THE DEVELOPMENT OF ENVIRONMENTAL COMPREHENSIVE MULTIPHASE
NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

by

Hussain Masoom

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Chemistry
University of Toronto

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Abstract

The Development of Environmental Comprehensive Multiphase Nuclear Magnetic Resonance Spectroscopy

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Doctor of Philosophy
Graduate Department of Chemistry
University of Toronto
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Soil is the most complex mixture on this planet and is central to the transport of contaminants, carbon cycling and sustainable agriculture. However, our understanding of soil is limited in large part due to the lack of analytical approaches that can provide detail information on molecular structure and interactions in the native state. Current pre-treatment practices remove key synergies between components that are responsible for soil’s reactivity, kinetics, interactions and numerous other characteristics. Nuclear magnetic resonance (NMR) spectroscopy has played an integral role in furthering soil research because of its versatility and non-destructive nature, but has not been able to study all phases of matter (solid, liquids, and gels) at once in a whole unaltered soil. Recently, comprehensive multiphase NMR (CMP-NMR) was introduced and combines traditional NMR techniques resulting in an instrument that has capabilities allowing for whole soil analysis.

After an introduction of soil and NMR, the first two research chapters focus on solving two key issues with environmental NMR, sensitivity and water suppression through experimental means. Sensitivity is increased by focusing NMR signals into one single spike and forgoing chemical shift. The result is an experiment that can determine experimental run time in low concentration samples as well as a rapid detection system for use in kinetics or dynamics. In the next chapter, a robust water suppression method is developed and applied to real environmental samples including soil by building on the current standard, SPR-W5-WATERGATE.

The latter two research chapters apply CMP-NMR to soil research by first characterizing the composition and structure of soil in its native state. It is found that aliphatic and carbohydrate components are available at the water interface while proteins from microbes and lignin are buried under the surface with hydrogen bonds and hydrophobicity playing a key role in their
protection. Finally, contaminant interactions are probed in all phases where kinetics, sorption orientation, and soil binding domains are characterized in all phases. As a whole, this thesis helps to develop CMP-NMR for use in soil research and positions it as an important novel NMR technology with potential widespread application in a range of fields including materials research, biology, biochemistry and medicine.
Acknowledgements

I would first like to thank my supervisor Dr. André Simpson for his unwavering support throughout my graduate degree. He has been a source for encouragement, mentorship, and friendship first, and a fantastic supervisor second. He gave me autonomy to explore the first class science that we do as well as the world outside of the lab. When I struggled with explaining my results and wasn’t sure where to sample, he’s been there with answers and guidance. It’s been the best five years of my life and I would not trade this experience for anything. You have played an integral role in making my tenure exceptional.

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recommended books or articles for me to read, that have put forward my name for leadership positions, and that have convinced me into doing things I wouldn’t normally have done. You all know who you are, and I am eternally grateful to have crossed paths with you and wish to cross paths again in the future.

There are also people in the world where upon first encounter, you know that you will get along with them, you will share stories with them, and you will feel comfortable to be yourself around them. You also know that they feel the same about you. These individuals are few and far between and are those that remain with you throughout your life. Alan, you appeared towards the end of my degree and have made a huge impact on my life in the short time that we’ve known each other. Words cannot describe the bond that I share with you.

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Charles Hwang is another companion that I have heavily relied on in the past five years. You’ve been there for me to navigate my life and have advised me on many important decisions. You’ve counseled me when I needed it and were there with your sympathy and comfort. Our video game sessions were more than games to me and I hope you felt the same. Although we only talk sparsely now, you’re a friend for life and that’s something I can smile about.

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You have shaped my entire being from my personality to my soul. You have fed my appetite for curiosity, for well-being, and for life. You gave me the strength to carry on, I would not be here without you. You motivated me through the worst, and celebrated with me during the best. I am me because you are you.
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List of Symbols

\begin{itemize}
  \item $\alpha$ lower energy state
  \item $\beta$ higher energy state
  \item $B_o$ external magnetic field
  \item $B_1$ applied magnetic field
  \item $B_{\text{eff}}$ effective magnetic field
  \item $B_i$ induced magnetic field
  \item $\delta$ arbitrary symbol for delay within an NMR experiment
  \item $\delta_{\text{ppm}}$ chemical shift
  \item $\Delta$ diffusion time
  \item $E$ energy
  \item $f_{\text{oc}}$ fraction of organic carbon
  \item $\gamma$ gyromagnetic ratio
  \item $\hbar$ simplified Plank’s constant ($\hbar/2\pi$)
  \item $H_D$ Hamiltonian for dipolar couplings
  \item $\theta$ angle between the internuclear vector and applied field
  \item $I$ spin quantum number
  \item $I(t)$ intensity dependant on time
  \item $k$ various constants
  \item $K_d$ soil distribution coefficient
  \item $K_{\text{oc}}$ soil organic carbon normalized distribution coefficient
  \item $m_{\pm 1/2}$ magnetic quantum numbers of a spin $\frac{1}{2}$ nucleus
  \item $n$ number of moles
  \item $n_\alpha$ number of nuclei in the lower energy state
\end{itemize}
\( n_\beta \)

number of nuclei in the higher energy state

\( NS \)

number of scans

\( NS_{CP} \)

number of scans in a cross polarization experiment

\( NS_{SS} \)

number of scans in a single spike experiment

\( r \)

distance between two nuclei

\( S/N \)

signal to noise

\( S/N_{CC} \)

signal to noise in a conventional \(^{13}\text{C}\) experiment

\( S/N_{CP} \)

signal to noise in a cross polarization experiment

\( S/N_{SS} \)

signal to noise in a single spike experiment

\( \tau \)

delay between CPMG echoes

\( T \)

temperature

\( t \)

time

\( T_1 \)

spin-lattice relaxation time

\( T_1^\rho \)

spin-lattice relaxation rate constant relative to the rotating frame

\( T_2 \)

spin-spin relaxation time

\( T_{HF} \)

cross polarization build up curve time coefficient

\( \mu \)

magnetic moment

\( \nu_{\text{reference}} \)

precessional frequency of the reference

\( \nu_{\text{sample}} \)

precessional frequency of the sample

\( \omega \)

Larmour frequency
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>1D</td>
<td>one dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two dimensional</td>
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<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>BFR</td>
<td>brominated flame retardant</td>
</tr>
<tr>
<td>BPLED</td>
<td>bipolar pulse longitudinal encode-decode</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>CMP-NMR</td>
<td>comprehensive multiphase nuclear magnetic resonance</td>
</tr>
<tr>
<td>CP</td>
<td>cross polarization</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell Meiboom-Gill</td>
</tr>
<tr>
<td>CPMG-SS</td>
<td>Carr-Purcell Meiboom-Gill single spike</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>db</td>
<td>decibel</td>
</tr>
<tr>
<td>DDT</td>
<td>dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DE</td>
<td>diffusion editing</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNP</td>
<td>dynamic nuclear polarization</td>
</tr>
<tr>
<td>DOM</td>
<td>dissolved organic matter</td>
</tr>
<tr>
<td>FID</td>
<td>free induction decay</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HR-MAS</td>
<td>high resolution magic angle spinning</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>HSTD</td>
<td>heteronuclear saturation transfer difference</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IDE</td>
<td>inverse diffusion editing</td>
</tr>
<tr>
<td>INEPT</td>
<td>insensitive nuclei enhanced by polarization transfer</td>
</tr>
<tr>
<td>MAS</td>
<td>magic angle spinning</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>ms</td>
<td>millisecond</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
</tr>
<tr>
<td>POP</td>
<td>persistent organic pollutant</td>
</tr>
<tr>
<td>PFC</td>
<td>polyfluorinated chemicals</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed field gradient echo</td>
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<tr>
<td>PFOA</td>
<td>perfluorooctanoic acid</td>
</tr>
<tr>
<td>PFP</td>
<td>pentafluorophenol</td>
</tr>
<tr>
<td>PPCP</td>
<td>pharmaceutical and personal care product</td>
</tr>
<tr>
<td>ppm</td>
<td>units for chemical shift</td>
</tr>
<tr>
<td>RADE</td>
<td>relaxation recovery arising from diffusion editing</td>
</tr>
<tr>
<td>RF</td>
<td>radio frequency</td>
</tr>
<tr>
<td>RHSTD</td>
<td>reverse heteronuclear saturation transfer difference</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SOM</td>
<td>soil organic matter</td>
</tr>
<tr>
<td>STD</td>
<td>saturation transfer difference</td>
</tr>
<tr>
<td>SPR-W5-WATERGATE</td>
<td>shaped presaturation W5 water suppression by gradient tailored excitation</td>
</tr>
<tr>
<td>TEMPO</td>
<td>(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl</td>
</tr>
<tr>
<td>TOCSY</td>
<td>total correlation spectroscopy</td>
</tr>
<tr>
<td>TOTAPOL</td>
<td>1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol</td>
</tr>
<tr>
<td>TWINS</td>
<td>tailored water suppression for inhomogeneous natural samples</td>
</tr>
<tr>
<td>µL</td>
<td>microlitre</td>
</tr>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
</tbody>
</table>
µs  microsecond
UV-VIS  ultraviolet-visible
W  Watt
WATERGATE  water suppression by gradient tailored excitation
Preface

This thesis is a collection of research performed by the author of this thesis with the important contribution of co-authors. Chapter 2 is published in a peer reviewed journal, while Chapters 3-5 are either currently under review or are in the process of submission. This work was designed collaboratively between Hussain Masoom and André J. Simpson. Data was collected and manuscripts were written by Hussain Masoom under the guidance of and with critical comments from André J. Simpson. Contributions from additional authors are listed below.

Rapid Estimation of NMR Experiment Time in Low Concentration Environmental Samples


Author Contributions

Experiments were designed by Hussain Masoom and André J. Simpson. Data collection and analysis was performed by Hussain Masoom with guidance from Denis Courtier-Murias and Hashim Farooq. The manuscript was written by Hussain Masoom with critical comments from Denis Courtier-Murias, Hashim Farooq, Ronald Soong, Myrna. J. Simpson, and André J. Simpson. Ronald Soong, Werner Maas, Rajeev Kumar, Martine Monette, and Henry J. Stronks provided technical assistance with the instrumentation.
Tailored Water suppression for Inhomogeneous Natural Samples (TWINS): NMR Analysis in the Native State


Author Contributions

Sample collection and preparation was performed by Hussain Masoom and André J. Simpson. Experiments were designed by Hussain Masoom and André J. Simpson. Data collection and analysis was performed by Hussain Masoom with guidance from André J. Simpson. The manuscript was written by Hussain Masoom with critical comments from André J. Simpson.

Soil Organic Matter: Unravelling the Most Complex Biomaterial on Earth


Author Contributions

Sample collection and preparation was performed by Chao Zhang and Brian Kelleher. Experiments were designed by Hussain Masoom, Denis Courtier-Murias, and André J. Simpson. Data collection and analysis was performed by Hussain Masoom with guidance from André J. Simpson. The manuscript was written by Hussain Masoom with critical comments from Denis Courtier-Murias, Hashim Farooq, Brian Kelleher, Rajeev Kumar, Myrna J. Simpson, and André J. Simpson. Ronald Soong, Werner Maas, Michael Fey, Howard Hutchins, Sridevi Krishnamurthi, Rajeev Kumar, Martine Monette, and Henry J. Stronks provided technical assistance with the instrumentation.
From Spill to Sequestration: The Molecular Journey of Contamination via Comprehensive Multiphase NMR


Author Contributions

Experiments were designed by Hussain Masoom, Denis Courtier-Murias, and André J. Simpson. Data collection and analysis was performed by Hussain Masoom with guidance from Denis Courtier-Murias and André J. Simpson. The manuscript was written by Hussain Masoom with critical comments from Myrna J. Simpson and André J. Simpson. Ronald Soong, Werner Maas, Michael Fey, Howard Hutchins, Sridevi Krishnamurthi, Rajeev Kumar, Martine Monette, and Henry J. Stronks provided technical assistance with the instrumentation.
Chapter 1

Introduction

Planet Earth has a thin fragile skin composed of bodies of water, sediment and minerals, and an outer semi-organic component that has only recently, according to celestial timescales, been terraformed into what we know today to be soil [1]. Soil contains the most complex organic material on the planet containing living and non-living components [2, 3]. It is integral in nutrient regulation for primary producers and is therefore a necessity for humans and all life on Earth. Soil is a sink and source of carbon, actively participating in the carbon cycle. In a continuously warming planet, whether the balance will shift to being more of a source or sink is debated in literature thereby potentially implicating soil in global warming [4, 5, 6, 2, 7]. Agricultural turnover relies on soil quality and health but unsustainable practices, erosion, and desertification continually threaten our global food production capacity [2, 8]. With agricultural, industrial, and household processes comes the release of toxic contaminants, which interact with soil in unique ways further convoluting environmental fate and ecotoxicity [9, 10]. On top of this, it acts as a habitat, a nutrient source, a carbon sink and mediator, a regulating body for water flow, a filter, a platform for infrastructure, and a source for minerals and resources [11, 2]. It is clear that soil is an essential asset and governs many important global processes, but these same processes are not fully understood and cannot be predicted accurately due to the chemical and structural complexity that soil exhibits.

Analytical limitations have prevented the molecular level analysis required to comprehend the interactions, dynamics, reactivity, and complexity of soil. Traditional optical spectroscopic techniques only inform about the abundance and presence of functionalities and many are ca-
pable of studying soluble fractions only [12, 13, 14, 15]. Other methods like mass spectrometry (MS) can provide details on composition [16, 17, 18]. Nuclear magnetic resonance (NMR) spectroscopy has been termed the most powerful analytical tool for environmental research because of its non-destructive nature and its ability to study structure and interactions at a molecular level [19, 20, 21].

This dissertation explores the advancement of NMR technology to address the limitations of current research in soil structure and chemistry by increasing environmental relevance. It will cover two central topics. The second and third chapters will focus on experimental advancements in NMR spectroscopy to develop new NMR technology that allows native state analysis. The second part of this thesis puts these and other spectroscopic methods to use in determining soil composition and structure as well as characterizing interactions with contaminants. The main goal of this work is to provide an analytical framework to breakdown the complexity that exists around soil research while retaining as much environmental relevance possible. This introductory chapter will first delve into the complexities of soil chemistry, which will allude to research gaps that exist because of analytical barriers. It will then discuss the analytical tools that are used in this dissertation which can fill those gaps in soil science.

1.1 An Introduction to Soil

While there are many fields of research within soil chemistry, the two fields of research that will be discussed in this dissertation are structural, chemical, and compositional characterization while the second adds a temporal variable and focuses on kinetics, dynamics, and reactivity of soil components. This section will focus on the first.

1.1.1 The Composition of Soil

Soil has been called the most complex biomaterial on Earth because of the vast complexity that it possesses [3]. There are some components of soil that are well characterized, some that are not well known, and others that are fiercely debated over. What all soil scientists can agree on is that soil contains inorganic and organic fractions with the latter being broken down into living and non-living material.
Inorganic Components

Inorganic matter in soil plays a foundational role by providing structural integrity and is composed of minerals from the parent bedrock [11, 22]. Minerals are often the most abundant component in soil. The mineral component is important as it provides mass, helps to retain water, and provides micronutrients to plants as they weather [11, 22]. The classification of inorganic components can be separated into primary and secondary minerals and differ based on the conditions under which they are formed. Primary minerals are those that form under conditions different from the surface of the Earth and most resemble the bedrock [11, 22]. These are largely composed of aluminosilicates such as quartz, mica, and feldspar. Secondary minerals have been formed by processes that occur at the surface of the soil such as weathering and oxidation and include kaolinite, montmorillonite, and illite, iron oxides like goethite and hematite, carbonates like calcite and dolomite, and sulfates like gypsum [11, 22]. Secondary minerals can be negatively charged, however in more weathered species, minerals can be positively charged at low pH [11, 22].

Organic Components

Although the organic fraction constitutes on average less than 15% of a soil’s mass, it is often considered the largest source of dynamic organic carbon on the planet weighing 1550 gigatons which is 3.3 times the size of the atmospheric pool and 4.5 times the size of the biotic carbon pool [2]. The composition of soil organic matter (SOM) can be divided into living and non-living, the later having been a subject of fierce debate for over 35 years.

Living Components The living components of SOM are plants and animals. At a macro scale, rodents, worms and bugs, and plant roots are all a part of the soil system. Earthworms ingest soil particles and organic residues resulting in an enhancement of the availability of plant nutrients in the material that passes through their bodies [11]. Burrowing species like ants, termites, earthworms, and larger organisms help with soil aeration and mixing. They transport soil materials from one horizon to another [11]. All of these processes help to increase water penetrability and diffusion of oxygen which ultimately assists in soil fertility.

At the microscale, the diverse microbial communities in soil are abundant and contain a greater variety of species than exists above the soil [11]. Therefore microbiota contribute to the
genetic variation that exists on Earth and humans have utilized that diversity for their gain in the production of goods such as beer, yogurt, and antibiotics [11]. Some research suggests that microbial populations may define soil structure by creating numerous levels of fine structure and organization for soil constituents [3]. Undeniably, a significant purpose of microbiota including fungi and bacteria is to help to degrade primary inputs like leaf and animal detritus in soil into the complex organic structures that give soil many of its important characteristics [11, 3, 23]. Without microbiota, soil fertility and health greatly decreases, an effect that illustrates their importance.

Non-Living Components  Soil scientists agree that the non-living component of SOM is biologically derived from plant and animal detritus. The chemical structure of these components after undergoing degradation and transformation is what has been under dispute. Early definitions of humic substances have been operational and relied on solubility under alkaline or acidic solutions [24]. The fraction that was soluble in alkaline solutions but not acid was termed humic acid, the fraction soluble in acid and alkaline solutions was termed fulvic acid, while the insoluble portion was termed humin [24].

There have historically been two discrete models of SOM structure, the polymeric model and the aggregate model. The polymer theory of SOM centers around new covalent bonds formed in the polymerization of bio-molecules in soil to create a whole new class of compounds called humic substances that exist in a continuum of chemical properties and molecular weight up to 1,000,000Da [24, 25] A proposed model of what humic substances as defined in the polymeric model is illustrated in Figure 1.1. Others have contributed to this theory but there has been no direct evidence describing the polymerization process to validate this model [26, 27].

In 1993, Wershaw described another theory called the aggregate model of SOM, which stated that SOM consisted of ordered aggregates where there was no polymerization through covalent bonds. Therefore, the molecules involved in aggregation consist of relatively unaltered plant polymer biomolecules such as lignin, carbohydrates, proteins, and lipids that have been oxidized [28, 29]. This model for the composition of SOM has become more widely accepted as a larger body of evidence emerged using advanced instrumentation techniques, such as NMR spectroscopy, to qualitatively identify macromolecules in SOM [29].
1.1.2 Soil Structure

While many soil minerals have repeating crystalline structure, the great amount of heterogeneity and complexity that exists in SOM prevents it from having a repeatable, crystalline structure. Therefore, the term soil structure throughout this dissertation is a loose definition of the organization of components, organic and inorganic, of soil. How these components are arranged and in what way they interact with one another are the questions that will be explored in this section.

Traditional Structural Models

Initial models of SOM in recent history were as described in Section 1.1.1, divided into two main classes, polymer and aggregate. The polymer model hypothesized that the weathered organic material formed covalent associations with one another creating a new polymer called humic substances [25]. This view was promoted by an ultracentrifugation technique which was often cited as direct evidence of the existence of humic substances [31]. A series of operational definitions came out of that publication stating that the humic acid molecules had a mass weighted average of 20,000 - 50,000Da, radii of 4-10nm, and random coil conformations [31]. Conformation dependence on pH was hypothesized and illustrated in Figure 1.2. Other spectroscopic,
microscopic, pyrolysis, and soft ionization studies were inconsistent with the polymer model [32, 33]. The model that is now more widely accepted is the aggregate model, which hypothesizes that the biomolecular inputs to SOM come together in supramolecular associations with one another held together by hydrogen bonds and hydrophobic interactions [32, 33, 34]. This model focuses largely on molecular interactions rather than chemical composition. The next two sections briefly explores this research.

Figure 1.2: A depiction of what was thought to be the conformational properties of humic and fulvic acid under different electrolyte and pH conditions under the polymer model. Repurposed with permission from reference [35].

**Organo-Mineral Complexes**

Clay surfaces have been known to have intimate relationships with SOM and have also been implicated in SOM recalcitrance [36, 37]. What is of greater importance is the characterization of species that interact with minerals as this yields insights into the overall structure of SOM. A 2007 review by Kleber et al. puts together several pieces of information from various studies which hypothesized a zonal model of organic matter binding to mineral surfaces [38]. In this model, several zones of organic matter exist on top of the mineral surface each with differing functionality and composition. The inorganic surface of the mineral binds to organic matter via van der Waals forces, ligand exchange, cation exchange, and cation bridging. This primary surface is called the contact zone. The aliphatic portion of these molecules then interact with the
aliphatic portion of other organic compounds, which again expose hydrophilic groups outwards away from the mineral surface. This zone is called the zone of hydrophilic interactions. Beyond this zone is the kinetic zone that is in fast exchange with the surrounding environment of the mineral molecule. It has been hypothesized that whole proteins can bind to the mineral and encompass the contact zone and zone of hydrophilic interactions as one unit [38].

Another body of research delves further into studying which specific chemical components of soil interact with minerals, critical to understand soil organic matter preservation mechanisms. Some research suggests that lignin interacts with clay fractions to the point where chemical oxidation of lignin is protected [39, 40, 41]. However, the organic matter content and composition of the soil fractions tested did factor into lignin protection highlighting the need to understand the interactions between organic components in soil as well as organo-mineral interactions.

Other research also suggests strong microbial interactions with minerals [42, 43]. Microbial-mineral interactions help in discerning the function and processes of microbially mediated activity including carbon and nitrogen turnover, degradation of pollutants, and soil-borne pathogenicity. Microbial-mineral interactions have been hypothesized to occur via a process known as active pre-conditioning where extracellular polysaccharides and proteins are excreted as adhesives [44, 45]. Overall, soil microorganisms and soil particle interactions have been described as bidirectional and numerous in quantity, but a qualitative understanding of interactions is scant. It is also important to note that when understanding mineral interactions, most studies use systems containing soil extracts or model compounds. Studying organo-mineral interactions in situ is more challenging, but is required to understand the environmental context of laboratory observations.

**Organic Matter-Water Interface**

Water plays a vital role in soil structure and reactivity acting as a medium for microbial activity, mineral and contaminant transport, and organic matter folding [11, 27, 38]. Soil components gain the ability to travel, fold, and arrange themselves using soil pore water.

Initial studies with SOM and water observed surfactant properties and the formation of micelles with soil extracts. These soil molecules would have hydrophobic interiors composed of subunits like lignin and aliphatics and hydrophilic exteriors composed of amphiphilic structures like carbohydrates and fatty acids [46, 47, 48]. Because amphiphilic molecules are required in the
creation of micelles, it was hypothesized that the majority of organic material must be composed of amphiphilic structures.

More recent research helps to illustrate the arrangement of specific biomolecular groups with respect to the water interface. These studies have illustrated that carbohydrates and fatty acids are available at the water-soil interface while other hydrophobic species were not visible [49, 50]. Aromatic groups like lignin and hydrophobic aliphatics like cutin and suberin have been speculated to be unavailable from the water surface, but became available upon the addition of a non-polar penetrating solvent like dimethyl sulfoxide (DMSO) [49, 51]. Protein also became prominent with the addition of DMSO [49, 51]. The origin of this protein, whether it was present in the soil from degraded material, or it was a part of whole microbial cells that were lysed has not been confirmed [49, 51].

1.2 Xenobiotics in Soils

Earlier it was stated that there are two main fields of basic soil research. Adding the variable of external xenobiotics, or foreign substances like contaminants, yields another more complex field of research that examines the interactions, kinetics, dynamics, and degradation of chemical contaminants in soil.

1.2.1 Sources of Contamination

There are many sources of soil contamination which have grown in number as technological advancement occurs. Direct sources of contaminants are those that are applied to the environment directly. Exposure to chemicals like dichlorodiphenyltrichloroethane (DDT), an insecticide used to kill mosquitoes to control malaria, poses more of a threat because of the exposure to humans and the surrounding environment. DDT was found to cause cancer in humans and was disruptive to avian ecosystems and was eventually banned [52, 53, 54, 55, 56]. Its banning in the United States of America has been correlated to the comeback of the American bald eagle and peregrine falcon [57, 58, 59]. DDT has also been found to sorb to soils and sediments and are found to have a diverse impact on soil with much of the literature finding that sorption and sequestration have been identified as key process for recalcitrance and warrant further study [53, 60, 61, 62]. Agrochemicals are another direct source of contamination and include a variety
Chapter 1. Introduction

of insecticides, herbicides, fungicides, nematocides, hormones, synthetic fertilizers, and other growth agents.

Indirect sources of contamination include the effluents of by-products from industrial and manufacturing processes, and household anthropogenic sources. Industrial processes like manufacturing and mining are known to cause concentrated contamination zones from their effluent locations [63, 64, 65, 66, 67, 68]. An example of an industrial contaminant are brominated flame retardants (BFRs) of which some have been labelled as persistent organic pollutants (POPs). The penta- and octa-congeners of the polybrominated diphenyl ether class of BFRs have been banned in countries across Europe and North America [69, 70, 71]. Point source contamination from factories are an issue for concentrated contamination within soil, while their widespread use in electronics, home furnishings, and plastics leads to widespread and continual release into soil.

The mining industry often uses many organic solvents and solutions containing harmful salts to isolate resources which results in concentrated soil contamination around the mining site. Oil contamination has been a major issue for soil resulting in long term irreversible infertility while mineral mining leaves unnaturally high levels of heavy metals and sulphuric acid in soil [63, 64, 65, 72].

Finally, human waste is a major concern for soil contamination. Pharmaceuticals and personal care products (PPCPs) often end up at waste water treatment plants where water is reclaimed and sometimes used in the agricultural industry. Compounds such as prescription medication, illicit drugs, DEET, triclosan and other antibiotics have been reported to leach into sources of drinking water [73, 74, 75, 76, 77, 78]. Many PPCPs and pharmaceuticals make it through the treatment process and those contaminants end up in the environment [79, 80, 81, 82, 83, 84]. Current research is focusing on the impact of PPCPs and pharmaceuticals in the soil at an ecotoxicology level. Soil studies have shown negative impacts on the germination and growth of crops, and there is concern over anti-bacterial resistance over long period of low level exposure [85, 86, 87, 88, 89].

1.2.2 Sorption Models

Sorption models have been used to describe contaminant interactions in soil starting in the early 20th century where the sorption process was thought to be simple and uniform. The molecular
theory was not introduced until the early 1980s. In this dissertation, three models will be discussed: soil as a partitioning medium, the rubbery glassy transition, and the molecular level approach to soil sorption.

**Soil as a Partitioning Medium**

Traditionally contaminant sorption to soil was seen as a partitioning property similar to that in chromatography where soil takes the role of the stationary phase and the dissolved contaminant takes the role of the mobile phase. This model assumes that the interaction between contaminant and soil is reversible and that there are only two phases that the contaminant can exist in, bound and free. This distribution coefficient that was developed was termed $K_d$, which is determined by equilibrium concentrations of contaminant in water and soil [90, 91]. A relationship between the fraction of organic carbon ($f_{oc}$) and $K_d$ was found to provide a more useful equilibrium coefficient that was normalized to the amount of organic matter in a soil. This assumes that the contaminant is only interacting with the organic fraction in the soil and therefore has limitations in explaining soil as a more complex system of components. This normalized coefficient is termed $K_{oc}$ and defined as

$$K_{oc} = \frac{K_d}{f_{oc}} \quad (1.1)$$

$K_{oc}$ was and still is predominantly used to describe contaminant interactions with soil organic matter. If one assumes that bimodal hydrophobic distribution is the only process governing the interaction with soil and contaminant, then the resulting isotherm would return a linear correlation. Under short time scales, this seemed to be the case and held true [90]. In some cases, the linearity of contaminant sorption began to falter as sorption experiments were performed for 24 hours or more which altered the perceived time required to perform an equilibrium experiment throughout soil sorption research [92, 93, 94]. The isotherms achieved were non-linear in nature and therefore the traditional two phase partitioning process was not the only mechanism governing contaminant sorption [93, 95, 96].

After the linear sorption isotherm, two other models that took non-linearity into account called Langmuir and Freundlich isotherms were employed [22, 98]. The Langmuir model assumes that a finite amount of sorption sites are available for sorption and that each sorption site requires
the same amount of energy to sorb. The result is an isotherm with a linear increase and a sudden shift to a flat equilibrium concentration once all sites have been occupied [22, 98]. The Freundlich isotherm does not have a mechanistic derivation behind it and is empirical in nature. Like the Langmuir isotherm, the Freundlich also accounts for non-linearity and fits many sorption curves better than the linear or Langmuir isotherms, but mechanisms that are consequent from these isotherms are speculative [22, 98]. The types of sites available was speculated to be derived from four main interaction as is illustrated in Figure 1.3. Newer mathematical models have been derived and are out of the scope of this thesis and are better described elsewhere [22, 98].
Rubber-Glassy Transition Model

Upon the realization that sorption was non-linear over longer equilibration times, newer models were required to explain interaction phenomenon that occurred in soil. Competitive sorption effects were observed as well [99] and when considered in conjunction with non-linearity, it was thought that site specific interactions were taking place rather than general partitioning which occurs in a “rubbery” sorbent [100]. This lead to the belief that if multiple different sorption sites existed in a system, then the resultant isotherm would be more representative of the curves that were found for soil. Experiments using model “rubbery” and “glassy” systems were used create sorption isotherms which indeed resulted in non-linear isotherms similar to what was achieved in soil [101]. Therefore, the “rubbery-glassy” model helped to illustrate that soil contained site specific interactions rather than general partitioning and was a step forward in understanding contaminant sorption in soil over long periods of time.

Soil Ageing

When field data on contaminant availability was collected over years, there seemed to be a hockey-stick (as illustrated in Figure 1.4) like trend where contaminant availability would sharply decrease linearly, followed by a specific turning point where the slope would gradually plateau [102]. This process is termed soil ageing where contaminants become less available over long periods of time [102].

This observation helped to illustrate the need for understanding soil sorption processes in more detail. Questions pertaining to the ageing process arose including the need to understand specific binding domains. The qualitative aspect of the kinetic mechanism was needed to answer how a contaminant becomes less bioavailable over long periods of time. These questions required a more detailed approach to understanding sorption processes.

Molecular-Level Approach to Soil Sorption

Progress was made to include some complexity of soil in describing sorptive phenomenon from the last two sections. Initially, soil was seen as a homogeneous partitioning medium, which later evolved into a bi-modal system where two partitioning systems existed with distinct properties. Continuing with this trend, compositional complexity of soil can be taken into account by
attributing characteristics of the rubber glassy model to properties of soil components. For example, the rubber phase where short term partitioning can occur has been described as loosely ordered organic material while the glassy phase is more rigid and tightly packed [103]. It has been reported that the loosely ordered domains are rich in aliphatic molecules while the tightly packed component is rich in aromatic moieties [104, 105]. There has been a great amount of debate in the literature centering on the relative importance of aliphatic versus aromatic molecules in soil sorption [104, 106, 107, 108, 109, 110]. Consensus has not been met over this debate [110, 111], but it is known that both play some role in soil sorption and are likely responsible for contaminant sorption in soil [110, 112]. While reductionist approaches are useful in identifying contaminant-structure relationships, an understanding of how contaminants behave in a whole soil is required to validate the ideas found through soil reduction studies.

