohydrates,[8, 9] lipids,[10] and proteins[11], as well as toxic[12] or inhibitory compounds.[9] BOD_5, COD, and TOC have previously been used to aid in the characterization of various complex compositions of organic matter, and specifically dissolved organic matter (DOM), which is a heterogeneous mixture of aromatic, amino, and aliphatic organic compounds containing various functional groups.[13, 14] Nonetheless, BOD_5, COD, and TOC still fail to provide information on the chemical composition of the DOM present and the analysis of these parameters is rather tedious and time consuming, and can require extensive equipment and instrumentation.[15, 16]

As the most effective analytical tool available for determining the structure of organic compounds, NMR spectroscopy is used to study both physically and chemically complex systems.[17] NMR is a non-targeted and fully quantitative approach used to obtain molecular-level data relating to the makeup of complex organic mixtures in only minutes.[12, 18] Environmental samples are most commonly characterized with reference to the targeted analysis of only specific chemical parameters. This makes it a difficult task to derive relevant information on the otherwise “known unknowns and unknown

unknowns” that are ubiquitous in environmental systems.[18] NMR is an extremely useful tool as it allows for simultaneous targeted and untargeted analysis of multiple chemical parameters. Despite this utility, the use of NMR in environmental science is relatively underdeveloped compared to other scientific disciplines. NMR has previously been used in the analysis of wastewater streams and has successfully identified macromolecular constituents,[19] organic contaminants,[20] as well as nutrients.[21, 22] These studies have shown NMR to have vast potential in the field of bioprocess control, and in particular have proven it to be an extremely useful tool for the identification of organics in wastewater. Although NMR has been successful in identifying macromolecular constituents, organic contaminants, and nutrients in wastewater, there are few studies that have gone beyond the scope of simply identifying and monitoring these compounds. There exists a need to further investigate how these individual components contribute to the biological and chemical attributes of the water itself.

Using NMR-based measurements, the overall objective of this study is to provide a rapid characterization of the organic content present in food waste samples. The specific objective of this study is to provide a chemical fingerprint that can be used as a proxy measurement to predict BOD_5 and COD. The goal is to develop enhanced methodologies and analytical tools to assist with the characterization of food waste. This research will seek to build upon the past uses of NMR in the analysis of waste streams, specifically with the intent to develop the use of NMR spectroscopy as a fingerprinting tool to quickly quantify and predict biodegradability based on macromolecular structure of the organic material in the wastewater. Having the ability to accurately characterize

the organic material present in wastewater is a crucial first step in any treatment process, and the need to know what is in the water before, during, and after treatment is essential for proper management. It is hypothesized that there will be correlations between the values obtained from the biodegradability tests (BOD_5, COD) and the NMR spectra, meaning that spectral regions associated with specific principle organic component will be observed to have distinct impacts on the chemical values obtained.

3.3 Materials and Methods

Samples of various food products, including 5 types of cheerios, 8 types of dairy and nut milk, 8 types of protein powder, and 5 different vegetables were chosen to generate simulated FW, emulating the major components of food waste; sugars, lipids, proteins, and fibers respectively. The nutritional facts for each component can be found in Appendix B.

3.3.1 Sample Preparation

Sample preparation was done in stages, dependent upon the analyte. Each substance had a unique preparation sequence before its consistent chemical and molecular analysis. Tables 3-1 – 3-4 show composition and concentration of each sample used in the analysis.

3.3.1.1 Cheerios

Original, honey nut, apple cinnamon, and 2 types of multi-grain cheerios were used for this experiment. For each type, a handful of cheerios were ground using a mortar and pestle. One (1) g of crushed cheerio was added to 100 mL of DI water and mixed using a Smart Stick Two Speed Hand Blender (Cuisinart) for 20 s on high speed to ensure homogeneity for the sample.

Table 3-1: Mixture composition for simulated sugar (cheerio) wastewater analysis.

Measurement	Original Mixture Mass of Cheerio / Volume of Water (g/mL)	Dilution Volume from Original Mixture Added to Water (mL/mL)	Ratio of NMR to COD/BOD_5 Dilution
NMR	1/100	- / -	1:1
COD	1/100	10 / 100	10:1
BOD	1/100	50 / 500	10:1

3.3.1.2 Milk

White (2 %), homogenized (3.25 %), skimmed (0 %) and lactose free diary milks, as well as almond, cashew, coconut and soy nut milks were used for this experiment. For each type, 1250 µL of milk was added to 100 mL of DI water and mixed using a hand blender (Cuisinart) on high for 20 s.

Table 3-2: Mixture composition for simulated lipid (milk) wastewater analysis.

Measurement	Original Mixture Volume of Milk / Volume of Water (µL/mL)	Dilution Volume from Original Mixture Added to Water (mL/mL)	Ratio of NMR to COD/BOD_5 Dilution
NMR	1250/100	- / -	1:1
COD	250/100	- / -	5:1
BOD	1250/500	- / -	5:1

3.3.1.3 Protein

A combination of whey protein, mass gainers, meal replacements, and plant-based proteins were used for this experiment. For each type, 1 g of protein powder was added to 100 mL of DI water and mixed using a hand blender (Cuisinart) on high for 20 s.

Table 3-3: Mixture composition for simulated protein (protein supplement) wastewater analysis.

Measurement	Original Mixture Mass of Protein Powder / Volume of Water (g/mL)	Dilution Volume from Original Mixture Added to Water (mL/mL)	Ratio of NMR to COD/BOD_5 Dilution
NMR	1/100	- / -	1:1
COD	1/100	5 / 100	20:1
BOD	1/100	25 / 500	20:1

3.3.1.4 Vegetables

Carrots, tomatoes, spinach, broccoli and asparagus were used for this experiment. For each type, a handful of vegetable was minced using the chopper / grinder attachment to the Smart Stick Two Speed Hand Blender (Cuisinart) on high for 10 s. One (1) g of minced vegetable was then added to 100 mL of DI water. The blender attachment was reinserted, and the solution mixed on high for 20 s.

Table 3-4: Mixture composition for simulated fiber (vegetable) wastewater analysis.

Measurement	Original Mixture Mass of Protein Powder / Volume of Water (g/mL)	Dilution Volume from Original Mixture Added to Water (mL/mL)	Ratio of NMR to COD/BOD_5 Dilution
NMR	1/100	- / -	1:1
COD	1/100	10 / 100	10:1
BOD	1/100	50 / 500	10:1

3.3.2 NMR Sample Preparation

To prepare samples for NMR analysis, 1.5 mL of each sample was placed into 1.5 mL centrifuge vials. Samples were then spun at 5000 rpm for 5 mins using Fisherbrand Gusto Mini Centrifuge (Heathrow Scientific). After centrifugation, 600 µL of the supernatant was passed through a 0.45 µm syringe filter to remove the fine particulates and combined with 60 µL of deuterium oxide (D_2O, Sigma Aldrich) that included 0.05 (w/v) 3-(trimethylsilyl)-propionic-2,2,3,3-d_4 acid, sodium salt (TMSP, Sigma Aldrich) as

an internal standard and chemical shift reference. Samples were transferred into a 5 mm diameter glass NMR tube (Wilmad) for the analysis to be carried out.

3.3.3 NMR Analysis

All NMR experiments were carried out on a Bruker Avance III 400MHz NMR spectrometer equipped with a Prodigy Probe. 1D $_1$H NOESY spectra were acquired using a 90º excitation pulse, 128 transients, 2 s recycle delay and 3 s acquisition time with 808,064 data points. All 1D spectra were processed using 262,144 points, Fourier transformed with 0.01 Hz line broadening, phased, and baseline corrected using the TopSpin 4.0.6 Software (Bruker BioSpin).

3.3.4 NMR Identification and Quantitation

Chenomx NMR Suite 8.3 professional (Chenomx Inc.), was used to identify and quantify compounds in each spectrum using reference spectra.

3.3.5 Chemical Oxygen Demand

To prepare samples for COD analysis, an amount from each original mixture (See Tables 3-1 – 3-4) was drawn, dependent upon the mixture type, and added to a 125 mL HDPE bottle with 100 mL of deionized water. These dilutions were prepared to ensure the COD values remained within the ‘mid-range’ zone (0 – 15,000 mg/L COD). Ten (10)

mL of each sample was then drawn from the diluted mixture and placed into a 15 mL conical centrifuge tube and spun at 5000 rpm for 5 mins. After centrifuge, the supernatant was passed through a 0.45 µm syringe filter prior to analysis. Standard methods for CODH00 from Thermo Scientific were then used to analyze the supernatant. Samples were digested using an Orion Thermoreactor COD165 (Thermo Scientific) and COD was measured using an Orion AQ4000 Advanced Colorimeter (Thermo Scientific).

3.3.6 5-Day Biochemical Oxygen Demand

To prepare samples for BOD_5 analysis, an amount from each original mixture (See Tables 3-1 - 3-4) was drawn, depending upon the mixture type, and added to a 600 mL HDPE bottle with 500 mL of DI water. These dilutions were carried out to match the previous dilutions for COD in order to normalize the values. BOD analysis was outsourced to a third party, ALS Environmental in Waterloo, ON, and conducted using method reference APHA 5210 B.

3.3.7 NMR, COD and BOD_5 Correlative Analysis

Regions of the NMR spectra identified as sugars, fats and amino acids were integrated using the TopSpin 4.0.6 software (Bruker BioSpin). These were compared to the values obtained for COD/BOD_5.

3.4 Results

Figure 3-1 compares the total signal measured in the $_1$H NMR spectrum and the COD.

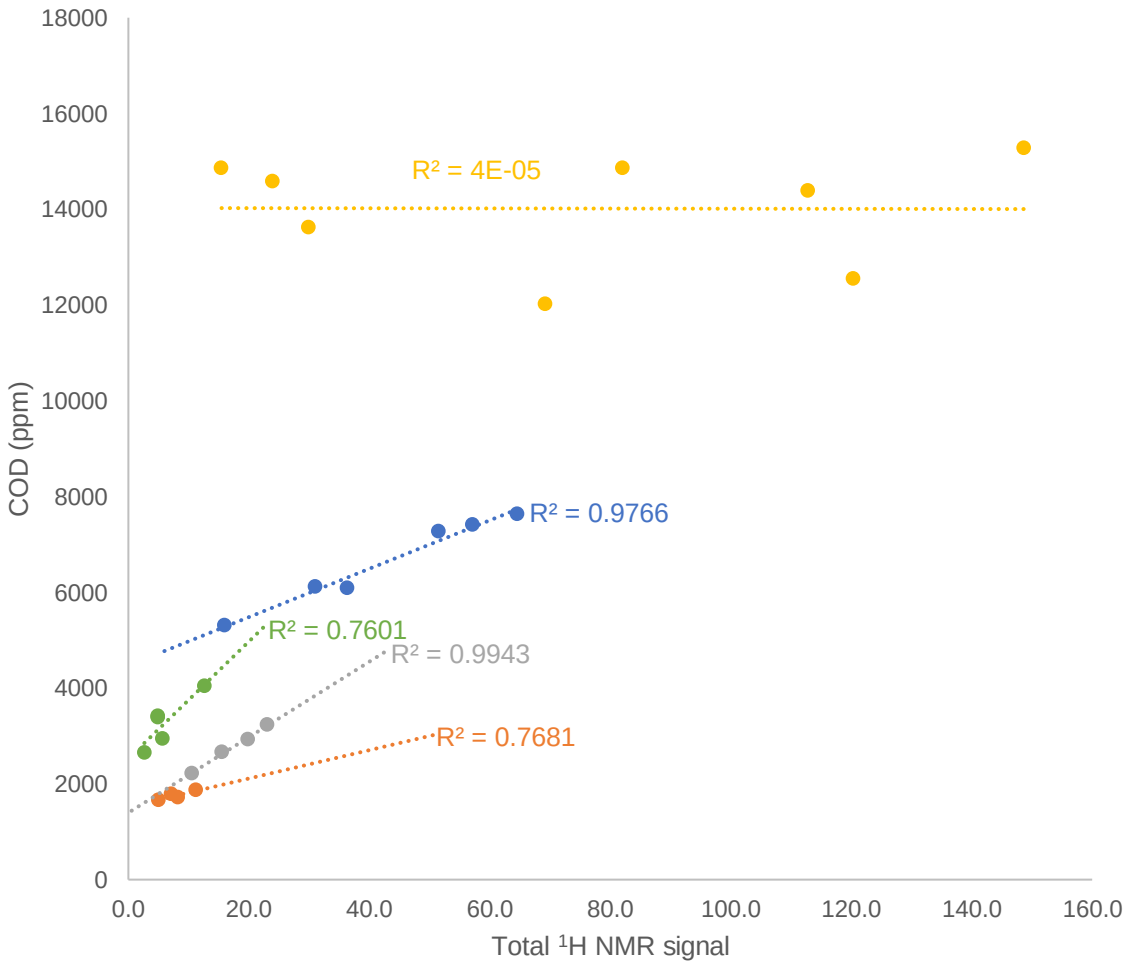

Figure 3-1: Correlations between total NMR signal in the $_1$H NMR spectrum and the Chemical Oxidation Demand (COD) for selected food materials; Vegetable (green); Cheerios (blue); Dairy milk (grey); Nut milk (orange); Protein (yellow).

Figure 3-2 compares the total signal measured in the $_1$H NMR spectrum and the BOD_5.

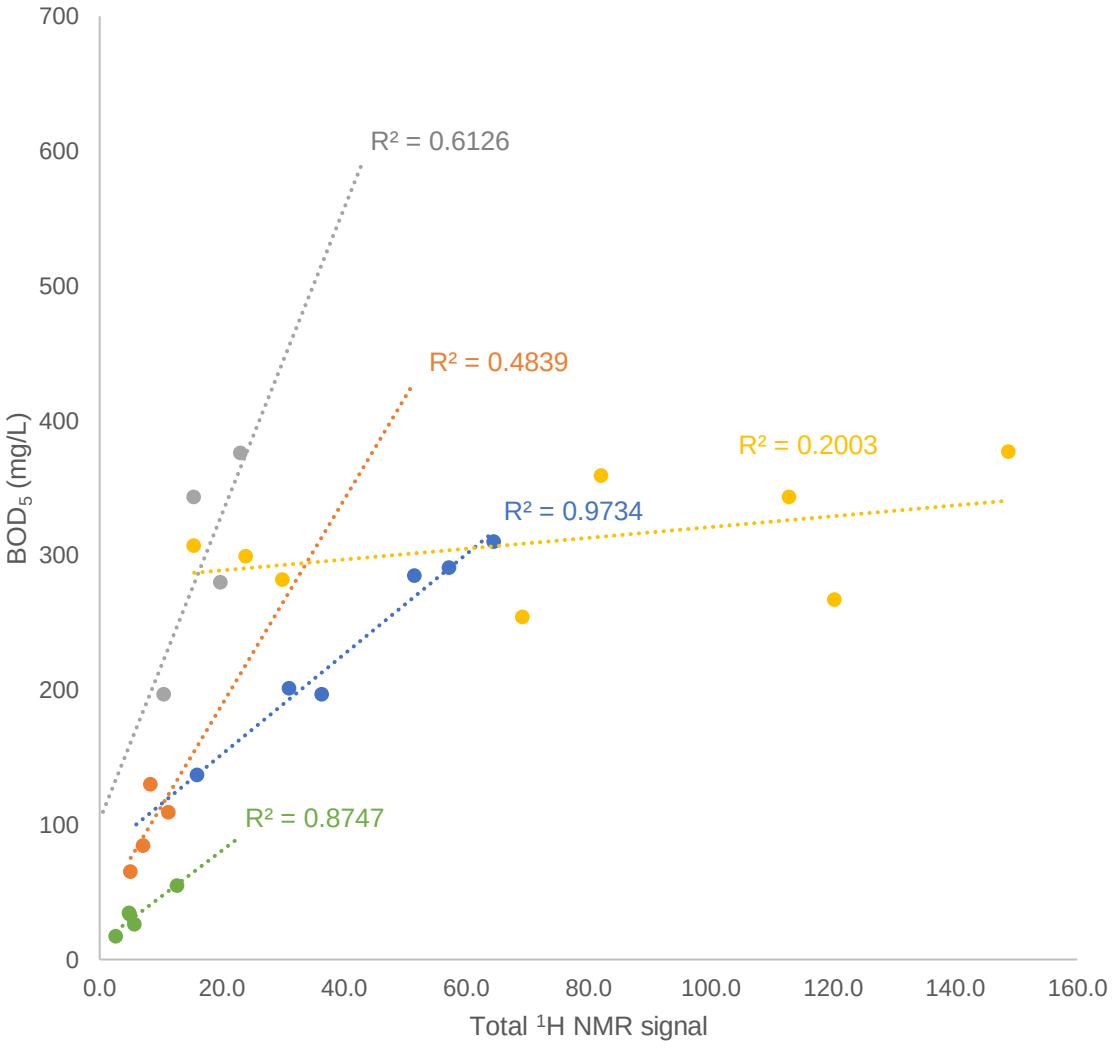

Figure 3-2: Correlations between total NMR signal in the $_1$H NMR spectrum and the 5-Day Biochemical Oxidation Demand (BOD5) for selected food materials; Vegetable (green); Cheerios (blue); Dairy milk (grey); Nut milk (orange); Protein (yellow).

Clear trends are observed between total signal in the ^{1}H NMR spectrum and the COD of mixture preparations of vegetables (R^2 = 0.7601), cheerios (R^2 = 0.9766), dairy milk (R^2 = 0.9943) and nut milk (R^2 = 0.7681). Clear trends are also observed between total signal in the ^{1}H NMR spectrum and the BOD_5 of mixture preparations of vegetables (R^2 = 0.8747), cheerios (R^2 = 0.9734), dairy milk (R^2 = 0.6126) and nut milk (R^2 = 0.4839). Each of these food types display positive trends, with the COD and BOD_5 increasing linearly with an increase on total signal in the ^{1}H NMR spectrum. Nevertheless, each food type displayed different slopes. This indicates that the relationship between NMR signal strength and oxidative demand is different for different food types, which can be attributed to different macromolecular compositions. The observed COD values for protein samples are significantly higher compared to the samples composed predominantly of sugars and fats. This was to be expected as wastewater containing high concentrations of protein are known to have high oxidative demands.[23, 24]
In general, the plot for total NMR signal in the ^{1}H NMR spectrum and BOD_5 follows very similar trends to COD. There exists a definite correlation between the two parameters as the values are highly waste dependent.[25, 26]

Figures 3-3 – 3-7 demonstrate spectral variation amongst samples, with significant regions of the spectrum being identified. The stacked spectra are scaled to the signal/noise ratio in order to clearly show variation and similarities amongst samples. Additional representation of these spectra can be found in Appendix B.

Macromolecular differences are evident in each NMR spectrum. Figure 3-3 shows the $_1$H NMR spectra for the simulated vegetable wastewater samples. Vegetables exhibit complex spectra primarily with sugars and amino acids present. There is high overlap and signal clustering between 3.0 – 4.4 ppm, which is characteristic of sugars. There is also scattered fats and amino acids found between 0.9 – 3.0 ppm with some consistent signals found throughout each different vegetable.