1.3 Gaps and Limitations in Soil Research

As the previous sections have been introduced, a clear pattern emerges illustrating gaps and limitations in soil research. There are two main gaps, one of which builds upon the other. The first is our limited understanding of soil structure. Soils are very complex media and previous
studies have simplified them to reduce complexity and improve lucidity of results obtained. The structural and chemical complexity of soil is responsible for its sorptive characteristics, reactivity, porosity, and fertility. Therefore, simplifying soil to better understand it can only take soil research so far, as the greater the degree of extraction, isolation and fractionation the less environmentally relevant the material becomes.

This leads to the second limitation. It has been a necessity to simplify soil because of fundamental limitations of most analytical approaches. Direct molecular level observations have been difficult as few instruments can provide such detailed information on whole intact samples. When instruments such as Fourier transform infrared (FT-IR) and ultraviolet-visible (UV-VIS) spectroscopy that are capable of providing such information are used, the results are often convoluted because of the soil complexity. These are the two limitations that will be discussed in this subsection, and are the core issues this dissertation tries to resolve.

Therefore, the research questions that arise from the literature review discussed above include developing instrumentation and experimentation to study complex environmental matrices in their natural state. Before this, is the necessity for technological advancement allowing for instrumentation to study complex environmental samples.

1.3.1 Soil Structure

It has been stated numerous times that “A major impediment to progress is the structural complexity of soil that presents major challenges in understanding its role as a habitat” [2]. Soil structure has been a monumental challenge to overcome. It is the most complicated mixture of molecular components in the world and how these components interact with one another, the specific molecular level interactions that exist between them (electrostatic forces, hydrogen bonding, van der Waal’s forces, dipole moments, etc.) are what govern the most important characteristics of soil including its reactivity, its porosity, its sorption capacity, and its fertility.

The body of evidence that was presented in Section 1.1.2 leaves many unanswered questions. We now know that traditional humic acid based structural models are not representative, and therefore surfactant and folding models are limited. For soil to be described as a surfactant, it would be necessary to determine a specific concentration that micelles are formed which has not been performed to this date. In addition, soil extracts were employed to describe these phenomenon and do not take into account minerals or microbes which are known to make up
large parts of the soil mass \cite{11, 113}. Figure 1.5 illustrates this issue clearly showing that direct measurements of soil has led to the understanding that “humic material” does not exist.

![Figure 1.5: A summary of the historical process and outcome of soil structure research (A) compared to the more recent techniques and understanding of soil structure (B). Soil is no more than a summation of the biological inputs that supply the organic matter and other ecosystem factors like climate and bedrock. Taken with permission from reference \cite{5}.](image)

Once minerals are considered, there is a broad range of evidence that shows multiple soil components interact with minerals and distilling this body of research into any kind of soil structure is difficult because of competing theories. Some data suggests that lignin and aliphatic components have strong sorption to mineral surfaces while other data suggests that microbes and proteins have strong mineral interactions. Which soil component forms a stronger interaction with minerals has found to be highly dependent on a variety of factors including pH, mineral composition, and soil component composition \cite{38, 114, 115}.

The reality is that there is no well defined soil structure. Therefore it is challenging to draw a soil particle and define how each component interacts with confidence because of the shear complexity of the components in soil as well as compounding environmental factors. Newer research has illustrated that soil structure is a function of the ecosystem that it supports \cite{5}. However, a broad understanding of which components are more bioavailable, or are at the water interface compared to other components may be possible since many soils largely share the types of biomolecular inputs. Direct molecular level elucidation of the general arrangement
of components with regards to their relative availability will help in modelling contaminant sorption and understanding key soil characteristics. This is the topic of the fourth chapter in this dissertation.

1.3.2 Instrumentation to Study Soil and Soil Interactions

The instrumentation that was traditionally available to study soil has lacked capabilities to resolve the molecular complexity. Early studies employed ultra centrifugation to shed light on molecular weight while elemental analysis allows for atomic content of nuclei including carbon and nitrogen [16, 116]. Both of these techniques measure bulk properties of soil rather than identifying discreet structures.

Early spectroscopic techniques included UV-VIS, fluorescence, and FT-IR spectroscopy. While these techniques are able to characterize specific functional groups within soil, the information gained was also of the bulk sample and the functionalities found could not be attributed to specific soil components without chemical extraction techniques [117, 118, 119]. MS was also applied to soil research and analyzes soil as one even mass rendering it unable to characterize the various components in soil. MS is a destructive technique and early studies required soluble samples once again which removed key structures in the soil [16, 17, 120, 121]. Overall, early spectroscopy and MS studies lacked the ability to study intact soil.

Scanning electron microscopy (SEM) is another tool that has been used to analyze soil and humic substances. SEM is able to study the fine micro-structure on the surface of solid materials. Information like pore size and even surface composition have been found using this tool [122, 123, 124, 125, 126, 127]. However, as was the norm with soil science, fractionation was often performed to make analysis easier to comprehend, or only surface properties of soil were able to be studied.

$^{13}$C cross polarization magic angle spinning (CP-MAS) NMR spectroscopy is a non-destructive and non-invasive analytical approach that has the ability to study whole samples with the spectral dispersion to separate general soil biomolecules including (alkyl, O-alkyl, aromatics, and carboxyl) [128]. This approach has been and still is very useful in determining the molecular composition and relative abundance of each component as a whole [16, 129, 130, 131, 132]. This was an advancement in soil science as many researchers were able to gain insights about specific soil components rather than bulk properties. Despite being an improvement compared to other
techniques in terms of chemical and compositional information, soil was still dried and analyzed and specific structural characteristics were hard to extract. In a way, the soil was still being studied as one whole mass while CP-MAS was able to separate bulk information into more isolated fractions without significant pretreatment. NMR is capable of analyzing structure and interactions, however, dipole interactions and anisotropy, themselves a source of information, lead to spectral broadening limiting the range of molecular information that can be extracted from the solid-state.

In 1996, a new form of NMR spectroscopy was found that allowed for the study of materials in their natural swollen state termed high resolution magic angle spinning (HR-MAS) NMR spectroscopy [133]. This was first applied to soil in 2001 where the soil water interface was characterized and contaminant interactions with specific components (isolated spectroscopically rather than chemically or physically) were studied on a whole soil swollen in D$_2$O [49]. This was likely the first time that whole soil in its swollen state was studied using an instrument with the capabilities to study structure, dynamics, interactions, and chemical characterization. While this instrumentation has been the most holistic and environmentally relevant technique used to study soil, the solid phase cannot be analyzed as HR-MAS probes cannot handle the power required to generate the intense RF fields required for high power decoupling and cross-polarization, elements essential in solid-state NMR. As such, HR-MAS is unable to detect structures, interactions, and dynamics that happen in the solid state, which has been hypothesized to be the final sorption domain for contaminants after sequestration.

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Figure 1.6: A comparison of the capabilities of multiple analytical approaches used to study soil. No current technique has the capabilities of analyzing all phases in soil in its whole natural state.
Figure 1.6 summarizes the capabilities of the mentioned analytical tools and their capabilities towards studying the structure and composition of soil in its natural state. While HR-MAS comes close, no analytical instrumentation has the ability to study all phases of soil at once. Significant advancement has been made towards studying soil in its natural state and studying it in a holistic manner where structure, dynamics, interactions, and kinetics could all be studied, but important details about soil are still being left out including solid phase analysis. Therefore, there is still the need for the ability to study all soil components in their whole natural state. The next section explores NMR further and discusses new technological advancements that allow for the analysis of soil in its whole swollen state which is used throughout this dissertation.

1.4 Nuclear Magnetic Resonance

NMR provides an unprecedented level of information for samples of all phases including crystalline solids, semi-solid or gel like materials, soluble compounds, and even gasses. Environmental complex matrices like soils contain a multitude of physical states, and given the requirement to study complex environmental media in their entirety, NMR is an invaluable tool to any soil researcher.

NMR is also a non-destructive and non-invasive approach. As was presented in Section 1.3.2, the need for analytical instrumentation that forgoes the requirement of sample simplification or pretreatment is highly valuable. Avoiding chemical or physical extractions retains the important chemical and physical bonds that give soil its characteristics. NMR is one of the few instruments that allows for whole sample analysis and is therefore necessary in soil chemistry research. However, limitations of NMR include the difficulties in studying samples with an abundance of paramagnetic nuclei and its insensitivity.

The amount of information that can be gained from NMR spectroscopy is extremely comprehensive including chemical characterization, molecular interactions and self-aggregation, dynamics and diffusion, and relative molecular motion. This unprecedented level of information aids in separating complex matrices using spectroscopic approaches rather than physical or chemical means. Therefore, components in soil can be studied in their natural environments, but the experimental resolution and versatility exists to be able to pick out any one of these components and better understand how it behaves and moves, what it interacts with, and other components
that it is close to. Based on these reasons, it is clear that NMR spectroscopy is very well suited to study soil in its whole natural state. However, there are some drawbacks which will be further explained, and then addressed later on in this chapter.

General NMR theory will first be presented to gain a basic understanding of its underlying principles after which traditional NMR approaches will be discussed. Then, limitations with current instrumentation will be described and addressed. Finally, relevant experiments and their importance to soil research will be reviewed.

1.4.1 Basic NMR Theory

This dissertation is concerned with the application of NMR spectroscopy to soil chemistry and thus will not go into a detailed understanding of NMR theory. For a detailed and accessible understanding, textbooks by Keeler [134] and Levitt [135] are recommended.

NMR is based on the fundamental quantum mechanical property of spin, or angular momentum, which each subatomic particle contains. This is an intrinsic property like mass or charge, but differs in that it is not scalable and does not translate into a physical property at a large scale. All nuclei have a spin value calculated by the number of protons and neutrons in their nuclei. An even number of protons and neutrons results in pairs for each spin leading to a net spin quantum number \( I \) of 0. An unpaired spin will result in a spin value of \( \frac{1}{2} \). In simple terms, when introduced into a strong magnetic field, spin active nuclei align either with or against the field following the Boltzmann distribution. Aligning with the external field is the lower energy state while aligning against the field is a higher energy state. Nuclei with spin states greater than \( \frac{1}{2} \) are termed quadrupolar nuclei and exhibit more than two spin states depending on the configuration. Examples of spin \( \frac{1}{2} \) nuclei that are relevant to environmental NMR include \( {}^1\text{H}, {}^{13}\text{C}, {}^{15}\text{N}, {}^{19}\text{F}, {}^{31}\text{P}, {}^{29}\text{Si}, {}^{111}\text{Cd}, {}^{113}\text{Cd}, {}^{199}\text{Hg}, {}^{207}\text{Pb} \).

Each nucleus has \( 2I + 1 \) possible magnetic quantum numbers \( (m) \) and range in values of \( +I \) to \( -I \) in integer steps. Therefore, a nucleus with \( I = \frac{1}{2} \) has 2 possible \( m \) values, \( +\frac{1}{2} \) and \( -\frac{1}{2} \) known as the \( \alpha \) and \( \beta \) states respectively. Because these energy states are quantized, each nucleus in the system must exist in either of these states. The vector quantity magnetic moment of these nuclei is calculated with the following equation

\[
\hat{\mu} = \gamma \hat{I}
\]
Where $\mu$ is the magnetic moment of a nuclei’s angular momentum and $\gamma$ is the proportionality constant termed the gyromagnetic ratio and is different for each isotopic nucleus. $I$ can be characterized by

$$\hat{I} = m\hbar$$

(1.3)

Where $\hbar$ is the simplified Plank’s constant ($h/2\pi$). Therefore

$$\vec{\mu} = \gamma m\hbar$$

(1.4)

This equation states that the angular momentum for a nucleus is proportional to the product of Plank’s constant, the magnetic moment of that nucleus, and the gyromagnetic ratio of the specific nucleus. Once this nucleus is placed in an external magnetic field ($B_o$) along the $z$-axis, $\mu_z$ interacts with the magnetic field removing degeneracy from $I$ and $m$ producing a Zeeman splitting. The energy of the resulting nucleus is therefore

$$E = \mu_z B_o$$

(1.5)

Which can be expanded to

$$E = \gamma m\hbar B_o$$

(1.6)

Therefore, when $m = +\frac{1}{2}$, the nucleus is aligned with the magnetic field in the lower energy state where the opposite is true for nuclei with $m = -\frac{1}{2}$. The difference in energy between these two states is given by

$$\Delta E = \gamma \hbar B_o$$

(1.7)

This is the energy that we detect with the NMR. However, this difference in energy can only be detected if there is a difference in the number of atoms aligned with and against the magnetic field. This separation populates itself according to the Boltzmann distribution

$$\frac{n_\alpha}{n_\beta} = e^{\frac{\Delta E}{kT}}$$

(1.8)
Where $n_{\alpha}$ is the number of nuclei in the lower energy state, $n_{\beta}$ is the number of nuclei in the higher energy state, $k$ is the Boltzmann constant, and $T$ is the temperature of the system in Kelvin. A difference in populated states allows for the presence of a small net magnetic moment in the direction of the higher populated quantum state. In NMR, this bulk magnetization is pointed towards the lower energy $\alpha$ state and has specific properties, notably a frequency ($\omega$), related to its gyromagnetic ratio and the external magnetic field:

$$\omega = \gamma B_o$$  

which results in

$$\Delta E = -\hbar \omega$$

Here, $\omega$ refers to the frequency of precession around the external magnetic field. This frequency is related to specific properties of the nucleus $\gamma$ and the strength of the external magnetic field. $\omega$ is the Larmour frequency and is the basis for resonance in nuclear magnetic resonance. The scale of this frequency is normally in the MHz range when discussing high field systems.

### 1.4.2 Detection in NMR

The principles of detection in NMR spectroscopy can be explained using quantum mechanics or a more tangible representation of the nuclei using arrows in a three dimensional coordinate plane, something called the vector model. The quantum mechanics behind NMR spectroscopy is very complex and thorough, and is outside the scope of this dissertation. For simplicity and approachability, the vector model will be used here to describe the processes that occur during experimentation.

NMR detection relies on the manipulation of the bulk magnetization through radio frequency (RF) pulses. Figure 1.7A illustrates the bulk magnetization precessing at the Larmour frequency along the $+z$-axis which aligns with the external magnetic field. The red arrows indicate the nuclei in the system as they are precessing at the Larmour frequency. It is important to note the difference in population of nuclei aligned with the field compared to against the field representing the Boltzmann distribution between $\alpha$ and $\beta$ states. In this case, the $x$-axis is the direction in which a detection, or receiver, coil is placed. This receiver coil often acts as a RF coil to
manipulate the spin system as well as receive the resultant energy and is placed just around the sample vessel. After a specific and known quantity of RF energy (as is illustrated with the blue arrow in Figure 1.7A), which can also be viewed as an applied magnetic field ($B_1$), is directed to the sample in the $+y$ direction, following the right hand rule, the magnetization aligns in the $+x$ direction (Figure 1.7B). The RF coil is now exposed to the precessing nuclei and can collect a time domain plot. This plot can then be Fourier transformed to convert the time domain into the frequency domain where individual signals are viewed at specific frequencies. After some time, the bulk magnetization relaxes and returns in alignment with the external magnetic field. How and why nuclei at specific positions on a molecule differ in frequency will be discussed in the next section.

![Figure 1.7: The general principles behind NMR detection are illustrated here. A) The nuclei in the applied magnetic field ($B_0$) align with or against the field according to the Boltzmann distribution and precess at frequency $\omega$. If an applied RF energy is delivered in the $+y$ direction, this acts as a $B_1$ field and will flip the signals along the $+x$-axis as is dictated by the right hand rule. B) With the detection coil able to observe along the $x$-axis, the signals are now observable.](image)

1.4.3 Chemical Shift

As we saw in section 1.4.2, Equation (1.9) illustrates the relationship of precessional frequency with magnetic field strength. Figure 1.8 illustrates a molecule of ethanol which has two equivalent chemical positions for $^1$H nuclei. All the protons in the CH$_3$ group share the same chemical environment with each other just as all the protons on the adjacent CH$_2$ group share the same chemical environment. These two environments differ in the electron density that is shared be-
between them because of their relative proximity to the electron withdrawing oxygen at the end of the alcohol. Hence, the CH₂ has less electron density compared to the CH₃ group. When simple concepts from Faraday’s law which states a moving charged particle generates a magnetic field are applied, it is then simple to understand that a presence of electrons causes an induced magnetic field \( B_i \) for each chemical environment resulting in an effective magnetic field strength \( B_{eff} \) as described by

\[
B_{eff} = B_0 - B_i
\] (1.11)

Since oxygen is electron withdrawing, relatively less electron density will exist around the CH₂ position compared to the CH₃ position. The CH₃ position will therefore have a higher \( B_i \), a lower \( B_{eff} \), and is more shielded while the CH₂ position has a lower \( B_i \), a higher \( B_{eff} \), and is deshielded. The proton that is at the OH group experiences the most amount of deshielding and is therefore further down-field on the NMR spectrum. It is through this process that molecules with similar chemical functionalities will resonate at similar frequencies, a concept which is used throughout this dissertation when interpreting complex NMR spectra. Another important side note to Figure 1.8 is the relative intensity of the signals. Each signal height will be proportional to the number of nuclei it represents. Thus, the CH₂ signal has twice the area than the OH signal and the CH₃ signal has three times the area. Chemical shift is simply a normalization to a specific reference frequency and follows this equation

\[
\delta_{ppm} = \frac{\nu_{sample} - \nu_{reference}}{\nu_{reference}}
\] (1.12)

Where \( \delta_{ppm} \) is the chemical shift, \( \nu_{sample} \) is the precessional frequency of the sample, and \( \nu_{reference} \) is the frequency of the reference. Since high field NMR is often performed in the MHz range, the common unit for chemical shift is ppm (parts per million). Consider a situation where \( \nu_{sample} \) is 1000Hz larger than the reference frequency of 500MHz

\[
\delta_{ppm} = \frac{1000\text{Hz}}{500 \times 10^6\text{Hz}} = 2 \times 10^{-6} = 2\text{ppm}
\] (1.13)

In this case, ppm is not representative of a concentration of a solution, but rather a denomination used to understand the scale of the chemical shift differences.
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Figure 1.8: This figure illustrates how chemical shift difference arise within the same nuclei. The more electron density an atom has, the more shielded it becomes and it occurs up-field. This is dependant on the proximity of electronegative groups. Note distances between resonances are not representative of the actual $^1$H NMR spectrum of ethanol.

1.4.4 Relaxation

After an RF pulse has been applied to a body of nuclei, those nuclei are perturbed from their equilibrium state along the direction of the magnetic field (z-axis). These nuclei being in a higher energy state will eventually relax to equilibrium over a period of time. Throughout this dissertation, a fundamental concept that is continuously exploited through experimentation is relaxation in NMR spectroscopy. NMR relaxation can be broken into two categories: spin-lattice relaxation, and spin-spin relaxation. Both are important to understand to fully grasp future chapters. Relaxation is an incredibly complex phenomenon and whole textbooks have been devoted to the topic [136, 137]. A general understanding of each of these processes will suffice for the rest of this dissertation.
Spin-Lattice Relaxation \((T_1)\)

Going back to the introduction of this section, once a body of nuclei are in the excited state, they need to release their energy to return to their equilibrium state. This is accomplished through coupling with the thermal energy of the system which is better known as the lattice in NMR terms. The lattice energy is tightly correlated with the motion of the molecules in that system. Since energy is quantized, the spin energy of the atom can only be relaxed through the lattice if the molecule is exhibiting molecular motion close to the frequency of the spin. Larger molecules, or molecules that are more rigid will have rotational motion that is of similar frequency to the spin energy and thus relax faster than a smaller molecule in the solution state. This process is called spin-lattice relaxation or longitudinal relaxation and is defined by the time constant \(T_1\). This type of relaxation governs the speed at which a scientist can obtain an experiment since in an ideal situation, all nuclei would return to the equilibrium state before another pulse is fired to allow for maximum sensitivity.

Another way of looking at spin-lattice relaxation is the amount of time it takes for nuclei to return to the \(z\)-axis after being excited to the \(x\)-\(y\) plane. This is portrayed in Figure 1.9B. Longitudinal relaxation becomes important in NMR of soil and other environmental matrices because different molecules will have different longitudinal relaxation rates. Because soil is so complex, it is important to measure spin-lattice relaxation to pick an appropriate delay between scans, known as the recycle delay, to ensure that all components in soil fully relax. This prevents unintentional signal losses from particular moieties in the sample.

Spin-Spin Relaxation \((T_2)\)

Another form of relaxation exists in NMR spectroscopy that defines a key property in environmental NMR is spin-spin, or transverse relaxation. This relaxation process comes about through the interaction of similar nuclei. Relaxation with nuclei that contact each other or are in close enough proximity to transfer energy to one another are able to relax through this mechanism. Again, because magnetization is quantized, only nuclei that have similar spin energy levels will be able to relax through one another. Like spin-lattice relaxation, larger and more rigid molecules have a faster spin-spin relaxation time, denoted by \(T_2\).
Figure 1.9: In following with the vector model, the individual spins as was displayed in Figure 1.7 can be represented as one arrow as the average of all spins in the system (A). $T_1$ relaxation can be visualized as the realignment of the bulk magnetization back to the $z$-axis from the $x-y$ plane (B). $T_2$ relaxation can be visualized as the decoherence of magnetization in the $x-y$ plane which averages itself out resulting in zero signal (C).
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Transverse relaxation can be visualized on a 3D NMR axis like longitudinal relaxation in Figure 1.9. As longitudinal relaxation can be thought of the net magnetization relaxing from the $x$-$y$ plane back to the $z$-axis, the transverse relaxation can be thought of as the decoherence of spins along the $x$-$y$ plane. When RF energy is applied to a spin system, they are all aligned at one point in the $x$-$y$ plane and are therefore coherent. Transverse relaxation allows these signals to fan out along the $x$-$y$ plane at different rates eventually becoming decoherent. This is illustrated in Figure 1.9C. Once these nuclei have become decoherent, the signal cannot be observed. Therefore, the amount of time available to collect signal from a sample is dependant on the $T_2$ relaxation time. The longer a signal is able to be collected, the more certainty exists about its frequency and therefore the signal is more resolved. If a signal has a short $T_2$ relaxation time, there is less certainty around the frequency of that signal which broadens the signal and reduces spectral resolution.

This property is important in soil research because soils are generally solid masses which means they have short $T_2$ relaxation times. Unfortunately, this also means that NMR spectra from soil are often characterized by broad spectral features leading to a huge amount of spectral overlap and a reduction in discernible spectral information. That being said, a distribution of relaxation rates can be advantageous in isolating components based on size or mobility.

1.4.5 Dipolar Coupling

Dipolar coupling is a complicated topic, and while being the basis of through space experiments in solution state NMR, it is integral in practically all solid-state NMR techniques. Here the basic principle is outlined to gain a broad understanding of its use and purpose in soil studies using NMR spectroscopy.

NMR active nuclei in a spin system have magnetic dipole moments because of their intrinsic spin. A good analogy often used to explain this phenomenon is interpreting nuclei as magnets. Because nuclei are charged, and they have some amount of spin energy, they exhibit magnetic dipoles and behave as magnets. When two magnets are brought near each other, they interact with one another. This interaction is called a magnetic dipole moment, and the two spins are said to be coupled. In a solution where molecules are tumbling in all directions, tend to average due to molecular tumbling.
In a rigid system, two nuclei may exist close to one another being a part of one larger rigid molecule. In this case, the magnetic dipole moments are strong, long-lived, and can be observed. To understand the benefits of dipolar couplings, the Hamiltonian for dipolar couplings becomes useful

\[
H_D = -\frac{\mu_0}{16\pi^3} \frac{\gamma_1 \gamma_2 h^2}{r^3} (3 \cos^2 \theta - 1) I_{1z} I_{2z}
\]

(1.14)

Where \(\gamma_1\) and \(\gamma_2\) are the gyromagnetic ratios for each nucleus, \(r\) is the distance between the two spins, \(\theta\) is the angle between the internuclear vector and the applied field, and \(I_{1z}\) and \(I_{2z}\) are the spin quantum numbers for each of the spins along the z-axis in the external field.

The important information to take from this equation is that the dipolar coupling strength is dependent on internuclear distance. Therefore, experiments that exploit dipolar couplings can be used to understand relative distances between molecules of a contaminant and binding site, which becomes important in characterizing contaminant sorption.

1.4.6 Traditional NMR Approaches

In the most traditional sense, NMR spectroscopy has been broken down into two main fields of study, solid state NMR and solution state NMR. HR-MAS was introduced in 1996 and focuses on gel-like components with the technology containing some elements of both solution and solid state NMR [133]. Each of these approaches have been used in soil science and their usage will be further discussed in this section.

Solid State NMR

Solid state NMR spectroscopy has attributes of traditional NMR where chemical shift differences can lead to characterization, but it also has special properties that are unique to the solid phase. As described in the previous section, dipolar couplings are common in crystalline solids. These couplings are dependent on nuclear distance, and thus physical structure of a crystal lattice can be calculated. Therefore, while chemical shift can produce information about different functionalities that exist and their abundance, dipolar coupling measurements can determine 3D spatial structures of crystalline compounds [138, 139, 140].
Solid state NMR is also sensitive to movement. There is a body of research that measures molecular motion and dynamics based on solid state NMR analysis. Without going into great detail, the solid state spectrum when static is reliant on the strengths of the dipolar couplings that exist. As a molecule changes or interacts over time, say as a solid is melting into a liquid, or a molecule is interacting with a substrate, the solid state spectrum will evolve and temporal studies are then possible to follow, for example, dynamics or kinetics [141, 142].

For soil NMR research, chemical shift has been the means by which characterization and relative quantification has occurred. As was stated in previous sections, there are limitations with this approach in that it is a bulk analysis rather than a true understanding of which components are crystalline, and which components are less rigid in a hydrated soil. Recent research has utilized solid state techniques to characterize contaminant interactions in soil [143].

Most if not all solid state NMR approaches require specific NMR probe capabilities to be able to perturb strong dipolar couplings. High power capabilities in the range of hundreds of Watts is necessary which can only be accomplished using special capacitors and RF coil designs.

**Solution State NMR**

Solution state NMR spectroscopy requires less power and has some benefits over solid state NMR. While chemical shift characterization can still be performed, solution state NMR offers better spectral resolution because of the long $T_2$ relaxation times that exist. To account for magnetic field drift, which is intrinsic to most NMR magnets, a solvent signal that the spectrometer can lock on is needed. Once the spectrometer locks onto this solvent signal, the spectrometer has the ability to take into account any magnetic field drift thus reducing the line broadening that would otherwise occur. Therefore, probe lock channels are required. On top of this, many solution state experiments rely on the use of magnetic field gradients. A gradient pulse is a pulse from a separate coil other than the RF coil that applies a magnetic field gradient along the sample. This can be used to intentionally distort signals as is done in water suppression techniques that use gradients or manipulate important signals by encoding their position in space as in diffusion based experiments.

Solution state NMR spectroscopy is mostly used for structure identification because of the relatively high resolution it offers as well as the enormous range of experiments offered in multiple dimensions to resolve overlapping signals [144]. In soil research, solution state NMR spectroscopy
has played a role in the characterization of soil extracts and natural organic matter [145, 146, 147, 148], in the analysis of soil extracts with contaminants [149, 150], and separation of components using diffusion based studies [30, 51, 151, 152, 153]. Overall, while studies like this provide a general understanding of soil extracts and their behaviour, their environmental relevance is lacking since soil is generally a gel-like mass, not a dissolved liquid. Extraction and separation techniques unfortunately omit large categories of compounds and associations that give soil its intrinsic properties. However solution state NMR provides such clear spectral resolution especially with the use of multi-dimensional NMR [144]. This was resolved with the introduction of HR-MAS NMR.

**High Resolution Magic Angle Spinning (HR-MAS)**

Semi-solid materials with some but limited mobility can be studied using solution state NMR methods, but the spectral resolution obtained is not as high as a freely diffuse molecule. This is because of residual dipolar couplings (dipolar couplings that are short lived) as well as bulk magnetic susceptibility [154]. To remove this broadness, magic angle spinning can be employed which helps remove dipolar couplings and other anisotropic effects. Referring back to Equation (1.14), the second term \((3 \cos^2 \theta - 1)I_1ZI_2Z\) can be brought to zero if the angle of the sample \((\theta)\) was approximately 54.7° to the external magnetic field. This nulls the entire dipolar coupling Hamiltonian, effectively removing dipolar interactions. Spinning the sample helps to average out anisotropic effects where the spinning speed is correlated to the degree that broadening factors are effective.

HR-MAS NMR has been applied to soil science sparsely but has significant potential. Soil can be studied in its natural swollen state with the sharp resolution that solution state has to offer. Characterization has been performed as well as interactions between contaminants and whole soil [49, 155]. Overall, HR-MAS NMR is highly underutilized for soil research [21].

The one drawback of HR-MAS NMR in soil research is its inability to study the solid state which is important because the solid phase is the final sequestration point for contaminants. The solid phase is also a major structural component in soil and is therefore imperative to study [19, 20].
Comprehensive Multiphase NMR (CMP-NMR)

Current NMR technology essentially forces the user to manipulate the sample in some way to meet the spectrometer’s requirements for analysis. For example, solution state NMR is performed most commonly in 5mm diameter glass tubes. These tubes must be filled with completely dissolved solution without any particulates or else one risks difficulties in shimming, which is the act of homogenizing the magnetic field within the instrument to the specific sample. Therefore, when a soil scientist has access to solution state NMR and needs to analyze their samples, extractions and filtrations must be performed to ensure complete dissolution.

In solid state NMR, samples must be completely dried and packed into zirconium rotors commonly 4mm in diameter. Samples that are not dry will obscure analysis and can sometimes cause spin failure during acquisition which can be detrimental to the probe and sample coil. For a soil scientist, this means extensive drying techniques on soil like freeze drying, and storing under vacuum and desiccant. With this technology comes the ability to study all soil components, and not being able to study it in its hydrated and swollen state. Therefore, compromises have to be made to the sample, to meet requirements for the instrumentation.

HR-MAS was one step closer in allowing whole swollen samples to be analyzed. Other than the fact that $D_2O$ is commonly used rather than $H_2O$, as a locking solvent, and to prevent the solvent signal from obscuring the entire spectrum. The issues with HR-MAS were outlined in the previous section and pertained to its lack of solid phase analytical capabilities.

Recently, new probe technology termed Comprehensive Multiphase NMR (CMP-NMR) was developed and built in the Bruker BioSpin US headquarters and tested in Dr. Andrée Simpson’s laboratory [156]. The author of this dissertation was heavily involved with the production, and testing of a variety of these probes [156]. This included an extended stay on two separate occasions at Bruker BioSpin headquarters and involved soldering and constructing the various electrical components to meet probe specifications. CMP-NMR probes incorporate a lock and susceptibility matching for ideal solution state line widths, a gradient coil and magic angle spinning for gel-phase analysis, and high power circuitry to generate intense $B_1$ fields.

This probe technology was used throughout the majority of this thesis. It solves the problem of being unable to study the solid phase in HR-MAS and permits the analysis of the solution and gel phases with high resolution as well. It is an all in one probe that has been tailored
to complex samples rather than altering the sample to fit the analytical tool. This is a necessity to better understand environmental matrices and their interactions in an environmentally relevant manner. In this dissertation, it was applied to soil research by studying soil structure and composition, as well as kinetics and interactions with foreign contaminants. However, the technology is widely applicable to any multiphase materials such as plants, soils, tissues and organisms.

1.5 Experiments Used in this Dissertation

There are a wide variety of CMP-NMR experiments used in this dissertation including many that have been developed specifically for use with complex matrices like soil. The following section provides a broad overview of many of the experiments used in this thesis.

Experiments using multiple different nuclei will be discussed. Different nuclei have their unique advantages and disadvantages in NMR spectroscopy. For example, $^1$H NMR can offer excellent sensitivity but has a narrow chemical shift range spanning 0-12ppm leading to overlapping signals. $^{13}$C has a wider 200ppm chemical shift range which helps to disperse signals but has reduced sensitivity mainly caused by a low natural abundance (1%). This creates the requirement of the use of indirect acquisition experiments which can be non-quantitative and will be discussed later on. $^{19}$F is also used in this dissertation because of two reasons. Organo-fluorine bonds are not naturally occurring in the environment and often exist because of contamination with a xenobiotic that has fluorine atoms. With this selectivity, interactions between the contaminant and the soil can be isolated through saturation transfer experiments and will be discussed later on. The second reason is that $^{19}$F is also a highly sensitive nucleus and has 100% isotope abundance.

This section will begin with $^1$H NMR and a discussion on water suppression and the spin echo, two experimental elements that are needed to be recognized for coming chapters. Then, experiments will be split into two categories: experiments used for molecular and structural characterization, and experiments used for interactions.
1.5.1 Water Suppression

The need for solvent suppression arises in solution state $^1$H NMR when solvents that are used have $^1$H nuclei. This need is even more pronounced in environmental samples because complete removal of water is often very difficult if not impossible. Normally, the solvent is the most abundant compound in the analyte leading to a solvent signal that is much more intense than any other signal. This can reduce the dynamic range of the instrument as the receiver gain will adjust to the most intense signal in the spectrum, thus reducing the detectability of weaker signals. Even with the use of deuterated solvents, exchangeable $^1$H from the sample may exchange and contribute to the solvent signal. As well, solution state samples are often created or extracted from other protonated solvents and completely removing those solvents is not trivial. This results in residual solvent that is observed once samples are analyzed.

Solvent suppression has evolved over the years to accommodate the need of a variety of sample situations [157]. Presaturation is a sequence that utilizes a long low power pulse on the solvent signal before the pulse sequence [158]. Some disadvantages of presaturation include its limited effectiveness on broad water signals and the loss of exchangeable groups. Improvements to this pulse sequence include the use of composite pulses [159], the use of lower flip angles to remove residual solvent humps [160], and phase modulated irradiation to improve phase properties of presaturation experiments [161].

Pulsed field gradients have opened up the possibilities of water suppression with techniques like WET [162], CHESS [163], SOGGY [164], WATERGATE [165], WEFT [166], and PURGE [167]. A pulse field gradient is used by first flipping the water signal using a selective pulse and then applying a gradient pulse to dephase the water signal helping to remove water from the detectable plane. A stronger and more complex water suppression sequence was developed to study dissolved organic matter at natural concentrations called SPR-W5-WATERGATE [145]. This sequence builds on the W5-WATERGATE sequence by adding a train of shaped water saturation pulses before the W5-WATERGATE sequence which helped to improve suppression and retain even baseline properties [145].
1.5.2 Spin Echo

Another concept worth mentioning that becomes important in a variety of experiments used in this dissertation is the spin echo. Figure 1.10A illustrates bulk magnetization that has been flipped to the $x$-$y$ plane on the observable $+x$-axis. The closer the sample is to the $+x$-axis, the more signal can be gained from it. As individual spins begin to evolve around the $x$-$y$ plane at differing rates based on a combination of their chemical shift and $T_2$ relaxation, they move farther away from the $+x$ axis and become less observable (Figure 1.10B). After some time $\delta$, a 180° refocusing pulse about the $x$-axis as illustrated in Figure 1.10C can be applied which manipulates the magnetization from the $+y$ direction to the $-y$ direction or vice versa. The magnetization then continues to evolve in the same direction it was traveling. After the same amount of time $\delta$ passes by, all the magnetization will have refocused onto the $+x$-axis once again (Figure 1.10D). This can be repeated over and over again to refocus signals multiple times for manipulation creating spin echoes.

1.5.3 Experiments Employed to Study Structures

This section will briefly review some of the main techniques used to study structure and composition of soil in this dissertation.

Diffusion Based Experiments

CMP-NMR allows for samples to be studied without the chemical or physical isolation of specific components and instead spectroscopically isolates groups of molecules based on chemical or physical properties. Diffusion experiments in NMR spectroscopy are one way of isolating components based on their diffusivities. Therefore, molecules can be isolated based on their size, shape, and interactions with other molecules. How this works is through gradient encoding and decoding sequences.

A pulsed field gradient echo (PFGEs) is a pulse used to manipulate spins in a given space or location in the sample vessel and is depicted in Figure 1.11. A PFGE will first flip the bulk magnetization along the observable $x$-$y$ plane and then dephase the spins with a first gradient pulse. This pulse is termed the encoding sequence and the degree to which the magnetization is perturbed along the $x$-$y$ plane depends on its position in the sample vessel. After half of a
Figure 1.10: (A) The bulk magnetization is brought to the observable $x$-$y$ plane from the $+z$ direction. (B) The individual spins begin to relax through spin-spin ($T_2$) relaxation for some time $\delta$. (C) A 180° pulse about the $x$-axis is applied which rotates the spins around the $x$-axis and the spins continue to relax in the same direction. (D) After the same amount of time $\delta$, the nuclei have aligned themselves back into their initial position on the $x$-$y$ plane. This process can be repeated creating echoes.

Preselected diffusion time ($\Delta$) passes by, the sample is able to evolve along the $x$-$y$ plane and a 180°$_x$ refocusing pulse is applied sending the evolution back in the opposite direction (see Section 1.5.2). After the complete diffusion period has passed by, a decoding gradient pulse is performed which is the same strength but opposite sign as the encoding pulse. Therefore, if the molecule remained in the same position, the decoding pulse would be adequate enough to refocus the magnetization back to the observable position. If the molecule has moved as is depicted in Figure 1.11B, the decoding gradient pulse would differ which would result in a partial refocusing. Therefore, the intensity of a molecule that diffuses quickly would decrease.
Since the magnetization is not completely aligned with the observable axis, signal is lost. On the other hand, those that do not diffuse during $\Delta$ will be aligned retaining signal intensity. The resulting spectrum is that of the large gel-like molecules and is called diffusion editing (DE) [156].

An equal experiment to this one can be conducted where the gradient power is turned off completely. This results in an identical experiment without selecting for slow diffusing molecules which produces a spectrum that contains both fast diffusing and slow diffusing molecules, a reference spectrum so to speak. The DE can be subtracted from this one to obtain only freely diffusing molecules in an experiment termed inverse diffusion editing (IDE). These experiments are discussed in further detail elsewhere [156].

**Relaxation Based Experiments**

Transverse relaxation can be used to a spectroscopist’s advantage through a couple of different methods. The spin echo (introduced in Section 1.5.2) is one such way that when applied consecutively can filter out large or fast relaxing species. When magnetization is brought to the $x$-$y$ plane and refocused, some signal from species that have fast $T_2$ relaxation times are lost if the delay ($\delta$) between echoes is sufficiently long enough. This occurs because species that are large have fast $T_2$ relaxation and become decoherent faster than small molecules or those that are dissolved. Therefore, if $\delta$ is long enough and an adequate number of echoes is applied, solution state molecules will continually be refocused while larger more rigid molecules will become decoherent and unobservable [168, 169, 170]. This loop of echoes applied before acquisition is called a $T_2$ filter and this element is often applied to many experiments that need to isolate solution like molecules.

Another example of relaxation based spectroscopy can isolate semi-rigid molecules through spectral subtraction. Section 1.5.3.1 described a diffusion editing experiment which intrinsically has many delays, including the diffusion delay. During these delays, semi-rigid molecules have the opportunity to relax mainly through transverse relaxation and become decoherent and unobservable. If these delays were minimized, the fast relaxing semi-rigid species will not have the chance to relax and are observed along with the slow and fast diffusing species. If the reference spectrum that was described in section 1.5.3.1 containing the slow and fast diffusing species was subtracted from a spectrum that contained everything (slow and fast diffusing as well as fast
Figure 1.11: (A) A depiction of the steps involved in diffusion editing when no diffusion occurs. The first step is an encoding gradient that manipulates the spins based on their position in the sample vessel. Then after half of a set diffusion time ($\Delta$), the spins have relaxed slightly. A spin echo brings them back to their original position after another $\frac{1}{2}\Delta$. If the nuclei did not diffuse and were in the same position they started in, the decoding gradient will align them back into the observable axis perfectly and there is no signal loss. In case the spins have moved in space (B), the decoding gradient pulse will be different at their new position resulting in magnetization that is not aligned with the observable axis. This causes signal attenuation of mobile species while retaining signal in non-diffusing components.
relaxing) then a spectrum of only the fast relaxing species would result and is representative of semi-rigid molecules. This experiment is termed relaxation recovery arising from diffusion editing (RADE) [156]. RADE is critically important as it allows the signals from fast relaxing components to be recovered that could be missed if diffusion editing approaches are used alone.

**Cross Polarization (CP)**

CP is a solid state experiment that uses dipole couplings to alter the Boltzmann distribution of an abundant spin system to a dilute spin system. Consider a situation where $^1\text{H}-^1\text{C}$ CP is desired. $^1\text{H}$ has a gyromagnetic ratio roughly four times that of $^{13}\text{C}$. This means that $^{13}\text{C}$ precesses at a slower rate (Equation (1.9)) and also has more frequent energy jumps between the $+1/2$ state and $-1/2$ state resulting in a lower Boltzmann distribution compared to $^1\text{H}$. The goal with CP is to get both nuclei to precess at the same frequency allowing for the spin states to mix and energy to be transferred between them. To do this, two separate RF pulses must be applied to each nucleus simultaneously to satisfy the Hartmann-Hahn condition

$$\gamma_{1\text{H}}B_{1\text{H}} = \gamma_{13\text{C}}B_{13\text{C}} \quad (1.15)$$

When the two spin states reach this condition, the Boltzmann distribution of the more abundant spin contributes more to the mixed spin state than the distribution of the less abundant spin. Therefore, considerable signal enhancements are seen in $^{13}\text{C}$ (roughly 4x the signal). This technique works best in systems where dipolar couplings are strong and rotational and translational motion is minimized. Because different functional groups have different forms of translational and rotational motion, CP efficiency is not identical throughout a molecule resulting in attenuation where there is more movement. Therefore, CP is not a quantitative technique. However, a benefit of using CP is that the longitudinal relaxation is reliant on the abundant spin system which allows for even greater sensitivity in the case of $^1\text{H}-^{13}\text{C}$ since $^1\text{H}$ relaxes faster than $^{13}\text{C}$.

In a soil, CP-MAS acts as an excellent experiment to characterize the types of carbons and their relative abundance (relative with respect to similar functionalities). The increased spectral dispersion of $^{13}\text{C}$ allows for more resolution. The sensitivity gains are also of great importance in soil research because $^{13}\text{C}$ spectra would take extremely long periods of time to acquire at natural abundance due to the low Boltzmann distribution of $^{13}\text{C}$ nuclei.
Heteronuclear Single Quantum Coherence (HSQC)

HSQC is a two dimensional experiment correlating two different nuclei. Originally HSQC was described for the use of $^1\text{H}$ and $^{15}\text{N}$ in 3D structure characterization [171], but it has also been used for detecting $^1\text{H}$ and $^{13}\text{C}$ correlations. This work will only be concerned with the $^1\text{H}$-$^{13}\text{C}$ HSQC although experimentally they are the same.

On a two dimensional plot where the horizontal axis is $^1\text{H}$ chemical shift and the vertical axis is $^{13}\text{C}$, an intersecting correlation on the plane indicates a proton that is bonded with a carbon atom. Effectively, each proton chemical environment can be matched with its corresponding carbon environment making structural and chemical elucidation much easier than with single dimension experiments.

How this experiment works is similar to the CP experiment in terms of magnetization transfer (see Section 1.5.3.3). Magnetization is delivered to the $^1\text{H}$ nucleus where it is then transferred to the $^{13}\text{C}$ nucleus using an insensitive nuclei enhanced by polarization transfer (INEPT) step. This polarization is very similar to the CP polarization transfer except $J$-coupling is used rather than dipolar coupling. After this polarization transfer, time ($\delta$) is allowed to pass where a frequency label is applied to the magnetization centered according to the offset of $^{13}\text{C}$. This magnetization is then transferred back to the $^1\text{H}$ signals through a reverse INEPT step and acquired. The experiment requires numerous slices where $\delta$ is incremented to allow for greater spectral resolution.

HSQC experiments are used predominantly for chemical composition determination because of their excellent ability to disperse all NMR signals. An HSQC signal of a particular kind of molecule is normally very unique to that subset of molecules. Therefore, HSQC correlations can be seen as fingerprints of specific molecular groups. For example, complex carbohydrates will all have similar resonances in the same regions save a few to account for their small differences. The same is true for aliphatic compounds, lignin, protein, etc. This procedure is used later on in chapter 4 to determine the broad chemical composition of a whole soil.

1.5.4 Experiments Employed to Study Interactions

The later part of this dissertation deals with interactions of xenobiotics to soil. The experiments used there are described in the following section and revolve around the spatial transfer of
magnetization from the contaminant to the soil and vice versa.

**Cross Polarization (CP)**

The point of CP is to transfer polarization from one nucleus to another and when the nuclei are chosen strategically, it can elucidate information specific to the interaction that is occurring in the system. It is important to keep in mind that CP relies on strong dipolar couplings to be efficient and thus only interactions happening in the solid phase are mapped with this approach. Therefore, if a contaminant contains a unique heteronucleus not present in the soil (for example $^{19}F$), and magnetization was transferred from the $^1H$ spin system of the soil to the contaminant, this would be a method to positively identify the strong binding of the contaminant to the soil. In fact, the degree of interaction can be mapped at various locations within the contaminant describing the binding mechanism specifically in the solid state. Another way to do this is through the use of CP build up curves which are described in Chapter 5. If the reverse is performed and magnetization is passed from the contaminant ($^{19}F$) to the soil ($^1H$). The components in the soil (protein, carbohydrates, lignin etc, responsible for binding in the solid-state can be directly identified. Therefore, CP is a versatile tool that also has the ability to study contaminant interactions that occur in the solid state.

**Saturation Transfer Difference (STD)**

STD is a solution and gel state experiment that probes interactions between a ligand and its receptor. Originally used in protein studies for ligand binding, it was adopted for the use in soil science by Shirzadi et al [172, 173, 174]. Saturation is a steady spin state where the length of the pulse is many times the duration of $T_1$. Therefore, a long drawn out low power pulse can produce this phenomenon. In essence it is the destruction of the Boltzmann distribution where an equal population of high energy and low energy spins exist. No signal can be gained from a spin population that has been equated. Recalling Section 1.5.1, saturation is also used in pre-saturation, a common water suppression technique.

Once saturation is accomplished, relaxation of the saturated pulses occurs through dipolar interactions which is a through space coupling rather than through bond like $J$-coupling. As such, any nuclei that are close to the saturated nucleus will receive saturation and will also...
Figure 1.12: This figure is a depiction of how STD works. A) is an illustration of a receptor molecule with a fictional ligand. For the purposes of explaining the concept, this molecule has fluorine atoms (purple), carbon atoms (grey), and an oxygen atom (red). B) shows the numbering of the fluorine atoms which are of importance because saturation is being transferred from $^1$H to $^{19}$F this case. C) shows two spectra, a saturation spectrum and a reference spectrum. The fluorine atom closest to the point of irradiation is attenuated the most through saturation processes while atoms that are farthest away are not affected. The reference spectrum does not saturate any signals so the intensities are unchanged. D) is an illustration of the result after subtraction and is the final result of STD experiments. Because of the subtraction, the position that is closest to the point of irradiation in the substrate is the tallest signal. This is how STD sheds insight on ligand binding orientation.
become attenuated. The degree of attenuation is correlated to the strength of the interaction, or the proximity in the case of dipolar couplings.

Figure 1.12 illustrates the concept of STD experiments using a fictitious compound with fluorine atoms as indicated with purple spheres. STD saturates the ligand receptor with a frequency selective pulse as is shown in Figure 1.12A. The ligand nuclei that are close to the receptor site are attenuated and the signal is acquired. As seen in Figure 1.12C, signal is attenuated closest to the position of interaction while less attenuation occurs farther away. After this, a spectrum where the saturation is far away from the observable frequency range is acquired as a reference spectrum and the saturation spectrum is subtracted from the reference, satisfying the “difference” aspect of the experiment. What is left is a spectrum where the tallest signals are the nuclei that are interacting the most with the receptor, while the signals that have decreased in intensity interact the least (Figure 1.12D). Ligand binding orientation can be extracted from this experiment illustrating that the fluorine atoms in position 1 are interacting the most while fluorine atoms in position 6 are interacting the least. When this experiment is performed on two nuclei that are different, it is termed heteronuclear saturation transfer difference (HSTD).

Reverse heteronuclear saturation transfer difference (RHSTD) also exists where the ligand is saturated, and the receptor (in this thesis, soil) is attenuated. Where HSTD allows for the determination of ligand binding orientation, RHSTD is the reverse in that the receptor involved in ligand binding is identified in the solution/gel phase. This experiment is essentially the same as HSTD except the nuclei are reversed.

STD experiments are useful in soil research because they help to shed light on the details of contaminant interactions such as which functional groups interact with the soil most, and which components in soil are involved in contaminant sorption.

1.6 Thesis Objectives and Summary

The introduction of CMP-NMR technology has many important applications for environmental NMR and resolves the issue with traditional NMR techniques with regard to sample pretreatment. This dissertation aims to develop experimentation and analytical procedures to fulfill its potential and to accentuate its benefits of whole sample analysis. Therefore, this work has
two central foci, development of CMP-NMR and its application in soil chemistry. The first two chapters are devoted to developing techniques to study soil and other environmental matrices and are useful tools for other applications as well. The last two chapters are the application of CMP-NMR technology and experimental techniques to first characterize the composition and structure of soil in its whole, natural, unaltered state, and then investigate contaminant interactions and kinetics.

1.6.1 Improving NMR Detection Using CPMG-SS

Chapter 2 begins with introducing a technique to improve the detection capabilities of solid state NMR. The title of the chapter is “Rapid Estimation of NMR Experiment Time in Low Concentration Environmental Samples” and introduces a new technique called the Carr-Purcell Meiboom-Gill Single Spike (CPMG-SS) experiment. A common NMR nucleus used in solid state analysis is $^{13}\text{C}$ which is not only insensitive but also has low natural abundance. As well, other sample properties may impede analysis such as the presence of paramagnetics which speed up spin-spin relaxation and broadens lines even further, or a low carbon content which adds to the insensitivity of the experiment. Therefore, estimating experimental run times to obtain an adequate spectrum can be difficult. CPMG-SS is added to CP which forgoes chemical shift differences and focuses all signals into one intense spikelet. Correlations between the intensity of this spikelet and the number of scans needed to obtain spectra of a predetermined $S/N$ are formed which was then used to estimate experimental run time. This experiment was applied to other nuclei as well and has applications as a detection technique where bulk signal intensity is required quickly, as in the case of kinetics experiments. Therefore, this experiment is also used in Chapter 5.

1.6.2 Water Suppression for Environmental Samples

Chapter 3 describes an important improvement in water suppression under MAS which is critical to obtain $^1\text{H}$ NMR of fully swollen soils without pretreatment. The chapter is titled “Tailored Water suppression for Inhomogeneous Natural Samples (TWINS): NMR Analysis in the Native State”. Soil in its natural state is swollen with water existing in a plethora of physical states including free water, water in exchange, and bound water. This produces a large and broad water
signal that often obscures the entire $^1$H chemical shift range. Chapter 3 address this problem with in situ soil analysis by creating a water suppression technique that completely eliminates the water signal in broad water samples. Experimental improvements were made to the current best performer of water suppression, SPR-W5-WATERGATE, including an exclusive 180° inversion pulse before the suppression pulse to remove far water, the addition of rotor synchronization, and improvements to phase cycling resulting in the TWINS sequence. This experiment was extensively tested in soil and a wide array of environmental media and was found to have increased water suppression capabilities as well as baseline effects without sacrificing spectral quality. Samples were collected from the environment and used as is in the sample vessel. An external D$_2$O lock capillary was created for the specific purposes of providing a solvent for the spectrometer to lock on without allowing it to interact with the sample further retaining environmental relevance. TWINS is an important addition to the arsenal of experiments required to study environmental samples in their natural, unaltered state.

1.6.3 Elucidating Soil Structure and Composition Using CMP-NMR

The next chapter delves into studying soil structure and composition using a full suite of CMP-NMR spectroscopy experiments. The title of chapter 4 is “Soil Organic Matter: Unravelling the Most Complex Biomaterial on Earth”. The title helps to point to the pivotal nature of this research in grasping the complexities of soil and distilling them into characteristics that are defined by spectroscopic means. 2D HSQC is used to characterize soil into biomolecular components and then CP is used to confirm those findings for the total soil carbon. As well, CP is used to identify components that are at the soil-water interface which include aliphatics and carbohydrates. Next, relaxation and diffusion experiments help to identify the relative mobility of different species which paints a picture of the structure of soil. Finally, different solvent conditions are assessed to determine what kind of interactions are preventing water from reaching into the more rigid parts of soil which are the aromatic and protein structures. This research provides direct observed evidence of the structure and composition of whole intact soil preserving the environmental relevance. The aim is for this work to help reveal the mysteries around soil and hopefully impact the direction of soil research on a broad scale.
1.6.4 Monitoring Dynamics and Interactions of Contaminants with Soil using CMP-NMR

The final research chapter is Chapter 5 entitled “From Spill to Sequestration: the Molecular Journey of Contamination Using Comprehensive Multiphase NMR”. In this chapter, CMP-NMR technology is highlighted once again this time for the use of kinetics, dynamics, and interactions of contaminants with soil. The two contaminants at question here are pentafluorophenol (PFP), and perfluorooctanoic acid (PFOA). Perfluorinated contaminants are pervasive throughout the environment being found in numerous environmental and biological matrices [175]. First the kinetics are mapped using the CPMG-SS technique in the solution, gel, and solid phases. The relative quantity of contaminant in each of these phases is also calculated and it is found that the transition between the solution and solid phase happens quickly since the gel phase is low in abundance. This was thought to be due to the physical surface area constraints of the gel phase which is defined as the transitional space at the soil-water interface. Then interactions are mapped using HSTD and CP and a two phase mode of action is found for PFP where interaction orientation differs in the gel phase from the solid phase, while PFOA interacts in a specific manner in both phases. Finally, sorption domains in soil were found using RHSTD and CP techniques and it was confirmed that PFP interacts with a variety of soil moieties with emphasis on aromatics while PFOA sorbs strongly to aliphatics which were hypothesized to be from protein. Overall, this is the first time a contaminant was mapped as it transfers between phases in a whole soil and represents another significant step in the direction of whole soil research with increased environmental relevance.

1.7 Future Work

The last chapter is a discussion on the future of CMP-NMR and its role in soil and environmental chemistry. This dissertation only presents one application of CMP-NMR, and its possibilities in other fields of research in environmental chemistry and beyond is considered in the final chapter.
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Chapter 1. Introduction


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Chapter 1. Introduction


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Chapter 2

Rapid Estimation of NMR Experiment Time in Low Concentration Environmental Samples

This material is adopted from:

2.1 Abstract

Nuclear magnetic resonance (NMR) spectroscopy is an essential tool for studying environmental samples but is often hindered by low sensitivity especially for the direct detection of nuclei such as $^{13}$C. In very heterogeneous samples with NMR nuclei at low abundance such as soils, sediments, and air particulates, it can take days to acquire a conventional $^{13}$C spectrum. This study describes a pre-screening method that permits the rapid prediction of experimental run time in natural samples. The approach focuses the NMR chemical shift dispersion into a single spike and even in samples with extremely low carbon content, the spike can be observed in two to three minutes or less. The intensity of the spike is directly proportional to the total concentration of nuclei of interest in the sample. Consequently, the spike intensity can be used as a powerful pre-screening method that answers two key questions: 1) Will this sample produce a conventional NMR spectrum? 2) How much instrument time is required to record a spectrum with specific signal to noise ($S/N$) ratio? The approach identifies samples to avoid (or pre-treat) and permits additional NMR experiments to be performed on samples producing high quality NMR data. Applications in solid and solution state $^{13}$C NMR are demonstrated and it is shown that the technique is applicable to a range of nuclei.
Chapter 2. Rapid Estimation of NMR Experiment Time

2.2 Introduction

Nuclear magnetic resonance (NMR) spectroscopy is arguably the most powerful tool for the study of organic structures and has been central in understanding a broad range of environmental systems including carbon cycling in the arctic [1, 2, 3], the effects of global warming on soil [4], unraveling the structure and interaction of humic substances [5, 6, 7, 8, 9], and elucidating organic components in air particles [10, 11]. In particular, cross polarization (CP) experiments have been used extensively because of the qualitative structural information that is gained and because of its non-destructive nature [12]. CP is now routinely used in environmental studies on a variety of samples including natural organic matter, wood, sediments, and black carbon [13, 14, 15, 16]. In most cases, $^{13}$C is the nucleus of interest which yields an overall profile of the organic composition of the sample, however studies involving CP of other nuclei such as $^{15}$N and $^{31}$P also have wide environmental application [17, 18].

Most environmental studies have to deal with rather limited amounts of sample. In some cases, obtaining enough sample in the first place is the limiting factor, for example, with studies that isolate dilute dissolved organics from water, air particulates from the atmosphere, or projects dealing with samples from remote locations (i.e. deep sediment cores, the Arctic or Antarctic), where logistically it is difficult to return with large sample volumes. In other cases samples become precious and limited due to extensive pretreatment or fractionation that may be required prior to NMR analysis [19]. Consider for example that a sediment low in organic carbon may require treatment by hydrofluoric acid many times to dissolve minerals, reduce paramagnetics and concentrate the organic material [20]. Such treatments can take months and when the organic carbon content is low, can consume a large amount of the original sample to produce the 100 mg required for common solid-state NMR analysis. Carbon combustion analysis may be performed to determine sufficient carbon content in a sample and helps determine if $^{13}$C NMR analysis is feasible. However, in many cases there is simply not enough material to permit accurate carbon analysis by combustion, which requires approximately five grams per measurement (for samples fewer than 10% organic carbon) and should be performed in triplicate which would require 15g total [21]. As such it is often the case that an environmental researcher desperately needs high quality NMR data and has spent months (possibly years) collecting and preparing the samples but cannot accurately gauge the feasibility of, or time involved in, ac-
quiring the NMR data. Additionally, most NMR centers have many users and the analysis of low carbon organic samples (especially by solids NMR) can routinely take one to four days per sample making the spectrometer demands for large projects considerable. In cases where there is no carbon data available, a lot of NMR time can be wasted on running a precious sample that produces no signal.

It would therefore be a considerable step forward for the field if it were possible to pre-screen natural samples in a nominal time frame (two to three minutes) and determine: 1) if a sample will produce an acceptable NMR spectrum and 2) how much time is required to generate a desired signal to noise ratio (S/N). This would identify samples to avoid and permit additional NMR experiments to be performed on samples producing high quality NMR data. Consequently, this approach greatly increases the amount of NMR information that can be obtained from natural samples in a set amount of time. Such an approach is introduced in this study. As the conventional NMR signal for a typical environmental sample is very weak (could take many days to observe), the prediction method is not based on the conventional NMR signal, but instead is based on a “spike-let” that represents the summation of all the nuclei in the sample in one extremely narrow and easily observed spike. The approach is based on manipulation of the Carr-Purcell Meiboom-Gill (CPMG) pulse sequence.

The CPMG sequence (Figure 2.1A and B) is commonly used in environmental studies for \( T_2 \) relaxation measurements to quantify contaminant binding in environmental media and in water suppression techniques as a relaxation filter [22, 23, 24]. It uses a series of spin echoes separated by a delay period (\( \tau \)). If this delay is shortened to the point where the echoes are applied at a faster rate than the chemical shift can evolve, the result is the chemical shift envelope is reduced to a single spike within the recorded bandwidth at the transmitter frequency [25]. In addition, to concentrating the signal into a single spike, the spin lock also perturbs \( T_2 \) relaxation, and removes the effect of inhomogeneous broadening which can be significant in complex heterogeneous samples [26, 27, 28]. As such, signal persists for much longer permitting increased sampling which leads to increased \( S/N \) (see Figure 2.1C and D as an example on hemoglobin). The signal intensity of the CMPG spike is proportional to the total carbon signal from the sample. Thus, it can be used not only to indicate the existence of observable nuclei, but to predict the time required to attain a particular \( S/N \) in a conventional NMR spectrum. The use of CPMG is well documented in many NMR applications and there are various papers
that describe the pulse sequence in greater detail [28, 29, 30, 31, 32].

Figure 2.1: A) An illustration of the solid state CPMG pulse sequence with cross polarization. An 18\(\mu\)s echo delay period (\(\tau\)) between each 180° pulse is used to avoid chemical shift evolution and is repeated 280 times (\(n\)). High power composite pulse decoupling (Spinal-64) is used during acquisition. B) The solution state CPMG experiment consists of a 90° on the X nucleus with Waltz 16 proton decoupling during acquisition. An 18\(\mu\)s echo delay period (\(\tau\)) between each 90° pulse is used to avoid chemical shift evolution and is repeated 2000 times (\(n\)). 180° pulses were not used in the solution state experiments as only a quarter of the power (essential on a solution state probe) is required for the corresponding 90° pulse and produced essentially the same result as a 180° pulse at fast repetition rate. See material and methods and for further details. The 90° flip angle could also be used for solid-experiment if required. C) FID from a standard CP experiment for hemoglobin. The FID is fully relaxed in approximately 4ms. D) FID from the CPMG-SS experiment for the same sample. The FID extends well beyond 80ms. Recording a solid state CPMG-SS spectrum for an acquisition this long is pushing the limits of the probe and was only produced once to demonstrate the concept. Readers should not try to reproduce this and are strongly encouraged to read the materials and methods for procedures on safe CPMG-SS optimization. If optimized as outlined in the materials and methods section the CPMG based solids experiment will actually use much less power than a conventional CP experiment and if applied appropriately can be carried out without stress to the spectrometer.

The single spike technique has been previously demonstrated by Morris and colleagues where it is explained as a method to improve sensitivity by reducing and controlling chemical shift scaling [33]. Lim et al. uses the single spike technique to enhance multiple quantum NMR experiments [25]. In this article, the use of the CPMG single spike (CPMG-SS) spectroscopy to predict if a sample will yield an adequate spectrum is demonstrated and how the intensity
of the CPMG spike correlates with the time required to obtain a full $^{13}$C NMR spectrum of a particular $S/N$ is evaluated. To illustrate the approach, the present study will analyze several environmental samples in solid and solution state NMR, and its use in $^{13}$C and $^{31}$P NMR is demonstrated.