Figure 3-4 shows the $_1$H NMR spectra for the cheerio wastewater samples. Cheerios exhibit signals primarily in the carbohydrate and sugar region. The overall profile of each cheerio is almost identical to one another with the major difference between samples being the total the amount of signal present for the different types of cheerio.

Figure 3-5 and 3-6 show the $_1$H NMR spectra for the dairy and nut milk wastewater samples. Both dairy and nut milks exhibit a mixture of sugars and fats, however the profiles for these types of milks are slightly different from one another, with more sugars evident in the dairy milks and a different profile of fats observed in the nut milks. The sugar region for white, homogenized and skimmed milks are almost identical to one another, whereas the sugar region of lactose free milk varies immensely because it contains other sugar alternatives.

Figure 3-7 shows the $_1$H NMR spectra for the prepared protein wastewater samples. The protein mixtures all exhibited similar COD values despite exhibiting a wide range of total NMR signals, indicating no correlation between the total amount of protein present

and the COD and BOD_5 values. The profile for all 8 of the protein samples are unique. In comparison to Figures 3-3, 3-4, 3-5, and 3-6, the spectra presented in figure 3-7 are noticeably different. The protein signals encompass the large underlying broad structural features that are observed throughout the spectra underneath the sharper, more defined peaks of carbohydrates and fat.

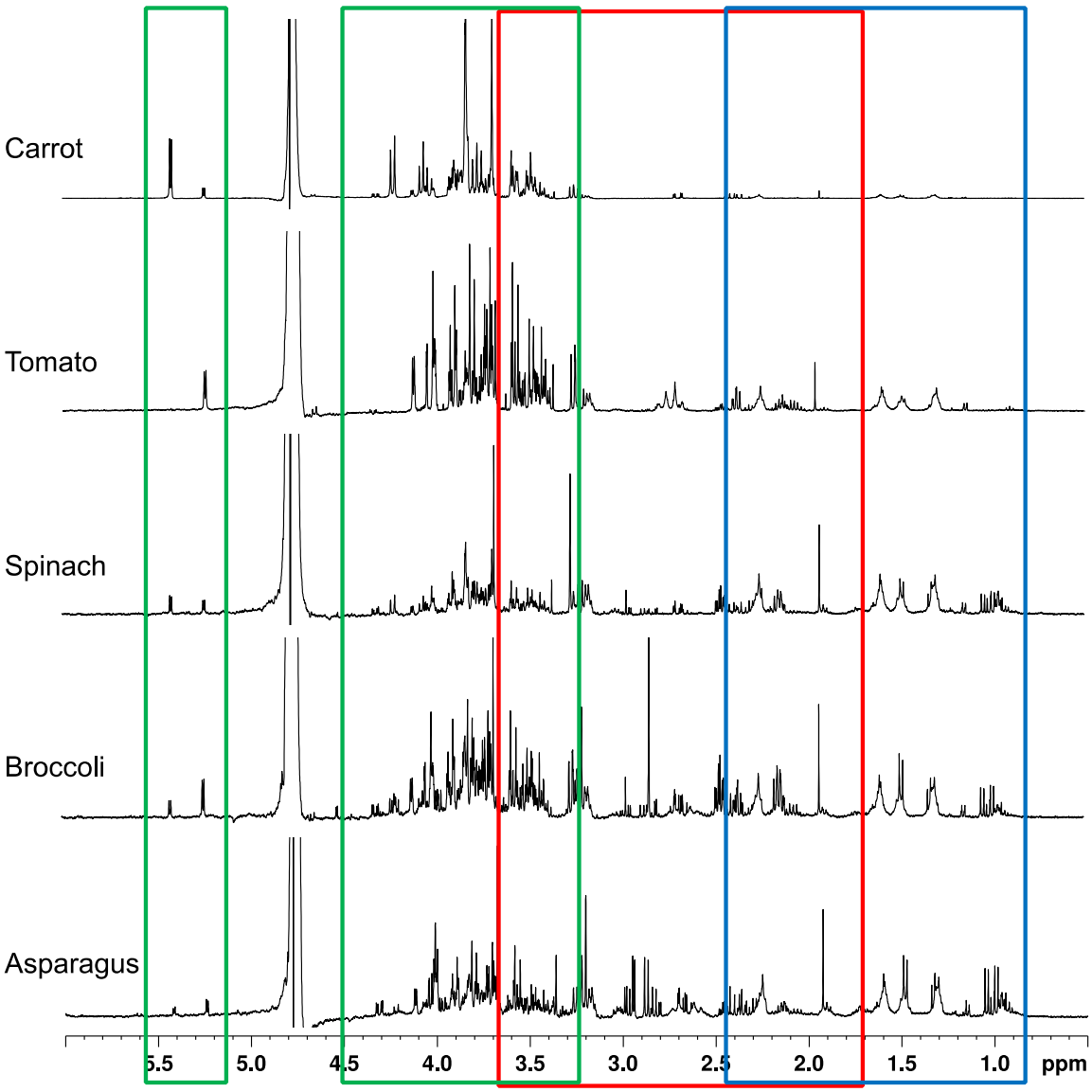

Figure 3-3: $_1$H NMR spectra of simulated vegetable food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue); Amino Acids (red).

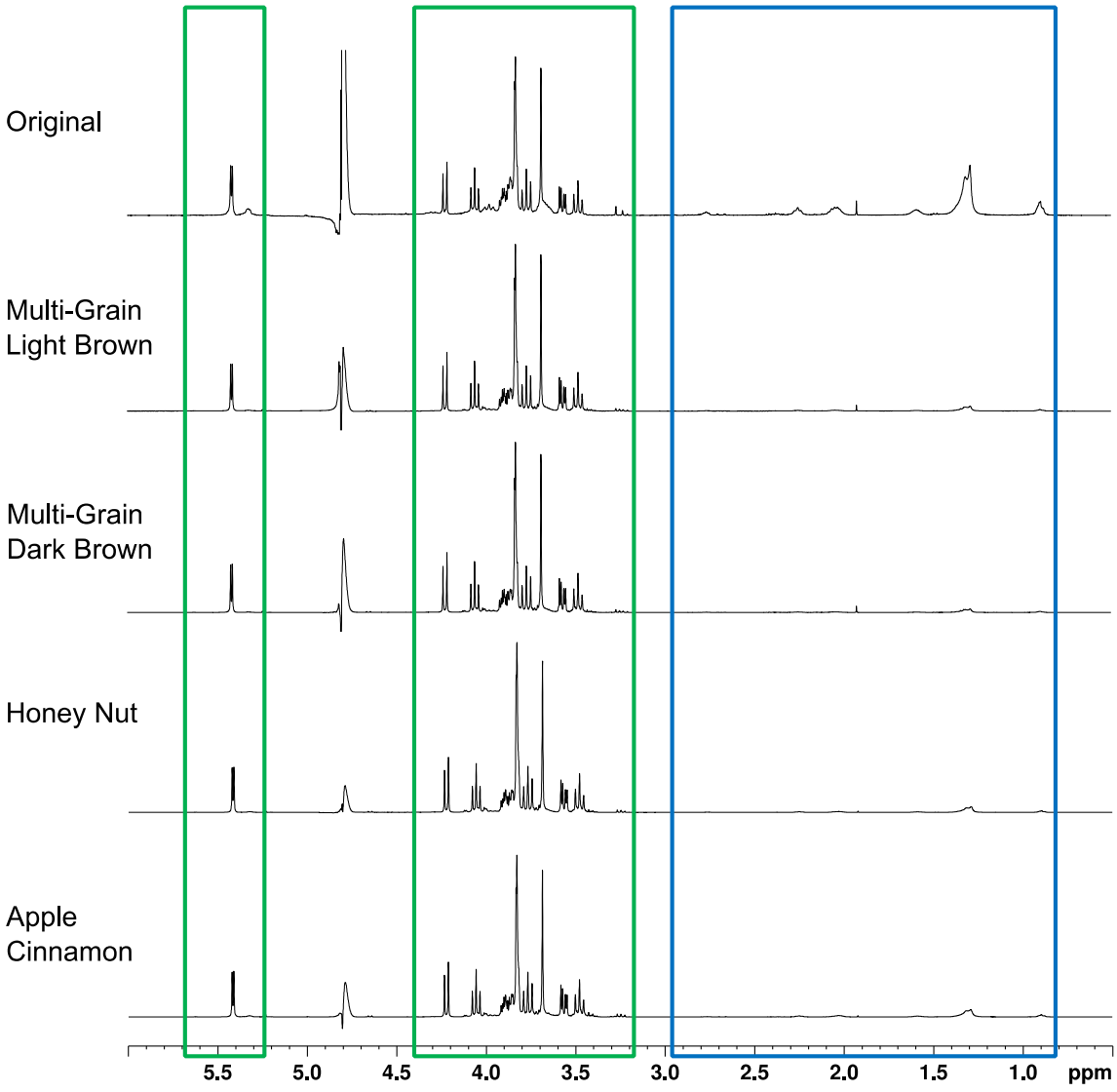

Figure 3-4: $_1$H NMR spectra of simulated cheerio food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue).

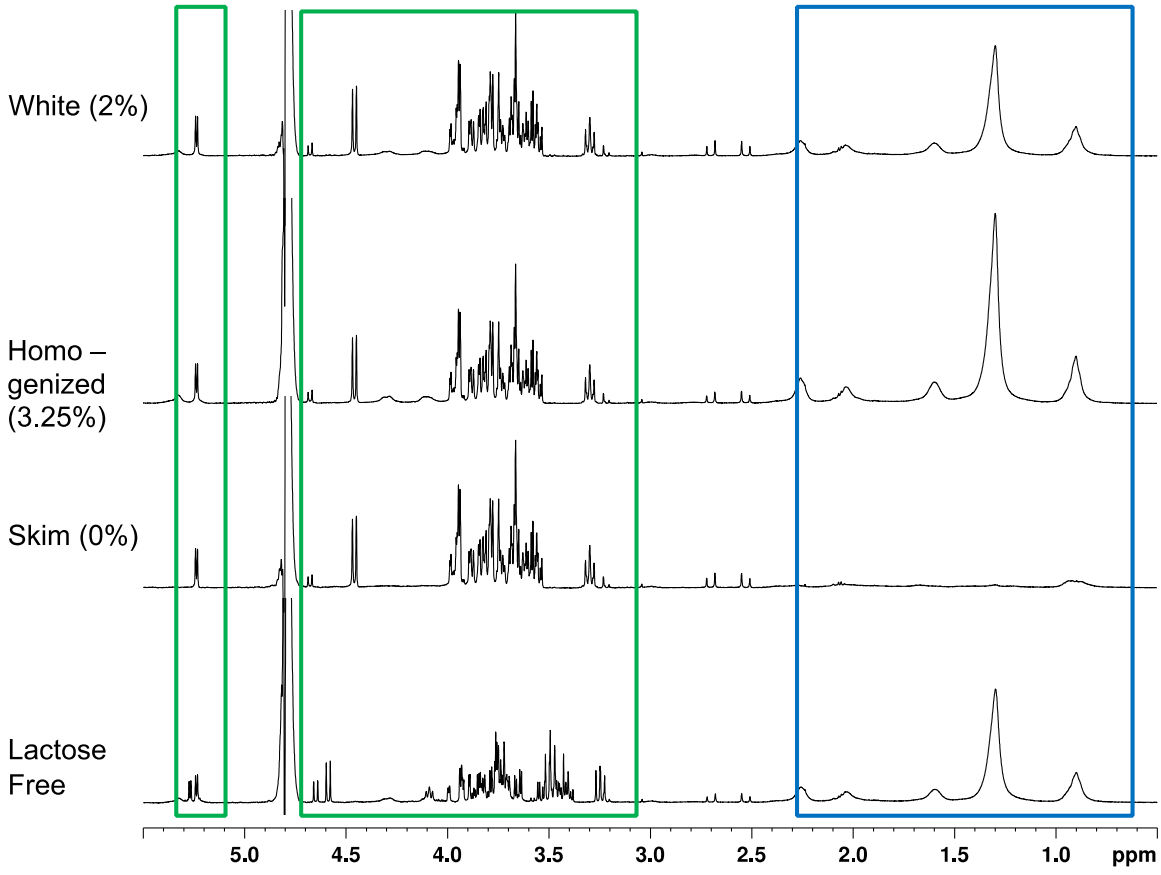

Figure 3-5: $_1$H NMR spectra of simulated diary milk food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue).

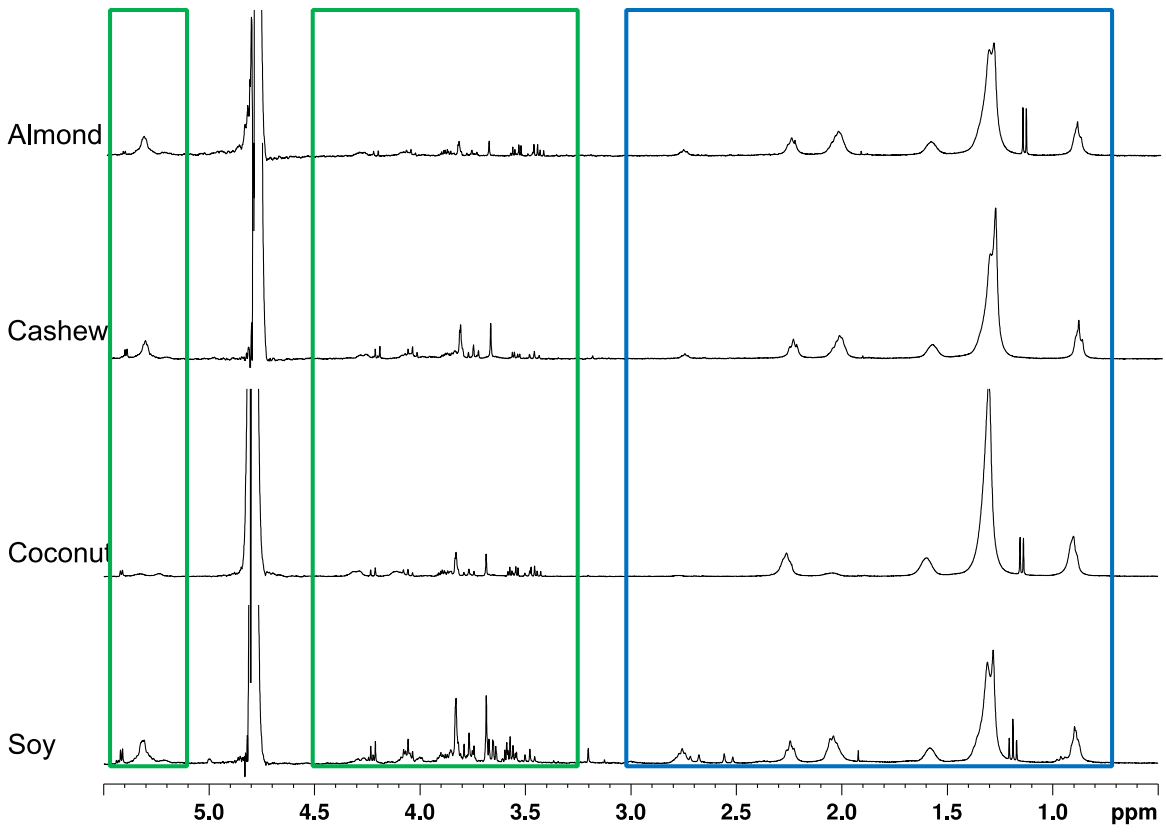

Figure 3-6: $_1$H NMR spectra of simulated nut milk food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue).

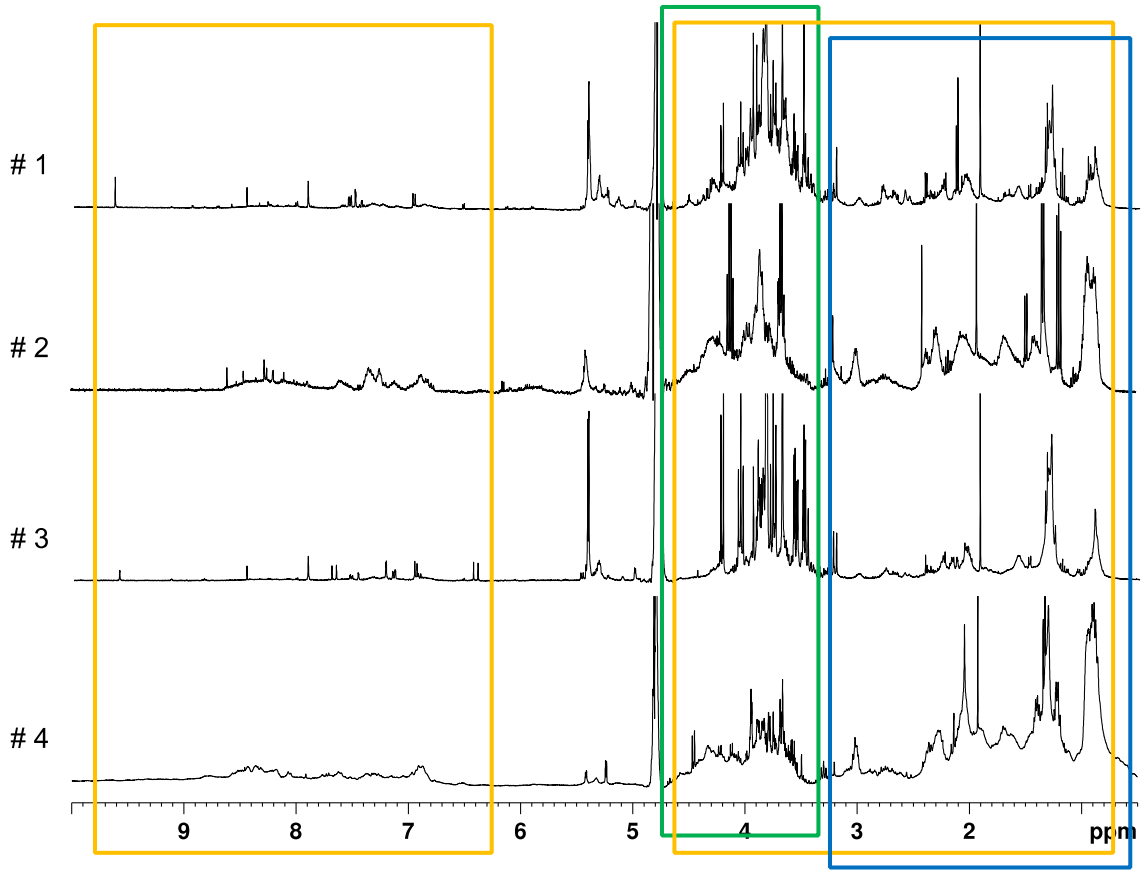

Figure 3-7: $_1$H NMR spectra of simulated protein food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue); Amino Acids / Protein (orange).

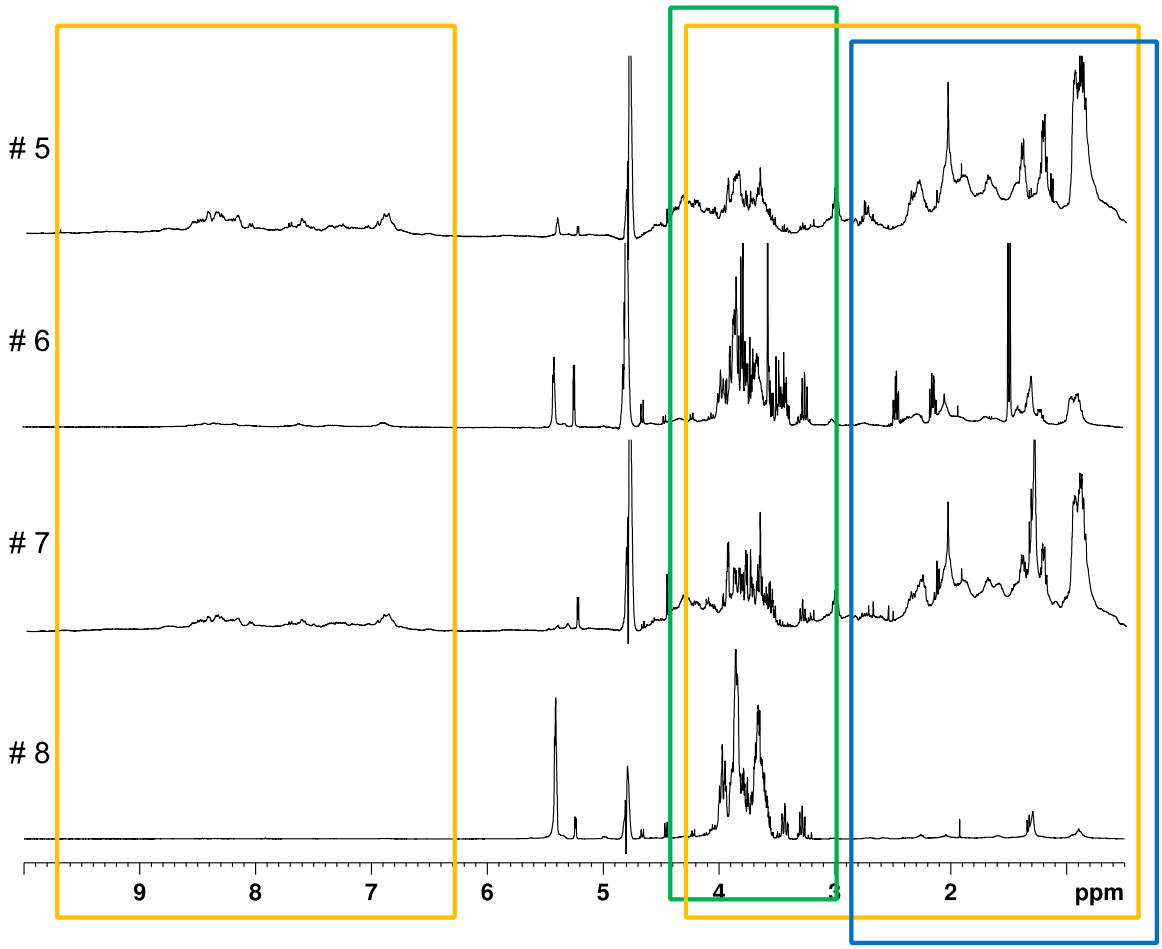

Figure 3-7 continued: $_1$H NMR spectra of simulated protein food wastewater with select groupings of organic components defined; Sugars (green); Fats (blue); Amino Acids / Protein (orange).

3.5 Discussion

Overall, it is observed that correlations exist between COD/BOD_5 and the total signal present in the NMR spectrum for individual food classes, excluding protein. These differences are due to the different degradability profiles of these foods based on different macromolecular compositions, which are evident in the NMR spectra.

Substrates for Anaerobic Digestion are composed of heterogeneous and complex organic matter, as seen in figures 3-3 – 3-7, and it is this variation in waste composition that makes characterization difficult. Organic matter can be fractionated into easily biodegradable compounds and poorly biodegradable compounds.[27] Simple carbohydrates, such as glucose, sucrose or lactose, and amino acids of VFAs are easily biodegradable and do not need any hydrolysis phase to be broken down.[27] Whereas, complex carbohydrates, such as cellulose, proteins, and long chain fatty acids (LCFAs) require hydrolyzation into degradable monomers before AD.[27]

The strongest correlations between NMR spectra and COD are exhibited by the cheerio and dairy milk wastewaters (see figure 3-1). These samples are predominantly composed of sugars (see figures 3-4 and 3-5) and the biodegradation of soluble carbohydrates (such as glucose and lactose) is generally faster and almost total in anaerobic conditions.[28]

Macromolecular differences are evident in each NMR spectrum, with sugars having the

greatest influence on oxidative demand followed by fats. Protein did not show any correlation and vegetable spectra displayed predominantly sugar and amino acids due to fiber being recalcitrant to hydrolyzation. Although fiber was not observed in the spectra, its presence was demonstrated in the oxidative demand. Oxygen demand tells the maximum amount of methane expected from organic matter.[29, 30] Substrates rich in lipids and easily degradable carbohydrates yield the highest methane potential, while more recalcitrant substrates with a high lignocellulosic fraction have the lowest.[31] Our results validate this as vegetables have the lowest recorded BOD_5 values (see figure 3-2).

Comparing dairy versus nut milks reveals the biodegradation of fat-rich (nut milk) wastes was slower than carbohydrate-rich (dairy milk) wastes, which is due to the slower hydrolytic step of fat. This resulted in higher BOD_5 and COD values for the dairy milks, which reflects the sugar and fat contents present in figures 3-5 and 3-6. The flow and characteristics of dairy wastewaters vary depending on product as well as the system and method of operation,[32] all with very different relative proportions of fats, proteins and carbohydrates.[23]

The breakdown of proteins into smaller peptides and amino acids is regulated by moisture, temperature, and bacteria[33] This process does not occur at a uniform rate and as such some proteins are degraded during early decomposition, while others are degraded during later stages of decomposition. ^{1}H NMR spectra of proteins is very difficult to interpret. Proton linewidth of proteins is characterized by homogeneous broadening, which is clearly demonstrated in figure 3-7, and it is this trait that makes a

comprehensive analysis nearly impossible for this class of macromolecules.

In a similar study conducted by Charnier et al. (2016), the correlation between biochemical composition and methane production was examined. Their results showed positive correlation between methane yield and methane production kinetics, but also unexpected independence of COD and BMP are shown. Sugars were correlated with readily biodegradable organic matter, while proteins were slightly anti-correlated with biodegradability. Using NMR to draw correlations, our findings suggest similar results. Simulated wastewaters with high carbohydrate content correlated nicely with biodegradability whereas wastewaters with high protein content did not show any correlation.

At present, it will likely be difficult to use this approach to predict the degradability of mixed food waste from the current dataset, as there is significant overlap in the key regions, including sugars, amino acids, and fatty acids. This approach to characterizing organics could only be applied to influents consisting of similar composition as the simplistic statistical analysis does not significantly differentiate amongst groups. Future work could explore the use of multivariate statistical tools, such as principal component analysis, to identify significant correlations between the NMR spectra and the degradability of the individual classes. This may be useful in predicting the degradability of unknown food mixtures and helping to identify correlations in complex mixtures.

3.6 Conclusion

The higher the biodegradable COD/BOD_5 content of an industrial effluent, the higher the pollution load it will have when discharged to receiving waters. Wastewater with high oxidative demands will result in oxygen deficiencies for aquatic organism and can lead to a serious deterioration of aquatic life. In order to form a more complete understanding of how the complex and variable composition of wastewater affects the treatment process, improved characterization of the full organic composition of wastewater during all stages of treatment is needed. This study has shown that NMR spectroscopy provides a complete, unbiased assessment of the organic constituents in simulated food wastewaters. The NMR spectra of different organic constituents show visible correlations to the selected operational parameters (COD, BOD_5,) that are most frequently applied during wastewater treatment. This approach allowed for a detailed investigation of the ways in which food waste composition affects the pollutant strength of wastewaters containing variable amounts of principle organic components. Improved knowledge of the macromolecular composition of wastewater based on NMR analysis will help to develop more effective strategies for the treatment and management of food wastewater by identifying areas for improvement. This research will also enhance the quality of the data used to model wastewater treatment processes, increasing their design capabilities and allowing for improved operation.

3.7 References

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Chapter 4 – Concluding Remarks and Continued Research

4.1 Conclusion

The overarching hypothesis of this dissertation is that the use of nuclear magnetic resonance spectroscopy to acquire a high-resolution molecular-level characterization of organic composition will improve our ability to better treat wastewater and optimize bioreactor operation. Improved understanding of the organic constituents present in waste streams will help in the application of optimal treatment technologies for systems with varying chemical composition and pollutant strength. Both physically and chemically complex environmental systems present analytical challenges when developing the most complete and high-quality data sets required to apply forensic tools, develop conceptual models and simulate wastewater treatment processes. Effective and efficient management of these systems can only be conducted once the chemical profile of these waste streams is fully identified. In order to address the overarching hypothesis of this dissertation, a series of projects were developed with the overall objective to develop and validate the use of NMR spectroscopy as a tool characterize the full organic composition of food waste influents, effluents, and process water.

The objective of Chapter 2 for this dissertation was to determine the effects quaternary ammonium compounds have on bioreactor systems by monitoring the evolution of the composition of an anaerobic digester in response to exposure to a quaternary

ammonium compound and relate those variations to the quality and quantity of the biogas produced. Bioreactor samples were characterized using both 1D and 2D NMR experiments as well as comparison to a spectral database. The results obtained from NMR generated a chemical profile which was used in contrast with biogas composition and concentration to determine the overall effect QACs had on the system. Direct comparison supported the hypothesis that the concentration of benzalkonium chloride is proportional with the magnitude of toxicity presented. Deviations from optimal operating procedures were also successfully identified by comparing the metabolic profiles obtained from the NMR fingerprint after exposure to different concentrations of QACs. Operators of environmental systems can suspect contamination in their waste streams but often are unable to identify and quantify the contaminant due to lack of appropriate analytical technologies. This study has shown that NMR spectroscopy provides an improved approach for both the monitoring and diagnostics of an anaerobic digester after exposure to a disinfection agent by providing a comprehensive characterization of the full organic composition, including fatty acids, amino acids, other metabolites, and the BAC signal itself in a single analysis. This improved approach for monitoring and diagnosing variations in the chemistry of industrial anaerobic digesters has the potential to help enhance the optimization of these systems by giving us the ability to better identify and respond to potential fouling events.

The objective of Chapter 3 for this dissertation was to explore the correlation between food waste composition and oxidative demand by providing a chemical fingerprint that could be used as a proxy measurement to predict BOD_5 and COD. NMR was used to

acquire a rapid characterization of the organic content present in food waste samples and then compared to the pollutant strength. Direct comparison supported the hypothesis that chemical values obtained from the biodegradability tests could be directly linked to spectral regions associated with specific principle organic components. Conventional analysis of operational parameters for wastewater treatment are predominantly conducted with reference to the macroscopic analysis of bulk properties, which often fail to accurately represent the molecular-level heterogeneity that exists within the composition of the organic constituents of wastewater. This study has shown NMR spectroscopy provides a detailed investigation of the ways in which food waste composition affects the pollutant strength of wastewaters containing variable amounts of sugars, fats, proteins, and fibers. Improved knowledge of the macromolecular composition of wastewater will form a more complete understanding of how the complex and variable composition of wastewater affects the treatment process. With further development, this non-targeted and unbiased approach could help to develop more effective strategies for the treatment and management of food wastewater by identifying areas for improvement.

Overall, the research presented in this dissertation supports the application of NMR spectroscopy to aid in food wastewater treatment. The use of NMR as an analytical tool for effective bioprocess monitoring was demonstrated by the works presented in Chapter 2. The use of NMR as an analytical tool for the characterization of food waste was validated by the works presented in Chapter 3. Having a high-resolution molecular characterization of the full organic composition of wastewater will lead to the future

development of new strategies and technologies to help monitor, mitigate and remediate sensitive environmental systems more effectively and efficiently. Improving the ability to generate high quality data will ultimately enhance the understanding of the underlying chemical parameters that are important for the development of upgraded management and optimal treatment of food waste.

4.2 Continuation of Research

4.2.1 Scale-Up to Industrial Anaerobic Digestor

The research discussed in Chapter 2 was conducted using a lab-scale anaerobic digester (10 L). The next step in this research is to scale up to an industrial anaerobic digestor (20,000 – 2,000,000 L).[1] NMR has shown the ability to characterize raw influents, treated effluents and process waters containing varying amounts of organics and contaminants. Scaling up industrial microbial processes, however, is a high-stakes endeavor, requiring time and financial investment. The disruption of a digester can be costly, resulting in heavy losses of biogas and prolonged recovery time. During the planning stages for the research in Chapter 2, a pilot study was constructed using mixed sludge from an industrial anaerobic potato digestor as seed for a lab-scale anaerobic digester system. This lab-scale system was injected with a minute concentration of QAC and continuously monitored. It was determined that the initial amount of QAC introduced to the system was insignificant, but it was elected to not inject a higher dosage into the system and instead mixed sludge was removed in order

to conduct microcosm experiments. The reasoning behind this was because the effects of a large concentration of QAC on the system was unknown, and the potential to shock and essentially disrupt and destroy the microbial communities was not worth the time invested in constructing and setting up the system. Bioreactors are extremely sensitive environmental systems and require a substantial amount of time and effort to get up and running. The financial implications in lab-scale versus industrial-scale digestors is incomparable. Research must be refined and reproducible before application to a larger scale. The limitations associated with a lab-scale bioreactor are that the operating parameters and conditions within the reactor are able to be monitored extremely closely and easily adjusted to achieve desired outputs. There was zero variation in the feedstock and very minimal variation from baseline conditions. This does not accurately reflect the varying compositions and flows that enter industrial systems, nor does it give an accurate representation of the systems full response to a known contaminant.

The research discussed in Chapter 2 only monitored biogas and chemical composition. Future research would benefit from expanding the operational parameters and variables monitored. No one parameter can give a complete assessment of the status of a wastewater treatment system. Incorporating measurements of BOD_5, COD, ammonia, nitrogen and phosphorus could help to draw further conclusions. Furthermore, beyond monitoring the initial effects QAC have on the system, the recovery period could also be investigated. Gaining knowledge of the fate and effect of QACs as they move through a system could help to model improved treatment systems as well as further understand how these sensitive environmental systems cope and adapt to contaminants.

4.2.2 Scale-Down to Benchtop NMR Spectrometer

The research discussed in Chapter 2 was conducted using a large high-field NMR spectrometer that is predominantly found in academic and research institutions. Future research will seek to scale down to a bench-scale low-field NMR spectrometer. The low-field is more cost effective and practical for use in an industrial setting when compared to the high-field, however it has limitations. Figure 4-1 will demonstrate the resolution between the same bioreactor sample analyzed on a 400 MHz high-field spectrometer and a 60 MHz low-field spectrometer. Peaks are sharper and more defined on the high-field, making their identification and quantitation less difficult. Water suppression techniques on the high-field are also more refined, allowing for the signal to be completely removed. Whereas on the low-field, the water signal is difficult to suppress, appearing as a large swath centered at around 4.7 ppm, often overlapping the signals from neighbouring compounds.

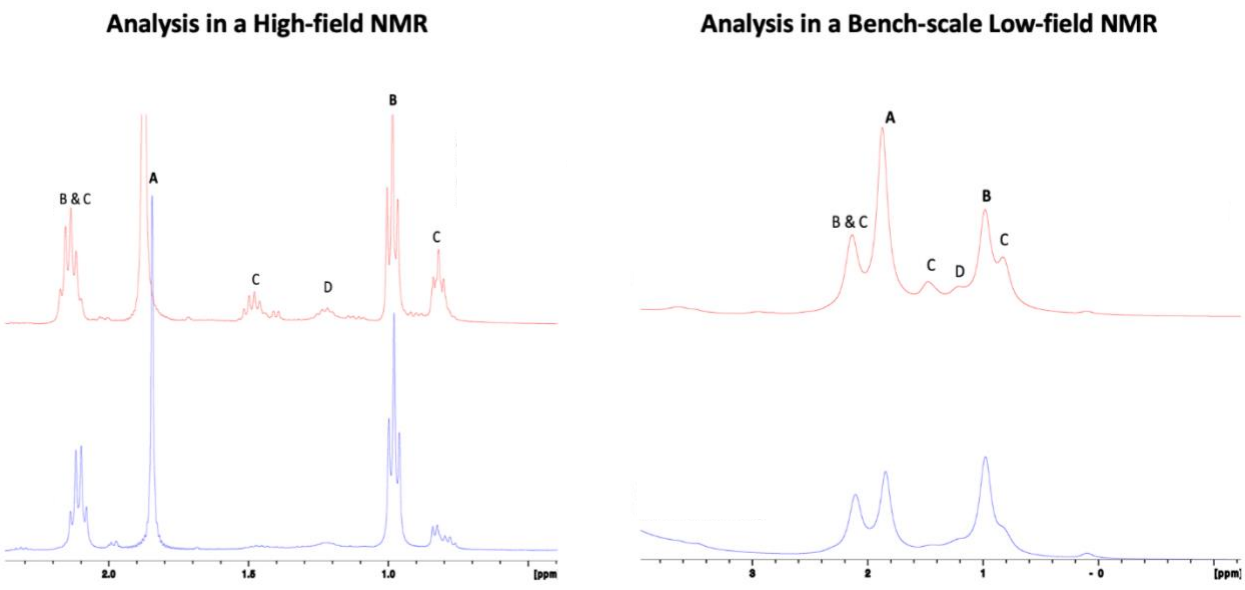

Figure 4-1: Bioreactor sample analyzed on a 400 MHz high-field NMR spectrometer (left) shown alongside the same bioreactor sample analyzed on a 60 MHz bench-scale low-field NMR spectrometer (right). Letters denote different fatty acids; (A) Acetic Acid, (B) Propionic Acid, (C) Butyric Acid, (D) Valeric Acid.