2.3 Materials and Methods

2.3.1 Sample Preparation

Crystalline hemoglobin from bovine blood was purchased from Sigma Aldrich and was used as is. Concentrations were varied by mixing with powered sodium chloride purchased from Fisher Scientific. An earthworm was selected from a healthy population of E. fetida maintained within our laboratory since 2006 as described in Brown et al. [34]. The original progenitor earthworms were purchased from The Worm Factory. The worm was flash frozen with liquid nitrogen, freeze dried, and stored at -20°C until required. Each solid sample was homogenized using a Wigi-L-Bug with a steel ball and pestle for approximately five minutes, and a full rotor was packed each time. Suwannee River dissolved organic matter (DOM) purchased from the International Humic Substances Society was dissolved in 600µL of D$_2$O (99.9%) and set to a pH greater than 12 using sodium deuterioxide (NaOD 99.5% D, 30% in D$_2$O) for solution state studies. D$_2$O and NaOD were purchased from Cambridge Isotope Laboratories.

2.3.2 NMR Spectroscopy

NMR experiments were performed on a Bruker Avance III 500MHz Spectrometer. Cross polarization (CP) and CPMG-SS experiments on hemoglobin and worm were performed on a 4mm broad-banded solid state probe. CP experiments were performed at a spinning speed 13kHz and a ramp of 80%-100% with a CP contact time of 2ms. 3072 points were used in the time domain with an acquisition time of 40ms and high power composite pulse decoupling (Spinal 64). Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 30Hz in the final spectrum.

CPMG-SS parameters were identical to the CP parameters with exceptions that the sample was static and that the decoupling power was attenuated by $1/2$ of its original value (see Section
While it is possible to run the CPMG-SS with magic angle spinning (MAS), rotor synchronization is required to ensure proper echo formation [35]. It was found that a static sample produced an adequate S/N in the CPMG-SS spectrum and indeed more than that observed in a non-synchronized experiment under MAS. Considering the approach here is to “pre-screen” samples, the use of a static sample is a considerable advantage in that the rotor does not have to be carefully balanced to ensure spinning, and time is not wasted in achieving a set spinning speed. As such, all the solid state CPMG-SS experiments were acquired without MAS. A 15\(\mu\)s 18W pulse (corresponding to a low power 180\(^{\circ}\)) was used for echo formation with 790 loops and a delay of 18\(\mu\)s. Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 30Hz in the final spectrum.

Once the CMPG echoes are optimized for a specific probe, recalibration on a per sample basis (even between very different samples) provided little to no improvement in the CPMG signal. Readers are strongly advised to read the materials and methods section on safely implementing this approach and how to safely calibrate the CMPG pulse.

Soils were run on a second 4mm broad-banded solid state probe. The acquisition time was 14ms with 1024 time domain points. The rotor spinning speed was 13kHz. CP experiments were performed with a ramp of 80%-100%, a CP contact time of 2ms, and high power composite pulse decoupling (Spinal 64). CPMG-SS parameters were identical to the CP experiments except they were acquired without spinning. A 12\(\mu\)s 12W pulse (corresponding to a low power 180\(^{\circ}\)) for echo formation was found optimal for the second probe and 280 loops were used with an 18\(\mu\)s delay.

Solution state experiments on DOM were conducted on a 5mm broad-banded probe. Carbon experiments were acquired with 16384 points, an acquisition time of 275ms, and low power Waltz-16 proton decoupling during acquisition. Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 50Hz in the final spectrum. CPMG-SS experiments in the solution state were acquired with 16384 points, a 1\(^{31}\)ms acquisition time and low power Waltz-16 proton decoupling (same power level as the conventional experiments). The CMPG pulse was calibrated to 18\(\mu\)s at 20W for the solution state broadband probe. This actually corresponds to a low power 90\(^{\circ}\) pulse because a 180\(^{\circ}\) pulse on this specific probe required considerably more power. The 180\(^{\circ}\) pulse was tested but only slightly enhanced S/N was obtained and therefore the lower power 90\(^{\circ}\) pulse was chosen.
to reduce stress on the solution state probe. However readers should not that because the delay between each pulse is so short, a spin lock condition is still satisfied and a single spike is still achieved as discussed in detail by de Andrade et al. [36]. Delay in the CPMG sequence was set to 18µs with 2000 loops. Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 50Hz in the final spectrum.

2.3.3 Practical Instrument Considerations

Readers should be extremely cautious when attempting to adapt these experiments and it should only be attempted by experienced users. They should be aware that under fast echo acquisition the FID of the sample will extend considerably (see Figure 2.1). The temptation will be to increase the acquisition time to ensure the FID fully relaxes. The main issue is not the CPMG echoes themselves but the high power decoupling in the case of solids experiments. We strongly recommend you do NOT reproduce the FID in Figure 2.1D as this was a “one off” to simply demonstrate the elongated FID. The 80ms acquisition time along with the high power decoupling and CPMG train are hard on the NMR probe. As such, we recommend that you keep the acquisition time shorter than this (ideally no longer than the ordinary acquisition time of any given system’s solids experiments) so that the FID is heavily truncated but that the experimental conditions are safe for the instrument. Furthermore, in solids experiments we recommend attenuating the high power decoupling by 3db (half the wattage). This will lead to a reduction in total CPMG signal intensity but we have found that even under these conditions, the CPMG signal collected in 2mins is larger than the conventional signal that can be collected in 4 days for the samples we tested. Therefore, the CPMG signal intensity is not the limiting factor and the conditions outlined are a safe compromise. It is worth noting that if the decoupler is reduced by 3db, the total RF power (including the CPMG power outlined below) is much less than a conventional experiment without CPMG and if applied appropriately can be carried out without stress to the spectrometer.

Calibration of the CPMG Pulse Power

Using high power for the CPMG 180° pulses is not only dangerous, as it deposits too much power into the probe, but it will also not give maximum signal due to significant saturation effects. We suggest taking a sample with a very strong signal. For example in the case of 13C
something at high concentration and/or $^{13}$C labeled and optimizing the pulse power in real
time. It is very important to use a sample with high signal to optimize in “real-time mode”.
This is because with such short delays between the pulse and acquisition there may be a tiny
breakthrough of signal from the pulse itself. This is completely eliminated if the phase cycle is
completed. However, if you optimize on a per-scan basis in “real time” the full phase cycle is
not being used and a tiny bit of the signal will be from the pulse itself. In a sample with strong
signals, this contribution from the pulse is so small that it becomes insignificant. However,
optimizing on a natural abundance sample at low concentration in real-time mode will give you
problems. If you must optimize on a low concentration sample then you should not optimize in
real-time and make sure that the phase cycle is completed to ensure the signal is real and not
pulse breakthrough. Note that even between drastically different samples we found no need to
re-optimize the pulse powers or lengths and found that one setting worked on all samples tested.
The following method was used for pulse calibration

Start at a very low power. Stay at least 12db (not more than 10% of the full power wattage)
below of the normal full power for the channel and work upwards. On our solids probe we found
that setting the 180° pulse length to 12-18µs and then varying the pulse power was most effective
as a starting point. You do not have full control over the pulse length because as you increase
the pulse length, the total echo time (which includes the pulse length) becomes too long and you
get more than one spike-let in the spectral window. Varying the pulse power is more effective
at least to get in the general region of the maximum signal. In the end, a compromise must be
struck between stressing the hardware and losing some signal. We recommend increasing the
pulse power until the gain in $S/N$ begins to increase less drastically. Once we found a rough
power setting that gives a reasonable signal, we then adjusted the length of the pulse within
the limitations described above. In our case the final solution on our solids instrument was
a pulse length of 15µms and corresponding power of 18.5W, which was the best compromise
between $S/N$, still retaining only one spike-let in the spectral window, and keeping the power to
a minimum. The acquisition time was restricted to 40ms corresponding to 790 loops. Increasing
the acquisition time further will give you a sharper and more intense spike, and the gains are
impressive, but you will eventually push the hardware beyond its limits. Here in our solids probe
we kept the acquisition time to an absolute max of 40ms (with the exception being Figure 2.1B,
which was for example only). Consider this; with the safe levels described here, we still see more
intensity with a soil sample in 2 minutes than can be acquired in about 4 days using normal CP. Therefore, you have to ask yourself “Do you really need more signal in the CMPG-SS?” at least for the applications outlined here. The additional stress on the spectrometer components may not be worth the additional $S/N$.

Finally, in the broadband solution probe we calibrated the pulse in the same manner. In this case we used 20W with a pulse length of 18$\mu$s to be the best compromise between power used and $S/N$ in the single spikelet. This actually corresponds to a 90° not a 180° pulse as outlined in Figure 2.1. However, the 90° pulse only required 20W whereas the corresponding 180° would have required $\sim$80W which is not feasible for a solution probe as the duty cycle is $\sim$20% during the actual sampling period. As such we chose the 90° pulse and found the signal to be only slightly lower that that with the 180°. This is because the delay between each pulse is so short, a spin lock condition is still satisfied, this is discussed in detail by Andrade et al [36]. Note that due to very long decay in the solution FID with CPMG-SS and the fact only low power waltz-16 decoupling was used we increased the acquisition time to 131 ms corresponding to 2000 loops.

### 2.4 How CPMG-SS Works

CPMG-SS focuses all signals from all nuclei in a sample into one spike in the recorded bandwidth. The result is a complete loss of chemical shift information but an intense spike that is directly proportional to the concentration of NMR active nuclei in the sample. The intensity of this spike can be used to estimate the amount of NMR experiment time required to record a conventional spectrum. However, before the technique can be applied directly to environmental samples it is important discuss some of the fundamentals behind the approach and demonstrate proof of principle. The present paper demonstrates the concept and proof of principle on hemoglobin. Hemoglobin was chosen as it has a heterogeneous structure, contains paramagnetic centers, and exhibits fast relaxation giving rise to broad NMR signals. As such it is difficult to work with in NMR spectroscopy (as are most environmental matrices), and represent a readily available and challenging surrogate for demonstration purposes. The approach is then applied to various soils (including a very low organic carbon soil from the Arctic), dissolved organic matter from Lake Ontario, and a freeze dried worm. The later represents whole tissues samples which are now being studied extensively by NMR to understand in situ environmental stress, metabolism
and toxicity [37].

2.5 Proof of Principle

A relationship between the $S/N$ of the CPMG spike ($S/N_{SS}$) and the number of scans required in a conventional $^{13}$C CP experiment to achieve a specific $S/N$ ($N_{SCP}$) can be determined. In any conventional NMR experiment, the $S/N$ is directly proportional to the square root of the number of scans, the amount of sample ($n$), as well as various other spectrometer and environmental conditions which are generally kept constant ($k$) [38]

$$S/N = kn\sqrt{N}$$

(2.1)

When implementing the CPMG-SS experiment, if the number of scans is kept constant, the spike intensity can be used as a comparison between samples. Equation (2.1) then reduces to

$$S/N_{SS} = kn\sqrt{N_{SS}} = k_1n$$

(2.2)

Where $N_{SS}$ is the number of scans of the CPMG-SS experiment and $k_1$ is equal to $k\sqrt{N_{SS}}$. If the goal is to acquire a CP experiment until a target $S/N$ ($S/N_{CP}$) is achieved, then $S/N_{CP}$ is kept constant and Equation (2.1) can again be rewritten for a CP experiment as

$$S/N_{CP} = k\sqrt{N_{CP}} = k_2n$$

(2.3)

where $k_2$ is equal to $k\sqrt{N_{CP}}$. Finally, to relate $S/N_{SS}$ and $N_{SCP}$, it is assumed that both experiments (CP and CPMG-SS) are run on the same sample, so $n$ is equal. By solving for $n$ in Equations (2.2) and (2.3) and equating them the final expression relating $S/N_{SS}$ with the $N_{SCP}$ is found

$$S/N_{SS} = \frac{K}{\sqrt{N_{SCP}}}$$

(2.4)

where $K$ is equal to $k_1k_2$. Equation (2.4) states that the relationship between the spike intensity for a given number of scans ($S/N_{SS}$) is inversely proportional to the square root of the number of scans in the CP experiment ($N_{SCP}$).
scans required for the CP experiment to obtain a certain $S/N$ value ($NS_{CP}$). This relationship is confirmed experimentally with hemoglobin as the surrogate.

Hemoglobin samples of differing concentration were used to create a standard curve. The number of scans required to achieve a $S/N$ of 10 and 20 in a conventional CP experiment ($NS_{CP}$) were found and then plotted against the $S/N$ in the CPMG-SS experiment using 64 scans. The correlation fit Equation (2.4) very well ($R^2 > 0.98$) as is shown in Figure 2.2. In addition to confirming the relationship between the $S/N_{SS}$ and $NS_{CP}$, the plot is also useful as a calibration curve from which the $S/N$ in a conventional $^{13}$C CP ($S/N_{CP}$) can be predicted from the $S/N$ in the CPMG-SS experiment ($S/N_{SS}$) for a hemoglobin sample of unknown concentration.

Figure 2.2: Four samples of hemoglobin (3.69mg, 5.81mg, 7.81mg, 12.07mg) were run until a $S/N_{CP}$ of 10 was reached. A second curve was created for a $S/N_{CP}$ of 20 (5.89mg, 7.81mg, 12.07mg, 25.69mg). Fitting the obtained data to Equation (2.4) demonstrates that the relationship between $S/N_{SS}$ and ($NS_{CP}$) hold experimentally; $S/N_{SS}$ is inversely proportional to the square root of ($NS_{CP}$). The slope of each line represents $K$ in Equation (2.4). These curves are used later as a calibration curve to predict $S/N_{CP}$ for hemoglobin samples of unknown concentrations.

To validate the predictive ability of the standard curve shown in Figure 2.2, the $S/N$ that would be obtained after a given experiment run time for hemoglobin samples with unknown concentration was predicted. Three samples of differing concentration were used and the $S/N_{SS}$ was determined for each. Depending on whether the intersection between $S/N_{SS}$ and experimental run time lies in the light grey, dark grey, or white region in Figure 2.2, the corresponding $S/N_{CP}$ can be estimated to be under 10, between 10 and 20, or over 20 respectively.

For example, from the $S/N_{SS}$ value attained in the CPMG-SS experiment after 64 scans and a given experiment time of 4 hours it can be predicted that the highest concentration
Figure 2.3: To demonstrate that the standard curve can estimate \( S/N_{CP} \), three hemoglobin samples with random quantities were used. A line from the \( S/N_{SS} \) and a predetermined experimental runtime formed an intersection (denoted with X’s) on the graph. If the intersection was below the 10 \( S/N_{CP} \) curve (light grey region), then a \( S/N \) lower than 10 would be achieved as illustrated by the green spectrum. An intersection between the 10 and 20 (dark grey region) or above the 20 \( S/N_{CP} \) curve (white region) meant the resulting conventional \(^{13}\)C CP spectrum would be 10-20 or >20 \( S/N \) respectively. Note that the \( x \)-axis has been renamed and shows the experimental run time equivalent to the \( \sqrt{NS_{CP}} \) to better illustrate the concept. Time indicates the CP experiment’s run time to acquire the spectrum shown.

Sample (red lines in Figure 2.3) should have a \( S/N >20 \). Experimentally, a \( S/N_{CP} \) of 25.50 was obtained which agrees with the prediction. The next sample (blue lines) was to be run overnight for 16 hours and falls in the dark grey region between 10 and 20 \( S/N_{CP} \). After the overnight CP experiment, the actual \( S/N_{CP} \) was 16.27. Finally, the last sample produced a very low \( S/N_{SS} \) (green lines) of only 5.26 and was to be a 16 hour experiment as well. Based on the standard curve, it was estimated that a \( S/N_{CP} <10 \) would be produced, and an experimental \( S/N_{CP} \) of 2.94 was obtained. This last example is a situation where running the CPMG-SS experiment prior to running the CP would have been enough to conclude that an adequate \( S/N \) would not be obtained overnight, and that more scans would be required to achieve a discernible spectrum. Each CPMG-SS spectrum took two minutes to run, thus determining if an adequate \( S/N \) would be obtained for each sample only took a few minutes to calculate. In a real life scenario, the spectrometer time for the last overnight run would have been saved if the CPMG-SS experiment was used to determine that an acceptable spectrum would not have been obtained in the allocated amount of spectrometer time.
2.6 Estimating Experimental Run-Time in Environmental Samples

The standard curve developed for hemoglobin cannot be used for samples that are significantly dissimilar in chemical composition because of the differences in heterogeneity. Other proteins could possibly use the standard curve developed in Figure 2.2, but samples with very different composition will require recalibration. Here the approach is tested using real environmental soil samples. A different standard curve for calibration is required because the composition of soil significantly differs with that of hemoglobin.

In this section, a single point calibration curve is used where only a single point (one sample from the study) along with the origin from the coordinate plane is plotted and used to predict experimental run time. This is much faster and only requires the acquisition of one conventional CP experiment from which the experiment time and $S/N$ for the other samples in a series can be predicted from the CPMG-SS (two to three minutes per sample). A single point calibration is adequate as the technique is most useful for estimation purposes and is helpful in answering the common questions; Will my sample give an acceptable spectrum? and How much spectrometer time do I need?

To demonstrate the concept a series of three forest soil samples were used. The $NS_{CP}$ and the CPMG spike intensity ($S/N_{SS}$) were determined for one of the samples at a $S/N_{CP}$ of 10. Using these values and the origin as the second point, the slope ($K$) was calculated. The slope can now be used to solve for $NS_{CP}$ given the $S/N_{SS}$ using Equation (2.4). Consequently, the $S/N_{SS}$ for the two other soils were obtained using only two to three minutes of spectrometer time. These were then used along with the calculated K value to determine how many scans were required ($NS_{CP}$) to obtain a $S/N$ of 10 in the conventional CP experiment. The number of scans required for a $S/N_{CP}$ of 10 was calculated to be 1148 scans for the first soil and 4110 scans for the second. The experimental $S/N_{CP}$ that was obtained after these number of scans were 11.60 and 8.16 respectively which is reasonably close to the predicted $S/N_{CP}$ of 10.

The variability mainly arises because of the chemical differences in the samples. While they are all forest soils, they are not chemically identical and tend to vary significantly with soil type, location, drainage, vegetation cover, microbial populations, and the ecosystem they support as a whole. As a result, the $S/N$ is calculated slightly differently for each of the conventional carbon
CP spectra. Despite this, it is clear that CPMG-SS spectroscopy is very powerful for estimation of runtime and S/N in unknown samples and can save spectrometer time if used efficiently.

It is important to stress that a calibration should be done for each particular series of samples to be studied. In this example, all three soils were forest soils and thus had broadly similar carbon chemical shift dispersion. However, it is unlikely that a single calibration curve will hold for all soils since they can drastically vary in chemical composition and heterogeneity. Similarly, in a biological setting, the calibration curve for hemoglobin is unlikely to hold for human blood since blood contains many other components other than hemoglobin. But once constructed from a single point, a human blood calibration curve should be fairly accurate for all blood samples.

2.7 Very Low Abundance Samples

Soil and sediment carbon contents can vary drastically so obtaining total organic carbon measurements can be very beneficial. In some cases however, the amount of sample required for this procedure may exceed the amount of sample available and the carbon contents remain unknown. The samples with high amounts of carbon can produce an acceptable NMR spectrum in minutes to hours, whereas the signals from samples with low carbon may require overnight or longer. These are samples that are most problematic and would benefit from a technique like the CPMG-SS which allows for rapid detection and experiment time estimation.

To really push the limits of detection, a sample with extremely low amounts of carbon nuclei was employed and observed the S/N_{SS} using 128 scans. The sample used was an Arctic watershed soil sample which had been previously reported to have a carbon content of approximately 1% \cite{2}. Here CPMG-SS is used in its most basic form simply attempting to ascertain whether this single sample will ever give rise to an acceptable NMR spectrum. The logic being that if there is no CMPG-SS signal after 128 scans then there is little hope of acquiring a conventional spectrum. However, if even a small CPMG-SS can be recorded then there is a chance to obtain a reasonable conventional NMR spectrum over an extended run. The spike obtained for this soil is very small (Figure 2.4A) when compared to the other forest soils used in the previous section as expected due to its very low carbon content. Because of this, it was not a surprise that the \textsuperscript{13}C CP experiment returned a barely discernible spectral profile overnight as illustrated by Figure 2.4B. In this case, the low CPMG-SS S/N is an indicator that considerably more ex-
experiment time is required to obtain a useful spectrum, and/or that pretreatment to concentrate this sample should be considered. After hydrofluoric acid treatment, Figure 2.4C shows that an acceptable spectrum could be acquired on this relatively precious Arctic soil. It is important to note however, that in just two minutes the $S/N$ of the CPMG-SS is higher than that obtained in an overnight run. From this some very crude, yet practically important conclusions can be drawn, even for single samples, that lack comparable partners on which a more accurate $S/N$ prediction can be based. These conclusions can be summarized as: 1) If a CPMG spike cannot be observed in two minutes it is very unlikely that a $^{13}$C conventional NMR spectrum can ever be recorded. 2) A CPMG-SS can be discerned in 128 scans but has a $S/N$ of 10 or less, it is likely the sample will require an extended run (weekend at least). If the $S/N$ in the CMPG-SS is greater than 10, it is likely an adequate spectrum can be acquired overnight.

Figure 2.4: Demonstration of CPMG-SS sensitivity in a very low concentration sample. An arctic watershed soil sample containing approximately 1% and a CPMG-SS spectrum (A) was seen within minutes (128 scans), but suggests a very low carbon content. Overnight the $^{13}$C CP spectrum (B) returned no discernible spectrum (black) as predicted from the CPMG-SS intensity. Running the CPMG-SS experiment informs the spectroscopist that a resolvable spectrum cannot be obtained in a reasonable amount of time and a pretreatment technique, if applicable, should be used to concentrate organic component, or the experiment should be run for a longer period of time. In this case, hydrofluoric acid treatment (C) was used to concentrate the sample and a resolvable spectrum (grey) was then produced with the same number of scans as spectrum.
2.8 Other Nuclei

A range of other spin-half NMR nuclei are of interest for environmental research from the cycling of $^{31}$P and $^{15}$N [17, 18], to the study of metal contaminants like $^{199}$Hg, $^{113}$Cd, $^{207}$Pb [24, 39, 40]. Recording NMR spectra is often challenging in part because the nuclei are relatively insensitive and in part due to the wide range of chemical environments present in heterogeneous environmental samples. To demonstrate the CPMG-SS approach is not just limited to $^{13}$C, $^{31}$P NMR is performed on a freeze dried worm.

Figure 2.5A displays the CPMG-SS spike (left panel) and corresponding $^{31}$P CP experiment of a freeze dried worm. Cross polarization was optimized for a $^{31}$P spectrum while the number of echoes and $\tau$ in the CPMG-SS experiments were kept the same as the $^{13}$C experiments since they met the requirements for a single spike within the bandwidth of the $^{31}$P spectrum. The CP spectrum shows sidebands at 13kHz which is equivalent to the spinning speed, and the spectrum itself is a broad signal characteristic of the inhomogeneity in the sample. As illustrated in the graph in Figure 2.5, a calibration curve similar to the one made for hemoglobin and soil in the previous sections can be constructed for $^{31}$P NMR using worm tissue (blue circle on Figure 2.5). To test the predictability of the calibration, a second worm sample was diluted 7.75 times using an inert filler. Using the constant $K$ (from the slope of the calibration) and Equation (2.4), the number of scans required to achieve a $S/N_{CP}$ of 15 is predicted to be 27,667 scans. When running the sample, the actual $NS_{CP}$ to achieve a $S/N_{CP}$ of 15 was 27,950 and is represented by the red square data point on the graph, a difference of 1% compared to the estimated value. Thus, experiment time can also be estimated for spin half nuclei other than $^{13}$C using CPMG-SS spectroscopy.

2.9 Solution State Experiments

The aqueous phase is important to environmental NMR studies including those involving soils, all bodies of water, plant studies and metabolomics [1, 6, 37]. The use of CPMG-SS to estimate experimental runtime can also be applied to solution state NMR as demonstrated in Figure 2.6. Here solution state $^{13}$C CPMG-SS spectra (left panel) of riverine dissolved organic matter (DOM) were obtained at various concentrations, and the corresponding conventional $^{13}$C experiments (top right) are shown in the corresponding color. The graph in the bottom
Figure 2.5: $^{31}$P CPMG-SS (left) NMR of a freeze dried worm and the corresponding $^{31}$P CP experiments (right) of the whole worm sample (A) and a worm sample with an inert salt filler (B). High power composite pulse decoupling (Spinal-64) was used with 790 loops and an 18µs delay similar to the $^{13}$C experiments. The $^{31}$P spectrum shows characteristic spinning side bands at 13kHz, and one broad signal representing the inhomogeneous chemical phosphorus in the worm. The calibration curve was created using the concentrated sample and the $(N_{SS}S_{CP})$ was then calculated for the dilute sample. It was found that the calculated $(N_{SS}S_{CP})$ was roughly 1% different from the actual value proving the applicability of this technique to spin half nuclei other than $^{13}$C.

right panel is a correlation between the $S/N_{SS}$ and the $S/N$ in the conventional $^{13}$C experiments ($S/N_{CC}$) of each of the three spectra and demonstrates that the CPMG-SS method has predictive capabilities in the solution state.

The sensitivity of the CPMG-SS technique is illustrated once again with the green spectra in Figure 2.6. Readers should note that in solution NMR the 128 scans CPMG-SS takes three minutes versus two minutes in solid-state NMR. This is due to the acquisition time being shortened in solid-state NMR to permit high power decoupling, which if applied too long can damage the NMR probe. In three minutes a CPMG-SS spectrum is obtained for the lowest concentration whereas the features are still indistinguishable from the noise in the conventional $^{13}$C spectrum after 16 hours. This is consistent with solid-state results that indicate a sample with a CPMG-SS $S/N$ of less than 10 in 128 scans will require much longer than overnight to record a reasonable spectrum. The approach provides the knowledge needed by the spectroscopist to pre-treat and concentrate the sample if necessary, or in some cases simply make the experimental run time long enough to achieve an acceptable spectrum. In addition, it provides a concrete method to identify samples that are simply too dilute for conventional NMR and avoids wasting instrument time.
Chapter 2. Rapid Estimation of NMR Experiment Time

Figure 2.6: Solution state conventional $^{13}$C and CPMG-SS spectra of DOM at three different concentrations (200mg/mL-red, 40mg/mL-blue, 10mg/mL-green). Each conventional $^{13}$C spectra was run for 16 hours while the CPMG-SS spectra took three minutes. Equating $S/N_{SS}$ and $S/N_{CC}$ by keeping the number of scans constant for each experiment reveals a linear correlation which is also predicted mathematically. This demonstrates that there is an obvious correlation with the amount of carbon in the sample and the $S/N_{SS}$ in solution state NMR which is all that is required to create a standard curve for experimental run time estimation.

2.10 Conclusion

Using the predictive single spike technique described in this study, it is relatively trivial to determine if a sample with unknown carbon content has enough to produce an acceptable NMR spectrum in a given amount of time. If the time required to obtain an adequate conventional spectrum is prohibitive, then the sample can be concentrated and/or pretreated and wasted NMR time is avoided. The CPMG-SS method is very rapid (two to three minutes) and does not require MAS in solids resulting in a rapid and simple pre-screening approach. To predict the runtime in a series of similar samples (note they only need to be roughly similar in terms of chemical composition, they can vary greatly in concentration), all that is required is one conventional NMR spectrum along with a CPMG-SS from any sample in the series. The intensity from CPMG-SS experiment (only requires two to three minutes per sample) is then used to predict the experimental runtime for other samples in the series. This approach yields enough accuracy ($\pm \sim 20\%$ of the true $S/N$) for estimation purposes. If a crude estimate only is required
to answer the question “will a specific sample produce an acceptable NMR spectrum at all”, an approximation can be made from the CMPG-SS S/N alone as was demonstrated with an Arctic soil. Finally, the CPMG-SS pulse program can be applied to other nuclei as demonstrated by the $^{31}$P experiments. The present study has demonstrated a novel approach to estimate required experiment time for the NMR analysis of various environmental samples, and has the potential to save spectrometer time and instrument costs.
REFERENCES


Chapter 3

From the Environment to the NMR: Water suppression for Whole Samples in their Native State

This material is under review for publication

Chapter 3. TWINS: NMR Analysis in the Native State

3.1 Abstract

Studying environmental samples in their native state is critical as drying, fractionating or extractions can alter composition, structure, conformation and biological activity, as-well as perturb essential interfaces and domains. NMR spectroscopy is a powerful and versatile tool that provides unprecedented levels of information regarding structure and interactions. Both High-Resolution Magic-Angle-Spinning and Comprehensive-Multiphase NMR probes facilitate the study of natural multiphase samples. $^1$H NMR is the most sensitive and provides unique information on swollen components and interfaces. However, samples such as plants, organisms, and soil have a high aqueous content and a range of free, exchanging, and bound water, leading to a broad and intense water signal that can span the entire $^1$H spectral region masking information from other components. In this chapter, a water suppression approach termed Tailored Water suppression for Inhomogeneous Natural Samples (TWINS) is developed out of a practical need to study samples in their native state. TWINS builds upon the most effective approach to date (SPR-W5-WATERGATE) for natural samples with the addition of various elements to make the approach effective in the most challenging systems. TWINS was tested on a range of environmental samples in both 1D and 2D NMR. A lock capillary was developed to separate the lock solvent from the sample, further reducing sample alteration. In summary the more challenging the sample, the more TWINS outperformed conventional approaches. In turn this increases the range and diversity of samples that can be studied in their natural state critical for a wide variety of fields and applications.
3.2 Introduction

Environmental reactivity or biological function is often a result of synergism between a wide array of organic and inorganic materials with key interfaces (aqueous/solid, gel/liquid), conformation, layering, hydrophobicity, hydrophilicity, localized pH all of which play critical roles. As scientific questions become more complex, the need to study samples in their natural state without pretreatments (i.e. fractionation, extraction and drying) is becoming increasingly important as these processes can disrupt the aqueous-organic interface, alter biological activity, and change structure and conformation.

NMR spectroscopy is arguably one of the most powerful and versatile tools in modern science. High Resolution Magic Angle Spinning (HR-MAS) probes [1] and Comprehensive Multiphase NMR probes [2] both permit $^1$H NMR HR-MAS can provide unique information as to swollen components and key structural interfaces such as the soil-aqueous interface. Furthermore, the potential to study samples in their biologically active state (without drying) opens the door to studies of soil metabolisms and real-time biotransformation of contaminants [3]. However, when applied to very complex samples such as soils, sediments and plants, the signal from water is ubiquitous and can be highly problematic by preventing receiver optimization. Such samples will contain water from multiple environments including, bound, gel-like and free. In some cases, the water base can span across the entire spectral region thereby concealing all other signals.

A wide range of water suppression approaches have been developed for NMR spectroscopy [4]. Previous work has compared these approaches by studying natural samples in the solution-state [5]. In this previous work, a sequence termed SPR-W5-WATERGATE was developed which combines a train of water selective shaped irradiation pulses with two gradient spin echoes surrounding a frequency selective element [5]. The authors showed that applying the SPR-W5-WATERGATE suppression allowed the water to be kept under the spectrometer noise for days on a solution-state spectrometer. This permitted the natural abundance analysis of river, lake, groundwater, seawater, and Antarctic glacial ice [6, 7]. However, even this sequence, which shows unsurpassed suppression in the solution-state NMR, is unable to cope with the extremely broad water observed under $^1$H MAS when whole natural samples such as soils that contain solids, gels and liquids are studied in their natural state.
This chapter describes the development of a novel water suppression technique termed Tailored Water suppression for Inhomogeneous Natural Samples (TWINS). Numerous elements are added to the SPR-W5-WATERGATE sequence with the goal to suppress the water effectively while still maintaining as much spectral information from the sample as possible.