The ultimate goal of the works presented in Chapter 2 is to form the basis for future research into in-line NMR instrumentation for real-time monitoring of bioreactors that can be conducted using a bench-scale spectrometer. Figure 4-2 shows a photo comparison between the size of a high-field and low-field instrument. Beyond resolution in the analysis, there exists extreme differences in both cost and portability. The high-field instrument displayed in Figure 4-2 is valued at approximately $1,000,000 whereas the low-field instrument is valued at approximately $60,000. The high-field instrument is also stationary, being extremely sensitive to its surroundings and requiring weekly maintenance. In contrast, the low-field instrument has portable capabilities, is less

sensitive to its surrounding environment, and needs less maintenance to stay running at optimal performance. It is difficult to use an off-line high-field instrument for analysis because it is not realistic to outsource samples. Bioreactor samples have a shelf life because they are biologically active and can change in composition and concentration over time. That is why the development of in-line NMR instrumentation is the crucial next step. Having a non-invasive approach with relatively quick and easy data acquisition that can provide information on a vast range of metabolites with a single experiment.[2, 3, 4]

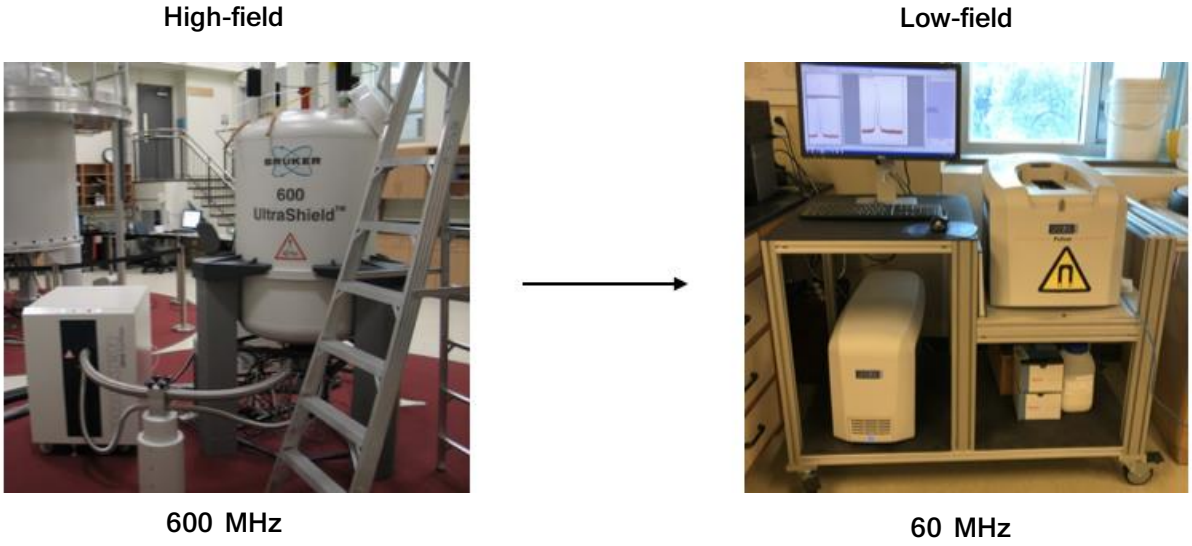

Figure 4-2: Picture of the 600 MHz high-field NMR spectrometer that was used for the research conducted in Chapter 2 (left) compared to a 60 MHz bench-scale low-field NMR spectrometer (right).

4.2.3 NMR Coupled with Multivariate Statistical Analysis

The research discussed in Chapter 3 highlighted a unique application of NMR to the characterization of food waste. Being able to quickly and concisely identify the macromolecular constituents present in varying compositions and concentrations of wastewater is invaluable. This characterization and subsequent application to operational parameter analysis, however, can be difficult due to the complexity and inherent similarities that exist amongst NMR datasets.[5] The richness of information displayed in NMR spectra of environmental samples is often too complex to be analyzed using simple statistical methods or compared visually. Therefore, the next step in this research is to apply multivariate statistical analysis (chemometrics) in order to extract useful and pertinent information in an efficient and effective manner.[6] With no prior knowledge of the compounds present in a spectrum, chemometric methods can be applied to identify variations amongst the NMR fingerprint of individual samples as well as groups of samples, relating their chemical composition with observed chemical parameters.[7] Having the ability to detect variation in wastewater treatment operations through the use of NMR coupled with chemometrics will greatly improve the characterization of sensitive environmental systems by correlating the change in chemical composition with the change in change in operational parameters.

The wastewater samples analyzed in Chapter 3 were made in the lab under controlled conditions with prior knowledge of composition and concentration. This approach allowed for a detailed investigation of the ways in which food waste composition affects

the pollutant strength of wastewaters containing known amounts of varying principle organic components. In order to scale-up this approach, samples from industrial food processing facilities or waste water treatment facilities are required. These real-world samples will be of unknown origin and composition, allowing for a blind analysis. This spectral fingerprinting will then be used in conjunction with chemometrics to obtain structural information and help understand the correlation between chemical components and chemical values in complex systems.[8, 9]

4.3 References

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Appendix A – Supplementary Material for Chapter 2

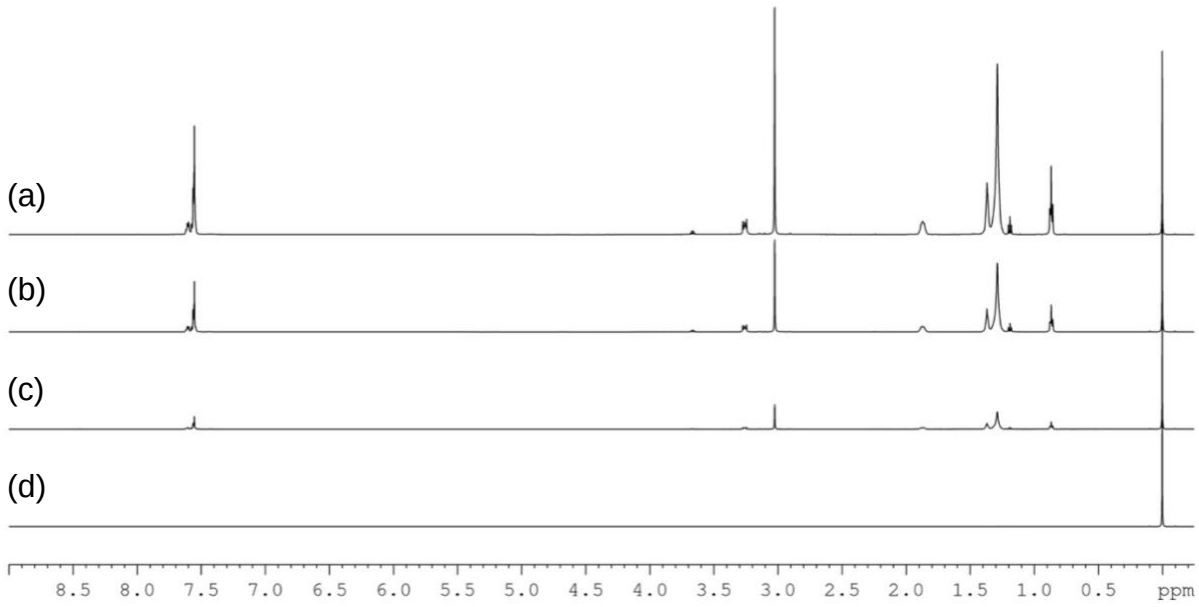

Figure A-1: Stacked $_1$H NMR spectra of (a) buffer solution. (b) 16.7 mg/L added BAC stock solution (c) 33.3 mg/L added BAC stock solution. (d) 66.7 mg/L added BAC stock solution.

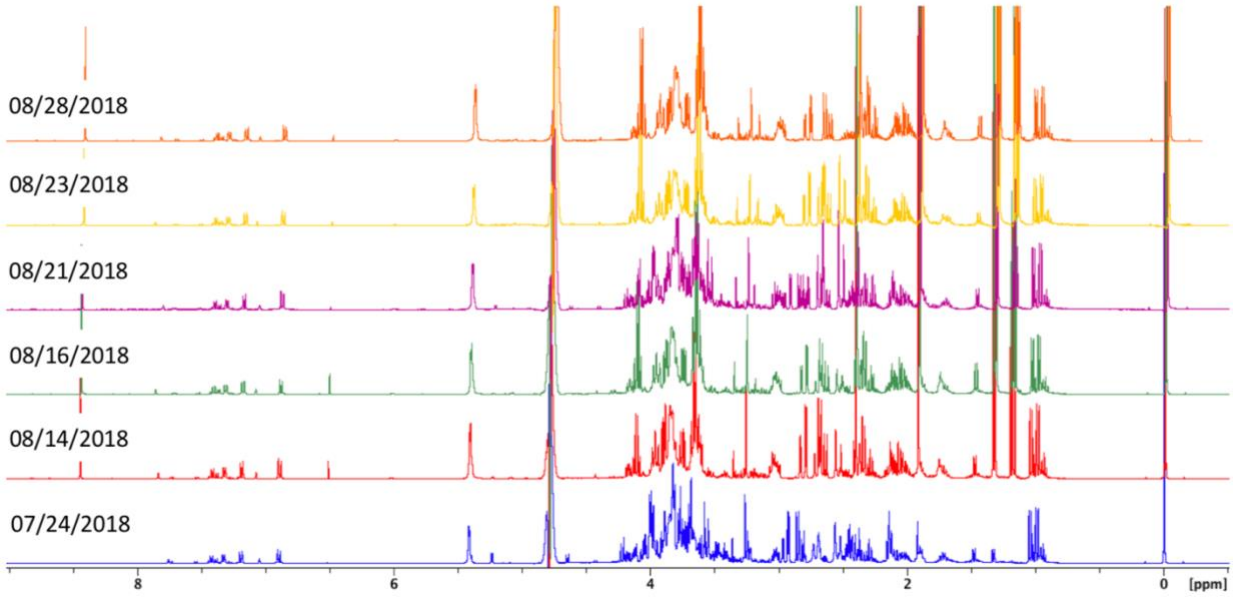

Figure A-2: **Stacked $_1$H NMR spectra of selected potato water influent samples from anaerobic digestor pilot study.**

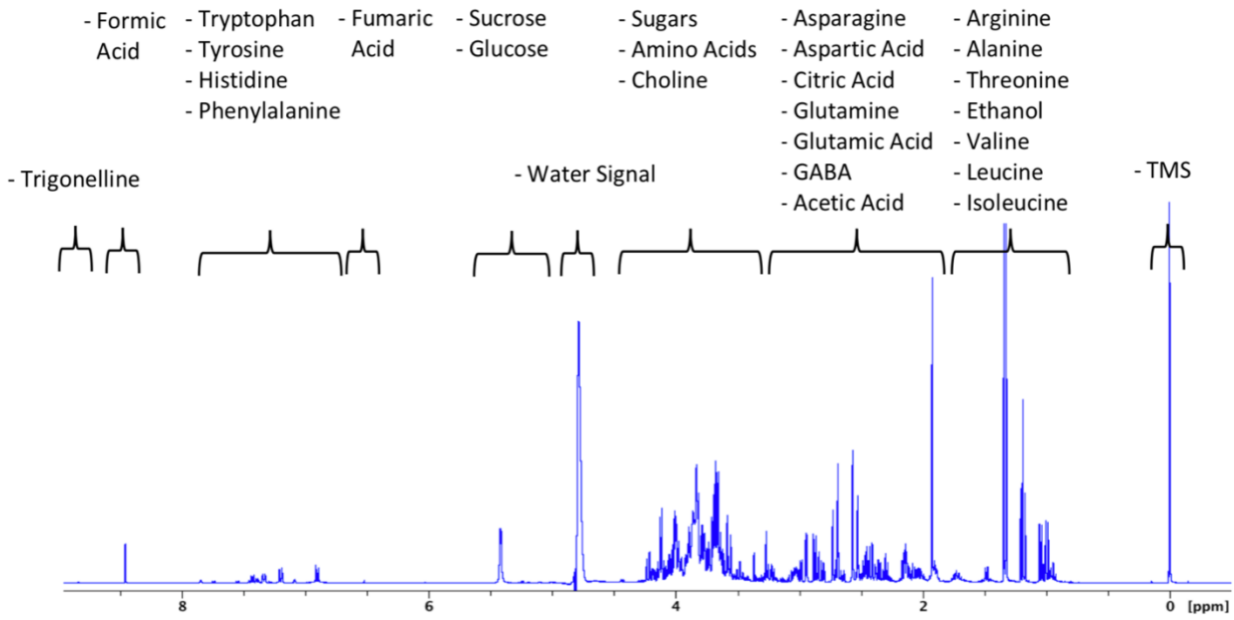

Figure A-3: $_1$H NMR spectra of potato water influent baseline from anaerobic digestor pilot study.

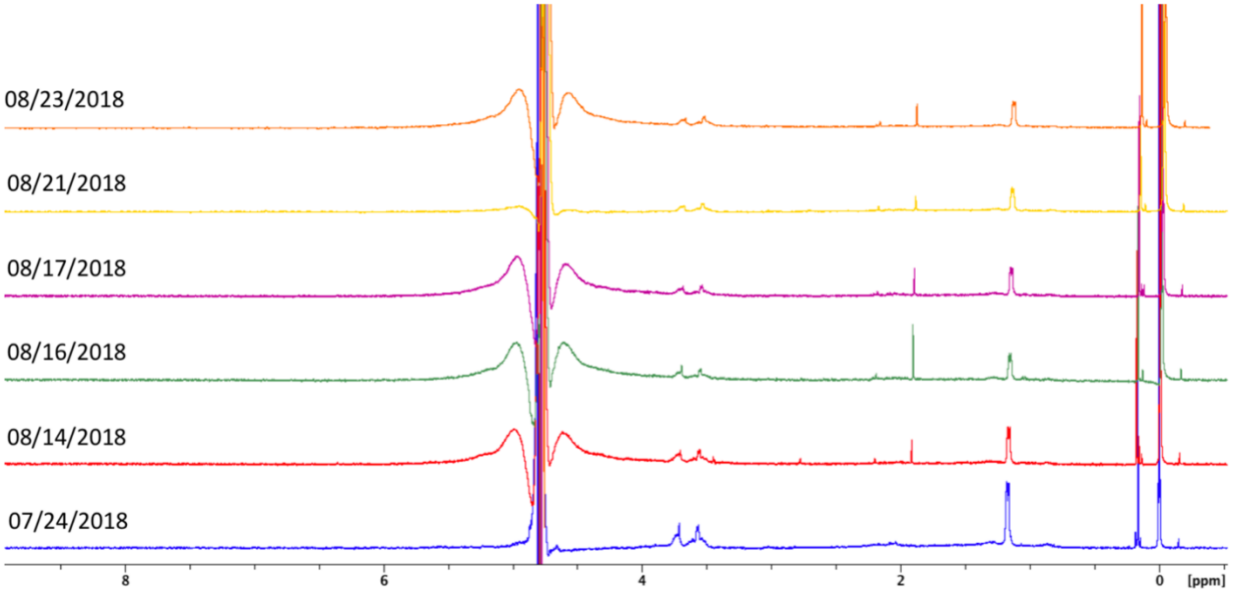

Figure A-4: Stacked $_1$H NMR spectra of selected bioreactor effluent samples from anaerobic digestor pilot study.

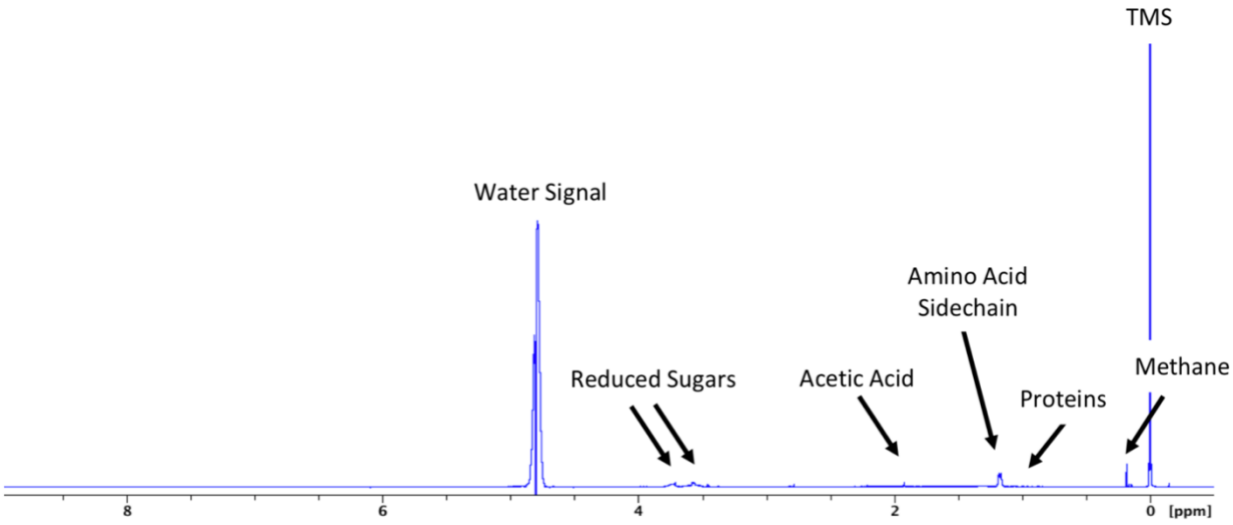

Figure A-5: $_1$H NMR spectra of bioreactor effluent baseline from anaerobic digestor pilot study.

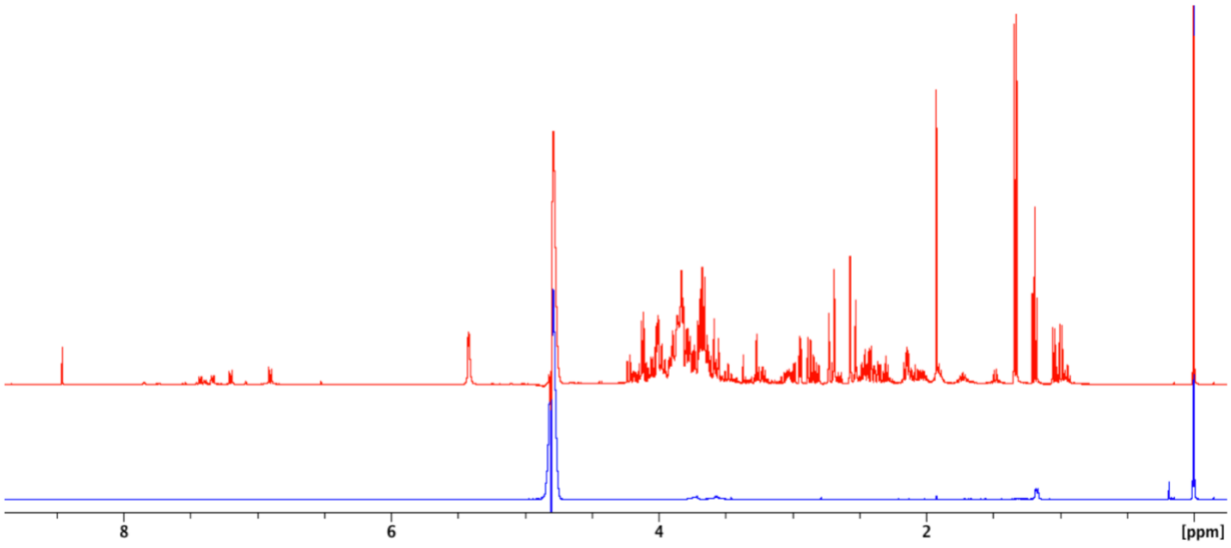

Figure A-6: Overlaid $_1$H NMR spectra of potato water influent and bioreactor effluent baselines from anaerobic digestor pilot study.

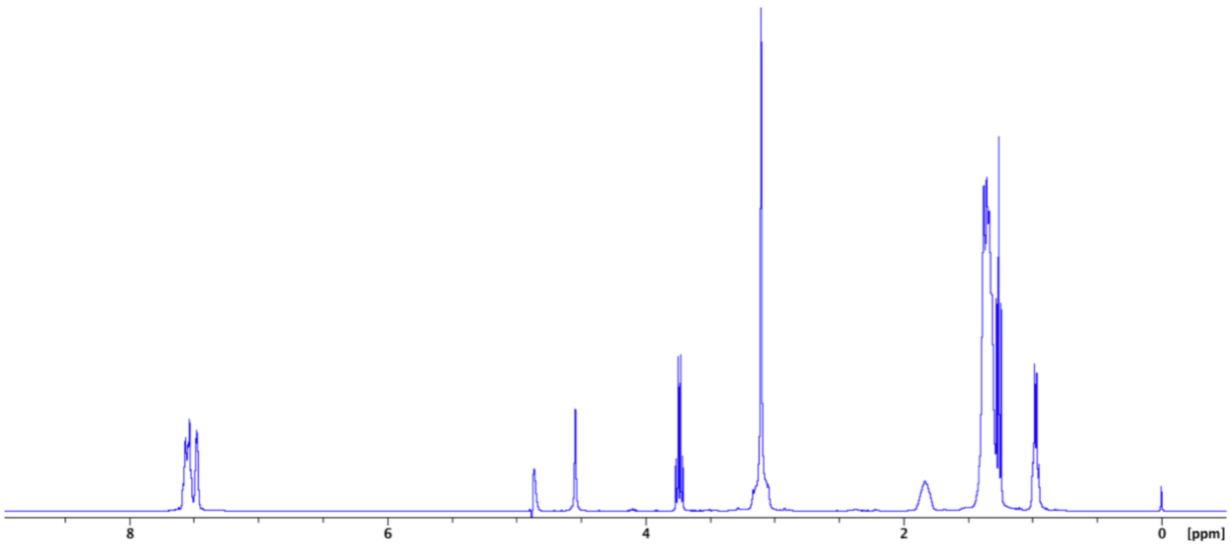

Figure A-7: $_1$H NMR spectrum of Ster-Bac (KQ-12), a liquid quaternary ammonium sanitizer.

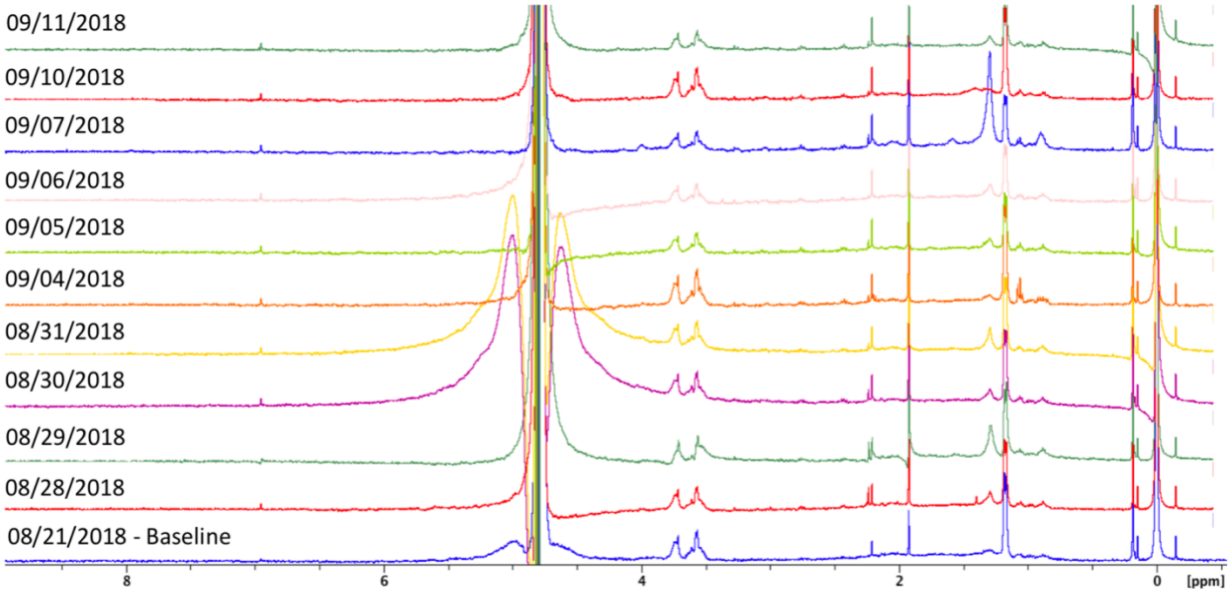

Figure A-8: Stacked $_1$H NMR spectra of effluent baseline and effluent after QAC has been added from anaerobic digestor pilot study.

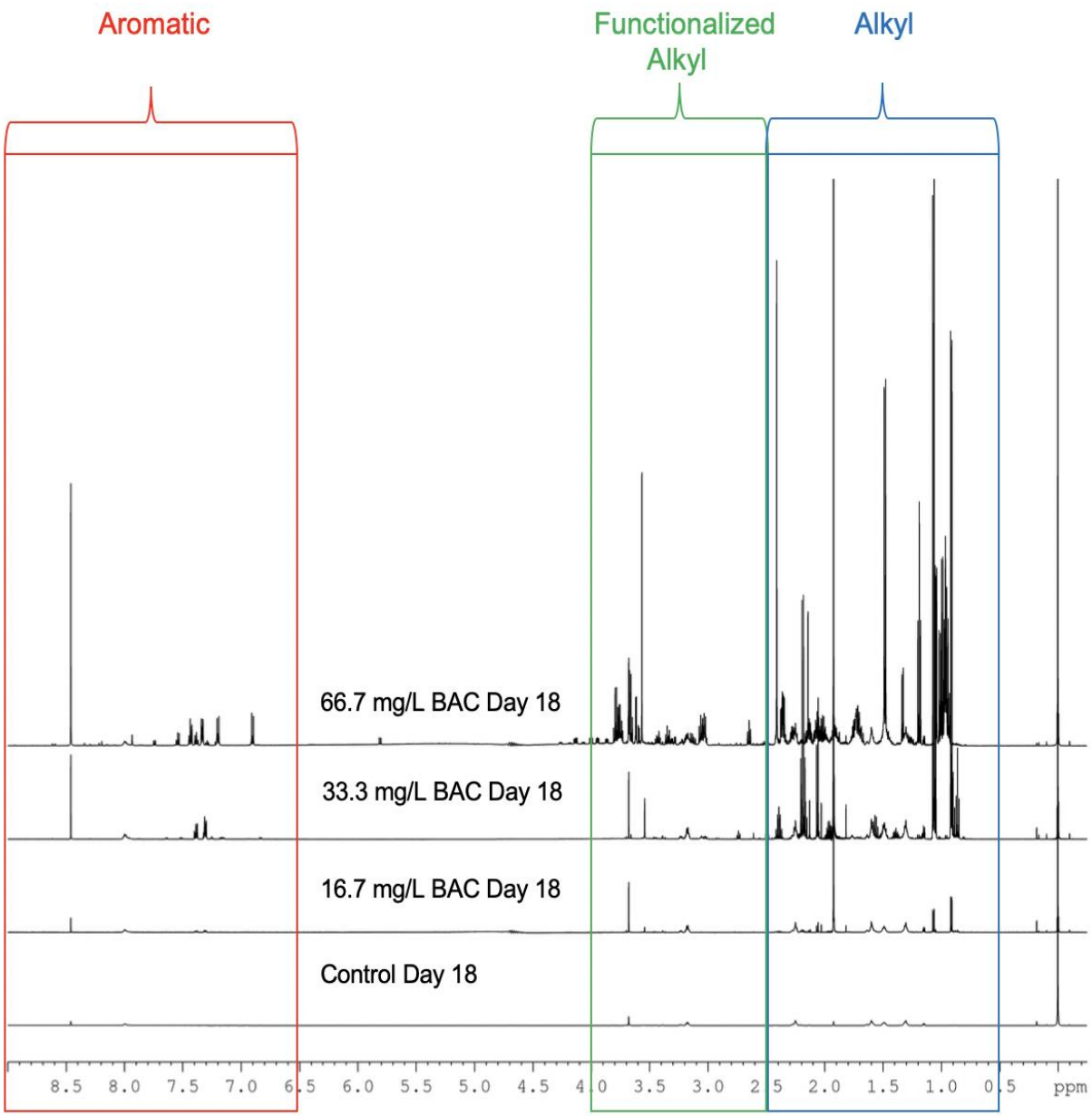

Figure A-9: Stacked $_1$H NMR spectra of Control Day 18, 16.7 mg/L added BAC Day 18, 33.3 mg/L added BAC Day 18, and 66.7 mg/L added BAC Day 18 with defined regions of interest; Alkyl, 0.5 – 2.5 ppm (blue); Functionalized Alkyl, 2.5 – 4.0 ppm (green); Aromatic 6.5 – 9.0 ppm (red).

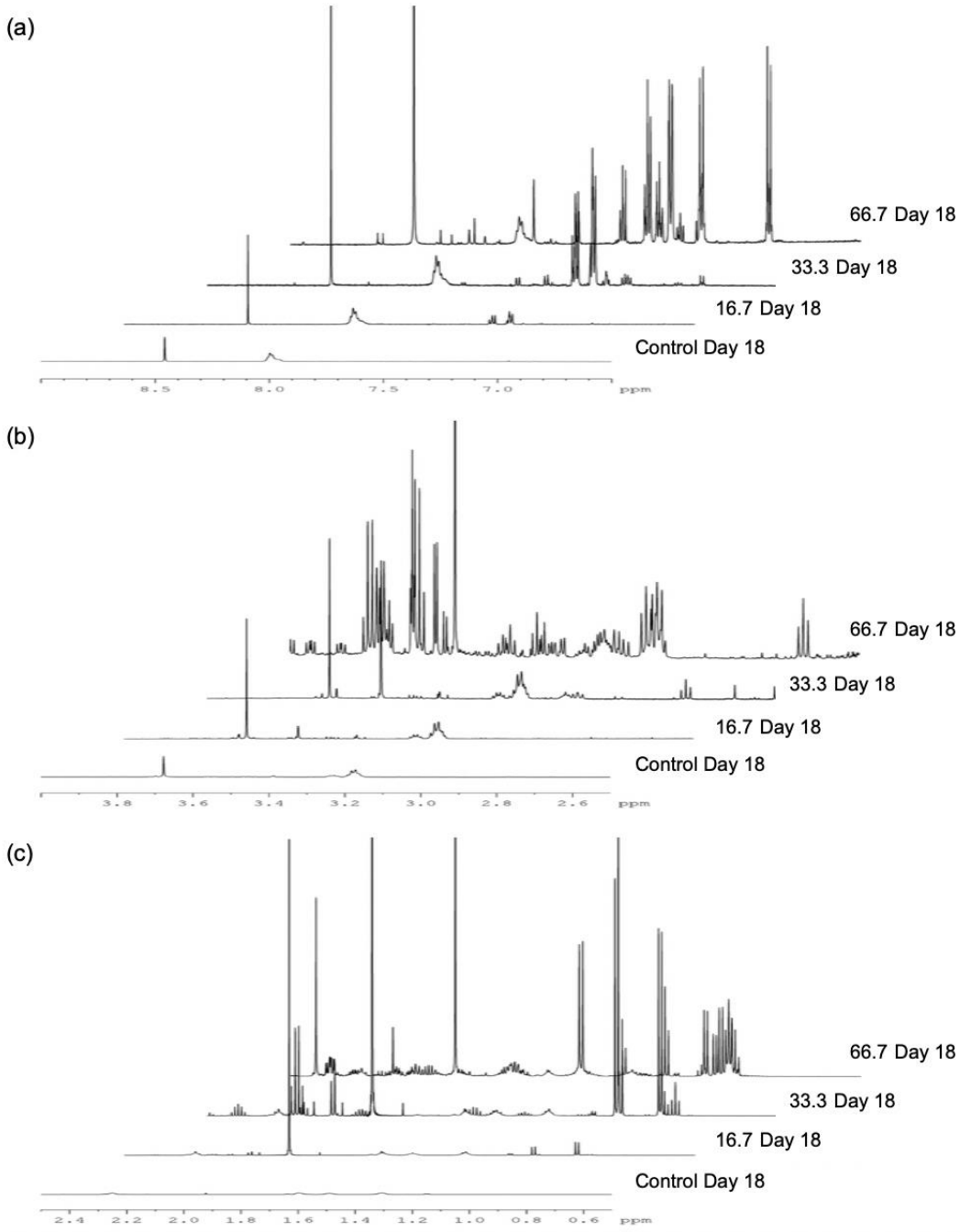

Figure A-10: Stacked $_1$H NMR spectra of control day 18, 16.7 mg/L added BAC day 18, 33.3 mg/L added BAC day 18, and 66.7 mg/L added BAC day 18. (a) Aromatic Region. (b) Functionalized Alkyl Region. (c) Alkyl Region.

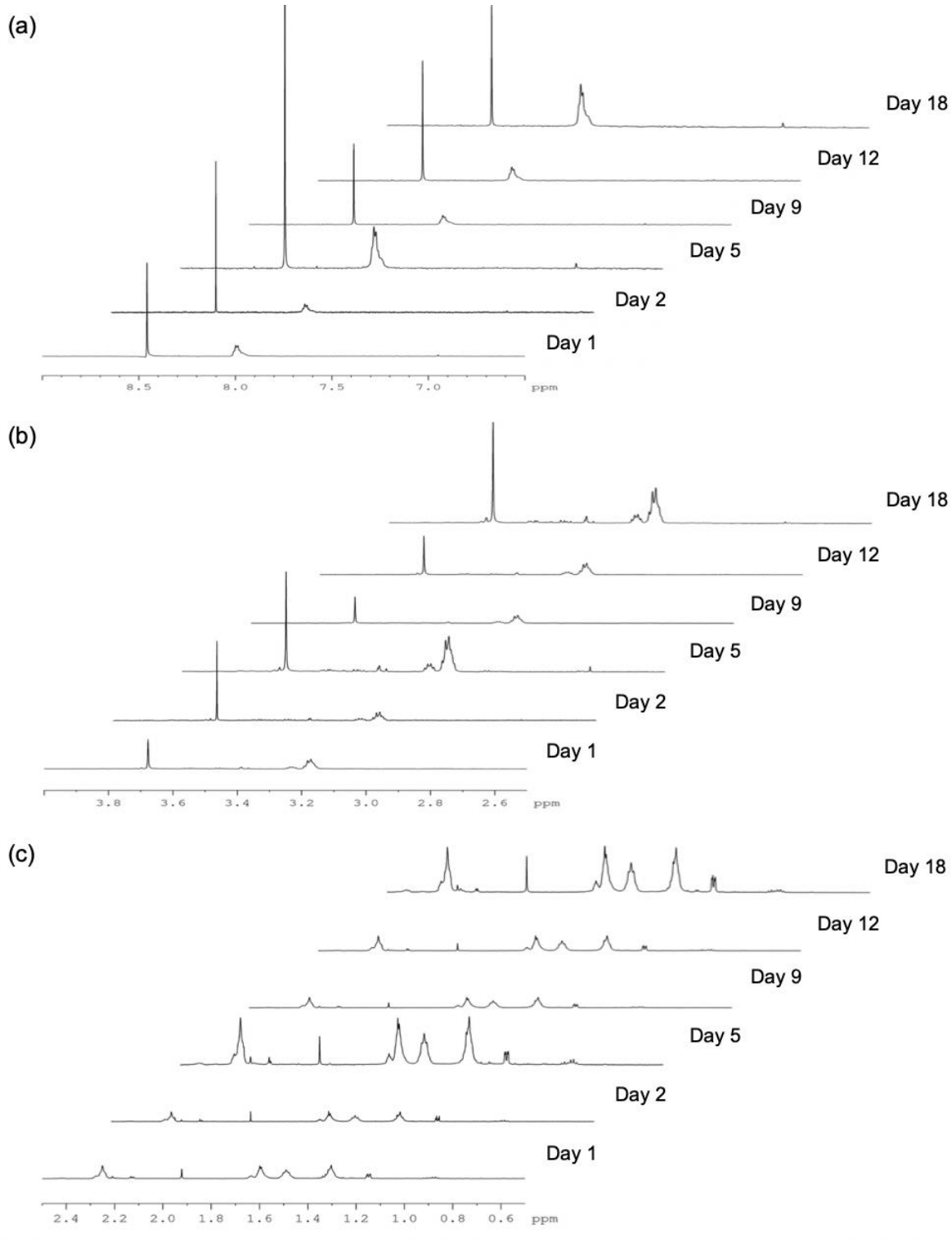

Figure A-11: Stacked $_1$H NMR spectra of control series day 1, 2, 5, 9, 12, and 18. (a) Aromatic Region. (b) Functionalized Alkyl Region. (c) Alkyl Region.

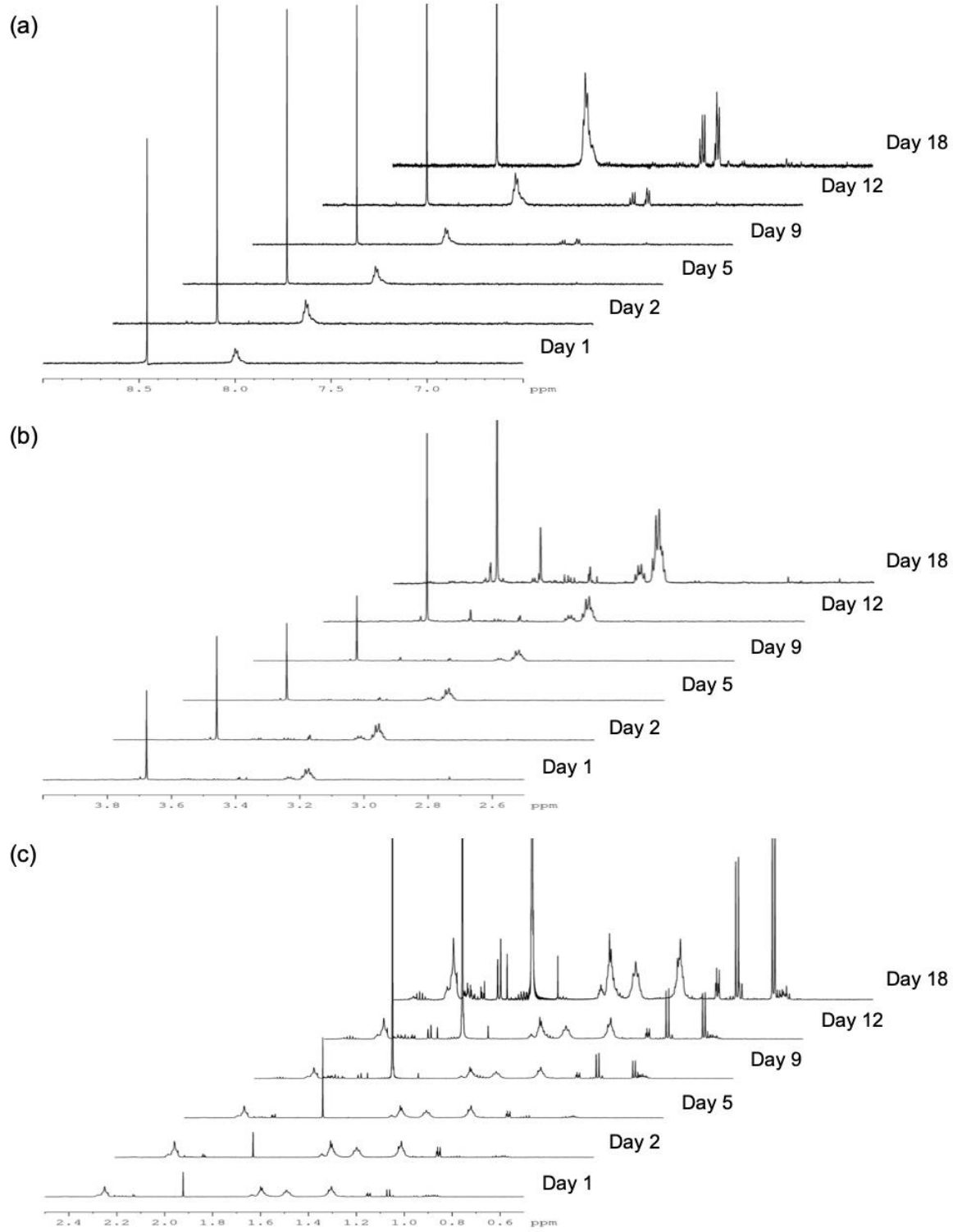

Figure A-12: Stacked $_1$H NMR spectra of 16.7 mg/L added BAC series day 1, 2, 5, 9, 12, and 18. (a) Aromatic Region. (b) Functionalized Alkyl Region. (c) Alkyl Region.