Considering that many environmental samples are highly heterogeneous, contain broad spectral profiles and can have low organic contents, $^1$H is sometimes the only possibility for extracting information in the native state. The TWINS approach permits the study of even the most challenging samples directly from the environment into an NMR spectrometer. TWINS is tested on a variety of natural samples in their natural state without any pre-treatment or alteration and is compared to SPR-W5-WATERGATE because of its widespread use to analyze natural samples with broad water [4, 8, 9, 10, 11, 12]. This includes aquatic samples where no addition of extra water for rotor balancing was required demonstrating true native state analysis. As such, the approach should provide a better understanding of processes and structure in the native state with a wide range of potential applications including direct metabolic profiling, real-time metabolism monitoring, contaminant/pesticide transformation in soils, swelling/drying processes, soil respiration and biological growth/decay processes.

### 3.3 Materials and Methods

#### 3.3.1 Sample Collection

**Samples in the native state**

A forest soil sampled from the surface of a mixed coniferous/deciduous forest, decaying wood, fern leaves, and pine needles were collected from Rouge National Urban Park (43°48'50.8"N, 79°09'23.0"W) in Toronto Ontario, Canada. Lake Ontario algae and marsh duckweed were sampled from Lynde Shores Conservation Area in Ajax, Ontario, Canada (43°50'23.0"N, 78°57'52.0"W). Forest soil, lake algae, and marsh duckweed samples were sampled directly from their native environment, collected in glass jars, and used without any alteration for analysis. No pre-treatment, freeze-drying, or solvent addition was used in these samples. Decaying wood, fern leaves, and pine needles were also collected in glass jars and were used with the addition of Millipore H$_2$O in the NMR rotors for sample balancing. No other modifications were performed to the samples.
other than cutting leaves to the appropriate size to fit into a 4mm diameter NMR rotor. A juvenile earthworm was selected from a healthy population of E. fetida maintained within our laboratory since 2006 as described in Brown et al [13].

**Lab samples**

In some cases the NMR profile of TWINS needed to be compared to that of more conventional approaches such as presaturation. In these cases as the conventional approaches were insufficient in the in-situ samples, alternative “dry” samples that could be swollen in D₂O were required. In these cases a commercial dried algae and lyophilized soil were used. 100% Chlorella Algae (Greengem) tablets used in Figure 3.3 were purchased from a local health and nutrition store and was ground into a powder before analysis. The lyophilized soil sample used was obtained from Hampstead Park in Dublin, Ireland. Hampstead Park soil is a Grey Brown Podzol with an organic carbon content of 6.8% +0.4%, retrieved from an open public area located within Albert College Park. In these specific cases pure D₂O was used such that it was possible to obtain acceptable NMR spectra using conventional water suppression sequences for comparison. In addition, a sucrose/glucose water standard was used for the excitation profile mapping containing 90% H₂O/10% D₂O.

### 3.3.2 D₂O Lock Capillaries

The natural samples were placed in rotors as-is. In some cases, the sample was wet enough to fill the entire rotor. In other cases, some additional water (Milli-Q, 18.2MΩ, pH 7.0) was added to remove air pockets required to improve magnetic homogeneity and to balance the rotor for spinning. To avoid adding D₂O (lock solvent) directly to the sample an external lock system was used. D₂O capillaries were made using 812µm inner diameter PTFE capillaries. A length of roughly 2cm was cut and approximately 5-10µL of D₂O (Cambridge Isotope Laboratories, Massachusetts, USA) was inserted into the capillaries using a micro pipette equipped with a gel loading tip. Both ends were folded over and sealed using heat. One sealed capillary was used for each rotor to provide a lock solvent. Figure 3.1 illustrates the D₂O lock capillary.

The commercial dried algae and lyophilized soil (mentioned in the previous section) were used for comparison of spectral profiles between TWINS and conventional sequences. In these
specific cases pure D$_2$O was used such that it was possible to obtain acceptable NMR spectra using conventional water suppression sequences for comparison.

![Figure 3.1: An illustration of the capillaries used to gain a better understanding of their scale in comparison to a 4mm NMR rotor.](image)

### 3.3.3 NMR Spectroscopy

NMR experiments were performed on a 500 MHz Bruker Avance III Spectrometer using a 4mm $^1$H-$^{13}$C-$^2$H comprehensive multi-phase NMR probe [2] fitted with an actively shielded magic angle gradient. All samples were spun using a spinning speed of 6666 Hz and all experiments were performed at 298K. 1D $^1$H experiments were performed with 256, 512 or 1024 scans depending on the sample. A recycle delay of $5 \times T_1$ and 16384 time domain points were used. Spectra were apodized through multiplication with an exponential decay corresponding to 0.3-3Hz line broadening in the transformed spectrum and a zero filling factor of 2.

Both TWINS and SPR-W5-WATERGATE were performed using Water suppression by Gradient Tailored Excitation (WATERGATE) and was carried out using a W5 train and a 150µs binomial delay [14] such that the sidebands occurred at approximately 12ppm and -2ppm and were outside the spectral window [14]. W5-WATERGATE was preceded by a train of selective pulses: 2000, 2ms, calibrated $\pi$ pulses were used, each separated by a 4ms delay [5]. Each selective pulse had a theoretical inversion profile of approximately 0.4ppm (calculated using Bruker Biospin’s, shape tool in TopspinTM 3.1) similar to that used for excitation sculpting [15]. Further modifications applied to create TWINS are discussed more in the main text.
2D $^1$H-$^1$H TOtal Correlation SpectroscopY (TOCSY) spectra were acquired in the phase-sensitive mode, using a mixing sequence with rotor synchronized constant adiabatic WURST-2 pulses within an X_M16 mixing scheme [16]. 64 scans were collected for each of the 128 increments in the F1 dimension. 2k data points were collected in the F2 dimension at a mixing time of 250 ms. F1 and F2 dimensions were processed using sine-squared functions with a $\pi/2$ phase shift and a zero-filling factor of 2.

### 3.4 Current Water Suppression Limitations

Water suppression has undergone many improvements including the introduction of composite pulses, the use of low flip angles, phase modulated irradiation, pulse field gradients and excitation sculpting [12]. However, current demands to study natural samples require a solvent suppression technique that can suppress a much broader and more intense water signal than previously required.

Figure 3.2 illustrates the current problem. Figure 3.2A shows an unsuppressed $^1$H NMR spectrum of a soil sample taken from the Rouge National Urban Park without any pre-treatment. The water resonance dominates the spectrum with no visible analyte signals. At $1000 \times$ zoom, a weak soil signal is barely observed albeit superimposed over the broad water resonance that spans the entire 10ppm chemical shift range (Figure 3.2B). The broad water arises due to a range of molecular states within the sample including, free water, water in various rates of exchange and water bound to both organic matter and minerals.

Figure 3.2C shows the same sample after the application of a basic water suppression technique termed pre-saturation. This technique does little to suppress the broad water and sample signals were not recovered. Figure 3.2D is the same soil sample with SPR-W5-WATERGATE suppression, which has been shown to be the most effective water suppression technique for challenging natural samples [5]. With SPR-W5-WATERGATE, the soil signals become visible but a significant water artifact prevents the user from analyzing signals that may reside in a chemical shift region roughly 4ppm wide centered on the water signal. Readers should note that the abundance of other water suppression approaches including excitation sculpting [15], WET [17], SOGGY [18], Watergate [14], PURGE [19], or WEFT [20] were not ignored. The majority were previously considered and found to be ineffective leading to the development of
Figure 3.2: A) A $^1$H NMR spectrum of an in situ soil sample without any water suppression. The water signal dominated the spectrum. B) The same spectrum magnified 1000×. A small analyte signal became visible but was still hidden under the broad water that spanned the entire spectral region. C) Pre-saturation applied to the same sample. Little was achieved in terms of retrieving analyte signals. D) The most powerful water suppression known to date still left a 4ppm bandwidth of residual water signal where sample signals could not be analyzed.

SPR-W5-WATERGATE [5]. Indeed these approaches were revisited here but in more challenging inhomogeneous environmental samples, these other sequences were ineffectual, with only SPR-W5-WATERGATE showing limited promise in successfully suppressing the water. Hence, SPR-W5-WATERGATE was used as the starting point for further improvement.

3.5 SPR-W5-WATERGATE Sequence Modifications

Three integral modifications were made to the SPR-W5-WATERGATE sequence to create the basic TWINS sequence, with the perfect echo16 approach being implemented as a fourth optional element described in greater detail towards the end of the chapter. Figure 3.3 shows a progression of the modifications applied to the SPR-W5-WATERGATE sequence as well as the corresponding reduction in water signal for the soil sample introduced in the previous section.
The original SPR-W5-WATERGATE sequence and the spectrum that results from the in-situ soil sample is shown in Figure 3.3A.

The first improvement was the introduction of a selective W5 water exclusive inversion train was introduced at the beginning of the sequence along with a corresponding 180° shift in the receiver phase. This selectively inverts all sample signals along z-axis, while leaving the main water unperturbed. This block was only applied during odd numbered scans, while even numbered scans are collected in the conventional manner. The net outcome was the subtraction of unperturbed water between odd and even scans, due to the receiver phase shift, while sample signals continue to add. The resulting spectrum (Figure 3.3B) produced a marked reduction in the wings of the water spectrum of roughly 50%. The approach was particularly effective because in addition to cancelling the main water signal, it also helped to cancel far water beyond...
the coil region, an idea central to other improved water suppression approaches such as pre-sat 180 [21].

Next, the sequence was fully rotor synchronized to refocus residual anisotropic interactions and reduce modulations caused by pulse imperfections [22]. In conventional W5-WATERGATE, the length of pulses are increased while the binomial delay (separating the pulses) is kept constant [14]. However, this leads to each of the five W5 sub-elements being applied at different positions in the rotor cycle. The simplest solution was to set the total time from the center of each pulse (encompassing the sandwiched binomial delay) to equal one rotor period. The binomial delay can then be adjusted to compensate for the continually changing pulse lengths while still retaining full rotor synchronization. In practical terms, the time adjustments required to compensate for the varying pulse lengths were relatively small (only a few microseconds) and brought the W5 train closer together reducing time allowed for $T_2$ relaxation. This led to a slightly improved excitation bandwidth (see next section for further discussion).

The result of the W5 rotor synchronization is shown in Figure 3.3C. The changes are exaggerated in the diagrammatic representation of the pulse sequence so that they can be easily visualized. Rotor synchronization of the W5 pulse train lead to a considerable improvement in the water suppression, reduction of artifacts and recovery of some of the sample signal close to the water.

The final addition was the alternation of the pre-saturation pulse phase cycle. The phase cycle was determined empirically and the cycle shown in Figure 3.3D worked best in combination with the aforementioned changes. Figure 3.3D shows the resulting spectrum with a significant reduction in water resonance wings. This spectrum was usable with a flat baseline and solute signals can easily be ascertained around the water resonance where they were initially very distorted and impossible to discern. Carbohydrates can be easily assigned to the large broad signal up-field of the water signal, while small signals possibly arising from the anomeric signal of carbohydrates were also discernible down-field of the water signal.

### 3.6 Excitation Profile

In this section, the excitation profile of TWINS was compared to SPR-W5-WATERGATE to demonstrate the slightly improved excitation profile TWINS exhibits. Figure 3.4 plots the
Figure 3.4: A comparison of the excitation profiles of TWINS and SPR-W5-WATERGATE.

Both profiles are very similar in terms of the central water suppression (0 KHz). The side-bands arise at 4KHz (8ppm at 500MHz) each side of central resonance. The excitation bandwidth with for TWINS is slightly more intense and covers a 3KHz window at almost full intensity up to 3KHz on each side of the water. This gives a total excitation of ~12ppm at 500MHz with an approximately 5% deviation from unity. As such, assuming analyte signals are not too close to the water (see Figure 3.5 and related discussion), those resonances should be detected relatively quantitatively with 5% variation due to the excitation profile. However, given that this is for signals that would be challenging or impossible to even observe by other water suppression approaches this is still quite an improvement over other techniques.

3.7 Area Attenuated Around the Water Resonance

To study the attenuation near the water signal, a detailed comparison between SPR-W5-WATERGATE and TWINS was performed. A sample of dry powdered algae was swollen with D$_2$O to better analyze analyte signals around the water signal that were attenuated by water suppression. Figure 3.5 illustrates an overlay of TWINS and SPR-W5-WATERGATE spectra zoomed into the spectral region around the water resonance. The percentages represent the amount of attenuation TWINS produced compared to the SPR-W5-WATERGATE experiment.

There are two main regions highlighted in Figure 3.5. The region outlined in red illustrates the region where signals were completely attenuated and no chemical shift information was
Figure 3.5: A comparison of the attenuation of the signals around the water signal in an algae sample between TWINS (grey) and SPR-W5-WATERGATE (black). Both experiments completely suppressed signals that were 0.4ppm away from the water signal, and both sequences slightly attenuated signals from 0.4ppm to 1.1ppm away from the water signal. While TWINS attenuated signals in this region slightly more than SPR-W5-WATERGATE, there was no additional suppression by TWINS beyond the 2.2ppm window.

retained. This occurred at a distance of up to 0.4ppm from the water resonance on each side as was reported elsewhere [5]. The second region was slightly attenuated at 1.1ppm on either side of the water signal in the SPR-W5-WATERGATE spectrum [5]. In this region, signals were still discernible but their intensity was reduced and thus, these resonances cannot be quantified. On the other hand, a conventional water suppression technique would not suppress the water signal to a degree where these signals would be visible. The TWINS and the SPR-W5-WATERGATE sequences were both equal in intensity at 1.1ppm away. Previous work has shown that SPR-W5-WATERGATE is not quantitative 1.1ppm either side of the water signal [5]. The identical profile between TWINS and SPR-W5-WATERGATE is very encouraging and demonstrates that the TWINS sequence did not attenuate signals beyond the central region (1.1ppm each side of water) already perturbed by SPR-W5-WATERGATE. This slightly higher suppression within the central region of TWINS is the consequence for improved water suppression but is easier to accept when you consider TWINS is often the only feasible choice to access information from samples in their natural state.
3.8 Comparison on an Environmental Sample

In this section, TWINS was compared to pre-saturation to determine how similar the spectral profile recovered with TWINS is when compared to the total sample. This is tested on the most sample challenging possible a whole soil, which exhibits very fast $T_2$ relaxation and very broad water. Unfortunately this is not possible on a natural sample in its native swollen state (see Figure 3.2C) as it is not possible to recover signals using pre-saturation. To test this, a soil that was lyophilized to remove residual water was swollen with D$_2$O and then TWINS and pre-saturation were both performed on the sample. Pre-saturation, simply uses an on-resonance irradiation field prior to sample excitation to suppress the water. As such there are no relaxation losses as the spectrum is acquired directly after excitation. With TWINS, every element in the sequence was designed to be a short as possible there will be unavoidable relaxation losses. Hence, the profile recovered from TWINS is broadly similar to that obtained from pre-saturation as is observed in Figure 3.6. The main losses occur in the aliphatic signals. This likely arises due to a fraction of the lipids being very rigid (for example cell walls in microbes) that relax very rapidly and are under-represented in the final spectrum. It is therefore important to stress in natural samples that contain components with very broad lines (for example semi-solids), these components may be underestimated with TWINS. Fortunately such solid and semi-solids can be easily recovered even for soils using a CMP-NMR probe and $^{13}$C detection cross-polarization based editing approaches are used [2]. As such, TWINS is ground breaking in that it permits information on the dynamic (dissolved, and gels) components and key interfaces that otherwise would be masked by water in the natural state.

3.9 Application to Unaltered Environmental Samples

TWINS can be applied to many environmental systems and different matrices were chosen to test the widespread applicability of this sequence. Figure 3.7 illustrates some of the samples that were tested here. All samples were taken directly from the environment and were not altered to highlight the capabilities of TWINS towards studying samples in their native and unaltered state. While SPR-W5-WATERGATE was fairly successful at removing the water signal from these samples, there were still some undesired artifacts remaining which made the spectra difficult to interpret. A residual water signal was present in all four spectra when
Chapter 3. TWINS: NMR Analysis in the Native State

Figure 3.6: A) A lyophilized soil sample re-hydrated with D$_2$O was studied using pre-saturation compared with B) the same sample studied with TWINS. The analyte signals in the TWINS sequence were similar to those in the pre-saturation experiment. This illustrated that TWINS did not significantly alter the spectrum.

SPR-W5-WATERGATE was used, whereas the TWINS sequence either removed or greatly reduced those imperfections. In two cases (marsh duckweed [A] and decaying wood [C]), the residual water signal remaining after SPR-W5-WATERGATE was enough to also distort the baseline causing great difficulty while phasing the spectra appropriately. TWINS was able to suppress the water signal and also exhibited superior baseline properties. This four panel figure includes samples taken from both aquatic and terrestrial sources to illustrate the versatility of this sequence on a variety of samples. The lower the shim quality and the more inhomogeneous a sample was, the better TWINS performed relative to SPR-W5-WATERGATE.
Figure 3.7: Four environmental samples taken from their natural habitat and studied without pre-treatment. In each instance, TWINS performed better than SPR-W5-WATERGATE with respect to removing the water signal as well as maintaining baseline properties. Baseline correction was not applied so that the true baseline resulting from the water suppression approach can be ascertained. The more challenging the sample and the broader the natural water line shape, the more TWINS outperformed SPR-W5-WATERGATE.

3.10 Two Dimensional TWINS NMR Spectroscopy

With the study of samples in their natural state, two-dimensional experiments are extremely helpful in providing additional dispersion and connectivity information to aid in signal assignment. Total correlation spectroscopy (TOCSY) is a very common 2D experiment in NMR spectroscopy providing connectivity information within a $^1\text{H}-^1\text{H}$ spin system. TOCSY was used here to demonstrate the application of TWINS in a multidimensional experiment. Here W5-TOCSY is directly compared to TWINS-TOCSY. W5-TOCSY is in our experience the most effective approach for suppressing water that is included in the standard sequences provided by Bruker BioSpin (Rheinstetten, Germany). In both the TWINS and W5-TOCSY, the conventional MLEV mixing scheme was replaced with a rotor synchronized X_M16 mixing scheme which has been shown to exhibit superior performance under MAS [16, 22].

In Figure 3.8A a rotor synchronized adiabatic TOCSY with the TWINS water suppression technique was applied to a pine needle sample. At first glance, the spectrum was cleaner and showed more $^1\text{H}-^1\text{H}$ correlations than W5-TOCSY (Figure 3.8B), which also had a water resonance at 4.7ppm. In Figure 3.8C, a 1D projection of the same slice of each TOCSY is shown. This slice was taken from the middle of the spectrum at the water resonance. A clear difference in water suppression can be seen in Figure 3.8C. The W5-TOCSY did not suppress the water signal completely and showed a considerable water signal while the TWINS-TOCSY suppressed the water signal effectively. It should be noted that no true TOCSY correlations coincided with the water slice (Figure 3.8C). Other signals beyond the water in this plane represent artifacts
Figure 3.8: A) TOCSY with TWINS water suppression compared with B) TOCSY with W5-WATERGATE water suppression. TWINS was much cleaner with more $^1$H-$^1$H correlations. As well, the water resonance did not occur in the TWINS-TOCSY. This is shown in C) where 1D projections of each spectra of the same slice were overlaid. W5-TOCSY shows a considerable water resonance, while TWINS-TOCSY fully suppressed the water resonance.
from incomplete cancellation. These same artifacts were also of lower intensity in the TWINS experiment. This example demonstrates that TWINS can be added to 2D experiments to allow for a more in depth analysis of samples studied in their natural state.

3.11 Perfect Echo TWINS

Recently, an ingenious perfect echo experiment was published that compensated for $J$-coupling during relatively long pulses or delays in some water suppression approaches [23]. The TWINS sequence however, does not incorporate long delays and as most natural samples exhibit a general broad line shape, such $J$-modulations are not observed in the majority of samples. However, in samples dominated by metabolites, line shape can be very sharp and $J$-modulations become apparent. Furthermore, it is feasible that a user may require extraordinarily long gradient pulses (a possibility in larger diameter rotors, not tested here) to dephase water. With increasing gradient pulse length, $J$-evolution becomes more significant. For completeness, the perfect echo version of the experiment and the significant improvement in the presence of exceptionally long gradient pulses is briefly considered. In the perfect echo approach, an orthogonal 90° pulse was placed between the two spin echoes of the SPR-W5-WATERGATE sequence (Figure 3.9A) [23]. This allowed $J$-couplings to refocus avoiding manifestation as signal distortions.

Figure 3.9B shows the TWINS spectrum of a whole juvenile worm with and without the perfect echo with a normal gradient pulse length (1.6ms). Even without a long gradient pulse, the sharp lines were slightly twisted without the addition of the perfect echo pulse. On the other hand, TWINS with the perfect echo pulse effectively eliminated the twisting without a loss in signal intensity or quality. Figure 3.9C shows the same sample with a longer gradient pulse (5ms) and marked twisting in the TWINS sequence without the perfect echo. Once the perfect echo was added, the twisting disappeared, with proper signal shape and intensities restored. Therefore, with the addition of the perfect echo, TWINS water suppression was further perfected to allow for longer pulse or delay times if desired.
Figure 3.9: A) A representation of the TWINS sequence with the addition of the perfect echo pulse (in red). B) The perfect echo did not distort the signal splittings found in the whole worm sample compared to the original TWINS sequence using normal gradient delay lengths (1.6ms). C) Longer gradient times (5ms) exacerbated the signal twisting in the original TWINS sequence while the sequence with the perfect echo retained the signals’ phase properties.
3.12 Conclusion

NMR is one of the most versatile tools in modern research but is often inhibited by requiring sample pre-treatment to remove the dominant water resonance that often render complex samples difficult to interpret. Environmental and biological research, in particular, require the understanding of complex environmental matrices as close to their natural state as possible. In this article, a pulse sequence developed specifically for the most challenging natural sample was reported and offers unmatched water suppression in both 1D and 2D NMR spectroscopy. In addition, its use was demonstrated on a range of environmental and biological samples to demonstrate its interdisciplinary versatility. TWINS water suppression represents a key step in the advancement of NMR spectroscopy and opens the possibility of studying a sample directly from the environment to NMR to determine structures, interactions and processes in their natural state that would otherwise be inaccessible. The robustness of TWINS water suppression in addition to its relative ease of implementation should allow for widespread application in many fields and opens up many novel applications in environmental research.
REFERENCES


Chapter 4

Soil Organic Matter: Unravelling the Most Complex Biomaterial on Earth

This material is under review for publication

4.1 Abstract

Since the isolation of soil organic matter in 1786, tens of thousands of publications have searched for its structure. Nuclear magnetic resonance (NMR) spectroscopy has played a critical role in defining soil organic matter but traditional approaches remove key information such as the distribution of components at the soil-water interface and conformational information. Here a novel form of NMR with capabilities to study all physical phases termed Comprehensive Multiphase NMR, is applied to analyze soil in its natural swollen state. The key structural components in soil organic matter are identified to be largely composed of macromolecular inputs from degrading biomass. Polar lipid heads and carbohydrates dominate the soil-water interface while lignin and microbes are arranged in a more hydrophobic interior. Lignin domains cannot be penetrated by aqueous solvents even at extreme pH indicating they are the most hydrophobic environment in soil and ideal for the sequestration of hydrophobic contaminants. Here, for the first time, a complete range of physical states of a whole soil can be studied. This provides a more detailed understanding of soil organic matter at the molecular level itself key to develop the most efficient soil remediation and agricultural techniques, and better predict carbon sequestration and climate change.
4.2 Introduction

Soil is the foundation of all life on Earth. It acts as a habitat, a nutrient source, a carbon sink and mediator, a regulating body for water flow, a filter, a platform for infrastructure, and a source for minerals and resources [1]. All living things rely on soil and its degrading health has a far reaching impact. With a warming planet comes the possibility of a release of carbon into the atmosphere from the largest active organic pool on the planet, further exacerbating global warming [2, 3, 4]. Soil erosion also contributes to global warming and food instability [4, 5]. Less soil equates to less area for agriculture. With agricultural, industrial and household processes comes the release of contaminants which irreversibly bind to hydrophobic components in soil organic matter (SOM). Contaminant interactions with soil are poorly understood because of our lack of understanding of soil structure leading to inefficient remediation strategies [6, 7, 8]. Remediating sites in the USA and the European Union are estimated to cost 1.4 trillion Euros citeOkx4. All of these processes are defined by a plethora of molecular level interactions within the soil itself that define its chemistry, reactivity and kinetics. Therefore, a deeper understanding of soil structure is desperately required.

Soil has been reported as the “most complex biomaterial on earth” and it is widely accepted that the structural complexity of SOM and lack of understanding thereof is a major impediment to progression in the field [9]. Traditionally SOM was thought to be comprised predominately of a unique category of cross linked structures termed humic substances. However, more recent work has shown that extracted SOM is better described as a complex mixture of plant and microbial inputs at various states of decay present at the time of extraction [10, 11]. In 2011 Schmidt et al. published a seminal review highlighting that a wide range of ecosystem properties will also define the persistent organic residues in soil [12]. As such, understanding the structure, water accessibility, conformation, interfaces, and reactivity of SOM with its surrounding environment and in its natural swollen state is now more imperative than ever.

Nuclear magnetic resonance (NMR) spectroscopy has traditionally been tailored to samples of a particular physical state which has proven to be disadvantageous for soils and other environmental media. In their natural state, soils exhibit a continuum of phases where everything from crystalline solids to solution like molecules are present. Section 4.4 elaborates on these issues.
4.3 Materials and Methods

4.3.1 Sample Preparation

Xylan, alkaline lignin extract, and bovine serum albumin were purchased from Sigma Aldrich and were used as is. The lipid extract was extracted from pine needles with the extraction procedure noted elsewhere [13]. These samples (35mg) were swollen with 60µL of DMSO-d₆. The soil sample used in all experiments was obtained from Hampstead Park in Dublin, Ireland. Hampstead Park soil is a Grey Brown Podzol with an organic carbon content of 6.8% ± 0.4%, retrieved from an open public area located within Albert College Park. 70µL of DMSO-d₆ was added to 35mg of soil for the 1D ¹H experiments and 2D HSQC. For the neutral soil the same procedure was repeated using D₂O. Acidic and basic soil samples were made by adding 65µL of D₂O to soil and then adding 5µL of either NaOD or DCl. The pH was measured using a glass electrode and adjusting for the deuterium isotope effect. In acidic conditions, the pH equivalent was 1.8 and in basic conditions, it was 9.3.

For solid state CP experiments, each sample was run with 35mg of soil. The D₂O and DMSO-d₆ samples were prepared as described above. The dry sample was homogenized with solid NaCl to dilute the total mass of soil to 35mg.

4.3.2 NMR Spectroscopy

NMR experiments were performed on a 500 MHz Bruker Avance III Spectrometer using a prototype MAS 4mm ¹H-¹³C-²H comprehensive multiphase NMR probe fitted with an actively shielded magic angle gradient. All samples were spun using a spinning speed of 6666Hz and all experiments were performed at 298K. 1D ¹H experiments were performed with 1024 scans, a recycle delay of 5 × T₁, and 4096 time domain points. The solvent signal (water or DMSO) was suppressed using presaturation water suppression. Spectra were apodized through multiplication with an exponential decay corresponding to 1Hz line broadening in the transformed spectrum and a zero filling factor of 2. Diffusion edited experiments were performed with a bipolar pulse longitudinal encode-decode (BPLED) sequence. 2048 scans were collected using encoding/decoding gradients of 1.2ms, 52.25 gauss/cm, sine shaped gradient pulse, a diffusion time of 180ms, and 8192 time domain points. Spectra were apodized through multiplication with an exponential decay corresponding to 10Hz line broadening in the transformed spectrum,
and a zero filling factor of 2. In the $T_2$ filtered experiments, the $T_2$ delay was set to 600µs and the pulse train was repeated 100 times resulting in a pulse train length of 120ms. Inverse Diffusion Edited (IDE), Inverse $T_2$, and Recovering relaxation losses Arising from Diffusion Editing (RADE) were created via subtraction from the appropriate controls as previously described [14]. For spectral editing, the spectra were scaled until the spectra being subtracted was nulled leaving a difference spectrum containing positive signals [14].

$^1$H-$^13$C CP experiments were performed with a contact time of 2ms, a recycle delay of 1s, and 512 points in the time domain. The number of scans performed was 102400. Soil spectra were apodized through multiplication with an exponential decay corresponding to 30Hz line broadening in the transformed spectrum, and a zero filling factor of 2. The summation spectrum of the model components was created by iterative fitting of individual components to create a spectrum most similar to that of the soil used.

All $^1$H-$^13$C Heteronuclear Single Quantum Coherence (HSQC) NMR experiments were performed using a second prototype MAS 4mm $^1$H-$^13$C-$^19$F-$^2$H comprehensive multiphase NMR probe fitted with an actively shielded magic angle gradient. Spectra were collected in phase sensitive mode using echo/anti-echo gradient selection, a $^1J_1^1$H-$^13$C (145Hz), and a relaxation delay of 1s. 1024 time domain points were collected for each of the 96 increments for all HSQC experiments. The F2 planes were multiplied by an exponential function corresponding to a 15Hz line broadening, while the F1 dimension was processed using sine squared functions with a $\pi/2$ phase shift and a zero filling factor of 2. 7782 scans were collected for each increment for the soil sample while the number of scans for the standard compounds varied from 256 to 716 scans per increment.

4.3.3 Integration

Spectral integration was performed using AMIX software and the regions were chosen based on the position of each of the specific regions for this soil (see Table 4.1 for a complete list). Overall integration percentages were calculated relative to the total integration value of the entire spectrum. Individual components were scaled appropriately to create the summation CP spectrum and the corresponding scaling factor was applied to each component for each integration region. The sum of all components for a given region was used to calculate relative percent contribution for each component.
Table 4.1: Chemical shift regions of integrations in the $^{13}$C CP

<table>
<thead>
<tr>
<th>Region</th>
<th>Chemical Shift Range (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aliphatic</td>
<td>0 - 49.1</td>
</tr>
<tr>
<td>Methoxy</td>
<td>49.1 - 61.7</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>61.7 - 95.2</td>
</tr>
<tr>
<td>Anomeric</td>
<td>95.2 - 114.1</td>
</tr>
<tr>
<td>Aromatic</td>
<td>114.1 - 167.2</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>167.2 - 200</td>
</tr>
</tbody>
</table>

4.4 Holes in Present Day NMR Technology

One major impediment to studying soil structure has been the lack of instrumentation to study complex environmental samples in their natural state [15]. This instrumentation must be able to provide the molecular level resolution required to identify and quantify the components present and their associations. NMR spectroscopy is a powerful tool to study complex environmental samples and their interactions at a molecular level [16]. Traditionally, however, NMR spectroscopy has developed largely as two separate fields, one dealing with solid crystalline structures and the other studying soluble compounds. Soil contains a broad continuum of structures ranging from crystalline solids to solution like compounds thus instrumentation to study all of these components is required.

Solid state NMR analysis requires least sample preparation, however $^1$H-$^1$H dipolar interactions in the solid state are considerable and reduce the information that can be obtained from $^1$H NMR resulting in the less sensitive $^{13}$C nucleus most commonly being used for detection [16, 17]. While solution state NMR can provide very high resolution fingerprints of soil organic matter, the technique is restricted to soluble components only.