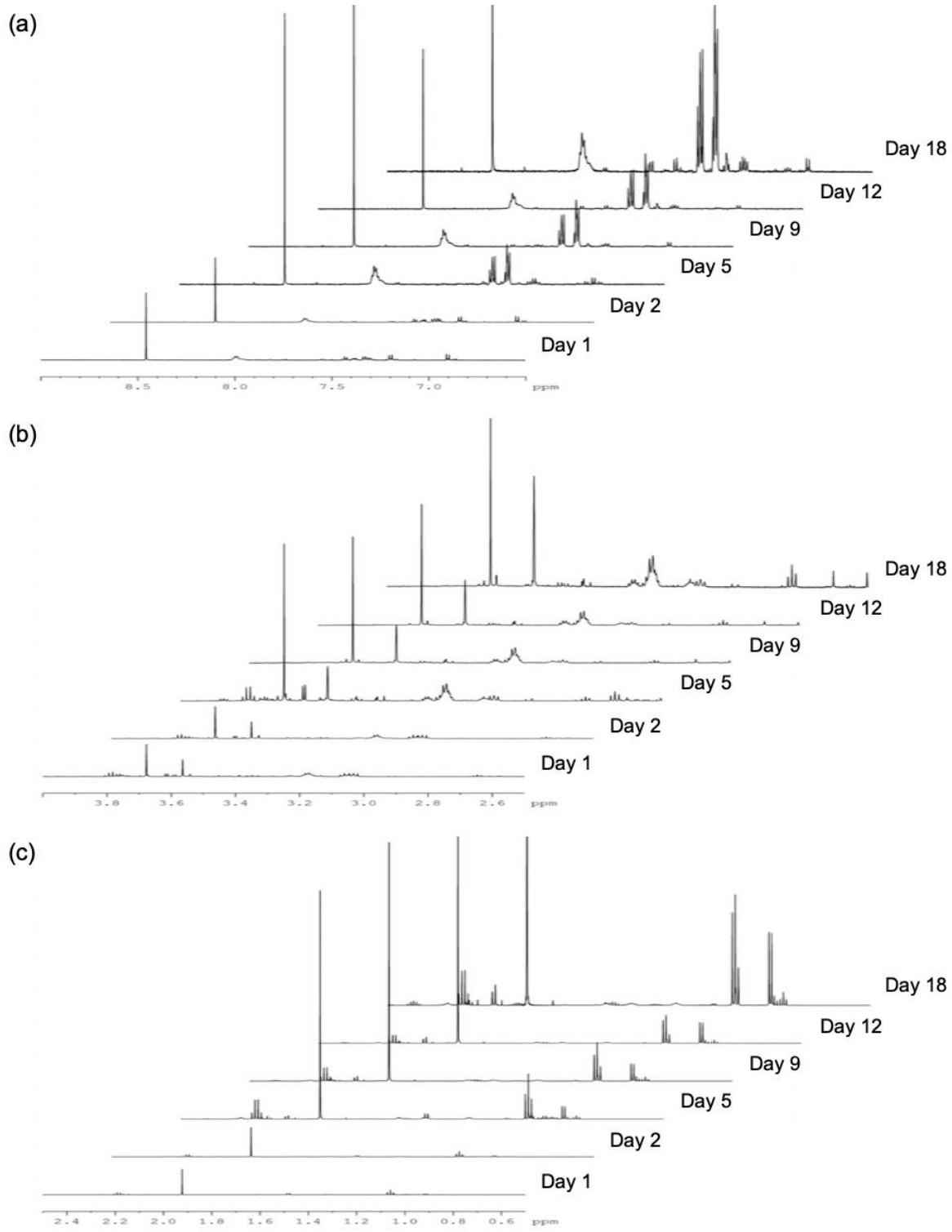

Figure A-13: Stacked ^{1}H NMR spectra of 33.3 mg/L added BAC series day 1, 2, 5, 9, 12, and 18. (a) Aromatic Region. (b) Functionalized Alkyl Region. (c) Alkyl Region.

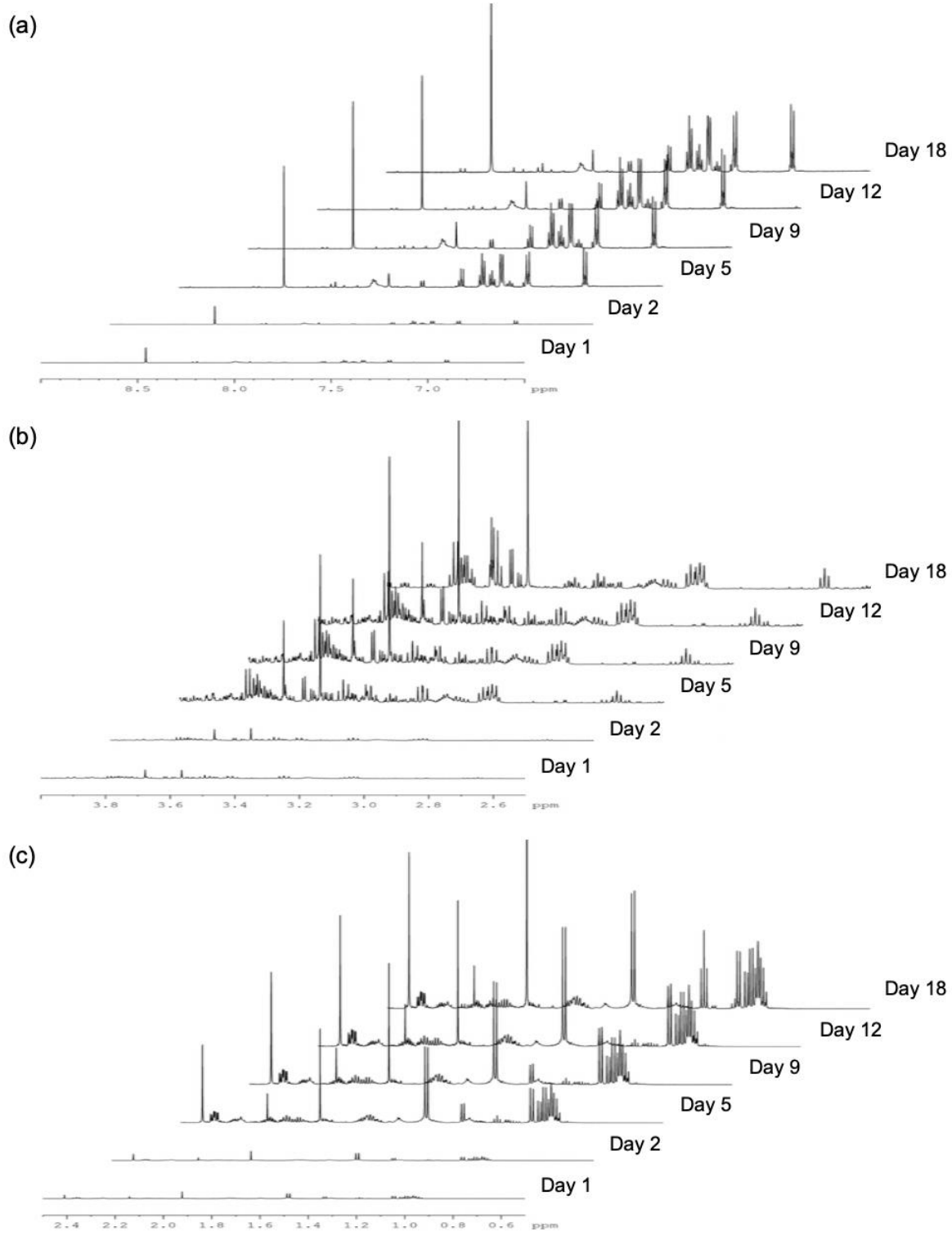

Figure A-14: Stacked $_1$H NMR spectra of 66.7 mg/L added BAC series day 1, 2, 5, 9, 12, and 18. (a) Aromatic Region. (b) Functionalized Alkyl Region. (c) Alkyl Region.

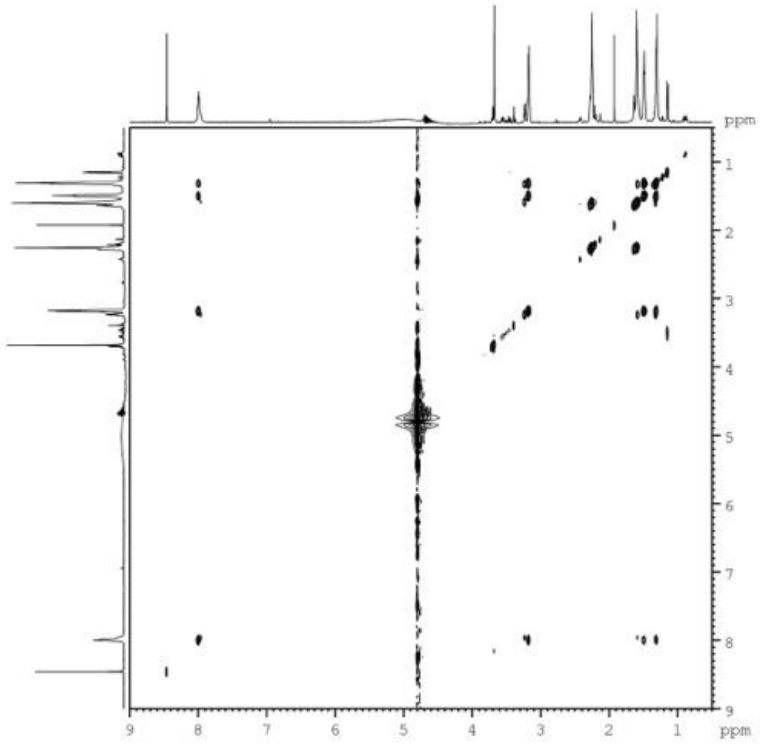

Figure A-15: $_1$H – $_1$H TOCSY NMR spectra of control day 18.

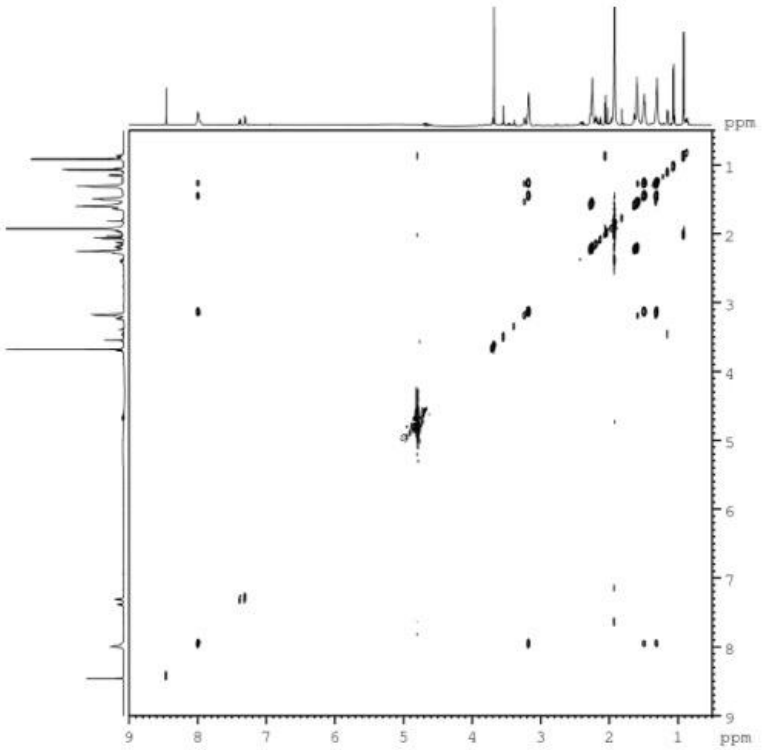

Figure A-16: $_1$H – $_1$H TOCSY NMR spectra of 16.7 mg/L added BAC day 18.

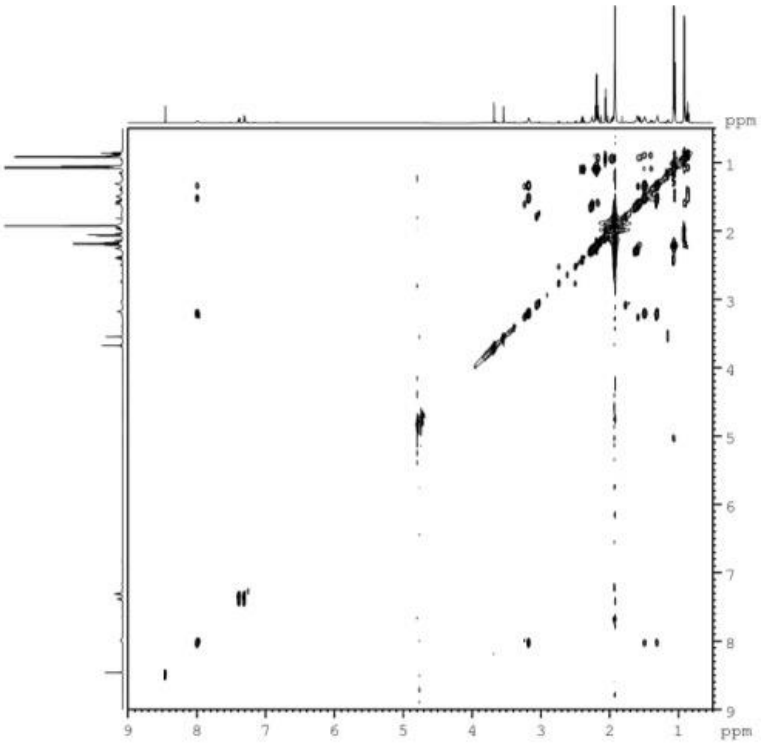

Figure A-17: $_1$H – $_1$H TOCSY NMR spectra of 33.3 mg/L added BAC day 18.

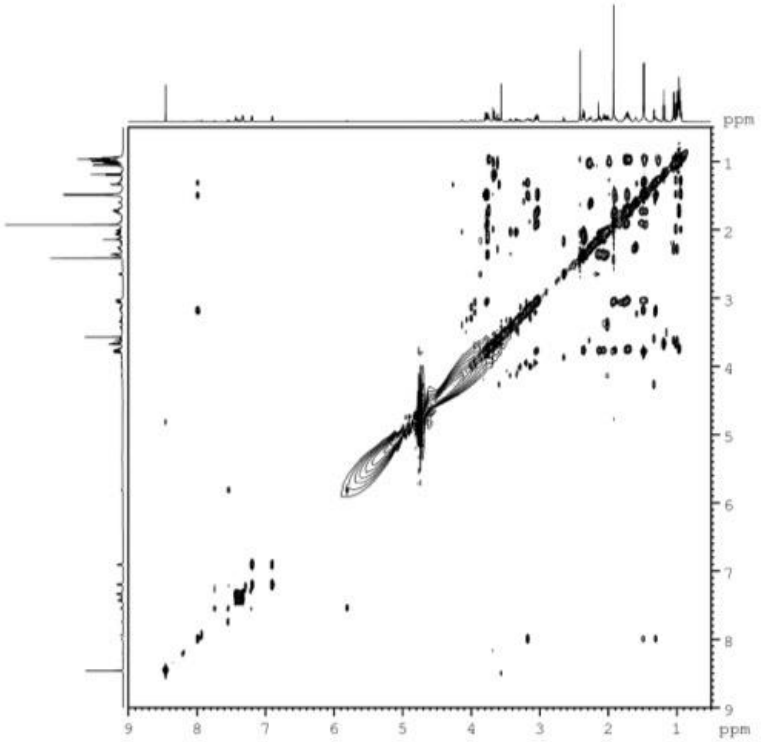

Figure A-18: $_1$H – $_1$H TOCSY NMR spectra of 66.7 mg/L added BAC day 18.

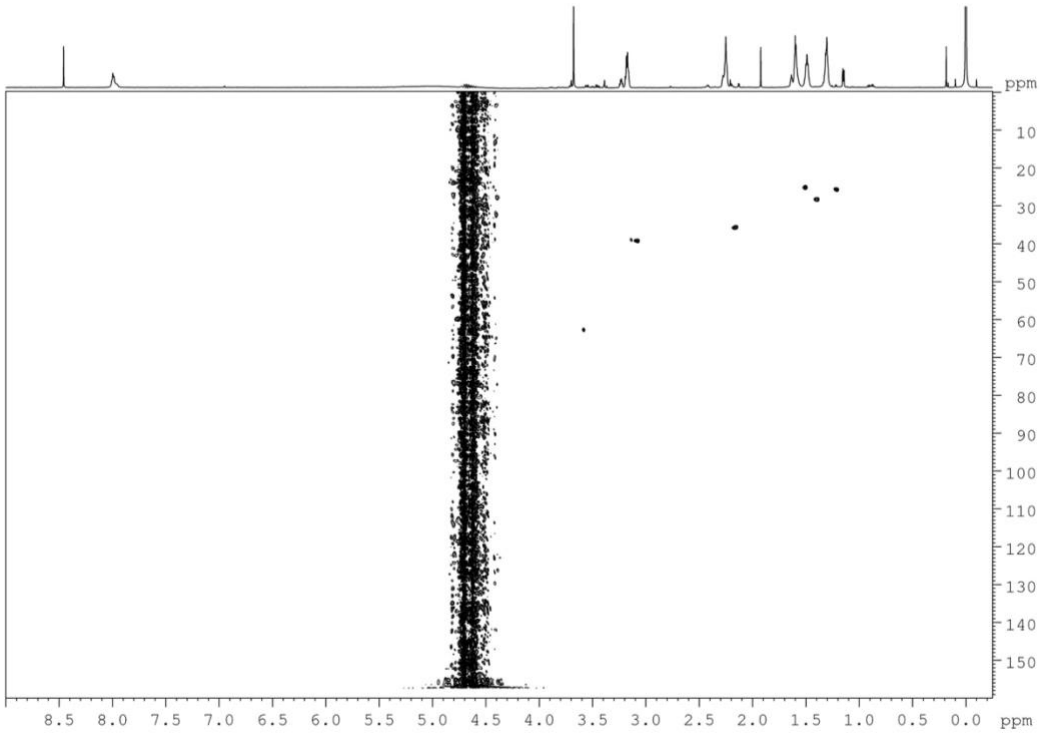

Figure A-19: $_{1}H$ – $_{13}C$ HSQC NMR spectra of control day 18.

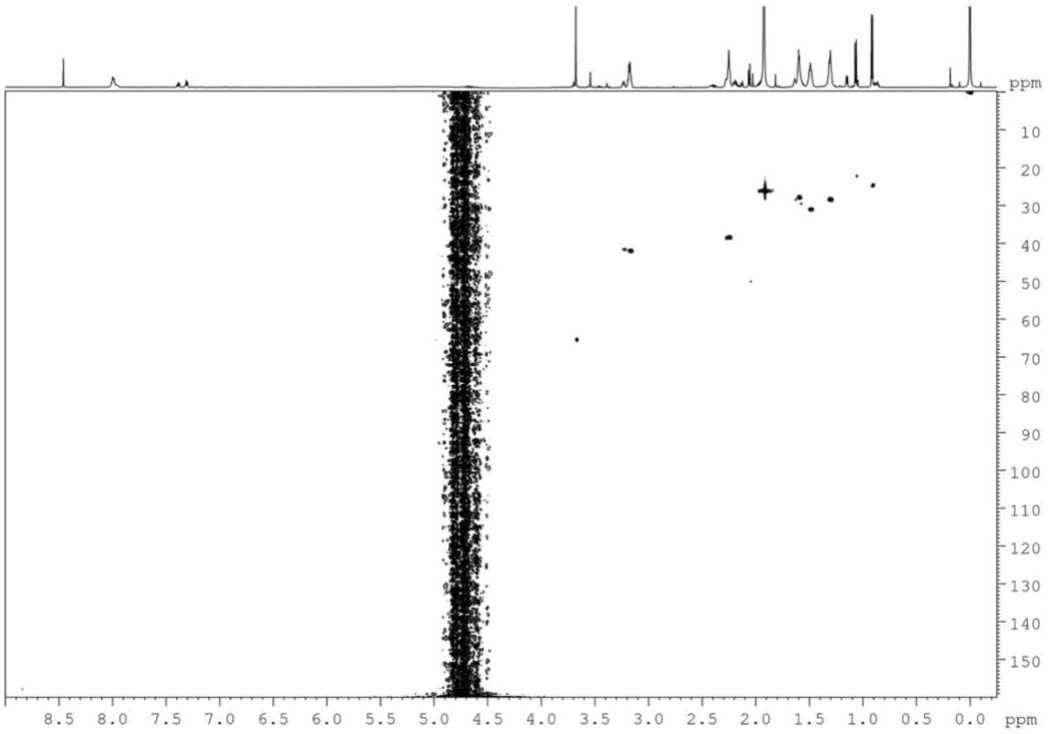

Figure A-20: $_{1}H$ – $_{13}C$ HSQC NMR spectra of 16.7 mg/L added BAC day 18.

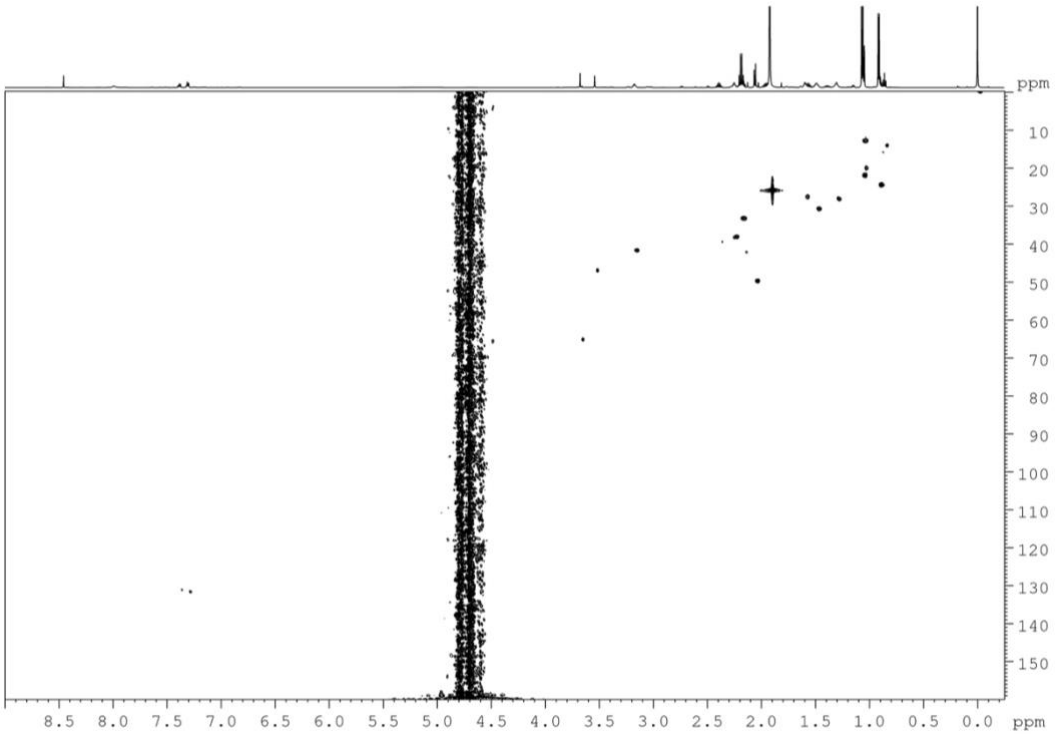

Figure A-21: $_{1}H$ – $_{13}C$ HSQC NMR spectra of 33.3 mg/L added BAC day 18.

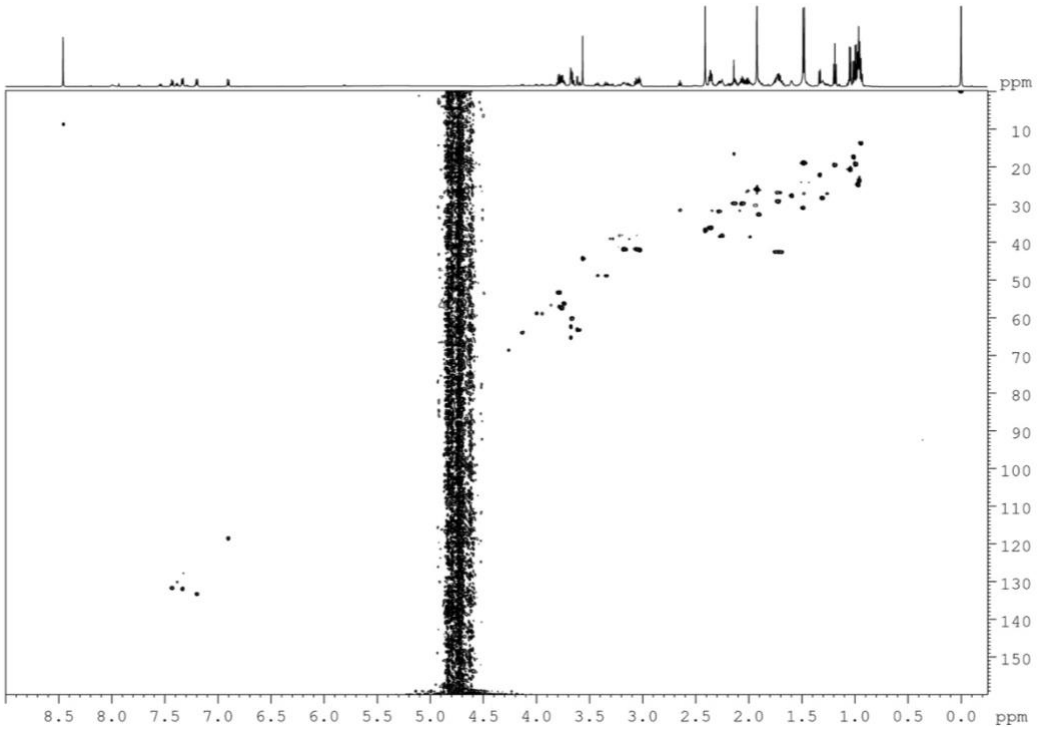

Figure A-22: $_{1}H$ – $_{13}C$ HSQC NMR spectra of 66.7 mg/L added BAC day 18.

Table A-1: Anaerobic digestor pilot study biogas monitoring numbers.

Date	Species	Retention Time	Peak Area	Composition
08/22/2018	CO2 CH4	2.233 1.466	5788.661 9306.546	38 % 62 %
08/30/2018	CO2 CH4	2.233 1.466	6507.024 8549.881	43 % 57 %
09/06/2018	CO2 CH4	2.233 1.466	7316.907 7840.924	48 % 52 %
09/13/2018	CO2 CH4	2.2 1.45	7051.645 7707.817	48 % 52 %
09/20/2018	CO2 CH4	2.216 1.45	7395.4995 8493.6055	47 % 53 %
09/27/2018	CO2 CH4	2.233 1.466	4787.903 8623.853	36 % 64 %
10/04/2018	CO2 CH4	2.233 1.466	7454.65 8174.241	48 % 52 %
10/11/2018	CO2 CH4	2.233 1.466	5637.936 6519.656	46 % 54 %
10/18/2018	CO2 CH4	2.216 1.45	4966.7535 6468.218	43 % 57 %
10/25/2018	CO2 CH4	2.25 1.466	4562.7545 9387.657	33 % 67 %
11/01/2018	CO2 CH4	2.216 1.45	6360.949 8174.252	44 % 56 %
11/08/2018	CO2 CH4	2.233 1.466	5994.601 7955.444	43 % 57 %
11/15/2018	CO2 CH4	2.233 1.466	6390.5905 7859.929	45 % 55 %
11/22/2018	CO2 CH4	2.2 1.45	5584.785 8257.525	40 % 60 %
11/29/2018	CO2 CH4	2.233 1.466	6658.9205 7874.196	46 % 54 %
12/06/2018	CO2 CH4	2.233 1.466	6975.1625 8888.856	44 % 56 %

Table A-2: pH of control, 16.7 mg/L added BAC, 33.3 mg/L added BAC, and 66.7 mg/L added BAC series.

Series	Day 1	Day 2	Day 5	Day 9	Day 12	Day 18
Control	6.90	6.95	6.88	6.94	6.84	6.86
16.7 mg/L	6.76	6.88	6.84	6.80	6.79	6.76
33.3 mg/L	6.79	6.81	6.84	6.89	6.86	6.78
66.7 mg/L	6.81	6.90	6.94	6.89	6.95	6.76

Table A-3: mM fatty acid concentrations from Chenomx for control series.

	Formate	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Succinate
Day 1	0.5701	0.0487	0	0	0	0	0	0
Day 2	0.6600	0.0357	0	0	0	0	0	0
Day 5	0.5650	0.0358	0	0	0	0	0	0
Day 9	0.7456	0.0396	0	0	0	0	0	0
Day 12	0.7082	0.0373	0	0	0	0	0	0
Day 18	0.3100	0.0871	0	0	0	0	0	0

Table A-4: mM fatty acid concentrations from Chenomx for 16.7 mg/L added BAC series.

	Formate	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Succinate
Day 1	0.5057	0.0776	0	0	0.0168	0	0	0
Day 2	0.7080	0.0622	0	0	0.0030	0	0	0
Day 5	0.6340	0.2585	0	0	0.0064	0	0	0
Day 9	0.5694	3.5220	0	0	0.0720	0	0.0546	0
Day 12	0.8296	5.0248	0	0	0.0970	0	0.0810	0
Day 18	0.3596	7.7161	0	0	0.1091	0	0.1827	0

Table A-5: mM fatty acid concentrations from Chenomx for 33.3 mg/L added BAC series.

	Formate	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Succinate
Day 1	0.5783	3.0116	1.0871	0.0276	0.0654	0	0.1141	0
Day 2	0.6325	4.4871	1.4018	0.0526	0.1647	0	0.1935	0
Day 5	0.7185	6.6072	2.3377	0.1032	0.3681	0	0.3448	0
Day 9	0.8586	10.1986	2.3106	0.3766	0.5786	0	0.6577	0
Day 12	0.4999	11.3637	1.5997	0.2688	0.6295	0	0.7995	0
Day 18	0.7868	15.6049	2.5665	0.3233	1.0504	0.5541	1.0764	0

Table A-6: mM fatty acid concentrations from Chenomx for 66.7 mg/L added BAC series.

	Formate	Acetate	Propionate	Butyrate	Isobutyrate	Valerate	Isovalerate	Succinate
Day 1	0.6441	0.4797	0.0124	0	0	0	0	0.1525
Day 2	0.8178	0.6505	0.0216	0	0	0	0	0.2942
Day 5	0.7739	0.8997	0.0208	0	0	0	0	0.5051
Day 9	0.8657	1.1376	0.0265	0	0	0	0	0.6937
Day 12	0.7809	1.2814	0.0322	0	0.0033	0	0	0.7693
Day 18	3.0579	4.2683	0.1652	0	0.1510	0	0	0.9452

Table A-7: mM amino acid concentrations from Chenomx for 33.3 mg/L added BAC series.

	Gly	Ala	Thr	Glu	Met	Val	Leu	Ile	Tyr	Phe	Trp
Day 1	0.1493	0.3766	0.1013	0	0	0	0.0668	0.0898	0.0698	0	0.0255
Day 2	0.1924	0.3656	0.0881	0	0	0	0.1356	0.0928	0.0719	0	0.0235
Day 5	0	0.2673	0	0	0	0	0	0.1139	0	0	0
Day 9	0	0	0	0	0	0	0	0	0	0	0
Day 12	0	0	0	0	0	0	0	0	0	0	0
Day 18	0	0	0	0	0	0.0137	0	0	0	0	0

Table A-8: mM amino acid concentrations from Chenomx for 66.7 mg/L added BAC series.

	Gly	Ala	Thr	Glu	Met	Val	Leu	Ile	Tyr	Phe	Trp
Day 1	0.2943	0.7343	0.2664	0.4966	0.1198	0.4001	0	0.2463	0.1231	0.1226	0.0392
Day 2	0.4453	1.0903	0.3412	0.7308	0.1736	0.5078	0	0.3660	0.2146	0.2265	0.0765
Day 5	0.7714	1.1694	0.4058	1.3446	0.2389	0.8311	0	0.5090	0.3062	0.3753	0.1115
Day 9	1.0460	1.4445	0.3918	1.5695	0.2573	0.9986	0	0.5967	0.3692	0.4007	0.1528
Day 12	1.1800	1.6840	0.3717	1.6905	0.2996	1.0741	0	0.6576	0.4027	0.4355	0.1864
Day 18	1.1200	1.4516	0.4618	1.9516	0.2987	1.2985	0	0.7657	0.4927	0.5022	0.1969

Table A-9: Biogas composition (CO_2 / CH_4) of control, 16.7 mg/L added BAC, 33.3 mg/L added BAC, and 66.7 mg/L added BAC series.

Series	Day 1	Day 2	Day 5	Day 9	Day 12	Day 18
Control	N/A	38/62	32/68	32/68	33/67	31/69
16.7 mg/L	N/A	36/64	28/72	30/70	29/71	31/69
33.3 mg/L	N/A	63/37	54/46	50/50	52/48	54/46
66.7 mg/L	N/A	71/29	68/32	69/31	72/28	76/24

Table A-10: Biogas concentration relative to control day 2.

Series	Day 1	Day 2	Day 5	Day 9	Day 12	Day 18
Control	N/A	0	0.55	0.86	0.94	1.06
16.7 mg/L	N/A	0.26	0.83	1.19	1.28	0.94
33.3 mg/L	N/A	-0.21	-0.03	0.13	0.18	0.09
66.7 mg/L	N/A	-0.4	-0.32	-0.37	-0.42	-0.56

Appendix B – Supplementary Material for Chapter 3

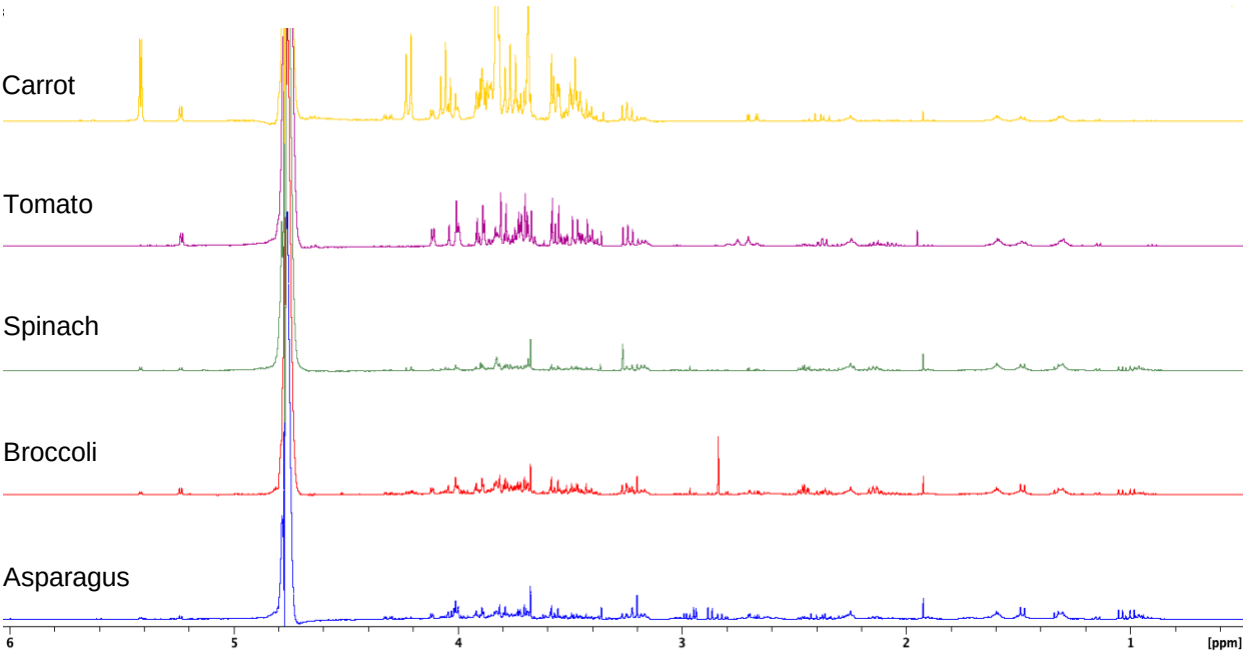

Figure B-1: Stacked $_1$H NMR spectra of simulated vegetable food waste. Scale 1:1.