The situation was improved in 1996 with the advent of high resolution magic angle spinning (HR-MAS) a technique that can study both soluble and semi-solid components [18]. HR-MAS NMR on whole soils yields high resolution spectra with the ability for multidimensional analysis, and has been applied to study whole soil interactions with contaminants at the soil-water interface [19]. However, HR-MAS probes do not have the radio frequency (RF) power handling capacities to study the solid component in soils which play a key role in long term contaminant
sequestration and soil stabilization [20, 21, 22].

Comprehensive multiphase nuclear magnetic resonance (CMP-NMR) spectroscopy contains the following technology to study a continuum of physical states between crystalline solids and solution like compounds: lock and susceptibility matching for ideal $^1$H lineshape employed in solution state NMR, magic angle spinning and magic angle pulsed field gradients used in HR-MAS NMR experiments, and high power circuitry to generate intense $B_1$ fields to accommodate for solid state NMR experimentation. In this chapter we employ the use of Comprehensive Multiphase (CMP) NMR which combines all the electronics from solution state, semi-solid and solid state NMR into a single NMR probe [14]. The resulting technology permits an uncompromised analysis of liquid, semi-solid and solid components within intact and unaltered samples.

### 4.5 Two Dimensional (2D) Structural Characterization by CMP-NMR

Before soil can be understood in its natural state it is critical to decipher the structural components present. This is best accomplished using a solvent to universally swell all components in soil. Dimethyl sulfoxide (DMSO) is a polar, aprotic solvent capable of breaking hydrogen bonds and penetrating both polar and hydrophobic domains. This allows a relatively comprehensive swelling of soil in turn permitting structural assignment through high resolution “gel state” 2D NMR experiments [23]. The identification of the main structural components in soil is a critical precursor to better understand confirmation and organization in more environmentally relevant aqueous systems later in the study. Figure 4.1 displays the two dimensional (2D) heteronuclear single quantum coherence (HSQC) spectra of four model compounds chosen to represent the main structural categories that are known to contribute to SOM [10]. To represent carbohydrates, xylan was used since it is found naturally in plant cell walls, bovine serum albumin was used to represent protein input, an alkaline lignin extract was used to represent lignin and a waxy lipid extract from pine needles to represent aliphatics in soil. Readers should note that the biopolymers chosen are not meant to represent the full diversity of components present in soil. For example, xylan will not represent the diverse range of carbohydrates including cellulose and hemicellulose. However, all carbohydrates are comprised of similar sugar sub-units. Therefore, these four components provide an indication as to where these major structural categories
Figure 4.1: The four panels on the left show 2D HSQC spectra of the four model compounds used to characterize SOM. The numbered boxes indicate regions where $^1$H-$^{13}$C correlations of important chemical bonds arise that are present in soils. Each numbered box represents:

1) Aliphatic single bonds and amino acid side chains, 2) Alpha proton on amino acids, 3) Methoxy signal from lignin, 4) $\text{CH}_2$ groups from carbohydrates, 5) Anomeric protons from carbohydrates, 6) Aromatic lignin and phenyl alanine, 7) Aromatic inputs from amino acids tyrosine and tryptophan. Once these four spectra are overlaid, each box is filled and the spectrum closely matches that of whole soil. This indicates that soil has strong contributions from carbohydrates, lipids/aliphatics, protein, and lignin. Readers should note that lignin does not contain strong aliphatic resonances and the aliphatic region in the Aldrich lignin sample likely indicates some aliphatic species have been co-extracted along with the lignin. Furthermore, the Aldrich lignin sample contains a relatively strong contribution from syringyl units (red box) commonly found in hard woods, whereas the lignin from the grassland soil has only a trace of these components.
resonate in various NMR experiments.

HSQC provides connectivity information between directly bonded $^1$H and $^{13}$C units. In simple terms it can be thought of as a high resolution ($\sim$200,000 signal capacity [24]) fingerprint of the H-C framework in a complex mixture. The overlay compared to an HSQC spectrum of a whole soil (Figure 4.1) shows that SOM is reasonably well represented by a complex mixture of various biopolymeric materials that will be abundant in plant and microbial soil inputs. Previous work based on NMR simulations of theoretical structures has demonstrated that traditional humic models based on novel cross linked molecules can be easily differentiated from plant and microbial soil inputs if they were present in abundance [10]. The HSQC data do not contain evidence for cross linked novel structures but instead indicate the vast majority of the SOM to be closely related to the logical plant and microbial inputs that are expected in soil likely present at various states of oxidation and decay.

4.6 Confirming the Molecular Composition Using $^{13}$C CP NMR

HSQC provides a high resolution fingerprint of soil components that allows relatively easy structural assignment. However, the drawback of HSQC is that only swollen domains are observed. Later in the manuscript the fraction of soil impenetrable by DMSO is considered in more detail. For now, the quantity of the total soil carbon that is consistent with the combination of the four main categories of compounds identified by HSQC in the previous section is explored. This is most easily investigated by employing solid state NMR.

In its most basic form, cross polarization (CP) when applied to a fully dried soil will provide a compositional overview of all the carbon in a sample. The CP NMR spectra of the four components overlaid (Figure 4.2A) closely matches that of the soil confirming that the soil is consistent with a complex mixture dominated by and derived from carbohydrates, proteins, lignin, and lipids.

It was also possible to determine the approximate relative contribution of each component. This was done by using the weighted contribution used to create the addition spectrum in Figure 4.2, and applying this to the integrals of the specific regions that have been identified (Table 4.2). This allows for determining the contribution of each of the components towards individual
Figure 4.2: (A) $^{13}$C CP comparison of the dry whole soil sample (brown) compared to the weighted summation CP spectrum of the individual components used in Figure 4.1 (black). The similarities between the two spectra suggest a large proportion of the soil spectral envelope is consistent with, or derived from, these biopolymers. (B) Comparison of the dry whole soil sample compared to the same sample with water added and (C) DMSO added. Carbohydrates, aliphatics and carbonyl carbons are the groups that have become attenuated, hydrated, and have gained mobility. DMSO impacts all regions demonstrating its indiscriminate nature compared to water.
regions found in the $^{13}$C spectrum of the soil as well as the overall contribution to the soil itself and is presented in Table 4.2.

Table 4.2: The relative percent contribution of each model component to the specified spectral region in CP.

<table>
<thead>
<tr>
<th>$^{13}$C Integration Regions</th>
<th>Model Components</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Aliphatic</td>
<td>46.9%</td>
</tr>
<tr>
<td>Lignin</td>
<td>44.0%</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>8.3%</td>
</tr>
<tr>
<td>Anomeric</td>
<td>1.8%</td>
</tr>
<tr>
<td>Aromatic</td>
<td>17.9%</td>
</tr>
<tr>
<td>Carbonyl</td>
<td>46.1%</td>
</tr>
<tr>
<td>Overall</td>
<td>27.4%</td>
</tr>
</tbody>
</table>

It was found that the protein, lignin and carbohydrates all contributed roughly 30% to the signal intensity from the overlay spectrum. It is also interesting to note the significance of protein in the aliphatic, lignin and carbonyl regions. The large contribution from protein in soil organic matter has been addressed previously and has been shown to be from microbial biomass [25].

### 4.7 Penetrability of SOM Components

With a reasonable estimate of the major structural components in soil, it is possible to use CMP-NMR spectroscopy to extract some basic information of the solvent accessibility of the various soil components. Solid state NMR provides an interesting start point in this endeavour. CP can be used to probe the components of a soil that are water accessible as these components will become attenuated compared to other SOM components. In a completely dry sample, CP is very efficient as it relies on permanent H-C dipoles which exist in solid structures. However, upon swelling, if water penetrates a structure then local dynamics are introduced which modulate the H-C dipolar interactions in turn reducing CP efficiency. In our previous work, we have shown that for solution like compounds no CP signal is observed while mobile gels are strongly
attenuated [14].

Figure 4.2B shows a $^{13}$C CP overlay of a dry and hydrated whole soil, the same grassland soil sample used in Figure 4.1. The dry soil represents the compositional overview of all the $^{13}$C in the sample. After the addition of water, the aliphatics, carbohydrates and the carbonyl carbons preferentially gain molecular mobility after hydration indicating they are readily available at the soil-water interface. Conversely, the aromatic region (mainly from lignin) does not change upon swelling indicating this material is not accessible to water. There could be three potential explanations for this. First, the lignin components could be mainly associated with large impenetrable plant fragments. Second, they could be buried away from the surface of the soil or lastly, the components are highly hydrophobic and water cannot penetrate them. It is important to note that the aliphatics, carbohydrates and carbonyl groups are not completely attenuated indicating a good portion of these components remain in the solid domain post swelling. Quantification demonstrates that $\sim 25\%$ of the SOM is exposed at the water interface leaving $\sim 75\%$ of the material not in contact with water.

The lower panel (Figure 4.2C) is similar to the one above except that DMSO was used as a solvent that can swell both hydrophilic and hydrophobic domains and penetrate deeper into the soil. Overall, $\sim 55\%$ of the signal has been attenuated by soil components swelling and becoming mobile while $\sim 45\%$ is still remains. However it is important to stress that many gels, especially rigid gels, can still undergo CP, and the fraction undergoing CP after DMSO addition, will represent both gel and rigid structures. Therefore, the actual true crystalline solid and inaccessible portion of soil is likely much smaller than 45\%. With DMSO as a solvent, the aromatic and aliphatic components are swollen while in the water hydrated soil, they were not. This demonstrates that these groups are solvent accessible to DMSO only and one reason water cannot penetrate this domain is because of their hydrophobic properties.

### 4.8 Investigating Soil Components Based on Mobility Using $^1$H CMP-NMR

Solid state CP based approaches provide an excellent overview of changes induced in the total carbon with swelling. Conversely, $^1$H detection methods can provide more detailed and selective information about the critically important soil-aqueous interface [14]. This arises as proton
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Figure 4.3: $^1$H spectra of the wet soil sample edited based on diffusion properties. (A) A reference spectrum illustrating all components at the water interface. (B) Inverse diffusion editing represents compounds that have unrestricted diffusivity and is dominated by carbohydrates. (C) Molecules that have restricted diffusion due to macromolecular size and/or sorption. Aliphatics have a large contribution likely being adsorbed at the surface of a soil colloid. (D) Recovering relaxation arising from diffusion editing experiment highlights fast relaxing semi-solid/rigid compounds and have strong contributions from both carbohydrates and aliphatics. The red brackets indicate an artifact from incomplete water suppression.

signals in true solids are too broad to observe using conventional low-power solution state experiments due to extensive spectral broadening resulting from strong $^1$H-$^1$H dipoles in the solid state. In swollen domains these interactions are modulated by solvent induced motion resulting in the swollen components being selectively detected [14].

Figure 4.3 compares spectral editing approaches based on molecular self-diffusion and is described in Section 1.5.3. In diffusion based editing it is the property of the entire molecules (not sub-units within the molecule) that give rise to spectral discrimination. The reference spectrum in Figure 4.3A contains signals from all components at the soil-water interface and is dominated by aliphatics and carbohydrates. Figure 4.3B highlights the molecules with free diffusion (i.e.
truly dissolved) and contains signals from carbohydrates but not lipids. Figure 4.3C highlights the species with restricted diffusion (i.e. macromolecules, surface bound components, swollen polymers). Lipids dominate indicating that they are exposed but not dissolved at the interface.

A significant contribution from carbohydrates is also apparent indicating swollen as well as dissolved carbohydrates also exist at the interface. Figure 4.3D represents components that are most rigid/semi-solid. This experiment termed Recovering relaxation losses Arising from Diffusion Editing (RADE) accounts for semi-solid components that relax too fast to be observed by the diffusion experiments [14]. The spectrum shows a relatively similar contribution from both lipids and carbohydrates in the semi solid state which is consistent with their large contribution to the CP after swelling (Figure 4.2B) and likely arises, in part, from cell walls and lipid membranes.

To further probe the soil-water interface, relaxation filtered NMR can be applied. Relaxation filtering differs from diffusion based editing by providing information about local dynamics within a molecule rather than the diffusion of the entire molecule (see Section 1.5.3)). Figure 4.4A shows the reference spectrum of the soil while Figure 4.4B shows the components with fast local dynamics. Consistent with the diffusion data the spectrum is dominated by carbohydrates. However, relaxation based editing also shows some signals from aliphatics which have been identified as lipids. First, it was important to note that no lipids were found to be freely diffusing and were likely bound at the soil-water interface. On the other hand, a disproportionately large signal of the hydrophilic end of the lipid is found in the free molecular motion spectrum indicating that the COOH group of the lipid is facing the water interface and has some degree of freedom. The hydrophobic tail is immobilized, likely interacting with a hydrophobic soil component. The inverse $T_2$ spectrum (Figure 4.4C) highlights components with fast relaxation (large molecules and semi-solids) and confirms that swollen carbohydrate and lipids with restricted dynamics are abundant at the soil-water interface.

Figures 4.2-4.4 collectively illustrate the continuum that exists in components of SOM with respect to molecular dynamics. More than half of the SOM has the potential to interact with DMSO while the rest is inaccessible possibly within larger physical fragments. Water can only penetrate roughly a quarter of all SOM and selectively interacts with specific groups in the soil, namely carbohydrates and lipids. These carbohydrates are found in all states, from free in solution to the bound state, while lipids are orienting themselves where the hydrophilic end is
towards the water interface and the hydrophobic tail is encapsulated by other soil components. Aromatic groups like lignin and protein are not penetrated by water. The pH dependence of the components in soil will reveal more information on their orientation and deserves consideration.

Figure 4.4: $^1$H spectra of the wet soil sample that show components with varying degrees of intramolecular motion. (A) Reference spectrum showing all components available at the water interface. (B) Illustrates compounds that have dynamic molecular motion ($T_2$ filtered), such as dissolved molecules or flexible domains. (C) Shows components with restricted mobility (inverse $T_2$ filtered, A minus B), such as macromolecules swollen biopolymers. The red brackets indicate an artifact from incomplete water suppression. *Acetic acid also contributes to this signal.

4.9 pH and the Soil-Water Interface

Figure 4.5 shows the $^1$H soil spectrum in four different chemical environments: natural pH (water), acidic, alkaline, and DMSO. The soil in acidic conditions has the lowest signal-to-noise ratio ($S/N$) of all the solvents. This suggests that in an acidic environment, it is more difficult to penetrate the SOM interface which is consistent with the negatively charged properties of the
majority of SOM components [11]. At higher pH these species will become charged and repel each other opening up the organic structure such that water can more easily penetrate. This hypothesis is supported by the spectrum at neutral pH which shows a considerable increase in $S/N$ in turn indicating that components are now more easily penetrated by water.

When exposed to base, more functional groups appear compared to the acid and water environments. In Figure 4.6A, the acetyl group from peptidoglycan appears at 2.1ppm in the diffusion edited spectrum. The shoulder at 1.5ppm (start of red arrow) is dominated by lipids (units $\gamma$ to a double bond and oxygen in an ester), while the 2.1ppm region is from the overlap of lipids and peptidoglycan. As the pH is raised this region appears to become more dominant and is difficult to assign to lipids alone, strongly suggesting additional contributions of peptidoglycan at the soil water interface. This indicates that microbial cell walls have more contact with the aqueous water phase as the pH is raised. This is confirmed with reference to the aromatic region. A $T_2$ filtered experiment is shown for the aromatic region as these experiments reduce baseline roll making spectral comparison easier. There are strong similarities between the spectral profile of pure microbes (cultured from soil) and the profile of the alkaline soil itself in this region (Figure 4.6B). These signals arise from microbial protein and indicate at high pH, water can penetrate and swell the microbial cells. At neutral pH few if any signals are seen indicating microbes are largely removed from the soil-water interface.

In summary, signals from peptidoglycan (from microbial cell walls [16, 26]) and microbial protein are abundant at the soil water interface at high pH but not at neutral pH. This suggests that microbes are not randomly distributed throughout soil which is consistent with previous work that indicates that microbes are largely associated with clay surfaces in whole soils [25, 27]. Even at high pH the profile of the aromatic region (Figure 4.6) of the soil is dominated by signals from microbial protein suggesting lignin has little exposure to aqueous solvents in the whole soil.

Figure 4.5D shows the spectrum in DMSO that has the ability to penetrate into both hydrophilic and hydrophobic domains. In comparison to the aqueous solvents used, the aliphatic portion of the spectrum is much more prominent and a clear spectral envelope for protein is also apparent. Proteins in the presence of strong lipid signals have been previously assigned in detail to soil microbial cells [28]. Indeed DMSO is known to lyse cell walls and will cause the content of microbial cells to be released [29]. Interestingly this process is less prominent in aqueous solvents (high pH can also lyse cells [30]) supporting the argument that soil microbes are less
Figure 4.5: $^1$H spectra representing different functionalities that arise in whole soil after being exposed to different solvent conditions. (A) Under acidic conditions the signal intensity and the carbohydrate contribution is lower relative to neutral pH conditions (B). When exposed to alkaline conditions (C), the intensity increases namely in the aliphatic region and the aromatics are discernible from the noise. After the addition of DMSO-d$_6$ (D), H-bonds and hydrophobic interactions are broken. The baseline of the spectrum closely resembles that of protein and could be a large contribution from microbes after being lysed by DMSO.
Figure 4.6: (A) $^1$H DE spectra of the aliphatic region of soil in different solvent conditions. The small acetyl signal from peptidoglycan of microbes downfield of the $\alpha$ lipid signal becomes more visible with the addition of basic conditions. This illustrates that microbes are available to the solvent in basic conditions. (B) $^1$H spectra of the aromatic region with relaxation filtering to even baseline effects. The aromatic region of microbes matches closely with that seen in basic soil conditions furthering the hypothesis that microbes are more available with the addition of base.

accessible to water in the whole soils and are in part protected by the hydrophobic nature of soil organic matter.

The triple peak pattern (~0.9pp, 1.3ppm and 2ppm) seen in the aliphatic region of Figures 4.4 and 4.5 is indicative of fatty acids or lipids. The signal residing at ~0.9ppm represents the terminal CH$_3$ groups of lipids. Since there is only one CH$_3$ group, it makes sense that this signal is the smallest in intensity. The tallest resonance (~1.3ppm) represents the long chain (CH$_2$)$_n$ groups of which there are many owing to its intensity. Of particular interest is the signal at ~2.0ppm. While some of this resonance is from acetic acid (a common degradation product in soil) a good portion of the intensity arises from CH$_2$ adjacent to the COOH in lipids. In Figure 4.4B,
the contribution from the mid chain \((\text{CH}_2)_n\) and terminal \(\text{CH}_3\) groups are relatively small, while the alpha \(\text{CH}_2\) is relatively large in intensity. No lipids were seen in the true solution phase (Figure 4.4B, free diffusion). When considered together this indicates that the polar groups are sticking out of the water-soil interface hence showing rapid local dynamics at the polar end (Figure 4.5B). Meanwhile, their hydrophobic tails are interacting with, or partially buried under, the soil surface. This is an interesting finding and suggests that lipids could be a key component of soil likely contributing to properties such as swelling, soil aggregate structure and possibly representing a conduit between the polar interface and the more hydrophobic interior for organic contaminants.

4.10 Piecing the Puzzle Together

Figure 4.7 is a graphical representation of the summation of the findings presented in this manuscript. Starting from the solution state, we have demonstrated that of the major components found in soil, carbohydrates are the only species to exist free in solution. These could be fragments of sugars that are being broken down. Going deeper into the gel phase, lipids and carbohydrates exist. The polar groups of the lipids are exposed to the water interface while the hydrophobic tail is less available. The semi-rigid phase would exist in the region slightly deeper from the water interface where some mobility exists. Here carbohydrates and lipids are once again found.

Microbes are partially exposed when ionic associations are broken under alkaline conditions and completely exposed when lysed in DMSO. This indicates at neutral pH the species are deeper in the soil where water is harder to penetrate, and they are protected from aqueous exposure by both ionic and hydrophobic interactions. Finally, lignin is only available once DMSO is introduced and the soil is uniformly and extensively swollen. This suggests lignin is either much deeper within the soil or that it is too hydrophobic to be available at the water interface. Either way, lignin seems to be a likely candidate for hydrophobic contaminant sequestration as a lignin moiety will offer protection from the polar water environment.

While protein is a main component of soil, it was not included in this final diagram because previous work has demonstrated that of the vast majority the protein in soil originates from the microbes themselves [28]. This is consistent with studies that have shown free protein to be
very labile and have short residence times within soil [31, 32]. This does not limit protein to only being within microbes, it certainly exists outside of the microbial cell wall at some level as there will be continual input and turnover from fresh organic deposition.

Figure 4.7: A representation of the possible arrangement of soil components based on the findings in this manuscript. Free in solution are carbohydrate (green) species while at the soil-water interface, carbohydrates and lipids (yellow) where the polar groups are faced towards the water. Not accessible to water are lignin (purple) and microbes (white) as well as other carbohydrate and aliphatic components.

Inorganic species like minerals are not considered here, as the NMR evidence present here does not provide direct information on the mineral component. This being said, minerals undoubtedly play a key role in determining how the soil is ordered. There have been studies suggesting that lignin binds strongly with minerals [33] making minerals the possible nucleus of a soil globule where lignin surrounds it followed by carbohydrates, aliphatics and microbes. Another area worth exploring is determining how organic components interact with one another within soil. These missing links need future investigation and will be required to complete our understanding as to the molecular organization of soil as a whole.

4.11 Implications to Soil Chemistry

There has been some controversy as to whether it is the aliphatic or aromatic component of soil that plays the most significant role in contaminant sorption [34, 35, 36, 37]. This study suggests that both are likely important and helps support the hypothesis that both aliphatic and aromatic
molecules each provide hydrophobic domains that facilitate sorption of hydrophobic xenobiotics [33, 38, 39, 40]. NMR evidence presented here indicates that lipid polar heads are at the soil-water interface suggesting that just beneath this there may be a hydrophobic layer of aliphatic chains that could be important for sorption of hydrophobic contaminants and soil building with subsequent SOM moieties. Species could feasibly become stuck in this layer itself and or potentially pass through (somewhat like a membrane) into a domain inaccessible by water. Similarly, lignin residues appear impermeable to water thus once a hydrophobic contaminant passes from the aqueous phase into a lignin domain it may be very difficult to release it. Lignin protection has been observed using other methods and has been shown to bind strongly to clay surfaces which could be a cause for its differential recalcitrance in soils with varying clay content and size fractions [41, 42, 43, 44, 45].

This research has shown how CMP-NMR has helped to uncover the complexity of soil by revealing the organization with respect to the water interface at the molecular level using samples that are in their unaltered state. Soil is a complex entity and needs to be studied in its natural state. Layering, associations and general solvent accessibility are critical to unravel and understand soil swelling, aggregate structure, contaminant sorption and are closely tied to carbon turnover and soil fertility. Previous work has demonstrated that microbial inputs are much higher than previously thought [28]. Interestingly, these species are not seen at abundance at the soil water interface at natural pH but are fully available in DMSO. This suggests in part the microbes are partially protected by the hydrophobic nature of SOM. Microbes may actively reside away from the aqueous-soil interface, possibly associated with mineral surfaces [46, 47], to prevent their erosion. Considering this, it is clear that the biology of soil is equally as important as the chemistry, both of which are controlled by water accessibility and the physical conformation of the materials in soil. Hence, tools such as CMP-NMR which provide the comprehensive molecular level information in the natural state are critically needed and will likely prove essential to address many of the big questions in environmental research and beyond.
REFERENCES


Chapter 5

From Spill to Sequestration: The Molecular Journey of Contamination via Comprehensive Multiphase NMR

This material is under review for publication

5.1 Abstract

Understanding xenobiotic sorption and sequestration at the molecular-level is essential for predicting transport, reactivity, bioavailability and ecotoxicology, as well as optimizing remediation. Nuclear Magnetic Resonance (NMR) spectroscopy has proven to be one of the most powerful tools for unravelling soil structure, however NMR has traditionally been developed as two different fields, one dealing with solid samples and the other with solutions. To truly understand soil reactivity, it is best to perform studies in the natural swollen state, as drying, fractionation and extraction may remove the aqueous-organo interface, negate biological activity and alter conformation and structure. Comprehensive Multiphase NMR (CMP-NMR) spectroscopy is a relatively new technique that permits all components (solutions, gels, solids) to be studied and differentiated in whole unaltered natural samples. A wide range of CMP-NMR interaction and editing-based experiments are combined to follow a contaminant from the solution state (after a spill) through the gel state and finally into the true solid state (sequestered). Two perfluoro contaminants pentafluorophenol (PFP) and perfluorooctanoic acid (PFOA) are used here as examples. Kinetics, contaminant orientation, and soil components responsible for binding are revealed for each phase in a fully swollen soil providing unprecedented molecular insight into the sorption and sequestration processes.
5.2 Introduction

Cost estimates for the clean-up of contaminated sites in the European Union and the United States are in the order of magnitude of 1.4 trillion euro [1]. A fundamental gap in our scientific knowledge as to how and why chemicals become sequestered in the environment hampers the development of optimal remediation strategies. This has led to a situation where 70% of soils from contaminated sites in Germany are being stored in vast waste disposal facilities until better clean-up technologies can be developed [2]. In addition, there are important debates over the molecular fate of perfluorinated chemicals (PFCs) in humans and the environment which are now ubiquitous [3]. Some scientists hypothesize the partitioning of PFCs into lipid membranes is the cause of PFC persistence in human blood whereas other groups argue the irreversible binding to human serum albumin is responsible [4]. These examples demonstrate the need for novel technologies that can provide an unprecedented molecular-handle on interactions from dynamic to permanent, and across the full spectrum of states including solution to solid in intact samples.

One major factor that impedes our understanding contaminant-soil interactions in whole samples is the lack of analytical methods that can provide comprehensive and detailed molecular-level information on unaltered samples in their native state. Soils are very challenging to study as they are highly heterogeneous and contain a range of components in different phases. For example, organic matter dissolved in soil pore behaves as a solution, swollen organic matter is more “gel like”, whereas organic matter sorbed to minerals surface is closer to a true solid.

Nuclear Magnetic Resonance (NMR) spectroscopy has shown great promise in uncovering molecular evidence regarding contaminant sorption in soils or soil extracts [5, 6, 7]. However, traditional NMR technologies namely solution state and solid state focus on analysis in a single physical state. Solid state NMR spectroscopy commonly requires dry packed soils which exclude water, thus removing information about the swollen state conformation and key interfaces such as the aqueous-solid interface, as well as halting biological activity. Conversely, solution state NMR requires samples to be isolated and dissolved, which removes materials from their native state (likely altering molecular-level interactions and conformation) and biases only extractable components. In 1996 an NMR technology termed High Resolution Magic Angle Spinning (HR-MAS) was introduced and allowed for swollen samples to be studied intact [8]. Unfortunately,
HR-MAS probes cannot handle the high power radio frequency pulses which are required for high power decoupling or cross polarization, both necessities for the study of solid components. In the case of a spill, a hydrophobic contaminant may enter the soil as a solution. As it interacts with the soil organic matter it may take on properties of a gel before being sequestered into hydrophobic pockets with more solid like character. To fully elucidate the process of chemical sequestration it is important to be able to investigate and differentiate the molecular interactions from all phases.

Comprehensive Multiphase NMR (CMP-NMR) introduced in 2012 is a novel technology which allows for the analysis of all phases in intact unaltered samples [9]. CMP-NMR incorporates a lock and full susceptibility matching (for ideal solution state line-width), magic angle spinning and pulsed field gradients (required to study the gel state), and high power circuitry (to generate intense $B_1$ fields required for solid state analysis). In combination, these features allow for all components to be studied and differentiated in soil analysis in its whole natural swollen state and thus maintaining the chemical and physical properties responsible for contaminant sorption in the environment.

In this study, the potential of CMP-NMR to study both kinetic transfer and molecular interactions during contaminant sequestration is demonstrated. Specifically, the fate of perfluorooctanoic acid (PFOA) and pentafluorophenol (PFP) in a grassland soil is tracked. Kinetic transfer between the solution, gel and solid states is monitored and binding orientation of the contaminant and the soil materials (protein, lignin etc.) responsible for sequestration are identified for each phase. PFOA is ubiquitous in the environment and has been found in humans and animals [3, 10, 11, 12]. It has also shown to bind strongly with soil [13, 14, 15]. Halogenated phenols like PFP have been detected in environmental systems because of their use as wood preservatives, pesticides, and herbicides [16]. Perfluoronated contaminants are studied here due to their increasing environmental importance and distribution [3]. In addition, the $^{19}$F nucleus is very helpful as it provides a sensitive nucleus unique to the contaminant important for some of the experiments described here. For other contaminants that do not contain fluorine, $^2$H labelled analogues could theoretically be used and while less sensitive should permit the study of practically any organic contaminant within a complex matrix.
5.3 Materials and Methods

5.3.1 Soil and Contaminant Information

The soil sample used in all experiments was obtained from Hampstead Park in Dublin, Ireland. Hampstead Park soil is a Grey Brown Podzol with an organic carbon content of 6.8% +/- 0.4%, retrieved from an open public area located within Albert College Park. PFP (99%) was obtained from Synquest Laboratories (Alachua, FL, USA). PFOA (96%) was obtained from Sigma Aldrich (Oakville, ON, Canada).

5.3.2 Sample Preparation

All sorption experiments were performed using 30mg of soil in 4mm zirconium NMR rotors. For kinetics experiments, the sample was run immediately after preparation and experiments were recorded as quickly as possible. The delay time from contaminant addition to the first scan in the spectrometer was roughly 2min. For PFP preparation, 51µl of D2O (Cambridge Isotope Laboratories, Tewksbury, MA, USA) was added to 30mg of pre-weighed soil in a 4mm zirconium NMR rotor and mixed well. 9µl of PFP was added after the water was added to simulate PFP entering a hydrated soil. PFOA (7.5mg) were dissolved in 60µL of D2O and the pD was adjusted to 7.0 using NaOD and a glass electrode. The deuterium ion effect was accounted for by adjusting to 7.4 on the pH scale [17]. The 60µL solution was added into 30mg of soil in a 4mm NMR rotor and lightly mixed. The rotor was then sealed and capped as quickly as possible to begin the kinetics experiments.

For the saturation transfer difference (STD) and cross polarization (CP) studies, sample preparation was exactly the same as the kinetics experiments with the addition of an equilibration time. Samples were prepared and stored at room temperature for 1 month before STD and CP experiments were performed. This was to ensure equilibrium conditions to better simulate the environment and to allow for the maximum amount of contaminant to sorb.

All experiments used in this manuscript are relatively low in sensitivity as they specifically target the interactions between the contaminants and the soils and only a finite number of contaminants will be interacting with any given binding site at any time [18]. Many of the experiments used here have been adapted from medical research to study drug binding in tissue [19, 20, 21]. The experiments such as CP and STD selectively detect only the sub-fraction
interacting with the receptor (soil) and it is recommended the ligand (contaminant) is always added in excess [19]. For this reason the quantities used here are not reflective of what may be found in the greater environment but more representative of spill conditions or agricultural application. PFOA is used commonly used in high concentration as an anti-foaming agent in the applications of agrochemicals [22]. In the case of PFOA the high concentration of PFOA had little effect on the aqueous-soil interface indicated by the $^1$H NMR spectrum of the soil (Figure 5.2). Conversely, the PFP did act like a solvent swelling the soil itself indicated by a changing $^1$H profile on addition. This indicates that the interactions may alter and many not necessarily be the same as trace concentrations, the implications of this are discussed in the main text. However, it also demonstrates that CMP-NMR with the ability to study the contaminants, interfaces, mechanisms etc. is an important tool to identify which chemicals impact the soil structure/interface itself fundamental truly understanding sorption. Considering that agrochemicals are rarely applied alone and are often mixed with soil surfactants it is probable that co-solvent effects are of environmental relevance and could be assessed using CMP-NMR.