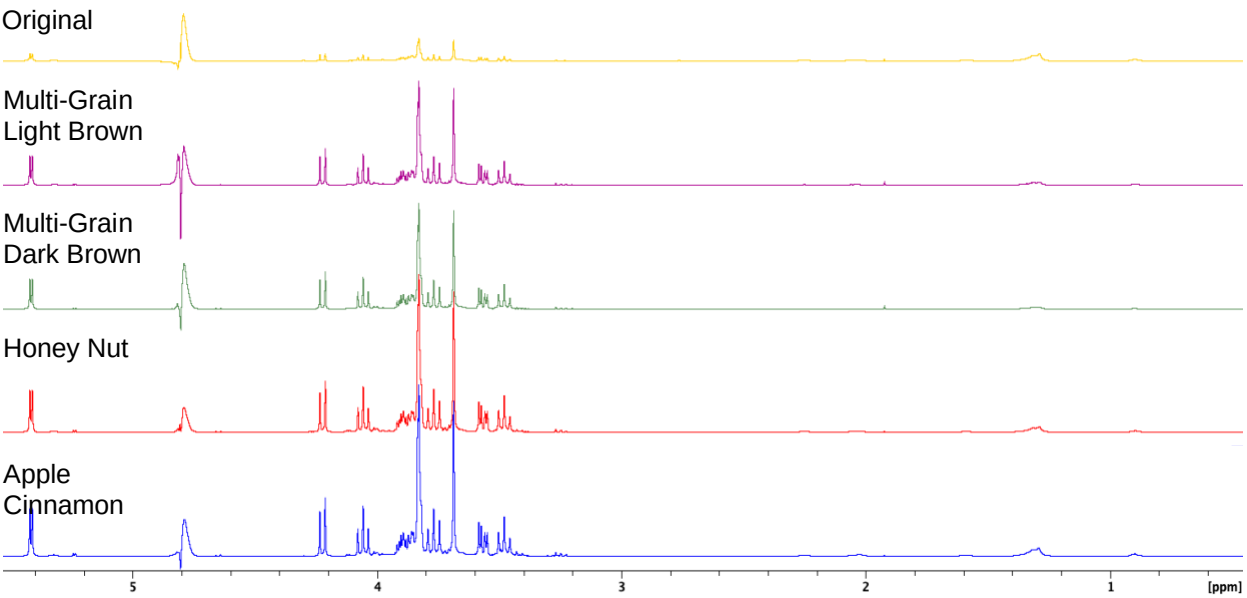

Figure B-2: Stacked $_1$H NMR spectra of simulated cheerio food waste. Scale 1:1.

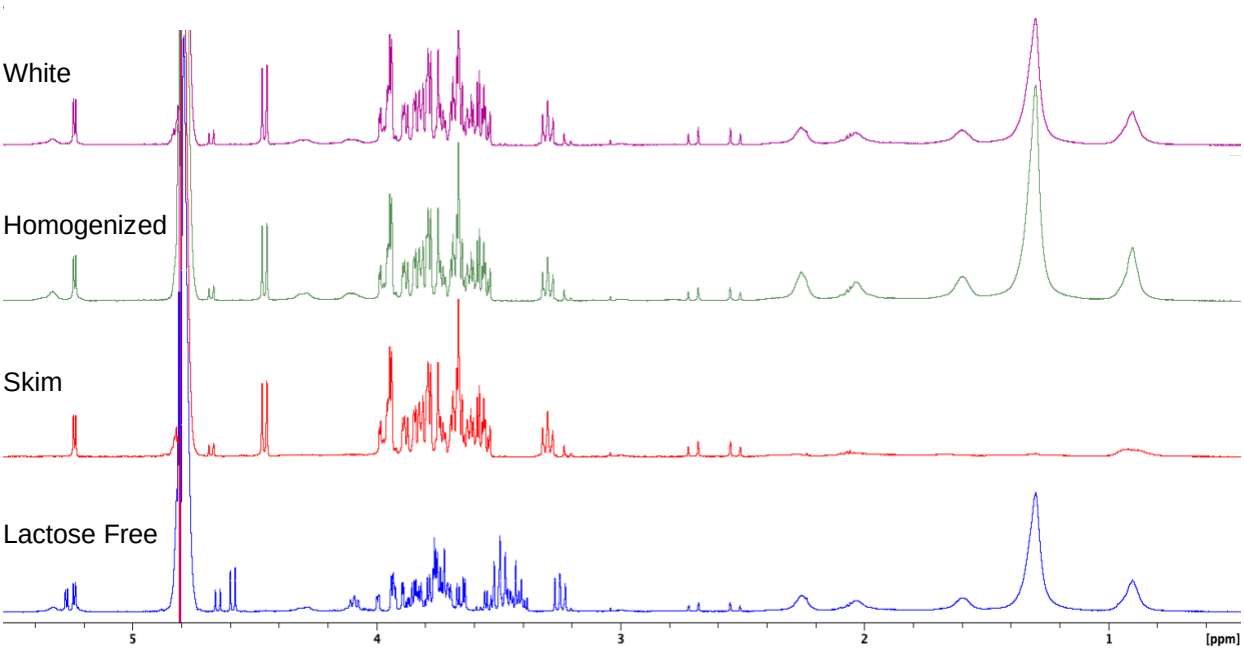

Figure B-3: Stacked $_1$H NMR spectra of simulated dairy milk food waste. Scale 1:1.

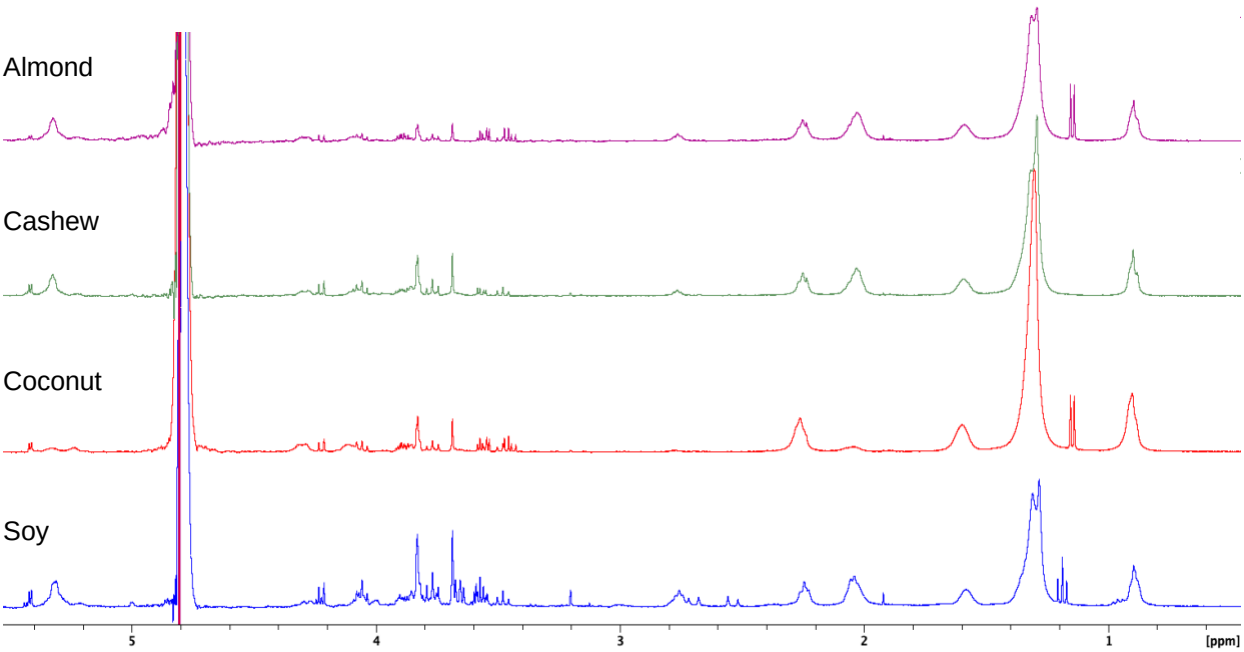

Figure B-4: Stacked $_1$H NMR spectra of simulated nut milk food waste. Scale 1:1.

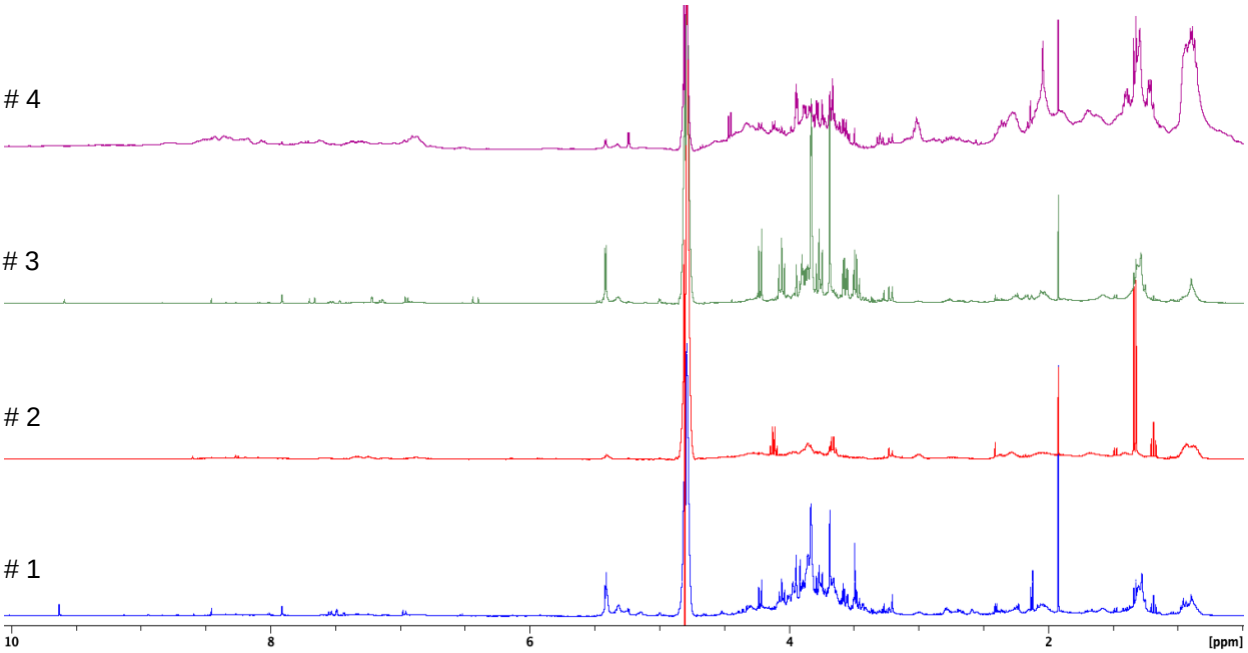

Figure B-5: Stacked $_1$H NMR spectra of simulated protein (1-4) food waste. Scale 1:1.

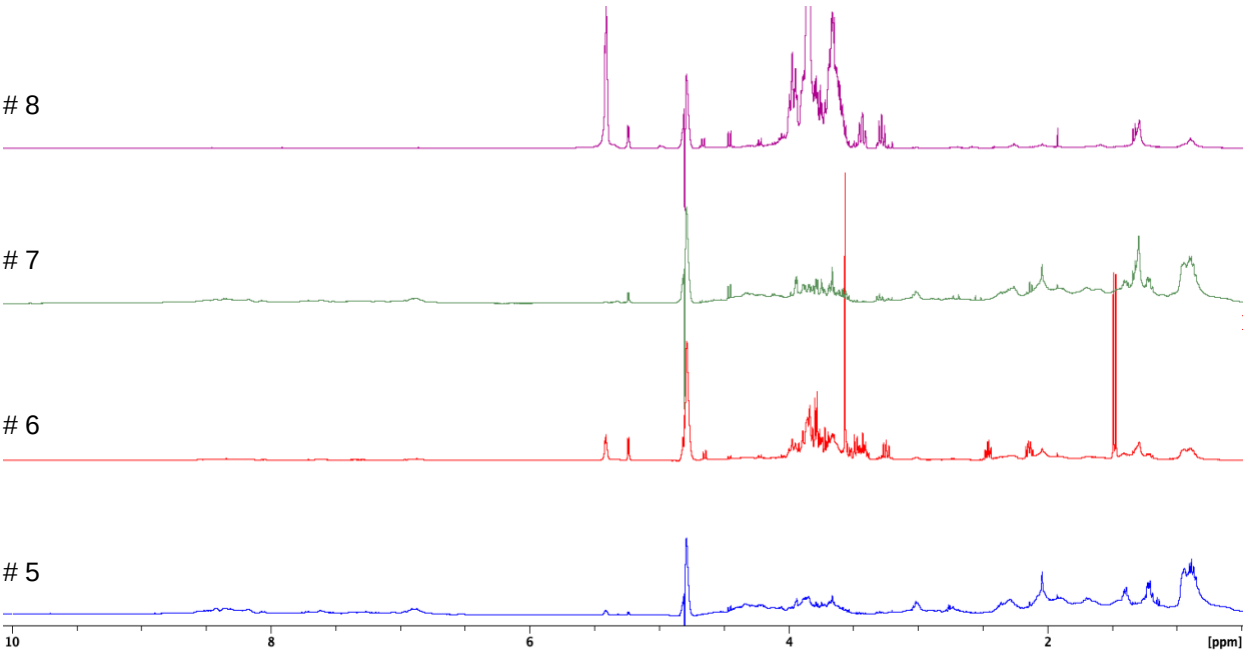

Figure B-6: Stacked $_1$H NMR spectra of simulated protein (5-8) food waste. Scale 1:1.

Table B-1: Nutritional Facts for simulated sugar (cheerio) wastewater.

Type	Fat (g)	Sugar (g)	Protein (g)	Fibre (g)	Serving Size
Original	2	1	3	3	27 g
Multi-Grain Light Brown	1.5	6	2	3	30 g
Multi-Grain Dark Brown	1.5	6	2	3	30 g
Honey Nut	1.5	9	2	2	29 g
Apple Cinnamon	2	10	2	2	30 g
Chocolate	1.5	9	2	2	29 g

Table B-2: Nutritional Facts for simulated lipid (milk) wastewater.

Type	Fat (g)	Sugar (g)	Protein (g)	Fiber (g)	Serving Size
White	5	12	9	0	250 mL
Homogenized	8	11	9	0	250 mL
Skim	0	13	9	0	230 mL
Lactose Free	6	10	16	0	310 mL
Almond	2.5	1	1	0	250 mL
Cashew	2.5	2	1	0	250 mL
Coconut	4.5	1	0.2	0	250 mL
Soy	2	3	6	1	250 mL

Table B-3: Nutritional Facts for simulated protein (protein supplement) wastewater.

Type	Fat (g)	Sugar (g)	Protein (g)	Fiber (g)	Serving Size
#1 - Muscle Mass Gainer Chocolate Fudge Brownie	4.3	6.4	20	2.9	100 g
#2 - Whey Protein Isolate Pineapple Coconut	1	0.5	25	0	31 g
#4 - Meal Replacement Banana Nut Bread	1	1	12	2	32.5 g
#6 - Whey Isolate Vanilla - Grass Fed	0	0	25	0	29 g
#9 - Undenatured Iso Whey Rich Chocolate	1.6	1	30	1	36 g
#10 - Plant-Based Protein Chocolate – No Artificial Colours, Sweeteners, Preservatives	3	2	22	4	36 g
#11 - Fermented Organic Vegan Proteins Unsweetened & Unflavoured	2	0	20	2	25.9 g
#13 - Vegan All-In-One Chocolate - Plant Based	5	1	20	7	46 g

Table B-4: Nutritional Facts for simulated fiber (vegetable) wastewater.

Type	Fat (g)	Sugar (g)	Protein (g)	Fiber (g)	Serving Size
Broccoli	0.4	1.7	2.8	2.6	100 g
Asparagus	0.1	1.9	2.2	2.1	100 g
Spinach	0.4	0.4	2.9	2.2	100 g
Tomato	0.2	2.6	0.9	1.2	100 g
Carrot	0.2	4.7	0.9	2.8	100 g
Mushroom	0.3	2	3.1	1	100 g

Table B-5: COD and NMR Correlation for simulated sugar (cheerio) wastewater.

Type	COD (ppm)	COD Corrected Total (x10)	Lipids NMR Integral	Total Carbs NMR Integral	Total NMR Signal	% Lipids	% Carbs
Original	530.3	5303	5.0	11.0	16.0	69	31
Multi-Grain Light Brown	610.6	6106	1.3	29.8	31.1	4	96
Multi-Grain Dark Brown	608.9	6089	2.3	34.1	36.4	6	94
Honey Nut	740.2	7402	3.7	53.5	57.2	7	93
Apple Cinnamon	763.2	7632	7.8	56.8	64.6	12	88
Chocolate	726.4	7264	4.1	47.4	51.5	8	92

Table B-6: COD and NMR Correlation for simulated lipid (milk) wastewater.

Type	COD (ppm)	COD Corrected Total (x5)	Lipids NMR Integral	Total Carbs NMR Integral	Total NMR Signal	% Lipids	% Carbs
White	584.1	2920.5	9.4	10.5	19.8	47	53
Homogenized	646.3	3231.5	13.2	9.8	23.1	57	43
Skim	443.4	2217	1.5	9.0	10.5	15	85
Lactose Free	530.4	2625	8.5	7.0	15.5	55	45
Almond	331.5	1657.5	4.3	0.8	5.1	85	15
Cashew	356.7	1783.5	5.6	1.5	7.1	79	21
Coconut	372.1	1860.5	9.7	1.6	11.3	86	14
Soy	343.4	1717	5.5	2.8	8.3	66	34

Table B-7: COD and NMR Correlation for simulated protein (protein supplement) wastewater.

Type	COD (ppm)	COD Corrected Total (x20)	Fatty Acid NMR Integral	Total Carbs NMR Integral	Aromatic NMR Integral	Total NMR Signal
# 1	719.0	14380	63.4	32.8	16.6	112.8
# 2	600.8	12016	26.1	39.5	3.6	69.2
# 3	743	14860	48.6	24.3	9.2	79.1
# 4	627.2	12544	12.0	106.3	1.9	120.2
# 5	680.9	13618	9.2	19.5	1.2	29.9
# 6	742.6	14852	7.4	6.4	1.6	15.4
# 7	728.6	14572	8.8	14.3	0.9	24
# 8	763.9	15278	81.8	46.4	20.6	148.8

Table B-8: COD and NMR Correlation for simulated fiber (vegetable) wastewater.

Type	COD (ppm)	COD Corrected Total (x10)	Fatty Acid NMR Integral	Total Carbs NMR Integral	Aromatic NMR Integral	Total NMR Signal
Broccoli	338.1	3381	0.8	4.0	0.1	4.9
Asparagus	340.7	3407	1.2	3.5	0.2	4.9
Spinach	263.9	2639	0.8	1.7	0.1	2.6
Tomato	293.4	2934	0.6	5.0	0.1	5.7
Carrot	404.2	4042	0.5	12.1	0.03	12.63
Mushroom	285.3	2853	0.6	7.0	0.3	7.9

Table B-9: BOD_5 for cheerio, milk, protein supplement, and vegetable wastewaters.

Component	BOD_5 (mg/L)
Cheerio	
Original	137
Multi-Grain Light Brown	201
Multi-Grain Dark Brown	197
Honey Nut	291
Apple Cinnamon	310
Chocolate	285
Milk	
White	280
Homogenized	376
Skim	197
Lactose Free	343
Almond	65.2
Cashew	84.4
Coconut	109
Soy	130
Protein	
# 1	343
# 2	254
# 3	359
# 4	267
# 5	282
# 6	307
# 7	299
# 8	377
Vegetable	
Broccoli	33.4
Asparagus	34.6
Spinach	17.2
Tomato	26.2
Carrot	55
Mushroom	22.9