5.3.3 NMR Spectroscopy

All NMR experiments were performed on a 500 MHz Bruker Avance III Spectrometer using a prototype MAS 4 mm $^1$H-$^{19}$F-$^{13}$C-$^2$H CMP-NMR probe fitted with an actively shielded magic angle gradient. All samples were spun at a rate of 6666 Hz and all experiments were performed at 288K.

Spectral Editing

Diffusion-edited $^{19}$F and spectra were collected using a bipolar pulse pair longitudinal encode-decode (BPLED) sequence with inverse gated decoupling [23]. Scans were collected using encoding/decoding gradients of 1.2ms at 50 gauss/cm and a diffusion time of 100ms. Inverse diffusion edited (IDE) and recovering relaxation losses arising from diffusion editing (RADE) were created via difference from the appropriate controls as previously described [9].

Kinetics Experiments

Solution state (inverse diffusion editing) and gel phase (diffusion editing) experiments used spectral editing techniques described elsewhere [9]. Considering the relatively low sensitivity of
the experiments the intensity was refocused into a single spike which greatly improves signal-to-
noise for detection. This technique is called CPMG single spike (CPMG-SS) described elsewhere
[24], and permits the kinetic experiments to be repeated more rapidly providing higher temporal
resolution. The drawback is loss of chemical shift information, but this is not an issue if the
goal is simply to see monitor the quantity of contaminant in each phase over time.

1D $^{19}$F solution and gel phase CPMG-SS experiments were performed with 16 scans, 8
dummy scans, a recycle delay of $5 \times T_1$, and 4096 time domain points. A $7\mu s$ 44W pulse was
used for echo formation with 988 loops and a delay of $12\mu s$. Spectra were zero filled by a factor
of 2 and processed using an exponential function corresponding to a line broadening of 10Hz in
the final spectrum.

Solid state $^{19}$F-$^{13}$C CP CPMG-SS experiments for kinetic curves were performed with a
80-100% ramp, a contact time of 2.5ms, and high power composite pulse decoupling (Spinal-64).
The number of scans were 320 with 8 dummy scans, a recycle delay of $5 \times T_1$ and 1024 time
domain points. A $15\mu s$ 6W pulse was used for echo formation with 250 loops and a delay of
$18\mu s$. The power of the CPMG train was reduced to protect the NMR probe as both high power
decoupling and the CPMG train had to be applied simultaneously. Spectra were zero filled by
a factor of 2 and processed using an exponential function corresponding to a line broadening of
50Hz in the final spectrum.

Experiments for Molecular Interactions

Solid state $^1$H-$^{19}$F CP experiments for the CP build up curves were performed with a linear
ramp defined by 100 points ranging from 80-100% of the full contact power. The contact time
was array from 0.05-5ms and high power composite pulse decoupling (Spinal-64) applied during
acquisition. The number of scans were 8192 with 16 dummy scans, a recycle delay of $5 \times T_1$
and 2048 time domain points. Spectra were zero filled by a factor of 2 and processed using an
exponential function corresponding to a line broadening of 50Hz in the final spectrum. Build up
curves were plotted and analyzed using SigmaPlot (Version 11, Systat Software, Inc. Germany).

Solid state $^{19}$F-$^1$H CP experiments were performed using a modified CP experiment with
$^1$H pre-saturation added for suppression of the residual water signal. An 80-100% ramp was
used with a contact time from 2ms. The number of scans were 28672 with 16 dummy scans, a
recycle delay of $5 \times T_1$ and 2048 time domain points. Spectra were zero filled by a factor of 2
and processed using an exponential function corresponding to a line broadening of 50Hz in the final spectrum.

$^1$H-$^{19}$F observe Heteronuclear Saturation Transfer Difference (HSTD) experiments were performed with an NMR experiment similar to the one described in Longstaffe et al [5]. 138240 scans were used for PFOA and 77824 scans for PFP were acquired with 128 dummy scans, 16384 time domain points, and a recycle delay of $5 \times T_1$. 0.05W of saturation power was delivered to all $^1$H nuclei by scanning the full range of $^1$H resonances from $+200$ to $+4000$ Hz in 200 Hz increments. Off resonance pulses were $+1000000$ Hz away. For PFP, a $T_2$ filter was used to isolate gel phase interactions because signal from semi-rigid signals complicated quantification. The $T_2$ filter employed 32 loops with a delay of 150$\mu$s between each loop and optimized empirically to suppress the rigid components only. Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 10 Hz in the final spectrum. Reference spectra were acquired with the same conditions except with 16 scans for PFOA and 512 scans for PFP both with 8 dummy scans.

$^{19}$F-$^1$H Reverse Heteronuclear Saturation Transfer Difference (RHSTD) was performed as previously described by Longstaffe et al. 128000 scans were used for PFOA and 46080 scans were used for PFP with 128 dummy scans for each, 4096 time domain points, and a recycle delay of $5 \times T_1$. 0.0001W of saturation power was delivered to $^{19}$F nuclei that were found to bind strongest in the $^{19}$F observe STD. This was -38923Hz in PFOA and -77895Hz for PFP. Off resonance pulses were $+1000000$Hz away. Spectra were zero filled by a factor of 2 and processed using an exponential function corresponding to a line broadening of 10Hz in the final spectrum. Reference spectra were acquired with the same conditions except with 1024 scans and 8 dummy scans.

5.3.4 Kinetic Curves

All kinetics curves were run in triplicate. Absolute signal integration was used to calculate solution and gel experiments and the quantities of each were based on the contribution of each sub-phase to the total $^{19}$F signal. The solid signal was hard to quantify directly as the CP process is not fully quantitative as such only relative change is reported for the solid components. Estimates of the amount in the solid phase are possible through difference which is discussed more in the main text. Integrals were taken using the multi-integrate tool in AMIX (Bruker
BioSpin, Rheinstetten, Germany), and relative integrals were taken using the first time point as the reference spectrum.

5.3.5 Epitope Maps

Epitope maps were calculated using relative signal integration through the multi-integrate tool in AMIX. HSTD and RHSTD epitope maps were created by comparing a saturation experiment to a standard experiment without saturation and intensities were normalized to the signal that had the greatest positive change in the difference spectrum (i.e. received most saturation) is denoted by the 100%. $^1$H-$^{19}$F CP epitope maps were derived through comparison to the $^{19}$F spectrum of the solid compound.

5.4 Spectral Editing of the Sub-Phases Within the Soil

To isolate individual phases using CMP-NMR spectroscopy a combination of diffusion filters were employed along with CP as a filter for true solids. These experiments have all been described before by Courtier-Murias et al [9] and are only briefly described here.

Freely diffusing soluble species can be selected through an inverse diffusion experiment (IDE). The IDE is created by the subtraction of the diffusion edited spectrum (diffusion gradient on, contains larger components with restricted diffusion) from a reference spectrum (diffusion gradient off, contains a profile of all components) leaving just the molecules that are free to diffuse (i.e. dissolved).

Molecules with restricted diffusion can be emphasized using diffusion editing (DE). The gradient encodes the spatial position of signals at the start of the experiment and then decodes them at the end [25]. The signal from molecules that diffuse and move positions are not refocused and are attenuated. Essentially the DE spectrum contains molecules that are bound and/or have restricted diffusion. To appear in diffusion editing the molecules must not move physical position, but have enough solvent induced bond dynamics to survive the relatively long delays in diffusion editing (see below for discussion of relaxation). As such, the molecules selected here in diffusion editing are going to be strongly biased towards those sorbed at the solid-water interface, which fulfills both of these conditions.
Due to the relatively long delays required for diffusion editing some molecules with fast relaxation are not observed. These signals can be recovered in a complimentary experiment termed Relaxation recovery Arising from Diffusion Editing (RADE). The experiment recovers the components that otherwise may be missed due to their fast relaxation if diffusion editing is used alone [9]. In conventional RADE, \(^1\text{H}\) is normally used for detection and true solids are not detected due to \(^1\text{H}-^1\text{H}\) dipole broadening [9]. However, \(^19\text{F}-^19\text{F}\) dipole couplings are generally weaker making perfluorinated compounds easy to detect using single pulse experiments. Therefore, the \(^19\text{F}\) RADE will include contributions from rigid-gels, semi-solids and some true solids.

A simple way to select the true solid components is to use cross-polarization. In a completely dry sample, CP-MAS is very efficient as it relies on permanent dipoles which dominate in solid structures. However, upon swelling, water introduces local dynamics which modulate the dipolar interactions in turn reducing CP-MAS efficiency. In previous work, it has been shown that no CP-MAS signal is observed for dissolved species while mobile gels are strongly attenuated. In this study either the dipole between the soil and the contaminant (\(^1\text{H}-^19\text{F}\)) or within the contaminant itself (\(^19\text{F}-^13\text{C}\)) can be used to monitor the solid phase. In both cases, CP is most efficient if the dipole is not modulated which occurs in the true solid phase. In this study, to demonstrate the applicability of both \(^1\text{H}-^19\text{F}\) and \(^19\text{F}-^13\text{C}\) CP, the \(^19\text{F}-^13\text{C}\) is monitored here for the purposes of kinetics and later in the chapter the more informative \(^1\text{H}-^19\text{F}\) dipole is exploited in regard to binding mechanism in the solid phase.

Figure 5.1 illustrates the decomposition of the spectra into the individual phases. PFP forms three distinct chemical shift bands corresponding to free (1), bound (2), and solid (3) clearest for the ortho fluorine. The IDE clearly highlights the free form (Figure 5.1B), while the DE highlights the form with restricted diffusion (Figure 5.1C). The RADE identifies a range of material from gel through solids (Figure 5.1D). CP (Figure 5.1E) selects only the broadest components consistent with the PFP that has been sequestered into the soil to form a true solid like phase. The use of spectral editing filters makes the assignment of the various bound states easier and less ambiguous. Unfortunately, for PFOA there are no chemical shift perturbations on binding but estimates of contributions from each phase can be performed by comparing the intensities of signal in each sub spectrum relative to the total (Figure 5.1A).
Figure 5.1: An overview of the spectral editing used in this manuscript. A) A conventional $^{19}\text{F}$ spectrum shows all phases. Using the ortho position as an example, solution (1), bound (2), and sequestered (3) signals can be discerned whereas this is not the case for PFOA. B) The solution phase is isolated using IDE. C) Diffusion editing illustrates the small number of contaminants that are at the interface of soil and water. D) The RADE accounts for any fast relaxing species lost in the delays of the diffusion experiments and encompasses a range of materials from rigid gels through to true solids. E) $^1\text{H}-^{19}\text{F}$ CP selects for components with rigid dipoles (most solid like material present). PFP shows much higher signal to noise in the CP experiment indicating more material sequestered into a solid like environment. F) CP-CPMG-SS was employed to focus the $^{19}\text{F}-^{13}\text{C}$ signals into a single spike to improve sensitivity and decrease experiment time. The single spike experiment permits the total amount in the solid fraction to be monitored with higher temporal resolution for kinetic studies.
The results indicate that PFP interacts to a greater extent with soil than PFOA. Only $\sim 9\%$ of the total $^{19}$F signal is from molecules in the dissolved state with $\sim 88\%$ sequestered into the rigid-gel, semi-solid, solid state (Figure 5.1D). Conversely over half of PFOA remains in the true dissolved state with 41% showing some signs of sequestration. The predicted $K_{oc}$ (ACD labs Phys. Chem. Suite 2012) at pH 7 are 36 for PFP and 3 for PFOA which are in line with a strong interaction for PFP. Interestingly the $K_{oc}$ at pH 5.5 is for PFP is predicted to be 578 while PFOA remains relatively unchanged. Therefore if lower pH micro environments exist within the soil these could show a strong preference towards PFP. The smaller size and roughly spherical shape of PFP may also aid in penetration into the soil. In addition, changes were noted in the $^1$H spectrum upon the addition of PFP. PFP acts like a solvent and is capable of swelling and penetrating a wider range of soil components (Figure 5.2). This behaviour is interesting, easily observable by NMR, but not easily predictable based on the physical-chemical properties of the molecule alone.

Unfortunately the absolute percent in the true solid phase cannot be accurately determined as CP cannot be quantified easily with respect to the total $^{19}$F signal. In the case of PFP as the solid components can be seen in the total spectrum, the contribution in Figure 5.1A can be used as a rough guide. In the case of PFP it appears that a considerable fraction ($\sim 50\%$ based on estimations from Figure 5.1A) is sequestered into the solid phase. Unfortunately, accurate estimates are not possible for PFOA as the solid components cannot be discerned in Figure 1a. However, there is $\sim 3$ times the signal-to-noise in the PFP spectrum than PFOA (Figure 5.1E) which suggest PFP sequesters $\sim 3$ times more than PFOA. The reason behind this is not completely clear but can be elucidated using other NMR techniques demonstrated in the rest of this manuscript starting with individual phase kinetics.

5.5 The Soil Swelling Power of PFP vs PFOA

Figure 5.2 illustrates the influence of PFP compared to PFOA. The experiment highlights the components at the soil interface [26]. Figure 5.2A is a control spectrum of soil swollen without the addition of contaminant and was performed with more scans than Figures 5.2B and 5.2C. Figures 5.2B and 5.2C were collected with the same number of scans, so the signal to noise ($S/N$) ratio can be compared in this case. In the case of PFOA the signal to noise is relatively
low, and this along with the generally similar profile to the control indicates the soil is not extensively swollen by PFOA. The main difference to the control is the reduced carbohydrates. This has been previously documented and is seen when the pH of the sample is decreased which in turn makes the carbohydrates more dense and less accessible to water [27]. While the pH of the PFOA was neutralized prior to addition, it is likely exchange within the soil or concentration of PFOA at the interface may lower the pH prevent swelling of the soil organic matter and reduces the accessibility of the carbohydrates to the aqueous solvent. Conversely PFP has a considerable affect on the soil-water interface. The S/N is much higher indicating more components are swollen in general. Interestingly some aromatic groups are exposed. Such groups are normally not available at the soil-water interface and are known to be buried under the surface in hydrophobic domains [26]. The swelling of the aromatic and aliphatic components, both considered the most hydrophobic in soil, indicate the liquid PFP behaves like a solvent and can penetrate into hydrophobic domains. This is interesting as it demonstrates that in the case of a spill it is not the properties of the soil alone that will determine its capacity to take up contaminant, but that specific physical action of the contaminant itself on the soil may also play a key role in some case. The ability of CMP-NMR to monitor all aspects of the soil provides a unique window to study such subtle processes.

5.6 Kinetics Between Phases

Kinetics studies are very useful to explain sorption properties including adsorption mechanisms, surface properties of the adsorbent, and degree of affinity for the adsorbent [28]. Most commonly the concentration of sorbent left in the solution phase is used to calculate the amount sorbed by difference. CMP-NMR is highly complementary as it can follow not just the free contaminant but also as it penetrates into the gel and becomes sequestered into the solid phase.

In this study, multiple phase kinetics were obtained in a whole soil through the use of CMP-NMR [9] and CPMG-SS [24] techniques, and absolute quantification was performed in a similar method as was done in Figure 5.1. Figure 5.3A plots the kinetics in the solution and gel for PFP and PFOA. The solution phase kinetics curve for PFP had a small and steady decline while the solution state curve for PFOA had a rapid decline and reached equilibrium quickly. In the case of PFOA, it is possible that the soil becomes saturated and cannot take on any
It is evident that PFP swells soil much more efficiently than PFOA as is indicated by the high S/N and broadness of the aliphatic and aromatic regions. More contaminant. On the other hand, PFP’s solvent like properties may aid the soil swelling allowing more molecules to penetrate.

The kinetic curve for the gel phase PFP and PFOA (as determined by diffusion editing) both declined continuously. Essentially these are molecules that have some bond dynamics and have relatively long relaxation, but show no physical movement and will therefore be dominated by molecules bound at the soil-water interface. This signal starts at ∼4-5% indicating a rapid initial sorption at the interface (before the first NMR experiment) followed by a slow decline caused by slow transfer across the interface into the more rigid soil interior. For both contaminants only a small fraction (<5%) of the contaminant was bound to the interface at any given time.

Finally, the solid state (Figure 5.3B), represent the fraction that has essentially become fully sequestered and taken on properties of a solid. Such soil domains are likely hydrophobic and removed from the water which itself would induce local dynamics in turn attenuating CP signal. While it is possible from Figure 5.1 to estimate that there is ∼3 times the PFP in the solid phase when compared to PFOA, unfortunately as mentioned above direct quantification from CP is not possible. As such, Figure 5.3 simply shows the relative change over time. For PFOA
Figure 5.3: The kinetics curves for PFP and PFOA interaction in whole soil for individual phases are plotted. A) Solution state curves show PFOA losing intensity quickly and reaching equilibrium while PFP exhibits a steady decline. Gel phase curves show slow declines for both contaminants while PFP has a stronger decline. B) Solid phase curves show a strong increase for PFOA while a smaller increase exists for PFP. Standard deviations were all smaller than the width of the representative markers and were excluded for figure clarity.

this is considerable, rising $\sim 80\%$ after the 2 hour window. This is correlated with a reduction in the solution fraction and demonstrates that the contaminant is crossing the soil interface and becomes sequestered into the hydrophobic interior. Interestingly the signal after the first point (10mins) is already significant (relative change 1.0) and demonstrates that more is sequestered in the first 10mins than over the next 2 hours (relative change 1.8 after 2 hours). In turn, this demonstrates that initially the soil can rapidly take up large quantities of contaminant and this contaminant becomes rapidly sequestered into the solid phase. A similar trend with PFP is observed but the increase in the solid fraction and the corresponding decrease in the solution phase are less pronounced. In addition, the solid fraction of PFP is still rising very slightly after 2 hours (along with a slight decrease in the solution fraction) indicating the kinetics for
PFP have yet to reach full equilibrium. This is likely in part due to the solvent effects of PFP which further swell the soil overtime permitting further sorption. While kinetics provides a deeper understanding of the sorption process it provides little direct evidence of the molecular-level phenomena behind the process. To address this, additional CMP-NMR experiments can be utilized to identify what soil components bind the contaminant as-well as the binding orientation of the contaminant itself.

5.7 Molecular Orientation of the Contaminant During Interaction

The molecular orientation of the contaminant in both the dynamic- and solid- phase are discriminated. As both approaches use different NMR techniques the dynamic interactions will be discussed first.

5.7.1 Orientation in the Dynamic Fraction

A technique called Heteronuclear Saturation Transfer Difference (HSTD) was employed. As $^1$H atoms from the soil are saturated, the saturation perturbates through spatial dipoles and is transferred to the $^{19}$F atoms that are in closest spatial proximity to the soil [4, 5, 29]. The $^{19}$F groups in the contaminant closest to the soil receive the most saturation, which can be quantified and converted into an epitope map. Each group is normalized to the strongest binding position (denoted 100%) and interactions at other positions are given relative to this. To suppress signals from solid signals, a short CPMG filter was determined empirically and was included prior to acquisition. Only components that show interaction with soil will be detected in HSTD while free components will be completely subtracted. Hence, the HSTD result can be thought of as the average binding orientation of the dynamic fraction. This will include molecules bound at the interface as well as more dynamic species partitioning with the soil.

Figure 5.4A illustrates the results of the epitope maps calculated for PFP and PFOA for the dynamic phase. The $^{19}$F position with the strongest interaction is normalized to 100% and other positions were reported relative to this. PFP displayed non-specific binding since each $^{19}$F atom is fairly close to 100%. However, there is a very slight preference towards the OH indicted...
by the strongest interaction of the $^{19}$F nuclei closest to the OH group. This is logical since the aqueous-soil interface has been shown to be relatively polar [26], and with a pKa of 5.5 the OH may be protonated in more acid micro-environments and could be more likely to associate with rather than repel the soil organic matter (itself mainly anionic). Conversely with a pKa of 3.8 [30], PFOA will be mainly negatively charged and largely repel organic matter. Indeed this is reflected in its epitope map which demonstrates a bias towards the hydrophobic tail end as the point of interaction. This consistent with previous work that illustrates that $^{19}$F rich functional groups play a key role for contaminant sorption to soil [4, 5, 31].

Figure 5.4: Epitope maps for PFP and PFOA in the gel and solid phases in whole soil were calculated using HSTD and CP. For PFP, there is a discriminant shift in binding mechanism from the gel to the solid phase where the OH group becomes less important in solid phase sequestration. Conversely, the CF$_3$ group in PFOA drives the sorption in both the dynamic and solid phases.
5.7.2 Orientation in Solid Fraction

The solid phase epitope map was derived from $^1\text{H}$$-^{19}\text{F}$ CP. In this case the $^1\text{H}$ magnetization is passed from the soil, which acted as a source of $^1\text{H}$, to the contaminant ($^{19}\text{F}$) via rigid dipoles exclusive to the contaminant in the solid phase. The inherent signal from $^{19}\text{F}$ is suppressed via phase cycling and the only way for signal to arise in the experiments is from magnetization transferred from the soil protons. As such, the signal in the resulting spectrum is proportional to the interaction with the soil from which an epitope map can be constructed.

Figure 5.4B shows the binding orientation for the molecules in the solid state. PFP in the gel state showed little orientation preference (Figure 5.4A, however in the solid phase there is a clear bias towards the most hydrophobic segment of the molecule (Figure 5.4B). This confirms that hydrophobicity is the overall driving factor for sequestration of PFP within soil. The reduced interaction at the OH strongly suggests the final sequestered site is removed from the water interface. The solid interactions of PFOA also support this finding. However, extracting information along the chain is challenging due to spectral overlap and low S/N, and therefore only an average for the CF$_2$ portion of the chain can be reported. It is clear that in the solid state the interaction further favors the hydrophobic end of the molecule and that the COOH group has little influence. This is consistent with previous binding studies that determine the halogenated functionalities in organic contaminants show the strongest preference for both soil and extracted soil organic matter [31, 32]. Combined kinetics and molecular orientation studies paint a detailed molecular pictures as to how and why contaminants become sequestered in soil. However, information regarding the exact soil components that bind the contaminants is missing.

5.7.3 CP Build Up Curves

To further support the binding orientation in the solid phase, CP $^1\text{H}$$-^{19}\text{F}$ build up curves were recorded for each $^{19}\text{F}$ chemical shift position. From these curves, the heteronuclear $^1\text{H}$$-^{19}\text{F}$ dipole couplings can be extracted. Essentially both build up curves and conventional $^1\text{H}$$-^{19}\text{F}$ CP produce the same binding orientations. CP build up curves were created by monitoring intensity of individual signals of the contaminants in the $^{19}\text{F}$ spectra as contact time, a CP parameter, is increased. When plotted and fitted according to Equation (5.1) then curves such
as those illustrated in Figure 5.4 result.

\[ I(t) = \frac{I_0}{1 - \frac{T_{HF}}{T_{1\rho}}} \left( e^{-t/T_{1\rho}} - e^{-t/T_{HF}} \right) \]  \hspace{1cm} (5.1)

CP efficiency is dependent on two variables, the strength of the dipole between two heteronuclei and the relaxation pathways that exist in a spin system. The first variable is represented by \( T_{HF} \) in Equation (5.1). It dictates how fast the curve is built up. The second variable is determined by \( T_{1\rho} \), or the relaxation rate constant relative to the rotating frame. The shorter the relaxation time, the quicker the curve will decay.

From this equation, extracting the \( 1/T_{HF} \) constants from each curve and comparing each provides information of the relative strength of the interaction that exists. Because this can be done for individual signals of the contaminant, CP build up curve data are another means to test the dynamics in the solid state and to assess the relative strength of binding each position within the molecule.

For PFP the \( 1/T_{HF} \) values were in agreement with what was found with the CP epitope map (Table 5.1 and Figure 5.5). The para fluorine had a largest \( 1/T_{HF} \) value which translates to a larger dipole strength and therefore contributes the most to PFP binding in soil relative to the other fluorine positions. The meta position had a slightly weaker dipole while the ortho position, closest to the OH group, had the weakest dipole strength. The PFOA build up curve data also followed the same trend that was observed with the CP epitope map. The CF\(_3\) group had a stronger dipole compared to the CF\(_2\) groups. Therefore, the CF\(_3\) group is interacting the strongest with the soil and is responsible for contaminant sequestration while the CF\(_2\) groups contribute relatively less to PFOA sorption in soil.

Table 5.1: List of \( T_{HF} \) values and errors calculated from plotting CP build up curves using Equation (5.1)

<table>
<thead>
<tr>
<th></th>
<th>( T_{HF} )</th>
<th>Error</th>
<th>( 1/T_{HF} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFOA CF(_3)</td>
<td>1668.671</td>
<td>210.2461</td>
<td>5.99\times10^{-4}</td>
</tr>
<tr>
<td>PFOA CF(_2) Chain</td>
<td>1947.775</td>
<td>614.2192</td>
<td>5.13\times10^{-4}</td>
</tr>
<tr>
<td>PFP ortho</td>
<td>1357.25</td>
<td>95.7044</td>
<td>7.37\times10^{-4}</td>
</tr>
<tr>
<td>PFP meta</td>
<td>1276.245</td>
<td>77.7812</td>
<td>7.84\times10^{-4}</td>
</tr>
<tr>
<td>PFP para</td>
<td>1217.832</td>
<td>273.734</td>
<td>8.21\times10^{-4}</td>
</tr>
</tbody>
</table>
Figure 5.5: After plotting intensity with contact time and fitting with Equation (5.1), $^{1}\text{H}^{19}\text{F}$ CP build up curves are formed and $1/T_{\text{HF}}$ constants can be extracted which describe the strength of interaction between different functional groups from the contaminants and soil. The results support the findings in the solid state epitope maps suggesting that the $^{19}\text{F}$ in the para position contributes the most to PFP sequestration while the CF$_3$ is responsible for binding in PFOA.

### 5.8 Elucidating Soil Components Responsible for Binding

#### 5.8.1 Orientation in Solid Fraction

To elucidate which sub-components of the soil bind the contaminant, the reverse of HSTD and CP experiments described above are employed. Reverse HSTD (RHSTD) is used to identify dynamic interactions. In this case the contaminant ($^{19}\text{F}$) is saturated with magnetization which is passed onto the soil $^{1}\text{H}$ for detection. $^{1}\text{H}^{1}\text{H}$ dipoles in the macromolecular soil components propagate the saturation such that the binding species are selectively coated. A difference approach is used for detection and in simple terms all components that are not interacting with
the contaminant cancel leaving a spectrum of components solely in contact with the contaminant. In the case of $^1$H detection, true solids are attenuated because they have wide spectral profiles. Therefore the RHSTD biases interaction with dynamic soil components at the interface, in exchange, or swollen sites.

The spectral profile of the dynamic components interacting with PFP is relatively intense and presents a wide range of soil components (lignin, lipids, protein, and carbohydrates) and is shown in Figure 5.6A. This is in line with PFP having solvent like properties that can indiscriminately swell all components in soil organic matter to some extent. Conversely, very little if any signal is observed for PFOA even if the experiment is run for many days. This is interesting as previous studies have shown with extracted soil organic matter in solution RHSTD spectra can be obtained and PFOA show a preference towards protein [29]. This suggests the protein fraction in extracted matter (known to be lysed from microbial cells [33]) is not available at the soil interface in whole samples, and reiterates the importance of studying samples in their unaltered state as well as demonstrating the importance of physical accessibility in the sorption process.

If all the data presented so far are considered, it can be seen that very little PFOA is in the gel state (Figure 5.1) with the majority is either not interacting at all (~60%), or in the semi-solid/solid phase (~40%, Figure 5.1D). As such, it seems that PFOA exhibits a more specific targeted interaction. If these locations are available it will bind and become sequestered as a solid, but if not PFOA will stay mainly in the solution state without swelling the soil interface. As mentioned above $^1$H-$^1$H dipoles lead to spectral components for solids many KHz wide. The lack of signal in the RHSTD suggests the components that PFOA interacts with have a strong solid like character.

5.8.2 Soil Components Involved in Solid Interactions

The solid phase components that are responsible for sequestration of contaminants are identified through $^{19}$F-$^1$H CP. In this case the $^{19}$F magnetization is passed from the contaminant to the soil ($^1$H) via rigid dipoles found in the solid phase. The result is a spectrum of the soil components that bind the components in the solid phase.

PFP demonstrates a markedly different spectrum than for the dynamic fraction. This spectrum is dominated by a broad aromatic resonance consistent with lignin in soil. This is in line with
the previous work that have shown perfluoroaromatics binding to lignin components in isolated organic matter and soil [7, 29]. It has been previously shown that lignin aromatics are buried under the soil surface away from the water-soil interface [26]. The mechanism of PFP interaction could be the following. First PFP indiscriminately swells soils such that the lignin component are uncovered and once exposed shows a strong preference towards these sub-components. This is in line with previous work which identifies that the overlap of the electrostatic potential density profiles between the prefluoroaromatic contaminants and the aromatic sub-units of lignin are responsible for the strong interaction in extracted organic matter [5].

PFOA displays a strong preference towards the aliphatic components of soil. The apex of the soil component is centered on 0.7ppm rather than 1.3ppm apex which would be expected for long chain aliphatics. Indeed previous work on soil organic matter has shown these components are from soil protein mainly derived from microbes [33]. Furthermore, extensive NMR based work in human blood and soil organic matter have shown that PFOA specifically binds to albumin proteins [4, 29]. Albumins are by design fatty acid transport proteins and PFOA is essentially a perfluoronated fatty acid and therefore binds to the same sites, specifically Sudlow's site I, but irreversibly [4]. In whole soil it appears the fate of PFOA is the same as in extracted organic matter and binds to the protein fraction. It is not clear whether the protein fraction is within living, dormant or microbial necromass. If PFOA is within living microbial cells, then the microbial community could have a much more direct and profound role on contaminant sequestration, fate and transformation than previously anticipated.

5.9 Conclusion

This manuscript has demonstrated that CMP-NMR is a versatile technique to better understand and monitor contaminant sequestration at the molecular-level using PFP and PFOA as examples. Interaction mechanism changes between phases are important to understand short and long term contaminant sequestration. This research has developed a framework that can be employed to study other contaminants in other soils eventually having applicability in specific scenarios where remediation techniques are required.

CMP-NMR is a versatile tool to follow complex processes in complex environmental media. Its ability to analyze individual physical states provides two important opportunities:
Figure 5.6: To determine which components in soil sequester PFP and PFOA, $^{19}$F-$^1$H RHSTD was employed in the gel phase (A) while $^{19}$F-$^1$H CP is used in the solid phase (B). PFP shows an indiscriminate profile in the gel phase showing interaction with most soil components. In the solid phase however, PFP shows a strong preference towards aromatics (lignin). PFOA shows few interactions in the gel state, but a strong preferential interaction towards proteins (microbial biomass) in the solid phase.

1. Separating physical states spectroscopically allows the user to study complex media without chemical or physical treatment thus maintaining the natural state and important facets such as the aqueous interface, the biologically active state, and native conformation.

2. Providing unprecedented molecular detail and experimental versatility to extract novel molecular information that is hard or impossible to decipher using more conventional approaches.

CMP-NMR has a wide variety of uses and great potential in environmental research and other fields. The major drawback of CMP-NMR is its relatively low sensitivity. In large part
this arises not from the probes themselves but studying samples in their native state. Consider for example if natural soil is 50% water. Then a researcher aims to study $^{13}$C in solid components only in a swollen sample. If it is argued that only 50% of the organic matter is truly solid then the signal from the solid-state in the native state is only $\sim 25\%$ when compared to a conventional solid state NMR study that packs the sample dry.

Methods to overcome this problem revolve around further advancement of NMR technology including the development of CMP-NMR cryoprobes and dynamic nuclear polarization [34, 35, 36]. However the simplest solution may be the development of larger diameter probes that simply introduce more sample. In turn this may permit lower ratios of contaminant to soil to be used with an eventual and future transition towards trace contaminant levels.

Perfluorinated contaminants were used here as they are both environmental relevant, and fortuitously, $^{19}$F is an excellent NMR nucleus in terms of both sensitivity and spectral dispersion. However while many potential contaminants (agrochemicals, pharmaceuticals, personal care products) do contain $^{19}$F, the majority do not. In some cases other heteronuclei such as $^{15}$N, $^{27}$Al, $^{29}$Si, $^{31}$P, $^{51}$V, $^{75}$As, $^{113}$Cd, $^{199}$Hg, $^{207}$Pb may be naturally present and could be used along with a suitably tuned CMP-NMR probe. In the case of organic contaminants one solution may be to isotopically label contaminants with $^{2}$H. $^{2}$H can replace $^{1}$H without significantly altering the chemical properties of a compound permitting a wide range of contaminants to be studied albeit with lower sensitivity afforded by $^{2}$H NMR [37, 38]. Interestingly $^{3}$H is the most sensitive of all NMR nuclei (more than $^{1}$H), but its radioactive nature would require stringent safety protocols and certification that may be out of reach for most multi-user NMR centers. In summary, however, the ability of CMP-NMR to provide unprecedented information on both structure and molecular interactions, for all components in natural unaltered samples, suggest it has an important and key role in unravelling and explaining complex environmental processes both now and in the future.
REFERENCES


Chapter 6

Summary and Future Directions

This dissertation was split into two main categories of research, providing both experimental tools and application examples that should facilitate the study of soils in the natural state. To do this, the main issues preventing soil from whole swollen analysis have to be addressed. The first is the shear complexity of soil and the second are analytical instrumentation limitations in elucidating a matrix with multiple phase components. Soil is known to be the most complex mixture in the world including both inorganic and organic components and this complexity is what makes chemical analysis difficult. There are multiple components in soil each with their own function, dynamics, and physical conformation. Studying whole soil in its entirety using traditional methods translates into spectral complexity and overlap which prevents analysis. Therefore, initial research in soil chemistry isolated specific components based on chemical or physical properties and analyzed those components individually. This however was a compartmentalized approach to a matrix that derives its qualities from the interactions between its components.

The second barrier to studying soil in its natural state is the instrumentation itself. Many analysis techniques have prerequisite sample requirements where samples are conformed to allow instrumental analysis. Early spectroscopic techniques like UV-VIS, fluorescence, and infrared required either solid state or completely dissolved samples. Bulk analyses like elemental analysis and centrifugation either destroyed the samples in the process of analysis or required extracts to get results that were discernible. Mass spectrometry has its limitations in the amount of isomeric information it can provide but has the theoretical capacity to provide chemical formulae for
individual species. However, as discussed, the complexity of soil forces some sample purification depending on the ionization technique used.

Nuclear magnetic resonance (NMR) is a technique that has huge spectroscopic power and is thus extremely versatile. NMR can study chemical structure, structural conformation, interactions, dynamics, and kinetics. It comes close to studying soil in its whole state but still has some drawbacks with analytical requirements of sample pretreatment. Solid state NMR requires dry and packed solid material to be studied while solution state NMR needs completely dissolved material without any bubbles or particulates. It was only in 1996 that high resolution magic angle spinning (HR-MAS) was introduced and swollen or semi-solid samples could be analyzed. HR-MAS was very close to being able to study all aspects of soil in its natural state, however there were still spectroscopic limitations leading to the inability to analyze the solid phase.

It is clear that the two problems preventing natural analysis that are outlined above are interconnected. Soil is too complex to understand without some kind of separation technique, and many analytical techniques cannot analyze whole swollen soil. This dissertation has presented a viable solution to both of these issues in one analytical instrument. It is a fact that soil complexity hinders analysis, however, instead of physically or chemically isolating components, spectroscopic editing based on rigidity, connectivity, through space interactions, diffusivity, relaxation, and chemical shift can be employed to provide spectroscopic discrimination. The second problem around sample preparation can also be resolved by combining all NMR electronics into one probe such that all phases (liquids, gels and solids) can be analyzed negating the need for sample preparation.

Comprehensive multiphase NMR (CMP-NMR) resolves this last problem in that it uses solid state electrical components and design to allow for the generation of intense $B_1$ fields in combination with pulse field gradients, magnetic susceptibility matching, and lock channel required for gel and solution studies. CMP-NMR addresses the issues of sample complexity through its spectroscopic resolving power and allows for whole sample analysis on intact soil. Work that was not discussed in this dissertation included the physical construction and development as well as spectroscopic development of the CMP-NMR probes, both of which the author assisted in. The next steps in introducing CMP-NMR to the scientific community were performed in this thesis and included experimental development and application to a real complex matrix, both of which are summarized in the next section.
6.1 Chapter Summaries

6.1.1 CMP-NMR Experimental Development

Chapters 2 and 3 focused on experimental development of CMP-NMR to study soil in its natural state by addressing two main concerns, sensitivity and water suppression. To address sensitivity, a novel experimental method was developed and implemented to better predict experimental run time for a given signal to noise (S/N) ratio. Chapter 2 largely dealt with the application of $^{13}$C cross polarization (CP) which is commonly used for soil analysis and exhibits low sensitivity because environmental samples often have very low carbon contents, and the abundance of $^{13}$C nuclei is roughly 1%. Therefore, it is challenging to predict how long an NMR spectrum needs to be performed to obtain an adequate S/N ratio, or if a spectrum would be obtained at all. Often times, when an evening of free spectrometer time presents itself, a sample will be “screened” by running a 16 hour experiment to test if any signal is obtained. Many times there are no signals after 16 hours and sometimes there are. When there are signals present, a tough question then enters a spectroscopist’s mind: How long does this sample need to be run to obtain an adequate spectrum? Another issue with this approach other than the great deal of uncertainty is the amount of time needed to simply “screen” the sample to determine experiment time. NMR spectrometers are very costly with expensive and limited cryogens continuously boiling off, and 16 hours is a lot of valuable time. Hence, the CP-CPMG-SS experiment was developed to aid with this issue. This experiment adds multiple quick spin echoes during acquisition which depletes chemical shift variation but intensifies signals into one sharp resonance. Therefore, a 16 hour “screen” for spectrum quality can be performed in under 3 minutes. A mathematical relationship was determined for the CP-CPMG-SS experiment that permits accurate prediction of experimental time. This was demonstrated for various nuclei and shown to be valid for both solution and solid state NMR. Overall, this experiment improves detection and has interesting applications in other NMR optimization protocols and has already been tested for the use with $T_1$ relaxation time determination which can also take lengthy amounts of time [1]. Although this experiment is primarily applied to insensitive nuclei, its applications are not limited to those nuclei. In cases where an experiment is needed rapidly simply for detection purposes, the CPMG-SS element can be inserted into that experiment and used for detection. This is illustrated in Chapter 5 where its use in kinetics experiments including one dimensional diffusion and $^{19}$F-
13C CP experiments. The CPMG-SS element is therefore a useful experiment to have in any environmental NMR spectroscopists arsenal.

Chapter 3 tackled another important problem in soil NMR research, water suppression. To study soil in its whole natural unaltered state means to study it as is from the environment into the spectrometer. This process is naturally hindered by the presence of water in the sample which is abundant and ubiquitous in nature. In soil, water is present in free, exchangeable, and bound forms which broadens out the already intense water signal leading to a base that can cover the entire spectral region. In turn this overlap masks information from components at lower concentrations than the water. 1H NMR is a necessity in environmental NMR because of the vast array of 1H detect experiments available, many of which provide correlations between 1H and X nuclei, which are integral for structural and compositional analysis. As well, 1H NMR offers unmatched sensitivity. As such water suppression is an essential element fundamental to the success of all these experiments. This chapter builds on the most effective water suppression technique currently available, SPR-W5-WATERGATE. Three main elements were added to this technique to improve its efficacy including the addition of an exclusive 180° W5 inversion train before the water suppression pulse to remove far water, the addition of rotor synchronization to remove anisotropy, and improvements to phase cycling resulting in the experiment termed tailored water suppression for inhomogeneous natural samples (TWINS). In addition, a D2O lock capillary was developed to provide the spectrometer with a lock solvent isolating D2O from the sample, which can lead to deuterium substitution in exchangeable groups, and exhibit toxicity. The combination of the external lock with TWINS was tested on a variety of samples including terrestrial and aquatic sources without any sample pretreatment at all. What was found was that TWINS outperformed SPR-W5-WATERGATE in all cases without any loss to signal intensity or increase in the region attenuated by water suppression. Effectively, an 8-10ppm wide water was effectively suppressed with only a 0.8ppm region centered on the water where no signals were discernible. From 0.4-1.1ppm, the signals are attenuated and therefore not quantifiable. TWINS was also applied in 2D homonuclear experiments and was tested for compatibility with a perfect echo sequence as well. The end result was a robust water suppression technique and lock capillary that when used together could study samples in their natural state using CMP-NMR spectroscopy.
The CPMG-SS and TWINS techniques solve two main hurdles encountered when trying to analyze soil in its natural state using NMR spectroscopy. The next part of the thesis implements some of these approaches in analyzing soil in its natural state.

### 6.1.2 Soil Analysis Using CMP-NMR

After developing necessary tools to study soil in its natural state using CMP-NMR spectroscopy, this instrumentation was tested on a whole soil sample to understand its capabilities. At the same time, other techniques were developed for these specific applications to aid in soil analysis. CMP-NMR spectroscopy’s ability to isolate components based on various chemical and physical characteristics including solution, gel, and solid phase analysis, diffusion editing, and relaxation filtering using spectroscopic approaches was implemented and refined on soil. Chapter 4 of this dissertation aimed to discern soil composition and how the identified components are arranged while Chapter 5 set out to determine how compounds interact with whole soil.

At the beginning of this dissertation in Section 1.3.1, it was stated that soil organic matter is far too inhomogeneous to have a set repeating structure: there is no such thing as a soil sub-unit. The aim of Chapter 4 then was to understand the general composition of soil, and then determine the relative placement of those molecules in their natural state using numerous properties. The main components that were determined to largely make up soil were carbohydrates, protein, aliphatic molecules, and lignin. This was found using a 2D approach termed heteronuclear single quantum coherence (HSQC) and essentially creates a fingerprint of a specific type of molecule. For example, all carbohydrates show characteristic patterns in HSQC. There are key resonances that exist throughout all carbohydrates, and different carbohydrate structures show only small variations in terms of their HSQC $^1$H-$^{13}$C correlations. The same applies for proteins, lignin, or aliphatic compounds. A soil HSQC was compared to an overlay of those four components and minimal differences were found. All major correlations in the soil HSQC were accounted for in the overlay. This was confirmed using $^{13}$C CP by once again adding the CP spectra for the four components and overlaying it with a soil CP. These were the techniques used to characterize soil composition. The first differentiating factor that was studied was mobility due to hydration. Using CP, it could be determined which compounds were available at the water interface by finding components that decreased in intensity (water disrupts rigid dipoles leading to a reduction in the CP efficiency). The main groups that decreased were carbohydrates, fatty
acids, and carboxyl groups. Aromatic groups like lignin and proteins did not decrease in intensity illustrating their lack of bioavailability. To determine the relative mobility of the species that were mobile, diffusion and relaxation editing approaches were employed. Carbohydrates resided in multiple phases from semi-rigid through to fully dissolved while the fatty acids were abundant at the interface but sorbed with only their polar heads towards the aqueous phase. Finally, to understand what types of interactions held these components together different solvents were used including acid, base, and DMSO, and routine $^1$H spectra were collected. Base expanded the soil organic matter with signatures characteristic of microbes being exposed at high pH. DMSO, a solvent that can break hydrogen bonds and swell both hydrophobic and hydrophilic domains, was able to penetrate all components in soil. This resulted in the conclusion that hydrophobic interactions and hydrogen bonding likely may a key role in the protection of organic matter in soil. In conclusion, this chapter illustrated a viable approach to understanding soil organic matter composition and structure, and provided an important understanding of how soil components could be arranged at least in the case of the soil studied.

The last research chapter of this dissertation, Chapter 5, set out to investigate contaminant interactions with soil in its whole natural swollen state. The title of Chapter 5 is “From Spill to Sequestration: The Molecular Journey of Contamination via Comprehensive Multiphase NMR”. In this chapter the fate of two perfluorinated contaminants, perfluorooctanoic acid (PFOA) and pentafluorophenol (PFP), was monitored using CMP-NMR on whole swollen soil. Therefore, the contaminant’s interaction was profiled in different physical states within the same system using spectral editing techniques. Using diffusion editing (DE), inverse diffusion editing (IDE), and relaxation recovery arising from diffusion editing (RADE), the approximate quantity of contaminant in each physical state was determined. Next, kinetic curves were plotted from each phase for both contaminants which allowed for an understanding of contaminant behaviour in the solution, gel, and solid phases. PFP transitioned from the solution to solid phase over a longer period of time illustrating a less targeted approach to sorption while PFOA sorbed very quickly showing more discriminate binding. Heteronuclear saturation transfer difference (HSTD) and CP experiments revealed binding orientation of the contaminants. PFP showed minimal biases while interacting in the gel phase with a slight emphasis on the OH group, however, in the solid phase, there was a clear preference for the more hydrophobic para end of the molecule with respect to soil interactions. PFOA showed discriminate sorption through very
biased interactions with the CF$_3$ group in the gel phase and even more pronounced in the solid phase. Finally, Reverse saturation transfer difference experiments were performed to determine the components in soil relevant to PFP and PFOA interactions. PFP was found to interact with carbohydrates, aliphatics, and an emphasis on aromatic groups in the gel phase, and had a magnified preference for the aromatic groups (predominantly from lignin) in the solid phase with some interactions occurring with aliphatics. This confirms the broad range of interactions that was hypothesized in earlier experiments for PFP. PFOA illustrated strong sorption to aliphatic components specifically from proteins which was in line with the discriminate binding that was observed through all phases of interaction. Overall, this work was the first of its kind in that it followed contaminant interactions through the various phases in an intact swollen soil sample similar to environmental conditions and it provided a framework for understanding contaminant interactions in whole soil.

These two studies serve two purposes. The first is the advancement in understanding soil and its interactions with anthropogenic contaminants. By improving experimental conditions to closely mimic the environment with the help of CMP-NMR, these two research chapters help to propel scientific knowledge of soil to a new level. The hope is that other soil researchers will gain meaningful insight from this work as well as understand the importance and potential of studying soil in its natural state. This leads to the second purpose of this research which is elaborated in the next section.

6.2 Limitations to the Research Presented

This dissertation has provided the scientific community with findings that impact the environmental, analytical, and NMR chemistry communities. However, this work is novel because of the instrumentation that was developed specifically for the applications discussed. Therefore, many of the studies here are largely introductory and need development from other fields and disciplines to establish standard procedures. The following is a description of specific drawbacks of each study.

In chapter 2, the main drawback for determining experimental runtime is the need for a standard. In cases where the sample is completely unknown, a standard may be difficult to procure. As well, the paramagnetic content of some natural samples may be unknown and
finding a sample with similar paramagnetic content would be required. Therefore, without a standard, accurate determination of experimental runtime is not possible.

Chapter 3 introduced TWINS, a water suppression technique that was better than the current best for natural systems, SPR-W5-WATERGATE. There are not many drawbacks in using this technique instead of SPR-W5-WATERGATE since TWINS was shown to be superior in suppressing water and creating spectra that were more use-able. The main drawback of this technique is that the region around the water cannot be quantified. However, this same bandwidth exists with SPR-W5-WATERGATE with an exception that TWINS attenuates signals slightly more.

The main drawbacks of chapter 4 included its limits in the diversity of soils studied as well as omitting the inorganic portion of the soils. Because soil composition differs all over the world, it will be important to study other soils to validate these findings. As well, the inorganic portion of soil should be considered when discussing soil structure if a complete picture of soil is desired. In addition, the arrangement of organic species in soil with respect to other organic species is imperative to understand the fine aggregate structure. One way to accomplish this is by labelling an input like leaves or bark with an NMR active nucleus like $^{13}$C, characterizing the labelled species as it degrades, and then perform magnetization transfer experiments like STD or CP to determine which soil components are near the labelled species. Doing this for multiple labelled species and different soils will help shed light into how organic components are arranged with respect to one another in a soil leading to a finer understanding of aggregate structure. Despite those drawbacks, this chapter illustrated a viable approach to understanding soil organic matter composition and structure, and provided an important understanding of how soil components could be arranged at least in the case of the soil studied.

In chapter 5, contaminants were sorbed to soil while observing kinetics and interactions in different phases. Drawbacks of this work include the necessity of $^{19}$F NMR to study these interactions. Not all contaminants have $^{19}$F nuclei so many contaminants are ineligible to be studied using the methods described. However, there is some potential for the use of $^2$D as a surrogate. $^2$D can replace $^1$H which many contaminants have. Furthermore, similar to chapter 4, these interactions were only probed in one soil and studies need to be performed to understand the behaviour of these compounds in soils with different compositions.
Once again, many of these drawbacks revolve around the lack of sample differentiation. However, since CMP-NMR has been released by Bruker BioSpin to the mass scientific community, different applications of CMP-NMR on soil and other environmental matrices will arise with developments in experimental procedures as well as standardizing measurements.

6.3 Future Directions of Research

6.3.1 Pushing the Limits of Soil Research

Characterizing Different Soils

While this dissertation provided insight into the chemical and aggregate structure of one soil obtained from a grassland in Ireland, there are many other varieties of soils to be characterized. There is great variation between soil organic matter based on the ecosystem they support, the climate they exist in, and nearby human activities. This variation could also lead to different aggregate structures and may provide key insights into how and why certain soils can support certain ecosystems. Different soils to study include forest soils, peat soils, and agricultural soils, the last of which has more immediate and measurable (from a human perspective) importance. Using the framework outlined in chapter 4 for other soils will be aide in a better understanding of soil as a resource and ecosystem supporter.

Contaminant Ageing Mechanism

Chapter 5 provided a starting point to better characterize how contaminants interact with soil from a multi-phase standpoint. Using the set of experiments that were introduced in that chapter, understanding which functional groups of specific contaminants are interacting with which soil components can be elucidated. When taking into account the aggregate structure of soil where loosely ordered material may exist (gel phase) as an exterior to a rigid center where water does not penetrate (solid phase), then a picture of how contaminants move from the solution phase, into the gel phase, and then end up in the solid phase can be drawn. This level of detailed information can be used in many levels from providing the basic knowledge needed to effectively remediate soil to creating contaminants that are more environmentally friendly.
This would provide a molecular level approach to understanding the movement of contaminants within a soil, however, a macroscopic look at contaminant movement through a soil column can also be conducted [2]. While CMP-NMR cannot perform imaging analyses yet, additional spectroscopic tools are required. Together, a complete macro and micro understanding of contaminant dynamics in soil can be formed and would have a great impact in the soil community. Different contaminants need to be analyzed as well as different soils to gain a comprehensive understanding about how contaminants interact in different soil environments. A large enough body of this work can lead to more detailed and accurate models of contaminant fate in soil which will aid in remediation techniques and “green” chemical synthesis.

6.3.2 The Importance of In situ Analysis

This dissertation strongly advocates for the analysis of complex environmental samples in their native state throughout each of the chapters. Many environmental matrices contain a wide range of physical states including, gases, liquid, gels or swell-able material, and solid crystalline components. In between each of them is a continuum of an infinite number of physical states and how many of these states is exhibited by any environmental matrix at any given time is a function of the chemical complexity within the sample itself. This complexity is often the source of many chemical and physical properties that the matrix exhibits including porosity, reactivity, diffusivity, and density all of which impact the main processes that occur including erosion, contaminant sequestration, carbon cycling, etc.

Removing those key interactions and components also removes key features of the sample which inhibits a true understanding of soil. For example, in Chapter 4, if the soil was extracted and physically isolated, the intricacies of each component including their relative availability and the types of bonds that were present preventing solvent from reaching those components would be lost. It is the synergy and and physical arrangement of components that dictate mobility through and interactions with soil. Chapter 5 also demonstrated some of the nuances that occur during soil contamination from the dynamic interaction profile of PFP in different environments. In the gel phase, there was no clear preference within PFP that directed its interaction with soil. However, the solid phase told a different story where the more hydrophobic para end was found to interact with soil to a greater extent. This difference would not be detected in only studying the solution or solid phase and may not exist if the soil was not intact. As well, the
differing interaction profiles with soil components between each of these contaminant could only be determined in a whole swollen soil where all components and phases were present. For these reasons it is important to study soil and other environmental matrices in their natural state.

### 6.3.3 Potential CMP-NMR Development

The second and third chapters of this dissertation touched on only a fraction of the experimental methods needed to develop CMP-NMR as an effective tool for environmental research. While there have been advancements forward in qualitative understanding, standardized quantitative measures are still required. There is a subjective definition of where one phase ends, and the other phase begins. Altering diffusion time, gradient pulse lengths, various delays, and other parameters often changes the limits of spectral editing. Having set definitions for a solution, a gel, a semi-solid, and a solid that can translate into set experimental parameters would help to standardize the analyses taking place through different individuals. This may eventually become an issue once CMP-NMR gains more traction and setting these standards early would help to prevent any discrepancies in analysis.

Another area of experimental improvement is using different heteronuclei to study contaminants. $^{19}\text{F}$ was used in the fifth chapter but is only applicable to a number of contaminants. Development of techniques to study insensitive nuclei like deuterium, which can translate to many different compounds as it is possible to replace $^1\text{H}$ which is fairly ubiquitous in many contaminants with $^2\text{H}$. As well, heavy metals like $^{27}\text{Al}$, $^{29}\text{Si}$, $^{51}\text{V}$, $^{75}\text{As}$, $^{113}\text{Cd}$, $^{199}\text{Hg}$, $^{207}\text{Pb}$ have a lot of applicability to the environment including contamination through mining sites and e-waste as well as providing a source of micro-nutrients to biota. Their transport and reactivity in the environment is therefore imperative to study. Performing this research while environmental media are in their natural state would provide an unmatched understanding of these processes. Many metals have very large signal dispersion spanning in the tens of kilo Hertz range which can be problematic when finding the resonance of interest, but on the flip side demonstrates the sensitivity of the nuclei to their chemical environment, which could be a rich source of novel information.

Sensitivity is a key issue that is of great importance in NMR spectroscopy. CMP-NMR suffers from even greater sensitivity impediments because of two main reasons, probe design, and sample concentration. It has been reported that the first CMP-NMR probe built ($^1\text{H}$-$^{19}\text{F}$-
\(^{13}\text{C}-^{2}\text{H})\) suffers from a loss in sensitivity of approximately 40\% on the \(^{1}\text{H}\) and \(^{13}\text{C}\) channels \cite{3}. When comparing the sensitivity of the \(^{1}\text{H}\) channel of the super probe to a conventional HR-MAS probe, there is a 40\% reduction because a conventional HR-MAS probe is optimized to the \(^{1}\text{H}\) channel whereas the CMP-NMR probe is optimized on the \(^{13}\text{C}\) channel \cite{3}. When comparing the \(^{13}\text{C}\) channels between the CMP probe and a solid state \(^{1}\text{H}\)-BB probe, a reduction of 40\% is seen because of the addition of so many other components in the CMP probe. With the addition of multiple channels and a gradient coil, avenues for energy loss within the circuit itself are increased which reduce probe sensitivity \cite{3}. The second reason that CMP probes have reduced sensitivity has to do with the premise of the probe itself. This probe was meant for the natural state analysis of inhomogeneous mixtures which eventually leads to lower concentrations of all compounds present. For example, in conventional solid-state NMR samples are dried and packed, essentially concentrating the carbon. However, if the sample is taken as is, the majority may be water resulting in a smaller amount of analyte. Lastly, environmental samples, especially soils and sediments, often have very small natural organic contents ranging as low as a fraction of a percent further exacerbating sensitivity concerns.

While experimental techniques, as illustrated in Chapter 2, can improve sensitivity, they often result in a compromise of spectral resolution. Many experiments are already optimized to achieve the most signal possible so there is little room for advancement. However, the symbiotic relationship between technology and science has helped to bolster NMR sensitivity. One obvious approach is the use of larger sample vessels. More sample equates to more signal with a few drawbacks. 7mm magic angle spinning (MAS) CMP-NMR probes are currently in the process of being developed and would be poised to improve sensitivity in the study of environmental samples in their whole swollen state. Some issues that exist with larger rotor diameters include the requirements for more robust NMR shims. As sample coils increase in diameter, the field homogeneity decreases requiring stronger shims for decent solution state line shape. Increased diameter coils also result in lower RF homogeneity and slower spinning speeds. Spinning speed is an important factor in homogenizing sample anisotropy in solid state or gel-phase samples. As spinning speed increases and begins to approach NMR pulse time scales, anisotropic effects average out more effectively. Without averaging, NMR line shape broadens resulting in low spectral resolution.
On the topic of spinning speed, faster spinning speeds lead to sharper lines in phases where anisotropic averaging cannot occur through motion. Therefore, solid and gel-phase NMR benefit from spinning samples faster and such technology has been produced to achieve spinning speeds upwards of 111,000Hz [4]. Having improved spectral resolution in the solid and gel phases can lead to a wide array of experimentation that is not currently possible including $^1$H solid state NMR spectroscopy. Proton NMR has excellent sensitivity but suffers from low spectral dispersion which makes solid and gel phase analysis complicated. Identifying and assigning signals can be difficult with broad lines so close together. Fast spinning speeds would achieve sharper line widths in solid state opening possibilities for other experiments [5, 6, 7, 8]. One of the downsides of fast NMR spinning speeds is the limitation in sample size. Small rotors from 1.3mm and below can achieve ultra fast spinning and these rotors reduce the sample volume. In addition, ultra fast spinning is reserved for solid state analysis where no solvent is present. If solvent was present, the centripetal forces at such high spinning speeds would force water out of the sample unless special seals were made. Overall, increasing spinning speeds may help to improve CMP-NMR analysis in the solid and gel-phases.

Another technology that directly addresses the inherent sensitivity problems in NMR spectroscopy is dynamic nuclear polarization (DNP). The main root cause for NMR insensitivity is because detection is largely based on the difference in spin populations based on the Boltzmann distribution. For NMR, this difference is quite low. Therefore, only a fraction of the total spins are being observed. DNP is a technology that transfers polarization from an unpaired electron source to the sample in a process similar to cross polarization [9, 10, 11]. The general idea of cross polarization, as described in Section 1.5.4, is to enhance an insensitive nucleus by creating a spin distribution similar to that of a higher sensitivity nucleus of close spacial proximity. Electrons have a much higher spin population distribution than atomic nuclei and a polarization transfer results in a spin population in atomic nuclei similar to that of electrons [9, 10, 11]. This can lead to sensitivity gains of over 10,000 times in solution state NMR [12]. Drawbacks of this technique are that the samples must be mixed with polarizing agents, often composed of organic compounds with free radicals present like (2,2,6,6-Tetramethylpiperidin-1-yl)oxy (TEMPO), or 1-(TEMPO-4-oxy)-3-(TEMPO-4-amino)propan-2-ol (TOTAPOL) [13] and often requires low temperatures cooled by cryogens [9, 12]. This would be detrimental to the purpose of CMP-NMR because natural conditions would no longer persist however, mechanisms
around this in the solution phase do exist where the polarizing agent is attached to a support while the sample flows past it [14, 15, 16, 17]. Overall, high field DNP is a rapidly developing field and many new technological advancements are needed to bring DNP to CMP-NMR. The marriage of these technologies will open many doors for environmental research in situ.

The last piece of technology to enhance sensitivity that should be mentioned here is cryo-probe technology. Thermal noise in electronic systems increases with temperature, resulting in lower S/N as temperature increases and vice versa for lower temperature systems. Hence, NMR probe technology that cools probe electronics with cryogens have been developed to improve spectral S/N. This is performed while the sample remains at room temperature. Cryo-probes are common for solution state NMR while MAS applications are still being developed. This technology when developed for MAS probes should be easily transferable to CMP-NMR probes resulting in S/N enhancements of a factor of two or more [18, 19].

6.3.4 The Versatility of CMP-NMR

With the great versatility of NMR also comes the versatility of CMP-NMR. Soil is not the only complex environmental matrix worth studying in its native state. Sewage sludge has a variety of applications including mine tailing remediation, vegetation amendment on contaminated sites, agricultural fertilizer, with research going into sewage sludge as a building material [20, 21, 22, 23, 24, 25]. An issue that plagues many developed nations is pharmaceutical and personal care products (PPCPs) entering waste treatment facilities. Because of this, sewage sludge often mixes with many PPCPs and their interaction is not well characterized. Therefore, while sewage sludge can be a useful resource and an excellent means of recycling, the potential impact of the release of contaminants over time is not well understood and could be detrimental to the environment [26, 27, 28, 29]. CMP-NMR has the potential to study sewage sludge in its natural state where the complexities of the interactions of organic components with organic contaminants could be characterized. This may lead to a better understanding of the ultimate fate of contaminants in sewage sludge as well as its efficacy in remediation over short and long temporal scales.

Another complex environmental matrix that has the potential applicability of CMP-NMR is plant material and animal tissue. Environmental metabolomics is a field that investigates the impact of a stressor on a host species by looking at changes in large quantities of metabolites over a period of time, or concentration, or any other relevant variable. It is an established
field that uses solution state NMR spectroscopy frequently because of its high resolution and reproducibility [30, 31, 32]. This is however limiting because only small dissolved molecules are observed whereas other larger metabolites that may be bound to a host are missed. CMP-NMR technology has the ability to study solid components such as shell, bone, as-well as gels (membranes, membrane proteins). As such, it opens the possibility to directly correlate metabolic responses to structures and even physical change (for example decrease in bone density) providing a holistic understanding of toxicity and environmental stress. Furthering the discussion of metabolomics is the emerging field of in vivo metabolomics. Studying organisms or plants in their whole natural state while they are alive offers an interesting temporal perspective of metabolic profiling. Changes induced by stressors may be monitored in real time providing an opportunity to answer questions relating to time scales such as how long it takes a contaminant to pass through an organism and how long an elevated stress response is felt. This type of research becomes possible with the use of CMP-NMR because of its application to whole unaltered samples. There have already been studies of whole organism characterization using this technology namely with plant and food items [33, 34].

This last point brings emphasis to another use of CMP-NMR; its application to other fields of research outside of environmental chemistry. Structure and composition are important to understand in many research streams including various disciplines in biology, materials science, medicine and pharmaceuticals, and others. Understanding native state interactions, structure, and composition is a fundamentally important concept to gain a more relevant understanding of the subject matter. While this dissertation has only applied and developed CMP-NMR to soil, its applications in science and research have enormous potential. It is with great hope that this work has successfully illustrated that potential, eventually leading to important scientific discoveries in other research streams to help humanity better understand the universe that we inhabit.
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Chapter 7

Appendix

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Rapid estimation of nuclear magnetic resonance experiment time in low-concentration environmental samples

Hussain Masoom, Denis Courtier-Murias, Hashim Farooq, Ronald Soong, Myrna J. Simpson, Werner Maas, Rajeev Kumar, Martine Monette, Henry Stronks, André J. Simpson

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